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Israel Vlodavsky
Ralph D. Sanderson
Neta Ilan *Editors*

Heparanase

From Basic Research to Clinical Applications

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Israel Vlodavsky is a Professor of Cancer and Vascular Biology at Technion – Israel Institute of Technology's Rappaport Faculty of Medicine and Technion Integrated Cancer Center (TICC). His discovery of the extracellular matrix as a reservoir for bioactive molecules provided the basis for the current appreciation of the tumor microenvironment and its significance in cancer progression and treatment. His pioneering achievement is the cloning and characterization of heparanase, the predominant enzyme that degrades heparan sulfate and fulfills important roles in tissue remodeling, cancer metastasis, angiogenesis, inflammation, diabetes, and kidney dysfunction. Through the combination of basic and translational research, he is a leading scientist in this area of research, offering basic insights and new treatment strategies for various cancers and other diseases. He earned his bachelor's and master's degrees from The Hebrew University of Jerusalem and his PhD from the Weizmann Institute of Science, Rehovot, Israel.

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doctorate in Animal Sciences, working on manipulation of the mammary gland of transgenic animals. He did his postdoctoral training at Yale University, focusing on tumor angiogenesis. His current research focuses on the mode of action and role of heparanase in cancer initiation, growth, and metastasis in order to address problems in the prevention, management, and treatment of cancer.

Part I
Historical and General Background

Chapter 1
Forty Years of Basic and Translational Heparanase Research

Israel Vlodavsky, Neta Ilan, and Ralph D. Sanderson

1.1 Historical Introduction

Initially, several apparently different heparanase enzymes and activities have been described. However, it soon became surprisingly apparent and now well documented that there is a single unique gene encoding for heparanase and that the enzyme is the sole heparan sulfate (HS) degrading endoglycosidase expressed by normal and malignant cells and playing a role not only in cancer metastasis and angiogenesis but also in inflammatory and autoimmune conditions. Although only one confirmed endoglycosidase (heparanase) had been cloned and characterized, there are nine different exoglycosidases that are involved in the ordered disassembly of HS in the lysosomes of all cells [5]. Genetic deficiencies in these exoenzymes result in a range of lysosomal storage disorders [5]. It has been generally assumed that heparanase is the first enzyme involved in the intracellular turnover of HS [5].

Enzymatic activity capable of cleaving glucuronidic linkages and converting macromolecular heparin to physiologically active fragments was first identified by Ogren and Lindahl in mastocytoma cells [6]. Yet, developments in the field were slow due to conflicting reports regarding the physicochemical properties and substrate specificity of the enzyme. Heparanase activity has been attributed to molecules ranging in molecular mass from 8 to 134 kDa [7], [8], [9]. It was claimed, for example, that based on their substrate specificities there are at least three types of endo-beta-D-glucuronidases and that the melanoma heparanase (Mr approximately 96,000) differ from the platelet and mastocytoma enzymes [10]. There have also

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been claims that the enzyme is a heat shock protein [11] or related to the CXC chemokine, connective-tissue-activating peptide III (CTAP) [8]. Other studies distinguished between the secreted and intracellular enzymes and suggested that there may be a family of heparanase proteins with different substrate specificities and potential functions [12]. Studies performed by Bame et al. focused on intracellular heparanase(s) and their involvement in normal catabolism of HS. It was claimed that inside cells, these enzymes are important for the normal catabolism of HS proteoglycans (HSPG), generating glycosaminoglycan fragments that are then transported to lysosomes and completely degraded. Characterization of the short glycosaminoglycans produced in Chinese hamster ovary (CHO) cells suggested that multiple heparanases are necessary for the formation of the short HS chains [13]. Based on their ability to bind ion-exchange resins and their elution from gel-filtration columns, four separate heparanase activities were partially purified. All four activities cleave free glycosaminoglycans over a broad pH range of 3.5–6.5, suggesting that they act in the endosomal/lysosomal pathway. It was further suggested that the formation of short HSPG inside CHO cells may be a result of the concerted action of multiple heparanases, and may depend on the proportions of the different enzymes and the environment in which the chains are degraded [13].

A large part of the lay summary presented below was taken from commentaries written by Drs. Eccles and Finkel [14, 15] *in response to the heparanase cloning papers published in 1999 by Vlodavsky et al.* [4] *and Hulett et al.* [1]. *These commentaries were found suitable for the current 'historic review' by virtue of their accuracy and high relevance to heparanase research performed since then.*

Tumor cell invasion and secondary spread through the blood and lymphatics is the hallmark of malignant disease and the greatest impediment to cancer cure. Two of the essential processes required for metastasis are neoangiogenesis and tumor cell invasion of the basement membrane (BM) and extracellular matrix (ECM). Prior to cloning of the heparanase gene, attention focused on serine and cysteine proteases and matrix metalloproteinases (MMPs). Although metastasizing cancer cells may produce as many as 25 different matrix-digesting metalloproteinases, cloning of the same heparanase gene by different groups [1–4] indicated that there is only one heparanase so that if its activity can be inhibited, other heparanases shouldn't be around to cover for it [15]. In addition to the structural proteins (i.e., collagens, laminin, fibronectin, vitronectin) cleaved by MMPs in the BM and ECM, the other chief components are glycosaminoglycans, mainly heparan sulfate proteoglycans (HSPG). HSPG are composed of a protein core covalently linked to HS glycosaminoglycan chains that interact closely with other ECM components. Endoglycosidase activity (heparanase) that degrades the HS side chains of HSPG is normally found mainly in platelets, placental trophoblasts, and leukocytes. Given their abundance in tumor tissues, it appears that the normal physiological functions of proteases and heparanases in embryonic morphogenesis, wound healing, tissue repair and inflammation have been effectively 'hijacked' by tumor cells.

Evidence indicates that heparanase not only assists in the breakdown of ECM and BM but also is involved in the regulation and bioavailability of growth factors

and cytokines [16]. For example, basic fibroblast growth factor (bFGF) and other heparin-binding growth factors (i.e., HB-EGF, VEGF, HGF) are sequestered by HS, providing a localized, readily accessible depot, protected from proteolytic degradation, yet available to activate cells and promote angiogenesis after being released by heparanase. It was suggested that the release of tissue-specific growth factors may be involved in organ selectivity of metastasis and the formation of a metastatic niche [17, 18]. Although these phenomena were well-documented, it has taken about 20 years to purify the heparanase protein and clone the respective gene mainly because of instability of the enzyme(s), its low abundance in normal cells and tissues and the lack of a robust, accurate and rapid assay for enzyme activity [14].

Researchers first made the connection between metastasis and heparanase in the mid-1980s. Three groups [Garth Nicolson's (M. D. Anderson Cancer Center in Houston), Christopher Parish (John Curtin School of Medical Research, Canberra) and Israel Vlodavsky (Hadassah-Hebrew University, Jerusalem)] were following up on the finding that the natural anticoagulant, heparin, inhibits the spread of cancer in animals [15]. The prevailing belief was that heparin worked because it prevented platelets from clotting around cancer cells, an event likely to help the cells lodge into, and ultimately penetrate the vessel wall. But "heparin" is a family of molecules, only some of which inhibit clot formation. The three groups independently showed that it still inhibits metastasis, even when depleted of its anticlotting activity [14]. Only about 15 years later it was demonstrated that heparin inhibits heparanase enzymatic activity through competition with HS on binding to the heparin/HS binding domains (HBD) of heparanase [19].

Both Parish [20] and Vlodavsky [21] had reported that heparanase helps immune cells traverse blood vessel walls on their way to infection sites. The evidence that it might be doing something similar for cancer cells immediately made researchers think about getting better heparanase inhibitors. But getting the pure enzyme proved to be difficult. Not only is heparanase unstable, but the only assay then available was slow and cumbersome [22, 23]. Nevertheless, the Israeli group finally managed to purify heparanase from a human liver cancer cell line and also from human placenta [4], while the Australian group purified it from human platelets [24]. In parallel, Toyoshima and Nakajima [3] reported the purification of a human heparanase from an SV40-transformed embryonic fibroblast cell line, and Kussie et al. [2] isolated and purified the enzyme from human SK-HEP-1 hepatoma cells. After determining peptide amino acid sequences derived from the purified proteins, the researchers then screened EST databases looking for gene sequences that could encode those amino acid sequences. Contrary to expectations that there might be more than one heparanase, the four groups found themselves with the same gene - the only one like it in the databases. Soon after, genomic organization and chromosome localization of the newly identified human heparanase gene was reported [25]. Cloning, expression, and purification of mouse heparanase that is 77% identical to the human enzyme was reported by Miao et al. [26]. The recombinant mouse heparanase protein was purified to homogeneity from cell lysates by a combination of Con-A affinity chromatography, heparin affinity chromatography, and size exclusion chromatography, purification steps that are commonly used nowadays. Experiments confirmed that the newly

cloned gene aids the spread of cancer cells. When Vlodavsky and his colleagues introduced the heparanase gene into nonmetastatic mouse melanoma and lymphoma cancer cells, they turned into rampantly malignant cells that colonized the lung and liver when injected into mice [4]. Parish, looking at several different types of rat cancer cells, found that their invasiveness correlates with the activity of their heparanase gene [1]. Conversely, inhibiting the enzyme inhibits cancer metastasis. Parish reported that a previously identified inhibitor of heparanase called PI-88 [27] decreased by 90% the number of lung tumors formed by breast cancer cells injected into rats. It also cut the blood supply of the primary tumors and slowed tumor growth [28]. The encouraging animal results have led Progen (Brisbane, Australia) to test the safety of the inhibitor in healthy volunteers, followed by clinical trials in cancer patients [29].

Notably, studies aimed at determining the role of heparanase in tumor progression relied on the use of heparin-mimicking molecules to inhibit heparanase activity [30–32]. Because the reagents used in these studies lacked specificity, the conclusions were somewhat debatable and inconclusive. It took another 12 years to generate heparanase neutralizing monoclonal antibodies (mAb) and demonstrate their ability to inhibit lymphoma tumor growth and dissemination [33]. Notably, the inhibitory activity of the mAbs was lower than that of the heparin mimetics (i.e., PG545) [33], further raising the issue of drug specificity vs. efficiency. Nowadays, heparanase is well established as a cancer drug target, and several inhibitors have progressed to clinical trials [31, 34]. In recent years, heparanase has also been implicated in a range of other diseases, such as diabetes and its complications [35–37], kidney disease [38], atherosclerosis [39, 40] and viral infections [41], to name a few, which continues to fuel research into this protein.

Studies performed before cloning of the heparanase gene contributed immensely to our understanding of key features in the biology of the enzyme, its mode of action and involvement in cancer metastasis and inflammation. Abstracts of selected 'Historical' studies referred to in the introduction are presented below in chronological order. The purpose of selecting these abstracts is to provide a historic perspective of how heparanase research was developed and progressed with time, rather than to highlight the most prominent and influential papers.

1.1.1 Key Observations Made Prior to Cloning of the HPSE Gene (Chronological Order)

Cleavage of Macromolecular Heparin by an Enzyme From Mouse Mastocytoma Heparinase was isolated from a transplantable mouse mastocytoma. The enzyme was shown to degrade macromolecular ^{35}S-labeled, mastocytoma heparin to products similar in size to commercial heparin apparently by nonrandom cleavage of a limited number of glycosidic linkages per molecule. Prolonged incubation times did not result in further degradation of the product. No significant depolymerizing activity was observed with any other glycosaminoglycan tested, including

chondroitin sulfate, dermatan sulfate, hyaluronic acid, heparan sulfate, and commercial heparin. The pH optimum for degradation of macromolecular heparin was around pH 5. Analysis of the degradation products showed a major radioactive component which behaved like L-gulonic acid. Since [^{3}H]gulonic acid would be the expected reduction product of a polysaccharide molecule, containing a glucuronic acid residue in terminal position, these results tentatively suggest that the heparinase is an endoglucuronidase. It appears that cleavage occurs in regions more abundant in N-acetylated glucosamine residues than other portions of the molecule [6].

**First paper stating that an endoglucuronidase is responsible for cleavage of heparin by mastocytoma cells. Notably, the presence of such heparin degrading activity was reported by the same authors already in 1971* [42].

A Heparan Sulfate-Degrading Endoglycosidase From Rat Liver Tissue Incubation of a rat liver lysosomal fraction with [^{35}S]heparan sulfate resulted in degradation of the polymer to oligosaccharides, demonstrating the presence of a heparan sulfate-degrading endoglycosidase. Judging from the size of the oligosaccharides, representing degradation end-products, only a limited number of the glycosidic linkages in the HS molecule would seem to be susceptible to the heparitinase. The pH-dependence of the enzyme (active at pH 5.6; inactive at pH 3.8) was found to differ from that of liver hyaluronidase (active at pH 3.8; inactive at pH 5.6), suggesting that the heparitinase is a previously unknown enzyme [43].

**Early (1975) study on the presence of HS-degrading endoglycosidase in liver tissue.*

Purification and Properties of Human Platelet Heparitinase An endoglycosidase which cleaves heparin and HS was isolated from outdated human platelets. The overall extent of purification of the platelet heparitinase is about 240,000-fold and the overall yield of the enzyme is about 5.6% as compared to the initial freeze-thaw solubilization preparation. The final product is physically homogeneous and exhibits an apparent molecular weight of approximately 134,000. Furthermore, our results indicate that the above enzyme is present within platelet lysosomes. The biologic potency of the endoglycosidase was examined as a function of pH and is maximally active from pH 5.5 to pH 7.5. The substrate specificity of the platelet endoglycosidase was determined by identifying susceptible linkages within the heparin molecule that can be cleaved by the above component. Our studies indicate that this enzyme is only able to hydrolyze glucuronsylglucosamine linkages. Furthermore, investigation of the structure of the disaccharide which lies on the nonreducing end of the cleaved glucuronic acid residue suggests that N-sulfation of the glucosamine moiety or ester sulfation of the adjacent iduronic acid groups are not essential for bond scission [9].

**Early (1982) study on the purification and properties of HS-degrading endoglycosidase in platelets.*

Heparan Sulfate Degradation: Relation to Tumor Invasive and Metastatic Properties of Mouse B16 Melanoma Sublines Mouse B16 melanoma sublines were used to determine the relation between metastatic properties and the ability of the sublines to degrade sulfated glycosaminoglycans present in the ECM of cultured vascular endothelial cells. Highly invasive and metastatic B16 sublines degraded matrix glycosaminoglycans faster than did sublines of lower metastatic potential. The main products of this matrix degradation were HS fragments. Intact B16 cells (or their cell-free homogenates) with a high potential for lung colonization degraded purified HS from bovine lung at higher rates than did B16 cells with poor potential for lung colonization. Analysis of the degradation fragments indicated that B16 cells have HS endoglycosidase. Thus the abilities of B16 melanoma cells to extravasate and successfully colonize the lung may be related to their capacities to degrade HS in the walls of pulmonary blood vessels [44].

**First study (1983) showing that HS endoglycosidase is associated with the metastatic potential of melanoma cells.*

Lymphoma Cell-Mediated Degradation of Sulfated Proteoglycans in the Subendothelial Extracellular Matrix: Relationship to Tumor Cell Metastasis Cloned lines of the low-metastatic T-lymphoma Eb line and its highly metastatic variant ESb line were compared for the ability to degrade proteoglycans in the subendothelial ECM produced by cultured endothelial cells. The ECM was metabolically labeled with $Na_2{}^{35}SO_4$, and the tumor cell-mediated release of labeled degradation products was analyzed by gel filtration. More than 90% of the labeled material released upon incubation of ESb cells with the ECM, either when exposed or covered with vascular endothelial cells, was in the form of low-Mr, HS-containing fragments compared to high-Mr nearly intact sulfated proteoglycans released by incubation with the low-metastatic Eb cells. The same high- and low-Mr degradation products were obtained by incubation of the ECM with a serum-free medium conditioned by the low (Eb)- and high (ESb)-metastatic sublines, respectively. The high-Mr proteoglycans released by incubation of the ECM with Eb-conditioned medium was further degraded into low Mr. GAG fragments upon subsequent incubation with ESb-conditioned medium. These fragments were smaller than intact glycosaminoglycan side chains released by treatment of the ECM with papain or alkaline borohydride, suggesting an ESb-specific endoglycosidase activity. The higher ability of the ESb over the Eb cells to solubilize the GAG scaffolding of the sub-endothelial ECM may, among other properties, facilitate their hematogenous dissemination and extravasation [45].

**First study (1983) showing that HS endoglycosidase is associated with the metastatic potential of lymphoma cells.*

Activated T Lymphocytes Produce a Matrix-Degrading Heparan Sulfate Endoglycosidase Circulating activated T lymphocytes specifically autosensitized to the basic protein of myelin (BP) penetrate blood vessels, accumulate in the nervous system and cause experimental autoimmune encephalomyelitis (EAE). To investigate how effector T cells reach targets outside the walls of blood vessels,

we have studied the interaction of anti-BP effector T lymphocytes with the basement membrane-like ECM produced by vascular endothelial cells. It was found that activated but not resting T lymphocytes produce an endoglycosidase (heparanase) capable of degrading HS side chains of the proteoglycan scaffold of the ECM. Moreover, the anti-BP T lymphocytes respond to BP presented by ECM by markedly enhanced elaboration of the endoglycosidase. These results suggest that tissue-specific antigens on blood vessel walls could direct lymphocyte homing by activating enzymes that facilitate penetration of the subendothelial basal lamina. They also suggest that effector T lymphocytes can recognize antigen which is not associated with a major histocompatibility complex signal [21].

**First study (1984) describing the expression and function of heparanase in activated T lymphocytes.*

Sequential Degradation of Heparan Sulfate in the Subendothelial Extracellular Matrix by Highly Metastatic Tumor Cells Both a protease and heparanase are synergistically involved in ESb-mediated degradation of ECM-bound HS and one enzyme produces a more accessible substrate for the next enzyme [46]. Briefly, it was found that degradation of HS in the ECM was markedly enhanced in the presence of plasminogen and inhibited by aprotinin, suggesting a role for plasminogen activator (PA) in sequential degradation of the ECM-HS [47]. Moreover, subsequent studies revealed that PA activity is residing in the ECM itself [48]. Thus, incubation of plasminogen on ECM resulted in plasmin generation and heating the ECM inactivated its ability to generate plasmin upon incubation with plasminogen. It was concluded that a proteolytic activity expressed by the tumor cells and/or residing in the tumor microenvironment (i.e., ECM) participate synergistically in sequential degradation of the EM' HS. This sequential cleavage is characteristic of degradation of a multimolecular structure such as the subendothelial ECM and hence cannot be detected in studies with purified HS as a substrate. [46, 47].

**First study showing that a proteolytic activity residing in the ECM is critical for subsequent cleavage of HS by heparanase.*

Endothelial Cell-Derived Basic Fibroblast Growth Factor: Synthesis and Deposition into the Subendothelial Extracellular Matrix The endothelium can store growth factors capable of autocrine growth promotion in two ways: by sequestering growth factor within the cell and by incorporating it into the underlying ECM. We hypothesized that release of ECM-bound basic FGF could stimulate the autocrine proliferation of adjacent endothelial cells. Moreover, tumor angiogenesis may in part be mediated by the action of tumor-derived HS-degrading enzymes, which would release basic FGF stored in capillary basement membrane. We further proposed that release of intracellular and extracellular stores of basic FGF may be a mechanism for the rapid mobilization of angiogenesis factors. This hypothesis was strengthened by the in situ experiments described below [49].

A Heparin-Binding Angiogenic Protein - Basic Fibroblast Growth Factor – Is Stored Within Basement Membrane The basement membranes of bovine cornea

were found to contain an angiogenic endothelial cell mitogen, basic fibroblast growth factor (FGF) that was bound to HS and released from the cornea by treatment with heparin, a hexasaccharide heparin fragment, HS, or heparanase. These findings indicate that basement membranes of the cornea may serve as physiologic storage depots for an angiogenic molecule. Abnormal release of this growth factor could be responsible for corneal neovascularization in a variety of ocular diseases. It was suggested that sequestration of heparin-binding proangiogenic mitogens in the basement membrane and their liberation by heparanase might be a general mechanism for regulating their accessibility to the vascular endothelium and that heparanase plays a key role in fulfilling this function [50].

*By now, the concept of ECM as a reservoir for bioactive molecules is well recognized, providing a strong basis to the current appreciation of the tumor microenvironment and its significance in supporting tumor growth and metastasis. Additional studies on the pro-angiogenic activity of heparanase and the contribution of heparanase residing in the tumor microenvironment are described later under 'key studies performed after cloning of the HPSE gene [50].

**The above two manuscripts are the first to demonstrate that heparanase functions in the liberation and mobilization of HS-bound growth factors, an activity that underlies the high significance of this enzyme in promoting tumor angiogenesis and growth, among other biological effects.*

Inhibition of Heparanase-Mediated Degradation of Extracellular Matrix Heparan Sulfate by Non-Anticoagulant Heparin Species The present study examined the heparanase inhibitory effect of nonanticoagulant species of heparin that might be of potential use in preventing heparanase mediated extravasation of blood-borne cells. For this purpose, we prepared various species of low-sulfated or LMW heparins, all of which exhibited less than 7% of the anticoagulant activity of native heparin. N-sulfate groups of heparin are necessary for its heparanase inhibitory activity but can be substituted by an acetyl group provided that the O-sulfate groups are retained. O-sulfate groups could be removed provided that the N positions were resulfated. Total desulfation of heparin abolished its heparanase inhibitory activity. Heparan sulfate was a 25-fold less potent heparanase inhibitor than native heparin. Efficiency of LMW heparins to inhibit degradation of HS in ECM decreased with their main molecular size, and a synthetic pentasaccharide, representing the binding site to antithrombin III, was devoid of inhibitory activity. Similar results were obtained with heparanase activities released from platelets, neutrophils, and lymphoma cells. We propose that heparanase inhibiting nonanticoagulant heparins may interfere with dissemination of blood borne tumor cells and development of experimental autoimmune diseases [51].

Evidence That Sulphated Polysaccharides Inhibit Tumour Metastasis by Blocking Tumour-Cell-Derived Heparanases Rat mammary adenocarcinoma 13,762 MAT cells produce a HS-specific glycosidase (heparanase) that degrades the HS side-chains of the ECM. The action of this enzyme, rather than that of other ECM-solubilizing enzymes, was inhibited by 5 antimetastatic sulphated polysaccharides but

not by 4 polysaccharides that failed to inhibit metastasis. Additional experiments indicated that the anti-coagulant activity of the polysaccharides probably plays a minor role in their anti-metastatic effects since heparin, almost completely depleted (98–99.5%) of heparin molecules with anti-coagulant activity by passage over an anti-thrombin III column, retained its ability to inhibit 13,762 MAT heparanases and was almost as effective as unfractionated heparin at inhibiting tumour-cell metastasis. Collectively, these data suggest that sulphated polysaccharides inhibit the metastasis of 13,762 MAT cells by inhibiting tumour-cell-derived heparanases involved in the penetration of the vascular endothelium and its underlying basement membrane by tumour cells. These results paved the way for the development and clinical testing of PI-88 (= phosphomannopentaose sulfate = Muparfostat) [52].

The above two studies demonstrate that both heparanase enzymatic activity and experimental metastasis are inhibited by non-anticoagulant species of heparin and other sulfated polysaccharides.

Inhibition of Allergic Encephalomyelitis in Rats by Treatment With Sulfated Polysaccharides A number of sulfated polysaccharides were tested for their ability to inhibit passively induced experimental allergic encephalomyelitis (EAE) in rats. Heparin and fucoidan both completely inhibited passive EAE even when treatment was begun 3 days after transfer of cells. Pentosan sulfate was partially inhibitory whereas chondroitin-4-sulfate had no effect. Inhibition was not merely due to killing of the cells since active sensitization 14 days after cell transfer resulted in an early onset of disease indicating the persistence of transferred cells as memory cells. Although all the inhibitory polysaccharides are anticoagulants, it would appear that this function alone is not the reason for inhibition since a heparin preparation devoid of anticoagulant activity also partially inhibited EAE. Actively induced EAE was also significantly delayed by treatment with heparin. The results are discussed in terms of the polysaccharides inhibiting the enzymatic dependent movement of lymphocytes across central nervous system vascular endothelium [53].

Suppression of Experimental Autoimmune Diseases and Prolongation of Allograft Survival by Treatment of Animals With Low Doses of Heparins Heparanase-inhibiting, nonanticoagulant species of heparin markedly reduced the incidence of lung metastasis in experimental animals. Low doses of these species of heparin also significantly impaired the traffic of T lymphocytes and suppressed cellular immune reactivity and experimental autoimmune diseases (allograft rejection, adjuvant arthritis, experimental autoimmune encephalomyelitis). The ability of chemically modified heparins to inhibit these immune reactions was associated with their ability to inhibit expression of T lymphocyte heparanase. There was no relationship to anticoagulant activity. Thus heparins devoid of anticoagulant activity can be effective in regulating immune reactions when used at appropriate doses [54].

The above two studies demonstrate that both heparanase and experimental autoimmune diseases are inhibited by non-anticoagulant species of heparin and other sulfated polysaccharides.

Molecular Behavior Adapts to Context: Heparanase Functions as an Extracellular Matrix-Degrading Enzyme or as a T Cell Adhesion Molecule, Depending on the Local pH Migration of lymphocytes into inflammatory sites requires their adhesion to the vascular endothelium and subendothelial ECM. Depending on the local pH, heparanase can function either as an enzyme or as an adhesion molecule. At relatively acidified pH conditions, heparanase performs as an enzyme, degrading HS. In contrast, at the hydrogen ion concentration of a quiescent tissue, heparanase binds specifically to HS molecules without degrading them, and thereby anchors CD4+ human T lymphocytes. Thus, the local state of a tissue can regulate the activities of heparanase and can determine whether the molecule will function as an enzyme or as a proadhesive molecule [55].

**Early article demonstrating that heparanase may function as cell adhesive molecule.*

CXC Chemokines Connective Tissue Activating Peptide-III and Neutrophil Activating Peptide-2 Are Heparin/Heparan Sulfate-Degrading Enzymes In this study, purification of a HS-degrading enzyme from human platelets led to the discovery that the enzymatic activity resides in at least two members of the platelet basic protein (PBP) family known as connective tissue activating peptide-III (CTAP-III) and neutrophil activating peptide-2. PBP and its N-truncated derivatives, CTAP-III and neutrophil activating peptide-2 are CXC chemokines, a group of molecules involved in inflammation and wound healing. SDS-PAGE analysis of the purified heparanase resulted in a single broad band at 8–10 kDa, the known molecular weight of PBP and its truncated derivatives. Gel filtration chromatography of heparanase resulted in peaks of activity corresponding to monomers, dimers, and tetramers. N-terminal sequence analysis of the same preparation indicated that only PBP and truncated derivatives were present, and commercial CTAP-III from three suppliers had heparanase activity. Antisera produced in animals immunized with a C-terminal synthetic peptide of PBP inhibited heparanase activity by 95%, compared with activity of the purified enzyme in the presence of the preimmune sera. The enzyme was determined to be an endoglucosaminidase, and it degraded both heparin and HS with optimal activity at pH 5.8. Sequence analysis showed that the two peaks contained identical protein, suggesting that a post-translational modification activates the enzyme [8].

Partial Purification of Heparanase Activities in Chinese Hamster Ovary Cells: Evidence for Multiple Intracellular Heparanases Our studies characterizing the short glycosaminoglycans produced in Chinese hamster ovary (CHO) cells suggested that multiple heparanases are necessary for the formation of the short HS chains. We examined whether this is the case by purifying heparanase activity from CHO cell homogenates. Based on their ability to bind ion-exchange resins and their elution from gel-filtration columns, four separate heparanase activities were partially purified. All four activities cleave free glycosaminoglycans over a broad pH range (3.5–6.5), suggesting that they act in the endosomal/lysosomal pathway. The sizes of the short HS chains generated by the partially purified heparanases ranged

from 6 to 9 kDa. Interestingly, all four enzymes generate short glycosaminoglycans with a sulfate-rich, modified domain at the non-reducing end of the newly formed chain. Our findings suggest that the formation of short HS glycosaminoglycans inside CHO cells may be a result of the concerted action of multiple heparanases, and may depend on the proportions of the different enzymes and the environment in which the chains are degraded [13].

As demonstrated in the above two studies, there were several serious attempts to purify the heparanase enzyme, yet subsequent studies failed to confirm the results.

1.2 Heparanase Gene Cloning

***Mammalian Heparanase: Gene Cloning, Expression and Function in Tumor Progression and Metastasis** We have purified a 50-kDa heparanase from human hepatoma and placenta, and now report cloning of the gene encoding this enzyme. Expression of the cloned cDNA in insect and mammalian cells yielded 65-kDa and 50-kDa recombinant heparanase proteins. The 50-kDa enzyme represents an N-terminally processed enzyme, at least 100-fold more active than the 65-kDa form. The heparanase mRNA and protein are preferentially expressed in metastatic cell lines and specimens of human breast, colon and liver carcinomas. Low metastatic murine T-lymphoma and melanoma cells transfected with the heparanase cDNA acquired a highly metastatic phenotype in vivo, reflected by a massive liver and lung colonization. This represents the first cloned mammalian heparanase and provides direct evidence for its role in tumor metastasis. Cloning of the heparanase gene enables the development of specific molecular probes for early detection and treatment of cancer metastasis and autoimmune disorders [4].

***Cloning of Mammalian Heparanase, an Important Enzyme in Tumor Invasion and Metastasis** We report the cDNA sequence of the human platelet enzyme, which encodes a unique protein of 543 amino acids, and the identification of highly homologous sequences in activated mouse T cells and in a highly metastatic rat adenocarcinoma. Furthermore, the expression of heparanase mRNA in rat tumor cells correlates with their metastatic potential. Exhaustive studies have shown only one heparanase sequence, consistent with the idea that this enzyme is the dominant endoglucuronidase in mammalian tissues [1].

The above two papers were published side by side (Nat Med. 1999; 5:793–809) preceded by a commentary entitled: Heparanase: Breaking down barriers in tumors. Cloning and functional characterization of the long sought-after heparanase opens a new chapter in the understanding and potential manipulation of metastasis and inflammatory processes [14].

Cloning and Functional Expression of a Human Heparanase Gene We have cloned a gene (HSE1) from a human placental cDNA library that encodes a novel

protein exhibiting heparanase activity. The cDNA was identified through peptide sequences derived from purified heparanase isolated from human SK-HEP-1 hepatoma cells. HSE1 contains an open reading frame encoding a predicted polypeptide of 543 amino acids and possesses a putative signal sequence at its amino terminus. Northern blot analysis suggested strong expression of HSE1 in placenta and spleen. Transient transfection of HSE1 in COS7 cells resulted in the expression of a protein with an apparent molecular mass of 67–72 kDa. HSE1 protein was detectable in conditioned media but was also associated with the membrane fraction following cell lysis. The HSE1 gene product was shown to exhibit heparanase activity by specifically cleaving a labeled heparan sulfate substrate in a similar manner as purified native protein [2].

Human Heparanase: Purification, Characterization, Cloning, and Expression We report the purification of a human heparanase from an SV40-transformed embryonic fibroblast cell line by four sequential column chromatographies. The enzyme was purified to homogeneity, yielding a peptide with an apparent molecular mass of 50 kDa when analyzed by SDS-polyacrylamide gel electrophoresis. Using the amino acid sequences of the N-terminal and internal heparanase peptides, a cDNA coding for human heparanase was cloned. NIH3T3 and COS-7 cells stably transfected with pBK-CMV expression vectors containing the heparanase cDNA showed high heparanase activitiy. The homology search revealed that no homologous protein had been reported [3].

Cloning, Expression, and Purification of Mouse Heparanase A full-length heparanase gene was cloned from a mouse embryo cDNA library and determined to encode a protein of 535 amino acids that is 77% identical to human heparanase. The full-length mouse gene was stably expressed in NS0 myeloma cells. The recombinant mouse heparanase protein was purified to homogeneity from cell lysates by a combination of Con-A affinity chromatography, heparin affinity chromatography, and size exclusion chromatography. The purified protein consisted of a non-covalent heterodimer of 50- and 8-kDa polypeptides, similar to the human homolog. The protein was enzymatically active in assays using radiolabeled ECM and HS as substrates. The maximum heparanase activity was observed at acidic conditions; however, significant activity was also detected at neutral pH. The enzymatic activity of mouse heparanase was blocked by known heparanase inhibitors [26] (see Gaskin et al., Chap. 7 in this volume, for more information about heparanase gene cloning).

1.3 Studies Performed Following Cloning of the HPSE Gene

1.3.1 Introductory Notes

Cloning of the heparanase gene boosted heparanase research thanks to the readily available recombinant enzyme, molecular probes and anti-heparanase antibodies. Notes on early developments obtained soon after the cloning of the HPSE gene are

presented below, followed by selected abstracts of key findings, arranged according to specific topics. Of the numerous publications focusing on heparanase, we have selected those we regard as important contributions to the heparanase field with preference to studies performed by scientists and groups that contributed to this book.

*The 'metastasis' paragraph presented below was taken from a commentary written by Drs. Nakajima and Boyd [56] in response to the heparanase gene silencing paper published in JNCI by Edovitsky et al. [57]. This commentary was found suitable for this 'historic review' due to its high relevance to the current status of heparanase research and the related questions that were raised back in 2004.

Metastasis Subsequent to the simultaneous cloning of the cDNA-encoding heparanase, Goldshmidt et al. [58] found that overexpression of the cDNA-encoding heparanase conferred a metastatic phenotype in lymphoma cells. Nevertheless, it was argued that the ability of increased heparanase levels to induce a metastatic phenotype does not necessarily imply that tumors make use of heparanase to drive tumor dissemination. In subsequent studies, Edovitsky et al. [57] have attempted to address this shortcoming by using ribozyme and small interfering RNA (siRNA) technology to knock down the levels of endogenous heparanase. The authors showed that, in models of experimental and spontaneous metastases, these strategies attenuated the ability of diverse tumor cells, including melanoma, mammary adenocarcinoma, lymphoma, and glioma cells, to invade in vitro and to colonize distant sites including the liver and lungs. This study [57] and an earlier study [59] performed by Uno et al. provided strong support for a role of heparanase in the metastatic process. Nevertheless, caution was taken in predicting clinical efficacy given that tumor cells have a remarkable system of redundant mechanisms that can efficiently overcome the targeting of single molecules [60]. This redundancy is one of the contributing mechanisms underlying the lack of clinical benefit seen with metalloproteinase inhibitors in cancer patients [61]. Taking into account that only one HS-degrading endoglycosidase was identified, redundancy is not considered a problem in targeting heparanase as compared to metalloproteinases.

An equally important issue raised by Boyed and Nakajima [56] relating to the utility of anti-heparanase and other anti-metastatic therapies in cancer treatment concerns the fact that, at the time of presentation, the majority of patients already have disseminated disease. Consequently, treatment of such patients with anti-heparanase regimens might be akin to closing the barn door after the horse has bolted [56]. Thus, how could a knock-out punch against heparanase be useful in the treatment of cancer patients? There are at least two options. First, with increasing public awareness, and as cancer screenings become more prevalent in the general population, the number of patients diagnosed with early-stage disease should increase. By definition, such tumors are still localized and are therefore more amenable to therapy with anti-metastasis agents. Second, considering the proangiogenic effects of heparanase first documented by Edovitsky et al. [62] and Elkin et al. [63], anti-heparanase drugs may have a static effect on both the primary tumor and distant

tumor lesions by preventing the establishment of tumor vasculature necessary for tumor growth beyond 1 mm^3. These considerations made a compelling case for the role of heparanase in tumor progression. The extent to which this information can be exploited in novel therapies depends on the development of specific inhibitors that target heparanase and the blockade of redundant mechanisms that compensate for the loss of heparanase in cancer. *This statement was rightly written (2004) in an editorial to the gene silencing* paper [56] *and is relevant nowadays as well.*

Gene Regulation de Mestre et al. reported the identification of the serum-inducible zinc finger transcription factor human early growth response gene 1 (EGR1), as a key regulator of inducible HPSE transcription in T lymphocytes [64] and cancer cells [65]. EGR1 is a nuclear phospho-protein that is rapidly induced in response to a variety of extracellular and environmental signals (including growth factors, cytokines, vascular injury, and hypoxia) [66, 67]. Studies using knockdown strategies have confirmed that EGR1 binds the HPSE promoter in vivo and plays a central role in tumor angiogenesis, growth, and metastasis in breast, bladder, colon and prostate adenocarcinomas [65, 68, 69], supporting a central role for EGR1 in regulating HPSE transcription in tumor cells (Gaskin et al., Chap. 7 in this volume).

Elkin et al. identified putative estrogen response elements in the heparanase promoter and demonstrated their functionality applying a luciferase reporter gene driven by the heparanase promoter [70]. Physical association between estrogen receptor (ER) and the heparanase promoter was confirmed by ChIP analysis. ChIP analysis, also revealed that wild type p53 inhibits transcription of the heparanase gene by direct binding to its promoter, while mutated, tumor-derived variants of p53 lose this inhibitory ability and in some cases even up regulate heparanase gene expression [71]. Examining a series of tumor-derived cell lines, we have found that cells which exhibit heparanase activity also harbor at least one unmethylated allele [72], while cell lines which exhibit no heparanase expression or activity were found to harbor fully methylated alleles. Treating these cells with demethylating agents such as 5-azacytidine restored heparanase activity accompanied by augmented metastatic capacity in vivo [69, 72]. Cathepsin L plays a critical role in the processing and conversion of latent heparanase into its active form [73]. Interestingly, promoter methylation [74] and EGR family members are also involved in cathepsin L activation [75], suggesting that heparanase and cathepsin L share some regulatory aspects. Applying the RIP1-Tag2 tumor model, Joyce et al. demonstrated that while cathepsin L expression was restricted to tumor cells, the majority of heparanase appeared to originate from infiltrating immune cells [76]. Thus, pro-heparanase secreted from one cell type (inflammatory cells) can be activated by cathepsin L secreted by another compartment (cancer cells) in cooperation that drives tumor development [18]. Notably, regulation of both heparanase and cathepsin L does not rely solely on gene transcription but rather involves complex regulatory mechanisms. Important regulatory elements of cathepsin L were identified in 5′, and 3′ untranslated regions (UTR) of the gene [77]. Likewise, Arvatz et al. revealed post-transcriptional regulation of heparanase gene expression by a 3′ AU-rich element in the 3′UTR of the gene [78].

1.3.2 Key Observations Made After Cloning of the HPSE Gene

(*Abstracts of papers arranged according to specific topics)*

Structural Aspects

Processing of the Human Heparanase Precursor and Evidence That the Active Enzyme Is a Heterodimer Human platelet heparanase has been purified to homogeneity and shown to consist of two, non-covalently associated polypeptide chains of molecular masses 50 and 8 kDa. Protein sequencing provided the basis for determination of the full-length cDNA for this novel protein. Based upon this information and results from protein analysis and mass spectrometry, we propose a scheme to define the structural organization of heparanase in relation to its precursor forms, proheparanase and pre-proheparanase. The 8- and 50-kDa chains which make up the active enzyme reside, respectively, at the NH (2)- and COOH-terminal regions of the inactive precursor, proheparanase. The heparanase heterodimer is produced by excision and loss of an internal linking segment. This paper is the first to suggest that human heparanase is a two-chain enzyme [79].

**Among the earliest studies demonstrating that the active enzyme is a heterodimer.*

Identification of Active-Site Residues of the Pro-Metastatic Endoglycosidase Heparanase Using PSI-BLAST and PHI-BLAST searches of sequence databases, similarities were identified between heparanase and members of several of the glycosyl hydrolase families from glycosyl hydrolase clan A (GH-A), including strong local identities to regions containing the critical active-site catalytic proton donor and nucleophile residues that are conserved in this clan of enzymes. Furthermore, secondary structure predictions suggested that heparanase is likely to contain an (alpha/beta) (8) TIM-barrel fold, which is common to the GH-A families. Based on sequence alignments with a number of glycosyl hydrolases from GH-A, Glu(225) and Glu(343) of human heparanase were identified as the likely proton donor and nucleophile residues, respectively. The substitution of these residues with alanine and the subsequent expression of the mutant heparanases in COS-7 cells demonstrated that the HS-degrading capacity of both was abolished. In contrast, the alanine substitution of two other glutamic acid residues (Glu(378) and Glu(396)), both predicted to be outside the active site, did not affect heparanase activity. These data suggest that heparanase is a member of the clan A glycosyl hydrolases and has a common catalytic mechanism that involves two conserved acidic residues, a putative proton donor at Glu(225) and a nucleophile at Glu(343) [80].

**First paper showing that heparanase is a member of the clan A glycosyl hydrolases and has a common catalytic mechanism that involves a putative proton donor at Glu(225) and a nucleophile at Glu(343).*

Biochemical Characterization of the Active Heterodimer Form of Human Heparanase (Hpa1) Protein Expressed in Insect Cells Hpa1 protein is initially synthesized as an inactive 65 kDa proenzyme that is then believed to be subsequently activated by proteolytic cleavage to generate an active heterodimer of 8 and 50 kDa polypeptides. By analysis of a series of Hpa1 deletion proteins we confirm that the 8 kDa subunit is essential for enzyme activity. We present here for the first time an insect cell expression system used for the generation of large amounts of recombinant protein of high specific activity. Individual subunits were cloned into baculoviral secretory vectors and co-expressed in insect cells. Active secreted heterodimer protein was recovered from the medium and isolated by a one-step heparin-Sepharose chromatography procedure to give protein of >90% purity. The recombinant enzyme behaved similarly to the native protein with respect to the size of HS fragments liberated on digestion, substrate cleavage specificity and its preference for acidic pH. A significant amount of activity, however, was also detectable at physiological pH values, as measured both by an in vitro assay and by in vivo degradation of cell-bound HS [81].

Similar observations were reported at the same time by Levy-Adam et al. [82] *in a paper entitled 'Heterodimer formation is essential for heparanase enzymatic activity'. Few months afterward, Nardella et al* [83] *published a paper entitled: 'Mechanism of activation of human heparanase investigated by protein engineering' and concluded that (i) the heparanase heterodimer (alpha/beta)* [8]*-TIM barrel fold is contributed by both the 8 and 50 kDa subunits with the 6 kDa connecting fragment leading to inhibition of heparanase by possibly obstructing access to the active site, (ii) proteolytic excision of the 6 kDa fragment is necessary and sufficient for heparanase activation, and (iii) Substituting the 6 kDa fragment with a spacer of three glycine-serine pairs resulted in constitutively active, single-chain heparanase which was comparable to the processed, heterodimeric enzyme*.

Involvement of Disulfide Bond Formation in the Activation of Heparanase The link between disulfide bond formation and the activation of heparanase in human tumor cells was investigated. Mass spectrometry analysis of heparanase purified from a conditioned medium of human fibrosarcoma cells revealed two disulfide bonds, Cys127-Cys179 and Cys437-Cys542, and one S-cysteinylation at the Cys211 residue. It was shown that although the formation of the Cys127-Cys179 bond and S-cysteinylation at Cys211 have little effect on heparanase function, the disulfide bond between Cys437 and Cys542 is necessary for the secretion and activation of heparanase. Thus, the present findings will provide a basis for further refinement of heparanase structural studies and for the development of novel heparanase inhibitors [84].

Processing and Activation of Latent Heparanase Occur in Lysosomes We generated an antibody (733) that preferentially recognizes the active 50 kDa heparanase form as compared to the non-active 65 kDa heparanase precursor. We have utilized this and other anti-heparanase antibodies to study the cellular localization of the

latent 65 kDa and active 50 kDa heparanase forms during uptake and processing of exogenously added heparanase. Interestingly, not only the processed 50 kDa, but also the 65 kDa heparanase precursor was localized to perinuclear vesicles, suggesting that heparanase processing occurs in lysosomes. Indeed, heparanase processing was completely inhibited by chloroquine and bafilomycin A1, inhibitors of lysosome proteases. Similarly, processing of membrane-targeted heparanase was also chloroquine-sensitive, further ruling out the plasma membrane as the heparanase processing site. Finally, we provide evidence that antibody 733 partially neutralizes the enzymatic activity of heparanase, suggesting that the N-terminal region of the molecule is involved in assuming an active conformation [85].

Early paper on the localization of heparanase in late endosomes and lysosomes.

Cathepsin L Is Responsible for Processing and Activation of Proheparanase Through Multiple Cleavages of a Linker Segment Applying cathepsin L knock-out tissues and cultured fibroblasts, as well as cathepsin L gene silencing and overexpression strategies, we have demonstrated that removal of the linker peptide and conversion of pro-heparanase into its active 8 + 50-kDa form is brought about predominantly by cathepsin L. Excision of a 10-amino acid peptide located at the C terminus of the linker segment between two functional cathepsin L cleavage sites (Y156Q and Y146Q) was critical for activation of proheparanase. Mass spectrometry revealed that the entire linker segment is susceptible to multiple endocleavages by cathepsin L and that an active 8-kDa subunit can be generated by several alternative adjacent endocleavages, yielding the precise 8-kDa subunit and/or slightly elongated forms. Altogether, the mode of action presented here demonstrates that processing and activation of proheparanase can be brought about solely by cathepsin L [73].

The above results corroborated an earlier publication by the same group entitled 'Site-directed mutagenesis, proteolytic cleavage, and activation of human proheparanase' presenting, among other aspects, a predicted structural model of the heparanase protein including a 1 kDa peptide of the linker segment that hinder accessibility of the HS substrate to the active site of the enzyme and hence inhibits heparanase enzymatic activity [86].

Structural Characterization of Human Heparanase Reveals Insights into Substrate Recognition Wu and al presented crystal structures of human HPSE at 1.6-Å to 1.9-Å resolution that reveal how an endo-acting binding cleft is exposed by proteolytic activation of latent proHPSE. We used oligosaccharide complexes to map the substrate-binding and sulfate-recognition motifs. These data shed light on the structure and interactions of a key enzyme involved in ECM maintenance and provide a starting point for the design of HPSE inhibitors for use as biochemical tools and anticancer therapeutics [87].

Resolution of the heparanase crystal structure. In a related study entitled 'Activity-based probes for functional interrogation of retaining β-glucuronidases' the same group reported that both the active and supposedly inactive heparanase

proenzyme can be labeled by the same activity-based (ABP) probes, leading to surprising insights regarding structural relationships between pro-heparanase, mature heparanase, and their bacterial homologs [88].

See Wu & Davies, Chap. 5 in this volume for more information on heparanase structural properties.

Gene Regulation

(See Gaskin et al., Chap. 7 in this volume, for more information on heparanase gene regulation)

Cloning and Characterization of the Human Heparanase-1 (HPR1) Gene Promoter: Role of GA-Binding Protein and Sp1 in Regulating HPR1 Basal Promoter Activity To understand the mechanisms of heparanase-1 (HPR1) gene expression and regulation, we first mapped the transcription start site of the heparanase (HPR1) gene and found that HPR1 mRNA was transcribed from the nucleotide position 101 bp upstream of the ATG codon. A 3.5-kb promoter region of the HPR1 gene was cloned. Sequence analysis revealed that the TATA-less, GC-rich promoter of the HPR1 gene belongs to the family of housekeeping genes. This 3.5-kb promoter region exhibited strong promoter activity in two thyroid tumor cell lines. Truncation analysis of the HPR1 promoter identified a minimal 0.3-kb region that had strong basal promoter activity. Truncation and mutational analysis of the HPR1 promoter revealed three Sp1 sites and four Ets-relevant elements (ERE) significantly contributing to basal HPR1 promoter activity. Binding to the Sp1 sites by Sp1 and to the ERE sites by GA-binding protein (GABP) was confirmed by electrophoretic mobility shift assay and competition and supershift electrophoretic mobility shift assays. Co-transfection of Sp- and GABP-deficient Drosophila SL-2 cells with the HPR1 promoter-driven luciferase construct plus the expression vector encoding the Sp1, Sp3, or GABP gene induced luciferase gene expression. Mutation or truncation of the Sp1 or ERE sites reduced luciferase expression in both SL-2 cells and thyroid tumor cell lines. Co-expression of GABPalpha/beta and Sp1 or Sp3 further increased luciferase reporter gene expression. Our results collectively suggest that Sp1 cooperates with GABP to regulate HPR1 promoter activity [89].

**Characterization of the human heparanase gene promoter and demonstration that Sp1 cooperates with GABP to regulate heparanase promoter activity.*

Regulation of Heparanase Gene Expression by Estrogen in Breast Cancer We identified four putative estrogen response elements in the heparanase promoter region and found that transcription of a luciferase reporter gene driven by the heparanase promoter was significantly increased in estrogen-receptor positive MCF-7 human breast carcinoma cells after estrogen treatment. Estrogen-induced heparanase mRNA transcription in estrogen receptor-positive, but not in estrogen receptor-negative, breast cancer cells, confirmed the promoter study data. The estrogen effects on heparanase mRNA expression levels were abolished in the presence of

the pure antiestrogen ICI 182,780, indicating that the classic estrogen receptor pathway is involved in transcriptional activation of heparanase. In vivo, exposure to estrogen augmented levels of heparanase protein in MCF-7 cells embedded in Matrigel plugs and correlated with increased plug vascularization. Collectively, our data suggest a new molecular pathway through which estrogen, independent of its proliferative effect, may induce heparanase overexpression and, thus, promote tumor-stromal interactions, critical for breast carcinoma development and progression [70].

Estrogen induces heparanase overexpression and, thus, promotes tumor-stromal interactions.

Role of Promoter Methylation in Regulation of the Mammalian Heparanase Gene To investigate the epigenetic regulation of the heparanase locus, methylation-specific and bisulfite PCR were performed on a panel of 22 human cancer cell lines. Cytosine methylation of the heparanase promoter was associated with inactivation of the affected allele. Despite lack of sequence homology, extensively methylated CpG islands were found both in human choriocarcinoma (JAR) and rat glioma (C-6) cells which lack heparanase activity. Treatment of these cells with demethylating agents (5-azacytidine, 5-aza-2′-deoxycytidine) resulted in stable dose- and time-dependant promoter hypomethylation accompanied by reappearance of heparanase mRNA, protein and enzymatic activity. An inhibitor of histone deacetylase, Trichostatin A, failed to induce either of these effects. Upregulation of heparanase expression and activity by demethylating drugs was associated with a marked increase in lung colonization by pretreated C-6 rat glioma cells. The increased metastatic potential *in vivo* was inhibited in mice treated with laminaran sulfate, a potent inhibitor of heparanase activity. We propose a model wherein expression of mammalian heparanase gene is modulated by the interplay between trans-activating genetic and cis-inhibitory epigenetic elements in its promoter [72].

Heparanase gene expression is modulated by the interplay between trans-activating genetic and cis-inhibitory epigenetic elements in its promoter.

Promoter CpG Hypomethylation and Transcription Factor EGR1 Hyperactivate Heparanase Expression in Bladder Cancer We hypothesized that promoter CpG hypomethylation with increased EGR1 expression could determine heparanase expression during the pathogenesis of bladder cancer. Bladder cancer cell lines significantly restored heparanase expression after 5-Aza-dC treatment. Transfection of EGR1 siRNA into T24 bladder cancer cell line significantly downregulated heparanase expression compared to the control siRNA transfection. In 54 bladder cancer and paired normal bladder samples, heparanase expression was significantly higher in bladder cancer than in normal bladder ($P < 0.01$). We performed methylation-specific PCR targeting the CpG sites within the core-binding consensus motifs of EGR1 (GGCG) and Sp1 (GGGCGG). Methylation prevalence was significantly higher in normal bladder than in bladder cancer ($P < 0.05$) and inversely correlated with heparanase expression ($P = 0.055$). In the total series of bladder cancer and normal bladder samples, the combination of promoter CpG

methylation and EGR1 expression regulated heparanase expression in a stepwise manner, where heparanase expression was the lowest in methylation-positive and EGR1-negative samples and the highest in methylation-negative and EGR1-positive samples. This is the first study demonstrating that increased heparanase expression during the pathogenesis of bladder cancer is due to promoter hypomethylation and transcription factor EGR1 [69].

Early Growth Response Gene 1 (EGR1) Regulates Heparanase Gene Transcription in Tumor Cells We identified the transcription factor early growth response gene 1, EGR1, as a key regulator of inducible heparanase transcription in T cells. Using chromatin immunoprecipitation, we demonstrate for the first time that EGR1 binds to the heparanase gene promoter in vivo. The important question of the role of EGR1 in regulating heparanase transcription in tumor cells was then assessed. Studies were carried out in four epithelial tumor lines of different tissue origin. Functional dissection of the heparanase promoter identified a 280-bp region that was critical for transcription of the heparanase gene. Transactivation studies using an EGR1 expression vector co-transfected with a reporter construct containing the 280-bp region showed EGR1-activated heparanase promoter activity in a dose-dependent manner in prostate or breast adenocarcinoma and colon carcinoma cell lines. In contrast, overexpression of EGR1 resulted in a dose-dependent repression of promoter activity in melanoma cells. Using site-directed mutagenesis the 280-bp region was found to contain two functional EGR1 sites and electrophoretic mobility shift assays showed binding of EGR1 to both of these sites upon activation of tumor cells. Furthermore, the heparanase promoter region containing the EGR1 sites was also inducible in tumor cells and induction corresponded to HPSE expression levels. These studies show that EGR1 regulates heparanase transcription in tumor cells and importantly, can have a repressive or activating role depending on the tumor type [65].

**The above two studies indicate that heparanase gene expression is due to promoter hypomethylation and interaction with transcription factor EGR1.*

Tumor Suppressor p53 Regulates Heparanase Gene Expression We demonstrate that wild-type (wt) p53 binds to heparanase promoter and inhibits its activity, whereas mutant p53 variants failed to exert an inhibitory effect. Moreover, p53-H175R mutant even activated heparanase promoter activity. Elimination or inhibition of p53 in several cell types resulted in a significant increase in heparanase gene expression and enzymatic activity. Trichostatin A abolished the inhibitory effect of wt p53, suggesting the involvement of histone deacetylation in negative regulation of the heparanase promoter. Altogether, our results indicate that the heparanase gene is regulated by p53 under normal conditions, while mutational inactivation of p53 during cancer development leads to induction of heparanase expression, providing a possible explanation for the frequent increase of heparanase levels observed in the course of tumorigenesis [71].

**Wild-type p53 binds to heparanase promoter and inhibits its activity.*

Post-Transcriptional Regulation of Heparanase Gene Expression by a 3' AU-Rich Element The purpose of the current study was to identify mechanisms responsible for heparanase induction. We provide evidence that heparanase expression is regulated at the post-transcriptional level by sequences at the 3′ untranslated region (3′ UTR) of the gene. Constructing the 3′ UTR immediately following the heparanase cDNA reduces heparanase enzymatic activity and protein levels, resulting in decreased cellular invasion capacity. We further identified a 185-bp sequence within the 3′ UTR that mediates heparanase down-regulation, and characterized an adenine (A)/uracil (U)-rich consensus element (ARE) within this region. Deletion of the entire 185-bp region or the ARE eliminated the inhibitory effect of the 3′ UTR, resulting in elevated heparanase levels and formation of larger tumor xenografts indistinguishable from those produced by heparanase-overexpressing cells in terms of size, vascularization, and Akt activation. These results suggest that loss of the ARE is an important regulatory mechanism contributing to heparanase induction in human cancer [78].

**Heparanase expression is regulated at the post-transcriptional level by sequences at the 3′ untranslated region (3′ UTR) of the gene.*

MicroRNA-1258 Suppresses Breast Cancer Brain Metastasis by Targeting Heparanase Heparanase (HPSE) is a potent protumorigenic, proangiogenic, and prometastatic enzyme that is overexpressed in brain metastatic breast cancer (BMBC). We hypothesized that HPSE gene expression might be regulated by micro RNA that might be exploited therapeutically. Using miR and a RNAhybrid, we identified miR-1258 as a candidate micro RNA that may directly target HPSE and suppress BMBC. We found that miR-1258 levels inversely correlated with heparanase expression, enzymatic activity, and cancer cell metastatic propensities, being lowest in highly aggressive BMBC cell variants compared with either nontumorigenic or nonmetastatic human mammary epithelial cells. These findings were validated by analyses of miR-1258 and heparanase content in paired clinical specimens of normal mammary gland versus invasive ductal carcinoma, and primary breast cancer versus BMBC. In regulatory experiments, miR-1258 inhibited the expression and activity of heparanase in BMBC cells, whereas modulating heparanase blocked the phenotypic effects of miR-1258. In functional experiments, stable expression of miR-1258 in BMBC cells inhibited heparanase in vitro cell invasion and experimental brain metastasis. Together, our findings illustrate how micro RNA mechanisms are linked to brain metastatic breast cancer through heparanase control, offering a strong rationale to develop heparanase-based therapeutics for treatment of cancer patients with brain metastases, BMBC in particular [90].

Genetic Variations in the Heparanase Gene (HPSE) Associate With Increased Risk of GVHD Following Allogeneic Stem Cell Transplantation: Effect of Discrepancy Between Recipients and Donors Graft-versus-host disease (GVHD) is the most common cause of nonrelapse mortality and morbidity after hematopoietic stem cell transplantation (HSCT). The well-documented involve-

ment of heparanase in the process of inflammation and autoimmunity led us to investigate an association between HPSE gene single-nucleotide polymorphisms (SNPs) and the risk of GVHD. The present study indicates a highly significant correlation of HPSE gene SNPs rs4693608 and rs4364254 and their combination with the risk of developing acute GVHD. Moreover, the study revealed that discrepancy between recipient and donor in these SNPs may elevate significantly the risk of acute GVHD. This association was statistically significant when the recipients possessed genotype combinations dictating higher levels of heparanase compared with their human leukocyte antigen (HLA)-matched donors. In addition, HPSE gene SNPs disclosed a correlation with extensive chronic GVHD, nonrelapse mortality, and overall survival. Our study indicates involvement of heparanase in the development of acute and extensive chronic GVHD. Moreover, it suggests a possible mechanism for the aggressive behavior of T lymphocytes leading to GVHD when the recipients possess genotype combinations that dictate high levels of heparanase mRNA compared with their HLA-matched donors expressing low levels of heparanase [91].

*See Ostrovsky et al., Chap. 8 in this volume for more information about heparanase gene SNPs.

Angiogenesis & Metastasis

Heparanase as Mediator of Angiogenesis: Mode of Action We demonstrate that heparanase is intimately involved in angiogenesis and elucidate its mode of action. Apart from its direct involvement in ECM degradation and EC migration, heparanase releases active bFGF from the subendothelial ECM, as well as bFGF-stimulating HS degradation fragments from the EC surface. Interestingly, ECM-derived HS fragments induced little or no potentiation of the growth-promoting activity of bFGF. The angiogenic effect of heparanase was demonstrated in vivo (Matrigel plug assay) by showing a three- to four fold increase in neovascularization induced by murine T-lymphoma cells after stable transfection with the heparanase gene. Increased tissue vascularity was also observed in a mouse wound-healing model in response to topical administration of recombinant heparanase. Immunohistochemical staining of human colon carcinoma tissue revealed a high expression of the heparanase protein in the endothelium of sprouting capillaries and small vessels, but not of mature quiescent blood vessels. The ability of heparanase to promote tumor angiogenesis and its involvement in tumor metastasis make it a promising target for cancer therapy [63].

**Early study demonstrating the ability of heparanase to promote tumor angiogenesis.*

Cell Surface Expression and Secretion of Heparanase Markedly Promote Tumor Angiogenesis and Metastasis The present study emphasizes the importance of cell surface expression and secretion of heparanase in tumor angiogenesis

and metastasis. For this purpose, nonmetastatic Eb mouse lymphoma cells were transfected with the predominantly intracellular human heparanase or with a readily secreted chimeric construct composed of the human enzyme and the chicken heparanase signal peptide. Eb cells overexpressing the secreted heparanase invaded a reconstituted basement membrane to a much higher extent than cells overexpressing the intracellular enzyme. Cell invasion was inhibited in the presence of laminaran sulfate, a potent inhibitor of heparanase activity and experimental metastasis. The increased invasiveness *in vitro* was reflected in vivo by rapid and massive liver colonization and accelerated mortality. In fact, mice inoculated with cells expressing the secreted enzyme succumb because of liver metastasis and dysfunction, as early as 10 days after s.c. inoculation of the cells, when their tumor burden did not exceed 1% of body weight. Cell surface localization and secretion of heparanase markedly stimulated tumor angiogenesis, as demonstrated by a 4–six-fold increase in vessel density and functionality evaluated by MRI of tumors produced by cells expressing the secreted vs. the non-secreted heparanase, consistent with actual counting of blood vessels. Altogether, our results indicate that the potent proangiogenic and prometastatic properties of heparanase are tightly regulated by its cellular localization and secretion. The increased potency of the secreted enzyme makes it a promising target for anticancer drug development [58].

Heparanase Gene Silencing, Tumor Invasiveness, Angiogenesis, and Metastasis Studies performed prior to HPSE gene cloning, have sought to determine the role of heparanase in tumor progression. However, such investigations relied on the use of heparin-mimicking molecules to inhibit heparanase activity. Because the reagents used in those previous studies lacked specificity, the conclusions drawn from the studies are somewhat debatable. Edovitsky et al. [57] applied ribozyme and small interfering RNA (siRNA) technology to knock down the levels of endogenous heparanase. The authors convincingly show that, in models of experimental and spontaneous metastases, these strategies attenuate the ability of diverse tumor cells, including melanoma, mammary adenocarcinoma, lymphoma, and glioma cells, to invade *in vitro* and to colonize distant sites including the liver and lungs. These and other results [59] provide strong support for a role for heparanase in the metastatic process. Moreover, these studies can be used to rationalize the development of anti-heparanase strategies for cancer patients.

* The above description was taken from a commentary written by Boyd & Nakajima [56].

Heparanase Induces Tissue Factor Pathway Inhibitor Expression and Extracellular Accumulation in Endothelial and Tumor Cells We have reported that heparanase stimulates tissue factor (TF) expression in endothelial and cancer cells, resulting in elevation of coagulation activity. We hypothesized that heparanase regulates other coagulation modulators, and found that heparanase over-expression or exogenous addition stimulated tissue factor pathway inhibitor (TFPI) expression by 2–3 folds. TFPI accumulation in the cell culture medium exceeded in magnitude the observed induction of TFPI gene transcription reaching 5- to six-fold increase.

Extracellular accumulation of TFPI correlated with increased coagulation activity. This effect was found to be independent of heparanase enzymatic activity and interaction with HS, and correlated with reduced TFPI levels on the cell surface. Interaction between heparanase and TFPI was evident by co-immunoprecipitation and resulted in TFPI displacement from the surface of the vascular endothelium. Thus, heparanase facilitates blood coagulation on the cell surface by two independent mechanisms: dissociation of TFPI from the vascular surface shortly after local elevation of heparanase levels, and subsequent induction of TF expression [92].

*See Nadir, Chap. 33 in this volume for more information on heparanase and the coagulation system.

Animal Models

Transgenic Expression of Mammalian Heparanase Uncovers Physiological Functions of Heparan Sulfate in Tissue Morphogenesis, Vascularization, and Feeding Behavior We have generated homozygous transgenic mice (hpa-tg) overexpressing human heparanase in all tissues and characterized the involvement of the enzyme in tissue morphogenesis, vascularization, and energy metabolism. Biochemical analysis of HS isolated from newborn mice and adult tissues revealed a profound decrease in the size of HS chains derived from hpa-tg vs. control mice. Despite this, the mice appeared normal, were fertile, and exhibited a normal life span. A significant increase in the number of implanted embryos was noted in the hpa-tg vs. control mice. Overexpression of heparanase resulted in increased levels of urinary protein and creatinine, suggesting an effect on kidney function, reflected also by electron microscopy examination of the kidney tissue. The hpa-tg mice exhibited a reduced food consumption and body weight compared with control mice. The effect of heparanase on tissue remodeling and morphogenesis was best demonstrated by the phenotype of the hpa-tg mammary glands, showing excess branching and widening of ducts associated with enhanced neovascularization and disruption of the epithelial basement membrane. The hpa-tg mice exhibited an accelerated rate of hair growth, correlated with high expression of heparanase in hair follicle keratinocytes and increased vascularization [93].

Transgenic or Tumor-Induced Expression of Heparanase Upregulates Sulfation of Heparan Sulfate In *Hpa-tg* liver showing excessive heparanase overexpression, HSPG turnover is accelerated along with upregulation of HS N- and O-sulfation, thus yielding heparin-like chains without the domain structure typical of HS. Heparanase overexpression in other mouse organs and in human tumors correlated with increased 6-O-sulfation of HS, whereas the domain structure was conserved. The heavily sulfated HS fragments strongly promoted formation of ternary complexes with fibroblast growth factor 1 (FGF1) or FGF2 and FGF receptor 1. Heparanase thus contributes to regulation of HS biosynthesis in a way that may promote growth factor action in tumor angiogenesis and metastasis [94].

Newly Generated Heparanase Knock-out Mice Unravel Co-Regulation of Heparanase and Matrix Metalloproteinases We report that targeted disruption of the murine heparanase gene eliminated heparanase enzymatic activity, resulting in accumulation of long HS chains. Unexpectedly, the heparanase knockout (Hpse-KO) mice were fertile, exhibited a normal life span and did not show prominent pathological alterations. The lack of major abnormalities is attributed to a marked elevation in the expression of matrix metalloproteinases (primarily MMP2 and MMP14) compensating for the lack of heparanase. Co-regulation of heparanase and MMPs was also noted by a marked decrease in MMP (primarily MMP-2,-9 and 14) expression following transfection and over-expression of the heparanase gene in cultured human mammary carcinoma (MDA-MB-231) cells. Generation of viable Hpse-KO mice lacking significant abnormalities may provide a promising indication for the use of heparanase as a target for drug development [95].

Mice Deficient in Heparanase Exhibit Impaired Dendritic Cell Migration and Reduced Airway Inflammation In this study, constitutive heparanase-deficient (Hpse(−/−)) mice were generated on a C57BL/6 background using the Cre/loxP recombination system, with a complete lack of heparanase mRNA, protein and activity. Although heparanase has been implicated in embryogenesis and development, Hpse(−/−) mice are anatomically normal and fertile. Interestingly, the trafficking of dendritic cells from the skin to the draining lymph nodes was markedly reduced in Hpse(−/−) mice. Furthermore, the ability of Hpse(−/−) mice to generate an allergic inflammatory response in the airways, a process that requires dendritic cell migration, was also impaired. These findings establish an important role for heparanase in immunity and identify the enzyme as a potential target for regulation of an immune response [96].

**The above four studies describe the generation and properties of heparanase over-expressing and knockout mice.*

Heparanase Uptake and Cellular Traffic

Heparanase Uptake Is Mediated by Cell Membrane Heparan Sulfate Proteoglycans We provide evidence that HS is not only a substrate for, but also a regulator of, heparanase. Addition of heparin or xylosides to cell cultures resulted in a pronounced accumulation of heparanase in the culture medium, whereas sodium chlorate had no such effect. Moreover, cellular uptake of heparanase was markedly reduced in HS-deficient CHO-745 mutant cells, heparan sulfate proteoglycan-deficient HT-29 colon cancer cells, and heparinase-treated cells. Notably, heparanase resides in the endosomal/lysosomal compartment for a relatively long period of time and is likely to play a role in the normal turnover of HS. Co-localization studies and cell fractionation following heparanase addition have identified syndecan family members as candidate molecules responsible for heparanase uptake, providing an efficient mechanism that limits extracellular accumulation and function of heparanase [97].

First paper describing syndecan-mediated cellular uptake of heparanase.

Heparanase Enhances Tumor Growth and Chemoresistance by Promoting Autophagy The protumorigenic properties of heparanase were found to be mediated, in part, by its proautophagic function, as demonstrated in tumor xenograft models of human cancer and through use of inhibitors of the lysosome (chloroquine) and heparanase (PG545), both alone and in combination. Notably, heparanase-overexpressing cells were more resistant to stress and chemotherapy in a manner associated with increased autophagy, effects that were reversed by chloroquine treatment. Collectively, our results establish a role for heparanase in modulating autophagy in normal and malignant cells, thereby conferring growth advantages under stress as well as resistance to chemotherapy [98].

First paper describing a role for heparanase in autophagy.

Heparanase Regulates Secretion, Composition, and Function of Tumor Cell-Derived Exosomes Emerging evidence indicates that exosomes play a key role in tumor-host cross-talk and that exosome secretion, composition, and functional capacity are altered as tumors progress to an aggressive phenotype. We have discovered that in human cancer cells (myeloma, lymphoblastoid, and breast cancer), when expression of heparanase is enhanced or when tumor cells are exposed to exogenous heparanase, exosome secretion is dramatically increased. Heparanase enzyme activity is required for robust enhancement of exosome secretion because enzymatically inactive forms of heparanase do not dramatically increase exosome secretion. Heparanase also impacts exosome protein cargo as reflected by higher levels of syndecan-1, VEGF, and hepatocyte growth factor in exosomes secreted by heparanase-high expressing cells as compared with heparanase-low expressing cells. In functional assays, exosomes from heparanase-high cells stimulated spreading of tumor cells on fibronectin and invasion of endothelial cells through ECM better than did exosomes secreted by heparanase-low cells. These studies reveal that heparanase helps drive exosome secretion, alters exosome composition, and facilitates production of exosomes that impact both tumor and host cell behavior, thereby promoting tumor progression [99].

First paper describing a role for heparanase in exosome formation and secretion.

Heparanase Activates the Syndecan-Syntenin-ALIX Exosome Pathway We showed that syndecans control the biogenesis of exosomes through their interaction with syntenin-1 and the endosomal-sorting complex required for transport accessory component ALIX. We investigated the role of heparanase in the syndecan-syntenin-ALIX exosome biogenesis pathway. Our findings identify heparanase as a modulator of the syndecan-syntenin-ALIX pathway, fostering endosomal membrane budding and the biogenesis of exosomes by trimming the heparan sulfate chains on syndecans. In addition, our data suggest that this mechanism controls the selection of specific cargo to exosomes [100]. It was further reported that heparanase tailors syndecan for exosome production and suggested that upregulation of syntenin and heparanase in cancers may support the suspected roles of exosomes in tumor biology [101].

*See: David & Zimmermann and Purushothaman and Sanderson Chap. 10 and 12 in this volume for more information on heparanase and exosomes.

**A recent review written by Sanderson* et al. [102] *focuses on the emerging role of exosomal surface enzymes in disease progression. Briefly, the review demonstrates that enzymatically active proteases and glycosidases are present on the surface of some exosomes. These enzymes can degrade the ECM, liberate growth factors and alter cell invasion, resulting in remodeling the extracellular space and regulating cancer progression, inflammation, and Alzheimer's disease*.

Chemotherapy Induces Secretion of Exosomes Loaded with Heparanase that Degrades Extracellular Matrix and Impacts Tumor and Host Cell Behavior We investigated the impact of anti-myeloma drugs on exosome biogenesis. When myeloma cells were exposed to the commonly utilized anti-myeloma drugs bortezomib, carfilzomib or melphalan, exosome secretion by the cells was dramatically enhanced. These chemotherapy-induced exosomes (chemoexosomes) have a proteome profile distinct from cells not exposed to drug including a dramatic elevation in the level of heparanase present as exosome cargo. Heparanase was present on the exosome surface where it was capable of degrading heparan sulfate embedded within an ECM. When exposed to myeloma cells, chemoexosomes transferred their heparanase cargo to those cells, enhancing their HS degrading activity and leading to activation of ERK signaling and an increase in shedding of the syndecan-1 proteoglycan. Exposure of chemoexosomes to macrophages enhanced their secretion of TNF-α, an important myeloma growth factor. Moreover, chemoexosomes stimulated macrophage migration and this effect was blocked by H1023, a monoclonal antibody that inhibits heparanase enzymatic activity. These data suggest that anti-myeloma therapy ignites a burst of exosomes having a high level of heparanase that remodels ECM and alters tumor and host cell behaviors that likely contribute to chemoresistance and eventual patient relapse [103].

**Anti-myeloma chemotherapy dramatically stimulates secretion of exosomes and alters exosome composition. Exosomes secreted during therapy (chemoexosomes) contain high levels of heparanase on their surface that can degrade ECM and also can be transferred to both tumor and host cells, altering their behavior in ways that may enhance tumor survival and progression* [103].

Nuclear Heparanase and Its Transcriptional Activity

Heparanase-Mediated Loss of Nuclear Syndecan-1 Enhances Histone Acetyltransferase (HAT) activity to Promote Expression of Genes that Drive an Aggressive Tumor Phenotype Heparanase acts as a master regulator of the aggressive tumor phenotype in part by enhancing expression of proteins known to drive tumor progression (e.g. VEGF, MMP-9, hepatocyte growth factor (HGF), and RANKL). However, the mechanism whereby this enzyme regulates gene expression remains unknown. We previously reported that elevation of heparanase levels in

myeloma cells causes a dramatic reduction in the amount of syndecan-1 in the nucleus. Because HS has been shown to inhibit the activity of histone acetyltransferase (HAT) enzymes in vitro, we hypothesized that the reduction in nuclear syndecan-1 in cells expressing high levels of heparanase would result in increased HAT activity leading to stimulation of protein transcription. We found that myeloma cells or tumors expressing high levels of heparanase and low levels of nuclear syndecan-1 had significantly higher levels of HAT activity when compared with cells or tumors expressing low levels of heparanase. High levels of HAT activity in heparanase-high cells were blocked by SST0001, an inhibitor of heparanase. Restoration of high syndecan-1 levels in heparanase-high cells diminished nuclear HAT activity, establishing syndecan-1 as a potent inhibitor of HAT. Exposure of heparanase-high cells to anacardic acid, an inhibitor of HAT activity, significantly suppressed their expression of VEGF and MMP-9, two genes known to be up-regulated following elevation of heparanase. These results reveal a novel mechanistic pathway driven by heparanase expression, which leads to decreased nuclear syndecan-1, increased HAT activity, and up-regulation of transcription of multiple genes that drive an aggressive tumor phenotype [104].

The Endoglycosidase Heparanase Enters the Nucleus of T Lymphocytes and Modulates H3 Methylation at Actively Transcribed Genes Via the Interplay with Key Chromatin Modifying Enzymes The methylation of histones is a fundamental epigenetic process regulating gene expression programs in mammalian cells. Here, we report the unexpected finding that heparanase enters the nucleus of activated human T lymphocytes and regulates the transcription of a cohort of inducible immune response genes by controlling histone H^3 methylation patterns. It was found that nuclear heparanase preferentially associates with euchromatin. Genome-wide ChIP-on-chip analyses showed that heparanase is recruited to both the promoter and transcribed regions of a distinct cohort of transcriptionally active genes. Knockdown and overexpression of the heparanase gene also showed that chromatin-bound heparanase is a prerequisite for the transcription of a subset of inducible immune response genes in activated T cells. Furthermore, the actions of heparanase seem to influence gene transcription by associating with the demethylase LSD1, preventing recruitment of the methylase MLL and thereby modifying histone H^3 methylation patterns. These data indicate that heparanase belongs to an emerging class of proteins that play an important role in regulating transcription in addition to their well-recognized extra-nuclear functions [105].

Two papers describing the involvement of nuclear heparanase in gene transcription.

Heparanase Non-Enzymatic and Signaling Function

Heparanase Induces Vascular Endothelial Growth Factor Expression: Correlation With p38 Phosphorylation Levels and Src Activation We examined the possibility that heparanase directly participates in VEGF gene regulation. We provide evidence that heparanase overexpression in human embryonic kidney 293, MDA-MB-435 human breast carcinoma, and rat C6 glioma cells resulted in a 3- to

six-fold increase in VEGF protein and mRNA levels, which correlated with elevation of p38 phosphorylation. Moreover, heparanase down-regulation in B16 mouse melanoma cells by a specific siRNA vector was accompanied by a decrease in VEGF and p38 phosphorylation levels, suggesting that VEGF gene expression is regulated by endogenous heparanase. Interestingly, a specific p38 inhibitor did not attenuate VEGF up-regulation by heparanase whereas Src inhibitors completely abrogated this effect. These results indicate that heparanase is actively involved in the regulation of VEGF gene expression, mediated by activation of Src family members [106].

**First paper on heparanase and VEGF gene expression.*

Structure-Function Approach Identifies a COOH-Terminal domain that Mediates Heparanase Signaling Heparanase exerts biological functions apparently independent of its enzymatic activity, enhancing the phosphorylation of selected protein kinases and inducing gene transcription. A predicted three-dimensional structure of constitutively active heparanase clearly delineates a TIM-barrel fold previously anticipated for the enzyme. Interestingly, the model also revealed the existence of a COOH-terminal domain (C-domain) that apparently is not an integral part of the TIM-barrel fold. We provide evidence that the C-domain is critical for heparanase enzymatic activity and secretion. Moreover, the C-domain was found to mediate nonenzymatic functions of heparanase, facilitating Akt phosphorylation, cell proliferation, and tumor xenograft progression. These findings support the notion that heparanase exerts enzymatic activity-independent functions, and identify, for the first time, a protein domain responsible for heparanase-mediated signaling. Inhibitors directed against the C-domain, combined with inhibitors of heparanase enzymatic activity, are expected to neutralize heparanase functions and to profoundly affect tumor growth, angiogenesis, and metastasis [107].

**First paper on the heparanase C-terminal domain and signal transduction.*

Heparanase Augments Epidermal Growth Factor Receptor Phosphorylation: Correlation With Head and Neck Tumor Progression We provide evidence that enzymatically active and inactive heparanase enhance epidermal growth factor receptor (EGFR) phosphorylation. Enhanced EGFR phosphorylation was associated with increased cell migration, cell proliferation, and colony formation, which were attenuated by Src inhibitors. Similarly, heparanase gene silencing by means of siRNA was associated with reduced Src and EGFR phosphorylation levels and decreased cell proliferation. Moreover, heparanase expression correlated with increased phospho-EGFR levels and progression of head and neck carcinoma, providing a strong clinical support for EGFR modulation by heparanase. Thus, heparanase seems to modulate two critical systems involved in tumor progression, namely VEGF expression and EGFR activation. Neutralizing heparanase enzymatic and nonenzymatic functions is therefore expected to profoundly affect tumor growth, angiogenesis, and metastasis [108].

**First paper describing the involvement of heparanase in epidermal growth factor receptor (EGFR) phosphorylation. The enzyme was also shown to induce Stat phosphorylation* [109].

Heparanase Inhibitors

Development of a Colorimetric Assay for Heparanase Activity Suitable for Kinetic Analysis and Inhibitor Screening We have developed a convenient assay based on the cleavage of the synthetic heparin oligosaccharide fondaparinux. The assay measures the appearance of the disaccharide product of heparanase-catalyzed fondaparinux cleavage colorimetrically using the tetrazolium salt WST-1. Because this assay has a homogeneous substrate with a single point of cleavage, the kinetics of the enzyme can be reliably characterized, giving a K(m) of 46 microM and a k(cat) of 3.5 s(−1) with fondaparinux as substrate. The inhibition of heparanase by the published inhibitor, PI-88, was also studied, and a K(i) of 7.9 nM was determined. The simplicity and robustness of this method, should, not only greatly assist routine assay of heparanase activity but also could be adapted for high-throughput screening of compound libraries, with the data generated being directly comparable across studies [110].

**The colorimetric assay is being used for screening of heparanase-inhibiting molecules.*

A Functional Heparan Sulfate mimetic Implicates both Heparanase and Heparan Sulfate in Tumor Angiogenesis and Invasion in a Mouse Model of Multistage Cancer Heparanase mRNA and protein expression are increased in the neoplastic stages progressively unfolding in a mouse model of multistage pancreatic islet carcinogenesis. Notably, heparanase is delivered to the neoplastic lesions in large part by infiltrating Gr1+/Mac1+ innate immune cells. A sulfated oligosaccharide mimetic of HS, PI-88 (= Mupafostat), was used to inhibit simultaneously both heparanase activity and HS effector functions. PI-88 had significant effects at distinct stages of tumorigenesis, producing a reduction in the number of early progenitor lesions and an impairment of tumor growth at later stages. These responses were associated with decreased cell proliferation, increased apoptosis, impaired angiogenesis, and a substantive reduction in the number of invasive carcinomas. In addition, we show that the reduction in tumor angiogenesis is correlated with a reduced association of VEGF-A with its receptor VEGF-R2 on the tumor endothelium, implicating heparanase in the mobilization of matrix-associated VEGF. These data encourage clinical applications of inhibitors such as PI-88 for the many human cancers where heparanase expression is elevated or mobilization of HS-binding regulatory factors is implicated [76].

SST0001, a Chemically Modified Heparin, Inhibits Myeloma Growth and Angiogenesis Via Disruption of the Heparanase/Syndecan-1 Axis The ability of SST0001 (glycol-split heparin = Roneparstat) to inhibit growth of myeloma tumors was assessed using multiple animal models and a diverse panel of human and murine myeloma cell lines. SST0001 effectively inhibited myeloma growth in vivo, even when confronted with an aggressively growing tumor within human bone. In addition, SST0001 treatment causes changes within tumors consistent with the compound's ability to inhibit heparanase, including downregulation of HGF, VEGF, and MMP-9 expression and suppressed angiogenesis. SST0001 also diminishes

heparanase-induced shedding of syndecan-1, and inhibited the heparanase-mediated degradation of syndecan-1 HS side chains, thus confirming the anti-heparanase activity of this compound. In combination with dexamethasone, SST0001 blocked tumor growth in vivo presumably through dual targeting of the tumor and its microenvironment [30].

*See Noseda & Barberi Chap. 21 in this volume for more information on Roneparstat.

PG545, a Dual Heparanase and Angiogenesis Inhibitor, Induces Potent Anti-Tumour and Anti-Metastatic efficacy in Preclinical Models PG545 (HS mimetic = Pixatimod) was shown to inhibit angiogenesis in vivo and induce anti-tumour or anti-metastatic effects in murine models of breast, prostate, liver, lung, colon, head and neck cancers and melanoma. Enhanced anti-tumour activity was also noted when used in combination with sorafenib in a liver cancer model. PK data revealed that the half-life of PG545 was relatively long, with pharmacologically relevant concentrations of radiolabeled PG545 observed in liver tumours. The anti-metastatic property of PG545, likely due to the inhibition of heparanase, may prove to be a critical attribute as the compound enters phase I clinical trials [111].

*See Hammond & Dredge, Chap. 22 in this volume for more information on Pixatimod.

M402, a Novel Heparan Sulfate Mimetic, Targets Multiple Pathways Implicated in Tumor Progression and Metastasis M402 (= Necuparanib) is a rationally engineered, non-cytotoxic HS mimetic, designed to inhibit multiple factors implicated in tumor-host cell interactions, including VEGF, FGF2, SDF-1α, P-selectin, and heparanase. A single s.c. dose of M402 effectively inhibited seeding of B16F10 murine melanoma cells to the lung in an experimental metastasis model. Fluorescent-labeled M402 demonstrated selective accumulation in the primary tumor. Immunohistological analyses of the primary tumor revealed a decrease in microvessel density in M402 treated animals. M402 treatment also normalized circulating levels of myeloid derived suppressor cells in tumor bearing mice. Chronic administration of M402, alone or in combination with cisplatin or docetaxel, inhibited spontaneous metastasis and prolonged survival in an orthotopic 4 T1 murine mammary carcinoma model [112].

**The above four papers represent heparin/HS mimetics that were or are being examined in clinical trials.* (See Chapters 19, 23, 22, 21 by Chhabra & Ferro; Hammond & Dredge; Gianini et al., and Noseda & Barbieri, for more information on heparanase-inhibiting compounds).

Various Tumors

Inhibition of Heparanase in Pediatric Brain Tumor Cells Attenuates their Proliferation, Invasive Capacity, and in Vivo Tumor Growth Levels of heparanase (HPSE) in pediatric brain tumors are higher than in healthy brain tissue and

treatment of pediatric brain tumor cells with HPSE stimulated their growth. Notably, the latent form of HPSE enhanced cell viability and rapidly activated the ERK and AKT signaling pathways, before enzymatically active HPSE was detected. The HPSE inhibitor PG545 efficiently killed pediatric brain tumor cells, but not normal human astrocytes, and this compound also reduced tumor cell invasion *in vitro* and potently reduced the size of flank tumors *in vivo*. These results indicate that HPSE in malignant brain tumors affects both the tumor cells themselves and their ECM [113].

*See Chapter 14 by Karin-Forsberg-Nillson for more information on heparanase and gliomas.

Involvement of Heparanase in the Pathogenesis of Mesothelioma: Basic Aspects and Clinical Applications Mesothelioma tumor growth was markedly attenuated by heparanase gene silencing and by heparanase inhibitors (PG545 and defibrotide). A marked increase in survival of the mesothelioma-bearing mice was recorded. Heparanase inhibitors were more potent *in vivo* than conventional chemotherapy. Clinically, heparanase levels in patients' pleural effusions could distinguish between malignant and benign effusions, and heparanase H-score above 90 was associated with reduced patient survival. Given these preclinical and clinical data, heparanase appears to be an important mediator of mesothelioma, and heparanase inhibitors are worthy of investigation as a new therapeutic modality in mesothelioma clinical trials [114].

Multiple Myeloma

Heparanase Promotes the Spontaneous Metastasis of Myeloma Cells to Bone Using a SCID mouse model, we demonstrate that enhanced expression of heparanase by myeloma cells dramatically up-regulates their spontaneous metastasis to bone. This occurs from primary tumors growing subcutaneously and also from primary tumors established in bone. Interestingly, tumors formed by subcutaneous injection of cells metastasize not only to bone, but also to other sites including spleen, liver, and lung. In contrast, tumors formed by injection of cells directly into bone exhibit a restricted pattern of metastasis that includes dissemination of tumor to other bones but not to extramedullary sites. In addition, expression of heparanase by myeloma cells (1) accelerates the initial growth of the primary tumor, (2) increases whole-body tumor burden as compared with controls, and (3) enhances both the number and size of microvessels within the primary tumor. These studies indicate that heparanase is a critical determinant of myeloma dissemination and growth in vivo [115].

Heparanase Influences Expression and Shedding of Syndecan-1, and its Expression by the Bone Marrow Environment Is a Bad Prognostic Factor in Multiple Myeloma Using Affymetrix microarrays we show that the gene encoding heparanase (HPSE) is expressed by 11 of 19 myeloma cell lines (HMCLs). In HSPE positive HMCLs, syndecan-1 gene expression and production of soluble

syndecan-1 were significantly increased. Knockdown of HPSE by siRNA resulted in a decrease of syndecan-1 gene expression and soluble syndecan-1 production without affecting membrane syndecan-1 expression. Thus, HPSE influences expression and shedding of syndecan-1. Contrary to HMCLs, HPSE is expressed in only 4 of 39 primary MMC samples, whereas it is expressed in 36 of 39 bone marrow (BM) microenvironment samples. In the latter, HPSE is expressed at a median level in polymorphonuclear cells and T cells; it is highly expressed in monocytes and osteoclasts. Affymetrix data were validated at the protein level, both on HMCLs and patient samples. We report that a gene's expression mainly in the BM environment (i.e, HSPE) is associated with a shorter event-free survival of patients with newly diagnosed myeloma treated with high-dose chemotherapy and stem cell transplantation. Our study suggests that clinical inhibitors of HPSE could be beneficial for patients with MM [116].

Heparanase Enhances Myeloma Progression Via CXCL10 Downregulation In order to explore the mechanism(s) underlying the pro-tumorigenic capacity of heparanase, we established an inducible Tet-on system. Heparanase expression was markedly increased following addition of doxycycline (Dox) to the culture medium of CAG human myeloma cells infected with the inducible heparanase gene construct, resulting in increased colony number and size in soft agar. Moreover, tumor xenografts produced by CAG-heparanase cells were markedly increased in mice supplemented with Dox in their drinking water compared with control mice maintained without Dox. Consistently, we found that heparanase induction is associated with decreased levels of CXCL10, suggesting that this chemokine exerts tumor-suppressor properties in myeloma. Indeed, recombinant CXCL10 attenuated the proliferation of CAG, U266 and RPMI-8266 myeloma cells. Similarly, CXCL10 attenuated the proliferation of human umbilical vein endothelial cells, implying that CXCL10 exhibits anti-angiogenic capacity. Strikingly, development of tumor xenografts produced by CAG-heparanase cells overexpressing CXCL10 was markedly reduced compared with control cells. Moreover, tumor growth was significantly attenuated in mice inoculated with human or mouse myeloma cells and treated with CXCL10-Ig fusion protein, indicating that CXCL10 functions as a potent anti-myeloma cytokine [117].

Chemotherapy Induces Expression and Release of Heparanase Leading to Changes Associated with an Aggressive Tumor Phenotype We discovered that drugs used in the treatment of myeloma upregulate heparanase expression. Frontline anti-myeloma drugs, bortezomib and carfilzomib activate the NF-κB pathway to trigger heparanase expression in tumor cells. Blocking the NF-κB pathway diminished this chemotherapy-induced upregulation of heparanase expression. Activated NF-κB signaling was also found to drive high heparanase expression in drug resistant myeloma cell lines. In addition to enhancing heparanase expression, chemotherapy also caused release of heparanase by tumor cells into the conditioned medium. This soluble heparanase was taken up by macrophages and triggered an increase in TNF-α production. Heparanase is also taken up by tumor cells where it induced expression of HGF, VEGF and MMP-9 and activated ERK and Akt signal-

ing pathways. These changes induced by heparanase are known to be associated with the promotion of an aggressive tumor phenotype. Importantly, the heparanase inhibitor Roneparstat diminished the uptake and the downstream effects of soluble heparanase. Together, these discoveries reveal a novel mechanism whereby chemotherapy upregulates heparanase, a known promoter of myeloma growth, and suggest that therapeutic targeting of heparanase during anti-cancer therapy may improve patient outcome [118].

*See Chapter 12 by Sanderson et al., for more information on heparanase and myeloma.

Antitumor Efficacy of the Heparanase Inhibitor SST0001 Alone and in Combination with Antiangiogenic Agents in the Treatment of Human Pediatric Sarcoma Models The present study focuses on the effect of SST0001 in a panel of pediatric sarcoma models, representative of various tumor histotypes (soft tissue and bone sarcomas). SST0001 treatment downregulated several angiogenic factors in the conditioned media of sarcoma cells, inhibited the pro-invasive effect of heparin-binding factors (VEGF, bFGF, HGF, PDGF), and abrogated PDGF receptor tyrosine phosphorylation. Subcutaneous administration of SST0001 was very effective, resulting in a significant growth inhibition (range, 64–95%) of all tested tumor xenografts. The efficacy of SST0001 was enhanced in combination with antiangiogenic agents (bevacizumab, sunitinib) as documented by the high rate of complete response. The synergistic effect of SST0001 in combination with antiangiogenic agents is consistent with the heparanase mode of action and with the relevant role of heparin-binding proangiogenic/growth factors in the malignant behavior of sarcoma cells [119].

*See Chapter 15 by Cassinelly & Lanzi for more information on heparanase in sarcomas.

Tumor Microenvironment

Heparanase Cooperates with Ras to Drive Breast and Skin Tumorigenesis Hpa-Tg mice overexpressing heparanase were far more sensitive than control mice to DMBA/TPA skin carcinogenesis, exhibiting a ten-fold increase in the number and size of tumor lesions. Conversely, DMBA/TPA-induced tumor formation was greatly attenuated in Hpa-KO mice lacking heparanase, pointing to a critical role of heparanase in skin tumorigenesis. In support of these observations, the heparanase inhibitor PG545 potently suppressed tumor progression in this model system. Our findings establish that heparanase exerts protumorigenic properties at early stages of tumor initiation, cooperating with Ras to dramatically promote malignant development [120].

**This study emphasizes the co-operation of heparanase with master oncogenes such as Ras.*

Heparanase-Neutralizing Antibodies Attenuate Lymphoma Tumor Growth and Metastasis We provide evidence that heparanase is expressed by human follicular and diffused non-Hodgkin's B-lymphomas, and that heparanase inhibitors restrain the growth of tumor xenografts produced by lymphoma cell lines. Furthermore, we describe the development and characterization of heparanase-neutralizing monoclonal antibodies that inhibit cell invasion and tumor metastasis, the hallmark of heparanase activity. Using luciferase-labeled Raji lymphoma cells, we show that the heparanase-neutralizing monoclonal antibodies profoundly inhibit tumor load in the mouse bones, associating with reduced cell proliferation and angiogenesis. Notably, we found that Raji cells lack intrinsic heparanase activity, but tumor xenografts produced by this cell line exhibit typical heparanase activity, likely contributed by host cells composing the tumor microenvironment [33].

**Anti-heparanase neutralizing monoclonal antibodies attenuate lymphoma growth by targeting heparanase in the tumor microenvironment.*

Inflammation and Cells of the Immune System

Heparanase Powers a Chronic Inflammatory Circuit that Promotes Colitis-Associated Tumorigenesis in Mice The research focuses on the importance of heparanase in sustaining the immune-epithelial crosstalk underlying colitis-associated tumorigenesis. Using histological specimens from ulcerative colitis (UC) patients and a mouse model of dextran sodium sulfate-induced colitis, we found that heparanase was constantly overexpressed and activated throughout the disease. We demonstrate, using heparanase-overexpressing transgenic mice, that heparanase overexpression markedly increased the incidence and severity of colitis-associated colonic tumors. We found that highly coordinated interactions between the epithelial compartment (contributing heparanase) and mucosal macrophages preserved chronic inflammatory conditions and created a tumor-promoting microenvironment characterized by enhanced NF-κB signaling and induction of STAT3. Our results indicate that heparanase generates a vicious cycle that powers colitis and the associated tumorigenesis: heparanase, acting synergistically with the intestinal flora, stimulates macrophage activation, while macrophages induce production (via TNF-α-dependent mechanisms) and activation (via secretion of cathepsin L) of heparanase contributed by the colon epithelium. Thus, disruption of the heparanase-driven chronic inflammatory circuit is highly relevant to the design of therapeutic interventions in colitis and the associated cancer [121].

**Highly coordinated interactions between the epithelial compartment and mucosal macrophages generate a vicious cycle that powers colitis and the associated tumorigenesis.*

Soluble Heparan Sulfate Fragments Generated by Heparanase Trigger the Release of pro-Inflammatory Cytokines through TLR-4 The study focuses on the role of heparanase in regulating the expression and release of cytokines from

human and murine immune cells. Ex vivo treatment of human peripheral blood mononuclear cells with heparanase resulted in the release of a range of pro-inflammatory cytokines including IL-1β, IL-6, IL-8, IL-10 and TNF. A similar pattern of cytokine release was also observed when cells were treated with soluble HS. Furthermore, heparanase-induced cytokine release was abolished by enzymatic-inhibitors of heparanase, suggesting that this process is mediated via the enzymatic release of cell surface HS fragments. As soluble HS can signal through the Toll-like receptor (TLR) pathway, heparanase may promote the upregulation of cytokines through the generation of heparanase-cleaved fragments of HS. In support of this hypothesis, mouse spleen cells lacking the key TLR adaptor molecule MyD88 demonstrated an abolition of cytokine release after heparanase stimulation. Furthermore, TLR4-deficient spleen cells showed reduced cytokine release in response to heparanase treatment, suggesting that TLR4 is involved in this response. Consistent with these observations, the pathway involved in cytokine upregulation was identified as being NF-κB-dependent. These data identify a new mechanism for heparanase in promoting the release of pro-inflammatory cytokines that is likely to be important in regulating cell migration and inflammation [122].

Heparanase triggers upregulation of pro-inflammatory cytokines through the generation of heparanase-cleaved fragments of HS.

Heparanase Is Required for Activation and Function of Macrophages We applied a genetic approach and examined the behavior and function of macrophages isolated from wild-type (WT) and heparanase-knockout (Hpa-KO) mice. Hpa-KO macrophages express lower levels of cytokines (e.g., TNFα, IL1-β) and exhibit lower motility and phagocytic capacities. Intriguingly, inoculation of control monocytes together with Lewis lung carcinoma (LLC) cells into Hpa-KO mice resulted in nearly complete inhibition of tumor growth. In striking contrast, inoculating LLC cells together with monocytes isolated from Hpa-KO mice did not affect tumor growth, indicating that heparanase is critically required for activation and function of macrophages. Mechanistically, we describe a linear cascade by which heparanase activates Erk, p38, and JNK signaling in macrophages, leading to increased c-Fos levels and induction of cytokine expression in a manner that apparently does not require heparanase enzymatic activity. [123].

Heparanase is a key mediator of macrophage activation and function in tumorigenesis and cross-talk with the tumor microenvironment.

Macrophage Polarization in Pancreatic Carcinoma: Role of Heparanase Enzyme Overexpression of heparanase is associated with increased TAM infiltration in both experimental and human PDAC. Moreover, macrophages derived from heparanase-rich tumors (which grew faster in mouse hosts), display pronounced procancerous phenotype, evidenced by overexpression of MSR-2, IL-10, CCL2, VEGF, and increased production of IL-6, an important player in PDAC pathogenesis. Furthermore, in vitro heparanase enzyme-rendered macrophages (stimulated by necrotic cells which are often present in PDAC tissue) pro-cancerous, as exemplified

by their enhanced production of key cytokines implicated in PDAC (including IL-6), as well as by their ability to induce STAT3 signaling and to augment pancreatic carcinoma cell proliferation. In agreement, we observed activation of STAT3 in experimental and clinical specimens of heparanase-overexpressing PDAC. These findings underscore a novel function of heparanase in molecular decision-making that guides cancer-promoting action of TAM and imply that heparanase expression status may become highly relevant in defining a target patient subgroup that is likely to benefit the most from treatment modalities targeting TAM/IL-6/STAT3 [124].

**Heparanase plays a key role in molecular decision-making that guides the cancer-promoting action of tumor associated macrophages.*

Heparan Sulfate Mimetic PG545-Mediated Anti-Lymphoma Effects Require TLR9-Dependent NK Cell Activation HS mimetics, such as PG545, have been developed as antitumor agents and are designed to suppress angiogenesis and metastasis by inhibiting heparanase and competing for the HS-binding domain of angiogenic growth factors. However, how PG545 exerts its antitumor effect remains incompletely defined. Here, using murine models of lymphoma, we determined that the antitumor effects of PG545 are critically dependent on NK cell activation and that NK cell activation by PG545 requires TLR9. We demonstrate that PG545 does not activate TLR9 directly but instead enhances TLR9 activation through the elevation of the TLR9 ligand CpG in DCs. Specifically, PG545 treatment resulted in CpG accumulation in the lysosomal compartment of DCs, leading to enhanced production of IL-12, which is essential for PG545-mediated NK cell activation. Overall, these results reveal that PG545 activates NK cells and that this activation is critical for the antitumor effect of PG545. Moreover, our findings may have important implications for improving NK cell-based antitumor therapies [125].

**PG545 activates NK cells and thereby exerts an antitumor effects.*

Heparanase Augments Inflammatory Chemokine Production from Colorectal Carcinoma Cell Lines To explore possible roles of heparanase in tumor-host crosstalk, we examined whether heparanase influences expression of inflammatory chemokines in colorectal cancer cells. Murine colorectal carcinoma cells incubated with heparanase upregulated MCP-1, KC, and RANTES genes and released MCP-1 and KC proteins. Heparanase-dependent production of IL-8 was detected in two human colorectal carcinoma cell lines. Addition of a heparanase inhibitor Heparastatin (SF4) did not influence MCP-1 production, while both latent and mature forms of heparanase augmented MCP-1 release, suggesting that heparanase catalytic activity was dispensable for MCP-1 production. In contrast, addition of heparin to the medium suppressed MCP-1 release in a dose-dependent manner. Similarly, targeted suppression of Ext1 by RNAi significantly suppressed cell surface expression of HS and MCP-1 production in colon 26 cells. Taken together, it is concluded that colon 26 cells transduce the heparanase-mediated signal through HS binding. We propose a novel function for heparanase independent of its endoglycosidase activity, namely as a stimulant for chemokine production [126].

NK Cell Heparanase Controls Tumor Invasion and Immune Surveillance NK cells are highly efficient at preventing cancer metastasis but are infrequently found in the core of primary tumors. Here, have we demonstrated that freshly isolated mouse and human NK cells express low levels of heparanase that increase upon NK cell activation. Heparanase deficiency did not affect development, differentiation, or tissue localization of NK cells under steady-state conditions. However, mice lacking heparanase specifically in NK cells (Hpsefl/fl NKp46-iCre mice) were highly tumor prone when challenged with the carcinogen methylcholanthrene (MCA). Hpsefl/fl NKp46-iCre mice were also more susceptible to tumor growth than were their littermate controls when challenged with the established mouse lymphoma cell line RMA-S-RAE-1β, which overexpresses the NK cell group 2D (NKG2D) ligand RAE-1β, or when inoculated with metastatic melanoma, prostate carcinoma, or mammary carcinoma cell lines. NK cell invasion of primary tumors and recruitment to the site of metastasis were strictly dependent on the presence of heparanase. Cytokine and immune checkpoint blockade immunotherapy for metastases was compromised when NK cells lacked heparanase. Our data suggest that heparanase plays a critical role in NK cell invasion into tumors and thereby tumor progression and metastases. This should be considered when systemically treating cancer patients with heparanase inhibitors, since the potential adverse effect on NK cell infiltration might limit the antitumor activity of the inhibitors [127].

Heparanase Promotes Tumor Infiltration and Antitumor Activity of CAR-Redirected T Lymphocytes Adoptive transfer of chimeric antigen receptor (CAR)-redirected T lymphocytes (CAR-T cells) has had less striking therapeutic effects in solid tumors than in lymphoid malignancies. Although active tumor-mediated immunosuppression may have a role in limiting the efficacy of CAR-T cells, functional changes in T lymphocytes after their ex vivo manipulation may also account for the reduced ability of cultured CAR-T cells to penetrate stroma-rich solid tumors compared with lymphoid tissues. We therefore studied the capacity of human in vitro-cultured CAR-T cells to degrade components of the ECM. In contrast to freshly isolated T lymphocytes, we found that in vitro-cultured T lymphocytes lack expression of the enzyme heparanase (HPSE), which degrades heparan sulfate proteoglycans, the main components of ECM. We found that HPSE mRNA is downregulated in in vitro-expanded T cells, which may be a consequence of p53 (officially known as TP53, encoding tumor protein 53) binding to the HPSE gene promoter. We therefore engineered CAR-T cells to express HPSE and showed their improved capacity to degrade the ECM, which promoted tumor T cell infiltration and antitumor activity. The use of this strategy may enhance the activity of CAR-T cells in individuals with stroma-rich solid tumors [128].

The above two papers show that heparanase plays a critical role in NK- and T- cell invasion into tumors. This might adversely limit the antitumor effectiveness of heparanase-inhibiting compounds.

See Chapter 17 in this volume for more information on heparanase in inflammation and cells of the immune system.

Vaccination

Heparanase: A New Metastasis-Associated Antigen Recognized in Breast Cancer Patients by Spontaneously Induced Memory T Lymphocytes Increased expression and secretion of heparanase (Hpa) by tumor cells promotes tumor invasion, tissue destruction, angiogenesis, and metastasis. Here, we show the existence in breast cancer patients of Hpa-specific T lymphocytes by fluorescence-activated cell sorting flow cytometry using Hpa peptide-MHC class I tetramers. We furthermore show memory T-cell responses in a high proportion of breast cancer patients to Hpa-derived HLA-A2-restricted peptides, leading to production of IFN-gamma and to generation of antitumor CTLs lysing breast cancer cells. Such CTLs recognized endogenously processed respective Hpa peptides on Hpa-transfected and Hpa-expressing untransfected breast carcinoma cells. According to these results and to the fact that such cells were not found in healthy people, Hpa seems to be an attractive new tumor-associated antigen and its HLA-A2-restricted peptides ought to be good candidates for peptide vaccination to reactivate memory immune responses to invasive and metastatic cancer cells [129].

H-2Kb-Restricted CTL Epitopes from Mouse Heparanase Elicit an Antitumor Immune Response In Vivo Heparanase is broadly expressed in various advanced tumors and can serve as a universal tumor-associated antigen. Although several epitopes of heparanase antigen are known in humans, the corresponding knowledge in mice is still rather limited. The present study was designed to predict and identify the CTL epitopes in the mouse heparanase protein. The results showed that, of the tested peptides, effectors induced by peptides of mouse heparanase at residue positions 398 to 405 (LSLLFKKL; mHpa398) and 519 to 526 (FSYGFFVI; mHpa519) lysed three kinds of carcinoma cells expressing both heparanase and H-2 K(b) (B16 melanoma cells, EL-4 lymphoma cells, and Lewis lung cancer cells). In vivo experiments indicated that mHpa398 and mHpa519 peptides offered the possibility of not only immunizing against tumors but also treating tumor-bearing hosts successfully. Our results suggest that the mHpa398 and mHpa519 peptides are novel H-2 K(b)-restricted CTL epitopes capable of inducing heparanase-specific CTLs in vitro and in vivo. These epitopes may serve as valuable tools for the preclinical evaluation of vaccination strategies [130].

**Two representative papers indicating that heparanase is an attractive new tumor-associated antigen and its HLA-restricted peptides are good candidates for peptide vaccination to reactivate memory immune responses to invasive and metastatic cancer cells.*

Diabetes, Diabetic Complications and Other Disorders

Heparan Sulfate and Heparanase Play Key Roles in Mouse β Cell Survival and Autoimmune Diabetes The autoimmune type 1 diabetes (T1D) that arises spontaneously in NOD mice is considered to be a model of T1D in humans. It is

characterized by the invasion of pancreatic islets by mononuclear cells (MNCs), which ultimately leads to destruction of insulin-producing β cells. Although T cell dependent, the molecular mechanisms triggering β cell death have not been fully elucidated. Here, we report that HS is expressed at extraordinarily high levels within mouse islets and is essential for β cell survival. In vitro, β cells rapidly lost their HS and died. β Cell death was prevented by HS replacement, a treatment that also rendered the β cells resistant to damage from ROS. In vivo, autoimmune destruction of islets in NOD mice was associated with production of catalytically active heparanase by islet-infiltrating MNCs and loss of islet HS. Furthermore, in vivo treatment with the heparanase inhibitor PI-88 preserved intra-islet HS and protected NOD mice from T1D. These results identified HS as a critical molecular requirement for islet β cell survival and HS degradation as a mechanism for β cell destruction. Hence, preservation of islet HS could be a therapeutic strategy for preventing T1D [131].

*See Chapter 24 by Simeonovic et al. for more information on heparanase and immune diabetes.

Heparanase Is Essential for the Development of Diabetic Nephropathy in Mice Diabetic nephropathy (DN) is the major life-threatening complication of diabetes. Abnormal permselectivity of glomerular basement membrane (GBM) plays an important role in DN pathogenesis. Loss of GBM HS in diabetic kidney was associated with increased glomerular expression of heparanase pointing to the essential involvement of heparanase in DN. With the use of Hpse-KO mice, we found that deletion of the heparanase gene protects diabetic mice from DN. Furthermore, by investigating the molecular mechanism underlying induction of the enzyme in DN, we found that transcription factor early growth response 1 (Egr1) is responsible for activation of heparanase promoter under diabetic conditions. The specific heparanase inhibitor SST0001 markedly decreased the extent of albuminuria and renal damage in mouse models of DN. Collectively these results underscore the crucial role of heparanase in the pathogenesis of DN and its potential as a highly relevant target for therapeutic interventions in patients with DN [132].

Endothelin-1 Induces Proteinuria by Heparanase-Mediated Disruption of the Glomerular Glycocalyx Diabetic nephropathy (DN) is the leading cause of CKD in the Western world. Endothelin receptor antagonists have emerged as a novel treatment for DN, but the mechanisms underlying the protective effect remain unknown. We previously showed that both heparanase and endothelin-1 are essential for the development of DN. Here, we further investigated the role of these proteins in DN, and demonstrated that endothelin-1 activates podocytes to release heparanase. Furthermore, conditioned podocyte culture medium increased glomerular transendothelial albumin passage in a heparanase-dependent manner. In mice, podocyte-specific knockout of the endothelin receptor prevented the diabetes-induced increase in glomerular heparanase expression, consequent reduction in heparan sulfate expression and endothelial glycocalyx thickness, and development of proteinuria observed in wild-type counterparts. Our data suggest that in diabetes,

endothelin-1 signaling, as occurs in endothelial activation, induces heparanase expression in the podocyte, damage to the glycocalyx, proteinuria, and renal failure. Thus, prevention of these effects may constitute the mechanism of action of endothelin receptor blockers in DN [133].

These and other studies underscore the crucial role of heparanase in the pathogenesis of diabetic nephropathy.

*See Chapters 27 and 28 by Massola et al., and by Abassi & Goligorsky, for more information on heparanase in fibrosis and kidney dysfunction [134, 135].

Endothelial Cell Heparanase Taken up by Cardiomyocytes Regulates Lipoprotein Lipase Transfer to the Coronary Lumen after Diabetes After diabetes, the heart has a singular reliance on fatty acid (FA) for energy production, which is achieved by increased coronary lipoprotein lipase (LPL) that breaks down circulating triglycerides. Coronary LPL originates from cardiomyocytes, and to translocate to the vascular lumen, the enzyme requires liberation from myocyte surface HS, an activity that needs to be sustained after chronic hyperglycemia. We investigated the mechanism by which endothelial cells (EC) and cardiomyocytes operate together to enable continuous translocation of LPL after diabetes. EC were co-cultured with myocytes, exposed to high glucose, and uptake of endothelial heparanase into myocytes was determined. Upon uptake, the effect of nuclear entry of heparanase was also investigated. A streptozotocin model of diabetes was used to expand our in vitro observations. In high glucose, EC-derived latent heparanase was taken up by cardiomyocytes by a caveolae-dependent pathway using HSPGs. This latent heparanase was converted into an active form in myocyte lysosomes, entered the nucleus, and upregulated gene expression of matrix metalloproteinase-9. The net effect was increased shedding of HSPGs from the myocyte surface, releasing LPL for its onwards translocation to the coronary lumen. EC-derived heparanase regulates the ability of the cardiomyocyte to send LPL to the coronary lumen. This adaptation, although acutely beneficial, could be catastrophic chronically because excess FA causes lipotoxicity. Inhibiting heparanase function could offer a new strategy for managing cardiomyopathy observed after diabetes [136].

*See also comment entitled **'**Heparanase shakes hands with lipoprotein lipase: a tale of two cells' [137]. See Chapter 30 by Chang et al., for more information on heparanase and cardiomyocytes.

In Vivo Fragmentation of Heparan Sulfate by Heparanase Overexpression Renders Mice Resistant to Amyloid Protein a Amyloidosis Amyloid diseases encompass >20 medical disorders that include amyloid protein A (AA) amyloidosis, Alzheimer's disease, and type 2 diabetes. A common feature of these conditions is the selective organ deposition of disease-specific fibrillar proteins, along with the sulfated glycosaminoglycan, HS. We have tested the susceptibility of *Hpa-tg* mice to amyloid induction. Drastic shortening of HS chains was observed in heparanase-overproducing organs, such as liver and kidney. These sites selectively escaped

amyloid deposition on experimental induction of inflammation-associated AA amyloidosis, whereas the same tissues from control animals were heavily infiltrated with amyloid. By contrast, the spleens of transgenic mice that failed to significantly overexpress heparanase remained susceptible to amyloid deposition. Our findings provide direct in vivo evidence that heparan sulfate is essential for the development of amyloid disease [138].

*See Chapter 25 by Li & Zhang, for more information on heparanase and amyloidosis.

Aterosclerosis & Thrombosis

Heparanase Regulates Thrombosis in Vascular Injury and Stent-Induced Flow Disturbance The purpose of this study was to examine the role of heparanase in controlling thrombosis following vascular injury or endovascular stenting. In the absence of vascular injury, wild type and heparanase overexpressing (*HPA-Tg*) mice had similar times to thrombosis in a laser-induced arterial thrombosis model. However, in the presence of vascular injury, the time to thrombosis was dramatically reduced in *HPA-Tg* mice. An ex vivo system was used to flow blood from wild type and *HPA-Tg* mice over stents and stented arterial segments from both animal types. These studies demonstrate markedly increased thromboses on stents with blood isolated from *HPA-Tg* mice in comparison to blood from wild type animals. We found that blood from *HPA-Tg* animals had markedly increased thrombosis when applied to stented arterial segments from either wild type or *HPA-Tg* mice [139].

**These results indicate that heparanase is a powerful mediator of thrombosis in the context of vascular injury and stent-induced flow disturbance.*

The Pulmonary Endothelial Glycocalyx Regulates Neutrophil Adhesion and Lung injury during Experimental Sepsis Sepsis, a systemic inflammatory response to infection, commonly progresses to acute lung injury (ALI), an inflammatory lung disease with high morbidity. We postulated that sepsis-associated ALI is initiated by degradation of the pulmonary endothelial glycocalyx, leading to neutrophil adherence and inflammation. Using intravital microscopy, we found that endotoxemia in mice rapidly induced pulmonary microvascular glycocalyx degradation via tumor necrosis factor-α (TNF-α)-dependent mechanisms. Glycocalyx degradation involved the specific loss of heparan sulfate and coincided with activation of endothelial heparanase, a TNF-α-responsive, heparan sulfate-specific glucuronidase. Glycocalyx degradation increased the availability of endothelial surface adhesion molecules to circulating microspheres and contributed to neutrophil adhesion. Heparanase inhibition prevented endotoxemia-associated glycocalyx loss and neutrophil adhesion and, accordingly, attenuated sepsis-induced ALI and mortality in mice. These findings are potentially relevant to human disease, as sepsis-associated respiratory failure in humans was associated with higher plasma heparan sulfate

degradation activity; moreover, heparanase content was higher in human lung biopsies showing diffuse alveolar damage than in normal human lung tissue [140].

*See additional studies on heparanase and the glycocalyx [133, 141].

Viral Infection

Heparanase Is a Host Enzyme Required for Herpes Simplex Virus-1 Release from Cells Herpesviruses exemplified by herpes simplex virus-1 (HSV-1) attach to cell surface HS for entry into host cells. However, during a productive infection, the HS moieties on parent cells can trap newly exiting viral progenies and inhibit their release. Here we demonstrate that a HS-degrading enzyme of the host, heparanase (HPSE), is upregulated through NF-kB and translocated to the cell surface upon HSV-1 infection for the removal of HS to facilitate viral release. We also find a significant increase in HPSE release in vivo during infection of murine corneas and that knockdown of HPSE in vivo inhibits virus shedding. Overall, we propose that HPSE acts as a molecular switch for turning a virus-permissive 'attachment mode' of host cells to a virus-deterring 'detachment mode'. Since many human viruses use HS as an attachment receptor, the HPSE-HS interplay may delineate a common mechanism for virus release [142].

*See Chapter 32 by Agelidis & Shukla, for more information on heparanase and viral infection.

Note added in Proofs: Given that many human viruses use HS as an attachment receptor, non-anticoagulant heparin/HS mimicking compounds (i.e., Roneparstat) may compete with HS and thereby inhibit viral infection. This may be relevant to the recent coronavirus (COVID-19) pandemic as HS has been found to function as adhesion molecule that increases the virus density on the cell surface, possibly facilitating the interaction between the virus (HCoV-NL63) and its receptor [143].

1.4 Concluding Remarks and Perspectives

Heparanase exerts strong pro-tumorigenic properties, promoting all aspects of tumor development (tumor initiation, growth, and metastasis) and chemo-resistance. The enzyme is expressed by tumor cells and cells of the tumor microenvironment and functions extracellularly as well as inside the cell. Collectively, the emerging premise is that heparanase expressed by tumor cells and cells of the tumor microenvironment is a dominant regulator of the aggressive phenotype of cancer, an important contributor to the poor outcome of cancer patients and a prime target for therapy.

As the investigation of heparanase proceeds, new roles for the enzyme in diverse processes such as signal transduction, gene regulation, exosome formation, autophagy, activation of innate immune cells, chemo-resistance, are emerging and thus

widening the impact of this enzyme [144]. It appears that heparanase functions extracellularly to remodel the ECM and regulate the bioavailability of ECM-bound factors, as well as inside the cell, augmenting, among other effects, gene transcription, and HS turnover. Unraveling these and other aspects of heparanase biology is ongoing and is critical to our understanding of its multiple functions in health and disease. Central to some of the downstream effects of heparanase is the enzyme's ability to regulate gene transcription. At the molecular level, nuclear heparanase appears, among other effects, to regulate histone 3 lysine 4 (H3K4) methylation by influencing the recruitment of demethylases to transcriptionally active genes [105]. Yet, our understanding of heparanase nuclear accessibility and mode of action is far from being complete. An important challenge in the field rests on structure-based rational development of clinically effective inhibitors (heparin mimics, neutralizing antibodies, small molecules) of heparanase that will be applied to treat cancer, inflammation and other diseases. A limiting factor in anti-heparanase drug development is the lack of a high throughput screening and validation assays. Most assays continue to use heterogeneous substrates prepared by derivatization of HS or heparin/LMWH in various ways. These assays are limited by their heterogeneity, semi-quantitative nature, multiple enzyme cleavage sites and inappropriateness for use in biological samples. Advances in the synthesis of simple synthetic oligosaccharide substrates with a single point of cleavage will ultimately lead to a "gold standard" assay for detailed kinetic analyses [23]. Also, undesirable effects of anti-heparanase therapy should be considered. For example, it was recently reported that heparanase plays a critical role in NK cell invasion into tumors and thereby tumor progression and metastases. It was shown that cytokine and immune checkpoint blockade immunotherapy for metastases were compromised when NK cells lacked heparanase [127]. Likewise, it was found that in contrast to freshly isolated T lymphocytes, HPSE mRNA is downregulated in *in vitro*-expanded T cells. This may explain the reduced ability of cultured CAR-T cells to penetrate stroma-rich solid tumors compared with lymphoid tissues. In fact, engineering the CAR-T cells to express HPSE resulted in their improved capacity to degrade the ECM, which promoted tumor T cell infiltration and antitumor activity [128]. It was suggested that the use of this strategy might enhance the activity of CAR-T cells in individuals with stroma-rich solid tumors. These results should be considered when systemically treating cancer patients with heparanase inhibitors, since the potential adverse effect on NK and CAR-T cells on cell infiltration might limit the antitumor activity of the inhibitors [128]. Yet, these effects appear negligible given that heparanase knock-out mice exhibit no obvious immunological and other deficits [95], implying that inhibition of heparanase will cause minimal side effects in cancer patients. Remarkably, heparanase inhibitors were effective even when the xenografted tumor cells were devoid of heparanase, emphasizing the significance of heparanase contributed by cells residing in the tumor microenvironment. It appears that targeting the tumor microenvironment by heparanase inhibitors enhances the antitumor activity of approved therapies, further providing a strong rationale for applying anti-heparanase therapy in combination with conventional anti-cancer drugs.

Another topic of interest is the crosstalk between heparanase and heparanase 2 (Hpa2), a close homolog of heparanase devoid of heparanase enzymatic activity [145]. Unlike heparanase, the role played by Hpa2 in normal physiology and pathological disorders is still largely obscure. Hpa2 appears to attenuate tumor growth via inhibition of heparanase, decreasing the expression of pro-angiogenic mediators and inducing the manifestation of genes involved in tumor suppression and cell differentiation (see chapters by Mckenzie and by Roberts & Woolf in this book). It appears that Hpa2 functions in heparanase activity- and HS- dependent and independent manners, and regulates the expression of selected genes that affect tumor vascularity, tumor fibrosis, cell differentiation, and apoptosis of cancer cells, resulting in tumor suppression [144, 146–149]. Clearly, further research is needed in order to appreciate the scope of Hpa2 function and crosstalk with heparanase.

Personal Notes I would like to close with a more personal note. I became interested in cell interaction with the ECM about 40 years ago just before finishing my postdoctoral research under the supervision of Prof. Denis Gospodarowicz (UCSF). We were among the first to realize that the ECM plays an active role in orchestrating cellular responses to both normal and pathological situations [149–152]. The emerging concept was one of an active interplay between cells and the ECM where cells synthesize matrix components which in turn dictate and regulate cell shape and function. The impact of these studies is clearly indicated by the current awareness of the ECM and the tumor microenvironment as key elements in the regulation of cell survival and cancer progression. It was only a few years after my return to Israel that I became interested in degradation of the ECM' HS as a mean to understand better how tumor cells enter and exit blood vessels in the process of cancer metastasis. I decided to focus on HS knowing that this polyanionic polysaccharide binds to other constituents of the ECM and plays an important role in assembling and stabilizing the entire supramolecular structure of the ECM. It was then that I became interested in the heparanase enzyme, a topic that kept me busy since then. Our first papers on heparanase and cancer metastasis were published in 1983 [152], in parallel to studies reported by Dr. Nicolson et al. [44]. Next, we reported on the sequential mode of heparanase action in degrading the ECM [46], its inhibition by species of heparin [51, 153] and expression by platelets [154], neutrophils [155] and activated cells of the immune system. An important concept was brought about in studies performed during a sabbatical with Michael Klagsbrun and the late Judah Folkman (Harvard Medical Center) revealing that HS in the ECM provides a storage depot for FGF2, and hence heparanase regulates its bioavailability [16, 49, 50]. The concept of ECM as a reservoir for bioactive molecules became well recognized and is of prime importance to the current appreciation of the tumor microenvironment and its significance in cancer progression and treatment.

These and other studies further emphasized the need to purify the heparanase protein and clone the respective gene, an objective that took a long time and was finally achieved at 1999 [4] and published back to back with a study performed by the group of Cristopher Parish in Canberra [1]. Gene cloning enabled studies on the modes of heparanase gene regulation, cellular uptake, lysosomal storage and activa-

tion [31, 34, 144, 156, 157]. We and others further revealed the modes of heparanase action in eliciting angiogenesis, tumorigenesis and signal transduction. Thus, 20 years following its cloning, the functional repertoire of heparanase and the complexity of the system are still being revealed. From activity mainly implicated in cell invasion associated with tumor metastasis, heparanase has turned into a multifaceted protein that appears to participate in essentially all major aspects of tumor progression and in the pathogenesis of other diseases. Importantly, the crystal structure of the heparanase protein was resolved [87], paving the way for rational design and optimization of site-directed heparanase-inhibiting small molecules and monoclonal antibodies. As a direct result of these and subsequent studies performed by other groups and us, heparanase was advanced from being an obscure enzyme with a poorly understood function to a highly promising, novel drug target, offering new treatment strategies for various cancers and other diseases (i.e., chronic inflammation, diabetic nephropathy). The significance of heparanase as a valid target for anti-cancer drug development was reinforced by studies indicating a marked inhibition of human myeloma, lymphoma, glioma, sarcoma, mesothelioma and pancreatic tumor growth in mice treated with heparin-like heparanase-inhibiting compounds (i.e., SST0001 = Roneparstat, PG545 = Pixatimod) that are being examined in clinical trials alone and in combination with other drugs [159, 160]. Unfortunately, we are not yet there raising the question/dilemma if it was wise to focus and invest so much time, energy and effort in the study of a single molecule. Do we really understand the mode of heparanase action in tumorigenesis?; Should we adopt another way of thinking?; Should we focus on other diseases?; Where we objective and unbiased in our interpretations of the results?; What mistakes, if any, were done along the way?; Should the final goal of a basic scientist is to translate his findings into a drug?; Was it worth? etc. (see also Ilan et al., Chap. 9 in this volume). Obviously, there are no simple and clear cut answers. Focusing on one molecule is risky and, nowadays in the era of 'big science', is regarded as an 'old fashion' approach. Yet, the heparanase journey is actively ongoing and should be evaluated in the broad context of cell interaction with the ECM and tumor microenvironment. In fact, heparanase research markedly reinforced the significance of the ECM in the control of cell proliferation and differentiation [149–152]. It led to important and often unexpected observations in diverse normal and pathological processes including, wound healing, angiogenesis [63], autophagy [98], signal transduction [107, 156, 161], protein trafficking [162], lysosomal secretion [144, 163], blood coagulation [92, 164], epithelial-mesenchymal transition [165], activation of immune cells [21, 121–123], exosome formation [99, 100, 102], drug resistance [34, 166], gene transcription [35, 104] and others. While most studies emphasize the involvement of heparanase in cancer progression, other pathologies were investigated. Among these are diabetes [35, 131], diabetic complications (i.e., diabetic nephropathy, diabetic cardiomyopathy) [132, 136, 167], kidney dysfunction [38], fibrosis [134, 168], inflammatory disorders (i.e., neuroinflammation, pancreatitis, ulcerative colitis, arthritis, sepsis) [121, 140, 169, 170], amyloidosis [138], atherosclerosis [39, 40, 171] and others. Interestingly, heparanase accomplishes all these by exerting both enzymatic and non-enzymatic functions that are

mostly HS-dependent yet in some cases are HS-independent [172]. In comparison with other ECM degrading enzymes (i.e., MMPs, cathepsins) [173, 174] the heparanase field is small, leaving enough room for other research groups to join and make significant contributions to basic and translational aspects. For example, little is known about a putative non-HS heparanase receptor [107, 175–178] that binds the enzyme and mediates its downstream signaling function. Likewise, in-depth research is needed to elucidate better the mode of heparanase nuclear translocation and transcriptional activity [104, 105], as well as its ability to activate macrophages and mediate their polarization [121–124]. An open area, not referred to in this review, has to do with heparanase-2 (Hpa2), a heparanase homolog devoid of heparanase enzymatic activity [146, 147, 178]. Of particular interest is to resolve the crystal structure of Hpa2, elucidate its mode of action as a tumor suppressor and reveal the significance of its crosstalk with heparanase [144]. Of prime importance from a translational point of view, is to apply the crystal structure of heparanase for rational design of heparanase-inhibiting small molecules, neutralizing monoclonal antibodies and HS-mimicking compounds endowed with good pharmacokinetics and examine their efficiency in animal models and cancer patients.

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Chapter 2
Heparanase – Discovery and Targets

Ulf Lindahl and Jin-Ping Li

2.1 Introduction

Heparin was discovered more than 100 years ago (1, 2) and heparan sulfate (HS) was defined as a distinct molecular identity about 40 years later (3). Their relationship in terms of structure and biosynthesis was only slowly elucidated during subsequent decades. The key protagonist of this volume, the enzyme heparanase, surfaced in the mid 70'ies as an unexpected outcome of attempts to define the macromolecular properties of native heparin. Subsequent development revealed heparanase as a major actor not only in polysaccharide metabolism and turnover but also in HS-proteoglycan (HSPG) pathophysiology, involving inflammation, cancer, and amyloid diseases. These aspects will be discussed in detail in the following chapters of this book. Here, we will attempt to describe the discovery of the enzyme, along with relevant reference to studies of heparin/HS structure and biosynthesis.

2.2 Heparanase, Early Findings

The first signs of the enzyme now known as heparanase emerged during attempts to define the macromolecular properties of native heparin. Both commercial heparin preparations (4) and glycopeptides isolated from chondroitin 4-sulfate cartilage proteoglycan (5) were found to contain the same -GlcA-Gal-Gal-Xyl- tetrasaccharide structure linking polysaccharide chains to serine units of a potential core protein, thus strongly suggesting the occurrence also of a heparin proteoglycan. Heparin extracted using mild methods from liver capsule contained covalently bound peptide

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(6), but also a reducible sugar end-group (7) collectively pointing to products of a heparin proteoglycan subjected to endoglycosidase cleavage. Accordingly, pulse labeling of mastocytoma cells with [^{35}S]sulfate showed initial incorporation into heparin of high molecular weight, followed by rapid intracellular degradation (8, 9). The enzyme responsible for degradation was recovered from a particulate fraction sedimenting at 20,000 x g. Reduction of the degraded heparin with sodium [^{3}H]-borohydride revealed a labeled L-gulonic acid end-group, corresponding to a D-glucuronic acid unit in the unreduced state (10). The degradation products averaged 15 kDa, approximately similar to commercially available heparin. The "macromolecular heparin" substrate was identified as a > 900 kDa proteoglycan with a polysaccharide-substituted portion of the core protein composed of predominantly alternating serine and glycine residues (hence resistant to pronase digestion), the substituent heparin chains ranging 60–100 kDa (11) (Fig. 2.1A). The proteoglycan (which could also carry chondroitin sulfate chains) was subsequently molecularly cloned (12) and coined "serglycin". The heparin-degrading enzyme thus identified as an endo-glucuronidase was initially termed "heparinase", but was later renamed "heparanase" to avoid mixup with microbial lyases.

2.3 Stereochemistry of Heparanase Target

Elucidation of the stereochemistry of the hexuronic acid components of heparin followed a winding path; the reader is recommended an entertaining essay on the topic by Lennart Rodén (in (13)). Still in the early 60'ies heparin was depicted as a polymer of alternating α-D-GlcA 2-O-sulfate and α-D-GlcN N,6-disulfate units (Fig. 2.2A). However, in 1962 Cifonelli and Dorfman reported on iduronic acid (IdoA) in heparin and HS (14), a finding subsequently corroborated by proton NMR analysis (15). The latter study further defined an α-L configuration of the IdoA residue. Moreover, analysis of deaminative cleavage products revealed IdoA as the major, and predominantly 2-O-sulfated hexuronic acid component of heparin (16) (Fig. 2.2B). Finally, the stereochemistry of the (relatively scarce) GlcA residues in heparin was clarified by biosynthesis experiments. A fraction of the nonreducing-terminal uronic acid residues of oligosaccharides recovered after deaminative cleavage of heparin could be released by digestion with ß-glucuronidase. Such a ß-glucuronidase-treated fragment served as acceptor for GlcA when incubated with UDP-[^{14}C]GlcA and a microsomal enzyme preparation derived from a heparin-producing mouse mastocytoma. On digestion of the radioactive product with ß-glucuronidase, all of the radioactivity was released as [^{14}C]GlcA (17). These findings established a ß-D configuration for GlcA in heparin (Fig. 2.2C), and thus identified heparanase as an endo-ß-D-glucuronidase. Notably, the α-L-*ido* and ß-D-*gluco* configurations show analogous arrangements around the corresponding anomeric carbon atoms, in accord with subsequent findings that IdoA units in heparin are generated by C5-epimerization of GlcA residues previously incorporated in the polysaccharide chain (18, 19).

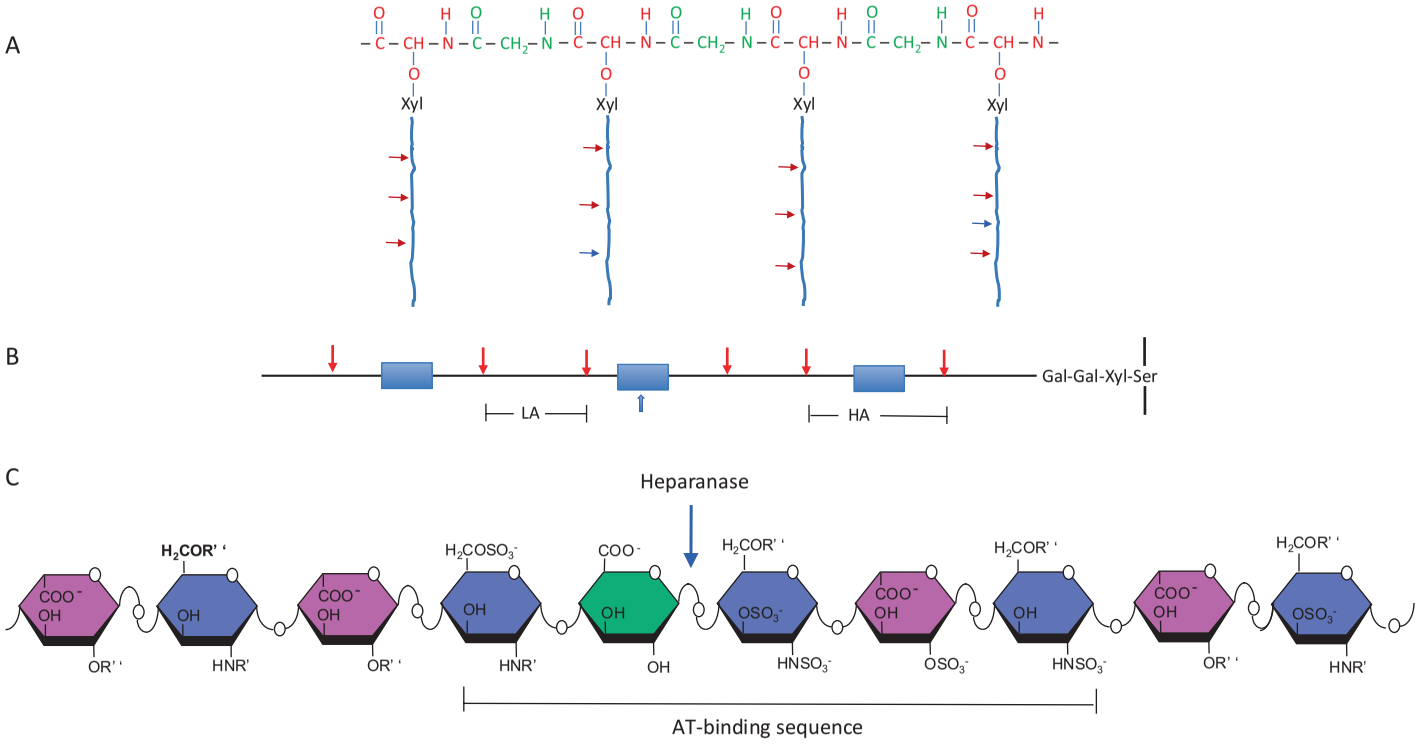

Fig. 2.1 Cleavage of heparin proteoglycan (serglycin) by heparanase. (**A**) Structure of serglycin, showing part of the polysaccharide-substituted portion of the core protein, composed of alternating serine (red) and glycine (green) residues. Serine residues are O-substituted with extended (60–100 kDa) heparin chains. The compact design renders the structure resistant toward commonly used proteolytic enzymes (such as papain or pronase). The red arrows indicate potential cleavage points by heparanase; the resultant products ranging ~7–20 kDa are similar to commercially available heparin. The blue arrows indicate "secondary" cleavage sites, within AT-binding pentasaccharide sequences. (**B**) Schematic illustration of heparanase attack on "macromolecular" heparin chain. The degradation products show high (HA) or low (LA) affinity for AT, depending on whether they contain or lack an intact AT-binding pentasaccharide sequence. Cleavage of such a sequence (blue arrow) will generate a LA-heparin product. (**C**) Segment of heparin containing the AT-binding pentasaccharide sequence. R' = $-COCH_3$ or $-SO_3^-$; R" = –H or $-SO_3^-$. The sulfate substituent on C3 of the internal glucosamine residue is a distinctive structural feature of the AT-binding region. The blue arrow marks a potential cleavage site for heparanase

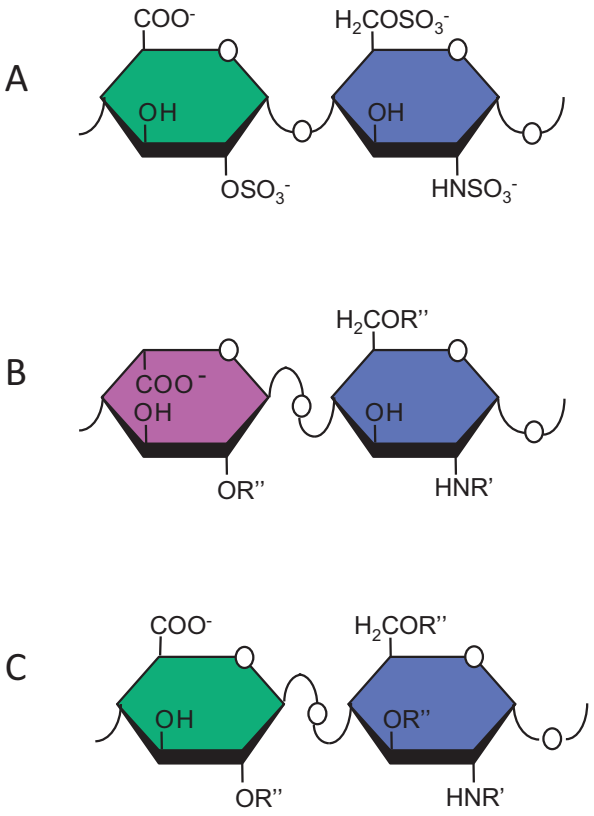

Fig. 2.2 Structures of disaccharide units in heparin/HS. (**A**) Disaccharide (incorrectly) conceived as the major building block in heparin around 1960. (**B**) IdoA-containing disaccharide unit of heparin/HS. (**C**) GlcA-containing disaccharide unit of heparin/HS. For further information, see the text. R' = $-COCH_3$ or $-SO_3^-$; R'' = –H or $-SO_3^-$

2.4 How Many Heparanases?

Enzyme activities reminiscent of heparanase were subsequently demonstrated in many different cells and tissues, including platelets, liver (20), T lymphocytes (21), bone marrow-derived mast cells (22) and placenta (23). Proposals of several distinct heparanases (24) were not supported in comparative studies of enzymes from different sources, that collectively identified a single endo-ß-D-glucuronidase (see e.g (25, 26)). This conclusion was corroborated by genetic studies following molecular cloning of the enzyme (27–29). So far, all heparanase activities have been attributed to products of a single gene. Whereas the early findings associated heparanase with mast-cell heparin proteoglycan, the overall role of heparanase in mast cell biology remains obscure. The only apparent effect of enzyme elimination from these cells was accumulation of proteases (30). The ubiquitous distribution of heparanase in tissues instead pointed to HS rather than heparin as the major target of enzyme action. An early finding by Vlodavsky et al. thus indicated heparanase-induced degradation of HS in the extracellular matrix (31). Moreover, recent work aimed at clarifying physiological and, in particular, pathophysiological aspects of the enzyme

revealed an unanticipated multitude of effects on development, homeostasis, and disease, including cancer, amyloid diseases, and inflammatory conditions. Whereas many of these effects were attributed to cleavage of HS chains, others appear dependent on non-catalytic mechanisms mediated by receptor interaction, which will be covered in subsequent chapters of this volume.

2.5 Heparanase and Polysaccharide Metabolism

Heparanase affects heparin and HS metabolism in diverse ways. Basic structural features of these polysaccharides are illustrated through the schemes in Fig. 2.3. A common [GlcA-GlcNAc-]$_n$ polymer is subjected to a series of modification reactions, involving N-deacetylation/N-sulfation of GlcNAc residues, C5-epimerization of GlcA to IdoA units, 2-O-sulfation of hexuronic acid, and finally 3-O- and 6-O-sulfation of GlcN residues. Modification is typically incomplete, such that HS chains exhibit relatively short domains of highly variable sulfate density and positioning (32). Moreover, it appears to be strictly regulated in a cell-specific fashion, different tissues containing HS of distinct (yet heterogeneous) composition (33). The mechanism behind this regulation, still poorly understood, appears designed to provide modulated interaction with various protein targets (34, 35). HS biosynthesis, accomplished by most mammalian cells, involves at least 11 distinct enzymes, including 6 glycosyltransferases and 5 polymer-modification enzymes (not including the numerous isoforms), all of which have been cloned (36). Heparin biosynthesis, restricted to mast cells, is catalyzed by the same set of enzymes but entails more extensive modifications, such that the final product is dominated by extended, heavily sulfated domains (Fig. 2.3). Notably, incorporation of a 3-O-sulfate group in a selected GlcN residue concludes generation of the antithrombin- (AT)-binding pentasaccharide sequence in heparin, essential for the blood anticoagulation activity of

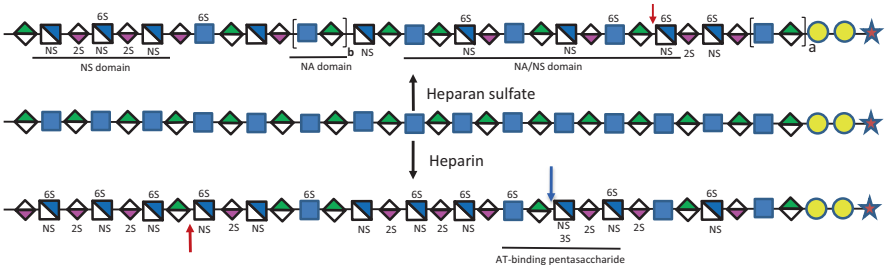

Fig. 2.3 Structures of (conjectural) heparin and HS chains. Both polymers are derived from a common [GlcA-GlcNAc]$_n$ precursor chain, through a series of N-deacetylation/N-sulfation, GlcA C5-epimerization, and O-sulfation reactions. Heparin is more extensively modified than HS (32). HS chains generally show a > b. The red and blue arrows indicate potential heparanase cleavage sites. NS, N-sulfated GlcN residues; 2S, 2-O-sulfated IdoA residues; 6S, 6-O-sulfated GlcN residues

the polysaccharide (Fig. 2.1C; 3). By comparison, 3-O-sulfate groups in HS are less frequent than in heparin but involve a number of 3-O-sulfotransferase isoforms, presumably designed to generate specific binding sites for selected proteins (37).

The role of heparanase in the intracellular processing of "macromolecular heparin" (i.e., the serglycin heparin proteoglycan; Fig. 2.1A) remains intriguing. GlcA accounts for only about 20% of the hexuronic acid residues in heparin, and one of these is consistently found in the AT-binding pentasaccharide sequence (Fig. 2.1C; 3). Commercially available heparin preparations would be products of heparanase action, yet only about one-third of the molecules show high affinity for AT (hence high anticoagulant activity) and contain an intact AT-binding site. The larger proportion of low-affinity species would presumably represent sequences between two AT-binding sequences, excised by heparanase cleavage, alternatively, products released by heparanase cleavage of the glucuronidic linkage within the pentasaccharide sequence (Fig. 2.1B). Indeed, a comparative study of heparanase substrates showed cleavage of the AT-binding pentasaccharide, occurring within a larger oligosaccharide sequence (Fig. 2.1C), but at a significantly lower rate compared to the actual pentasaccharide devoid of adjacent saccharides (38). Accordingly, an incubation product of commercial heparin size (~15 kDa) retained the specific pentasaccharide sequence, although oligosaccharides (3–4 kDa) containing this sequence could be degraded by the same enzyme. Indeed, commercial heparin was found to be a powerful inhibitor of heparanase action toward AT-binding oligosaccharides (26, 39, 40). The functional significance of these relations is unclear, as is, in fact, the pathophysiological role of anticoagulant heparin generated by the extravascular mast cells.

Whereas the proportion of GlcA residues is higher in HS than in heparin, the potential targets for heparanase attack are restricted by the requirement for sulfate substituents for substrate recognition. However, a minimal substrate recognition structure has not been defined; instead, it has been suggested that heparanase is capable of varying its substrate recognition depending on the saccharide structures around the cleavage site (41). Such "plastic substrate specificity" may facilitate an adaptation of heparanase action to highly diverse functional scenarios.

Early findings ascribed a role for heparanase in the metabolism and turnover of HS, as initial endo-hydrolase cleavage generates oligosaccharide substrates for subsequent exo-hydrolase action (42). The functional significance of this step is however somewhat unclear, as HS lacking 2-O-sulfate groups and thus resisting heparanase cleavage was found to undergo intracellular degradation with normal kinetics (43). Apparently, efficient degradation of the polysaccharide may be achieved by exolytic glycosidase and sulfatase action alone.

2.6 Heparanase and the GAGosome

Transgenic or tumor-induced overexpression of heparanase led to an unexpected upregulation of HS sulfation. Liver from mice transgenically overexpressing heparanase thus showed accelerated turnover of HSPG along with upregulation of N-

and O-sulfation, yielding heparin-like chains lacking the domain structure typical of HS (44). Likewise, Spalax, a subterranean blind mole rat, synthesized HS of overall higher sulfation degree, compared to that of normal murine tissues. This animal is adapted to life in an extreme hypoxic environment, by up-regulated expression of growth factors and enzymes, including heparanase, for ensuring sufficient oxygen supply. Accordingly, human embryonic kidney cells exposed to hypoxic conditions showed up-regulation of heparanase and a structural change of HS similar to that observed in the Spalax (45). These findings highlight a fundamental question in HS biology – what is the mechanism(s) behind regulation of HS biosynthesis, capable of fine-tuning saccharide structure? The action of heparanase so far has been localized to the extracellular matrix, cellular surface, and the endosome/lysosome organelles. However, it cannot be excluded that the enzyme is also functionally involved during the biosynthesis process. The "GAGosome" concept, still conjectural, features complexes of HS biosynthesis enzymes interacting with each other, their substrates and, potentially, additional auxiliary proteins (35, 46). How would heparanase fit into such machinery?

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Chapter 3
Heparanase: Historical Aspects and Future Perspectives

Mayank Khanna and Christopher R. Parish

3.1 Introduction

In this Chapter, a historical overview of our understanding of the functional properties of heparanase (also known as heparanase-1) is provided followed by a general discussion of unanswered questions and future areas of heparanase research. To assist the reader, a Table is provided (Table 3.1) that chronologically lists important advances in our understanding of heparanase. It is also important to note that although heparanase was initially thought to be an endoglycosidase that cleaves HS chains, there are a number of studies showing that heparanase can also perform non-enzymatic functions. Thus, this review will, after an initial historical overview of the general properties and substrate specificity of heparanase, consider the multiple enzymatic functions mediated by heparanase and then, in a separate section, review non-enzymatic processes performed by heparanase.

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Table 3.1 Chronology of major advances in heparanase research

Date	[a]Major advance	References
1975–1977	First evidence that endoglycosidase (heparanase) exists that can degrade HS/heparin	[1–7]
1982–1983	Demonstration that metastatic tumors express heparanase, the level of the enzyme correlating with metastatic potential	[8–10]
1982-present	Development of heparanase assays	Reviewed in [48]
1984	Demonstration that T cells, following activation, upregulate heparanase	[76]
1986–1987	First heparanase inhibitors developed	[106, 107, 109]
1989	First evidence that heparanase can release pro-angiogenic growth factors sequestered by HS in ECMs	[38]
1995	First demonstration that heparanase has non-enzymatic functions, i.e., can act as cell adhesion molecule	[130]
1999	Mammalian heparanase cloned	[12, 13]
1999	Heparanase shown to exist as a pro-enzyme that is protease processed to form an enzymatically active heterodimer	[30]
1999	Discovery that there is only one heparanase enzyme in the mammalian genome	[12, 13]
2000	Discovery of heparanase-2 gene	[35, 36]
2000	Heparanase shown to be a family 79 glycoside hydrolase, with $(\alpha/\beta)_8$ TIM-barrel fold, Glu^{225} and Glu^{343} in active site	[35]
2000-present	Heparanase upregulated in all cancer types, correlated with poor prognosis and can be derived from tumour microenvironment	Reviewed in [73]
2000-present	Heparanase shown to be a key player in inflammatory responses	Reviewed in [71, 85, 89]
2004	First evidence that heparanase can activate signaling pathways	[134]
2004	First reports that heparanase can translocate to the nucleus	[144, 146]
2008	Cathepsin L identified as proheparanase processing enzyme	[32]
2010	First evidence that heparanase-2 can inhibit heparanase function	[155]
2012	Demonstration that heparanase controls transcription by regulating the methylation of histone H3 tails	[148]
2015	Crystal structure of heparanase solved	[42]
2015	Discovery that heparanase can promote autophagy	[56, 57]
2016	Heparanase is required for the activation and function of macrophages	[140]
2015–2019	Heparanase shown to facilitate the spread of HS-binding viruses	[103–105]

[a]Advances in italics represent functions independent of heparanase enzymatic activity

3.2 Historical Overview and General Properties of Heparanase

Heparin and HS degrading activity was first reported in mammalian cells during the mid- to late-1970s, with rat liver lysosomes [1, 2], skin fibroblasts [3, 4], a mouse mastocytoma [5] and platelets [6, 7] being shown to contain such activity, although the functional relevance of HS degradation was unclear (Table 3.1). However, interest in heparanases increased dramatically in the early 1980s when studies by Nicolson and colleagues revealed that metastatic B16 melanoma cells contain a HS-specific endoglycosidase that releases HS fragments from the ECM of cultured vascular endothelial cells [8, 9]. Of particular interest was the observation that the heparanase activity of different B16 melanoma sublines positively correlated with the metastatic potential of the sublines [10]. Similarly, Vlodavsky and colleagues reported that a highly metastatic variant of a T lymphoma cell line very efficiently released HS fragments from a subendothelial ECM whereas the poorly metastatic parent line did not possess this activity [11]. These findings led to the proposal that heparanase(s) may aid tumor metastasis by degrading HS chains in the walls of blood vessels.

Unfortunately, further progress in understanding the biological relevance of heparanase was severely hampered by the inability to purify and clone the enzyme. In fact, it took another 16 years before the enzyme was finally cloned and characterized [12, 13]. This extraordinary delay was mainly due to the lack of a rapid, simple and reliable heparanase assay, most studies measuring by gel filtration the release of radiolabelled HS fragments from radiolabelled ECM [14–16]. This assay approach is semi-quantitative and consequently did not allow accurate estimates of heparanase recovery and purity. Solid phase heparanase assays using radiolabelled HS attached to solid support were also developed, the release of radiolabelled HS from the solid support being a measure of heparanase activity [17–19]. However, these assays suffered from difficulties in coupling HS to the solid support and potentially poor accessibility of the HS substrate to the heparanase enzyme. The assay problem was further compounded by the fact that most tissues contain very low levels of heparanase, only human placenta and platelets eventually being identified as containing sufficient quantities of heparanase for enzyme purification and characterization. The situation was made worse by HS-binding proteins in tissue homogenates binding to HS substrates and blocking HS cleavage. In fact, in the absence of reliable heparanase assays, vastly different molecular masses for heparanase were reported, ranging from 8–137 kDa [18, 20, 21], and claims were also made that heparanase had sequence homology, in one case, to heat shock proteins [22, 23] and in another report to the chemokine β-thromboglobulin [20]. A further complication was the proposal that at least three mammalian heparanases exist, based on the ability of different heparanase preparations to degrade a mast cell heparin precursor, heparin and/or HS [20, 24, 25].

Thanks to an outstanding effort, Craig Freeman in our laboratory developed a rapid and highly quantitative heparanase assay that was instrumental in us purifying human platelet heparanase to homogeneity [21, 26]. The assay took advantage of our finding that the HS/heparin-binding plasma protein, histidine-rich glycoprotein

(HRG), binds across heparanase cleavage sites in HS. Thus, when HRG was immobilized on beads radiolabelled HS bound strongly to the beads whereas if HS chains had been cleaved by heparanase they failed to bind to the HRG coated beads. One major advantage of the assay was that its ability to detect heparanase activity was unaffected by the presence of large quantities of irrelevant proteins, as occurs with tissue homogenates.

Of course, another reason for the long delay in cloning heparanase was that the cloning procedures employed in the 1980s and 1990s were tedious and technically difficult. They involved initially purifying the protein of interest to homogeneity, obtaining some amino acid sequence of the protein and then designing PCR primers, based on the available amino acid sequence, to eventually deduce the nucleotide sequence of the protein. We eventually obtained the N-terminal amino acid sequence as well as the sequence of 10 heparanase tryptic peptides but were still struggling to clone the enzyme. The breakthrough came with the emergence of expressed sequence tags (ESTs) as part of the human genome project, ESTs being short mRNA sequences generated by a single sequencing reaction from randomly selected clones from cDNA libraries [27]. Initially, the public EST database was incomplete and so many transcripts, including that of heparanase, were not represented. However, the database was being continually updated, and eventually, an EST appeared, derived from a human placenta cDNA clone, that contained the predicted nucleotide sequence of five of the heparanase peptides we had identified and encoded the 3′ end of the gene. Once this information was available, we were then able to use standard techniques to rapidly deduce the complete nucleotide sequence of human heparanase and, subsequently, rat and mouse heparanase.

In August 1998, one of the authors of this Chapter, Chris Parish, attended the Xth International Vascular Biology Meeting in Cairns, Australia. We were well aware of the major contributions Israel Vlodavsky and his colleagues were making to the heparanase field and so were excited to hear that he would be attending the conference. When Chris and Israel presented their heparanase research to the meeting, it was obvious that both groups had successfully cloned heparanase and were close to submitting their findings for publication. Also, following their conference presentations Chris and Israel compared their heparanase sequences, without giving away too much information, and it became abundantly clear that both laboratories had cloned the same protein. The question then arose how to proceed. Both Chris and Israel were not comfortable with a ‘race to the summit’ scenario and decided to submit their findings as back-to-back papers in *Nature Medicine*. In due course, the two manuscripts were submitted simultaneously and there followed an anxious wait. When the decision was received from the *Nature Medicine* Editor it was short and sweet – accept without change and a single sentence from the reviewers stating that ‘this work must be published’!

The two *Nature Medicine* articles [12, 13] reported that human heparanase consists of a polypeptide of 543 amino acids and has a molecular mass of 61.2 kDa, a finding confirmed by four other groups soon afterward [28–31]. However, the N-terminus of the enzymatically active enzyme was found to be 157 amino acids downstream of the initiation codon, implying that heparanase initially exists as a pro-enzyme that is proteolytically cleaved to yield the active enzyme. Indeed, it was

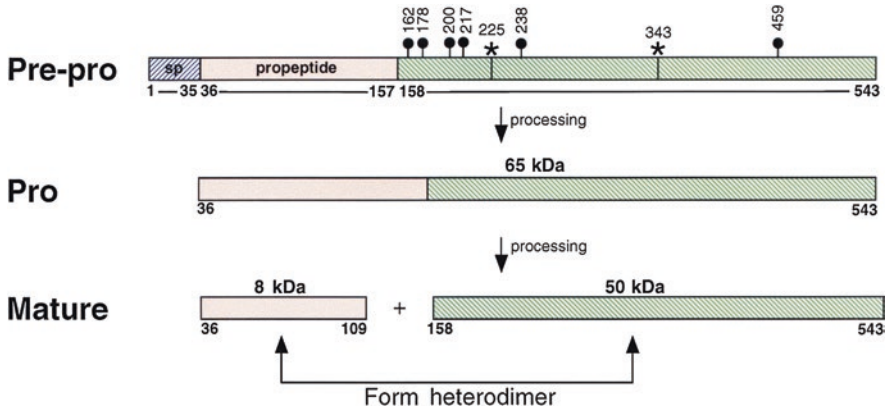

Fig. 3.1 Predicted structure and processing of human heparanase (*circa 2001)*. A schematic representation of the domain structure of the heparanase protein and the proposed processing steps to produce the active form of the enzyme are shown. The enzyme is predicted to be synthesized in a pre-pro-form, which is processed to an inactive pro-form upon removal of the putative signal peptide. The pre-pro form is then processed into the active mature enzyme by removal of amino acids 110–157 to give two polypeptides of 8 kDa (residues 36–109) and 50 kDa (158–543), which form a heterodimer. The locations of the six putative N-linked glycosylation sites (N162, 178, 200, 217, 238 and 459) are indicated by the solid circles, and the putative catalytic proton donor (Glu225) and nucleophile (Glu343) residues by asterisks. The domain boundaries are numbered. (Figure reproduced from Parish, et.al [41]. with permission)

subsequently reported that the enzyme is a heterodimer composed of a 50 kDa subunit (Lys^{158}-IIe_{543}) associated non-covalently with an 8 kDa peptide (Gln_{36}-Glu_{109}), with an interconnecting 6 kDa peptide (Ser_{110}-Gln_{157}) being excised by proteolysis (Fig. 3.1) [30]. Another 9 years elapsed before cathepsin-L was identified as the key protease that processes pro-heparanase to form the active heparanase enzyme [32].

One of the most intriguing aspects of the cloning of mammalian heparanase is, however, that there appears to be only one heparanase encoding gene in the mammalian genome. This surprising conclusion has remained unchanged for the last 20 years, in humans the gene being located on chromosome 4q21.3 [33], spanning 40 kb and being composed of 12 exons separated by 11 introns [13, 34]. We did discover, however, based on exhaustive sequence homology studies, that heparanase is a clan A glycoside hydrolase (family 79), with secondary structure predictions suggesting that heparanase contains an $(\alpha/\beta)_8$ TIM-barrel fold, which is characteristic of clan A glycoside hydrolases [35]. Soon afterward a human cDNA was identified by McKenzie et al. that encodes a protein, designated **heparanase-2**, that has 40% overall identity and 59% sequence resemblance with heparanase (heparanase-1), and resembles a family 79 glycoside hydrolase [36] although it was subsequently shown to lack enzymatic activity [37]. Recent research indicates, however, that although heparanase-2 lacks endoglycosidase activity it has a higher affinity for HS than heparanase-1 and thus, via competition for HS, inhibits heparanase-1 enzymatic activity [38]. Furthermore, heparanase-2 regulates a range of genes associated with tumor suppression, implying that heparanase-2 acts as a tumor suppressor, a truly remarkable finding (reviewed in [37]).

Additional, molecular modeling studies of heparanase-1 established critical active site residues, Glu^{225} being identified as the proton donor and Glu^{343} as the nucleophile (Fig. 3.1). Site-directed mutagenesis studies with human heparanase confirmed these predictions [35]. Thus, by 2001 we had a reasonably detailed understanding of the secondary structure of heparanase (Fig. 3.1), although at that stage the position of disulfide bonds within the molecule had not been determined, a deficiency that was rectified in 2007 with the report that heparanase has two disulfide bonds, namely Cys127-Cys179 and Cys437-Cys542, with the latter disulfide being essential for enzymatic activity [39]. We also predicted six glycosylation sites in human heparanase (Fig. 3.1) and relatively soon after this prediction was made it was reported that all 6 sites were glycosylated, with glycosylation not being required for enzymatic activity, but is required for heparanase secretion [40].

We then constructed a space-filling model of heparanase based on the crystal structure of the endo-1,4-β-xylanase from *Penicillium simplicissium*, a member of the glycoside hydrolase 79 family, the active site residues of heparanase being shown to be surrounded by patches of basic residues that could potentially bind to negatively charged HS [41]. Following these initial studies of the structure of heparanase, we keenly awaited the determination of the crystal structure of the molecule. Unfortunately, another 14 years elapsed before the 3D structure of heparanase was determined (Table 3.1) [42], although we were pleased to discover that the deduced structure did confirm essentially all of our predictions.

3.3 Overview of Substrate Specificity of Heparanase

Since HS is the substrate of the endoglycosidase heparanase, some structural and functional features of HS should be highlighted prior to discussing the enzymatic functions of heparanase. HS is a linear glycosaminoglycan consisting of repeating disaccharides of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) (reviewed in [43]). During biosynthesis of HS substantial changes are made to the molecule, sulfate groups being attached to specific hydroxyl groups, GlcNAc residues being N-deacetylated and N-sulfated and GlcA residues undergoing epimerization to become iduronic acids (IdoA). Such changes are not evenly distributed throughout HS molecules but tend to be concentrated in 'hot spots' of sulfation and epimerization and result in HS molecules that exhibit great structural diversity. In fact, >300 proteins have been shown to interact with HS, many of these proteins binding specifically to unique structural motifs in HS (reviewed in [43]).

HS is ubiquitously expressed on cell surfaces and in the extracellular matrix (ECM) of most animal species, with some studies suggesting that HS can also accumulate in the nuclei of cells (reviewed in [44]). A major function of HS is to provide a scaffolding with which HS-binding proteins can interact and become stably positioned within 3-dimensional space. A classic example of this process is the establishment of chemokine gradients, HS-binding chemokines interacting with HS and forming such gradients [45]. Similarly, HS molecules can oligomerize HS-binding proteins and act as a scaffold that promotes protein-protein interactions

(reviewed in [43]). These unique structural features of HS should be kept in mind when analyzing the functional consequences of heparanase degradation of HS chains.

In terms of substrate specificity we now know that heparanase is an endo-β-glucuronidase that cleaves HS chains at a limited number of sites, usually the non-reducing side of highly sulfated regions of HS [46], to yield fragments of ~3–5 kDa in size. Hydrolysis of HS chains proceeds via a double displacement mechanism, with the anomeric configuration of the substrate being retained and, consequently, heparanase being classified as a retaining glycosidase [47]. A number of studies indicate that the HS cleavage site consists of the linkage between GlcA and *N*-sulfoglucosamine that is either 3-*O*- or 6-*O*-sulfated, with the minimum sequence being a trisaccharide (reviewed in [48]). However, the substrate specificity of heparanase is not fully resolved, recent studies suggesting that the specificity of the enzyme is somewhat plastic, being dependent on saccharide structures around the cleavage site [49].

3.4 Functions Dependent on Heparanase Enzymatic Activity

3.4.1 HS Turnover

As discussed earlier, heparanase is the only known *endo*glycosidase in mammals that can cleave heparan sulfate (HS) chains, either free or attached to HS proteoglycans (HSPG) (reviewed in [37, 41, 50]). In contrast, there are at least 9 mammalian *exo*glycosidases present in lysosomes that very specifically and sequentially degrade HS chains from their non-reducing termini. The importance of these exoglycosidases in degrading HS is highlighted by their deficiency resulting in the massive accumulation of HS in lysosomes in various tissues and disease syndromes called 'mucopolysaccharidoses' (reviewed in [51]). It was originally thought that heparanase plays an important role in initiating degradation of HS chains, particularly as heparanase accumulates in lysosomes like the exoglycosidases that degrade HS [52, 53]. However, to date, there have been no reports of a mucopolysaccharidosis in humans resulting from heparanase deficiency [54]. Furthermore, heparanase deficient mice, although producing HS chains of higher molecular mass than their wild type counterparts, exhibit no signs of HS accumulation in their tissues [55]. These data imply that lysosomal heparanase is not required for HS turnover but it has been proposed that lysosomal heparanase promotes **autophagy** and thereby maintains cellular homeostasis in damaged cells [56, 57].

3.4.2 Involvement in Cell Invasion

It has been proposed for many decades that the subendothelial basement membrane represents a major barrier to the passage of cells through the blood vessel wall and entry into tissues. Basement membranes are a specialized type of ECM that separate

different cell types and tissues and are composed predominantly of collagen type 4, laminin and HSPGs [58]. To overcome this barrier it was discovered that invading cells use a battery of degradative enzymes that disassemble the basement membranes and allow the passage of cells [59, 60]. By the early 1980s, a number of proteases had been identified that degrade ECMs/basement membranes and aid tumor metastasis. Thus, when it was first reported in 1983 that heparanase activity correlated with melanoma and lymphoma metastasis [10, 11], the finding was received with much enthusiasm as it implied that heparanase aids **tumor metastasis** by cleaving HS chains, rather than polypeptide chains, in basement membranes. In fact, it was anticipated that proteases and heparanase(s) acted cooperatively to degrade BMs and allow the passage of cells, a view that was supported by a study some years later [61]. Furthermore, additional investigations confirmed that the metastatic potential of tumor cells correlated with their content of heparanase [62–64] and heparanase inhibitors were found to be very effective at inhibiting tumor metastasis (see Sect. 3.4.6 below). However, developments in this area were severely hampered by the heparanase enzyme not being cloned and characterized.

The situation changed dramatically in 1999 with the cloning of heparanase (Table 3.1). It was immediately shown that heparanase mRNA is highly expressed in metastatic rat and human mammary adenocarcinoma cell lines whereas the poorly metastatic parent cell lines contained little or no heparanase mRNA [12, 65]. In situ hybridization and immunohistochemical studies confirmed the mRNA results, with heparanase transcripts and protein expression being upregulated in highly invasive breast and colon cancers when compared to non-cancerous tissue nearby [65, 66]. It was also shown that stable transfection of lymphoma and melanoma cell lines with the heparanase gene increased the metastatic ability of the cell lines [13, 65]. Conversely, knockdown of heparanase transcripts reduced the metastatic potential of highly metastatic tumors [67–69]. These early studies also confirmed that most normal tissues contain very low levels of heparanase, the exceptions being lymphoid organs, peripheral blood leukocytes and the placenta [12, 28]. Thus, the results were consistent with the view that heparanase is mainly expressed by cells that are in an invasive rather than a resting state. In fact, subsequent studies have demonstrated that heparanase expression is enhanced in all major cancer types, namely carcinomas, sarcomas and hematological malignancies [50, 70–74]. Furthermore, numerous clinical studies have shown that upregulation of heparanase expression correlates with increased metastasis and poor prognosis [37, 50, 71, 74, 75]. However, it is increasingly being recognized that some tumors rely on heparanase being provided by components of the tumor microenvironment (e.g., fibroblasts, platelets), the tumor cells in these cases being essentially heparanase deficient (reviewed in [37]).

Initially, the focus of heparanase research was on the ability of the enzyme to facilitate tumor metastasis by degrading and remodeling ECM and basement membranes. Under normal circumstances, however, the function of heparanase is to aid the invasion of non-malignant cells through ECM barriers, with cells of the immune system entering sites of **inflammation** being major participants. In fact, it was demonstrated as early as 1984 that following activation T lymphocytes produce heparanase [76] and, soon after, neutrophils were shown to also release heparanase [77]. In both studies, it was proposed that the released heparanase plays a key role in leucocyte extravasation through subendothelial basement membranes. Support for

this view was the demonstration that heparanase inhibitors exhibit anti-inflammatory activity (see Sect. 3.4.6 below).

Subsequent studies demonstrated that in inflammatory responses heparanase is not only expressed by leucocytes, inflammatory cytokines inducing epithelial and/or endothelial cells to produce the enzyme and aid leucocyte entry into inflammatory sites [78–81]. In fact, in addition to cytokines a range of other stimuli are able to augment heparanase expression, e.g., high glucose, reactive oxygen species [78, 82] and estrogens [83, 84]. Based on these observations it is not surprising that heparanase has been shown to play a key role in the pathogenesis of a range of inflammatory disorders, notably inflammatory lung disease, rheumatoid arthritis and chronic colitis (reviewed in [70, 71, 85, 86]). However, inflammatory reactions are complex, involving multiple cell types and cytokines, and so the precise role of heparanase in inflammation remains to be elucidated. There is also the intriguing observation that inflammation is associated with cancer progression [87, 88] and mounting evidence that heparanase may play a central role in the induction of **inflammation-associated cancers** (reviewed in [71, 85, 89]).

3.4.3 Involvement in Release of ECM Bound Proteins

During both tumor progression and inflammatory responses, heparanase enzymatic activity not only contributes to the breakdown of ECM barriers and cell invasion but also results in the release of ECM bound proteins. Examples of such proteins are HS-binding growth factors, such as basic fibroblast growth factor, hepatocyte growth factor and vascular endothelial growth factor [90]. These factors are sequestered by HS in the ECM and are also protected from degradation by proteases. Following liberation from the ECM by heparanase, however, the growth factors become available to stimulate nearby cells, this process being particularly important for the induction of endothelial cell proliferation and resultant **angiogenesis** as well as wound healing (reviewed in [91–93]) [38, 94]. Released growth factors also remain associated with HS-fragments that can crosslink growth factors to their receptors and thereby enhance signaling by the receptors [95, 96]. Since >300 proteins have been identified that bind heparin/HS [43] and, consequently, can potentially bind to ECM- and cell surface-associated HS chains, the influence of heparanase on the functional behavior of these proteins must be immense and would be expected to underpin many biological processes, not just the release of HS-binding growth factors from the ECM.

3.4.4 Involvement in Depletion of Intracellular Anti-Oxidant Stores of HS

Usually, HS is predominantly expressed outside cells either in the surrounding ECM or on cell surfaces, with relatively little in the cytoplasm and nucleus of cells (see Sect. 3.3). In collaboration with Charmaine Simeonovic, we discovered,

however, that the insulin-producing β-cells in the Islets of Langerhans of the pancreas contain extremely high levels of intracellular HS [97]. We also discovered that this intra-islet HS made the β-cells extremely resistant to reactive oxygen species (ROS) by a mechanism that is being currently elucidated [97]. It is not surprising that β-cells possess such potent anti-oxidant activity as they are one of the most biosynthetically and metabolically active cells in the body. In Type-1 Diabetes (T1D) in mice and humans, however, autoreactive T cells against islet auto-antigens enter the islets and, via depletion of intracellular HS by T cell-dependent heparanase, render the insulin-producing β-cells susceptible to ROS killing [97, 98] (reviewed in [99, 100]). In addition, we found that T1D is particularly dependent on heparanase as auto-reactive T cells are required to not only pass through the subendothelial basement membrane of pancreatic blood vessels but also to traverse a basement membrane surrounding the Islets of Langerhans. This conclusion was further supported by the finding that a heparanase inhibitor (PI-88) markedly reduces diabetes incidence in NOD mice that spontaneously develop T1D (Simeonovic et al., Chap. 24 in this volume). An intriguing question that arises from these findings is whether other tissues/cells use cytoplasmic HS as an anti-oxidant and, as a result, are susceptible to heparanase exposure.

3.4.5 Facilitator of Spread of HS-Binding Viruses

Many viruses bind HS and use it as a co-receptor for infecting cells (reviewed in [101]). This observation provides an intriguing paradox, namely, if viruses bind HS how do they escape from a primary site of infection and spread to other organs, particularly as HS is ubiquitously expressed on cell surfaces and the ECM. A similar situation also occurs with the influenza virus, in this case, the hemagglutinin of the virus binding sialic acid, an interaction that facilitates influenza virus infection of cells but also impedes the spread of progeny virus. Influenza overcomes this problem by expressing neuraminidase, a glycosidase that removes sialic acid from neighboring molecules and allows virus spread (Fig. 3.2a) (reviewed in [102]). Thus, an attractive hypothesis based on these data is that HS-binding viruses recruit

Fig. 3.2 Schematic representation of the role of neuraminidase in influenza virus spread and the participation of heparanase in HSV-1 and VACV dissemination. (**a**) Neuraminidase, a major protein expressed on the surface of influenza virus, cleaves sialic acid from neighboring molecules, which prevents the influenza hemagglutinin from interacting with sialic acid and, consequently, promotes virus release. In contrast, heparan sulfate (HS) binding viruses like HSV-1 and VACV rely on heparanase (HPSE) mediated cleavage of HS in order to spread. However, unlike influenza, HSV-1 and VACV do not encode for such enzymatic activity. (**b**) In fact, HSV-1 has been shown to induce the expression of HPSE in infected cells, release of which results in degradation of extracellular matrix (ECM) and cell surface HS and allows localized spread of HSV-1. (**c**) In contrast, VACV being highly cytopathic attracts platelets to sites of infection, which in turn release pre-formed stored HPSE to promote inflammation in response to vascular injury. VACV released from the infection site ECM by platelet HPSE would also be expected to carry HS fragments, which could potentially block the virus from further interactions with ECM HS. This would allow an easier long-range spread of VACV (Figure reproduced from Khanna et al. [105] with permission)

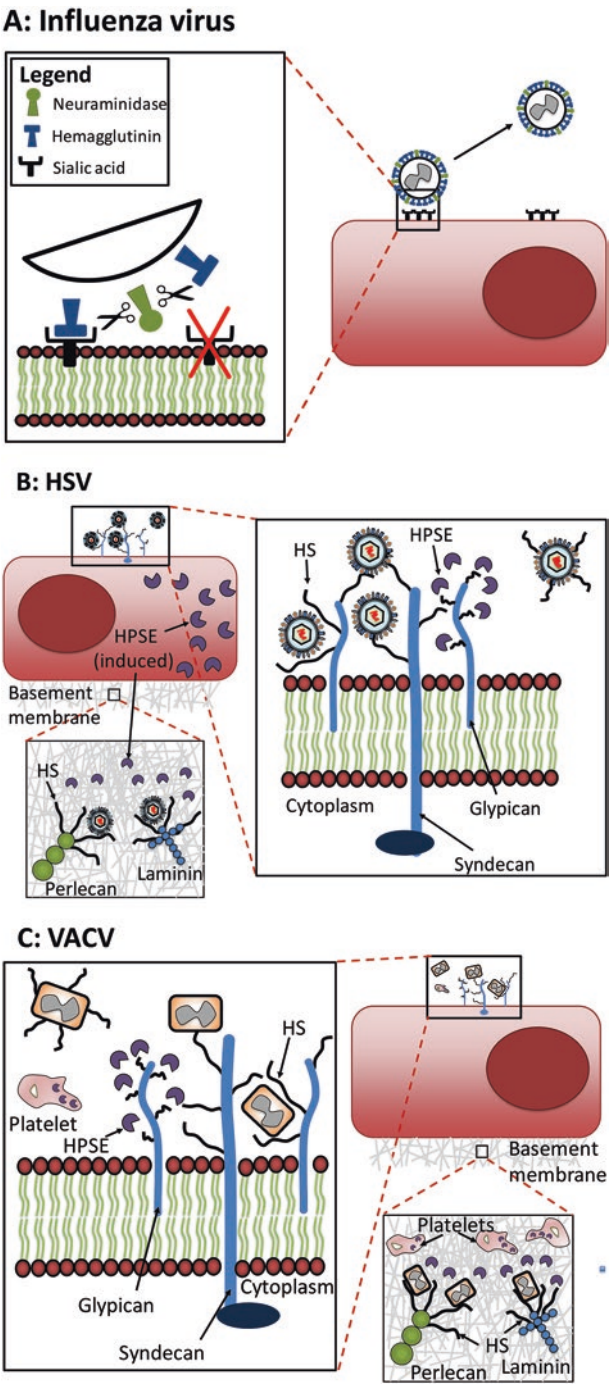

Fig. 3.2 (continued)

heparanase to degrade HS in the vicinity of the virus, although in this case, the heparanase must be host rather than virus derived.

In fact, herpes simplex virus-1 (HSV-1), an HS-binding virus, has been shown recently to induce the expression of heparanase in HSV-1 infected cells, the enzyme then allowing the localized spread of the virus (Fig. 3.2b) [103, 104]. We have undertaken similar studies with vaccinia virus (VACV), an HS-binding virus that very effectively spreads to distant organs. Using heparanase deficient mice we discovered that spread of the virus was substantially reduced and in some cases, depending on the inoculation site, delayed by up to 3 days (Fig. 3.2c) [105]. In contrast, heparanase deficiency had no effect on virus replication at the inoculation sites. Additional *in vitro* experiments showed that heparanase treatment released VACV from the ECM of infected HS-expressing cells but not from infected HS-deficient cells. We were also unable to find any evidence of VACV inducing heparanase expression in infected cells as was seen with HSV-1. In this case, we suggest that since VACV is highly cytopathic it attracts platelets to sites of infection, platelets being a rich source of pre-formed heparanase (Fig. 3.2c) [105]. It is also highly likely that VACV liberated from the ECM by heparanase carries HS-fragments that block any further interaction of the virus with the ECM. These findings suggest that heparanase inhibitors may interfere with the spread of HS-binding viruses, particularly if administered in the first few days after virus infection (Agelidis and Shukla, Chap. 32 in this volume).

3.4.6 Inhibitors of Heparanase Enzymatic Activity

Soon after heparanase had been implicated in tumor metastasis the search for heparanase inhibitors began, an endeavor that became more attractive following the discovery that there is only one heparanase gene in the mammalian genome. At the outset it was already known that heparanase enzymatic activity could be inhibited by heparin [10, 11], so initial inhibitors were derived from heparin and involved the production of heparin preparations with reduced anticoagulant activity, this being achieved by chemical modification [106, 107]. Further development of heparin-based inhibitors lacking anticoagulant activity has ensued since these early studies, although structural heterogeneity of heparin has made quality control difficult [50]. Sulfated polysaccharides were also identified that inhibit tumor metastasis via heparanase inhibition [108, 109], these studies leading to the production of sulfated oligosaccharides or HS mimetics with substantial heparanase inhibitory activity but much better safety profiles than sulfated polysaccharides [110]. Totally synthetic HS mimetics were also developed, such as sulfated linked cyclitols [111] and variants of suramin, polysulfated naphthyl urea, that have a better safety profile than the parent compound [112].

PI-88 (Muparfostat) resulted from the sulfated oligosaccharide approach and is the first heparanase inhibitor that has reached Phase III clinical trials (reviewed in [113, 114]). It contains a mixture of sulfated mannose-based oligosaccharides (Fig. 3.3) [110]. It was designed to simultaneously inhibit heparanase and a number of HS-binding pro-angiogenic growth factors, in the case of growth factors the drug blocking the cross-linking of growth factor/growth factor receptors by HS. PI-88

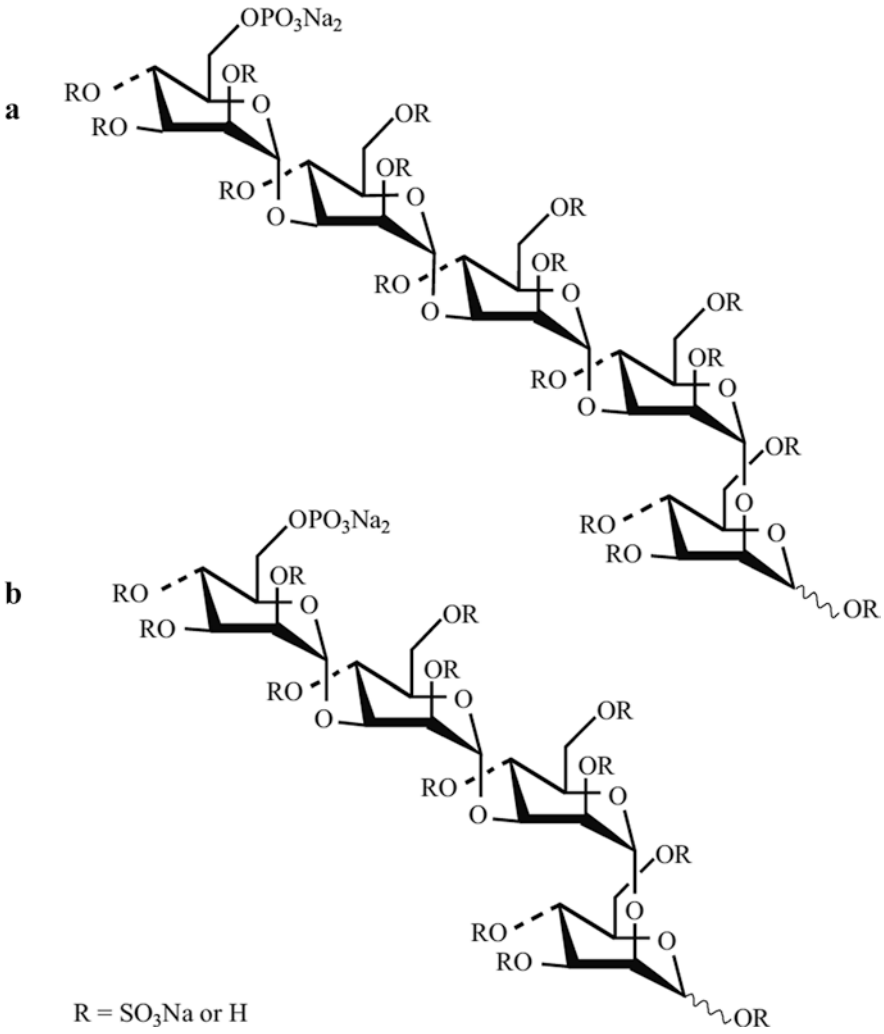

Fig. 3.3 The chemical structure of PI-88, the first heparanase inhibitor to reach a Phase III clinical trial. PI-88 is composed predominantly (~90%) of **(a)** phosphomannopentaose and **(b)** phosphomannotetraose sulfates, with the ratio between the pentasaccharide and tetrasaccharide ranging from approximately 2:1–3:2. This structural heterogeneity resulted in a drug that simultaneously inhibits heparanase and the pro-angiogenic activity of a number of HS-binding growth factors) (Figure reproduced from Khachigian and Parish [113] with permission)

has also been shown to block the enzymatic activity of endoglucosamine 6-sulfatases, these enzymes having pro-angiogenic activity [115]. The structural heterogeneity of PI-88 potentially increases the number of HS-binding proteins it interacts with and, thereby, reduces the chances of treatment escape variants arising in cancer patients. The most impressive preclinical data was obtained in the RIP-Tag2 tumor model, a mouse model of multistage pancreatic islet carcinogenesis. It was found that PI-88 acted at several stages of carcinogenesis from the formation of early progenitors to

invasive carcinomas, the drug inhibiting tumor cell proliferation, increasing tumor cell apoptosis, impairing angiogenesis and, ultimately, reducing the number of invasive carcinomas [94]. Based on this excellent preclinical data and acceptable Phase I clinical trial safety, the drug entered a randomized Phase II clinical trial in hepatocellular carcinoma patients, the drug exhibiting preliminary efficacy as adjuvant therapy following tumor resection [116]. A subsequent Phase III clinical trial, however, failed to significantly improve disease-free survival (DFS) in the overall treatment group but did significantly prolong DFS in the microvascular invasion group that constituted 40% of the trial population and includes patients with the poorest prognosis [117, 118]. The drug was also shown to have a good safety profile. These data indicate that Muparfostat has potential as a mono-therapy but, as an anti-metastatic and anti-angiogenic drug, is much more likely to exhibit efficacy when combined with other anti-cancer agents, such as checkpoint inhibitors. A second generation PI-88, PG545, has been developed that is a cholesterol-conjugated maltotetraose sulfate that has a much longer half-life *in vivo* than PI-88 and is structurally more homogeneous [119, 120]. PG545 has shown efficacy in acute kidney injury as a nephroprotective agent [121], inhibits colon cancer initiation and growth [122] and is a potent anti-lymphoma drug [123]. For more information see Chapters by Chhabra and Ferro; Hammond and Dredge; and Abassi and Goligorsky, Chaps. 19, 22 and 28 in this volume.

More recently a plethora of heparanase inhibitors have been identified based on high throughput screening of small molecule libraries and of natural products (reviewed in [124]. In addition, there have been successful approaches using biologicals, such as peptides [125] and oligonucleotide-based inhibitors [126], neutralizing antibodies [127], RNA interference [128] and immunotherapy [129] (Fig. 3.4).

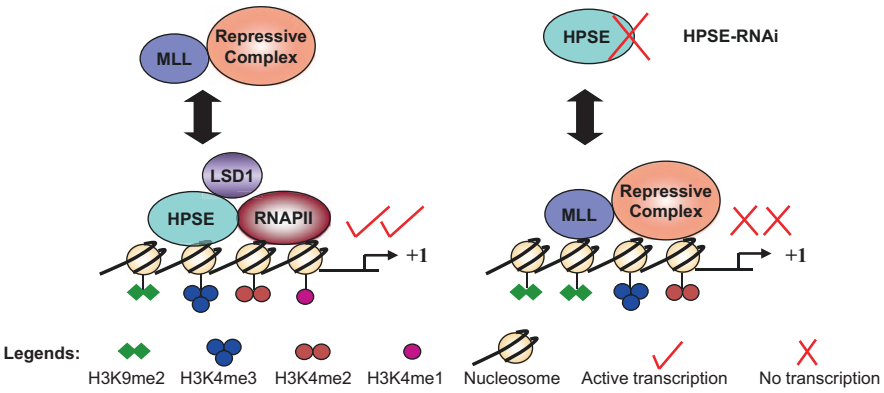

Fig. 3.4 Proposed model of the interplay of nuclear heparanase (HPSE) with LSD1, RNAP II, MLL, and histone methylation marks. In the left schematic, heparanase displaces the MLL-Repressive Complex and recruits the demethylase LSD 1 and RNAP II to the promoter of genes and imposes histone methylation marks that result in transcription. In contrast (right schematic), in the absence of heparanase (induced by heparanase-specific RNAi in this case), the methyltransferase MLL-Repressive Complex binds to gene promoters and changes the histone methylation marks such that transcription is halted

LSD1: lysine-specific histone demethylase 1; MLL: mixed lineage leukemia methyltransferase; RNAP II: RNA polymerase II (Figure reproduced from He et al. [148] with permission)

Despite all this activity, however, PI-88 (Muparfostat) appears to be the only heparanase inhibitor that has reached Phase III clinical trials, a drug that was developed over 20 years ago and is now off patent, the original PI-88 patent being filed in 1995 (Chhabra and Ferro, Chap. 19).

3.5 Functions Independent of Heparanase Enzymatic Activity

3.5.1 Cell Adhesion Molecule

The first evidence that heparanase performs functions independent of its enzymatic activity was reported in 1995, prior to the cloning and characterization of heparanase [130]. In this study, it was shown that at neutral pH, which is suboptimal for the enzymatic activity of heparanase, the enzyme acted as a cell adhesion molecule for $CD4^+$ T cells. In contrast, at acidic pH that is optimal for the glycolytic activity of the enzyme, heparanase aided $CD4^+$ T cell invasion of the ECM. In a related investigation in 2003, expression of heparanase in non-adherent lymphoma cells resulted in the cells becoming adherent and migratory regardless of whether the cells were transfected with either active or point mutated inactive enzyme, i.e., active site residues Glu^{225} and Glu^{343} were mutated [131]. This observation immediately implied that heparanase is able to act as a cell adhesion molecule, independent of its capacity to be an endoglycosidase, with other analyses correlating enhanced adhesion and migration of heparanase transfected cells with β1-integrin and Rac activation [131–133].

3.5.2 Promoter of Signal Transduction

At the same time that the cell adhesion results were being obtained using heparanase transfected cell lines, it was noted that exogenous addition of heparanase to endothelial cells resulted in enhanced protein kinase B (Akt) phosphorylation that was independent of cell surface HS and heparanase enzymatic activity [134]. Subsequent investigations (reviewed in [135]) found that enzymatically inactive heparanase was able to aid proliferation and survival of cancer cells by not only activating the signaling molecule Akt, but also other molecules such as signal transducer and activator of transcription (STAT), steroid receptor co-activator (Src) and extracellular signal-regulated kinase (Erk), as well as hepatocyte growth factor, insulin-like growth factor and epidermal growth factor receptor (EGFR) [50, 135]. Furthermore, this signaling function is mediated by the C-terminal domain of heparanase, which is totally devoid of endoglycosidase activity, overexpressing this domain in cancer cells augmenting signaling pathways and tumor growth [136–138]. A recent study has also shown that targeting either heparanase or the C-terminal domain of heparanase to mammary epithelium increases both mammary gland

development and tumor growth and metastasis [139]. Another recent report has revealed that heparanase is required for the activation and function of macrophages [140]. Collectively, these studies indicate that heparanase is a remarkably versatile molecule and a major facilitator of many aspects of inflammation and tumor progression, not just endoglycosidase-mediated leukocyte migration, tumor metastasis and angiogenesis.

3.5.3 Transcription Factor

A number of studies have detected heparanase in the nucleus of cells [141–144]. In fact, in patients with lung, neck and gastric cancers, the presence of nuclear heparanase is associated with a favorable prognosis, whereas patients with cytoplasmic heparanase have a poor survival [145]. There is also some evidence that nuclear heparanase expression is linked to cell differentiation [142–144, 146, 147]. Based on these observations we examined whether nuclear heparanase can regulate transcription in resting and activated T lymphocytes, using the human Jurkat T cell line as a well-researched model of T cell activation [148]. Initially, we noted that heparanase associated with transcriptionally active euchromatin, with T cell activation resulting in increased localization of heparanase to the nucleus, and heparanase being recruited to both the promoter and transcribed regions of a unique subset of transcriptionally active genes. Knockdown and overexpression studies showed that heparanase is required for the transcription of a number of immune response genes by associating with the lysine-specific histone demethylase 1 (LSD1), preventing recruitment of the mixed lineage leukemia (MLL) methyltransferase and, consequently, modifying the methylation pattern of histone H3, allowing recruitment of RNA polymerase II and transcription of the genes [148]. A schematic model of this process is depicted in Fig. 3.4. Based on these data it is clear that heparanase can enter the nucleus of cells, associate with both the promoter and transcribed region of a number of transcriptionally active genes, and enhance transcription via changing the methylation state of histone H3.

3.6 Future Perspectives

3.6.1 How Does Heparanase Initiate Signalling Pathways?

Although several studies have shown that the addition of exogenous heparanase to cells results in the induction of signaling pathways the actual cell surface receptor(s) that bind heparanase and initiate this response have not been identified. A number of receptors, however, have been defined that mediate the endocytosis and targeting of exogenous heparanase to lysosomes, namely the HS side chains of syndecan-1 [52], low-density lipoprotein receptor-related protein [149] and the cation-independent

mannose-6-phosphate receptor [150]. The rate of uptake of heparanase via these lysosome-targeting receptors is very high, which raises the possibility that leakage of a small percentage of endocytosed heparanase into the cytoplasm of cells could be sufficient to activate signal transduction pathways. If this hypothesis is correct it implies that heparanase 'hitches a ride' into the cytoplasm of cells and then interacts directly with signaling pathway molecules, rather than binding to and activating a specific cell surface receptor on plasma membranes.

3.6.2 Do Nuclear Heparanase and HS Interact?

It is well established that both heparanase and HS/HSPG can enter the nucleus of cells and, in both cases, influence transcription (see Sect. 3.5.3 and review [44]). There is, however, no information about whether or not these two molecules interact with each other in the nuclei of cells. We have already shown that heparanase is recruited to both the promoter and transcribed regions of a unique subset of genes that are transcriptionally active. Whether HS interacts with a similar subset of genes and either enhances or suppresses transcription would be of particular interest, with changes in the methylation status of histone H3 being the likely outcome of such an interaction. There are also studies showing that the structure of nuclear HS changes during the cell cycle [44]. How such changes influence the effects of HS on transcription would be a research area worth pursuing. It also appears that nuclear heparanase can be enzymatically active [44] and, therefore, would be able to liberate HS fragments from HSPG molecules that have entered the nucleus. It would be interesting to see whether these HS fragments are more able to interact with transcription factor complexes than HS chains linked to HSPGs.

3.6.3 Relationship Between Heparanase-1 and Heparanase-2

One of the most exciting recent development in the heparanase field is the realization that heparanase-2 can interact with heparanase-1, both directly and indirectly, and counteract many of the biological effects of heparanase-1 (reviewed in [37]). When heparanase-2 was first cloned in 2000 it was regarded as a distant relative of heparanase-1 and also, based on mRNA expression, appeared to have a different cellular distribution pattern to heparanase-1 [36]. Subsequent studies, however, have revealed that although heparanase-2 has no glycosidase activity it inhibits the enzymatic activity of heparanase-1, suppresses tumor growth and angiogenesis, and maintains cells in a differentiated state. Thus, heparanase-2 appears to counteract the pro-tumorigenic properties of heparanase-1 and behaves like a tumor suppressor. There is much to be done to understand the molecular basis of this intriguing interaction and eventually harness it for the development of new therapies. For further information on Hpa 2, see Chapters by E. Mckenzie and by Roberts and Woolf, Chaps. 34 and 35 in this volume.

3.6.4 Drug Development: Where to Next?

The discovery that there is only one heparanase gene in the mammalian genome and, thus, only one endoglycosidase that can degrade ECM HS, made heparanase a very attractive target for drug development. The subsequent finding, however, that heparanase deficient mice are essentially normal was a surprise, particularly as heparanase has been identified as a major contributor to many biological processes, not just tumor metastasis and angiogenesis. Certainly, these developments have made highly specific inhibitors of heparanase enzymatic activity much less attractive as cancer therapeutics. In fact, in heparanase knockout mice, upregulation of certain matrix metalloproteinases (MMP) occurs which probably aid migration of cells through basement membrane barriers and compensate for heparanase loss [55, 151]. Based on this conclusion, it would be expected that heparanase inhibitors would rapidly select for tumors that exploit this evasion strategy. There are also recent studies that indicate enzymatic degradation of basement membranes is not the only way cells can navigate their way through basement membrane barriers, cells passing through preformed entry and exit sites in basement membranes and/or providing mechanical forces that trigger basement membrane breaches (reviewed in [152]). Nevertheless, there may be specific situations where inhibitors of the enzymatic activity of heparanase are effective therapeutics, the most likely being localized inflammatory responses. Also, it appears that the microenvironment of tumors is a rich source of heparanase so targeting heparanase inhibitors to these sites, which are non-cancerous and, consequently, less prone to developing treatment resistance, is worthy of investigation.

On the other hand, the encouraging clinical trial data obtained with the HS mimetic PI-88 demonstrates that this class of drug has potential as an anti-cancer treatment, a key feature of PI-88 being that multiple HS-dependent processes were simultaneously targeted, not just inhibition of the enzymatic activity of heparanase. Similar considerations hold for PG545 (= Pixatimod) and SST0001 (= Roneparstat). Combining HS-mimetics with other, complementary therapies, such as checkpoint-inhibitors and conventional cytotoxic drugs also deserves attention. The discovery that heparanase can act as a promoter of signaling pathways and as a transcription factor obviously opens up the possibility of completely different heparanase-based therapeutics, although how these will be identified will be a major challenge. Finally, the demonstration that heparanase-2 counteracts the multifaceted pro-tumorigenic properties of heparanase-1 raises the possibility of therapeutics that enhance the tumor suppressive activities of heparanase-2. Inhibition of the pro-inflammatory features of heparanase, which may or may not have anti-tumor effects, is another factor that needs to be considered when developing heparanase-based therapeutics. Thus, despite heparanase inhibitors being developed for almost 40 years, overall heparanase still represents an attractive target for multiple therapeutic strategies.

3.7 Concluding Remarks

There is now overwhelming evidence that heparanase belongs to that rapidly expanding class of proteins that perform multiple tasks and are termed 'multifunctional proteins' [153]. In the case of heparanase, multifunctionality is evident from the ability of the molecule to simultaneously act as an endoglycosidase, a signaling molecule and a transcription factor (Table 3.1). But since the substrate specificity of heparanase is HS this results in the enzymatic activity of the molecule also having multiple functional effects. As discussed earlier in Sect. 3.3, over 300 proteins interact with HS and as a result of this interaction often form oligomers in 3-dimensional space that would be disassembled following exposure to heparanase, resulting in a diverse range of functional consequences. Another unique feature of heparanase is that it is present in multiple locations within and outside cells, namely in the ECM, on cell surfaces, in the cytoplasm, within lysosomes, endosomes and exosomes and, finally, within the nuclei of cells. In fact, an analysis of 235 immune-related proteins that could potentially bind HS revealed that heparanase was *the only HS-binding protein* that is found at intracellular, extracellular and plasma membrane locations [154]. Based on these findings it is not surprising that heparanase influences so many biological processes and, for example, is claimed to promote all aspects of tumour development [37]. Such discoveries indicate that heparanase research has a bright future indeed!

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Chapter 4
Involvement of Syndecan-1 and Heparanase in Cancer and Inflammation

Felipe C. O. B. Teixeira and Martin Götte

4.1 Introduction

4.1.1 The Syndecan Family of Heparan Sulfate Proteoglycans

Glycosaminoglycans (GAG) are sulfated polysaccharides composed of unbranched chains of repetitive disaccharide units of uronic acid and glucosamine. One of the most studied GAGs is Heparan sulfate (HS), a complex GAG present in virtually all animal cells [1, 2] (Fig. 4.1). Via a linkage tetrasaccharide, HS can be O-glycosidically attached to serine or threonine residues of proteins in the cell membrane, or to proteins which are secreted into the extracellular matrix (ECM), thus forming Heparan Sulfate Proteoglycans (HSPG)[1, 2]. The HS chains present a vast structural complexity due to the possibility of inserting different disaccharide units during biosynthesis. The insertion of sulfate radicals residues largely contributes to its negative charge characteristic, and the degree of sulfation usually ranges between 0.6–1.5 sulfates per disaccharide [3]. The iduronic or hexuronic acid can be sulfated at the position 2, while the glucosamine can be N-sulfated, N-acetylated and/or O-sulfated in positions 6 and 3. Besides its sulfation pattern, the molecular weight can widely vary between HS chains, ranging frequently between 5–70 kDa [4]. Another important characteristic is the considerable amount of non-sulfated regions of glucuronic acid and N-acetyl glucosamine, allowing the HS chains to organize itself in

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N-acetylated and N-sulfated domains. Each domain has distinct structural characteristics that dictate the kinds of molecules that can interact with them [2]. The synthesis of HSPGs occurs in the Golgi apparatus and is dependent on many enzymes which catalyze the different steps of HS elongation and modification in a sequential manner. The majority of HS deregulation in disease occurs as a result of alterations in the expression of enzymes involved in its synthesis, however, in some cases, the deregulation is also due to alterations in the core protein [5].

Syndecans (Sdc) are a family of transmembrane HSPGs expressed at the surface of several cell types, although they have also been found in the nucleus [6]. Moreover, their intact extracellular domains can be shed into the extracellular environment [7]. In mammals, this family is composed of four members that are expressed in a highly regulated cell-specific manner [8]. Among the syndecans, Sdc1 is the most highly expressed HSPG on the cell membrane of epithelial and plasma cells. Both the GAGs heparan sulfate and chondroitin sulfate (CS) are bound to its protein core [9]. Neuronal cells and cartilage express mainly Sdc3, while mesenchymal cells express Sdc2 and Sdc4, but have Sdc1 as well [10]. Sdc4 is expressed by most cell types [8]. All syndecans are composed of three different domains: an extracellular N-terminal domain where several glycosaminoglycan chains are covalently attached, a single transmembrane domain and a cytoplasmic C-terminal domain, which is subdivided into two constant domains separated by a variable region (Fig. 4.1). The extracellular domains of syndecans mediate various cell-cell

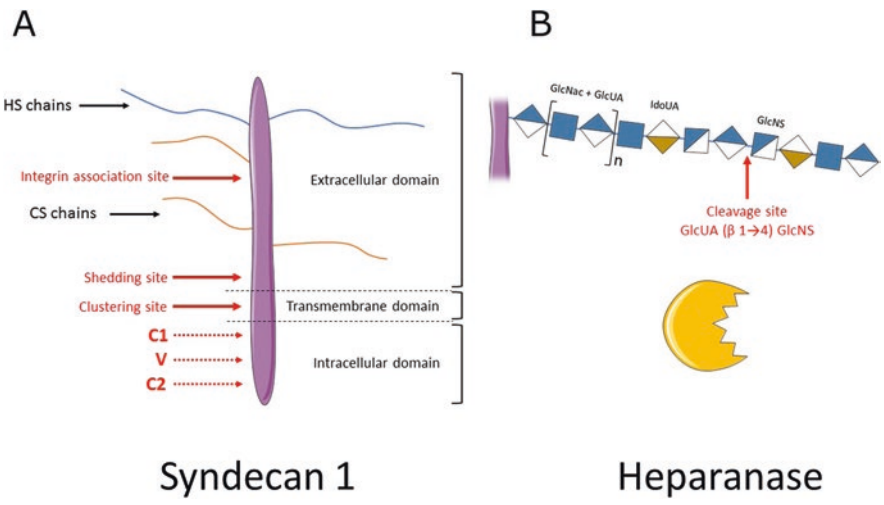

Fig. 4.1 Structure and specific protein and glycosidic domains of Syndecan-1 and Heparanase function. **(A)** The representative structure of the Syndecan-1 core protein. The different domains are represented on the right side and the GAG chains are represented in blue (Heparan Sulfate) and orange (Chondroitin Sulfate). The red arrows represent the various protein domains important for the interaction with other proteins. **(B)** Example of a Heparan Sulfate chain. The different monosaccharidic components are represented. The Heparanase cleavage site is represented in red

and ECM-cell interactions dependent on their GAG composition. This domain, in turn, varies significantly between the members of the syndecan family, unlike the transmembrane and cytoplasmic domains, which are highly conserved [11]. Although most studies focus on describing the importance of the extracellular and intracellular domains of syndecans, the transmembrane region was described to be sufficient in reducing cell migration via alterations in focal adhesion dynamics [12]. The GAGs attached to syndecans have a structure capable of directly interacting with many growth factors, cytokines, chemokines, and other macromolecules and enzymes found in the ECM, leading to the presentation to their receptors on the cell membrane. These interactions mediated by the GAG chains imply several physiological activities attributed to syndecans, such as cell proliferation, migration, and invasion [13]. The cytoplasmic domains of syndecans are capable of interacting with various intracellular kinases and the cytoskeleton, promoting important intracellular signaling [11]. Syndecans can also interact with other family members as structural analysis has shown that Sdc1 is less likely to form homodimers than the other syndecans, and can form heterodimers with Sdc2 and Sdc3 but not with Sdc4 [14, 15]. Further studies are needed to better understand the implications of these interactions. These diverse intra- and extracellular interactions with different molecules characterize the syndecans as key molecules in various physiological and pathological cellular functions. In this review, we will mainly focus on the role of the prototype member of the family, Sdc1, which is best characterized for its functional interplay with heparanase in the context of inflammation and cancer.

The extracellular domain of Sdc 1 harbors five GAG-attachment sites. Three are close to the N-terminus and two are close to the cell membrane. While HS and CS can be located near the N-terminus, only CS is found near the plasma membrane. Cells can vary the number of HS and CS chains added to Sdc1, but it always has at least one HS chain at its N-terminus [10]. Studies have shown that the number of GAGs attached to Sdc1 also affects its function, such as reducing its ability to mediate cell-cell interaction and cell invasion through the ECM [16]. The extracellular domain of Sdc1 can be shed by many proteases, such as matrix metalloproteinases (MMPs) and A-disintegrin and matrix metalloproteinases (ADAMs) [17]. Currently, there are no reports of endogenous extracellular trimming of CS chains in proteoglycans of mammalian cells. The presence of extracellular CS chains close to the plasma membrane can alter its susceptibility to cleavage and shedding by proteases, as well as change its ability to associate with receptors and other syndecans. Injuries or bacterial infections may lead to upregulation of Sdc1, heparanase, and proteases that can release the ectodomain fragment of Sdc1 [18–20]. In vitro, Sdc1 ectodomain has been shown to decrease proliferation of tumor cells [21], whereas another study using tumor cells expressing uncleavable membrane-bound Sdc1 showed increased proliferation and decreased invasion [22]. These events are regulated by HS chains in Sdc1. The length and sulfation of these chains can vary between cell types due to the action of several enzymes that regulate glycan elongation and modification [23]. Of these enzymes, cleavage by heparanase is of particular importance.

4.1.2 Heparanase – A Key Enzyme in ECM Remodeling

Heparanase is an endo-β-glucuronidase that degrades specific domains of HS chains (Fig. 4.1). After being produced in the rough endoplasmic reticulum, it is sent to the Golgi apparatus and secreted to the ECM in its inactive, latent form [24]. Subsequently, it can interact with Sdc1 at the plasma membrane and be endocytosed to reach the lysosomes, where it can be proteolytically activated [25]. Following this processing step, heparanase can reach the ECM again, where it executes extracellular functions [25, 26], or act inside the cell and modulate intracellular processes [26]. Despite its enzymatic activity, studies using mutated inactive heparanase have demonstrated that it can also exert activities unrelated to HS degradation. Enzymatically inactive heparanase can facilitate adhesion and migration of endothelial cells [27], promote phosphorylation of signaling molecules such as Akt and Src [27–29], as well as activate receptor tyrosine kinases such as EGFR [30]. For example, in head and neck carcinoma, heparanase was also shown to facilitate the formation of lymphatic vessels and the migration of tumor cells toward these vessels [31].

Most of what is known about heparanase function are based on its activity in cancer, where heparanase RNA and protein levels are increased in many different forms of malignant diseases [32] (Table 4.1). By degrading HS chains, heparanase alters many regulatory paths, mainly by augmenting the bioavailability of growth factors previously bound to the HS. This diffusion of growth factors and cytokines affects many different physiological processes, such as cell migration, angiogenesis, inflammation, coagulation, autophagy, and exosome production [33, 34]. Most members of the syndecan family are involved in these activities. In angiogenesis, for instance, Sdc1, Sdc2, and Sdc3 have important effects related to their ability to bind and present pro- and anti-angiogenic factors [35–37].

4.2 The Heparanase-Syndecan Axis

There is ample evidence that heparanase can affect cell behavior by regulating the structure and function of HSPGs. The Heparanase/Syndecan Axis (commonly referred as the Heparanase/Syndecan-1 axis due to most evidence showing a major role for Sdc1 on this process) has been shown to affect signaling cascades in healthy or malignant cells and biological systems (Fig. 4.2). The most studied growth factors that are strongly regulated by this axis are hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF). Other than just stimulating syndecan shedding, the cleavage of heparan sulfate chains by heparanase also makes it easier for additional proteins to recognize and bind to syndecan [38]. One example is the binding of the protein lacritin, a prosecretory epithelial mitogen that is expressed in the tear ducts and can bind directly to syndecan-1 core protein only after the trimming of HS chains by heparanase [39]. The heparin-binding domains of heparanase also

Table 4.1 Dysregulation and prognostic value of Syndecan-1 and heparanase in cancer

Cancer type	Syndecan-1			Heparanase
	Cell-surface	Stromal	Serum	
Bladder	Decreased expression related to poor prognosis [202, 203]	Higher expression related to poor prognosis [203]	Higher expression related to poor prognosis [203]	Higher expression related to poor prognosis [204, 205]
Breast	High expression is associated with poor prognosis in most subtypes [206–210]	High expression is associated with poor prognosis [206]		Higher expression related to poor prognosis [211, 212]
Colon	Downregulation is associated with more aggressive clinicopathological parameters [213–215]	Syndecan-1 is upregulated in the stroma of a fraction of tumors [214]		Higher expression related to poor prognosis [216]
Gallbladder	Higher expression related to poor prognosis [217]			Higher expression related to poor prognosis [219]
Gastric	Decreased expression related to poor prognosis [219, 220]	Higher expression related to poor prognosis [219, 220]		Higher expression related to poor prognosis [221]
Head and neck	Decreased expression related to poor prognosis [222, 223]	Higher expression related to poor prognosis [224]		Higher expression related to poor prognosis [225]
Kidney	In clear-cell renal cell carcinomas, decrease with increasing nuclear grade [226].			Higher expression related to poor prognosis [227, 228]
Liver	Decreased expression related to poor prognosis [229]		Higher expression related to poor prognosis [230]	Higher expression related to poor prognosis [231]
Lung	Decreased expression related to poor prognosis [232]		Higher expression related to poor prognosis [233, 234]	Higher expression related to poor prognosis [235, 236]
Melanoma		Low infiltration with Sdc1-positive plasma cells is related to good prognosis [237]		Higher expression related to poor prognosis [238–240]

(continued)

Table 4.1 (continued)

Cancer type	Syndecan-1			Heparanase
	Cell-surface	Stromal	Serum	
Oral	Decreased expression related to poor prognosis [241]	Higher expression related to poor prognosis [242]		Higher expression related to poor prognosis [243]
Ovarian	Decreased expression related to poor prognosis [244]	Higher expression related to poor prognosis [244, 245]		Higher expression related to poor prognosis [246]
Pancreas	Higher expression related to favorable prognosis [247]	Higher expression related to poor prognosis [247]		Higher expression related to poor prognosis [248]

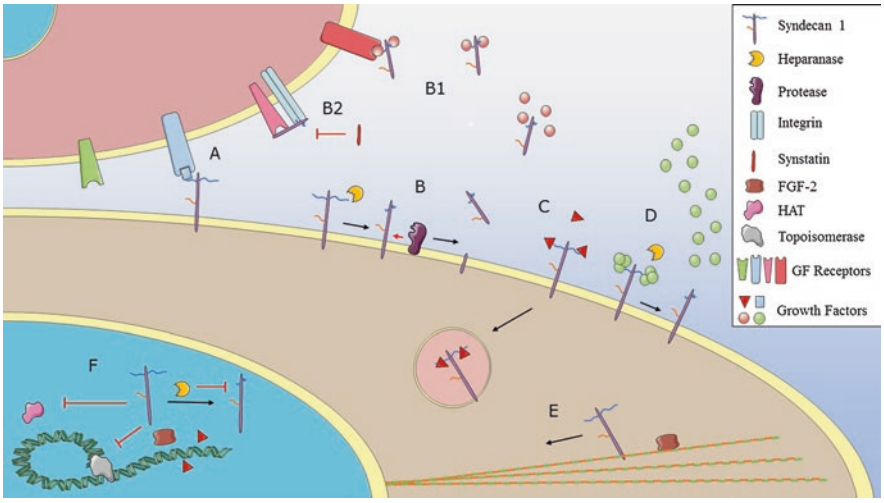

Fig. 4.2 The different functions of Syndecan-1 and how it can be affected by Heparanase. Syndecan-1 participates in many important physiological functions, such as (**A**) presenting growth factors to receptors on the cell surface or on the membrane of neighboring cells. (**B**) The trimming of HS chains mediated by heparanase exposes Sdc1 to the recognition and shedding by proteases, what can lead to (**B1**) binding and accumulation of factors in the extracellular matrix and presentation to receptors in distant cells, or (**B2**) binding to both integrins and directly to some receptors, activating them and leading to activation of intracellular pathways, what can be inhibited by Synstatins. (**C**) Some extracellular factors can trigger Sdc1 endocytosis, this endosome can be degraded or translocated into the cell nucleus and the factors can act as transcription factors. (**D**) Sdc1 can sequester factors from the extracellular environment, binding them to their GAG chains. Heparanase releases them into the extracellular matrix allowing them to act on their specific receptors. (**E**) The translocation of Sdc1 to the nucleus is highly dependent on tubulin, and it is also responsible for FGF-2 shuttling to the nucleus. (**F**) Sdc1 known nuclear actions include inhibiting DNA topoisomerase I activity and blocking the enzyme histone acetyltransferase (HAT) activity, controlling cell proliferation and differentiation. The trimming of the HS chains by heparanase decreases the level of nuclear Sdc1

facilitate the clustering of Sdc1 and Sdc4 on the membrane of human glioma cells, initiating signaling cascades that lead to augmented cell adhesion and spreading. Notably, this activity does not require the HS degrading activity of heparanase [40].

4.2.1 Heparanase Mediated Sdc1 Shedding

The proteoglycans present in the cell membrane have many important roles in health and disease. One example is their strong interaction with the tumor stroma, allowing the activation of numerous pathways in cancer cells [7]. Several of these proteoglycans can also undergo an enzymatic shedding of their extracellular domains by proteases known as 'sheddases'. In this process, the intact ectodomain of the proteoglycan is converted to a soluble molecule, allowing them to stay functional even after solubilization [7]. The shedding of membrane-bound proteoglycans is a controlled process that regulates many physiological and pathological conditions. With the exception of glycosylphosphatidyl-inositol (GPI)-anchored proteoglycans, such as the Glypican family, all membrane-bound proteoglycans are shed by a protease [1, 7].

The shedding of proteoglycans can be regulated at different levels. One of the mechanism is the regulation of the expression of sheddases and proteases inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), or the binding of these inhibitors to the GAG chains, either inhibiting or enhancing the proteoglycan activity, or even protecting them from degradation [41, 42]. The shedding occurs constitutively in cells, but it can also be enhanced by several stimuli, such as growth factor signaling, bacterial and viral stimuli, cell stress, among others [43, 44]. One of the most well-characterized actions of heparanase is its ability to facilitate Sdc1 shedding [45–47]. By trimming the HS chains on Sdc1, the core protein becomes more vulnerable to the actions of proteases [38]. All syndecans can be shed in vivo and in vitro by a process mediated by plasmin, thrombin, MMPs and/or ADAMs, and its proteolytic cleavage can be modulated by different signaling pathways such as protein kinase C (PKC), and the protein tyrosine kinase (PTK) MAP kinase [41]. After Sdc1 shedding, its remaining cytoplasmic C-terminal fragment (cCTF) undergoes proteolysis by γ-secretase and the proteasome. Nevertheless, this fragment has also been shown to inhibit lung cancer cell migration and invasion through the phosphorylation of Src, FAK, and Rho-GTPase, which blocks Sdc1-dependent migration and invasion, and also reduces lung tumor formation by these cells [48].

In tumors, shed Sdc1 plays multiple roles such as the delivery of growth factors to both tumor and host cells and triggering of signal transduction events at the cell surface [1, 41]. Most known functions of Sdc in the tumor context are mediated by the membrane-bound Sdc on cancer cells. However, tumor stroma syndecan has been shown to also have an important role in this context, either bound in the membrane of stromal cells or soluble ectodomains, which can be generated by cleavage from the tumor cells or other cell types such as cancer-associated fibroblasts, endothelial cells or leukocytes. In myeloma cells, the up-regulation of heparanase or

the addition of recombinant heparanase to the media was shown to lead to enhanced Sdc1 expression and shedding [45, 46, 49]. This was mostly due to the heparanase mediated activation of ERK signaling, leading to enhanced expression of MMP-9. This proteinase cleaves Sdc1 in the juxtamembrane region, releasing an intact ectodomain to the extracellular matrix [50, 51]. This effect in myeloma cells is interesting because it is highly dependent on the HS degrading activity of heparanase, whereas in other cells types ERK signaling can be triggered by heparanase, independent of its enzymatic activity [52, 53]. In a lung inflammation model in mice, soluble Sdc1 has been shown to be required for the formation of chemotactic gradients regulating leukocyte-endothelial interactions and angiogenesis [54, 55]. Another interesting finding regarding the shed ectodomain of Sdc1 is the observation that it can be transported to the nucleus, where it can influence transcription by the inhibition of histone acetylation [6, 10, 56] (see also Purushothaman and Sanderson, Chap. 12 in this volume).

4.2.2 Heparanase and Sdc1 in the Nucleus

Sdc1 has been found in the nucleus of many cell types such including malignant mesothelioma, myeloma, neuroblastoma, lung adenocarcinoma, and breast carcinoma [57]. In addition, Sdc2 has been found in the nucleus of injured cerebral cortex neurons, astrocytes, and chondrosarcoma [58, 59]. Apart from localization to the cytoplasm and cell membrane, heparanase can also be found in its active form in the nucleus and this localization is correlated with cell differentiation [60]. From a mechanistic perspective, a lot remains to be uncovered about how HSPGs enter the nucleus and regulate nuclear processes. In the nucleus of mesothelioma cells, Sdc1 has been shown to co-localize with heparanase and fibroblast growth factor-2 (FGF-2)[61]. The amount of HSPGs in the nucleus is increased after inhibition of PKC and drastically diminished after its stimulation [62]. Apparently, FGF-2 seems to trigger its nuclear translocation by stimulating the dephosphorylation of the protein core [63].

The nuclear translocation of Sdc1 has been shown to be highly dependent on tubulin both in malignant and normal cells. Sdc1 co-localizes with tubulin at the mitotic spindle during all phases of mitosis and the interference with tubulin integrity blocks the transport of Sdc1 to the nucleus [6]. During mitosis in malignant mesothelioma cells, Sdc1 accumulates in the nucleus by associating with tubulin structures and inhibits cell cycle progression, proliferation, and migration [57]. Nuclear FGF-2 and HS also seem to regulate cell cycle in many cell types. The nuclear entry of FGF-2 happens in the G1 restriction point of the cell cycle and exogenous HS arrests cells in the G1 phase in a transient manner [64, 65]. A decrease in nuclear HS allows the regular cell cycle progression. The arrest of cells in the G2/M phase of the cell cycle efficiently blocks the nuclear translocation of Sdc1. It has been shown that the entire molecule is present in the nucleus, with both ecto- and endodomains and the HS chains [6]. The HS chains of HSPG were found to be

important for the uptake and nuclear translocation of many different molecules, including heparanase [60]. The mechanism of FGF-2 shuttling into the nucleus is dependent on both the FGF receptor and HSPGs [66]. Sdc1 and FGF-2 share the same tubulin-mediated route to the nucleus, where they co-localize with heparanase. The minimal peptide sequence required for the tubulin-dependent nuclear translocation of Sdc1 is the juxtamembrane RMKKK motif that acts as a nuclear localization signal (NLS) [61]. Replacement of arginine (R) in the RMKKK motif dramatically reduces the amount of nuclear Sdc1, and complete deletion of this sequence abrogates the nuclear translocation of Sdc1 [67].

Many of the known nuclear functions of Sdc1 are associated with the HS interaction with various growth factors and nuclear structures. It is known that Sdc1 can be found in the nucleus as an intact HSPG, but it is still unclear if its actions are dependent only on the GAG chains or the entire molecule. The HS chains are important for nuclear translocation or degradation of Sdc1 and its negative charges facilitate their nuclear interactions. HS can compete with DNA for the binding of proteins such as transcription factors and enzymes. Sdc1 main known nuclear actions are to shuttle FGF-2 into the nucleus, inhibit DNA topoisomerase I activity and inhibit the activity of the enzyme histone acetyl transferase (HAT), controlling cell proliferation and differentiation [63, 64, 68]. Trimming of the HS chains by heparanase decreases the level of nuclear Sdc1, leading to an enhanced HAT activity and, consequently, augmented expression of genes that promote an aggressive phenotype in tumor cells such as MMP-9, VEGF, HGF and RANKL [69, 70]. It has been shown that HS competes with the DNA for topoisomerase I binding in the nucleus and is also able to dissociate the DNA-topoisomerase I complex, that is necessary for the unwinding of supercoiled DNA during transcription. This would suggest a negative effect of nuclear HS on gene transcription [68]. Activation of EGFR leads to nuclear translocation of heparanase and degradation of HS, increasing the activity of topoisomerase I and cell proliferation [71].

4.2.3 Effects on Exosome Formation and Function

Exosomes are secreted to the extracellular matrix by virtually all cell types [72]. They are very small vesicles ranging between 30–100 nm in diameter, but their classification comes from their endosomal origin rather than size. In mammalian cells, the biogenesis of exosomes begins with the invagination of the cell membrane forming the primary endocytic vesicles, which leads to the fusion of these vesicles and the formation of the early endosomal compartment [72]. During its maturation into late endosomes, it changes the protein composition of its membrane and receives many components derived from the trans-Golgi network. In the meantime, a second invagination occurs at the interior of the endosome forming the intraluminal vesicles (ILVs), that, consequently, have the same topology of the cell membrane (inside-in/outside-out) [73]. The late endosome containing many ILVs is called a multivesicular body (MVB). When it fuses with the plasma membrane, their ILVs

are released to the extracellular environment and, upon release, the ILVs are called exosomes [72, 73]. Both MVB and ILV formation is dependent on the endosomal sorting complex required for transport (ESCRT), but also on specific membrane domains and oligomerization/clustering processes [74, 75]. ESCRT-0 leads the clustering of mono-ubiquitinated cargo proteins into the endosome. ESCRT-I and ESCRT-II are related to the membrane deformation and the intraluminal budding of these cargos. ESCRT-I recruits ESCRT-III via ESCRT-II or through the adaptor protein ALG-2 interacting protein X (ALIX), resulting in ESCRT-III stabilization. ESCRT-III induces vesicle scission, and the vacuolar protein sorting (Vps) 4 ATPase mediates the final step of dissociation and recycling of ESCRT-III leading to IVL formation [74].

The way by which Syndecans influence the biogenesis of exosomes is through the interaction with the syntenin/ALIX complex[76–78] (Fig. 4.3). Syntenin is a small cytosolic protein that contains two PSD95/Dlg/Zonula occludens 1 (PDZ) domains. Both PDZ domains are necessary for syntenin membrane localization and the high-affinity interaction with syndecans [79, 80]. Syntenin can interact with both Sdc core protein and ALIX, which leads ALIX to bind to ESCRT-III, the complex required for the IVL formation in multivesicular bodies. The HS chains in Sdc are essential for this activity [76]. In some human cancer cell types, when the cells are exposed to exogenous heparanase or the endogenous expression of heparanase

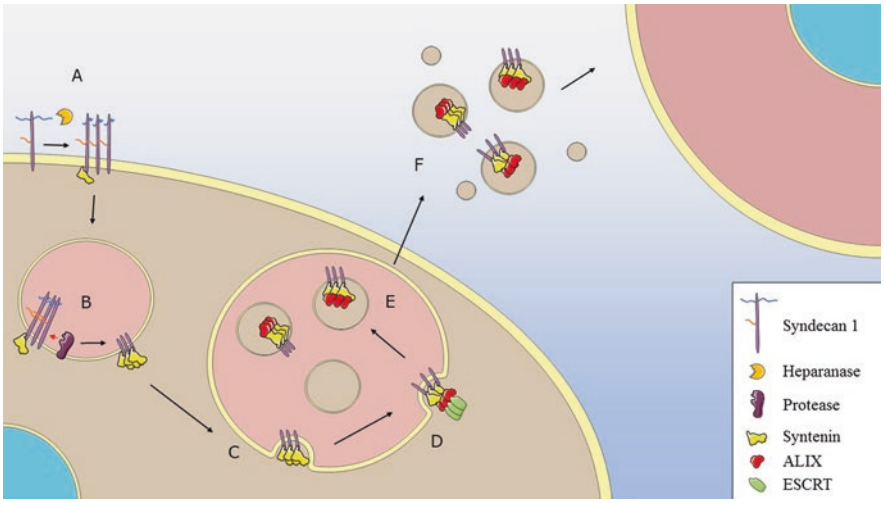

Fig. 4.3 Syndecan-1 and Heparanase mediated exosome formation. (**A**) Heparanase acts by trimming the HS chains in Sdc1, which facilitates its clustering. This, stimulates the binding of the cytoplasmic domains of syndecan to syntenin and its internalization. (**B**) This cluster of Sdc1 is recognized by proteases and cleaved. (**C-D**) After cleaving of the core protein, syntenin interacts with both Sdc1 and ALIX, which leads ALIX to bind to ESCRT-III and (**E**) intraluminal vesicles formation in the multivesicular bodies. (**F**) After releasing to the extracellular environment, these vesicles are called exosomes and can carry many different factors to distant sites throughout the body

is enhanced, exosome secretion increases dramatically. This process is dependent on the HS trimming activity of heparanase, as enzymatically inactive forms of the enzyme do not have the same effect [81]. Heparanase acts in this pathway by trimming the long HS chains in Sdc into shorter ones, which facilitates the clustering of syndecans [82, 83]. This clustering stimulates the binding of the cytoplasmic domains of syndecan to syntenin, driving ALIX-ESCRT-mediated sorting into exosomes [82–84]. This action of heparanase also facilitates the recruitment CD63 to exosomes, in a syntenin dependent fashion [82, 83] (Fig. 4.3). Consequently, heparanase inhibitors or syntenin inhibitors could be of particular interest for the treatment of cancer patients, as both exosome release and heparanase expression are frequently elevated in more aggressive subtypes [47, 81]. Heparanase has been shown to up-regulate the biogenesis of exosomes and affect its composition and function in myeloma cells [81]. Heparanase has also been found in exosomes isolated from ascites of ovarian cancer patients [85], and the levels of Sdc1, VEGF and HGF in exosomes derived from heparanase high expressing cells seem to be higher compared to heparanase low expressing cells [81]. Interestingly, exosomes secreted from heparanase high expressing cells were shown to better stimulate spreading of tumor cells on fibronectin and also stimulate invasion of endothelial cells compared to exosomes from heparanase low expression cells, suggesting a role in cancer angiogenesis and the spreading of cancer cells [81] (see also David and Zimmermann; Purushothaman and Sanderson, Chaps. 10 and 12 in this volume).

4.2.4 Effects on Growth Factor Signaling

The shedding of syndecans exposes domains in the core protein that can bind to different receptors. One well-described mechanism that affects signaling processes in the cell membrane is the one mediated by syndecan binding to integrin and to a tyrosine kinase receptor such as HER2, EGFR or IGF-1R. This tertiary complex activates various functions in the cells such as cancer progression and angiogenesis [86–90]. In these cases, the signaling mechanism can be disrupted by synthetic peptides called Synstatins (SSTNs), that mimic the binding sites of syndecan and compete with coupling to the tyrosine kinase receptors. Sdc1 shedding exposes a domain on the core protein that can bind to very late antigen 4 (VLA-4) integrin and vascular endothelial growth factor receptor 2 (VEGFR2). When Sdc1 binds and couples these receptors, VEGFR2 becomes activated and stimulates invasion in tumor cells [49]. The same mechanism potentiates endothelial tube formation and angiogenesis. Synstatin peptides based on either the VEGFR2 or VLA-4 binding site in Sdc1 can prevent invasion of tumor cells and endothelial tube formation [49]. Interestingly, the heparanase inhibitor Roneparstat, a chemically modified nonanticoagulant heparin, can decrease tumor invasion and angiogenesis by preventing Sdc1 shedding [49] (see also Noseda and Barbieri, Chap. 21 in this volume).

HSPGs are also involved in the uptake and nuclear translocation of growth factors and cytokines [91]. The vast majority of growth factors with nuclear translocation

bind to HSPGs, which can efficiently deliver molecules to their intracellular targets [92, 93]. Apart from growth factors, a myriad of ligands, viruses, nucleic acids, peptides, lipoproteins, and exosomes enter the cells via HSPG-mediated endocytosis [94]. The juxtamembrane MKKK motif is the required peptide sequence for the efficient raft dependent endocytosis, and this sequence is also part of the same RMKKK motif necessary for Sdc1 nuclear translocation, being crucial for both endocytosis and nuclear transport of Sdc1 and any associated molecule [95].

Both heparanase and Sdc1 can regulate HGF function. In myeloma cells, heparanase enhances HGF expression [96]. This growth factor binds strongly to Sdc1 in the membrane, augmenting the interaction with the c-met receptor and facilitating tumor growth [97, 98]. Shed Sdc1 can also bind to HGF and some evidence suggests that c-met signaling in osteoblasts is stimulated by shed Sdc1/HGF complexes [99]. Heparanase also stimulates VEGF secretion in tumor cells [31, 50]. Secreted VEGF can form a complex with shed Sdc1 that positively regulates VEGF receptor by activating the extracellular signal-regulating kinase (ERK) signaling pathway, leading to augmented endothelial invasion and angiogenesis [50]. Immuno-depletion of the VEGF/Sdc1 complex or treatment with heparinase III, a HS degrading enzyme derived from bacteria, blocks the augmented phosphorylation of ERK. It is interesting to notice that Sdc1 also activates αvβ3 integrin in endothelial cells, which is a key regulator of endothelial activation and angiogenesis [50, 100, 101]. In that manner, Sdc1 promotes endothelial cell activation, angiogenesis, and tube formation. This is mediated not only by αvβ3 integrin activation but also by binding to VEGF presenting it to its high-affinity receptor as a tertiary complex described above. Heparanase plays a central role in this process by up-regulating Sdc1 shedding. Heparanase also inhibits FGF2 signaling in melanoma cells by degrading membrane-bound HS [102]. Modification of these chains is required for effective binding of FGF2 to the cell surface and subsequent stimulation of ERK and FAK phosphorylation [102]. FGF2 high-affinity binding requires HS chains of a minimum size and some specific structural features. Upon cleavage of HS by heparanase, specific sequences in the HS chains that bind to FGF2 could be either removed or revealed [103, 104]. In addition, interplay between heparanase and Sdc1 is required for renal tubular cells to undergo epithelial to mesenchymal transition induced by FGF2 [105] (see also Masola et al.; van der Vlag and Buijsers, Chap. 26 and 27 in this volume).

4.3 Functional Cooperation of Syndecan-1 and Heparanase in Inflammation

Inflammation is a complex process that involves interactions of various cell types, most notably leukocytes and endothelial cells, which exchange signals via cytokines and chemokines and their respective transmembrane receptors. Physical interactions between these cells are mediated through cell surface receptors of the selectin, integrin, and the immunoglobulin superfamily of cell adhesion molecules

(CAMs) such as ICAM and VCAM [106–108]. In order to fight pathogens and toxins, leukocytes need to be recruited from the circulation to inflammatory sites via a controlled hierarchy of low- and high-affinity interactions with the endothelium, ultimately resulting in leukocyte transmigration, or diapedesis [108]. A role for HS in leukocyte recruitment has been documented in numerous studies and involves all key steps of this process (reviewed in [54, 108]). At early stages of leukocyte recruitment, HS promotes proinflammatory signaling processes by providing binding sites for growth factors and chemokines, resulting in the formation and stabilization of ternary complexes with their receptors [109, 110], and the establishment of chemokine gradients that are a crucial element in the leukocyte recruitment cascade [55, 111, 112]. Moreover, HS itself shows a distinct basolateral gradient pattern across blood vessels [113]. HS is also needed to ensure directional transendothelial chemokine transport, thus allowing for their presentation at the luminal surface endothelium [114]. Activated endothelial cells express adhesion molecules of the selectin family, which mediate low-affinity interactions with leukocytes that result in leukocyte rolling along the vessel wall [107]. Indeed, HS was shown to interact with L- and P-selectin, and L-selectin-HS complex formation appears to play an important role in enhancing leukocyte rolling mediated by P-selectin-PSGL-1 interactions [108, 114]. As will be pointed out in detail in the following section, Sdc1 plays a crucial role in modulating the next step of leukocyte adhesion, which is mediated by interactions of leukocyte β2 integrins with ICAM-1. Moreover, HPSE-mediated degradation of HS is a means of regulating intraluminal crawling of leukocytes within blood vessels, a process that involves interactions between the leukocyte β2 integrin Mac-1 and endothelial ICAM-1 [115]. Finally, HS modulates leukocyte diapedesis not only by facilitating chemokine gradient formation, but also via interactions with the leukocyte integrins LFA-1 and VLA4, and their binding partners ICAM-1 and VCAM-1 (reviewed in [55, 108]). Notably, several of the HS binding molecular and cellular mediators of inflammation are regulated by Sdc1 and heparanase, respectively, as will be detailed in the following section.

4.3.1 Lessons from Mouse Models

The relevance of Sdc1 and HPSE as regulators of inflammatory processes in vivo has been explored with the help of transgenic and knockout mouse models in a variety of experimental models of inflammation and repair [32, 54, 55, 108]. Early studies in Sdc1 knockout mice revealed that these mice show increased recruitment of leukocytes to the endothelium of the ocular vasculature [116]. Bone marrow transplantation experiments demonstrated that the increased adhesion was due to the lack of Sdc1 on leukocytes rather than the endothelium. Notably, intravital microscopy of TNFα-stimulated mesentery venules demonstrated that loss of Sdc1 was associated not only with a massively increased adhesion of leukocytes to blood vessels, but also with a substantial increase in leukocyte diapedesis [116]. Further mechanistic studies revealed that increased adhesion of Sdc1-deficient

polymorphonuclear cells (PMNs) and monocytes to human umbilical vein endothelial cells (HUVEC) in vitro could be inhibited by heparin if the endothelium was not activated. The increased adhesion was not altered by heparin when Sdc1-deficient leukocytes were allowed to adhere to TNFα-stimulated HUVECs, suggesting that the Sdc1-dependent adhesion phenotype involves HS/heparin-sensitive and insensitive steps, that depend on the activation state of the endothelium [117]. Further studies employing Sdc1-deficient and WT leukocytes in vitro revealed that increased adhesion of Sdc1-deficient PMNs to ICAM-1 could be inhibited by heparin, suggesting a role for HS in this process [118]. Moreover, increased adhesion of Sdc1 KO PMNs to ICAM-1 could be efficiently blocked with antibodies directed against the leukocyte integrin CD18 [119]. In summary, these data suggest that the lack of Sdc1 on leukocytes may allow them to interact with non-activated endothelium, and enhances leukocyte-endothelial interactions in HS-dependent manner at the level of ICAM-1-CD18 interactions.

In addition to Sdc1, the role of heparanase in leukocyte recruitment has been studied in vitro and in vivo. In vivo studies in rats demonstrated that intraperitoneal injection of heparanase resulted in increased recruitment of inflammatory cells to the peritoneal cavity, and an increase in leukocyte rolling and adhesion in postcapillary venules, as evidenced by intravital microscopy of mesentery microvessels [120]. Moreover, in vitro adhesion assays showed that heparanase treatment increased neutrophil and mononuclear cell adhesion to HUVEC cells [120]. Surprisingly, in contrast, a study utilizing heparanase knockout and overexpressing mice found that monocyte, but not neutrophil, recruitment into peritoneal cavities inflamed by zymosan treatment depended on heparanase [121]. Moreover, although heparanase was upregulated in effector T-cells, it was not required for extravasation inside inflamed lymph nodes or skin in adoptive transfer experiments. However, in an experimental mouse model of sepsis-associated acute lung injury, inhibition of heparanase prevented endotoxemia-associated glycocalyx loss and neutrophil adhesion [122]. Moreover, in an experimental model of inflammation that utilized heparanase overexpressing vs WT mice, heparanase was shown to interfere with the process of intraluminal crawling of leukocytes within blood vessels [115]. Cleavage of HS by heparanase perturbed an experimentally applied gradient of the chemokine CXCL2 in the cremaster muscle, resulting in a loss of directionality of intraluminal leukocyte crawling. Finally, a study on transendothelial migration of hepatocellular carcinoma cells revealed that cell lines expressing high levels of heparanase show a higher transendothelial migration rate compared to cells with lower expression and that heparanase inhibition or downregulation suppressed this process both in vitro and in vivo [123]. In conclusion, in spite of context- and cell-type-specific effects, the aforementioned studies suggest that heparanase has a similar effect on leukocyte recruitment as the knockout of Sdc1.

As mentioned above, increased recruitment of Sdc1-deficient leukocytes to sites of inflammation was observed in a variety of experimental models of inflammation [55]. Interestingly, a range of similar or even identical disease models has been used to study the function of heparanase in inflammation employing transgenic and knockout mice with altered heparanase expression. In the following section, we will

discuss the effect of Sdc1 and heparanase on four selected experimental models of inflammation (contact allergy, colitis, kidney inflammation, and experimental autoimmune encephalomyelitis), as they will allow us to compare the individual functions of Sdc1 and heparanase in different inflammatory diseases.

Role of Sdc1 and Heparanase in Delayed-Type Hypersensitivity

Delayed-type hypersensitivity (DTH) is a mouse model for allergic contact dermatitis, which consists of a sensitization phase involving covalent modification of surface proteins with haptens such as oxazolone or TNCB (2,4,6-trinitro-1-chlorobenzene), which are subsequently taken up and processed by dendritic and Langerhans cells. These cells migrate to lymph nodes and prime hapten-specific T cell populations, which are recruited and activated during the elicitation phase, resulting in cytokine and chemokine release, mast cell degranulation and massive leukocyte infiltration of the skin [124, 125]. Consistent with the previously described increase in leukocyte recruitment in Sdc1-KO mice, elicitation of an oxazolone-mediated DTH response resulted in increased leukocyte recruitment, and increased and prolonged edema formation. Compared to wild-type mice, expression of ICAM-1, cytokines (i.e., TNFα and IL-6), and chemokines (i.e., CCL5/RANTES, CCL-3/MIP-1α) was increased in Sdc1-deficient animals [119]. Interestingly, loss of Sdc1 can compensate for the loss of another proteoglycan, decorin, in this model: decorin-deficient mice show reduced DTH responses, associated with attenuation of leukocyte infiltration, which is overcome in the absence of Sdc1 [125]. However, while the in vivo data suggest that loss of Sdc1 releases the block in diapedesis observed in decorin-deficient mice, the detailed mechanisms underlying this observation still need to be elucidated. In the TNBS model of DTH, Sdc1-deficient dendritic cells migrated at a higher rate and faster to draining lymph nodes, resulting in an increased DTH response both in Sdc1 KO mice and in WT mice subjected to adoptive transfer of Sdc1 KO dendritic cells [126]. Moreover, upregulation of CCL2, CCL3, VCAM1 and talin, and a prolonged presence of CCR7 at the cell surface was observed on Sdc1 KO vs WT cells during dendritic cell maturation. Notably, Sdc1-KO dendritic cells showed an increased migration towards CCL21 and CCL19 compared to WT cells [126]. In addition to Sdc1, the role of heparanase during DTH has been studied in vivo. While Sdc1 expression is downregulated prominently in the epithelium of inflamed skin during oxazolon-induced DTH response [119], heparanase is upregulated particularly by the endothelium at the site of DTH-induced inflammation [127]. Moreover, heparanase-overexpressing mice showed a substantially increased DTH response compared to WT animals, whereas heparanase inhibition in WT mice resulted in a reduced DTH response and less vascular leakage [127]. In summary, these data suggest that upregulation of heparanase and absence of Sdc1 generate a similar, pro-inflammatory phenotype during DTH in mice. However, while Sdc1 negatively regulates endothelial leukocyte recruitment and dendritic cell migration during DTH responses, heparanase appears to primarily act at the level of the endothelium, where it regulates vascular permeability.

Role of Sdc1 and Heparanase in Anti-Glomerular Basement Membrane Glomerulonephritis

Experimental anti-glomerular basement membrane (anti-GBM) nephritis is a model of inflammation that mimics aspects of the autoimmune disease Goodpasture syndrome [128]. This experimental animal model is based on the injection of antibodies directed against antigenic material from the GBM of mice into recipient mice. An initial heterologous phase, where leukocyte influx peaks within hours and albuminuria become apparent can be observed within 24 h. This phase is followed by an autologous phase during which endogenous anti-GBM IgG is produced, leading to persistent albuminiuria [128]. In Wild-type (WT) mice, induction of anti-GBM glomerulonephritis resulted in an upregulation of Sdc1, and Sdc4 protein 2 and 18 h after induction of the disease, which normalized after 4 days [129]. In contrast, heparanase is upregulated both in the heterologous and autologous phase of the disease [130]. 4 and 8 days after administration of rabbit anti-GBM IgG, glomerular deposition of mouse anti-rabbit IgG was higher in Sdc1 KO compared to WT mice [129]. Notably, the numbers of PMNs and macrophages were significantly higher in inflamed glomeruli of Sdc1 KO mice in the heterologous phase, whereas the numbers of CD4+ and CD8+ T-cells were higher in Sdc1 KO mice in the autologous phase compared to WT. As a result, Sdc1 KO mice developed more severe albuminuria and showed worsened kidney function compared to WT mice. These changes were accompanied by significant increases of ICAM-1, L-selectin, IL-1β, MCP-1, IL-6 and IL-10 expression in Sdc1 KO mice vs WT during the heterologous phase, and in numerous ECM proteins (fibronectin, collagen IV, collagen XVIII, laminins, MMP7 and MMP9) along with L-selectin and MCP-1 during the autologous phase of the disease. Overall, Sdc1 KO mice showed a shift of the Th1/Th2 balance towards a Th2 response [129]. Compared to WT mice, heparanase-deficient mice showed better kidney function in this experimental model, which was accompanied by a reduced influx of PMNs and macrophages, reduced glomerular damage, and a reduction of the expression of numerous inflammatory factors along with reduced Sdc1 levels [130]. Heparanase KO mice showed reduced expression of both Th1 and Th2 cytokines. Reduced degradation of basement membrane HS was identified as a mechanistic aspect of the beneficial effects of heparanase deficiency, as shown in vitro using heparanase-silenced mouse glomerular endothelial cells, which displayed a lower transendothelial albumin passage compared to controls. It appears that Sdc1 and heparanase may play distinct mechanistic roles in this experimental model, although it is conceivable that heparanase deficiency may result in reduced Sdc1 ectodomain shedding, which may dampen the inflammatory response by reducing the influx of leukocytes [129, 130].

Sdc1 and Heparanase in Experimental Autoimmune Encephalitis (EAE)

Experimental autoimmune encephalitis (EAE) is an experimental T-cell dependent in vivo model of multiple sclerosis. In EAE, antigen-specific $CD4^+$ Th1 cells cause inflammatory damage in the central nervous system, thus mimicking the

demyelination, axonal loss and progressive paralysis caused by autoreactive T-cells in human multiple sclerosis [131, 132]. In the EAE model, Sdc1-deficient mice showed a higher severity of the disease compared to their WT counterparts and recovered more slowly [133]. Mechanistically, Sdc1 is upregulated at the transcriptional level, but shed from epithelial cells of the choroid plexus to the cerebrospinal fluid in WT mice, resulting in a loss of cell-surface bound CCL20 chemokine, which showed a partial co-localization with Sdc1 in naïve WT mouse brain. Notably, in Sdc1 KO mice, early recruitment of leukocytes, and levels of IL-6 were enhanced, resulting in recruitment of Th17 cells and aggravation of the inflammatory reaction. Moreover, enhanced plasma cell levels and higher levels of myelin oligodendrocyte glycoprotein–specific antibodies – a driving factor of the disease – in Sdc1 KO mice may be the underlying cause for delayed recovery from EAE [133]. The role of heparanase in EAE was studied applying recombinant heparanase via daily intraperitoneal administration starting from the day of immunization with proteolipid protein until day +7 or day +17 [134]. In this study, heparanase ameliorated the clinical signs of the disease. Moreover, the formation of clusters of inflammatory cells, as seen in WT mice in the white matter zone of the spinal cord, was not observed in heparanase-treated mice. Mechanistic in vitro and in vivo experiments revealed that heparanase treatment caused a shift of the cytokine spectrum toward Th2 cytokines (IL-4, IL-6, IL-10), resulting in an inhibition of a mixed lymphocyte reaction and mitogen-induced splenocyte proliferation [134]. While these data clearly demonstrate a role for both Sdc1 and heparanase in EAE, both molecules acted via different mechanisms in the two studies presented, and the focus on different mechanistic aspects and application of different analytical assays impedes a direct comparison. Further investigations may help to clarify the possible interplay of Sdc1 and heparanase in EAE. For example, it could be envisaged that heparanase is involved in the shedding of Sdc1, however, it is not clear if it is expressed in the epithelium of the choroid plexus during the EAE reaction. Moreover, while upregulation of IL-6 in Sdc1 KO mice was seen as a factor promoting disease progression in the study by Zhang et al. [133], it was presented as part of an anti-inflammatory Th2 signature in the study by Bitan et al. [134]. Differences in the time-courses of IL-6 expression, and in the tissues the cytokine is derived from, may account for the deviating interpretations of the respective phenotype.

Role of Sdc1 and Heparanase in Inflammatory Bowel Disease and Colitis-Associated Colon Cancer

Inflammatory bowel diseases are complex diseases that constitute a major health burden, which is characterized by an aberrant immune response in the gastrointestinal tract [135]. An important experimental model of inflammatory bowel diseases like Crohn's disease and ulcerative colitis is the dextran sodium sulfate (DSS) colitis murine model, which is based on DSS-induced epithelial cell injury, which is followed by the entry of luminal bacteria and associated antigens into the mucosa

and a subsequent inflammatory reaction [136]. Of note, reduced expression of Sdc1 has been observed in patients with ulcerative colitis, which has been linked to disrupt healing of colonic ulcers [137].

When the mechanistic role of Sdc1 in colitis was studied by inducing the disease with 3% DSS in Sdc1 KO mice, a substantial increase in mortality was observed compared to WT animals (61% versus 5%) [118]. Sdc1 KO mice showed prolonged recruitment of leukocytes and impaired mucosal healing, which were accompanied by an upregulation of TNFα, CCL3/MCP1, and VCAM-1 in the inflamed tissue. Notably, treatment with enoxaparin improved mucosal wound repair and reduced lethality of Sdc1 KO mice, suggesting that heparin may be able to compensate for the loss of the heparan sulfate chains of Sdc1 and the associated poor outcome in this disease model [118]. Of particular clinical relevance, chronic inflammatory bowel disease increases the risk of colon cancer [138], a disease that is also associated with a downregulation of Sdc1 expression in the colonic epithelium [139, 140]. Colitis-associated cancer can be experimentally modeled in mice by application of the carcinogen azoxymethane (AOM) and subsequent induction of chronic colitis with DSS[141]. Sdc1 KO mice developed more severe inflammation during chronic DSS colitis, associated with increased recruitment of inflammatory cells, increased crypt damage, and increased weight loss compared to WT mice [142]. IL-6 expression and activation of STAT3 were increased in the inflamed colon tissue of Sdc1 KO vs. Wt mice. Notably, Sdc1 KO mice formed larger tumors than their WT controls in the AOM-DSS model, which was attributed to increased activation of STAT3, and an upregulation of cyclin D1, CCL2, and c-Myc in the tumor tissue [142]. Overall, these data suggest that the increased inflammation and tissue damage in the absence of Sdc1 drive colon cancer progression via enhanced signaling through the IL-6/STAT pathway. While Sdc1 is downregulated in IBD and colon cancer, heparanase is upregulated in the inflamed and tumor tissue in these diseases [143, 144], and in the experimental AOM / DSS and the acute and chronic DSS animal models [145]. Notably, when mice that transgenically overexpress heparanase were subjected to DSS colitis, high heparanase expression preserved the inflammatory conditions, along with increased expression of TNFa and Cyclin D1 (thus showing a resemblance to Sdc1 KO mice in this model) [142], and a substantially increased recruitment of TNFa-expressing macrophages [145]. Similar to Sdc1 KO mice, tumors were larger in heparanase transgenic mice [142, 145]. Moreover, the number of tumors was higher compared to WT mice, and tumor angiogenesis was enhanced. Overall, these data suggest that heparanase drives a vicious cycle that promotes colitis and chronic-inflammation-related tumorigenesis. The phenotype shows undeniable similarities to the Sdc1 KO mouse in this experimental model, however, the interrelation between these molecules still awaits experimental investigation. Table 4.2 summarizes the phenotypes and molecular mechanisms of the mouse models that were presented in this section, revealing similarities and disparities in the mechanisms by which Sdc1 and heparanase modulate inflammatory processes in vivo.

Table 4.2 Phenotype of Sdc1- and heparanase-manipulated mice in experimental models of inflammation

Disease model	Gene manipulated	Phenotype compared to WT	Molecular changes
Contact allergy (DTH) [119, 126, 127]	Sdc1 knockout	Increased leukocyte recruitment, increased and prolonged edema formation, increased dendritic cell migration and chemotaxis in vivo and in vitro	Sdc1 downregulated in WT; increased expression of ICAM-1, TNFα, IL-6, CCL5 and CCL-3 in inflamed KO tissue; increased expression of CCL2, CCL3, VCAM1 during KO dendritic cell maturation
	Heparanase overexpression and inhibition	HPSE overexpression leads to increased DTH response, whereas HPSE inhibition in WT mice resulted in a reduced DTH response and less vascular leakage.	HPSE upregulated in WT endothelium; IFNγ induces HPSE in vitro.
Goodpasture syndrome (anti-GBM nephritis) [129, 130]	Sdc1 knockout	Increased glomerular deposition of mouse anti-rabbit IgG, and of early infiltration with PMNs and macrophages and late infiltration with CD4+ and CD8+ T-cells. More severe albuminuria and worsened kidney function. Shift of the Th1/Th2 balance towards a Th2 response.	Increased ICAM-1, L-selectin, IL-1β, CCL2, IL-6 and IL-10 during early phase, and increased fibronectin, collagen IV, collagen XVIII, laminin, MMP7, MMP9, L-selectin and CCL2 during late phase.
	Heparanase knockout	Better kidney function, reduced influx of PMNs and macrophages, reduced glomerular damage. Lower transendothelial albumin passage in vitro.	Reduced expression of numerous inflammatory factors (both Th1 and Th2) and of Sdc1. Reduced degradation of basement membrane heparan sulfate.
Multiple sclerosis (EAE) [133, 134]	Sdc1 knockout	Increased early recruitment of leukocytes, recruitment of Th17 cells aggravates inflammatory reaction. Enhanced plasma cell levels.	Increased Sdc1 shedding from choroid plexus of WT mice leads to loss of cell-surface bound CCL20. Higher IL-6 levels in KO mice. Higher levels of myelin oligodendrocyte glycoprotein–specific antibodies in KO mice.
	Heparanase enzyme therapy	Heparanase ameliorates clinical signs of EAE. Reduced formation of inflammatory cell clusters. Inhibition of a mixed lymphocyte reaction and of mitogen-induced splenocyte proliferation ex vivo.	Heparanase treatment causes shift toward Th2 cytokines (IL-4, IL-6, IL-10).

(continued)

Table 4.2 (continued)

Disease model	Gene manipulated	Phenotype compared to WT	Molecular changes
Ulcerative colitis (DSS colitis)[218, 142, 145]	Sdc1 knockout	Substantial increase in mortality, prolonged recuitment of leukocytes and impaired mucosal healing.	Upregulation of TNFα, CCL3 and VCAM-1 in KO mice.
	Heparanase overexpression	High heparanase preserves the inflammatory conditions, substantially increased recruitment of TNFα-expressing macrophages.	Increased expression of TNFa and Cyclin D1 in heparanase overexpressing mice.
Colitis-associated colon cancer (AOM-DSS) [142, 145]	Sdc1 knockout	More severe inflammation during chronic DSS colitis (increased recruitment of inflammatory cells, increased crypt damage, and increased weight loss) compared to WT mice. Sdc1 KO mice form larger tumors.	IL-6 expression and activation of STAT3 are increased in inflamed Sdc1 KO colon tissue. Increased activation of STAT3, and upregulation of cyclin D1, CCL2, and c-Myc in Sdc1 KO tumor tissue.
	Heparanase overexpression	Larger size and higher incidence of tumors, enhanced tumor angiogenesis.	Increased expression of TNFa and Cyclin D1.

4.4 Syndecan-1 and Heparanase as Pathogenesis Factors and Therapeutic Targets in Malignant Disease

The investigation of biopsies and blood from cancer patients has revealed that Syndecan-1 and heparanase are mis-expressed in a large number of cancers, underscoring their clinicopathological relevance (see Table 4.1). As of August 2019, close to 1000 publications for each molecule have described a role for Syndecan-1 or heparanase in different types of cancer in the PubMed database, generating a need to present selected examples of their molecular functions in malignant disease. The molecular mechanisms that govern tumorigenesis and cancer progression have been conceptually summarized by Hanahan and Weinberg in their landmark article 'The Hallmarks of Cancer', which originally included sustained proliferation, evasion of growth suppression, death resistance, replicative immortality, induced angiogenesis, and initiation of invasion [146]. Additional hallmarks were defined 10 years later in an updated version of the article and included the aspect of chronic inflammation (see Sect. 4.3) and avoidance of immune destruction [147] (see reference [148] for a recent review on the role of cell surface proteoglycans in immunotherapy). Notably, proteoglycans such as Sdc1 and enzymes like heparanase, which utilize HS proteoglycans as substrates have been shown to modulate most, if not all of these Hallmarks [148, 149]. For example, regarding the cancer hallmark of *sustained proliferation*, Syndecan-1 acts as a co-receptor for receptor tyrosine kinases,

thus contributing to proliferative signaling and tumor growth, as demonstrated for example in breast cancer, colon cancer, and multiple myeloma [22, 97, 108]. Notably, it has been shown that the soluble ectodomain of Syndecan-1 can competitively inhibit mitogenicity of the cytokine FGF2, whereas platelet heparanase is able to convert the HS chains of soluble Syndecan-1 from an inhibitor into heparin-like HS fragments that substantially activate FGF-2 mitogenicity [150]. Moreover, both latent heparanase and its mature active form promote signaling through multiple pathways with relevance to tumor progression, including the Src, MAPK, HGF-, IGF-, and EGF-receptor pathways [151]. The *insensitivity to antigrowth signals* has been defined as an additional hallmark of cancer. An important growth-inhibitory cytokine and mediator of epithelial-to-mesenchymal transition is TGFβ which has been shown to be negatively regulated by Syndecan-1 during liver fibrogenesis [152]. Interestingly, heparanase-mediated shedding of Syndecan-1 has been shown to result in an upregulation of TGFβ in hepatocellular carcinoma, providing a link between heparanase and Syndecan-1 in the regulation of growth-inhibitory signals [153]. The *enabling of replicative immortality* adds to the six original hallmarks of cancer [146]. Replicative immortality is a shared feature of cancer stem cells and is partially linked to the activity of telomerase, which prevents the shortening of chromosome ends [154]. Syndecan-1 has emerged as an important regulator of the cancer stem cell phenotype: The reduction of a wnt-responsive precursor cell population has been identified as a molecular mechanism underlying the resistance of juvenile Sdc1 KO mice to breast cancer and additional forms of experimentally induced cancer [154–156]. Moreover, siRNA mediated knockdown of Sdc1 in human breast cancer cell lines representative of different molecular classifications resulted in a reduction of stem cell properties, including the expression of typical cancer stem cell markers ($CD44^{+}/CD24^{low}$, side population, ALDH) as well as colony and mammosphere formation [157, 158]. Modulation of the stemness-related notch, wnt, and IL-6/STAT signaling pathways by Syndecan-1 was identified as the mechanistic basis for this finding. Notably siRNA knockdown of Syndecan-1 also resulted in an upregulation of heparanase expression in this model system [159]. Indeed, modulation of stem cell properties in the context of malignant disease has not only been ascribed to Syndecan-1, but also to heparanase, which acts in a context-dependent manner. In breast cancer, nuclear heparanase has been shown to induce tumor cell differentiation [160]. Moreover, heparanase-mediated modifications in the bone marrow microenvironment regulate the retention and proliferation of hematopoietic progenitor cells [161], modulate clonogenicity, proliferative potential and migration of mesenchymal stem cells in the bone marrow [162], and shift the differentiation potential of osteoblast progenitors within the myeloma bone microenvironment from osteoblastogenesis to adipogenesis [163]. Moreover, embryonic stem cells overexpressing heparanase proliferated faster than wild-type controls in culture and formed larger teratomas in vivo [164], indicating an important role for heparanase in (cancer) stem cells and the modulation of replicative immortality. Closely linked to the previous hallmarks of cancer is the *resistance to cell death*, as it allows for the proliferation of tumor cells which carrying mutations, ultimately leading to another enabling characteristic, genome instability.

Several studies have documented a role for Syndecan-1 as a regulator of apoptosis in an oncological setting. For example, Syndecan-1 suppresses apoptosis in multiple myeloma by activating the IGF1 receptor [88], and in endometrial cancer by enhancing Erk and Akt activation [165]. In contrast, Syndecan-1-dependent MAPK-signaling was shown to be of importance for the pro-apoptotic effect of the n-3 polyunsaturated fatty acid docosahexaenoic acid in prostate and breast cancer cells. Likewise, heparanase modulates cancer cell apoptosis in a context-dependent manner [166, 167]. Early studies indicated a positive correlation between heparanase expression and spontaneous apoptosis in hepatocellular carcinoma[168]. Along these lines, an apoptosis-enhancing function of heparanase was revealed in growth-hormone secreting pituitary tumor cells [169]. In contrast, orthotopic xenograft experiments utilizing heparanase overexpressing breast cancer cells identified heparanase as a survival factor for breast cancer cells in vivo [170]. Similarly, overexpression of heparanase inhibited apoptosis in cervical cancer cells [171]. Since Syndecan-1 and heparanase modulate similar signal transduction pathways, it can be envisaged that they cooperate in the regulation of cell death, however, more studies are needed to confirm the mechanistic involvement in more detail. The *induction of angiogenesis* is another hallmark of cancer that is modulated both by Syndecan-1 and heparanase. The sprouting and growth of blood vessels from existing blood vessels is a carefully orchestrated physiological process, that is hijacked by tumor cells via secretion of angiogenic factors and by causing an imbalance between pro-and anti-angiogenic factors [89, 172]. Tumor angiogenesis is a prerequisite for supplying the tumor with nutrients and oxygen, and for promoting metastatic spread via the circulation. Of note, syndecan-1 expression has been identified as part of a molecular signature marking an angiogenic switch in early stages of breast cancer [173] and stromal syndecan-1 expression was shown to correlate with microvessel density and blood vessel area both in human breast cancer specimens and in xenograft models [174]. In vivo studies have demonstrated that absence and overexpression of syndecan-1 modulate angiogenesis via enhanced leukocyte recruitment and by promoting proteolysis, respectively [42, 116]. Moreover, syndecan-1 promotes angiogenesis as a classical co-receptor for angiogenic factors such as FGF-2, VEGF, and c-Met [97, 173, 175]. In addition, MMP9-induced syndecan-1 is part of a mechanism that is responsible for radiation-induced angiogenesis in medulloblastoma [176]. Moreover, syndecan-1 regulates tumor angiogenesis via lateral association of its extracellular core protein domain with proangiogenic integrins [86, 89]. Notably, in multiple myeloma, heparanase stimulates shedding of syndecan-1, which leads to a deposition of VEGF that is bound to the syndecan-1 HS chains in the ECM, where it stimulates endothelial invasion [50]. Heparanase-induced syndecan-1 shedding also promotes hepatocarcinoma lymphangiogenesis via the VEGF-C/ERK pathway [175]. However, heparanase promotes tumor angiogenesis not only via induction of syndecan-1 shedding but also via the release of proangiogenic factors from heparan sulfate on cell surfaces and the ECM, through Src-dependent upregulation of VEGF expression, enhancement of Akt signaling and stimulation of PI3K- and p38-dependent endothelial cell migration and invasion [177].

Finally, the *activation of invasion and metastasis* has been defined as a hallmark of cancer, which is of utmost importance as cancer-associated metastasis to vital organs is the leading cause of cancer-related mortality [178]. The misexpression of Syndecan-1 that is observed in numerous tumor entities (Table 4.1) contributes to metastatic behavior in several ways. While the membrane-bound form of Syndecan-1 had an invasion-inhibiting effect on the human breast cancer cell line MCF-7, overexpression of the soluble ectodomain and of the intact proteoglycan resulted in a substantial increase in Matrigel invasion chamber assays [22, 159]. The increase in invasiveness could be attributed to a downregulation of the MMP inhibitor TIMP-1 and upregulation of urokinase-type plasminogen activator receptor (uPAR) in cells overexpressing soluble Syndecan-1. Moreover, a downregulation of the anti-invasive homotypic cell adhesion molecule was observed both in MCF-7 breast cancer cells overexpressing soluble Syndecan-1, and in the highly invasive breast cancer cell line MDA-MB-231 upon Syndecan-1 depletion [22, 159]. While this observation may suggest a potential involvement of syndecan-1 in regulating the pro-metastatic process of epithelial-to-mesenchymal transition (EMT) in breast cancer, no consistent upregulation of mesenchymal markers was observed upon Syndecan-1 depletion in the same experimental system [157]. However, an indirect regulatory impact on EMT of prostate cancer cells was recently proposed, involving alterations in EMT-regulating microRNA processing that was modulated by syndecan-1-dependent changes in expression of the miRNA processing enzyme, Dicer [179]; Syndecan-1 promotes EMT in hepatocellular carcinoma by enhancing TGFbeta function [180]. In breast cancer, time-lapse microscopy analysis revealed that silencing of Syndecan-1 in human MDA-MB-231 breast cancer cells results in a substantial increase in cell motility [159] and migration through fibronectin-coated filter membranes [181]. Inhibitor studies and signal transduction analysis revealed that this increase could be attributed to increased activation of focal adhesion kinase and dysregulation of Rho-GTPases. In lung cancer, Syndecan-1 was shown to inhibit metastasis in vivo and invasiveness in vitro via a mechanism that relies on the cleavage of Syndecan-1 core protein by ADAM17 and gamma-secretase, and the generation of a cytoplasmic fragment with biological activity [48]. Moreover, similar to its function during inflammation, Syndecan-1 promotes chemotaxis of breast and lung cancer cells as a co-receptor for chemokines such as MIP1 [182]. Of note, heparanase-mediated processing of Syndecan-1 has an important role in the regulation of metastasis. Heparanase promotes shedding of syndecan-1 in myeloma and breast cancer cells, whereas syndecan-1 promotes processing of heparanase [45, 183]. Moreover, heparanase regulates the secretion of tumor exosomes, including their Syndecan-1 content, thus driving metastatic behaviour [81] (Fig. 4.3). Finally, Syndecan-1 can inhibit heparanase-mediated in vitro invasion of melanoma cells in an HS-dependent manner, indicating complex regulatory circuits between heparanase and Syndecan-1 [184]. Apart from the mechanisms involving Syndecan-1 processing, heparanase has been shown to promote invasive growth and metastasis of a wide range of tumor entities [185] (Table 4.1). Indeed, heparanase promotes metastasis via several mechanisms, as demonstrated

in transgenic mouse models overexpressing heparanase and in tumor cell lines subjected to heparanase silencing of inhibition. These mechanisms including a degradation of basement membrane HS, thus removing a physical barrier of metastasis in epithelia and endothelia [186, 187], through the release of stored growth factors and chemokines from the ECM and cell surfaces, thus promoting chemotaxis and pro-invasive signaling [103, 188, 189], and through the impact on exosome secretion and function [190], as mentioned above (Fig. 4.3).

Given the importance of Syndecan-1 and heparanase as prognostic markers in various tumor entities, and as mechanistically relevant factors in driving cancer progression, it is no surprise that these molecules have been identified as therapeutic targets in malignant disease [188, 191]. Regarding a therapeutic targeting of Syndecan-1, it has to be considered that it is highly expressed on several healthy epithelia, including the gut, skin, and lung [8, 41], thus increasing the danger of an unfavorable side effects profile upon therapeutic targeting. Nevertheless, promising results have been achieved upon Syndecan-1-targeting in preclinical models. One approach includes the interference of Syndecan-1 integrin interactions with the Syndecan-1-derived peptide synstatin, which successfully disrupted tumor angiogenesis in vivo [86, 89]. Moreover, Syndecan-1-conjugates with cytotoxic drugs (i.e., indatuximab and ravtansine) have shown efficacy in preclinical in vivo models of triple-negative breast cancer [192]. In patients with relapsed or refractory multiple myeloma, this antibody-drug conjugate entered phase I/IIa clinical trials and showed efficiency with respect to clinical activity, although side effects on epithelial tissues were also observed [148, 193]. Finally, CAR-T cell therapy has been employed to target Syndecan-1, showing efficacy in a preclinical model of multiple myeloma, and promising results in patients, including stable disease for over 3 months, and a reduction of myeloma cells in the peripheral blood, respectively [148, 194, 195]. Compared to Syndecan-1, heparanase represents an even more attractive drug target, as it is only expressed in a few cell types in healthy adults (mainly leukocytes), thus limiting potential therapy-associated side effects [189]. A recent comprehensive review provided an overview of different heparanase inhibitors that have been explored in preclinical studies and partially also in clinical trials [196]. These inhibitors include heparin and its derivatives (e.g., roneparstat/SST0001 and mupofastat/PI-88), nucleic acid-based inhibitors (e.g. defibrotide), synthetic inhibitors (e.g., suramin) and heparanase-neutralizing antibodies. Apart from targeting tumor cell-derived heparanase, these inhibitors can also have an impact on the tumor stroma and inhibit processes that support tumor progression, e.g. tumor-promoting inflammation and angiogenesis. Indeed, several of these inhibitors have demonstrated anti-tumor, anti-angiogenic and anti-metastatic efficacy in preclinical animal models of the disease, including e.g. myeloma [51], lymphoma [197], and sarcoma models [198]. Moreover, heparanase inhibitors have shown to be largely well-tolerated in phase I and phase II clinical trials [196, 199–201], providing a positive outlook for progress in targeted cancer therapy in the near future (see Noseda and Barbieri; Hammond and Dredge; Chhabra and Ferro, Chaps. 19, 21 and 22 in this volume).

4.5 Concluding Remarks

Syndecan-1 and heparanase fulfill important and pleiotropic physiological functions during development and tissue homeostasis. Notably, several processes regulated by Syndecan-1 and heparanase are also relevant in the context of inflammatory and malignant disease. These processes include the regulation of cell proliferation and survival, cell motility and cell invasion, leukocyte recruitment and angiogenesis, which are achieved by modulation of growth factor-, chemokine- and morphogen-mediated signaling processes and via regulation of the composition and functional properties of the ECM. While Syndecan-1 and heparanase retain some autonomous functions, many of their properties are functionally linked. Syndecan-1 can act as a substrate for heparanase, and heparanase promotes Syndecan-1 shedding as an important mechanism of conversion of this membrane-bound molecule into a soluble paracrine effector. In turn, Syndecan-1-dependent signaling mechanisms regulate heparanase expression, and the cytoplasmic domain of Syndecan-1 plays an important role in heparanase processing. Moreover, ligands bound to the HS chains of Syndecan-1 can be released by the action of heparanase, resulting in an altered functionality. In contrast, the HS chains of Syndecan-1 can have an inhibitory impact on heparanase activity, depending on their fine structure. In most cases, synergistic effects of Syndecan-1 and heparanase are observed, which contribute to the pathogenesis of inflammatory diseases and tumor progression. Therefore, Syndecan-1 and heparanase have emerged as important targets for therapeutic approaches. Due to their pleiotropic functions and their mechanistic involvement in inflammatory and malignant diseases, their targeting represents a highly promising approach, as it can be expected that therapeutics simultaneously inhibit multiple processes related to disease progression. Substantial evidence from preclinical models and promising results from phase I/II clinical trials provide a promising perspective regarding the translation into a clinical setting.

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Part II
Crystal Structure, Substrate Specificity and Gene Regulation

Chapter 5
An Overview of the Structure, Mechanism and Specificity of Human Heparanase

Liang Wu and Gideon J. Davies

5.1 Introduction

Heparan Sulfate proteoglycans (HSPGs) are ubiquitous components of the extracellular matrix (ECM), where they mediate diverse structural and signaling interactions between cells and proteins of the ECM [1]. HSPGs are comprised of a core transmembrane, membrane-anchored, or extracellular protein attached to one or more chains of the glycosaminoglycan (GAG) polysaccharide heparan sulfate (HS). Interactions between HSPGs and their binding partners primarily occur via the HS chains which decorate the core HSPG protein [2].

The chemical composition of HS is complex and dynamically regulated in response to stimuli *via* a process of continual turnover [3–5]. HS composition has been shown to vary in relation to development [6–8], cancer stage [9–11], and general age [8, 12]. While biosynthesis of HS is a multistep process involving the concerted action of a host of polymerases, sulfotransferases, and epimerases [13], breakdown of HS in mammals is primarily carried out by a single enzyme – heparanase (HPSE) [14–15].

HPSE is an *endo*-acting glycoside hydrolase, which cleaves within long HS chains to release product fragments of HS ~5–7 kDa in size [14]. The HS degrading activity of heparanase is essential for ECM remodeling, affecting diverse processes such as inflammation, angiogenesis and cell migration [16–18]. HPSE activity can also release growth factors sequestered within networks of HS, which subsequently promote angiogenesis and wound healing [19]. Whilst normal HPSE function is essential for physiological processes which involve ECM remodeling, the HS degrading capability of HPSE can also be co-opted by cancerous cells to promote

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malignant growth and dissemination. Accordingly, upregulation of heparanase is a hallmark of aggression and metastasis in a wide range of cancers [20–24].

A full summary of the many functions of HPSE in health and disease is beyond the scope of this article, and will be covered elsewhere in this book. Instead, we aim here to provide a structure/function-centric review of HPSE, drawing from insights gained from crystal structures of HPSE and its related proteins. From these, we hope to provide the reader with an appreciation of the structural features that underlie the many biological and biochemical insights obtained from decades of research on HPSE.

5.2 Heparan Sulfate – The Biochemical Basics

Chemically, HS is a linear glycosaminoglycan polysaccharide comprising of alternating 1,4 linked units of hexuronic acid (HexUA) and glucosamine (GlcN) [25]. HS chains can display high complexity due to the number of permutations possible for the core HexUA and GlcN building blocks. The HexUA of HS can be either β-D-glucuronic acid (GlcUA) or α-L-iduronic acid (IdoUA), and GlcN can be either N-acetyl-α-D-glucosamine (GlcNAc) or N-sulfo-α-D-glucosamine (GlcNS). These core residues are further decorated by varying degrees of O-sulfation (Fig. 5.1a).

Biosynthesis of HS is non-templated, allowing HS composition to vary substantially along a single polysaccharide chain (typical HSPGs contain HS chains between 40–300 sugar units (20–150 nm) in length) [1]. Variations in HS structure occur across broad macromolecular regions, leading to the formation of N-acetyl (NA) domains (characterized by poorly sulfated GlcNAc-GlcUA repeats) and N-sulfo (NS) domains (characterized by highly sulfated GlcNS-IdoUA repeats), separated by mixed NS/NA domains (Fig. 5.1b). This structural heterogeneity is crucial for HS function, enabling a single polysaccharide chain to interact with a host of different binding partners. HS heterogeneity is also of central importance for its breakdown by HPSE. As will be further discussed below (Sect. 5.4.2.), cleavage of HS by HPSE is limited to only certain GlcUA residues within the sugar chain, depending on the local sulfation pattern around the target site.

5.3 Historical Developments in HPSE Research

5.3.1 Identification of a Specific Heparan Sulfate Degrading Enzyme

The existence of a specific mammalian HS degrading factor was first demonstrated in 1975 by Ogren and Lindahl [26], and Höök *et al* [27], who described the isolation of enzyme preparations from mouse mastocytoma and rat liver respectively, which

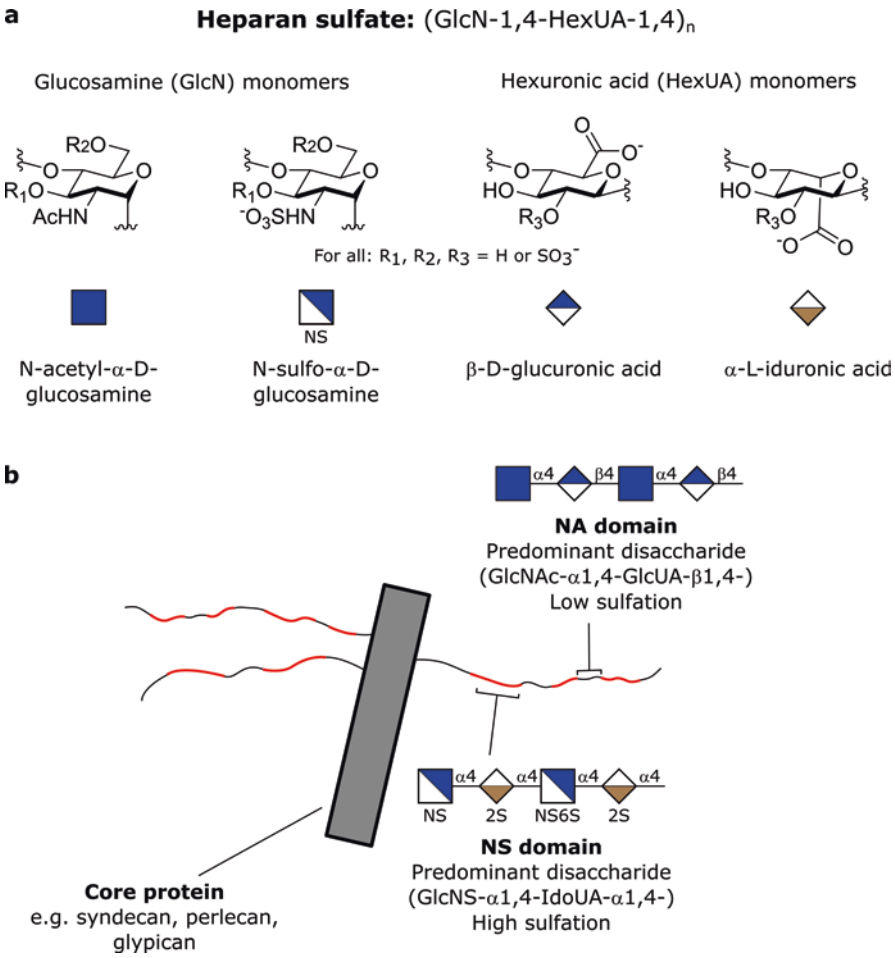

Fig. 5.1 **(a)** Chemical structures of HexUA and GlcNX building blocks of HS, with possible sites of sulfation shown. **(b)** Representative HSPG illustrating the domain structure of HS chains, and the predominant disaccharide units found within NA and NS domains. Mixed NS/NA domains separating NS and NA domains have not been shown here

were capable of degrading heparin and HS to low molecular weight fragments (heparin is structurally similar to highly sulfated HS). These studies were closely followed by reports of similar heparan sulfate degrading activities in a number of different cell and tissue types (Vlodavsky et al., Chap. 1 in this volume).

Heparan sulfate degrading activity in platelets was first demonstrated by Wasteson et al., who found that cultured human glial cells exposed to platelet lysates released low molecular weight HS into their culture medium [28]. Similar HS degrading activity was subsequently identified in placental tissue by Klein and von Figura [29]. Nicolson and coworkers demonstrated that B16 mouse melanoma cells utilized a HS degrading enzyme to assist with breakdown of ECM like barriers *in*

vitro [30], and that the HS degrading capabilities of B16 subpopulations positively correlated with their metastatic potential *in vivo* [31]. This direct relation between heparanase activity and metastatic potential in cancer cells was further demonstrated by Vlodavsky et al., who showed that the poorly metastatic T-lymphoma cell line Eb and its spontaneous highly metastatic variant ESb differed strongly in their ability to degrade HSPGs [32].

A number of observations from these early studies have since become recognized as hallmarks of HPSE activity. Chemical analysis of enzymatically degraded HS products found that cleavage occurred only at the glucuronic acid of HS, not at the glucosamine, indicating that the responsible enzyme was a glucuronidase [26, 29, 33]. Enzymatic HS cleavage was also found to be limited, leading to the formation of intermediate-sized oligosaccharide products resistant to further degradation, consistent with an *endo*-glucuronidase that targets specific HS sites [26, 28, 30–32]. Although commonalities between these early studies indicated researchers were studying the same enzyme activity, it would take more than a decade for the enzyme responsible to be identified unambiguously.

5.3.2 Isolation of Heparanase Enzyme and Cloning of the HPSE Gene

The identity of the HS degrading enzyme was controversial for a number of years, with proteins ranging from 8 kDa to 137 kDa mass being reported as possessing HPSE activity [34–36]. These discrepancies were resolved in the late 1990s, following several independent reports describing the purification of the same HS degrading protein from various sources. Goshen et al. first reported the purification of a ~50 kDa HS degrading enzyme from human placenta [37], followed by Freeman and Parish, who isolated an enzyme of similar size and biochemical profile from platelets [38]. These reports were closely followed by seminal studies from Toyoshima and Nakajima, Vlodavsky et al., Kussie et al. and Hulett et al., who all carried out peptide sequencing of the isolated HS degrading protein, and used this information to identify and clone the responsible HPSE gene [39–41]. These groups all noted the strange observation that whilst the HPSE gene encoded for a ~65 kDa protein; purified HPSE appeared to be ~50 kDa in size, with its N-terminus apparently beginning at Lys158. Furthermore, expression of the full HPSE gene was found to be required for activity, with expression of the sequence corresponding to the ~50 kDa subunit alone failing to endow cells with HS degrading activity [41].

The discrepancy between HPSE gene and protein size was resolved by Fairbanks et al., who demonstrated the existence of a previously undetected 8 kDa subunit in HPSE purified from platelets [42]. This 8 kDa subunit was found to tightly associate with the 50 kDa subunit, only being separable under denaturing conditions, indicating the existence of a non-covalently associated heterodimer. MALDI-TOF analysis identified the 8 kDa subunit of HPSE as Gln36-Glu109, corresponding to an

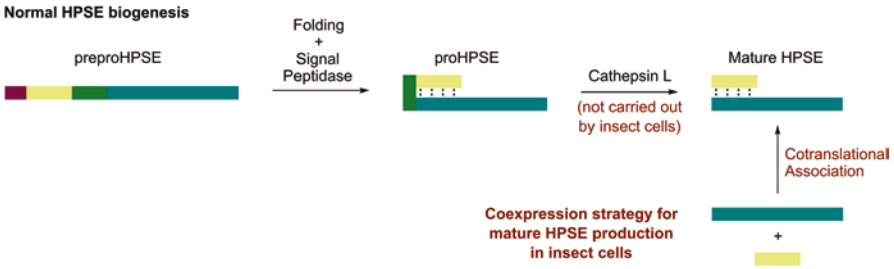

Fig. 5.2 HPSE biogenesis pathway. Steps pertinent to baculoviral expression of pro- and mature HPSE in insect cells are highlighted in red

N-terminal fragment encoded by the HPSE gene. Based on these results, Fairbanks et al. proposed the now widely accepted maturation pathway of the HPSE protein. HPSE is initially expressed as a single chain pre-proenzyme (pre-proHPSE), comprising an N-terminal signal peptide (Met1-Ala35), followed by the 8 kDa (Gln36-Glu109) and 50 kDa (Lys158-Ile543) subunit sequences, separated by a 6 kDa linker peptide (Ser110-Gln158). Loss of the signal peptide from pre-proHPSE following signal peptidase cleavage [43] leads to formation of the inactive HPSE proenzyme (proHPSE). Active HPSE is only produced following proteolytic excision of the 6 kDa linker peptide from proHPSE, leading to formation of the mature enzyme, which exists as a non-covalent heterodimer of 50 kDa and 8 kDa subunits (Fig. 5.2).

5.3.3 Production of Homogenous Recombinant HPSE

Structural biology studies rely on the availability of large amounts of purified homogenous protein. In this regard, the production of recombinant HPSE presents an unusual challenge, due to the complex process of HPSE maturation. Recombinant expression of HPSE in mammalian cells often leads to a mixture of both 65 kDa proHPSE as well as mature HPSE heterodimer [39, 41], rendering these expression systems unsuitable for structural biology purposes.

Protein production in insect cells using the baculovirus expression vector system (BEVS) [44] has proven an invaluable tool for the study of recombinant HPSE. BEVS is a bipartite gene expression platform utilizing recombinant baculovirus for gene delivery and cultured insect cells for protein production. Because insect cells are eukaryotic animal cells (albeit non-mammalian cells), gene expression using BEVS usually allows for the faithful reproduction of native mammalian protein folds. Serendipitously, insect cells lack the cellular apparatus to carry out proHPSE maturation, thus precluding the production of pro- and mature HPSE mixtures [41]. Although this feature of insect cell protein production provides an obvious route towards proHPSE, the problem of accessing mature HPSE was not addressed until McKenzie *et al* demonstrated that co-expression of the 50 kDa and 8 kDa subunits

under two different promotors led to co-translational association of the two subunits, allowing for direct access to mature HPSE [45] (Fig. 5.2).

A markedly different approach to tackling the HPSE linker problem was reported by Nardella *et al* [46], who engineered HPSE to replace the linker region with much shorter sequences. Expression of engineered proHPSE in which the linker sequence was replaced by either an artificial (GSGSGS) repeat or the analogous sequence from a *Hirudinaria manillensis* hyaluronidase (AFKDKTP) gave a single chain variant of HPSE with activity comparable to wild type enzyme. The key role of the 6 kDa linker peptide in controlling (pro)HPSE activity is discussed further below (Sects 5.4.3 and 5.5.1).

5.4 Heparanase – Insights from Crystal Structures

We reported the crystal structure of mature HPSE in 2015, revealing the overall 3-dimensional protein structure of HPSE, and also (via several ligand complexes), the mode of interaction between HPSE and its substrates [47]. This was followed by the solving of the proHPSE crystal structure in 2017 [48]. In this section of the review, we aim to provide an overview of the main insights into the HPSE structure from these two studies, and how the structural features of HPSE relate to its biochemical and biological properties.

5.4.1 3-Dimensional Structure of Mature HPSE

Several features were immediately apparent upon initial solving of the HPSE crystal structure (PDB accession code: 5E8M). The HPSE protein comprises two major domains: a predominant $(\beta/\alpha)_8$ barrel domain, flanked by a smaller β-sandwich domain. The 8 kDa HPSE subunit contributes a single β-sheet towards the β-sandwich domain, as well as the first β-α-β elements of the $(\beta/\alpha)_8$ domain, with the rest of the protein structure being contributed by the 50 kDa subunit. Such a division of structure between the 8 kDa and 50 kDa subunits of HPSE was postulated by Nardella et al., based upon the predicted secondary structure elements within the HPSE sequence [46]. The $(\beta/\alpha)_8$ barrel domain is commonly found in glycoside hydrolases, and usually contains the active site of these enzymes [49]. Visual inspection of the $(\beta/\alpha)_8$ barrel of HPSE revealed a clear cleft in the domain, spanning ~10 Å in diameter, suggesting a binding site for chains of HS. This cleft was lined with a number of basic Arg and Lys residues, which are commonly found in HS interacting protein domains [50–55]. Notably, HS binding "domains" (HBDs) I (Lys158-Asp162) and II (Pro271-Met278), previously identified by Levy-Adam *et al* [56], were found to lie around the HPSE binding cleft, supporting a role for these two domains in facilitating HPSE-HS interactions (Fig. 5.3).

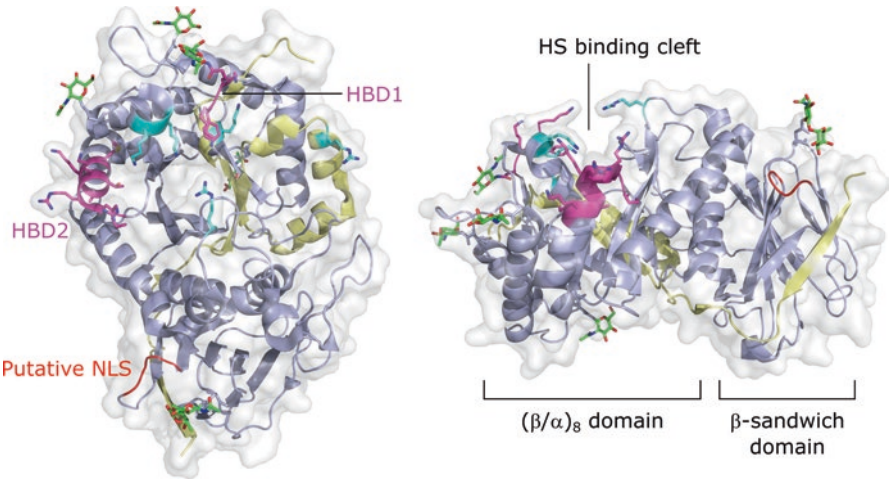

Fig. 5.3 Three-dimensional structure of unliganded human HPSE, showing 'top' (left) and 'side' (right) views. The 50 kDa subunit is colored in blue, and the 8 kDa subunit is colored in yellow (colors correspond with Fig. 5.2). Two domains can be discerned in the HPSE structure, a $(\beta/\alpha)_8$ barrel domain containing the HS-binding cleft, and a smaller β-sandwich domain. HBDs I and II identified by Levy-Adam *et al* [56] are highlighted in pink, other basic residues around the binding cleft are highlighted in cyan. A putative NLS sequence in the β-sandwich domain is highlighted in red. N-glycans are shown in green

N-glycosylation of HPSE is known to be essential for its proper cellular trafficking, and its secretion by cells into the extracellular space [57]. Of the six N-glycosylation sites predicted by analysis of the HPSE sequence, five were visible in the crystal structure of unliganded HPSE, albeit endoglycosidase H digestion carried out prior to protein crystallization meant that most of these were only visible as a single N-linked GlcNAc.

One of the more curious findings of HPSE biology has been the discovery of HPSE in cell nuclei, where it appears to co-localise with highly transcribed euchromatin regions of the genome [58]. Nuclear HPSE can alter the expression of tumor-promoting genes such as matrix metalloproteinase 9 (MMP-9), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [58–61]. Two putative nuclear import signals were noted by Schubert et al. in the HPSE sequence: residues 271–277 (PRRKTAK) and residues 427–430 (KRRK) [62]. While the Pro271-Lys277 sequence forms an alpha helix (and also corresponds to HBD II), Lys427-Lys430 appears in the HPSE crystal structure as a disordered loop near the β-sandwich domain of HPSE. Lack of secondary structure renders this loop-free to interact with the importin machinery involved in nuclear trafficking [63] and is thus consistent with a role for Lys427-Lys430 as a nuclear import signal.

5.4.2 Structural Insights into HPSE Substrate Interactions

The defining feature of HPSE mediated HS cleavage is the high degree of sequence discrimination displayed by HPSE, rendering only certain sites in a HS chain susceptible to enzymatic attack. This behavior is in marked contrast to the bacterial heparin lyases, which carry out a much more complete breakdown of HS with little regard for the sequence of the sugar chain [64–66]. The high specificity of HPSE cleavage was noted as early as the 1970s, with observations that HPSE mediated cleavage of HS produces products intermediate in size between the initial substrate and fully depolymerized HS, and that these oligosaccharide products are resistant to further hydrolysis by HPSE [26, 28, 30–32].

Although sulfation of HS substrates had long been suspected to be important for recognition and cleavage by HPSE, early studies on this topic were hampered by a lack of pure enzyme preparations and chemically defined HS substrates. In this light, the pioneering 1999 study by Pikas et al. on HPSE cleavage site specificity stands as an impressive milestone given the state of HPSE research at the time [67]. Subsequent advances in cloning and recombinant expression of purified HPSE, and the advent of chemoenzymatic HS synthesis have contributed to several reassessments on this topic (summarized in Table 5.1) [68–71]. Whilst studies of HPSE cleavage site specificity agree on a few central points: that HPSE is an *endo*-β-D-glucuronidase, and that sulfation around the HPSE target site is essential for cleavage, the finer details of substrate recognition, especially regarding the specific sulfation patterns required for cleavage, have been a source of disagreement.

Structural biology can help to address such questions regarding enzyme/substrate specificities, *via* the direct visualization of enzyme-substrate complexes. Given the heterogeneous nature of HS itself, the development of specific chemoenzymatically driven HS oligosaccharide synthesis by Petersen and Liu was a crucial foundation for our work to characterize well-defined HPSE-HS complexes [70].

We utilized 3 distinct synthetic, commercially-available, HS oligomers with different sulfation states to probe HPSE-HS interactions with both non-sulfated and sulfated substrates. M04 S00a and M04 S02a contain no sulfates and 1 N-sulfate, respectively and are not HPSE substrates. In contrast, M09 S05a contains 4 N-sulfates and 1 O6-sulfate, endowing this oligosaccharide with a consensus HPSE cleavage site (Fig. 5.4a). Soaking crystals of HPSE with these defined HS oligosaccharides enabled the capture of HPSE-HS complexes *in crystallo*, allowing the molecular basis for interactions between HPSE and its substrates to be mapped (Fig. 5.4b).

HPSE Interactions at the −1 Subsite

The −1 subsite of a glycosidase enzyme is the position occupied by the sugar which directly undergoes glycosidic bond cleavage by the enzyme [72]. In all HPSE-HS complexes we obtained (PDB accession codes: 5E97, 5E98, 5E9B; complexes with

Table 5.1 Summary of studies on HPSE-HS cleavage site specificity

Study	Source of HS	Source of HPSE	Analytical technique	Identified optimal cleavage site	Other notes
Pikas 1998 [67]	Chemically or enzymatically modified *E. Coli* K5 capsular polysaccharide (GlcNAc-α1,4-GlcUA-β1,4-)$_n$. Purified antithrombin binding heparin octasaccharide.	Purified platelet and hepatoma enzyme	Size exclusion chromatography. HPLC	GlcNAc(6S)–GlcUA−/-GlcNS-HexUA(2S)	
Okada 2002 [69]	Defined oligosaccharides purified from porcine intestinal heparin or bovine kidney HS.	Recombinant	HPLC	GlcNS(6S) –GlcUA−/-GlcNS(6S)	O3 sulfation at the +1 subsite promotes HPSE cleavage in low sulfation contexts, and inhibits HPSE cleavage in high sulfation contexts. Given similar cleavage sites, longer HS oligosaccharides are preferred over shorter oligosaccharides.
Peterson 2010 [71]	*E. Coli* K5 oligosaccharide, sulfated using purified HS biosynthetic enzymes.	Recombinant	HPLC Chemical analysis of degradation products.	GlcNS–GlcUA−/-GlcNS(3S or 6S)	If +1 GlcNS is not O3 or O6 sulfated, HPSE searches at the −3 or + 2 GlcUA subsites for 2O sulfation. IdoUA(2S)-GlcNS repeats inhibit HPSE.
Peterson 2012 [70]	Chemoenzymatically synthesized defined oligosaccharides.	Recombinant	HPLC LC-MS	GlcNAc/S(6S) –GlcUA−/-GlcNS(6S)	Only GlcNAc(6S)-GlcUA or GlcNS(6S)-GlcUA repeats were studied. HPSE cleaves consecutive sites if the −2 subsite is GlcNAc(6S) and carries out 'gapped' cleavage if the −2 subsite is GlcNS(6S).
Mao 2014 [68]	Porcine intestinal mucosa HS, bovine kidney HS and recombinant syndecan-4.	Recombinant	LC-MS	GlcUA at boundary between NS/NA domain (non-reducing side) and NS domain (reducing side).	

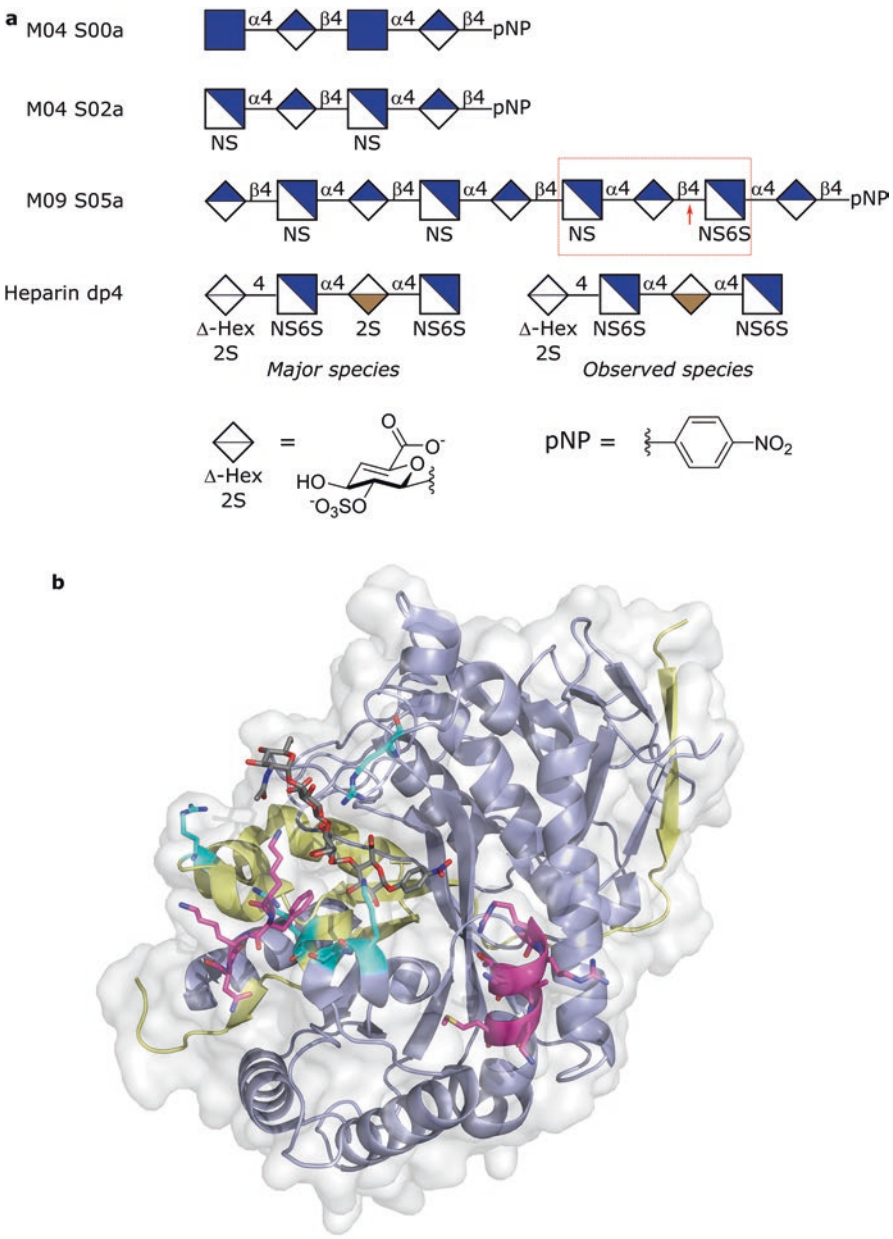

Fig. 5.4 (a) HS and heparin oligosaccharides used to obtain ligand complexes with HPSE. Carbohydrate symbol nomenclature as in Fig. 5.1. M09 S05a contains a consensus HPSE cleavage site – highlighted in the red box, with the cleaved bond indicated by the red arrow. pNP - *para*-nitrophenol. **(b)** Ribbon and surface figure of an M04 S00a oligosaccharide bound within the active site cleft of HPSE (grey sticks). HBDs and other basic residues around the HPSE binding cleft are highlighted pink and cyan respectively

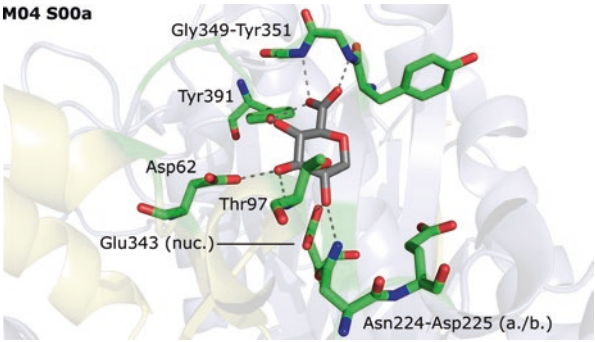

Fig. 5.5 HPSE-HS interactions at the −1 enzyme subsite with M04 S00a. For clarity, only the −1 subsite ligand atoms have been shown

M04 S00a, M04 S02a and M09 S05a respectively), the −1 subsite was occupied by a GlcUA, making identical interactions to the enzyme active site in all cases, illustrating the invariant nature of GlcUA binding at this position. Glycosidases such as HPSE utilize two key catalytic residues to facilitate substrate hydrolysis, a nucleophile and a general acid/base (detailed reviews of glycosidase mechanisms can be found in Refs. 73–77). GlcUA at the −1 subsite of HPSE positions its anomeric center proximal to the catalytic residues Glu343 (nucleophile) and Glu225 (acid/base), in a position ready to undergo attack by the enzyme.

The HPSE −1 enzyme subsite is also characterized by a dense network of H-bonding interactions, made to the C6 carboxylate of the GlcUA from Gly349, Gly350, and Tyr391. These H-bonding interactions appear to be highly conserved amongst HPSE and its homologs [78–79], and likely function as a specificity filter to recognize and bind GlcUA over superficially similar sugars such as glucose (Fig. 5.5).

HPSE Interactions at the −2 Subsite

Whilst −1 subsite interactions in HPSE were observed to be invariant between M04 S00a, M04 S02a and M09 S05a complexes, differences at the −2 subsite of HPSE could be discerned, highlighting the interactions employed by HPSE to recognize different HS sulfation patterns. M04 S00a, which contains no sulfation and is not cleaved by HPSE, places its −2 GlcNAc N-acetyl moiety near residues Ala388-Tyr391 and Asn64, making direct H-bonds to Tyr391, Asn64, and an ordered water molecule. M04 S02a, which differs from M04 S00a by the presence of an N-sulfate, places its −2 GlcNS in the same orientation as the GlcNAc of M04 S00A. However, the larger N-sulfate of GlcNS can make an additional H-bonding interaction to the backbone amide of Gly389, thus rationalizing the preferred interaction of HPSE with GlcNS at the −2 subsite.

The role of O6 sulfation at the −2 subsite was probed by the M09 S05a complex, which showed that the O6 sulfate of GlcNS(6S) was placed towards the ‘upper’ portion of the −2 subsite, proximal to some of the basic residues lining the substrate binding cleft (Lys158 and Lys159). Although we could not observe ordered interactions between O6 sulfate and these basic residues, non-directional electrostatic

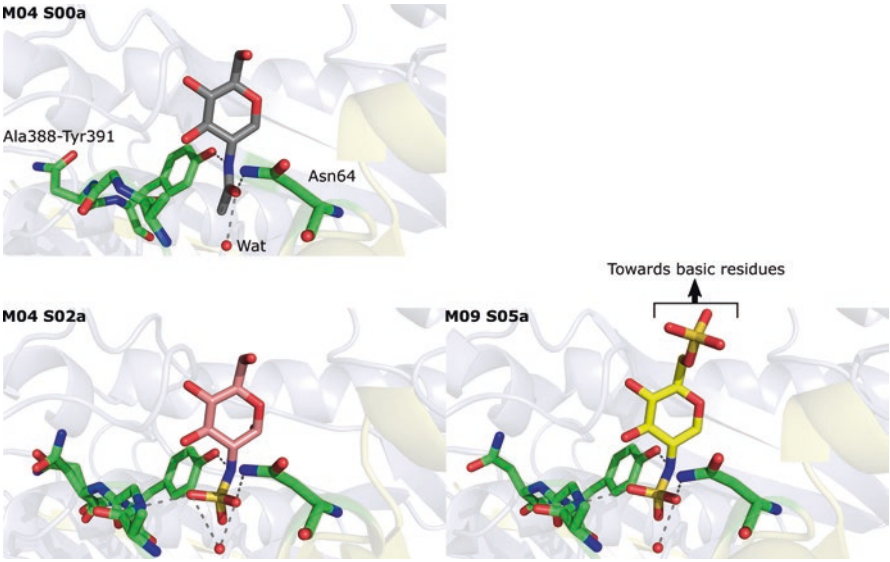

Fig. 5.6 HPSE-HS interactions at the −2 enzyme subsite with M04 S00a, M04 S02a, and M09 S05a. For clarity, only the −2 subsite ligand atoms have been shown

interactions likely play a role in stabilizing the −2 subsite complex between HPSE and O6 sulfated HS substrates (Fig. 5.6).

HPSE Interactions at the +1 Subsite

The primary disadvantage of employing HS oligosaccharides to generate HPSE complexes is the propensity of the enzyme to turn over substrates that match the requirements for HS cleavage. Thus whilst M04 S00a and M04 S02a were observed *in crystallo* to place non-hydrolysed pNP groups at the +1 subsite of HPSE, the presence of a consensus HPSE cleavage site in M09 S05a led to the +1 subsite of the enzyme in this complex to be poorly occupied, due to enzymatic cleavage of the aglycon fragment.

To circumvent this problem, we turned to heparin, a close structural analog of HS, and a known inhibitor of HPSE activity. Soaking HPSE crystals with a heterogeneous heparin dp4 oligosaccharide (obtained through heparin lyase cleavage of polymeric heparin; Fig. 5.4a) yielded a structure with interpretable heparin dp4 electron density within the HPSE active site cleft (PDB accession code: 5E9C). This observed density likely corresponded to a minor component of the heparin dp4 mix, as it was substantially weaker than the electron density observed for the pure HS oligosaccharides. However, this heparin dp4 density spanned the −2, −1 and (crucially) +1 positions of the HPSE active site, thus providing insight into the nature of HPSE +1 subsite interactions.

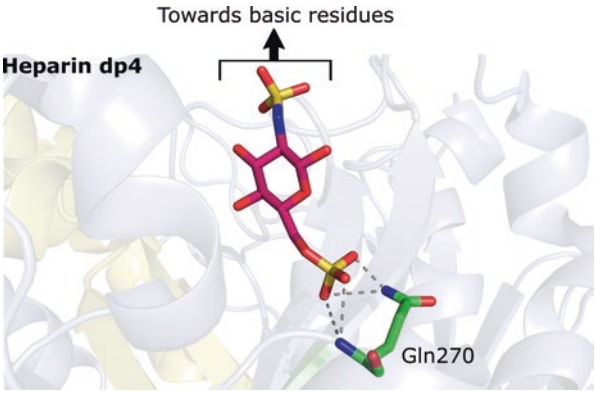

Fig. 5.7 HPSE-HS interactions at the +1 enzyme subsite with heparin dp4. For clarity, only the +1 subsite ligand atoms have been shown

HPSE +1 subsite interactions with heparin dp4 were broadly similar to those observed at the −2 subsite with M09 S05a, except the helical nature of HS and heparin substrates reversed the roles of N- and O6 sulfates at the +1 subsite. Analogous to the role of −2 subsite N-sulfate, we observed H-bonds from +1 subsite O6 sulfate to the sidechain and backbone amide of Gln270. Electron density for the +1 subsite N-sulfate of dp4 was too poor to be observed directly. However, a + 1 subsite N-sulfate could only plausibly be modeled towards the 'top' of the HPSE binding cleft, in position to make electrostatic contacts with the basic residues lining this region (Arg303 and Arg232) (Fig. 5.7).

Taken together, the combined structural data from complexes with M04 S00a, M04 S02a, M09 S05a, and heparin dp4 indicate that HS sulfates in the 'upper' portion of the HPSE binding cleft (−2 subsite O6 sulfate, +1 subsite N sulfate) electrostatically interact with the basic residues around the cleft. In contrast, sulfates 'lower' in the HS-binding cleft appear to make direct H-bonding interactions with HPSE residues and ordered water molecules. Our structures indicate a 'dual mode' of interaction between HPSE and its substrates, with the 'lower' H-bonds likely acting as specificity filters for sulfation (due to the directional nature of H-bonding), while the 'upper' electrostatic interactions stabilize the binding of HS within the active site cleft (Fig. 5.8).

Beyond the +1 Subsite

Although the crystal structures of HS oligosaccharide complexes point to a maximally favored trisaccharide cleavage site, they do not rationalize all findings from biochemical studies of HPSE cleavage specificity. Observations that HS hexasaccharides are preferentially cleaved by HPSE over shorter tetrasaccharides [69], and the ability of HPSE to cleave substrates lacking −2 or + 1 subsite O-sulfation, but containing −3 or + 2 sulfation [71], hint at interactions beyond the core −2, −1 and + 1 subsites that were not captured in our HPSE crystal structure complexes.

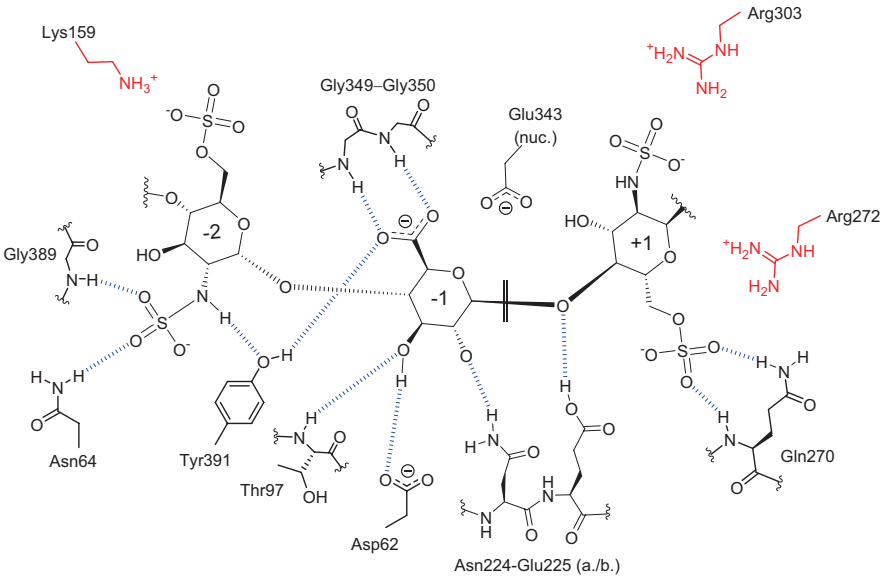

Fig. 5.8 HPSE-HS interactions across the −2, −1 and + 1 subsites of the HPSE binding cleft, as mapped by complexes with HS oligosaccharides and heparin dp4. The site of enzymatic cleavage is shown (//). H-bonding interactions between HPSE and HS are illustrated in blue. Basic residues in place to make electrostatic interactions with HS substrates are shown in red. Nuc. – catalytic nucleophile (Glu343). (a./b.) – catalytic acid/base (Glu225)

The most likely candidates for mediating additional interactions between HPSE and HS are the HBDs postulated by Levy-Adam et al., which may help to bind sulfates outside of the core trisaccharide cleavage site. Modeling studies of HPSE with either the highly sulfated HPSE inhibitor SST0001 [80], or the synthetic HS pentasaccharide fondaparinux [81], suggest that a +2 subsite GlcUA(2S) could interact with basic residues in HPSE HBD2, and thus contribute toward enzyme-substrate binding [82]. It may be the case that HPSE-HS interactions under native contexts are less strictly defined than those captured by static crystal structures, with more distant sulfates potentially being able to compensate for lack of sulfation around the core trisaccharide cleavage site.

5.4.3 3-Dimensional Structure of proHPSE

Proteolytic excision of the 6 kDa linker peptide of proHPSE is required for its maturation to HPSE, indicating a role for this peptide in inactivating proHPSE towards HS substrates. Based on the positions of the 8 kDa and 50 kDa subunit C- and N-termini (respectively) in mature HPSE, we postulated that the 6 kDa linker peptide of proHPSE would likely lie near the HPSE substrate binding cleft, implying a

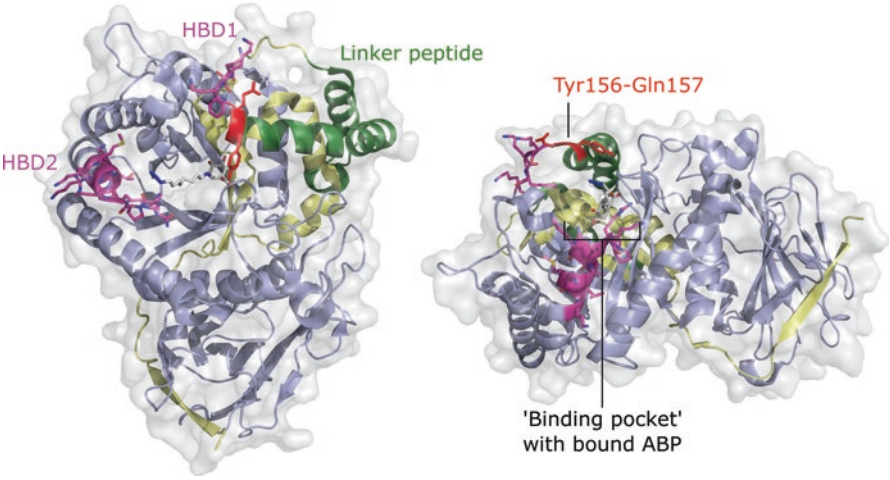

Fig. 5.9 Three-dimensional structure of human proHPSE, showing 'top' (left) and 'side' (right) views. The 6 kDa linker peptide of HPSE (green) sterically occludes the HS-binding cleft (compare with Fig. 5.3). HBDs are highlighted in pink. Tyr156 and Gln157, which form part of the CTSL cleavage site involved in HPSE maturation, are highlighted in red. CTSL cleavage occurs between Gln157 and Lys158 (part of HBD I). A 'binding pocket' structure can be discerned on the surface of proHPSE, and is shown here in complex with a glucuronidase specific activity-based probe (grey sticks)

steric occlusion mechanism for inactivation. This steric mechanism for proHPSE inactivation by its 6 kDa linker peptide was confirmed by the 2017 crystal structure of proHPSE (PDB accession code: 5LA4). Broadly speaking, the proHPSE 6 kDa linker peptide forms a predominantly α-helical domain which sits 'atop' the HPSE binding cleft, thereby preventing the HPSE active site from binding HS [48]. Occlusion of HS-binding appears to be the only mechanism whereby the 6 kDa linker peptide inactivates HPSE (Fig. 5.9). Indeed, protein engineering to 'shrink' the proHPSE linker peptide produces an enzyme with HS degrading activity similar to wild type HPSE [46].

ProHPSE readily binds to cell surface HSPGs and undergoes internalization and trafficking to the lysosome, whereupon it undergoes processing to produce mature HPSE [83–85]. This process of proHPSE sequestration has been proposed to contribute to aggression and metastasis in cancer cells, by providing a mechanism for these cells to capture extracellular proHPSE and increase their own stores of mature HPSE. Although the substrate binding cleft is occluded in proHPSE, HBD1 and HBD2 remain freely accessible on the surface of proHPSE, and electrostatic interactions between these HBDs and cell surface HSPGs may facilitate proHPSE binding to cell surfaces, with subsequent internalization and processing. Proteolytic processing of proHPSE is mediated by cathepsin L (CTSL), with one key cleavage occurring at Gln157−/−Lys158, directed by CTSL recognition of the nearby Tyr156 residue [86–87]. Tyr156-Lys158 in the proHPSE structure reside within a highly

disordered turn towards the end of the 6 kDa linker sequence, where they would be freely accessible for interaction with CTSL (or another protease).

One of the most surprising discoveries upon solving of the proHPSE structure was that the 6 kDa linker peptide only obscures part of the HPSE binding cleft, with a substantial 'binding pocket' still remaining on the protein surface. Whilst large HS substrates are occluded from proHPSE, the 'binding pocket' of proHPSE renders its active site residues fully accessible to smaller 'monosaccharide' like molecules. We confirmed the catalytic competency of proHPSE (at least towards artificial substrates) by labeling the protein with aziridine based activity-based probes, which are highly activated substrate mimics we have previously utilized to study many classes of glycoside hydrolase (PDB accession code: 5LA7; Fig. 5.9) [88–91]. It remains to be seen whether there are biologically relevant substrates *in vivo* which are turned over by the proHPSE 'binding pocket', or whether this motif is an evolutionary relic from an ancestral enzyme (discussed further in Sect. 5.5.1.).

5.5 HPSE Within the Broader CAZy Classification

As an enzyme which catalyzes the hydrolytic breakdown of a carbohydrate substrate, HPSE falls within the general enzyme class known as the glycoside hydrolases (or glycosidases). Glycoside hydrolases are a diverse group of enzymes, which facilitate the hydrolytic breakdown of carbohydrate-containing biomolecules (e.g., glycoproteins, polysaccharides, small molecule glycoconjugates) in varied contexts across all domains of life [92].

Reflecting the central importance of carbohydrate-containing molecules in biology, it has been estimated that ~1–3% of the protein-coding genome of a (non-archael) organism corresponds to enzymes involved in carbohydrate processing (both for synthesis and breakdown) [93]. The Carbohydrate Active enZymes (CAZy) classification aims to classify these carbohydrate processing enzymes into sequence-based families [93–104]. Given that protein sequence largely dictates structure and function, CAZy families typically contain enzymes with similar structural folds and enzyme mechanisms, although the specific substrates processed by enzymes within a family can vary. Under the CAZy classification, HPSE belongs to the GH79 family, itself further classified into the broader GH-A clan (clans are based on groupings of GH families with similar overall topologies and conservation of active site residues) [100, 105]. The GH79 family primarily consists of retaining β-D-glucuronidases, although the substrate contexts of these glucuronic acid residues are diverse, including HS [47, 79], but also chondroitin sulfate [79], hyaluronic acid [46], β-D-glucuronides linked to plant arabinogalactan proteins [106–107], and small molecule β-D-glucuronide glycoconjugates [108].

5.5.1 *Structural Determinants of* exo vs. endo-*Glycosidase Activity in the GH79 Family*

The GH79 family contains representatives of both *endo*-acting and *exo*-acting β-D-glucuronidases, raising the question of how a single 'scaffold' can be adapted to process substrates in either an *exo*- or *endo*- acting fashion. To date, 3 GH79 enzymes have been structurally characterized: HPSE [47–48], *Ac*GH79 from *Acidobacterium capsulatum* [78], and *Bp*Hep from *Burkholderia Pseudomallei* [79]. In keeping with the scope of CAZy classification, there is substantial sequence and structural homology between these three enzymes and all three act as β-D-glucuronidases, although the natural substrates of the two bacterial enzymes are not known.

One area of major variability in GH79 family enzymes is a loop which we have termed the '*exo*-pocket' loop, which connects the 2nd β-sheet of the $(\beta/\alpha)_8$ barrel domain to the 2nd α-helix. Comparison of the three structurally characterized GH79 enzymes demonstrates that the '*exo*-pocket loop' can vary dramatically in size, and appears to act as a key structural determinant of whether an enzyme of the GH79 family behaves as an *exo*- or an *endo*- acting glycosidase.

AcGH79

The crystal structure of the *exo*-acting β-D-glucuronidase *Ac*GH79 was reported by Michikawa et al. in 2012, and was the first enzyme of the GH79 family to be structurally characterized (PDB accession code: 3VNY) [78]. Although the function of *Ac*GH79 in its native biological context is not well understood, the authors determined that this enzyme could not hydrolyze 4-O-methyl GlcUA containing substrates. *Ac*GH79 may be involved in the catabolism of plant arabinogalactan proteins, which typically contain both GlcUA and 4-O-methyl GlcUA substitutions on the main arabinogalactan polymer [106–107].

The '*exo*-pocket' loop of *Ac*GH79 is 23 residues long, extending from Phe86 to His108 (limits defined on the basis of homology to BpHep and proHPSE; Fig. 5.10). This sequence adopts an extended turn that occludes the 'rear' face of the *Ac*GH79 active site, delimiting an *exo*-acting substrate binding pocket that can only accommodate a single GlcUA residue. Discrimination of 4O-methyl GlcUA *vs.* GlcUA is facilitated by Glu45, Pro104, and His327, which together form a tight binding pocket around O4 of GlcUA, which does not allow for further methyl substitution.

BpHep

*Bp*Hep was the first *endo*-acting GH79 enzyme to be structurally characterized, and the second structurally characterized GH79 enzyme overall (PDB accession code: 5BWI) [79]. *Bp*Hep is an *endo*-β-D-glucuronidase which can degrade both heparan

sulfate and chondroitin sulfate, suggesting it may be a general glycosaminoglycan breakdown enzyme. Saturation-transfer difference NMR binding experiments using defined HS oligomers suggest that *Bp*Hep prefers to interact with HS cleavage sites rich in GlcNAc rather than GlcNS, indicating a different HS substrate specificity to that displayed by HPSE.

Compared to *Ac*GH79, the '*exo*-pocket' loop of BpHep is substantially shorter in length (Gly92 to Asp99; 8 residues long; Fig. 5.10), and is not long enough to occlude any part of the enzyme active site. Instead, this very short '*exo*-pocket' loop of B*p*Hep results in opening of the enzyme active site, revealing an extended *endo*-acting binding cleft, well suited for interaction with glycosaminoglycan substrates.

(pro)HPSE

ProHPSE to HPSE maturation provides the most direct example of the role of the 'exo pocket' loop in controlling *exo−/endo-* activity in GH79 family enzymes. The '*exo*-pocket' loop of (pro)HPSE (110 to 157; 48 residues long) directly corresponds to the 6 kDa linker peptide and is substantially increased in size compared to corresponding '*exo*-pocket' loop sequences in AcGH79 and BpHep (Fig. 5.10).

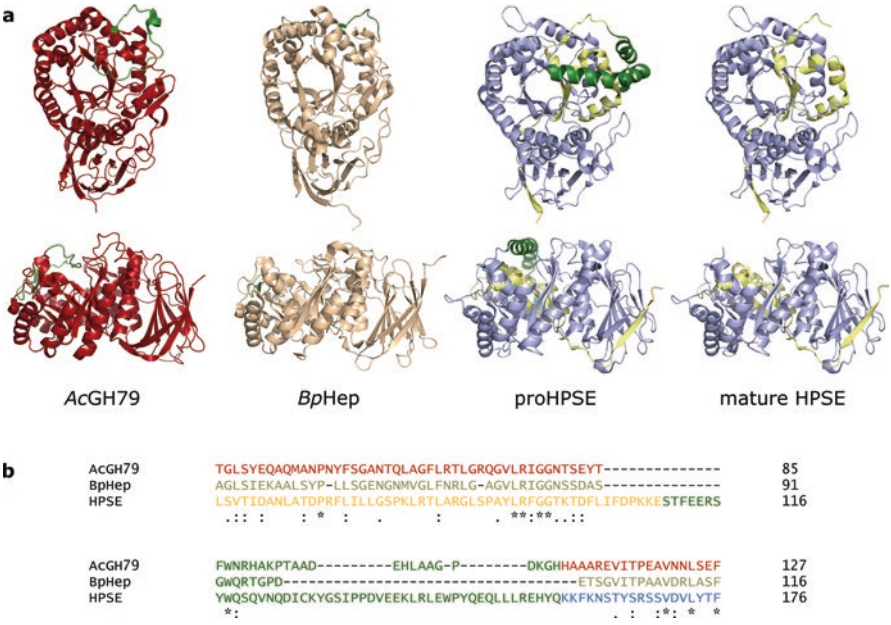

Fig. 5.10 (**a**) '*Exo*-pocket' loop structures for *Ac*GH79, *Bp*Hep and proHPSE (highlighted green) showing their role in delineating an *exo*-acting binding pocket structure in *Ac*GH79 and proHPSE, or alternatively, an *endo*-acting binding cleft structure in *Bp*Hep. Proteolytic removal of the '*exo*-pocket' loop of proHPSE (i.e. the 6 kDa linker peptide), reveals the *endo*-acting binding cleft of mature HPSE. (**b**) Clustal ω [149] alignments of *Ac*GH79, *Bp*Hep, and HPSE showing the variation in '*exo*-pocket' loop lengths between these three proteins

As mentioned in Sect. 5.4.3., the 6 kDa linker of proHPSE forms an alpha-helical domain that acts as a direct steric block 'above' the HPSE binding cleft, preventing interaction of the enzyme with HS substrates. Removal of the proHPSE linker is required for unmasking of the mature HPSE binding cleft, and can be considered analogous to the effect of the minimal '*exo*-pocket' loop sequences of *Bp*Hep [79] and engineered single chain HPSE mutants [46].

Comparison of proHPSE with *Ac*GH79 and *Bp*Hep places the exoglycosidase like binding pocket of proHPSE into an understandable evolutionary context (see Sect. 5.4.3.). We hypothesize that expansion of the '*exo*-pocket' loop sequence from an ancestral *exo*-acting GH79 enzyme led to formation of the 6 kDa proHPSE linker, without formal loss of the *exo*-acting binding pocket architecture. The retention of such *exo*-glycosidase like structural features on proHPSE warrants consideration whether there are genuine substrates that are processed by this protein species *in vivo*. Supporting this hypothesis, mature HPSE has been demonstrated to possess *exo*-glycosidase activity against terminal glucuronides within certain HS contexts [109]. There is no *structural* reason why proHPSE would not also possess such activity, and thus it may play a role in, e.g. trimming terminal glucuronides from certain HS chains.

5.6 Concluding Remarks and Future Challenges

HPSE has captured the interest of researchers for over four decades, with efforts to understand its function ranging from fundamental biochemical studies on HPSE enzymatic activity to complex biomedical characterizations of its role in cancer and other diseases. Structural studies of HPSE and proHPSE provide a framework on which to place these biochemical and biomedical insights, allowing them to be related to features on the HPSE protein itself.

There are still a number of unresolved challenges in the HPSE field, which will likely be the subject of substantial research efforts in the coming years.

Most pressingly, despite intense interest in HPSE as an anti-cancer target, there are few effective HPSE inhibitors known, and none in use clinically. Various small molecule HPSE inhibitors have been reported, based on benzoxazole, furanylthiazole [110], isoindole [111], benzimidazole [112–113], and other scaffolds. However, none of these small molecule inhibitors appear to have progressed beyond initial enzyme inhibition and invasion/angiogenesis studies. More recently, four HPSE inhibitors have entered clinical trials: PI-88 [114–119], SST0001 [82, 120–122], M402 [123–124] and PG545 [125–129], although an interim analysis of PI-88 phase III clinical trials showed a failure to meet its primary endpoint (disease-free survival) [130] (Chhabra & Ferro; Noseda et al., Hammond & Dredge, Chaps. 19, 21 and 22 in this volume). All HPSE inhibitors currently under clinical trials are highly sulfated oligosaccharide molecules, and of these only PG545 possesses a well-defined molecular structure. Such oligosaccharide-derived molecules are less likely to possess desirable pharmacokinetic properties, and a renewal of efforts to develop novel small molecule HPSE inhibitors may be timely.

The HPSE field also lacks a reliable, sensitive, and facile method for quantitation of HPSE enzymatic activity [131]. The development of routine activity assays, often relying on artificial chromogenic and fluorogenic substrates, has been essential for enzyme discovery and enzyme characterization efforts in the glycosidase field [132–136]. Robust assays are vital for effective inhibitor development since a potential inhibitor cannot be quantitatively characterized if there is no suitable assay available to inhibit. The lack of 'gold standard' assays for HPSE probably reflects the complex nature of its interaction with HS, which may be difficult to recapitulate in artificial substrates.

Finally, the discovery of a close homolog of HPSE, termed HPSE2, which can bind HS but lacks glycosidase activity [137], raises questions regarding the biological functions of HPSE2, and how they might relate to HPSE. HPSE2 expression inversely correlates with the size and grade of tumours [138–139], and it appears to act as an anti-tumorigenic factor [140], possibly through antagonism of HPSE activity [141]. Biallelic mutations in HPSE2 have been linked to the rare genetic condition urofacial syndrome (UFS; also known as Ochoa syndrome) [142–145], a disease characterized by symptoms of facial grimacing, coupled to loss of adequate urinary voiding [146]. Such symptoms may indicate that HPSE2 plays a role in urinary tract and/or neurological development [147–148]. (Mckenzie; Roberts and Woolf, Chaps. 34 and 35 in this volume).

We anticipate that meeting the above (and other) challenges in the HPSE field will greatly benefit from an improved understanding of HPSE structure/function relationships. Structure-guided development of new methods to assess and modulate HPSE activity will doubtless lead to improvements in our ability to treat HPSE driven cancers and other HPSE related diseases. More fundamentally, improved molecular understanding of HPSE activity will also help us better understand the many varied roles of this enzyme in the regulation of HSPGs and the ECM.

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Chapter 6
Molecular Aspects of Heparanase Interaction with Heparan Sulfate, Heparin and Glycol Split Heparin

Stefano Elli and Marco Guerrini

6.1 Introduction

Heparanase (Hse) is a sub-class of Glycosyl Hydrolase enzymes (GH) that catalyze the hydrolysis of glycosidic bonds of different geometries and connectivity, in di-, oligo- and poly- saccharides. The first mention of heparanase in the literature was in 1975 when Ögren and Lindahl [1] reported the cleavage of heparin by an enzyme isolated from mastocytomas of tumor-bearing mice. The enzyme was cloned and expressed more than two decades later by five independent groups: Vlodavsky et al. [2], Hulett et al. [3], Kussie et al. [4], Fairbanks et al. [5], and Toyoshima et al. [6] In human, two isoforms of heparanase are known: heparanase 1, here referred simply as heparanase, is responsible for the hydrolysis of the glycosaminoglycan heparan sulfate (HS). In 2000 the group of McKenzie (McKenzie et al.) [7] discovered a second isoform of heparanase: heparanase 2, characterized by a 40% similarity with Hse 1, but lack of enzymatic activity, whose roles in physiology and pathological states are until now poorly understood (Rivara et al. [8]).

More specifically, Hse is an endo-β-glucuronidase that belongs to the GH79 family; Hse is the principal enzyme that degrades HS in both normal (HS turnover) and pathological (tumor metastasis and/or inflammation) conditions, catalyzing the hydrolysis of the β-1-4 glycosidic bond in -GlcUA-β(1-4)-GlcNX-. HS is a ubiquitous component of cell surfaces and tissues, being together with fibrous proteins one of the main components of the extracellular matrix (ECM). The biosynthetic precursor of HS is a polymer composed by the -4) D-GlcUA β(1-4) D-GlcNAc α(1- disaccharide repeating unit, which by the coordinated action of several enzymes (e.g., glycosyl- and sulfo-transferases, endo-sulfatases, deacetylases, and epimerase), gives rise to a complex polymer implicated in various biochemical and physiological activities. HS is assembled with different core proteins, and its structure varies

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both in terms of sulfation degree and organization of sulfated and not sulfated domains, according to organisms and cell types (Yates et al. [9]). As well as contributing to the structural integrity and self-assembly of the ECM, HS acts as a reservoir of enzymes, growth factors, cytokines, and chemokines, providing extracellular storage of these biomolecules and modulating their bioavailability. In inflammatory and cancer diseases, Hse expression is markedly increased, contributing to the release and activation of growth factors and facilitating cell migration through intact capillary walls into the tissues, promoting angiogenesis and tissue remodeling. Inhibition of heparanase is, therefore, a promising target to prevent cleavage of HS in the extracellular matrix and attenuate tumor growth and angiogenesis. Despite considerable efforts invested in the development of different heparanase inhibitors, the mechanism of molecular recognition by which Hse interacts with natural and chemically modified HS substrates is still not fully elucidated. Since the human Hse crystal structure has recently been solved (Wu L. et al. [10]) (Wu and Davies, Chap. 5 in this volume), some early proposed binding modes of heparan sulfate as well as the design of novel inhibitors have been developed initially by homology models, used as template for different GH proteins of known 3D structure.

This chapter aims to review the state-of-the-art knowledge about the structural aspects of Hse activities in terms of substrate recognition, mechanism of hydrolysis, and inhibition, starting from the recently published details on the 3D structure of Hse (Wu L. et al.) and previous studies based on homology modeling approach and our own structural data.

6.2 Mechanism of Enzymatic Hydrolysis

The enzymatic hydrolysis of GH works through a mechanism of acid catalysis, which from a stereochemical point of view implies the retention or inversion of the anomeric carbon configuration. This mechanism requires two key residues: a proton donor (acidic in accord to Bronsted-Lowry) and a nucleophile (base) (Fig. 6.1). Interestingly, the retention mechanism requires, as a geometrical constraint, a distance smaller than 6 Å between the side chains of the two catalytic pairs. Differently, enzymes with inversion mechanism have catalytic pair residues at greater distances (approximately 10 Å), due to the necessarily involved water molecule, located between the anomeric carbon of the substrate and the nucleophile attacking residue (Davies G. et al.) [11]. From a general point of view, the “architecture” of the active site in GH enzymes contributes, by means of different strategies, to reduce the energy barrier on the reaction coordinate systems. In some cases, the geometry of the substrate glycan ring will be deformed to a half-chair or sofa-conformation, as shown in the neuraminidase family.

In other cases, the geometry of the enzyme active site contributes to deformation of the glycosidic bond that will be hydrolyzed, for example, protonating the glycosidic oxygen. Davies et al. [11] classified the GH in families (more than 45) in accord to similarities of the amino acid sequence, with the idea that inside each

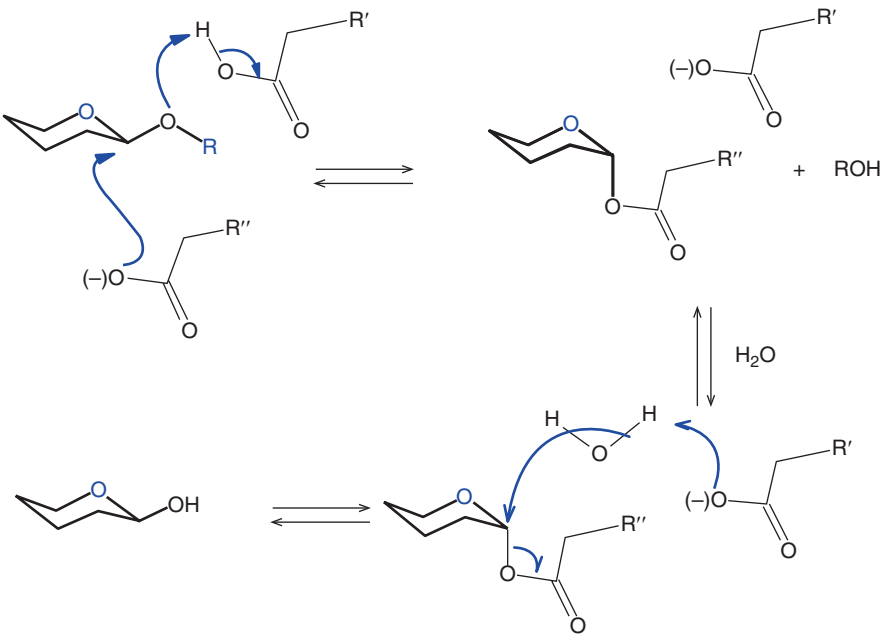

Fig. 6.1 Mechanism of the hydrolysis reaction with retention of the anomeric configuration

family, the different enzymes should fold similarly. From another point of view, the same authors suggested to classify the GH enzymes in three different topology types, in accord to the shape/geometry of the active site and the sub-structures that surround it, and independently from the stereochemistry of the catalysis. The first topology is the *pocket* or *crater*, in which the active site is found at the bottom of a deep hole and recognizes a saccharide residue at the non-reducing end. This topology of the active site is optimized to degrade non-reducing end branched polysaccharides such as starch by β-amylase, or the receptors of the influenza virus terminated at their non-reducing end by sialic acid, that will be removed by the neuraminidase enzyme. GH with this active site topology are termed *exo* acting GH. The second topology of GH active site is the *cleft* or *groove*, an exposed channel structure at the bottom of which the catalytic pair is located; this site allows a random binding of the polysaccharide chain and is typical of the *endo* acting GH. The third topology is the *tunnel*, thought to be evolved from the previous topology by a loop extension that covers part of the cleft. This topology allows the enzyme to remain firmly bound to the polysaccharide chain during degradation, improving the efficiency of the process, but without giving a unique interpretation of how the substrate binds the enzyme, namely in an *exo*-GH (tunnel opening), or *endo*-GH (by one side of the tunnel) manner.

Hse is a stereospecific enzyme that acts with retention of the anomeric carbon configuration [12, 13, 18]. Since the end of the 90s, biochemical investigations were initiated by independent groups (Pikas D. S. et al. [12] and Okada Y. et al. [13]) that

applied a set of HS-like and Hep-like oligosaccharides and investigated the amount of the hydrolyzed substrate or the possible inhibitory effects. They established that Hse recognizes preferentially the high sulfation domains of HS and that the smallest HS-like sequence recognized by Hse is a trisaccharide GlcNX-GlcUA-GlcNX, in which the two GlcNX residues flanking the target GlcUA, need to be sulfated in N- and/or 6-O- positions. Considering that Hse acts as an *endo*-enzyme, and its active-site topology is a binding cleft (Fig. 6.2, center), the HS chain should approach in parallel to the enzyme active site, before the recognition stage that immediately precedes the hydrolysis. The binding cleft must recognize the series of residues that precede and follow the target GlcUA, where the glycosidic bond will be hydrolyzed. Davies et al. [14] proposed a labeling system for the residues in glycan chains serving as a substrate of GH enzymes: the target residue sequence is labeled from -n to +n in going from the non-reducing to the reducing end, while the hydrolyzed glycosidic bond is located exactly between -1 and + 1, respectively. In this system, the target GlcUA is labeled -1, while the two flanking GlcNX at the non-reducing and reducing end, will be labeled -2 and + 1 in this order. Interestingly, these investigations indicate that the high sulfation degree in this trisaccharide moiety is necessarily required to be targeted by Hse, but some variability in term of positions of these sulfo groups is allowed, probably reflecting the variability of the HS and heparin glycosaminoglycans (GAGs). Okada et al. [13] found that human recombinant Hse recognizes the trisaccharide sequence GlcNAc6S-GlcUA-GlcNS, in which, in addition to the required 6-O-sulfo and 2-N-sulfo groups, an extra sulfo group located at +1 or in an additional neighbor sequence (+2), is also necessary for substrate recognition. Pikas et al. [12] and Okada et al. [13] both established that the 3-O sulfo group in GlcN residue located at +1 position, typically found in the Hep antithrombin (AT) specific binding moiety, promotes Hse hydrolysis when it is part

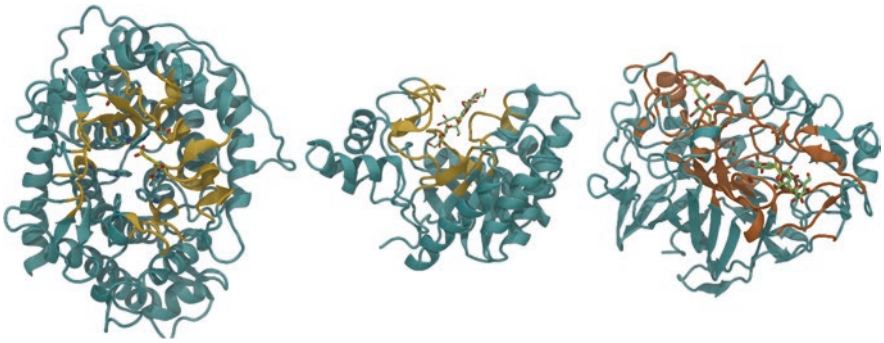

Fig. 6.2 3D structure of GH obtained by X-ray diffraction pattern resolution. The protein is represented in ribbon, and the substrate is drawn in tubes. Presented is glucoamylase from Awamori PDB (ID 1GLM; left); Catalytic domain of endo-1,4-glucanase Cel6A (Fusca PDB ID 2BOD center); and Cellobiohydrolase Cel7A from T. Atroviride PDB (ID 5O59; right). The active site sub-units are underlined in yellow (left and center complexes) and orange (right complex). Shown are examples of GH with the active site in pocket topology (left), clef or groove (middle), and tunnel (right) topologies, as suggested in Davies et al. [11]

of a longer and sulfated GAG fragment (octasaccharide). Later studies (Bisio et al. [15], in agreement with Pikas et al. [12]), demonstrated that even the shorter synthetic pentasaccharide fondaparinux (GlcNS,6S-GlcUA-GlcNS,3S,6S-IdoUA2S-GlcNS,6S-OMe, here labeled AGA*IA, corresponding to the AT binding sequence) is hydrolyzed by Hse between GlcUA and GlcNS,3S,6S. Pikas et al. [12] also indicated a slightly different trisaccharide recognition moiety for Hse obtained from human hepatoma and platelets; in their study the 2-N-sulfo groups are considered compatible but not strictly required, while the 6-O-sulfo groups on GlcN at both positions -2 and + 1, and additionally the 2-O-sulfo on IdoUA2S or GlcUA2S at +2 position, are essential for the HS or Hep sequence to be recognized and hydrolyzed by Hse. A more recent study, using a series of chemo-enzymatically modified polysaccharides, confirmed the observation of Pikas and Okada demonstrating that O-sulfation of the GlcNS residue at position +1 conferred susceptibility to cleavage by Hse, independently by its position (i.e., 3-O or 6-O sulfation). By using [35] S-labeled polysaccharide substrates and the combined action of Hse and heparinase enzymes, authors also demonstrated that if the O-sulfation of GlcN at position +1 is absent, Hse recognizes the 2-O sulfation on GlcA at position +2, cleaving the GlcA-GlcNS sequence. On the contrary, the exclusive presence of 2-O-sulfation on IdoA residue caused complete inhibition of the enzyme, comparable to the synthetic PI-88 (Peterson et al. [16]); some of these observations will find an explanation when considering the structural details nowadays available for the Hse active site (Wu and Davies, Chap. 5 in this volume), as summarized in the following paragraphs.

6.3 Heparanase and its Active Site Structure as Predicted by Homology and x-Ray Diffraction Models

Description of Hse structure and synthesis is presented in several excellent reviews (Rivara *et al* [8] and Vlodavsky et al. [17]). Hse is produced as a pre-pro-enzyme of 543 amino acids, the sequence M1-A35 is then removed to generate the pro-heparanase, with molecular weight of approximately 65 kDa. The pro-heparanase is activated after excision of a linker peptide S110-Q157, generating a dimeric protein formed by a small (N-terminal Q36-E109) and large (K158-I543) sub-units, with molecular weights of 8 and 50 kDa, respectively. The two sub-units remain non-covalently linked, and both are required for Hse catalytic activity. The 3D structure of Hse was first investigated by homology models generated using GHs of known 3D structure as templates and characterized by high sequence identity/similarity and similar stereochemistry of reaction as an additional constraint. Hulett et al., [18] applying protein sequence alignment and secondary structure prediction approach, revealed that mammalian Hse is related to the families 10, 39, and 51 of the clan A of GH (GH-A). Interestingly, the GH-A presents a common sub-structure: the $(\beta/\alpha)_8$-TIM-barrel domain, a distinctive characteristic which is also predicted by the secondary structure analysis, that underlines an alternating α-helix and β-sheet

motif. The high degree of sequence identity between the active sites of GH-A family 10, 39, 51, and Hse suggests a similar catalytic mechanism and allows to identify E225 and E343 as the common putative proton donor and nucleophile acting residues, respectively. This result was also supported by the mutations E225A or E343A, that in both cases generate a complete inactive enzyme. Additionally, the authors showed that the **W**xxx**NE** sequence, which immediately precedes the putative proton donor E225, is conserved in retaining GH configurations. Analyzing the human Hse sequence, Hulett et al. [18] identified several amino acids with positive side chains in the proximity of E225 and E343, that are supposed to play a role in HS recognition and binding. Two clusters of basic amino acids were detected, that include a known HS binding sequence (xBBBxxBx or xBBxBx) QKKFKN (157-162) and PRRKTAKM (271-278), respectively. The identification of these clusters on the sequence and 3D structure of endo-β-1-4-Xylanase from P. *simplicissimum* (PDB ID 1BG4), allowed to localize them in proximity to the active site, supporting their contribution to the recognition/binding of HS. Zhou Z. et al. [19] (2006) published a homology model of Hse in an attempt to design and characterize a set of inhibitors. The homology procedure applied as template the Xylanase (PDB ID 1BG4), that shows a sequence identity and similarity of 20% and 57% respectively, indicated that both the template and target sequence shared the $(\beta/\alpha)_8$-TIM barrel sub-structure. In fact, also in this case, the secondary structure prediction algorithms depict the repeating α-helix and β-sheet motif that is exactly at the base of this sub-unit, as previously reported by Hulett et al. [18]. In accord to Hulett et al. [18], Zhou et al. [19] proposed a catalytic mechanism that involves, in the center of the active site cleft (groove), a proton donor (E225) and a nucleophile (E343), at the hedge and in the middle of the TIM-barrel, respectively, where they protrude from opposite directions. Interestingly, the local sequences that include E225 and E343 show a good alignment with the corresponding Xylanase template and the alternative reference Glycanase (PDB ID 1EXP), reproducing their 3D structure at least locally. In the second part of their work, Zhou et al. [19] applied automatic docking, augmented by an "induced-fit" procedure, to dock a set of 2,3-dihydro-1,3-dioxo-1H-isoindole-5-carboxylic acid (DDICA) derivatives, that have been previously reported to be inhibitors of heparanase [20, 21]. The observation that the "induced-fit" docking improved the score of the solutions and the quality of their final Structure-Activity Relationship (SAR) suggested that the Hse binding cleft presents some degree of flexibility surrounding a possible ligand. Particularly, in the absence of the ligand, the conformation of side chain residues in the binding cleft is optimized, reducing the accessibility to the cleft itself. A subset of the ensemble of ligands was used as a "training set" to estimate the parameters of the Free Energy of Binding (FEB) model, that was expressed in term of IC50. The estimated IC50 was then compared to the measured values, revealing a qualitatively good linear correlation and thus supporting the whole procedure as a structure-activity prediction method. Later, Sapay et al. [22] proposed a homology model of Hse based on the endo-1,4-β-D-Xylanase of *P. simplicissimum* (GH10 family), whose 3D structure was co-crystallized with a series of xylan oligosaccharides. In this case, the sequence identity and similarity between the query and the target were 20% and 40%,

respectively. Contemporaneously, Gandhi et al. [23] applied homology modeling to estimate the 3D structure of Hse, focusing on its active site, using as templates the endoglucanase belonging to the GH family 44 and corresponding to the β-retaining endo-acting GH (PDBID 3IK2 and 2E4T). The sequence identity between Hse and these two GH44 templates were 14% and 16%, respectively. The VERIFY 3D test applied on the binding domains and the catalytic site suggested that this model is of reasonable quality, despite the low sequence identity/similarity. In the second part of their work, Gandhi et al. [23] applied automatic docking to map the contacts between different oligosaccharides and residues of the modeled Hse active site. The set of evaluated ligands included GAGs that are known to be hydrolyzed (Pikas et al. [12], Okada et al. [13]) and poly-sulfated oligosaccharides that are proven to be inhibitors of Hse (Maltohexaose sulfate, PI-88 [24, 25]). This investigation was the first attempt to systematically correlate results already established by biochemical experiments on HS- and heparin-binding domain of human Hse, with those represented by the early models of this enzyme generated by homology procedures.

The first X-ray resolved 3D structure of Hse in both apo and complexed with HS oligosaccharide mimics was published only recently by Wu et al. [10] (Wu and Davies, Chap. 5 in this volume). The quaternary structure of Hse is organized in two sub-units: a $(\beta/\alpha)_8$-TIM barrel domain, in which a secondary structure motif β/α is replicated eight times to form a cylindrical assembly, and a β-sandwich domain formed by eight β-strands. The first three secondary structure elements of the TIM barrel (β-α-β) and one of the eight β-strands of the β-sandwich domain belong to the 8 kDa sub-unit, while the remaining elements are part of the 50 kDa sub-unit [8]. The binding cleft is part of the $(\beta/\alpha)_8$-TIM barrel domain, which extends in liner size between 15-20 Å, as measured on the 3D model (PDB ID 5E9B), giving the possibility to bind/recognize from six to seven residues of a GAG chains (Fig. 6.1b, Fig. 6.1c in Wu et al. [10] and Fig. 6.3). In this model of Hse, the excised S110-Q157 sequence of Pro-Hse should be near the active cleft, in accord to previously reported observations on homology-built models. It was assumed that the presence of this sequence should hinder HS chains from approaching the binding cleft, leaving the pro-enzyme inactive. The Hse binding cleft is surrounded at its border by several positively charged amino acid side chains, whose role is to attract and orient from a large distance the negatively charged chains (GAGs) and to fit them in the cleft. Analysis of the late-appearing homology model of Hse, built using the 3D structure of the GH 79 β-glucuronidase from *A. Capsulatum* (Pala et al. [26]), indicated that the active site cleft accommodates heparin and glycol-split heparin (gsHep) oligosaccharides with the non-reducing end oriented toward the set of residues known as: "Heparin Binding Domain 1" HBD1 (R70, R93, K98, K108, K159, K161, R303); while "Heparin Binding Domain 2" HBD2 (K231, K232, R272, R273, K274, K325) will host the reducing end of HS. The hypothesis of a preferred orientation of GAGs was also reported in earlier homology models of Hse (Sapay et al. [22], and Gandhi et al. [23]) even if not explicitly discussed, and in a conformational characterization by molecular dynamic simulation of heparin-like octasaccharides in the bound state with Hse (Elli et al. [27]). Noteworthy, the 3D structure of Hse for this investigation was obtained in collaboration with the groups of Livnah and Vlodavsky (Vlodavsky

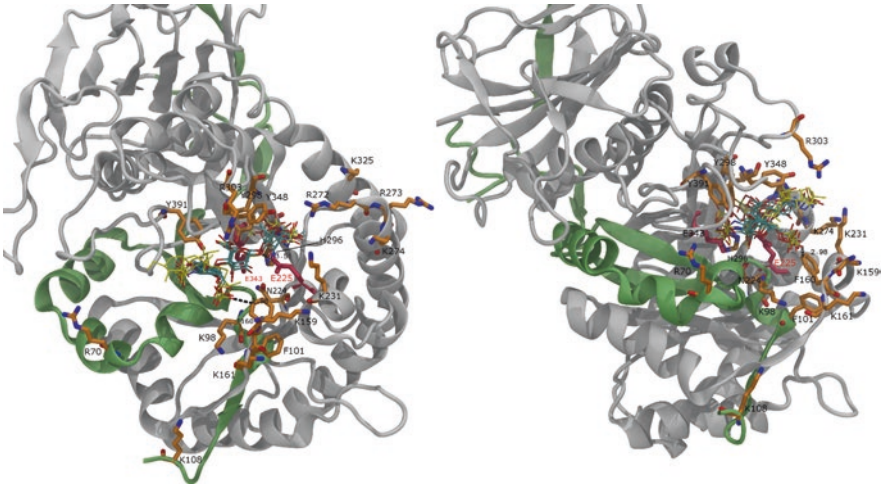

Fig. 6.3 The trisaccharide GlcNS6S-GlcUA-GlcNS6S in the bound state with Hse generated by MD simulation of 30 ns length; the trisaccharide is represented by three snapshots sampled every 10 ns and drawn with cyan tubes for the carbon skeleton. The gray and green ribbon indicates the 50 and 8 kDa subunits, respectively of Hse, corresponding to the 30 ns pose of the simulation. The complex geometry includes an X-ray resolved tetrasaccharide -GlcNS-GlcUA-GlcNS6S-GlcUA, residual of a nona-saccharide GlcUA-[GlcNS-GlcUA]$_3$-GlcNS6S-GlcUA-pNO$_2$-Ph, co-crystallized with Hse (PDB ID 5E9B, Wu L. et al. [10]). The geometry of the X-ray resolved complex is superposed to the four MD generated snapshots, matching two selected sequences of eleven residues each, that are centered on the two catalytic residues E225, E343 (backbone RMSD <0.6 Å). Left and right panels correspond to the top and side view respectively of the $(\beta/\alpha)_8$ TIM-barrel and β-sandwich heterodimer structure of Hse. The MD simulation was run using NAMD 2.12 in explicit solvent (TIP3P water model) and the images were generated using VMD 1.9.2. The Force Field used to describe the protein and the glycan was Amber [31] and GLYCAM06 [32] respectively; the former is commonly accepted in protein description, and the latter corresponds to the "state of the art" for glycan characterization. To run the simulation the NAMD 2.12 software was applied [33]. The initial geometry of the complex was obtained superposing their GlcUA on the corresponding residue in the X-ray resolved complex between a tetrasaccharide -GlcNS-GlcUA-GlcNS6S-GlcUA-, residual of a nona-saccharide, and Hse (PDB ID 5E9B, Wu et al. [10])

et al. 2010, unpublished results) [28], before the nowadays public available structure of Wu et al. [10]. Briefly, a single-chain constitutively active heparanase was obtained by connecting the 8 and 50 kDa subunits with a spacer of three glycine-serine pairs. This preparation (HepGS3) was comparable to the processed, heterodimeric enzyme with regard to specific activity, profile of hydrolysis products, and inhibition by heparin (Nardella C. et al. [29]). Crystallization of the HepGS3 protein (Golan G., Livnah O., Vlodavsky I. et al. unpublished results) revealed structural features (i.e., TIM-barrel domain that contains the two catalytic glutamic acids and two heparin-binding domains, attached to a β-sandwich C-domain) identical to the recently resolved crystal structure of the native enzyme [10]. An earlier investigation by Levy-Adam et al. [30] demonstrated the ability of this domain to bind HS and heparin, applying NMR titration experiments using a peptide "to sketch" the

HBD1 (K158-N171) domain and the pentasaccharide sequence (AGA∗IA). This experiment and site-directed mutagenesis studies underlined the key role of the electrostatically charged K158, K159, K161, the hydrophobic F160, and the polar N162. This result was corroborated by the observation that this peptide inhibits Hse enzymatic activity, possibly by competition with the substrate. Levy-Adam et al. [30] also showed that Hse missing the sequence K158-N171 (HBD1) or N270-K280 (HBD2), lost completely its enzymatic activity, suggesting that both domains HBD1 and HBD2 act cooperatively in binding the HS chains, for example being part of the same recognition system (cleft). This prediction becomes clearly conceivable considering the 3D structures of the different Hse homology models here reported (Sapay et al. [22], Gandhi et al. [23], Pala et al. [26]) and the resolved X-ray structure (Wu et al. [10]), which described the binding region formed by the HBD1 and HBD2 domains that host the non-reducing and reducing ends of HS, leaving the catalytic site at the centre of the cleft as part of the $(\beta/\alpha)_8$-TIM barrel domain. As predicted by models [7, 19, 22, 23, 26], the X-ray structure of Hse shows that the catalytic pair of residues E225 and E343, positioned in the middle of the cleft, could be found in a hydrophobic but also hydrogen bond forming "pocket" that include Y298, Y348, and Y391 (Fig. 6.3). These residues are supposed to be important for the enzyme activity; for example, upon replacing Y298 with "A", the Hse catalytic activity was drastically reduced (Fig. 6.4). Yet, additional investigations are required to unravel the role of these residues in Hse activity.

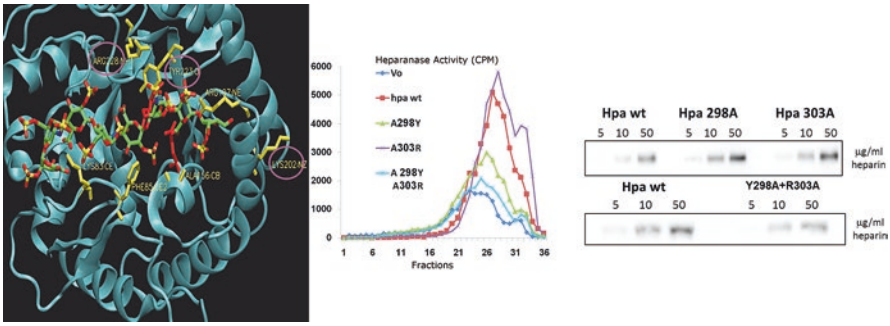

Fig. 6.4 Heparanase 298Y (=223Y) and 303R (=228R) are essential for its enzymatic activity and substrate binding. Docking and molecular dynamic (MD) studies revealed several amino acids that appear critical for interaction of the heparanase protein with its substrate/inhibitor (left panel). Each of these residues was subjected to site-directed mutagenesis to verify its significance in heparanase substrate binding (right) and enzymatic activity (middle). Briefly, cells were stably transfected with the point mutated heparanase constructs and cell lysates were analyzed for heparanase enzymatic activity (middle panel). The mutated forms were also tested for their substrate (heparin) binding capacity (i.e., secretion and accumulation in the culture medium upon addition and binding to heparin) (right panel). There was no effect to point mutation A303R alone, but almost complete inhibition of heparanase enzymatic activity by A298Y and even more so by the double mutant A298Y and A303R. Interestingly, these results show that each point mutation alone did not affect substrate binding, while the double mutant exhibited a marked reduction in its heparin-binding capacity, suggesting that these residues act cooperatively in the catalytic mechanisms. However, a molecular interpretation of these effects requires further investigations

In the X-ray model of Wu et al. [10] the distance between the carboxylic groups of the two catalytic residues is approximately 5.5 Å, typical of a GH working as configuration retaining enzyme [11]. The substrate binding site modeled by Sapay et al. [22] showed a good correspondence with that resolved by the X-ray diffraction pattern, however, the predicted residue distributions inside the cleft of Hse present some discrepancies in comparison to the X-ray reference structure, showing a smaller set of hydrophobic residues: F101 in HBD1 and L300 in HBD2. Despite this, the "hydrophobic pocket" Y298, V268, L300 at the +1 subsite predicted by Sapay et al. [22], corresponds to the "pocket" formed by Y298, Y348, and Y391 on top of both the -1 and + 1 subsites described in the X-ray model, and even if L300 is not too far from this hydrophobic area, its side chain is oriented outside the cleft. The distribution of amino acids with positive side chains at the border and in proximity to the cleft predicted by Sapay et al. [22] is roughly in accord to that observed in the X-ray model of Hse. In fact, even if in the homology complex residues K107, K108, R193, and the following K277, K280, and R307 were predicted to be in proximity of HBD1 and HBD2, all these residues were found far from the binding cleft in the reference structure. Additionally, Sapay et al. [22] predicted that the protein-ligand electrostatic interactions are localized on K231, K277, and R382, while the hydrophobic contacts involve residues F101, F385, Y298, Y348, and Y391. This finding is supported by the X-ray model, which shows that the K231 electrostatically driven contacts and the hydrophobic cupola formed by Y298, Y348, Y391 and F160 (Levy-Adam et al. [30]) were found to interact with the trisaccharide GlcNS6S-GlcUA-GlcNS6S whose residues are placed at the -2, -1, and + 1 subsites, respectively (Fig. 6.3 and Fig. 6.5). More accurately, the X-ray model (Wu et al. [10]) showed also that F160 (HDB1) is one of the few hydrophobic amino acids that interact with 2-N-sulfo of GlcNS6S at the -2 subsite (distance smaller than 5 Å, Fig. 6.3 and Fig. 6.5). In the same model, K231 interacts with 2-N-sulfo of GlcNS6S at +1, while the hydrophobic cupola covers part of GlcUA, and GlcNS6S at -1 and + 1, respectively.

Analysis of the amino acid residues in vertebrate Hse showed that only a few were preserved in the cleft, such as the catalytic pair E225, E343, and Y298, compared to the HS-binding domain (Sapay et al. [22], Gandhi et al. [23]). This indicates that conservation of the amino acid residues between vertebrates and in the GH79 family has the main scope to preserve the catalytic activity device, while the substrate recognition/interaction systems are less preserved, reflecting (presumably) the variability of the polysaccharides as substrates of the GH enzymes.

6.4 Hse/HS Binding

Wu L. et al. [10] mapped, for the first time, the electronic density of selected HS-like oligosaccharides co-crystallized within the active site cleft of the enzyme. The selected oligosaccharides include two tetrasaccharides, made by -GlcNAc-GlcUA- and -GlcNS-GlcUA repetition units and a nona-saccharide obtained by the repeating

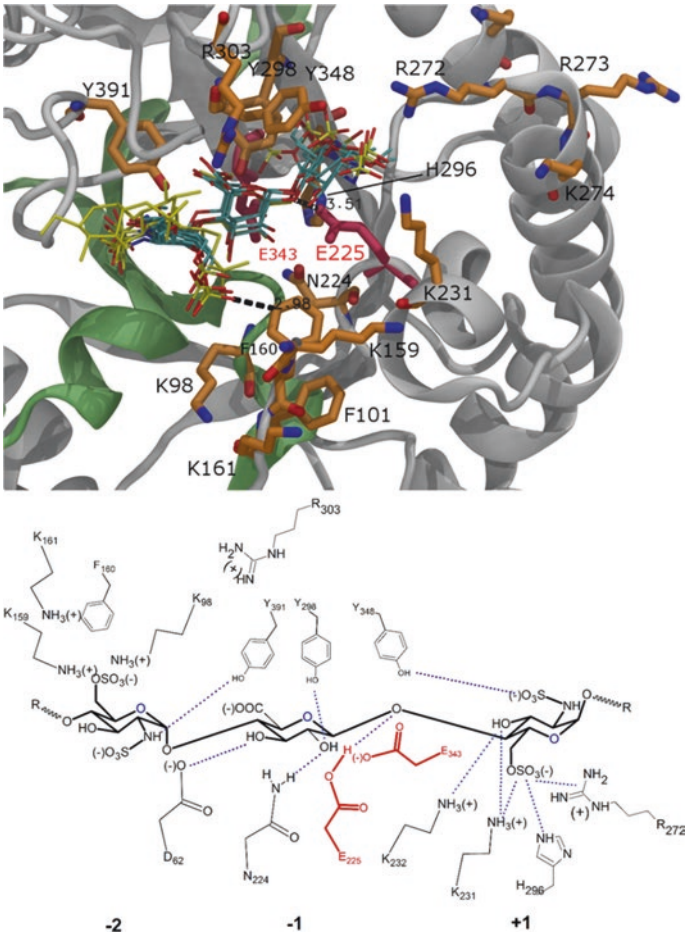

Fig. 6.5 Contacts between the Hse active site and the trisaccharide GlcNS6S-GlcUA-GlcNS6S described through a zoomed view of the complex previously reported in Fig. 6.3 (top), and by a synthetic scheme in which selected basic contacts cited in the text are reported. The catalytic pair E225, E343 is underlined in red, selected hydrogen bonds are underlined by blue dotted lines (bottom). (Artwork inspired by Fig. 6.4 of Wu et al.10)

unit GlcUA-GlcNS-. All these glycans are terminated by 1-O-(para-nitrophenyl)-glucuronide (GlcUA-pNO2-Ph) group, initiator of the chemoenzymatic synthesis (Xu et al. [34]). In this last case, only four of the nine residues were mapped at subsites -4, -3, -2, and -1 (PDB ID 5E9B). The substrate residues at subsites -2, -1, and the principal contacts in the Hse cleft, together with details on the active site geometry, were determined. Additionally, electronic density of the substrates allowed to detect the conformation of each glycan residues. In all the complexes, the GlcUA residue occupies the subside -1 of the Hse active site cleft, assuming a $^4C_1$ chair conformation. The GlcUA anomeric carbon approaches the nucleophile E343

(3.2 Å), while the side chain of D62, N224, Y391, and the NH backbone of T97, G349, and G350 form h-bonds with GlcUA involving OH and COO(-) groups (Fig. 6.4a in Wu et al. [10]). The "protonated" E225 side chain, is left at h-bond distance from the glycosidic oxygen of GlcUA just before the hydrolysis reaction takes place. As previously said, the three Y298, Y348, and Y391 side chain residues form a "hydrophobic pocket" on top of the GlcUA at subsite -1. Interestingly, the presence of a 2-O sulfo group on GlcUA in -1 subsite does not allow such residue to interact with the side chain of N224, due to steric interaction. These structural details correlate with all the previously known evidence that oligosaccharides including GlcUA2S and/or IdoUA2S bound to GlcNX, were not hydrolyzed by Hse (Pikas et al. [12], Okada et al. [13]). Subsite -2 is occupied by GlcNX (X = Ac, or S) or GlcNS6S in $^4C_1$ conformation. Considering the proximity of the positive side chain of K98 and K159 to GlcNS6S, Wu et al. [10] suggested that a 6-O-sulfo group of a GlcNS6S residue is favored compared to GlcNS, and also, in turn, compared to GlcNAc, in accord to Okada et al. [13]. Differently, the 2-N-sulfo group of GlcNS at -2 subsite contributes to the recognition, forming an h-bond with Y391 (bottom of the cleft) in accord to Pikas et al. [12]. This representation also suggests a contact between subsites -4 and -3 with R303 in the central part of the cleft, forming a lid on the groove entrance (Fig. 6.3, Fig. 6.5). The role of R303 was also investigated, showing that replacing both R303 and Y298 with "A", partially decreased the activity of Hse, indicating the involvement of R303 strictly in cooperation with Y298 in Hse catalytic activity (Fig. 6.4). To determine the structure at subsite +1, not visible in the previously cited complexes, a mixture of two heparin-like tetrasaccharides: ΔHexUA2S-GlcNS6S-IdoAU-GlcNS6S, and ΔHexUA2S-GlcNS6S-IdoAU2S-GlcNS6S, were allowed to interact with Hse. Only the former tetrasaccharide was co-crystallized with Hse and, even if both glycans include the trisaccharide moiety GlcNS6S-IdoUA/GlcUA-GlcNS6S, the presence of 2-O sulfation does not allow the uronic acid residue to assume subsite -1. Noteworthy, the IdoUA at -1 was found in $^2S_0$ conformation, and the eventual $^1C_4$ conformation will leave the axial 2-OH of IdoUA, to clash with E343 (see the complex ΔHexUA2S-GlcNS6S-IdoAU-GlcNS6S-Hse in PDB ID 5E9C). It is important to note that only rarely the nonsulfated IdoUA residue was found in $^2S_0$ conformation, particularly in the bound state with a protein. One example concerns a recently described hexasaccharide that binds and activates antithrombin, and that includes a particular antithrombin binding sequence (AGA*IA), in which IdoUA replace IdoUA2S (Stancanelli et al. [35]). Wu et al. [10] suggested that IdoUA in $^2S_0$ conformation should prevent the substrate from acquiring the favored geometry once in the bound state with Hse, thus reducing the rate of hydrolysis. This observation will explain the inhibitory effect observed in heparin-like GAGs, that are characterized by a larger percentage of IdoUA or IdoUA2S compared to HS.

Another important aspect of GAG-Hse interaction is the effect of binding on the conformation of the substrate itself. Comparing the conformations of the tetrasaccharides co-crystallized with Hse, with those assumed by the heparin chain in the free state, as determined by NMR spectroscopy and MD simulation (Mulloy et al. [36], Wu et al. [10]), revealed a significant conformational change in the surrounding

of IdoUA residue, indicating a heparin chain bending and an helical pitch alteration induced upon the binding event. This conformational change that is driven also by the 2-N-sulfo and 6-O-sulfo of the -2 and + 1 subsites, respectively, is supposed to help the catalytic residues of Hse (E225, E343) to approach the glycosidic oxygen and the anomeric carbon of the IdoUA at the -1 subsite. Authors suggested that this mechanism could be extended to HS-like GAGs, in which IdoUA is replaced by GlcUA, being part of the GAG-Hse recognition/binding event. Additional efforts in this direction are strongly encouraged to improve the probability of success in the design of new strategies and/or drugs for Hse inhibition.

To further characterize the -2, -1, and + 1 subsites of the Hse cleft, MD simulation of a trisaccharide GlcNS6S-GlcUA-GlcNS6S in the bound state with Hse in explicit solvent was run by our group (unpublished results), starting from the 3D structure of Wu et al. [10]. Four snapshots of this MD simulation sampled in the range from 0 to 30 ns, at steps of 10 ns, were superposed to the previously cited X-ray resolved complex (PDB ID: 5E9B) and reported in Fig. 6.3 and Fig. 6.5. The MD simulation showed that the GlcNS6S at -2 subsite preserves the contacts between NS and Y391, and between 6-O-sufo and K98, K159, while the GlcUA at -1 maintains the contacts observed in the X-ray structure (Wu et al. [10]). The GlcNS6S at +1 showed the 2-N-sulfo group in contact with both K231 and R272, confirming their importance in contributing to the binding interaction, as described by Okada et al. [13] In this simulation, the 6-O-sulfo group of GlcNS6S at +1 approaches both H296 and Y298 (cleft bottom), the former by h-bond, while the latter by induced dipole intermolecular forces. Analyzing these MD simulation results and the x-ray resolved complex PDB ID 5E9C, we can presume that an eventual 3-O-sulfo group (GlcNS6S at +1) could be accommodated in the cleft establishing contacts with the positive side chains of K231 and K232 (Fig. 6.3 and Fig. 6.5). This last analysis supports (for the first time) the observation of Pikas et al. [12], Okada et al. [13], and Liu et al. [16] that reported how the 6-O-sulfo or the 3-O-sulfo of a GlcN at the +1 site, are recognized by Hse active site, improving the hydrolysis of the glycan (see "Mechanism of enzymatic hydrolysis"). A targeted structural biology characterization, involving the glycan-Hse interaction by complementary approaches (STD-NMR, tr-NOESY, titrations, mutagenesis experiments) are strongly needed to support these new hypotheses.

The trisaccharide GlcNS6S-GlcUA-GlcNS6S in the bound state with Hse was also studied by Gandhi et al. [23] in a docking study based on the homology-built model of Hse. Despite some inaccuracies of the predicted active site structure, the top-ranked pose of the docking (lowest free energy of binding) showed the GlcUA residue of the trisaccharide at the -1 subsite position, characterized by a distance between the glycosidic linkage oxygen and E225 of 3.4 Å, and that between the C1 of GlcUA and E343 of 4.4 Å, in accord with Wu et al. [10] and the MD simulation results reported in Fig. 6.3 and Fig. 6.5. The 6-O-sulfo group of GlcNS6S at +1 position forms an h-bond with the side chain of Y298, while the GlcNS6S at -2 is supposed to interact with residues that belong to HBD1, in accord to the previously cited characterizations summarized in Fig. 6.3 and Fig. 6.5. Interestingly, Gandhi et al. [23] in their set of oligosaccharides included also the heparin-like pentasaccharide

AGA*IA and the corresponding glycol-split variant (gsAGA*IA), the former shown to be a substrate (Bisio et al. [37]), while the latter was an inhibitor of the enzyme (Naggi et al. [38]). The predicted set of contacts between the AGA*IA and the homology model of Hse were found in accord to the trisaccharide in Fig. 6.3 and Fig. 6.5, with the GlcUA occupying the subsite -1, the 6-O-sulfate of the GlcNS6S at -2 interacting with K159, K161, and the GlcNS3S6S at the +1 subsite forming an h-bond between the 6-O-sulfo group and Y298. The authors correlated the possibility of cleavage with the distances of the glycosidic oxygen and the C1 of GlcUA from E225 and E343, respectively, that in this docking solutions were smaller than 4.5 Å.

6.5 Glycol Split Heparin Inhibitors

Differently, when the gsAGA∗IA was docked into the Hse active site, the greater flexibility of the gsGlcUA residue affects the whole glycan conformation generating a greater number of docking solutions compared to the intact pentasaccharide (Fig. 6.6). The glycol-split substructure (gsGlcUA) introduces a flexible joint compared to a regular heparin chain, allowing for a greater number of available conformations, in comparison to GlcUA, as was previously documented by Casu et al. [39] using NMR spin-lattice relaxation times (T_2) and NMR NOESY (Nuclear Overhouser Effect SpectroscopY) experiments. Later, Ni et al. [40] showed clearly how the "ring opening" increases the number of dihedral angles on the glycan backbone, improving the whole glycan conformational flexibility. Authors speculated on the possibility that this improved flexibility could help the glycan to fit the Hse active site, i.e., optimizing its h-bonds with the active site of Hse, in term of number and geometries (Fig. 6.6). Gandhi et al. [23] run the first attempt to characterize the interaction between glycol-split heparin-like oligosaccharides and Hse. Unfortunately, characterization of these docking solutions was missing in the paper. Maltohexaose, Maltohexaose sulfate (MHS) and the analog PI-88, representative of high sulfation degree oligosaccharides, were also considered by Gandhi et al. [23] as ligands for their Hse active site model. The docked solutions showed similar properties, the most important of which was the ability of these pentasaccharides to interact with both HBD1 (K159, K161) and HBD2 (R272) by electrostatic interactions, and the involvement of Y298 residues by h-bonds.

In the development of Hse inhibitors, a most important significance is attributed to SST0001 = Roneparstat® (Sigma-Tau Research Switzerland S. A.) corresponding to a high molecular weight N-acetylated, glycol-split heparin, proved to be active in several xenograft models and being examined in phase I clinical trial for patients with multiple myeloma[8] (Noseda and Barbieri, Cassinelli et al., Chaps. 15 and 21 in this volume). Pala et al. [26] reported a kinetic investigation of Hse inhibition by Roneparstat in which data were fitted using two Hill type curves measured in two different ranges of concentration and corresponding to the formation of complexes inhibitor-Hse of 1:1 and 2:1 stoichiometry, respectively. The authors assumed that in the 1:1 inhibitor-Hse complex, a glycol-split Hep chain occupies the Hse active site,

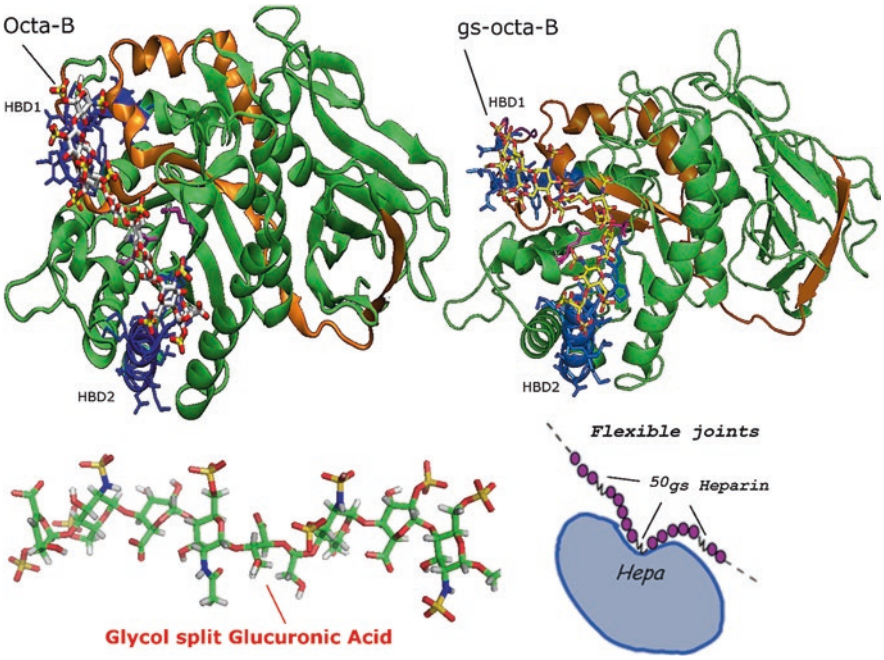

Fig. 6.6 Glycol-splitting (arrow) of N-acetylated heparin (SST0001 = Roneparstat) introduces flexibility (arrow) that enhances binding to heparanase. The open "G" ring gives more flexibility to the oligosaccharide structure (gs-Octa-B, right panel), which allows a better adjustment in the active site of HPSE (vs. the rigid Octa-B, left panel), bridging HBD1 and HBD2 (blue color). Crystallization of HepGS3 was performed by Golan G., Livnah O., Vlodavsky I. et al., unpublished results

HBD1, and HBD2, while in a 2:1 stoichiometry two glycol-split heparin chains bind HBD1 and HBD2 separately, giving rise to the possible formation of complexes with different stoichiometry, or the oligomerization of Hse. Pala D. et al. [26] also suggested the existence of a third GAG binding site of Hse, previously hypothesized by Levy-Adam et al. [30], that corresponds to the sequence K411-R432 enriched by amino acids with basic side chains; unfortunately, no data to confirm this hypothesis were presented.

For a molecular interpretation of the inhibitor/Hse complex, a homology model of Hse was built by Pala et al. [26] targeting the sequence of the catalytically active GS3 construct of human Hse, using as template the previously resolved 3D structure of GH 79 β-glucuronidase from *A. Capsulatum*. The generated model of Hse was characterized by a high structural similarity to the X-ray structure of Wu et al. [10]. In fact, superposing the two structures, the RMSD calculated on the Cα backbone of the TIM-barrel subunit was 3.32 Å, reduced to 1.76 Å considering the residues of the ligand binding site that surround E225 and E343 at distances smaller than 4 Å. (See paragraph *"Comparison between heparanase model and crystal structure"* and Fig. 6.7 A, B and C in Pala D. et al. [26]). The modeled Hse was submitted to docking

studies with the antithrombin binding pentasaccharide AGA*IA (fondaparinux), that was used as a reference compound, and a glycol-split heparin-like heptasaccharide representative of Roneparstat. A customized "induced-fit" protocol that considers both ligand and receptor flexibility was required to fit these ligands in the active site of Hse, suggesting that the latter presents some flexibility degree, as pointed out previously (See "*materials and methods*" in Pala D. et al. [26]). This allows the residues AGA* of the AGA*IA to occupy the -2, -1, and + 1 subsites, matching the glycan residues co-crystallised with Hse (RMSD of 0.52 Å). Additionally, the interaction patterns of the docked fondaparinux, compared to that of the glycan co-crystallized with Hse in the paper of Wu et al. [10], forms an h-bond between the K159 and the 6-O-sulfo group of GlcNS6S at the -2 subsite. The docking of Roneparstat on Hse gave rise to three different clusters of complex geometries: the first accommodates the glycol-split GlcUA in the catalytic site between E225 and E343, while the flanked residues at the non-reducing and reducing ends interact with HBD1 and HBD2 domains of the binding cleft, respectively. The inter-molecular forces that drive the recognition 'Roneparstat-Hse' are, as discussed earlier, electrostatic, polar, and hydrophobic interactions. The residues of Hse cleft involved in this recognition are K158, K159, F160, and R272, K274, T275, that belong to HBD1 and HBD2, respectively (Fig. 6.8A and B in Pala D. et al. [26]). Similar to the first cluster, the second cluster of poses presented a recognition pattern of HBD1 and HBD2, however, similarly to the previous case, the glycol-split GlcUA remained above the pair E225, E343, avoiding fitting to the catalytic pocket. The third cluster, differently from the first two, does not fit the central part of the cleft (catalytic pocket) due to the bent conformation, and for this reason, it was not able to bind both HBD1 and HBD2. The pose reported in Fig. 6.8C of Pala et al. [26]showed half of the Roneparstat molecule interacting with HBD1, while the remaining portion was exposed toward the bulk solvent. Interestingly, all the three clusters presented the Ronaparstat molecule oriented with the non-reducing and reducing terminals interacting with HBD1 and HBD2, respectively, in agreement to previously cited data obtained with oligosaccharides mimicking heparin and HS. The docking solutions of Roneparstat in the active site of Hse, also confirmed by 150 ns of MD simulation, indicated that different binding modes are possible, some of which matching with a 1:1 stoichiometry (they correspond to the first and second cluster, respectively), while others are in agreement with n:1 ligand-Hse ratio with $n > 1$ (third cluster). The former cases were observed at a low concentration of inhibitors and indicated a kinetic inhibition curve with a Hill coefficient approximately equal to 1. The latter event corresponds to high inhibitor concentration and shows a kinetic law with a Hill coefficient greater than 1.

6.6 Conclusions

Hse is the principal enzyme that degrades HS in both physiological (HS turnover) and pathological (tumor metastasis, inflammation) cell conditions, catalyzing the hydrolysis of the β-1-4 glycosidic bond in -GlcUA-β(1-4)-GlcNX-. Hse is an endo-

β-glucuronidase (GH79 family) that acts through an acid catalysis mechanism with retention of the anomeric carbon configuration. Since the first evidence of an enzyme able to hydrolyze heparin (Ögren et al. [1]), important advances were achieved, including gene cloning, protein expression, and biochemical investigations to decipher the mode by which the active site of Hse recognizes HS and heparin (Vlodavsky et al., Chap. 1 in this volume). The driving force of this effort was the causal involvement of heparanase in various human pathologies and the associated development of strategies and compounds to inhibit Hse, and thereby tumor progression and metastasis. Important progress in understanding the molecular features of the HS sequence for recognition and hydrolysis by the enzyme was obtained by several groups during the last 15 years. These efforts allowed to establish the minimum sequence (trisaccharide) in term of residues and sulfation pattern, that allows glycans to be recognized by Hse. Interestingly, similar to other protein targets (i.e., FGFs) a rigorous "molecular code" by which Hse enzyme read and degrade HS chains was not detected. We thus assume that Hse permits some degree of degeneracy in its action, considering the vast variability of HS substrates. Given that the 3D structure of native Hse resolved by X-ray diffraction was published only in 2015 (Wu et al. [10]), earlier steps in the analysis of the 3D structure of Hse and its active site required the application of homology modeling approaches. Comparing the secondary structure of Hse with that of different GHs of known 3D structures (families 10, 39, and 51 of clan A), together with mutagenesis experiments, disclosed the basic structural details and the enzymatic catalysis mechanism of Hse. In particular, the earlier homology modeling allowed to predict the heterodimeric organization of Hse, that includes a $(\beta/\alpha)_8$-TIM-barrel domain and a β-sandwich domain. Both subunits are required for the enzyme catalytic activity, the former hosting the binding cleft (or groove) that recognizes the HS chains (endo-acting), as well as the catalytic pair E225, E343. These investigations successively identified several amino acids with positively charged side chain in the proximity of E225 and E343, which are clustered in two opposite peptides (HBD1, HBD2) located at the beginning and end of the binding cleft (groove) of Hse. NMR titration and mutagenesis experiments suggested that these two clusters act cooperatively in binding to the long HS chain. The availability of these models, together with molecular docking and molecular dynamics (MD) simulation, have made it possible to study the interactions between oligosaccharide mimicking heparin (i.e., glycol split heparin) and Hse. Specifically, these studies showed that a glycol-split heptasaccharide (mimicking one of the most potent Hse inhibitor drug: Roneparstat®) binds Hse in a 1:1 or 2:1 ratio, depending on its concentration, giving rise to different inhibition kinetics for different concentrations of inhibitor.

The X-ray diffraction model of Hse described by Wu et al. [10] and Golan. et al. (Golan G., Livnah O., Vlodavsky I. et al. unpublished results) represented key steps in the characterization of Hse structure and molecular recognition pattern. The observed electron density of the apoenzyme alone and in complex with HS-like oligosaccharides allowed to detect at a nearly atomic scale resolution the active site residues involved in recognition of the substrate, as well as the position and conformations of the co-crystallized glycans. This model supports the pioneering identification of the minimum sequence of HS recognized by Hse (Pikas et al. [12] Okada

et al. [13] Peterson et al. [16]), but more importantly, represents a starting point to advance ligand-Hse molecular recognition investigation. That is the case with the never observed before complex between the trisaccharide GlcNS6S-GlcUA-GlcNS6S and Hse, whose geometry was predicted by MD simulation. Interestingly, this complex is in accord with the criteria of the minimum sequence of HS, supporting the reason by which the AGA*IA pentasaccharide or HS-like oligosaccharides with GlcNSXS at +1, could be recognized and hydrolyzed by Hse, regardless of the position of the extra sulfate group. Additional efforts are required to support these results and to better understand how HS mimetics longer than three or four residues are recognized by Hse before being hydrolyzed or inhibit the enzyme. It is also important to consider the flexibility of the Hse active site, a feature that opens up the development of novel Hse inhibitors with a structure significantly different from HS or heparin. Other approaches should necessarily involve complementary structural and biological techniques such as mutagenesis, ligand-receptor interactions (thermodynamic and NMR titrations), MD simulation, and more recent cryo-EM imaging approaches. Results of these investigations will be useful to design new and more efficient inhibitors with therapeutic efficacy in diseases in which Hse plays a determinant role.

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Chapter 7
Heparanase: Cloning, Function and Regulation

Shaun M. Gaskin, Tatiana P. Soares Da Costa, and Mark D. Hulett

7.1 Introduction

7.1.1 Identification of Heparanase

Heparanase (HPSE) is an endo-β-glucuronidase that cleaves heparan sulfate (HS) polysaccharide chains at the cell surface and in the extracellular matrix. It was first described in 1952 as heparinase due to its heparin cleavage functionality [1]. However, it was not until 1975 that its HS cleavage activity was observed whilst investigating the metabolism of cell surface glycosaminoglycans [2]. This discovery was followed by studies describing HS degradative activity by platelets in 1976 [3]. The enzyme was renamed heparanase (HPSE, a.k.a. HPA, HPA1, HSE1) in 1983 when it was found to be expressed in the metastatic B16 melanoma cell line, suggesting a possible role in cancer [4]. To this day, HPSE remains the only known endoglycosidase enzyme able to directly cleave HS.

7.1.2 Normal Expression of Heparanase

HPSE is expressed ubiquitously across different animal species, including invertebrates, with high amino acid sequence conservation ranging from 53 to 98% [5]. Despite the prevalence of *HPSE* genes in the database for various animal species, studies have primarily focussed on humans and the common laboratory mouse,

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Mus musculus, given its use as a model for understanding human disease (mouse and human *HPSE* share 77% amino acid sequence identity).

Human tissue analyses reveal that expression of *HPSE* is low to undetectable in most non-immune tissues, including the kidney, pancreas, heart, brain, endometrium, lung, testis, trachea, colon, adrenal glands, salivary glands, and skeletal muscle, whereas the placenta exhibits high levels of transcript expression [6–10]. Investigations into HPSE expression in immune cells reveal high expression levels (constitutive or inducible) in nearly all haematopoietic cells, including platelets, monocytes, macrophages, neutrophils, mast cells, dendritic cells, activated lymphocytes, and eosinophils, with lower expression levels observed in the lymph node, bone marrow, spleen, liver and thymus [6, 11, 12]. This indicates a key role for HPSE in the function of the immune system. HPSE expression is also upregulated in a number of pathological settings, such as cancer and inflammation-related disease, which will be explored in Sect. 7.3.4.

7.2 Gene Cloning

7.2.1 Race of Four: The Cloning of Human Heparanase

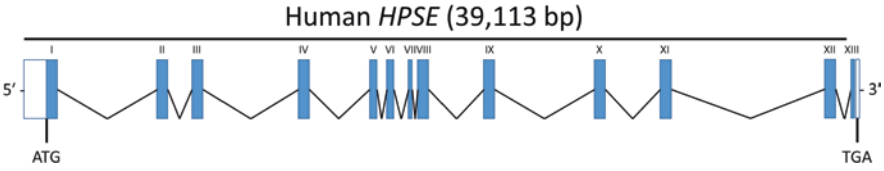
The year 2019 marks the 20th anniversary of the cloning of human HPSE. The *HPSE* gene was initially cloned in 1999 from a number of different cell lines and tissues, including placenta and the SV-40 transformed embryonic lung fibroblast cell line WI38/VA13 [6, 13–16]. Such was the high significance and strong competition of the 'race' to clone human HPSE, Hulett et al. (1999), Vlodavsky et al. (1999), Kussie et al. (1999), Toyoshima and Nakajima (1999) and Fairbanks et al. (1999) all published papers within a few months of each other [6, 14–17].

Using platelet-purified HPSE, Hulett et al. (1999) sequenced the purified 50 kDa protein via *in situ* tryptic digest and N-terminal sequencing [6, 18]. The tryptic digest generated 10 peptides, which were used in a BLASTN database search, resulting in the identification of a placenta-derived cDNA clone. This clone encoded for 5 of the observed HPSE peptides and matched to a 1.1 kb sequence that encodes for the 300 C-terminal HPSE amino acids. PCR amplification of a human placental cDNA library using primers based on the BLASTN-identified clone was successful in amplifying an 800 bp sequence containing the 5′ end of the gene and the corresponding 3′ overlapping sequence of the HPSE cDNA. This revealed an open reading frame of 1629 bp, encoding for a 543 amino acid protein. The identified sequence allowed for the prediction of six *N*-linked glycosylation sites, a second potential initiation site, and hydrophobic regions at the N- and C-terminus; characteristic of transmembrane and signal peptide sequences. The identical 1629 bp cDNA sequence was also identified in the human T-cell lymphoma Jurkat cell line. Comparison of this sequence to the 50 kDa N-terminal sequencing results initially suggested that the N-terminal domain may represent the active enzyme. However, this hypothesis

was dismissed when functional assays demonstrated that the 50 kDa domain was inactive, thus indicating the importance of amino acids 1–157 in producing a functional HPSE enzyme.

HPSE purified by Vlodavsky et al. (1999) from human hepatoma cell line SK-HEP-1 and placenta underwent tryptic digest, followed by HPLC and sequencing, resulting in the identification of a corresponding cDNA clone from a placental cDNA library [6, 14, 19]. This clone contained an insert of 1020 bp; a 973 bp open reading frame, a 27 bp 3′ UTR, and a 20 bp poly-A tail. The 5′ sequence was obtained using PCR amplification of DNA from placenta Marathon RACE cDNA composite, resulting in a 930 bp amplicon, containing the start AUG codon and overlap with the original clone. This yielded a 1758 bp cDNA with an open reading frame encoding a 543 amino acid protein of 61 kDa calculated molecular weight, which was consistent with the hypothesis from Hulett et al. (1999) that the open reading frame encodes for a proenzyme form of HPSE [6]. Vlodavsky et al. then screened a human genomic library in Lambda phage in an attempt to clone the *HPSE* gene. The coding region, except for a 2 kb sequence, was identified from three isolated plaques, and the missing 2 kb gap was completed using PCR amplification from human genomic DNA. Analysis of the *HPSE* gene and a 2.3 kb region upstream of the start site revealed a region of 39,113 bp, which was segregated into 13 exons and 12 introns (Fig. 7.1). The HS-cleavage functionality of the cloned *HPSE* gene was confirmed using radiolabelled HSPGs purified from the extracellular matrix (ECM) that were analyzed by gel filtration and gel shift assays.

Later in the same year, Kussie et al. (1999) published the cloning of active human HPSE from a placental cDNA library [15]. Using a similar experimental pipeline to Hulett et al. and Vlodavsky et al., HPSE was purified from the SK-HEP-1 cell line by sequential chromatography, and the resulting protein subjected to tryptic digest. A single EST clone was identified as a match to one of the peptides, with sequence



Exon	Exon Size (bp)	Intron	Intron Size (bp)	Exon	Exon Size (bp)	Intron	Intron Size (bp)
I	106	**1**	234	**VIII**	94	**8**	450
II	258	**2**	12191	**IX**	107	**9**	2527
III	146	**3**	2749	**X**	115	**10**	3934
IV	126	**4**	6056	**XI**	119	**11**	1043
V	174	**5**	2223	**XII**	147	**12**	5456
VI	169	**6**	643	**XIII**	3043		
VII	48	**7**	535				

Fig. 7.1 Gene structure of human *HPSE*: The human *HPSE* gene is 39,113 bp long on chromosome 4, and is composed of 13 exons (boxes, roman numerals) and 12 introns (connecting lines) [20]. The gene encodes for a predominant 1.7 kb transcript comprising a 224 bp 5′ UTR (empty box), a 1629 bp open reading frame (start ATG codon, filled boxes, stop TGA codon), and a 27 bp 3′ UTR (empty box). The sizes (bp) of the exons and introns are indicated in the table

analysis revealing it contained a 975 bp open reading frame, a stop codon, a poly-A tail, and when translated, matched with the remaining 5 peptide sequences. Absent from this clone was a start ATG codon and a Kozak sequence. To clone the complete *HPSE* gene, a pooled array placental cDNA library was screened using PCR primers designed against the identified EST clone. From this study, a 1.7 kb fragment was obtained and sequenced. Analysis of the sequence revealed a 1629 bp open reading frame encoding for a 543 amino acid enzyme, with the predicted molecular weight of HSPE. Expression of this 1.7 kb clone in COS-7 cells confirmed it encoded for a protein with the ability to cleave HS chains using ^{35}S radiolabelled HS.

In the same year, cloning of HPSE was also achieved by Toyoshima and Nakajima utilizing enzyme purified from SV-40 transformed WI38/VA13 embryonic fibroblast cells [16]. The 50 kDa fragment of the purified HPSE was subjected to N-terminal sequencing or *in situ* Lys-C endoproteinase digest. Sequences of the resulting peptides were used as search queries utilizing a TBLASTN database akin to the workflow undertaken by Hulett et al. (1999), Vlodavsky et al. (1999) and Kussie et al. (1999), yielding two overlapping ESTs (yw97a02.r1 and yw70a03.s1). PCR analysis identified a 731 bp amplicon in the WI38/VA13 cell line, corresponding to the overlapping ESTs. The amplicon was used as a probe to screen for the full-length cDNA sequence of HPSE from a WI38/VA13 cDNA library, resulting in the identification of a 3762 bp insert containing a 1629 bp open reading frame encoding for a 543 amino acid protein. HS-degradation assays confirmed the protein as HPSE.

7.2.2 Identification of an 8 kDa Peptide in the Active HPSE Enzyme

The cloning studies outlined in the previous section were successful in defining the gene arrangement and the open reading frame encoding the HPSE enzyme. However, the composition of the active enzyme, including the precise role of the 157 N-terminal residues, remained to be elucidated.

The apparent absence of the N-terminal domain 157 amino acids, predicted from the full-length open reading frame in the purified 'active' 50 kDa enzyme, led to the hypothesis that HPSE was synthesised as a proenzyme; containing a signal peptide and 'pro-domain' that regulate post-translational processing and intracellular trafficking [6, 14, 15]. Given that the 65 kDa pro-form of the protein was enzymatically inactive, it was proposed that upon proteolytic cleavage, the 50 kDa domain was released as the active form of HPSE [14]. As discussed in Sect. 7.2.1, the 50 kDa domain of HPSE alone has no HS-degrading activity, indicating that the 157 amino acid N-terminal domain is vital for enzymatic function [6].

The critical observation that the active form of HPSE contains an 8 kDa fragment derived from the N-terminal 157 amino acid domain, in addition to the previously described C-terminal 50 kDa peptide, was first suggested by Fairbanks et al. (1999) employing a novel sequential chromatography workflow for the purification of

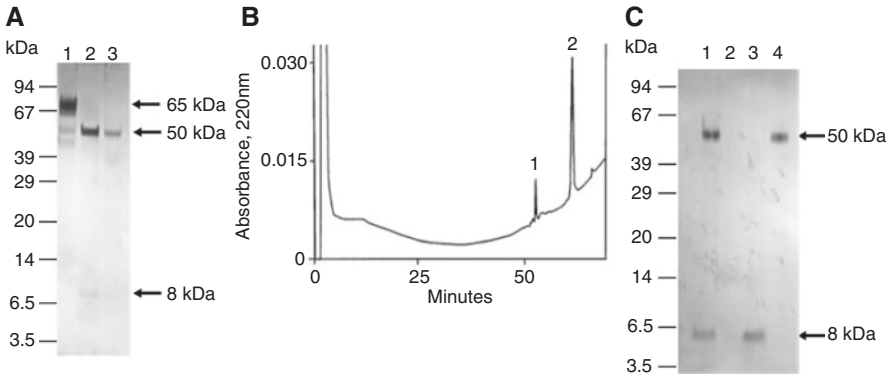


Fig. 7.2 Identification of an 8 kDa peptide in active HPSE: (**A**) SDS-PAGE of 3 distinct fractions from the purification of platelet-derived HPSE, obtained under non-reducing conditions, that exhibit HPSE activity. (**B**) Proteins were resolved on a Jupiter C4 reverse-phase column over 70 min with a linear gradient of increasing acetonitrile concentration from 0 to 70% in 0.15% TFA. (**C**) SDS-PAGE of the RP-HPLC peaks shows that peak 1 correlates with the 8-kDa (lane 3) and peak 2 with the 50-kDa chain (lane 4) identified in the load [1]. Adapted from [17]

HPSE from platelets (Fig. 7.2) [17]. RP-HPLC analysis revealed a 1:1 molar ratio of 50 kDa to 8 kDa peptide in the active form of HPSE. Alignment of the digested peptide sequences corroborated previous findings that the 50 kDa peptide resides at the C-terminal portion of the HPSE open reading frame, while the 8 kDa domain was found at the N-terminal end adjacent to the predicted signal peptide domain [6]. Analysis of the N- and C-terminal processing of the newly identified 8 kDa peptide led to the proposal that this domain consists of amino acids Gln^{36}s– Glu^{109}, with the interdigitating 6 kDa peptide unable to be observed in SDS-PAGE, predicted to be removed by proteolytic processing. Fairbanks et al. (1999) proposed that the 8 kDa and 50 kDa peptides non-covalently interact, forming a heterodimer, yet lacked the evidence to prove their hypothesis.

Following the hypothesis postulated by Fairbanks et al. (1999), Levy-Adam et al. (2003) were able to confirm the formation of a heterodimer using immunoprecipitation of tagged 8 kDa and 50 kDa domain peptides (Fig. 7.3) [21]. Further experiments identified that the amino acids responsible for the interaction among the 8 kDa and 50 kDa reside between residues 288 and 417. Expression of the 8 kDa and 50 kDa domains in cells was also found to be sufficient to increase HS degradation compared to expression of either of the domains alone, with the effect similar to that of transfection with full-length HPSE. Overall, these studies led to the proposal that the HPSE enzyme is synthesized as a pre-proenzyme, consisting of a signal peptide domain, followed by an 8 kDa domain (Ala^{35}-Glu^{109}), a 6 kDa linker region (Ser^{110}s–Gln^{157}) and a 50 kDa domain (Lys^{158}-Ile^{543}) (Fig. 7.4). The pre-proenzyme is initially processed to remove the signal peptide domain, then further processed to excise the 6 kDa linker domain, resulting in the formation of a heterodimer, which will be discussed in Sect. 7.4.4.

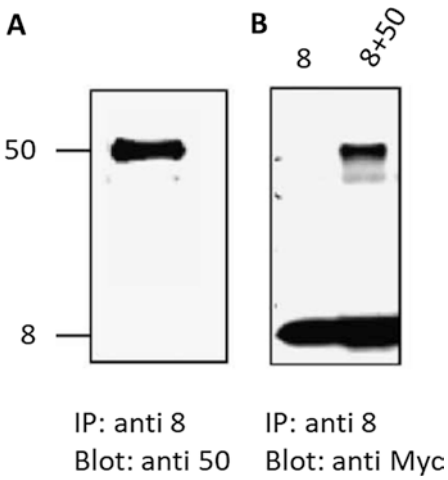


Fig. 7.3 The 8 and 50 kDa heparanase domains associate: **(A)** Total cell lysates of HEK293 cells stably transfected with the full-length heparanase were subjected to IP with anti-8 kDa heparanase antibodies, followed by immunoblotting with anti-50 kDa heparanase antibodies. (**B**) HEK293 cells were transfected with the c-Myc-tagged 8 kDa plasmid [8] or co-transfected with the c-Myc-tagged 8 and 50 kDa (8s+s50) plasmids. Total cell lysates were subjected to IP with anti-8 kDa antibodies, followed by anti-c-Myc immunoblotting. Adapted from [21]

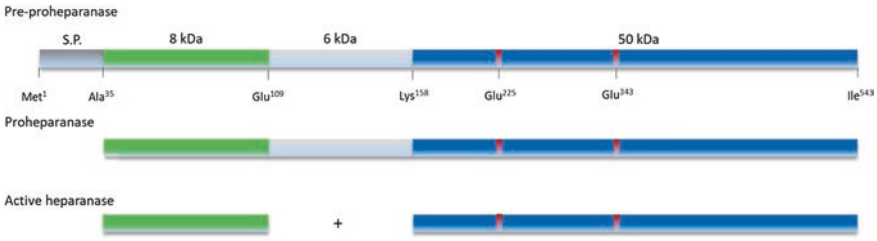


Fig. 7.4 Processing of the 65 kDa pre-proheparanase into the active heparanase enzyme: HPSE is translated as a pre-proenzyme including a C-terminal signal peptide sequence (S.P., dark grey), the 8 kDa domain (green), the 6 kDa linker domain (light grey), and the 50 kDa domain (blue) where the active site residues Glu^{225} and Glu^{343} sare located (red). Proteolytic processing removes the signal peptide sequence and the linker domain to produce the active enzyme

7.2.3 Cloning of Heparanase From Other Organisms

Using the human HPSE cDNA sequence identified in placenta and Jurkat cells, Hulett et al. (1999) attempted to clone mouse HPSE [6]. The cDNA sequence was screened against the dbEST database using BLASTN, which identified a homologous mouse HPSE. The database search returned five mouse ESTs corresponding to the 280 amino acids in the C-terminus of HPSE. Using first-strand cDNA derived from the RNA of activated mouse spleen cells, PCR amplification produced a 1368 bp cDNA sequence corresponding to the human HPSE sequence from amino acid

Table 7.1 HPSE is conserved across species: HPSE identified from various animal species, highlighting the size of the translated protein and the identity to human HPSE

Species	Protein size (amino acids)	AA identity to human HPSE
Gorilla gorilla	543	98%
Gallus gallus	523	62%
Bos taurus	545	78%
Sus scrofa	542	80%
Spalax	534	85%

158 to the poly-A tail. This sequence extended the combined EST sequence a further 532 bp in the 5′ direction, resulting in the identification of amino acids 158–543 in mouse HPSE. The use of primers based on the human HPSE sequence did not allow for the isolation of the remaining N-terminal sequence of mouse HPSE. The cloned sequence contained only the coding region of the 50 kDa domain that was shown not to be active when expressed. The complete mouse HPSE coding sequence was eventually cloned in 2002 [22]. A cDNA library from day 12.5 mouse embryo was prepared using oligo dT primers, resulting in the identification of a single clone containing an 1800 bp insert. Sequencing of this clone revealed a 1605 bp open reading frame encoding for a 535 amino acid protein, displaying a 77% amino acid homology to human HPSE.

While identifying and cloning the human and mouse HPSE proteins, Hulett et al. (1999) also attempted to clone HPSE from rat [6]. Using first-strand DNA from the highly metastatic rat mammary adenocarcinoma cell line MAT 13762, PCR amplification identified a cDNA fragment encoding for the same 386 amino acid C-terminal sequence of the protein identified in mouse HPSE. As expected, the rat HPSE sequence did not exhibit activity upon transfection into COS-7 cells (Table 7.1). To date, genes from 97 different species have been annotated as *HPSE* orthologues [23].

7.2.4 Cloning of Heparanase for In Vitro Analysis

The identification of a suitable expression system to produce functional recombinant HPSE has long been a challenge due to necessary post-translational modifications required for generating the active enzyme, including the proteolytic processing of the immature peptide as well as glycosylation required to facilitate the efficient secretion into the extracellular space [14, 16, 24–26]. Two approaches have been implemented to generate active recombinant heparanase. The first approach focused on expressing the 8 kDa and 50 kDa domains in bacteria [27, 28]. However, the putative Asn residues were unable to be glycosylated, which could explain why the 50 kDa domain was commonly found in inclusion bodies [27, 29]. The second approach utilized an insect cell expression system, allowing for glycosylation and increased solubility [29, 30].

Bacterial Expression Systems

Winkler et al. (2014) attempted to express the heterodimer peptides in separate vectors in *E. coli* BL21 (DE3)plys*S* cells [28]. The lysates were then mixed, revealing an increase in degraded HS chains, indicating the formation of active HPSE (Fig. 7.5). To further investigate the ability of this method to produce functional recombinant HPSE, both domains were purified using the hexa-histidine incorporated in the cloning and metal affinity chromatography. Mass spectrometry analysis revealed protein masses consistent with the expected mass of the domains. This mixture was unable to degrade HS, indicating that the cellular context and content, even upon cell lysis, was important for the 8 kDa and 50 kDa domains to adopt the active HPSE structure. Nevertheless, yields of approximately 20% and 15% for the purification of the 8 kDa and 50 kDa domains, respectively, were achieved, indicating the utility of this system for efficient purification of these domains.

Similarly, Pennacchio et al. (2017) expressed the 8 kDa and 50 kDa domains from separate vectors while incorporating a hexa-histidine onto the 50 kDa domain. Both plasmids were expressed separately in BL21 (DE3) *E. coli* cells and upon native lysis, approximately 85% of the 8 kDa domain was found in the soluble fraction, while most of the 50 kDa domain was insoluble. Despite this, the soluble fractions of both lysates were combined, and protein purified utilizing a heparin-sepharose column, followed by size exclusion chromatography. Mass spectrometry of the purified protein revealed molecular masses consistent with that of the 8 kDa and 50 kDa domains and activity was confirmed by assessing its ability to degrade HS [27].

Insect Cell Expression Systems

Expression of human HPSE in insect cells facilitates the post-translational processing and modification of recombinant HPSE. Accordingly, McKenzie et al. (2003) cloned the human 8 kDa and 50 kDa HPSE domains into a single expression vector, incorporating a GP67 secretory sequence to facilitate efficient purification, and created baculovirus containing these sequences in Sf9 insect cells [29]. The virus particles were then used to infect Tni cells to stably express the domains and the enzyme purified using a heparin-sepharose column. Yields of approximately 1 mg/L at >90% purity was achieved, and the enzyme was confirmed to be active.

The use of insect cells to produce recombinant HPSE has been utilized to significantly advance the understanding of HPSE, with this approach used to generate protein crystals to determine the structure of the human enzyme to 1.75 Å in 2015 [30] (Fig. 7.6). In this study, cDNA encoding the 8 kDa and the 50 kDa domains were cloned into a single bacmid under the control of separate viral promoters. Mellatin signal peptide was incorporated into the N-terminus of each domain, and baculovirus produced in Sf21 insect cells. Tni cells were infected and the secreted HPSE purified utilizing a 3-step protocol. While this protein was used to solve the structure of human HPSE for the first time (for more information, see chapter by Wu & Davies, Chap. 5; in this volume), no indication of yield, purity or activity was provided.

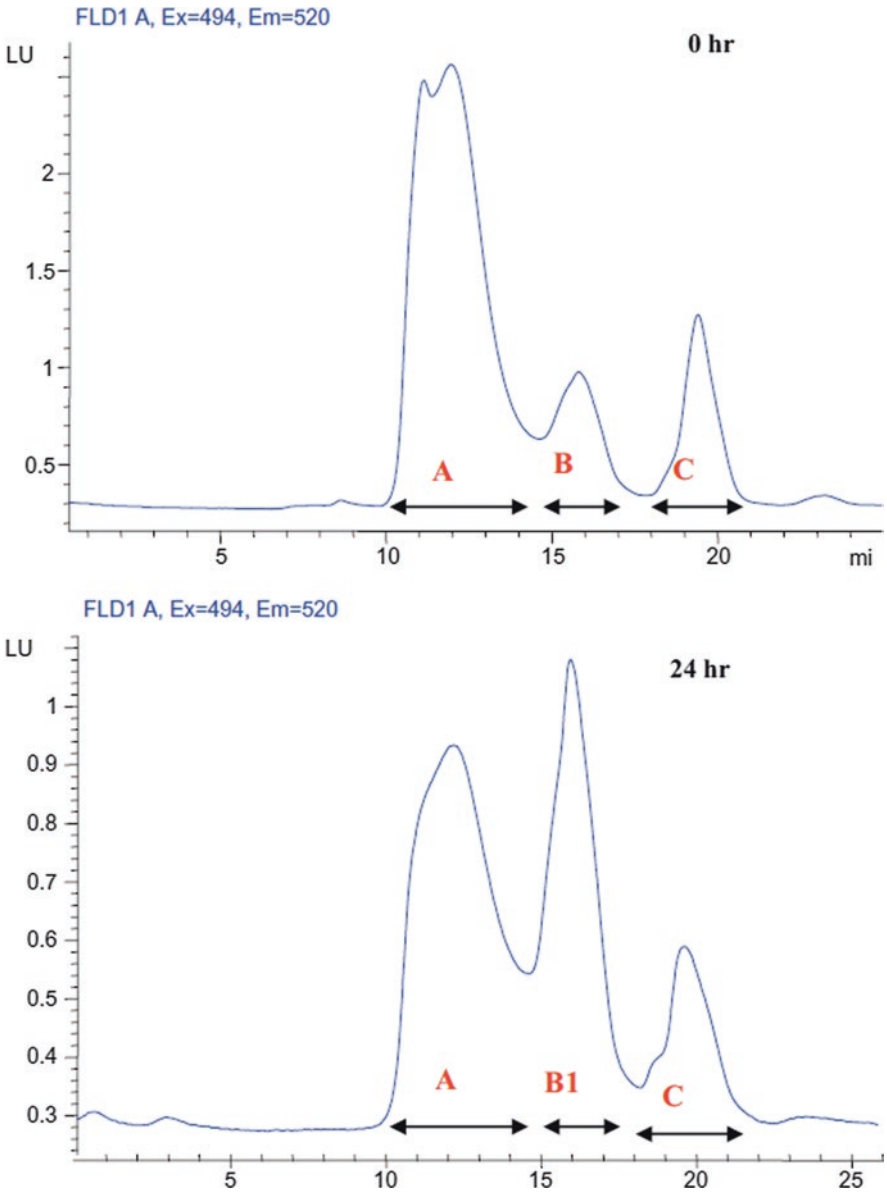


Fig. 7.5 Activity assay of the two recombinant HPSE subunits from mixed bacterial lysates: (0sh) Peak A represents the fluorescence of intact FITC-labelled heparan sulfate. Peaks B and C represent the fluorescent materials from the bacterial lysates; the control profile is not shown here. (24sh) Peak B1 contains the degraded amount of FITC heparan sulfate. Comparing the 0sh and 24sh of digestion, the peak of intact FITC-HS (**A**) decreases and the peak containing degraded heparan sulfate (**B1**) increases. Adapted from [28]

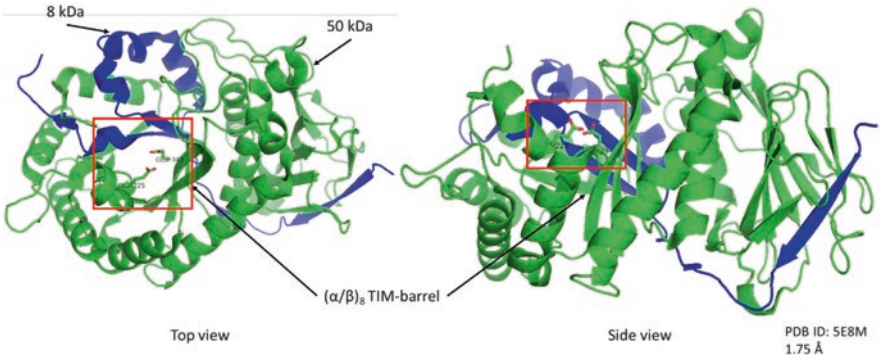


Fig. 7.6 HPSE adopts a (α/β)8 TIM-barrel conformation: The active HPSE enzyme consists of the 8 kDa and 50 kDa domain that form a $(\alpha/\beta)_8$ TIM-barrel structure, where the active site glutamate residues reside at the top of the barrel. The 8 kDa domain contributes two β-sheets, while the 50 kDa domain contributes six β-sheets. PDB ID: 5E8M [30]

7.3 Function

The role of HPSE is multi-faceted, performing a number of different functions both intra- and extracellularly. Various functions of HPSE require enzymatic activity, while others can be performed independent of this activity.

7.3.1 Heparan Sulfate Proteoglycans as HPSE Targets

The enzymatic activity of HPSE is localized predominantly to the extracellular space, where HS chains are ubiquitously found [31]. The ECM is a collection of secreted macromolecules, which form a three-dimensional lattice-like network implicated in biomechanical (e.g., providing structural scaffolding and a compression buffer to the tissue) and biochemical (e.g. regulation of cellular processes such as cell growth, migration, differentiation, and homeostasis) functions [32]. Approximately 300 proteins contribute to the composition of the ECM, with the majority of these being collagens, elastin, fibronectin, laminins, and glycosaminoglycans. Thirty-six of these constituents are proteoglycans, including heparan sulfate proteoglycans (HSPG) [33]. HS chains are linked to a protein core in the Golgi apparatus through a galactose-xylose linker, covalently bound to a serine residue in the protein core [34]. Generally, HSPGs contain between one and four chains of HS, consisting of a repeating glucuronic/iduronic acid and glucosamine disaccharide unit, which range from 40–300 residues in length (Fig. 7.7).

The glucuronic/iduronic acid and glucosamine disaccharide units share a 1 → 4 bond and allow for *N*- and *O*-sulfation and acetylation of the residues, resulting in

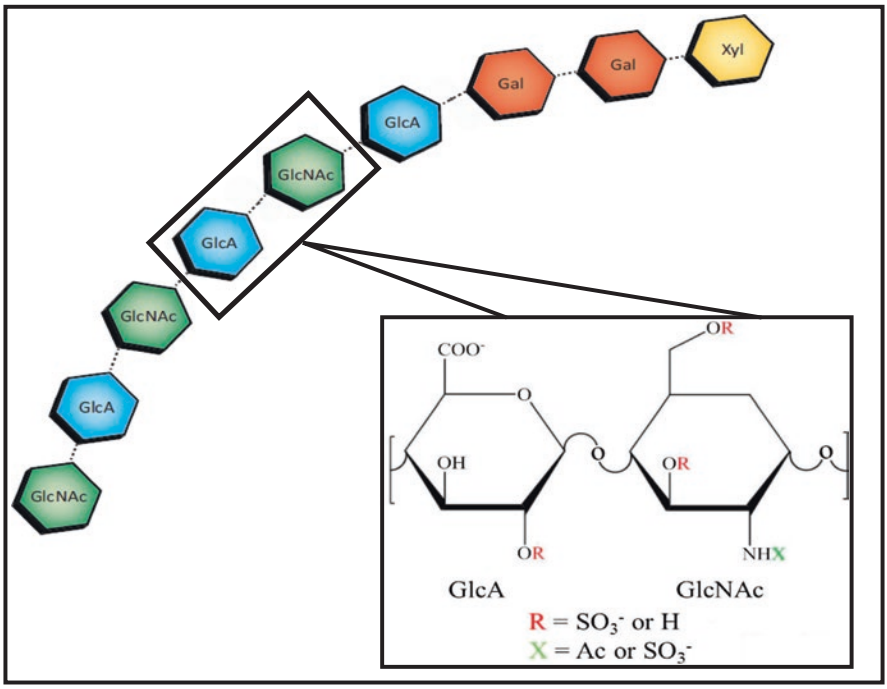


Fig. 7.7 Schematic representation of heparan sulfate chain structure: The repeating glucuronic/iduronic acid and glucosamine disaccharide units are bound to the protein core via a xylose-galactose linker and a serine residue in the protein core. Each of the saccharide units can undergo sulfation and/or acetylation. GlcAs=sglucuronic acid, GlcNs=sglucosamine, Acs=sacetylated. Adapted from [35, 36]

HS chains being amongst the most highly negatively charged biopolymers. Diversity in the structure and anionic character is provided by sulfation and acetylation of the HS chains [37, 38]. This diversity allows HS chains to act as an ECM reservoir by binding to approximately 300 different proteins, including growth factors, cytokines, and enzymes [39].

Extensive analysis of the HS chain structure revealed that HPSE recognizes inherent chain modifications for cleavage [18, 40, 41]. HS polysaccharides require a minimum -GlcN-GlcA-GlcN- trisaccharide in a highly sulfated region for HPSE recognition, with cleavage occurring between the GlcA and the GlcN carrying a 6-*O*-sulfo group [41, 42]. Conjecture surrounds the importance of 3-*O*-sulfation of the GlcN for HPSE activity [41, 42]. The sequence -GlcA-GlcNS- is also able to be cleaved by HPSE, reliant on the proximal presence of -GlcA2S-GlcN- [38]. Carboxyl-reduced HS is not a HPSE substrate, while the presence of 2-*O*-sulfated uronic acids is preferred for cleavage [18, 42, 43]. The recognition requirements for HPSE cleavage of HS result in the formation of ~5–12 kDa products (see also the chapter presented by Wu and Davies, Chap. 5; in this volume).

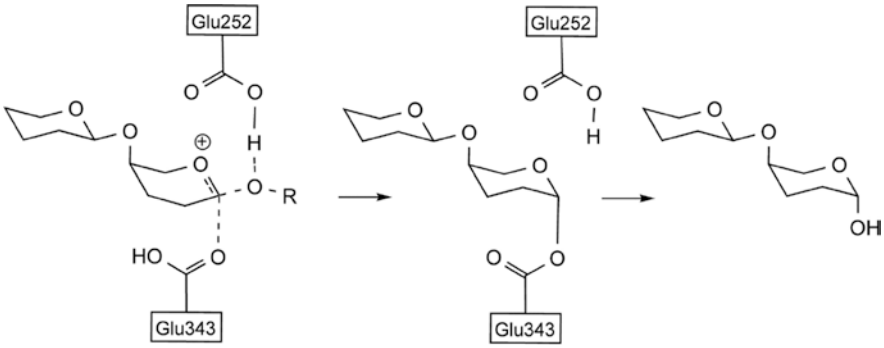


Fig. 7.8 HPSE cleaves HS using a hydrolytic mechanism: Glu^{225} donates a proton to the exocyclic oxygen within the glycosyl link, activating it as a leaving group. Glu^{343} then acts as a nucleophile by donating an electron pair, completing the cleavage of HS. Adapted from [46]

The HPSE active site contains two key residues; Glu^{225} which acts as a proton donor, and Glu^{343} which acts as a nucleophile [44]. Structural analysis reveals that these residues are located at the top of the TIM-barrel domain, allowing them to readily participate in HS cleavage [30, 45]. The cleavage of HS chains occurs via hydrolysis in a cleft that allows for flexibility in relation to configurational variations (Fig. 7.8).

The degradation of HS by HPSE not only begins to remove the physical barrier preventing cell invasion but also releases various proteins that bind to HS, promoting activation of cellular signaling pathways and responses. Interestingly, the protection of HSPGs from degradation by the intracellular scaffolding protein syntenin-1 is also able to promote these signaling events [47]. Seemingly counter-intuitive given that HS degradation by HPSE increases cell signaling, syntenin-1 is proposed to facilitate the recycling of HSPGs through trafficking to the cell surface or facilitating their inclusion in exosomes, resulting in increased receptor signaling activation [47].

Although not the focus of this chapter, it should be acknowledged that HPSE2 has been identified as a homolog of HPSE. HPSE2 is 592 amino acids in length, and the two proteins share 47% amino acid identity [9]. HPSE2 can bind to heparin and heparan sulfate but lacks the heparan sulfate cleavage ability [48] (Mckenzie; Roberts and Woolf, Chaps. 34 and 55; in this volume).

7.3.2 Regulation of Syndecan Function by HPSE

Exosomes are extracellular vesicles between 40 and 100 nm in diameter released from the endosome of cells [49]. Exosomes can carry payloads of cytoplasmic and membrane components, including DNA, proteins, enzymes, mRNA, miRNA, lipids and activated receptors [50–54]. Recipient cells endocytose the exosome, releasing their contents and therefore playing a role in cell-cell communication. A role for HPSE in exosome activities has been identified through its regulation of HS cleav-

age. Syndecans are proteoglycans with sulfated GAGs of either HS or chondroitin sulfate and have been strongly implicated in the formation of exosomes [55–57]. Binding of proteins to the HS chains on multiple syndecan HSPGs causes clustering of syndecans, resulting in the recruitment of adaptor proteins to facilitate intraluminal budding and formation of exosomes [55]. The enzymatic activity of HPSE results in cleavage of syndecan-1 HS chains, allowing clustering of syndecan-1, causing an increase in the production of exosomes [55, 57]. Increased HPSE expression also results in changes in the composition of exosomes from cancer cells [57] (David and Zimmermann, Chap. 10 in this volume).

The interplay between HPSE and syndecan-1 extends beyond the formation of exosomes. Increased HPSE expression results in an ERK signaling-dependent increase in MMP-9 expression; a protease able to cleave syndecan-1 from the cell surface [58–60]. Released syndecan-1 can be transported to the nucleus where the bound HS chains in conjunction with HPSE trafficked into the nucleus can influence numerous events, including the promotion of mitotic spindle formation and subsequent chromosome stability, inhibition of DNA topoisomerase I activity, and regulation of cell proliferation [58, 61–67]. In addition to these roles, HS has been shown to inhibit histone acetyltransferase (HAT) in a chain length and sulfation pattern dependent manner, resulting in decreased gene expression from the associated chromatin [68, 69]. Despite the optimal pH for HPSE activity being 5.5–6, HPSE can cleave the HS sidechains of syndecan-1 in the nucleus. High levels of HPSE causes low levels of nuclear syndecan-1 and HS, resulting in increased HAT activity [70, 71]. This increase in HAT activity causes an increase in tumor-proliferative genes including *VEGF*, *HIF1*, *HGF*, *VIM*, and *TGF*-β, while repressing expression of the major cellular iron transporter, *hepcidin*, indicative that HPSE can be a potential master regulator of pro-tumourigenic genes [72–75]. HPSE may also regulate gene expression by binding directly to gene promoters, with evidence of promiscuous binding to DNA independent of its enzymatic activity [58].

7.3.3 HPSE in the Immune System

As previously mentioned, several different immune cell types express HPSE. The role of HPSE in these cells is multi-faceted, with effects on immune cell gene regulation, differentiation, and migration/invasion of the cells. HPSE enters the nucleus of activated T lymphocytes, regulates histone methylation, and co-localises with RNA polymerase II at promoters of actively transcribed immune genes, including *CD69*, *IFNγ*, *IL-2* and *TNFα* [76, 77]. The nuclear localization of HPSE was found to be dependent on HSP90 activity and resulted in differentiation of leukemic cells *in vitro* [78]. HPSE is also vital for macrophage activation and function [79–81]. Incubation of peripheral mononuclear blood cells and splenocytes with purified HPSE resulted in increased release of proinflammatory cytokines, including IL-8, IL-10, and TNF, with a significant role for TLR4 identified in this effect [81]. This effect relied on HPSE enzymatic activity, as treatment with HPSE inhibitors prevented increased cytokine release, while addition of HS fragments to the system

drove cytokine release. These results were in contrast to previous findings by Blich et al., who discovered that addition of HPSE to macrophages was sufficient for their activation and increased cytokine release, yet their findings showed that the response by macrophages was independent of the enzymatic activity [80, 81]. Regarding function, macrophages from WT and HPSE-KO mice have been assessed for their ability to express cytokines and their effect on tumor cells. The WT macrophages exhibited higher cytokine expression than the HPSE-KO macrophages, while the knockout macrophages remain in the periphery of tumors in HPSE-KO mice due to their ability to cleave HS and invade the tumor microenvironment [79]. This results in tumor growth being unaffected by exposure to HPSE-KO macrophages, in contrast to the inhibitory effect on tumor growth by WT macrophages [79]. Dendritic cells (DCs), a key cell type in initiating innate and adaptive immune responses, possess migratory abilities to move between the various tissues to lymphatic vessels. Expression of HPSE by these cells is vital for their ability to migrate, with an absence of *HPSE* resulting in decreased migration of DCs from the skin to lymph nodes compared cells expressing *HPSE* [82]. Furthermore, HPSE is strongly implicated in the invasion/migration function of natural killer (NK) cells [83], Expression of HPSE is low, but is increased upon NK cell activation, facilitating NK cell invasion into tumors and tumor surveillance. Abolishment of HPSE in NK cells does not cause a difference in proliferation, survival, cytokine release, or degranulation of the cells, but does result in impaired migration/invasion, and inability to control metastases [83]. As seen here, the loss of HPSE activity results in the inability of various immune cells to maintain proper function. Further to the examples presented here, HPSE is vital for macrophage activation and polarisation, neutrophil granulocyte function, activity of T lymphocytes, and leukocyte recruitment to sites of inflammation, to name a few [79, 84–88]. Loss of HPSE function for these cells results in decreased ability for immune surveillance, inability to elicit an inflammatory response, and the overall depletion of immune system defenses.

7.3.4 HPSE Function in Pathogenesis

The enzymatic activity of HPSE contributes to various pathologies, including cancer and inflammatory diseases, for which supporting evidence is provided below. Other pathologies to which HPSE contributes have been extensively reviewed elsewhere [85, 89–103].

HPSE research has predominantly focussed on its role in cancer. This is not surprising, given that it is implicated in at least 5 of the 10 hallmarks of cancer, and is up-regulated in 90% of all cancers [10, 104–114]. Pre-clinical and clinical data demonstrate that high HPSE expression correlates with increased tumor size, tumor progression, tumor cell metastasis, lack of cell heterogeneity and poor patient prognosis [112, 114–118]. In the extracellular environment, HPSE cleaves HS chains from HSPGs, resulting in ECM remodeling and release of HS-bound cytokines and

growth factors [56]. The effect of this HS degradation is paired; the destruction of the ECM provides passage for tumor cells to begin invasion/metastasis, while the release of the cytokines and growth factors facilitates up-regulated receptor signaling, driving angiogenesis and tumor growth. The ability for HPSE to induce metastasis is demonstrated by increasing HPSE activity, through either transfection or treatment with exogenous enzyme, to induce increased metastasis, while knockout of HPSE or inhibition of HPSE activity decreases the metastatic nature dramatically [113, 119–125]. Expression of HPSE drives upregulation of VEGF, HGF, and MMP-9, promoting aggressive tumour behaviour [59, 126–130].

High expression of HPSE in tumor cells is also responsible for an increased shedding of syndecan-1 [123, 129, 131]. These shed HPSGs allow the formation of activated signaling complexes at the cell surface, driving tumor development [132]. Increased HPSE expression also allows for enhanced cleavage of syndecan-1 HS side-chains, promoting the formation of exosomes [57]. High HPSE expressing cells have been shown to produce increased amounts of exosomes than low HPSE expressing cells, facilitating enhanced inter-cellular signalling [57]. HPSE present in these exosomes may provide a mechanism for the establishment of a niche where metastatic tumor cells colonize. Strong evidence supports a role for HPSE in chemoresistance, with HPSE expression increased in resistant cancers and the application of a HPSE inhibitor sufficient to attenuate resistance [133]. Finally, increased HPSE expression has also been implicated in promoting chemoresistance through exosome binding to the recipient cell surface and increased autophagy [133–136].

As described in Sect. 7.3.3, HPSE plays a vital role in the immune system function and inflammation. It is, therefore, no surprise that HPSE functions in many inflammation-related pathologies, including atherosclerosis and diabetes. Using a mouse model of atherosclerosis, inhibition of HPSE employing a selective inhibitor resulted in lowered blood pressure (both systolic and diastolic), a modest increase in kidney function, decreased HDL-cholesterol levels, a decrease in oxidative stress, and induced weight loss in mice [137]. This provides evidence for a multi-faceted role for HPSE in atherosclerosis and provides reasoning for further investigation into the role of HPSE in this context.

The role of HPSE and HS in diabetes is quite broad and context-dependent. Islet beta cells in the pancreas are protected from free-radicals by a HS barrier. Increased HPSE activity in the pancreas leads to the degradation of this protective barrier, resulting in increased islet beta cell death. This causes a decrease in insulin production, leading to the development of type I diabetes [77] (Simeonovic et al., Chap. 23 in this volume). In contrast, it has also been suggested that high expression of HPSE can inhibit the development of diabetes, while also inducing glucagon resistance [138] (Shang et al., Chap. 30 in this volume).

Given HPSE has multiple roles in numerous pathologies, it is an attractive target for inhibitor development. Accordingly, a series of HPSE inhibitors have been developed, with some of these being discussed in Sect. 7.4.7.

7.4 Regulation of Heparanase

Regulation of HPSE expression and activity is achieved at the gene, transcript and protein level through a variety of mechanisms. In this section, we will explore these mechanisms.

7.4.1 A Lack of Methylation at the HPSE Promoter Increases HPSE Expression

DNA methylation is the epigenetic process of enzymatically adding a methyl group to the cytosine rings of DNA. These modifications are dynamic and can be added or removed to drive or suppress gene expression based on intracellular and extracellular signaling. Methylation of the *HPSE* promoter was identified as a potential regulator of *HPSE* expression in 2003, with a CpG island immediately upstream of the translation initiation site found to be methylated in a glioma cell line lacking HPSE activity [139]. The promoter of *HPSE* was found to be methylated in cell lines with low expression levels, with inhibition and removal of DNA methylation in these cell lines resulting in an increase in *HPSE* expression and activity (Fig. 7.9) [139, 140]. Demethylation of the *HPSE* promoter results in a translational effect *in vivo*, with glioma cells treated with the demethylation agent colonizing the lung nearly six-fold better as a result of increased HPSE expression and activity in mice [140].

The role of methylation of the *HPSE* promoter has been well investigated in cancer models, with demethylation in prostate, breast, and bladder cancers resulting in increased *HPSE* expression, activity, and pro-invasive and metastatic characteristics [109, 141–143]. Interestingly, no difference in *HPSE* promoter methylation is observed between normal colon cells and colon carcinoma cells, indicating other factors play a role in regulating *HPSE* expression [144].

7.4.2 Regulation of HPSE Expression by Transcription Factors

Various transcription factors have been shown to regulate the expression of *HPSE* and will be discussed in this section.

The binding of specificity protein 1 (Sp1) transcription factor to promoter recognition sequences has been shown to both maintain basal expression and induce or inhibit expression of a large number of target genes [145–147]. HPSE has been demonstrated to be one of these target genes, with three recognition sequences identified in the *HPSE* promoter, and binding and activity of Sp1 at the *HPSE* promoter required for *HPSE* expression (Fig. 7.10) [148].

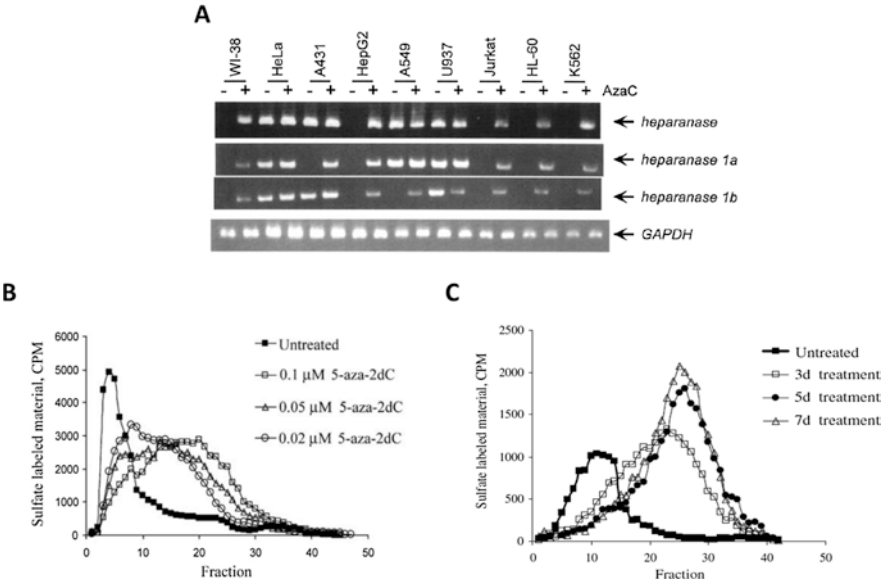


Fig. 7.9 Loss of *HPSE* methylation induces heparanase expression and activity: (**A**) Human tumor cell lines were treated with 10smM AzaC for 3sdays. The mRNAs were prepared from cells, and the relative levels of heparanase, heparanase 1a, heparanase 1b, and GAPDH mRNAs were analysed by semi-quantitative PCR. (**B**) Untreated human JAR cells (■) and cells treated with 0.02 (□), 0.05 (△), or 0.1 (○) mM 5-aza-2dC, were lysed and analysed for HPSE activity. (**C**) Untreated C-6 rat glioma cells (■); and C-6 glioma cells exposed to 0.2smM 5-aza-2dC for 3 (□), 5 (●), or 7 (△) days, were lysed and analysed for HPSE activity. Adapted from [139, 140]

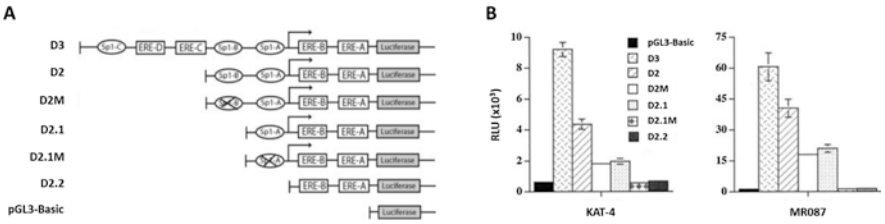


Fig. 7.10 Identification of putative Sp1 sites in the HPSE promoter required for the basal promoter activity of the *HPSE* gene: (**A**) schematic presentation of the luciferase reporter gene driven by the *HPSE* promoter containing mutated or truncated Sp1 sites. (**B**) Luciferase activity analysis of *HPSE* promoter activity in thyroid tumor cell lines KAT-4 and MR087. Adapted from [148]

As Sp1 sites are removed from the *HPSE* promoter sequence, promoter activation is decreased. Sp1 activity at the *HPSE* promoter has been shown to facilitate regulation of *HPSE* expression by various means, such as the binding of chemokine CCL19 to the CC chemokine receptor 7 (CCR7) [149]. The binding of CCL19 to CCR7 has been implicated in the invasive capacity of a variety of immune and tumor cells, including non-small cell lung cancer and oesophageal squamous cell

carcinoma, where overexpression of CCR7 correlates with increased metastasis, [150–153]. Incubating A549 squamous lung cancer cells with CCL19 resulted in an increase in HPSE mRNA and protein levels, while blocking CCR7 decreased HPSE expression [149]. Sp1 expression was also shown to be increased with CCL19 incubation and was found to be directly responsible for the increase in HPSE mRNA and protein expression upon exposure to CCL19. Another example is the regulation of *EGFR* expression, with binding of Sp1 to *EGFR* promoter recognition sites required for *EGFR* transcription [154]. EGF activation of the EGFR has been shown to create an environment conducive to tumor metastasis [155]. To facilitate this, it is not surprising that EGFR activation results in increased HPSE expression, promoting tumor metastasis [62].

Interestingly, Sp1 recognition sites are found within the promoter sequence for a variety of proteins which have been implicated in regulating *HPSE* expression, including EGFR, vitamin D, p53, and estrogen [156]. This may allow for rapid induction of *HPSE* via an initial increase in expression of a single transcription; Sp1. T-cell activation is required for differentiation and proliferation of naïve T-cells into cytotoxic CD8 T-cells and helper CD4 T-cells, facilitating normal immune system function. The ETS transcription factors are vital in differentiation of these types of T-cells and are responsible for the rapid induction of *Egr1* observed upon differentiation [157, 158]. T-cell activation has also been shown to induce *HPSE* expression, albeit in a more gradual and robust manner [95, 158]. Egr1 binds directly to a recognition sequence within the *HPSE* promoter, resulting in activation of *HPSE* expression in T-cells and tumor cell lines [109, 158, 159]. This was the first example of inducible *HPSE* expression. Interestingly, Egr1 was found to inhibit transcription of *HPSE* in melanoma cells, providing evidence that the role of Egr1 in regulation of *HPSE* expression is cell type dependent and more complex than initially thought [160]. In addition to the induction of *HPSE* expression by Egr1, ETS transcription factors ETS1 and ETS2 have also been demonstrated to bind to two functioning ETS binding sites in the *HPSE* promoter and strongly drive *HPSE* expression [161].

The vitamin D receptor (VDR) transcription factor binds 1,25-D3 (active vitamin D), interacts with retinoid X receptor (RXR) and subsequently binds to vitamin D response elements in DNA. Recently, two studies have identified an interaction between 1,25-D3 and HPSE expression in the renal system [162, 163]. Vitamin D has been shown to exert anti-proteinuric effects in experimental and human glomerular diseases. The development of proteinuria has been strongly attributed to the loss of HS in the glomerular filtration barrier and given that HPSE is the only human enzyme able to cleave HS, implies that HPSE is important in this pathology [164, 165]. Increased expression of HPSE has been observed in experimental and human glomerular diseases, with inhibition of HPSE resulting in decreased formation of proteinuria. This provides direct evidence for a role of HPSE in the development of proteinuria. Using rodent models, 1,25-D_3 has been shown to bind directly to the *HPSE* promoter, resulting in decreased HPSE transcript and protein levels (Fig. 7.11) [162]. This decrease in HPSE expression led to decreased transendothelial albumin passage, demonstrating a reduction in proteinuria formation. (Van der Vlag, et al., Chap. 26 in this volume).

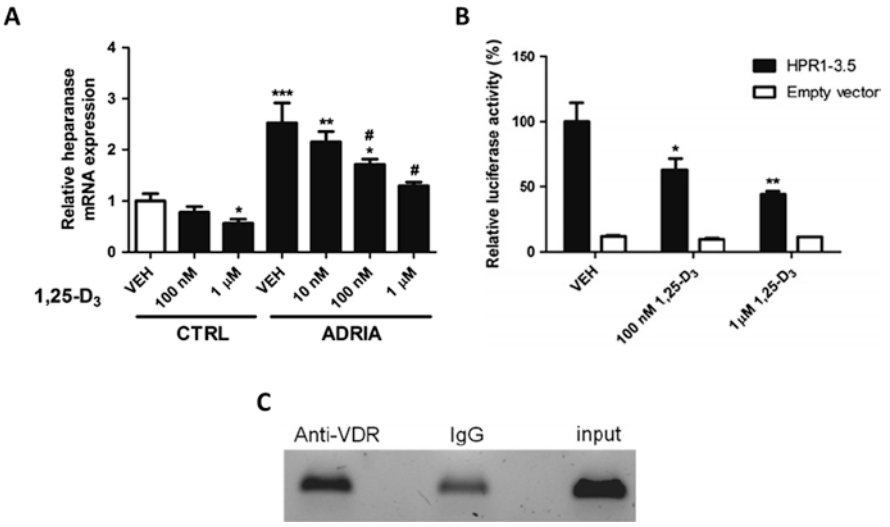


Fig. 7.11 Vitamin D receptor decreases *HPSE* expression by binding directly to the *HPSE* promoter: (**A**) *HPSE* mRNA levels in cultured mouse podocytes (CTRL) or stimulated with adriamycin (ADRIA, to induce a cellular FSGS model) in the absence or presence of 1,25-D_3 for 24sh. Statistical analysis conducted treatment VEH versus ADRIA. (**B**) Heparanase promoter activity determined by a luciferase reporter assay. Statistical analysis conducted treatment versus VEH (vehicle). *=<0.05, **=<0.01, and ***s=<0.001 versus VEH CTRL; #=<0.05 (**C**) ChIP using anti-VDR antibody compared with isotype control (IgG lane). Two percent of the chromatin used for the immunoprecipitation is shown (input lane). Adapted from [162]

Recently, the transcription factor SMAD4, a key protein in the TGF-β signaling pathway, was found to repress expression of *HPSE* by binding to the *HPSE* promoter region [166, 167]. In analyzing the *HPSE* promoter of neuroblastoma tissue, a potential SMAD4 binding site was identified approximately 2300 bp upstream of the *HPSE* transcription start site (Fig. 7.12A) [166]. Analysis of SMAD4 expression in normal dorsal ganglion (DG) and various neuroblastoma cell lines revealed an inverse correlation between SMAD4 and HPSE expression (Fig. 7.12B). Utilizing the two lowest SMAD4-expressing neuroblastoma cell lines, the effect of over-expression of SMAD4 on HPSE expression was explored. Over-expression of SMAD4 in these cell lines caused a decrease in HPSE expression when compared to the mock-transfected cells (Fig. 12C). shRNA knockdown of SMAD 4 confirmed a role for SMAD4 in regulation of HPSE expression, and the direct nature of this regulation was demonstrated using luciferase assays. An inverse relationship between SMAD4 and HPSE expression levels was also observed in gastric cancer cell lines compared to primary stomach epithelial cells [167]. ChIP analysis revealed enrichment around the SMAD4 binding site, confirming that SMAD4 is a direct negative regulator of *HPSE* expression.

The p53 transcription factor plays a vital role in the control of the cell cycle at the G1/S interface. It also plays an important role in apoptosis in response to severe

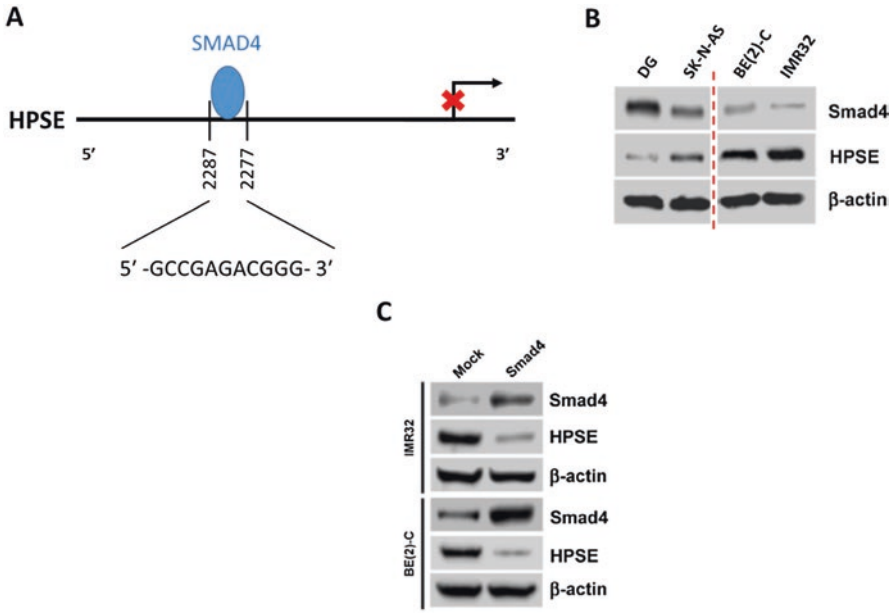


Fig. 7.12 SMAD4 potentially regulates expression of HPSE: (**A**) Scheme of the potential binding sites of Smad4 within HPSE promoter, locating at bases −2287/−2277 upstream the transcription start-site (arrow). (**B**) Western blot showing the expression levels of Smad4 and HPSE in normal dorsal ganglia (DG) and NB cell lines. (**C**) Western blot indicating the protein levels of Smad4 and HPSE in IMR32 and BE(2)-C cells stably transfected with empty vector (mock) or Smad4. Adapted from [166]

cellular DNA damage and is often mutated to provide cells with pro-oncogenic function. In 2006, HPSE transcript and activity were first observed to be increased in $p53^{-/}$ MEF cells, with this observation replicated in WT cells transfected with p53-targeting shRNA [168]. A non-functional conformational p53 mutant was created, rendering p53 protein functional at 32 °C and inactive at 37 °C. This mutant was shown to increase expression of *HPSE* in cultures grown at 37 °C, while p53 was shown to bind to the *HPSE* promoter between 2400 and 2700 bp upstream of the transcriptional start site (Fig. 7.13, HPSE-4). The observed inhibition of *HPSE* expression by p53 binding to the promoter also resulted in a decrease in HPSE activity, demonstrating that p53 is a potent regulator of *HPSE* expression and that loss of this protein alone is sufficient to increase *HPSE* transcript levels.

Estrogen signaling has been described as one of the key factors in the development of breast cancer, while HPSE has been shown to increase growth and invasion rates of breast cancer when over-expressed [14, 169, 170]. Estradiol, the predominant form of estrogen produced during reproductive years, has been demonstrated to be responsible for increasing *HPSE* expression in healthy endometrial and breast cancer cells [171–173]. Binding of estradiol to the estrogen receptor (ER) results in ER activation and relocation to the nucleus where it functions as a transcription factor [174]. The ER has been shown to bind to an ER recognition sequences

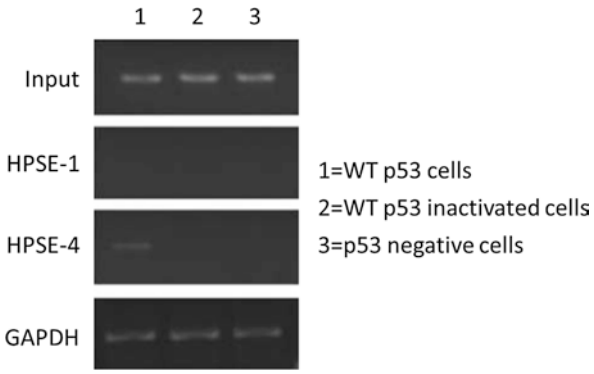


Fig. 7.13 p53 binds to the HPSE promoter: ChIP analysis was performed for WI-38/hTERT (WT p53), WI-38/hTERT/GSE56 (WT p53 inactivated), or H1299 (p53 negative) cells to demonstrate p53 binding to each of the heparanase promoter sites. Following crosslinking of proteins to DNA, DNA was fragmented, and the p53 protein was immunoprecipitated. PCR analysis was performed on the immunoprecipitated DNA samples using primers specific for regions of the heparanase promoter. Adapted from [168]

identified in the *HPSE* promoter and induce expression of *HPSE* at physiological levels of estradiol both in vitro and in vivo. This increase in *HPSE* expression results in increased cell surface HS degradation and tumor angiogenesis; typical of high HPSE expression. Tamoxifen (an antagonist of the ER) is the preferred treatment option for ER-positive breast cancer, yet treatment of breast tumor cells with tamoxifen still elicited an increase in HPSE expression, representing the first evidence of tamoxifen acting as an ER agonist [173]. Given the role of HPSE in tumor progression and metastasis, there appears to be a compromise in treating tumors with tamoxifen, with more attention to potential metastatic events required.

Hypoxia response pathways are activated under low oxygen conditions and have been shown to increase *HPSE* expression in the retina via NF-κβ signaling. This signaling has also been shown to induce tumor cell invasion in a HPSE-dependent manner. However, no definitive evidence has been provided for NF-κβ binding directly to the *HPSE* promoter and driving *HPSE* expression [60, 72, 175]. Contrary to this, NF-κβ localization to the nucleus has been shown to result in decreased *HPSE* expression in melanoma [58].

7.4.3 Regulation of Heparanase by MicroRNAs

MicroRNA (miRNA or miR) are short single-stranded non-coding RNA sequences 21–25 bp in length that regulate mRNA translation and mRNA stability predominantly via binding to the 3′ UTR [176]. Despite the short nature of the *HPSE* 3′ UTR (27 nucleotides), three miRs have been identified as regulators of HPSE expression [177].

The over-expression of miR-1258 in brain metastatic breast cancer (BMBC) cells results in a three-fold decrease in HPSE activity levels, while activity more than

doubles in cells treated with miR-1258 inhibitor [178]. Consequently, the over-expression of miR-1258 can decrease the number of brain metastases in HPSE-dependant manner, with invasive ductal carcinoma breast cancer patient samples revealing an inverse correlation of expression between miR-1258 and HPSE in both the primary tumor sections and metastatic lymph node sections. Patients with undetectable miR-1258 expression also experienced shorter postoperative survival than those with detectable expression, typical of patients with high HPSE expression [179, 180]. An inverse correlation was also observed in non-small cell lung cancer patient samples, and increased expression of miR-1258 in epithelial cell lines resulted in decreased HPSE expression and cell invasion [181]. It has also been suggested that HPSE expression is regulated by miR-1258 in gastric cancer cells [182].

Recently, miR-558 has been identified as a direct regulator of HPSE expression, functioning differently to other miRs [167, 183]. As mentioned earlier in this chapter, SMAD4 is a negative regulator of *HPSE* transcription, repressing LEF1-facilitated *HPSE* transcription [166]. Increased levels of HPSE mRNA, protein and degradative activity as a result of increased miR-558 expression have also been observed in neuroblastoma patient tissue and cell lines, and gastric cancer cell [167, 183] (Fig. 7.14A & B). Studies in gastric cancer cell lines have identified a miR-558 binding site adjacent to the SMAD4 binding site in the *HPSE* promoter, with over-expression of miR-558 resulting in decreased SMAD4 binding (Fig. 7.14C). Mechanistically, it is proposed that binding of miR-558 adjacent to the SMAD4 binding site in the *HPSE* promoter physically prevents SMAD4 binding, resulting in the increase of *HPSE* expression [167] (Fig. 7.14D). The effect of miR-558 on *HPSE* expression can be quenched by circHIPK3 expression, which contains two miR-558 binding sites [184].

Identified as a tumor suppressor gene in breast cancer, and correlating with decreased cisplatin sensitivity of endometrial endometrioid carcinoma, miR-429 is predicted to bind to the 3′ UTR of HPSE and decrease HPSE expression [185–187]. An inverse correlation between miR-429 and HPSE in gastric cancer tumor samples has been demonstrated, with expression of miR-429 observed to be low in tumors, compared to increased expression of *HPSE* [187]. The over-expression of miR-429 caused a decrease in HPSE mRNA and protein expression, while it also decreased the invasion capacity of gastric cancer cells, mirroring the effect of silencing HPSE mRNA [187].

A summary of the key transcription and miR mechanisms that regulate *HPSE* expression is presented in Fig. 7.15.

7.4.4 Regulation of Heparanase Activity by the Presence of the Linker Domain

Fairbanks et al. (1999) were the first to identify the 8 kDa domain in the active HPSE enzyme [17], leading to the proposed domain structure of the protein outlined in Fig. 7.4.

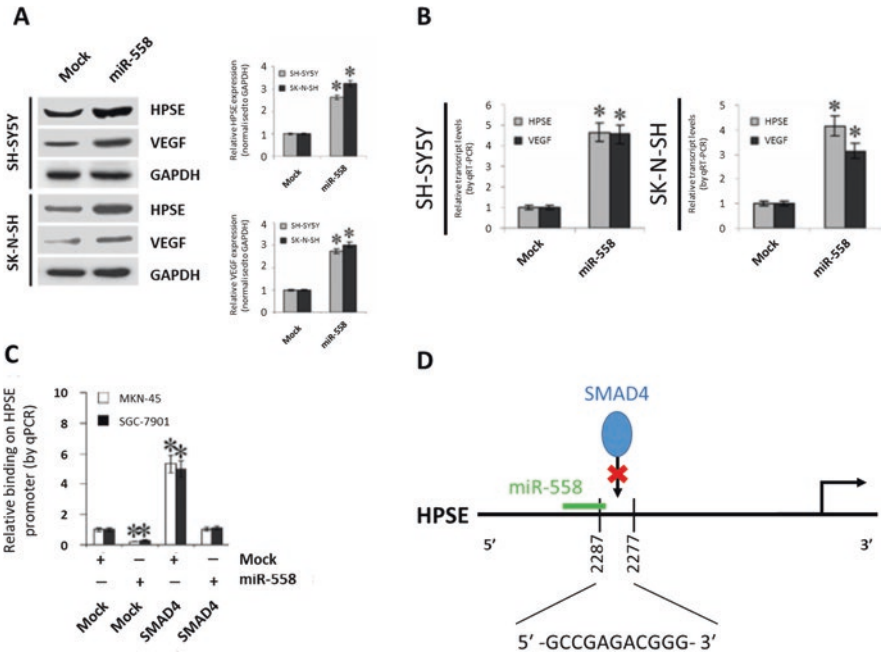


Fig. 7.14 Expression of miR-558 promotes HPSE expression by preventing SMAD4 binding to the *HPSE* promoter: (**A**) and (**B**) Western blot and real-time quantitative RT–PCR indicated that the stable transfection of the miR-558 precursor increased the HPSE and VEGF levels in SH-SY5Y and SK-N-SH neuroblastoma cells than those in mock cells (*P<0.01 versus mock). Adapted from [183]. (**C**) ChIP and qPCR assay showing the binding of SMAD4 to the HPSE promoter in MKN-45 and SGC-7901 gastric cancer cells, and those stably transfected with empty vector (mock), SMAD4 or miR-558 precursor. *p<0.01 versus mock. Adapted from [167]. (**D**) Schematic representation of the proposed mechanism for miR-558-driven expression of HPSE by inhibiting SMAD4 binding to the HPSE promoter

Proteolytic processing of the HPSE pre-pro form is required to form the active heterodimer, with the N-terminal signal peptide and 6 kDa linker domain not contributing to the active enzyme. HPSE is secreted from the cell initially as the inactive proenzyme (lacking the signal peptide domain), but upon binding to syndecan-1, LRP1 or the mannose-6-phosphate receptor, undergoes endocytosis into the cell for activation [188–190]. Cathepsin L was identified as responsible for the excision of the 6 kDa domain, with a bulky hydrophobic amino acid at position 156 found to be vital for the cleavage event at Gln^{157}-Lys^{158} [191, 192]. Structural determination revealed the 6 kDa domain resides in the substrate cleft of HPSE, blocking access of HS chains (Fig. 7.16). [207–210]. Cleavage of the 6 kDa domain was found to be confined to the lysosome and confirmed using multiple approaches, resulting in activation of HPSE [193]. Release of active HPSE from the cell is a tightly regulated process based on extracellular cues from molecules such as ATP and TNFα [194, 195].

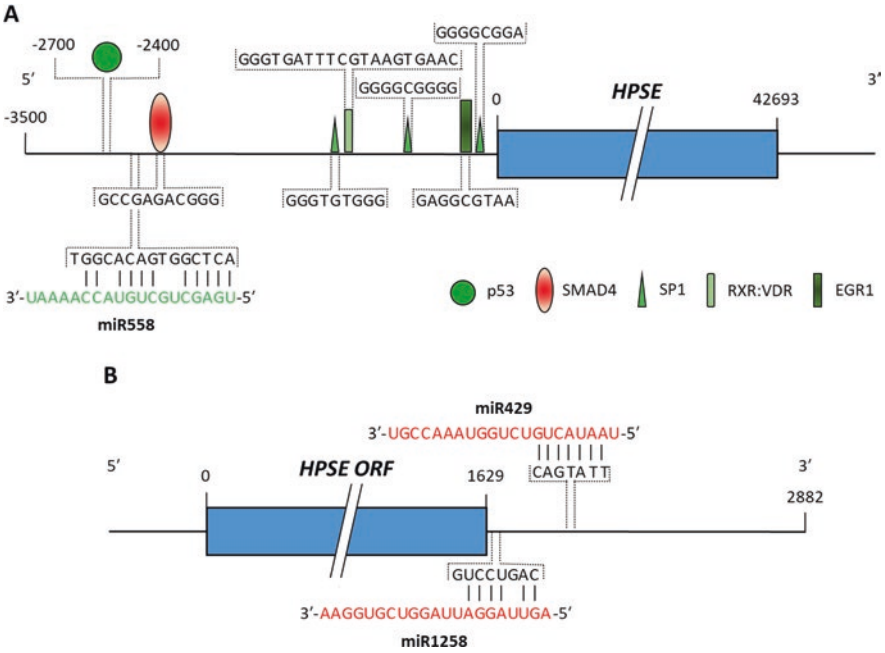


Fig. 7.15 Nucleotide level regulation of HPSE expression**: (A**) p53, SMAD4, SP1, RXR:VDR, and EGR1 are transcription factors that influence *HPSE* expression through the *HPSE* promoter region (up to 3.5 kb 5′ of the *HPSE* transcription start site), with miR558 also influencing *HPSE* expression through the promoter. (**B**) miR1258 and miR429 alter *HPSE* expression via the 3′ UTR. Green symbols and text indicate factors increasing *HPSE* expression, red symbols and text indicate factors inhibiting *HPSE* expression. Schematic is not to scale

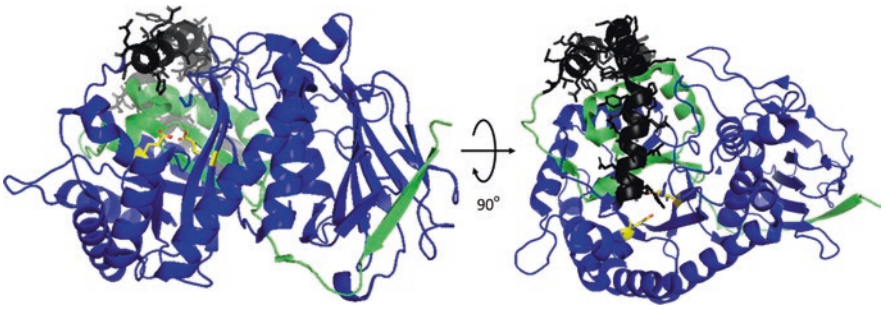


Fig. 7.16 Structural analysis of proHPSE reveals that the 6 kDa linker domain prevents binding to HS: The 6 kDa linker domain (black ribbon) resides in the substrate binding groove of proHPSE (green and blue ribbons, with cataytic Glu^{225} and Glu^{343} residues in yellow). This is proposed to inhibit binding of the HS substrate, inhibiting HS cleavage by the proenzyme. Adapted from [196]. PDB ID: 5LA4

7.4.5 Regulating HPSE Activity by HS Masking

Histidine-rich glycoprotein (HRG) is a multidomain protein and is one of the most abundant plasma proteins; found at concentrations of approximately 1.5μM. HRG is important in a large number of processes, including the formation of immune complexes, facilitating the removal of dying and dead cells, angiogenesis regulation, coagulation and cancer progression [197]. HRG binds to a variety of different molecules, including heparin, HS and HPSE. HRG can regulate the activity of HPSE by binding to HS and masking the HS cleavage sites, preventing the release of small HS fragments bound with growth factors and cytokines [198]. An interaction between HRG and HPSE has also been identified, with this interaction resulting in increased HPSE activity in neutral and acidic conditions [199]. Due to the abundance of HRG in the plasma and the ability for HRG to both inhibit and promote HPSE activity, HRG is thought to be an important regulator of HPSE activity [199].

7.4.6 The Effect of Small Biological Molecules on HPSE Expression

A number of non-protein biological molecules have been implicated in regulating HPSE expression, including reactive oxygen species (ROS), glucose, and fatty acids.

ROS were first identified to regulate expression of HPSE in 2006 during studies investigating the effect of proteinuria on renal damage [200]. As previously mentioned, the development of proteinuria is associated with the loss of HS attributed to the hydroxyl radical [201]. Kramer et al. (2006) investigated whether this loss of HS was a result of HPSE expression by treating rats with a hydroxyl radical scavenger. After 3sweeks of treatment, HPSE expression was decreased and HS loss was recovered [200]. Although a mechanism is not defined in this study, the evidence suggests that ROS can increase the expression of HPSE. The regulation of HPSE expression by ROS in the renal system was further proposed by Hoven et al. (2009) [202]. While ROS lack the functionality to exhibit a direct effect on the *HPSE* promoter, they have been shown to mediate an increase in HPSE expression during hyperglycaemic conditions [203].

For over a decade, the role of HPSE in diabetes has been of interest, with increasing glucose levels leading to an increase in HPSE expression and activity [75, 204]. Studies have revealed that glucose regulation of HPSE expression causes altered cell signaling and intestinal epithelial barrier damage [205–211]. HPSE secretion has also been shown to be regulated by high glucose levels, and that insulin cooperates with glucose to increase HPSE expressions and secretion [209, 211].

Fatty acids have been implicated in increased HPSE expression, subcellular localization, and secretion from endothelial cells [195, 209, 212]. Analysis of the media from oxidized low-density lipid-treated endothelial cells revealed a two-fold

increase in HPSE expression compared to untreated cells [195]. Cell exposure to oleic acid also resulted in an increase in HPSE expression, with elevated transcript and protein expression observed in a dose- and time-dependent manner [195]. This regulation is likely to occur through the activity of the Sp1 transcription factor, at least in part, as mutation of the Sp1 binding site within the *HPSE* promoter results in decreased promoter activation [195].

7.4.7 Active Site Inhibitors of Heparanase

Given the importance of HPSE in cancer progression, tumor metastasis, and immune cell migration/invasion, there has been a focussed effort on developing inhibitors of HPSE. Heparin, produced in humans exclusively by mast cells, was the first identified inhibitor of HS degradation, resulting in complete inhibition of HPSE activity at 5 μg/mL [213–215]. The sulfation of the heparin is critical for HPSE inhibition, with no inhibition of HPSE activity observed in desulfated heparin [214]. Given that heparin polysaccharides are HPSE cleavage targets and the inability of other glycosaminoglycans to inhibit HPSE activity, heparin is hypothesized to bind to the active site and compete for substrate binding [216]. Although the potent anti-coagulant activity of native heparin prevents its use as HPSE inhibitor *in vivo*, it provides the framework for the development of HPSE inhibitors lacking the anti-coagulant activity of heparin. While numerous inhibitors of HPSE have been developed in recent times, this section will focus on the most well-characterized molecules.

Therapeutic regulation of HPSE activity is an area of ongoing, competitive, and constantly evolving research [217]. As inhibition of HPSE is not cytotoxic, HPSE inhibitors would be utilized in the clinic in combination therapies to slow tumor progression and metastasis. Several compounds have been developed as HPSE inhibitors, including symmetrical benzazolyl derivatives [218, 219], triazolothiadiazoles [220], arylamidonaphtalene sulfonate analogs [221], sulf-2-endosulfatase inhibitor OKN-007 [222], various polymers of HS [223, 224], and suramin [225–228]. A recent report also suggests the regulation of HPSE activity by aspirin [229].

The HPSE inhibitors that have entered clinical trials have all been heparin derivatives, namely PI-88, PG545, and SST0001. PI-88 is a complex mixture of highly sulfonated oligosaccharides, SST001 is a mixture *N*-acetyl-reduced oxyheparins with 100% N-acetylation and 25% glycol split, while PG545 is single molecular identity composed of a fully sulfated tetrasaccharide with a cholestanyl aglycon [230–232]. In addition to inhibiting HPSE enzymatic activity, these compounds also compete with HS for binding of VEGF and FGF, resulting in a decrease in angiogenesis, while presenting with only minor anti-coagulation effects and resulting in a decrease in metastatic events. Complementing these features, PG545 has been shown to induce apoptosis in HPSE-independent manner [217]. SST0001 has also been shown to function by disrupting the HPSE/syndecan-1 axis [123]. PI-88 is the most successful HPSE inhibitor clinically tested to date, with the first phase I

studies published in 2002 [233]. The inhibitor mixture has twice entered phase III clinical trials in hepatocellular carcinoma patients (2008 [NCT00568308] and 2015 [NCT01402908]), but these trials had to be terminated early due to significant side-effects, most notably immune-related thrombocytopenia [233]. PG545 has been studied in two phase I clinical trials since 2011 ([NCT01252095], 2016 [NCT02042781]), and is currently recruiting for a third phase I study. Concerns over the emergence of unexpected site reactions caused the early termination of the trial in 2011 while undertaking multiple exposure studies (observed at both 25smg/week and 50smg/week for 7sweeks). To date, SST0001 has completed one phase I study in multiple myeloma patients in 2016, yet no results of this study have been published (NCT01764880) (Chhabra and Ferro; Hammond and Dredge; Giannini et al., and Noseda and Barbieri; Chap. 19, 21, 22, and 23 in this volume).

Although significant progress has been made in the past two decades into understanding heparanase biology, there is still much to be learned. More information into the structure, function, and regulation of heparanase will allow the development of new strategies for inhibition, and ultimately, the design of better therapeutic opportunities.

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Chapter 8
Mechanism of HPSE Gene SNPs Function: From Normal Processes to Inflammation, Cancerogenesis and Tumor Progression

Olga Ostrovsky, Israel Vlodavsky, and Arnon Nagler

8.1 The HPSE Gene SNPs Characterization, Distribution, and Linkage Disequilibrium

According to the definition of SNP, the frequency of such polymorphism is greater than 1%, in at least one population. SNPs are located in different regions of genes such as promoters, exons, introns, and 5′ and 3′ untranslated regions (UTR) and may affect gene expression and regulation. Thereby, SNPs contribute to disease susceptibility, various responses to medication treatment and differences in outcomes within a disease population [8, 9].

Ninety percent of all human variations are SNPs, which appear every 300 bp. Majority of nucleotide substitutions are C to T as a result of easy deamination of C nucleotide. SNPs are useful tools in various aspects of molecular biology, anthropology, and history. Polymorphisms may help to investigate population genetics, tracing of migration, human evolution, and tolerance to different enzymes. SNPs are good genetic markers when correlated with disease phenotype, are located in known genes and are linked to the disease-caused mutations [8, 16]. In common complex diseases, SNPs may help to identify genes or loci that contribute to disease susceptibility. Genetic association studies are the primary method for analysis of the effect of SNPs on disease outcomes [9, 52].

Identification of functional SNPs, including those of heparanase, among healthy persons, allows elucidating the normal functions of a gene in various activated and non-activated cells and involvement of the protein encoded by this gene in normal

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processes [2]. Functional SNPs take part in pathological processes such as cancer development and progression, acute and chronic inflammation, resistance to treatments, and elevated risk of treatment complications [3]. Functional SNPs, which lead to high or low gene expression among healthy populations help to identify actual biological processes in comparison to studies performed with cell lines. Each cell line has a different cell and disease origin (for example, lymphoma cell lines originate from different types of lymphomas) and is derived from a different population (i.e., Caucasian or African populations). Overexpression or silencing of appropriate genes and treatment of cell lines with different reagents do not reflect what happened during disease development or treatment due to the exclusion of many factors and cells that are involved in a given process. The disadvantage of the SNP approach is the necessity to include a large number of subjects in each group and perform an accurate selection of each participant in the analyzed group.

Up today 9746 SNPs were found in the HPSE gene. The frequencies of 1680 SNPs were determined. Five hundred and fifty-three SNPs are located in coding regions, and 71 SNPs have a double hit. The first characterization of the HPSE gene SNPs was performed by us in 2006 in four Israeli Jewish populations [38]. During 12 years 21 SNPs were analyzed in normal and pathological samples. Figure 8.1 shows all SNPs, which were investigated in different studies. The most prominent SNPs are marked in bold. The frequencies of the HPSE gene SNPs differed between Jewish and non-Jewish populations, excluding a partial similarity between Caucasian and Israeli populations [40]. It should be noted that Japanese, Chinese, and Sab-Saharan Africans differ from Caucasians in these SNPs (Table 8.1 according to NCBI SNPs database, http://www.ncbi.nlm.nih.gov/projects/SNP/).

Detailed examination of linkage disequilibrium (LD) among all analyzed SNPs showed the presence of two blocks [40] (Fig. 8.1). These data were congruent with the information regarding the HPSE gene in Haploviewer and Ensemble genome browser. The first block includes SNPs from rs4693612 to rs11099592 and the second block starts from SNP rs6856901. The most prominent SNP rs4693608, located

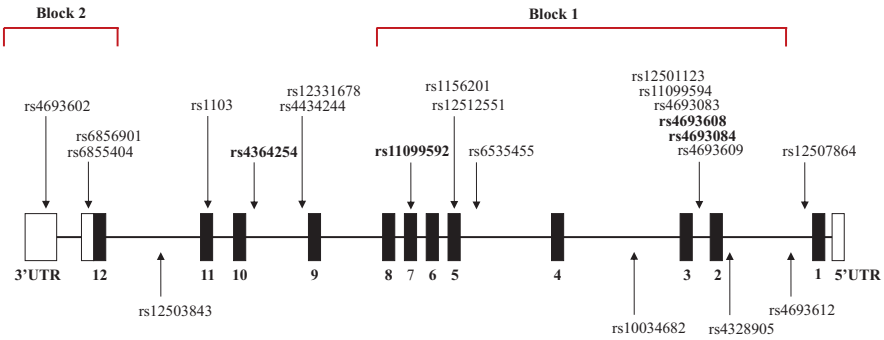

Fig. 8.1 HPSE gene SNPs map. Numbered boxes represent the 12 exons. Filled boxes represent open reading frame, and open boxes represent the 5′- and 3′-untranslated regions. The most prominent SNPs were marked in bold. Arrows show the locations of SNPs. Red brackets represent a block area

Table 8.1 Frequencies of HPSE gene SNPs among different populations

Name of SNP	Population	Frequency, %	
		1st allele	2nd allele
rs4693608 A → G	European	60	40
	Asian HCB	87	13
	Asian JPT	82	18
	Sub-Saharan African	77	23
rs4693084 G → T	European	78	22
	Asian HCB	92	8
	Asian JPT	90	10
	Sub-Saharan African	77	23
rs11099592 G → A	European	78	22
	Asian HCB	92	8
	Asian JPT	89	11
	Sub-Saharan African	87	13
rs4364254 T → C	European	69	31
	Asian HCB	77	23
	Asian JPT	79	21
	Sub-Saharan African	49	51

HCB – Han Chinese in Beijing
JPT – Japanese in Tokyo, Japan

in intron 2 and included in the first block, was found in LD with the other SNPs in spite of the presence of two blocks. Analysis of SNPs that form an anchor of the association revealed that the combination of two SNPs rs4693608 and rs4364254 significantly correlates with heparanase mRNA and protein expression. Polymorphism rs4364254 is located in intron 9 between the two LD blocks. In this genotype combination, the rs4693608 SNP is the leading polymorphism. The rs4364254 SNP helps to identify heterozygote AG individuals with low mRNA HPSE expression levels (AG-CC genotype) [40].

Our more recent study [43] shows that a region in intron 2 of the HPSE gene exhibits enhancer activity in both the sense and antisense directions (chr4: 84,241,177-84,242,376). This region includes the most prominent rs4693608 SNP. Additional rs4693609, rs4693084, and rs4693083 SNPs are also located in the enhancer region. These SNPs were found in strong LD with rs11099594 (intron 2), rs6535455 (intron 4), and rs11099592 (intron 7) (Fig. 8.1). We genotyped additional SNP rs10034682, which is located in intron 3 (unpublished data) and found that this SNP is also in strong LD with 6 other SNPs (rs4693609, rs4693084, rs4693083, rs11099594, rs6535455, and rs11099592). According to the UCSC Genome Browser (http://genome.ucsc.edu/), additional putative enhancer is located in intron 3, and it is active in keratinocytes. NCBI SNPs database includes many other SNPs which were mapped in the first block and have the same allele frequencies as the above-mentioned polymorphisms among Caucasians. An association between rs11099592 and the risk of acute lymphoblastic leukemia (ALL) development [39], and poor outcome of gastric cancer [28, 58] was detected previously. Given the strong LD between rs11099592 with SNPs located in the enhancer regions, the

observed disease associations may result from modification in the enhancer activity. According to EMSA and luciferase assays, rs4693084 may also affect the intron 2 HPSE gene enhancer activity [43]. The distance between rs4693608 and rs4693084 is only 17 bp, and under appropriate conditions both SNPs may be part of a common DNA/protein complex. Importantly, any of the 7 SNPs mentioned above may be a marker for SNP-associated studies. As a result of strong LD between SNPs in this region, identification of a causative SNP needs additional investigations.

8.2 Correlation Between the HPSE Gene SNPs and Heparanase Expression Among Healthy Individuals

Up today an association between the HPSE gene SNPs and heparanase expression was performed only among healthy Israeli population [40]. The known similarity in HPSE gene SNPs frequencies between Caucasians and Israeli Jewish populations allows assuming that the correlation between HPSE gene SNPs and heparanase expression also exists in the Caucasian population. Without SNP association analysis it is difficult to predict what happened in Asian and African populations as a result of different selection pressures (resource availability, environmental conditions, and biological factors) on various populations.

Our previous study [40] has demonstrated a significant correlation between at least five HPSE gene SNPs (rs4693608, rs11099594, rs6535455, rs11099592, rs4364254) and the expression level of heparanase, SNP rs4693608 being the most prominent. Haplotype analysis indicated the existence of significant differences between groups with relatively low and high heparanase expression levels. Notably, best results were obtained when the combination of two SNPs (rs4693608 and rs4364254) was assessed. This approach allowed distribution of all possible HPSE genotype combinations into three groups (LR, MR, and HR) correlating with low, intermediate and high heparanase mRNA and protein expression levels. Group LR included four genotype combinations (GG-CC, GG-CT, GG-TT, and GA-CC), while groups MR and HR included two genotype combinations each (GA-CT, GA-TT, and AA-TT, AA-CT, respectively). Genotype combinations were not casually distributed. These combinations were grouped according to the first SNP rs4693608, except the GA-CC genotype. Presumably, the A-C haplotype of the GA-CC genotype originates from single ancestor recombination between this variation and rs4693608 SNP and correlates with low levels of heparanase.

Although HPSE mRNA and plasma protein levels correlated with the same SNPs, an inverse association was observed [40]. For example, the rare genotype GG of rs4693608 SNP was associated with low HPSE mRNA expression level and high plasma heparanase concentration. In contrast, the frequent AA genotype of this SNP was associated with high HPSE mRNA expression and low plasma protein level. In this study mRNA expression was analyzed in total leukocytes of peripheral blood (PB). In another investigation [42] the correlation between rs4693608 and mRNA HPSE expression level was performed in mononuclear cells (MNC) from PB of

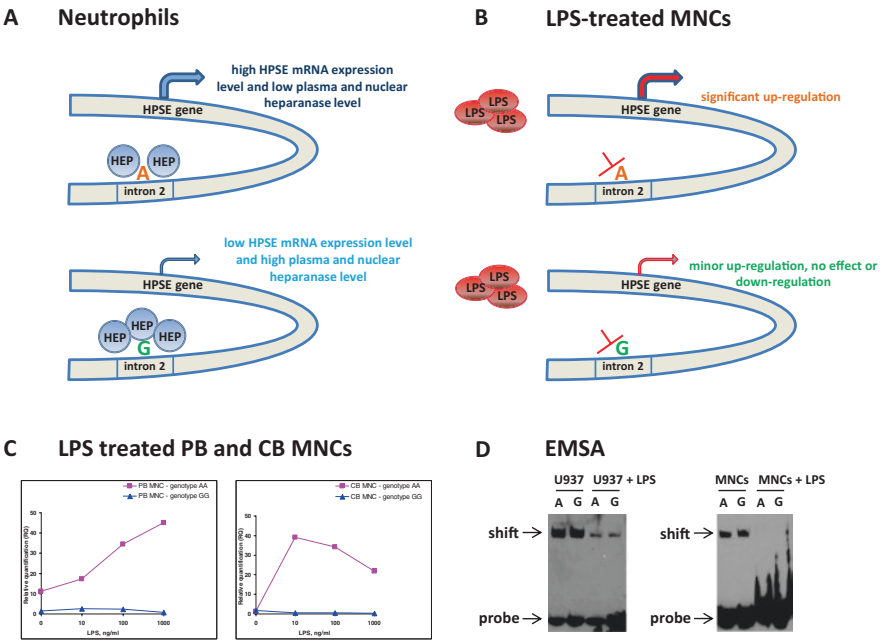

Fig. 8.2 A model of the HPSE gene enhancer regulation in normal leukocytes (neutrophils) and LPS treated mononuclear cells. A. Normal cells. Heparanase binds to the enhancer region of intron 2 and regulates HPSE expression by negative feedback in rs4693608 SNP dependent manner. Additional molecules of heparanase bind to the enhancer region in carriers of allele G in comparison to possessors of allele A. As a result, the expression level of the HPSE gene will be higher in persons with genotype AA than in individuals with genotype GG. B. LPS-treated MNCs. LPS treatment leads to a decrease in the ability of heparanase to bind to the enhancer region and modify heparanase expression in rs4693608 SNP dependent manner. Possessors of the AA genotype disclose up-regulation of heparanase with high ratio in MNCs, while individuals with genotype GG showed non-responsiveness or down-regulation of the HPSE gene in response to LPS. C. PB and CB MNC with genotypes AA and GG were exposed to increasing concentrations of LPS for 18 h and relative quantification (RQ) of the HPSE gene expression was determined. MNC with the AA genotype exhibited up-regulation of the HPSE gene, while MNC with the GG genotype disclosed non-responsiveness to increasing amounts of LPS in both PB and CB samples. D. Effect of LPS on the ability of DNA/protein complexes to bind to the enhancer. LPS treatment resulted in disappearance of DNA/protein complexes in normal MNCs and decreased affinity of DNA/protein complexes in monocytic U937 cell line

healthy adults and umbilical cord blood (CB). Analysis of heparanase expression in resting MNC did not reveal differences among individuals with various HPSE gene genotypes in both PB and CB samples. We, therefore, assume that this type of correlation in resting leukocytes is restricted to neutrophils (Fig. 8.2A).

The level of nuclear heparanase is also correlated with rs4693608 SNP [43]. It was higher in possessors of the GG genotype in comparison to carriers of the AA genotype. EMSA analysis of normal blood samples revealed the binding of a DNA/protein complex to both alleles with higher affinity to allele G (Fig. 8.2A). DNA pull-down assay followed by Western blot verification showed that heparanase

binds to the enhancer region of intron 2 and regulates HPSE gene expression via negative feedback in rs4693608 SNP-dependent manner. This may explain an inverse correlation between HPSE gene SNPs and heparanase expression.

Heparanase is normally expressed in PB neutrophils, monocytes, macrophages, NK and activated T-lymphocytes. In cord blood, heparanase (both mRNA and protein) is expressed in neutrophils, monocytes, macrophages and NK cells. Heparanase expression in T and B lymphocytes is very low. The pattern of heparanase expression in cord blood NK cells differs from its expression in other hematopoietic cells. While in NK cells heparanase appears on the cell membrane in clusters and is widely expressed in the nucleus, in neutrophils and monocytes heparanase is more uniformly distributed on the cell membrane and is hardly detected in the cell nucleus [42].

Functional assay for NK cell activity revealed that susceptibility of Hela cells to lysis by fresh NK cells from different healthy persons correlates with rs4693608 and rs4364254 SNPs. Percent of specific lysis in individuals possessing HR genotype was higher (50.4%) in comparison to possessors of the MR (37.6%) and LR (23.3%) genotypes ($p = 0.009$). Moreover, treatment of NK cells with heparanase led to their increased cytotoxic ability. In contrast, HPSE gene silencing in NK92 cells resulted in low-efficiency killing of their K562 target cells as compared to control NK cells treated with non-specific siRNA (unpublished results).

8.3 HPSE Gene SNPs and Inflammation

The connection between inflammation and heparanase was first shown more than 20 years ago, prior to cloning of the heparanase gene, when HS-degrading activity was detected in immunocytes (neutrophils, activated T-lymphocytes) and found to contribute to their ability to extravasate and accumulate in target organs [30, 31, 53]. Up-regulation of heparanase in response to inflammatory and autoimmune stimuli was noted in various pathologies including arthritis [29], colitis [21, 27], autoimmune diabetes [61], sepsis [47] and experimental encephalomyelitis [30].

In our previous study [42] we tried to mimic the influence of the recipient pro-inflammatory milieu on heparanase expression in donor cells by stimulation of PB and CB MNC with LPS, revealing a significant increase in HPSE expression, which was mediated by TLR4. We found that heparanase expression is modified differently in MNC in accordance with their rs4693608 SNP genotype. Possessors of the AA genotype disclosed up-regulation of heparanase with high ratio in both PB and CB MNC, while individuals with genotype GG showed little or no responsiveness or down-regulation of the HPSE gene in response to LPS (Fig. 8.2B). PB and CB MNC with genotypes AA and GG were exposed to increasing concentrations of LPS, and relative quantification (RQ) of HPSE gene expression was determined. While MNC with the AA genotype exhibited up-regulation of the HPSE gene, MNC with the GG genotype disclosed non-responsiveness to increasing amounts of LPS in both PB and CB samples (Fig. 8.2C).

In a recent study [43] we analyzed the effect of LPS on the ability of DNA/protein complexes to bind to the strong HPSE gene intron 2 enhancer. LPS treatment resulted in disappearance of DNA/protein complexes in normal MNC and decreased affinity of DNA/protein complexes in U937 monocytic cells (Fig. 8.2D).

Pro-inflammatory genes can be regulated by epigenetic mechanisms such as methylation of gene regulatory regions or post-translational modification of histone proteins in chromatin [18]. Increase of methylated DNA decreases transcription factor-binding activity and affects promoter activity and gene transcription [44, 49]. Post-translational histone modifications alter the open or closed chromatin configurations [20]. The relaxed chromatin exposes DNA for transcription factor binding, leading to increased gene expression [56]. LPS treatment attenuates the ability of heparanase, in normal cells, and of the helicase transcription factor (HLTF), in malignant U937 cells, to bind the enhancer region and modify heparanase expression in rs4693608 SNP dependent manner [43].

Heparanase is involved in several inflammatory/autoimmune processes including leukocyte recruitment, immune cell extravasation and migration, release of HS-bound cytokines and chemokines and activation of innate immune cells [54]. Heparanase modulates inflammatory reactions in neuroinflammation [60], sepsis-associated lung injury [47] and inflammatory bowel disease [27], and is an important player in coupling inflammation and tumorigenesis, particularly in colitis-associated colon carcinoma.

Osterholm et al. [37] showed that heparanase expression was increased in carotid plaques and elevated in symptomatic lesions. They analyzed different combinations of the two SNPs (rs4693608 and rs4364254) and found associations between these polymorphism combinations and HPSE expression in carotid plaque. Heparanase gene expression was higher in carotid endarterectomies with HR genotype ($n = 27$) in comparison to samples with MR ($n = 48$) and LR ($n = 29$) genotypes ($p = 0.029$ using a linear regression model). The authors concluded that heparanase expression is increased in human atherosclerosis associated with inflammation, coagulation and plaque instability. Additional investigations are needed to clarify if possessors of the AA genotype have a higher risk to develop atherosclerosis-related complications in comparison to carriers of AG and GG genotypes.

Another study performed by Seifert et al. [48] revealed the involvement of heparanase SNPs in development of sinusoidal obstruction syndrome (SOS), previously known as veno-occlusive disease (VOD; referred to as SOS/VOD). This is a common, potentially life-threatening complication observed after hematopoietic stem cell transplantation (HSCT). SOS/VOD is characterized by ongoing inflammation of terminal hepatic venules and sinusoids in zone 3 of the hepatic sinus, which contributes to occlusion. Histopathologically, the epithelium injury is accompanied by secretion of vasoactive mediators, activation of the coagulation cascade, and subendothelial deposition of clotting factors (i.e., large von Willebrand factor multimers, factor VIII, fibrin) with progressive obstruction. More recent studies indicate that the toxic damage primarily relates to sinusoidal endothelial cells and leads from ischemia and structural destruction, up to hepatocellular necrosis. Furthermore, hepatic stellate perisinusoidal cells are also involved in the pathogenesis of SOS/

VOD [7, 23]. This study [48] indicated that patients with genotypes GG and AG of rs4693608 had a significantly reduced incidence of SOS on day 100 after HSCT compared to patients with genotype AA (4.7 vs. 14.3%, p = 0.038). In addition, the incidence of SOS in patients with genotype TT of rs4364254 was significantly higher in comparison to patients with genotype CC and CT (14.7 vs. 2.3%, p = 0.004). The authors concluded that HPSE gene polymorphism (rs4693608 and rs4364254) is a significant independent risk factor (p = 0.03) for development of SOS/VOD.

Pemphigus is a group of potentially fatal autoimmune blistering diseases of the skin and/or mucous membranes caused by IgG autoantibodies, which predominantly target two transmembrane desmosomal cadherins: desmoglein Dsg 1 and Dsg3 [17]. Higher incidence of pemphigus has been described in the Mediterranean population, Ashkenazi Jewish population, and Macedonian Roma population. Both genetic and environmental factors are involved in the development of pemphigus. Analysis of acantholytic lesions in the epidermis (one of them is pemphigus Vulgaris) showed a pronounced loss of syndecan-1 expression, suggesting that loss of syndecan-1 may be a prerequisite for loss of cell adhesion. Syndecan-1 is a transmembrane heparan sulfate proteoglycan (HSPG). One of the enzymes that modulate its function is heparanase [4, 34]. In our pilot study, we analyzed frequencies of rs4693608 and rs4364254 SNPs in 29 patients with pemphigus Vulgaris and compared them to 210 healthy individuals. Frequencies of LR genotype were significantly higher in the group of patients compared to healthy individuals (46.4% vs 23.3%, p = 0.028) (unpublished data). Studies in a large cohort of patients will clarify the significance of the results and involvement of heparanase in the development of pemphigus Vulgaris. As mentioned earlier, according to the UCSC Genome Browser, the additional putative enhancer is located in intron 3 and is active in keratinocytes. Analysis of HPSE SNPs located in this region is therefore needed.

8.4 Involvement of HPSE Gene SNPs in Cancer Development and Progression

Published data reveal a role of heparanase in dictating tumor progression. It is well supported by *in vitro* and *in vivo* studies as well as extensive research focusing on human cancer patients [55]. On the other hand, the significance of heparanase in the early phase of tumor initiation and development was not fully elucidated. Twelve HPSE gene SNPs were analyzed in different malignancies (Table 8.2). Part of them showed significant correlation with the risk of developing hepatocellular carcinoma and hematological malignancies. Additional studies in different populations are needed to further support these results. Analysis of SNPs in correlation with cancer progression and patient survival yielded clear results (Table 8.2). Allele A of rs4693608 and allele G of rs11099592 revealed significant association with poor disease survival and worse prognosis in gastric cancer and multiple myeloma (MM)

Table 8.2 Summary of analyzed HPSE gene SNPs in different malignancies

Type of cancer	Analyzed SNPs	Number of patients	Results	Allele	References
Epithelial ovarian cancer	rs4328905, rs12501123, rs6535455, rs11099592, rs6855404, rs6856901	136	rs4328905 was found associated with stage of disease (p = 0.0148)	C	[45]
Hematological malignancies	rs4693608, rs6535455, rs11099592, rs4364254, rs4693602, rs6856901	AML – 80 MDS – 24 ALL – 43 CML – 50 MM – 44 HD – 18	rs11099592 and rs6535455 revealed correlation to ALL (p = 0.026); rs4364254 exhibited significant association to AML (p = 0.044); rs4693602 was found in correlation to MM (p = 0.026)	A, C C A	[39, 40]
Gastric cancer	rs4328905, rs4693608, rs11099592, rs6856901	155	rs11099592 was associated with a Borrmann type classification (p = 0.015) and invasion depth (p = 0.02); rs6856901 was correlated with better tumor-related survival	A C	[58]
Gastric cancer	rs4328905, rs4693608, rs11099592, rs4364254, rs4693602, rs6856901	404	rs4693608 was found in association with poor disease survival (p = 0.049); rs4364264 was correlated with well cell differentiation (p = 0.002); haplotype AG of rs4693608 and rs11099592 had greater distribution in the group of Borrmann type 3 and 4 (p = 0.037), the group of a greater number of lymph node metastases (p = 0.046) and was correlated to poor survival (p = 0.044).	A T AG	[28]
Multiple myeloma	rs4693608, rs6535455, rs11099592, rs4364254	348	rs4693608 A-allele had a higher frequency of vertebral fractures (p = 0.02); carriers of the rs6535455 variant T-allele had better survival (p = 0.002)	A T	[1]
Hepatocellular carcinoma	rs4328905, rs4693608, rs11099592, rs12331678, rs4364254, rs12503843	400	rs12331678 and rs12503843 correlated with the risk of hepatocellular carcinoma (p = 0.0046 and p = 0.005, respectively); significant interaction between rs12331678 and rs12503843 and hepatitis B virus carrier status was observed.	A T	[57]
Breast cancer	rs4693608, rs11099592, rs4364254	209	rs4364254 revealed significant correlation with expression of progesterone receptor: High expression was correlated with allele C, and low expression with allele T (p = 0.002)	C	Our unpublished data

Significant SNPs were signed in bold

(Table 8.2). Our recently published article [43] showed that rs11099592 is in strong linkage disequilibrium (LD) with rs4693084, both located close to rs4693608 (17 bp only) in the enhancer region. We assume that common rs4693608 and rs4693084 SNP-dependent enhancer activity affect cancer progression and outcomes.

SNP rs4364254, located in intron 9, revealed a significant correlation with progesterone receptor expression among patients with breast cancer. High expression of progesterone receptor was correlated with allele C, and low expression - with allele T, respectively ($p = 0.002$) (unpublished data). The progesterone receptor (PR), a member of the nuclear receptor family, is a well-known estrogen receptor (ER)-regulated gene that is expressed in over two-thirds of ER-positive breast cancers. PR is highly expressed in the luminal A breast cancer subtype and is associated with tumor grade, ER expression, Nottingham Prognostic Group and negative HER2 status in early breast cancer. Multiple studies have demonstrated improved prognosis of PR-positive breast cancers [15, 25, 32]. Allele C of rs4364254 is in LD with allele G of rs4693608, allele T of rs4693084 and allele A of rs11099592, which were found in correlation with better prognosis in other types of malignancies. However, the only correlation to rs4364254 was found in breast cancer. We have previously reported that rs4364254 SNP polymorphism helps to identify heterozygote AG individuals for rs4693608 with low heparanase expression [40] and low risk of acute GVHD development [41]. As mentioned above allele C of this SNP was also found in association with low risk of VOD development. This rs4364254 SNP is located between two main blocks of SNPs, and no regulatory elements were found in intron 9. Additional studies are needed to clarify the role of rs4364254 SNP in different processes.

Multiple myeloma is a B cell malignancy characterized by destructive bone lesions, chemoresistance, tumor relapse, and poor patient outcome. Heparanase is an important driver of myeloma progression [34, 46] (Purushothaman and Sanderson, Chap. 12 This book). Our recent results showed that HPSE gene enhancer is highly active in multiple myeloma (MM) cell lines (CAG, RPMI8226, U266). Andersen et al. [1] observed that rs4693608, which modulates the enhancer activity, correlates with bone morbidity and survival in MM patients. The authors found that additional SNP rs6535455, located in intron 4, is associated with the outcome of 348 MM patients in Denmark [1]. According to our previous studies, rs6535455 was found in complete LD with rs11099592. However, the authors did not identify any correlation to rs11099592. Notably, DNA for analysis was purified from PB MNCs by the salting out method or from paraffin-embedded tissue by phenol extraction. We assume that DNA extracted from paraffin-embedded tissues is enriched in myeloma cells. In another study, Huang et al. [26] using a custom SNP microarray demonstrated a high frequency of loss of heterozygosity (LOH) in the HPSE gene of hepatocellular carcinoma. Allele loss in paraffin-embedded tissue samples of MM patients may explain the observation of Andersen et al. [1] that homozygous carriers of the variant T-allele of rs6535455 had better overall survival in comparison to carriers of the C-allele.

Malignant cells escape immune-mediated cell death by deploying epigenetic mechanisms to evade host immune recognition and immunogenicity. This acquired

immune evasive phenotype is achieved by epigenetic down-regulation of many critical molecules required for efficient cancer and immune cell interactions, such as suppression of tumor-associated antigens (TAAs), reduced expression of many antigen processing and presentation machinery (APM) components, and low cell surface levels of accessory/co-stimulatory molecules, death receptors, and stress-induced ligands [5, 50]. Malignant cells use chromatin remodeling for their purpose and alter gene transcription by post-translational modification of histone proteins, which include acetylation, methylation, phosphorylation, deimination, and ubiquitination. Also, the 3D nuclear architecture in normal and malignant cells contributes to transcriptional regulation and malignant cells escape. Chromatin domains containing transcriptionally active genes can form chromatin loops that extend away from compact chromosome territories to reposition near transcriptional factors at the center of the nucleus. Organization of the nuclear architecture is thought to mediate gene transcription by controlling the accessibility of regulatory DNA elements to transcription factors and RNA polymerases through subnuclear gene positioning and intra−/inter-chromosomal interactions [18].

Strong enhancer recently identified in intron 2 of the HPSE gene [43] may be involved in epigenetically regulated malignant cells escape. Applying electromobility shift assay (EMSA), we analyzed the binding of nuclear proteins, extracted from normal leukocytes, malignant cell lines (12 hematological cancer cell lines and 14 solid tumor cell lines) and primary leukemia samples, to DNA probes that contained the rs4693608 SNP. We observed SNP-dependent and SNP independent binding. Analysis of healthy control samples demonstrated gel shift bands for both allelic probes (Fig. 8.3C). In all 26 analyzed cancer cell lines and primary leukemia samples, the band of DNA-protein complex was shifted significantly in comparison to normal samples. The affinity of the complex to the G allele probe was slightly higher in comparison to the A allele probe (Fig. 8.3C). At least 5 extra shifted bands were incorporated to allele A probe while allele G probe was bound to only one main DNA/protein complex (Fig. 8.3C).

DNA pull-down assay followed by Western blot verification showed that in normal leukocytes heparanase binds to the enhancer region and regulates HPSE gene expression via negative feedback in rs4693608 SNP-dependent manner (Fig. 8.3A). In all analyzed malignant cell lines and primary leukemia samples, heparanase halted self-regulation of the enhancer region. Instead of heparanase, the Helicase-like transcription factor (HLTF) binds to the regulatory region (Fig. 8.3B) [43]. HLTF is a member of the yeast mating SWItch/Sucrose Non-Fermenting (SWI/SNF) family of proteins involved in chromatin remodeling. HLTF plays a significant role in gene transcription, DNA repair, ubiquitination, and genome stability (Hishiki et al. 2015, Cheng et al. 2016). Two different consensus sequences recognized by HLTF were proposed: (C/A)C(T/A)TN(T/G) and (A/G)G(T/C)(G/T)G [14]. Analysis of the HPSE enhancer region included in rs4693608 SNP indicated that in the case of allele A, HLTF recognizes the first sequence CC**A**TTG. A to G alteration does not result in loss of the transcription site but rather a change to the second variant of the HLTF recognition site (C**G**TTG), except the first nucleotide. Our EMSA results disclosed that not only HLTF binds to the HPSE enhancer region in allele A, but other proteins may form a common DNA-protein complex.

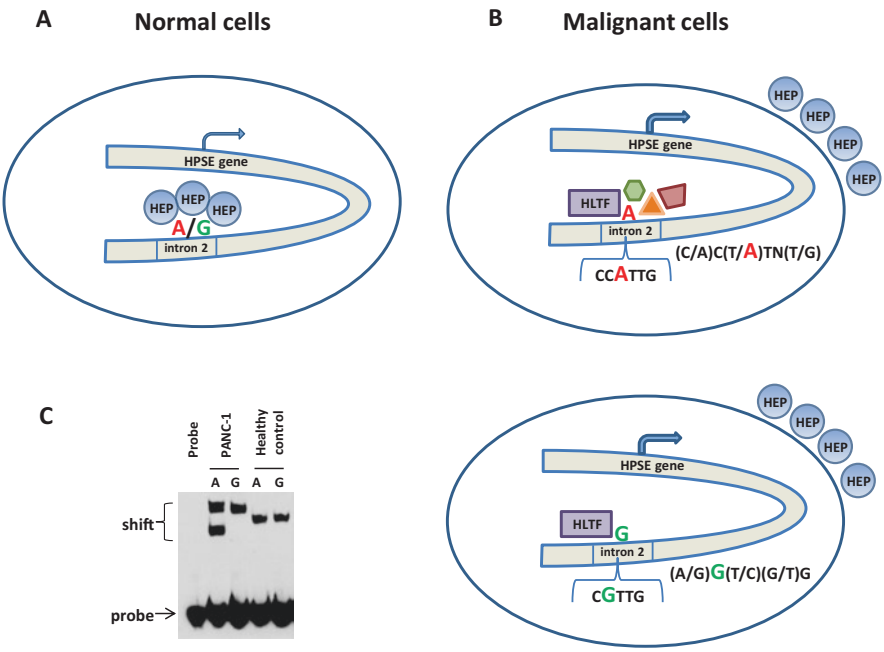

Fig. 8.3 A model of the HPSE gene enhancer regulation in normal and malignant cells. (**A**). Normal cells. Heparanase binds to intron 2 enhancer region and regulates HPSE expression by negative feedback in rs4693608 SNP dependent manner. (**B**). Malignant cells. In malignant cells, heparanase is not recruited to the enhancer region, and hence its negative self-regulation is discontinued. Instead, the helicase-like transcription factor (HLTF) binds to the regulatory enhancer region. Two different consensus sequences that are recognized by HLTF were identified in the enhancer region: (C/A)C(T/A)TN(T/G) for allele A and (A/G)G(T/C) (G/T)G for allele G. While in carriers of allele A, HLTF binds to the enhancer region together with other proteins, possessors of allele G bind the HLTF alone. (**C**). Electromobility Shift Assay (EMSA) using DNA probes containing rs4693608 in normal leukocytes and PANC-1 cells. Rs4693608 SNP-dependent and SNP-independent binding were observed. Healthy control samples demonstrated gel shift bands for both allelic probes. In malignant cells, the band of DNA-protein complex was shifted significantly in comparison to normal samples. Additional shifted bands were incorporated to allele A probe while allele G probe was bound to only one main DNA/protein complex. We speculate that discontinuation of heparanase normal self-regulation and binding of the HLTF to the HPSE gene enhancer region results in translocation of heparanase from the cell nucleus to the cytoplasm and extracellular matrix. This may function in malignant cell escape from immune-mediated cell death and contribute to the process of cancerogenesis and tumor progression

In contrast, in the case of allele G, only HLTF binds to the HPSE gene enhancer region (Fig. 8.3B). These results may explain worse prognosis and poor survival of patients with genotypes AA/AG in comparison to possessors of genotype GG [43].

HLTF is thought to be a tumor suppressor supported by the detection of HLTF promoter hypermethylation in various types of cancer tissues and cell lines [14]. However, recent observations of increased expression of HLTF in transformed cells and cancer tissues suggest that in some cases HLTF could be associated with cancerogenesis and may act as an oncogene. Investigation of HLTF expression in a hamster model of kidney tumors revealed that HLTF gene activation is linked to

initial steps of cancerogenesis and should be analyzed in an early stage of other neoplasms [13]. Taken together we speculate that cessation of heparanase normal self-regulation and binding of HLTF to the HPSE gene enhancer region results in translocation of heparanase from the nucleus to the cytoplasm and ECM and may function in malignant cell escape from immune-mediated cell death, thereby contributing to the process of cancerogenesis and tumor progression. Our hypothesis is supported by Cohen et al. [12] showing that cytoplasmic staining of heparanase is associated with a poor prognosis whereas nuclear heparanase predicts a favorable outcome of patients with lung cancer.

8.5 HPSE Gene SNPs and the Risk of Acute Graft Vs. Host Disease (aGVHD)

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially effective curative therapy for a variety of malignant and non-malignant hematological diseases [3, 6, 11, 35]. However, transplant-related complications remain a major obstacle [2, 19, 51]. The outcome of HSCT is affected by several variables including disease and disease status, general condition, patient and donor age, and patient and donor HLA matching [22, 33]. Disease relapse and transplant-related complications including graft-versus-host disease (GVHD), major infection, and organ toxicities such as interstitial pneumonitis and hepatic veno-occlusive disease (sinusoidal obstruction) are the major causes for transplant failure [22, 33]. Long-term causes of morbidity include chronic GVHD and infection [36]. In addition to HLA matching, genetic diversity among patients and donors contribute to differences in individual responses to tissue injury, inflammation, and severity of acute and/or chronic GVHD.

We have demonstrated a highly significant association between rs4693608 and rs4364254 combinations and the risk of acute GVHD. The genotype combination HR, associated with increased heparanase mRNA expression [41], correlates with a high risk of acute GVHD. Conversely, the genotype combination LR, associated with decreased level of heparanase mRNA, correlates with a low risk of GVHD. Moreover, disparities between recipient and donor pairs in HPSE gene SNPs combinations significantly increased the probability of developing acute GVHD post-HSCT (Fig. 8.4C).

Association between increased risk of extensive chronic GVHD and either the recipient genotype combination HR or the D1 group was found. Moreover, a significant correlation between rs4693608 and rs4364254 combinations and both transplant-related mortality (TRM) and overall survival (OS) post HSCT were revealed [41].

We investigated the effect of pre-transplant conditioning regimens on heparanase expression levels in transplant recipients, as well as the predictive value of rs4693608 polymorphism for post-transplant engraftment and GVHD occurrence [42]. Pre-transplant conditioning led to a significant over-expression of the HPSE gene, followed by a gradual decrease thereafter. Notably, the increase in HPSE gene expression post-conditioning was higher in patients harboring the AA genotype vs. the GG genotype.

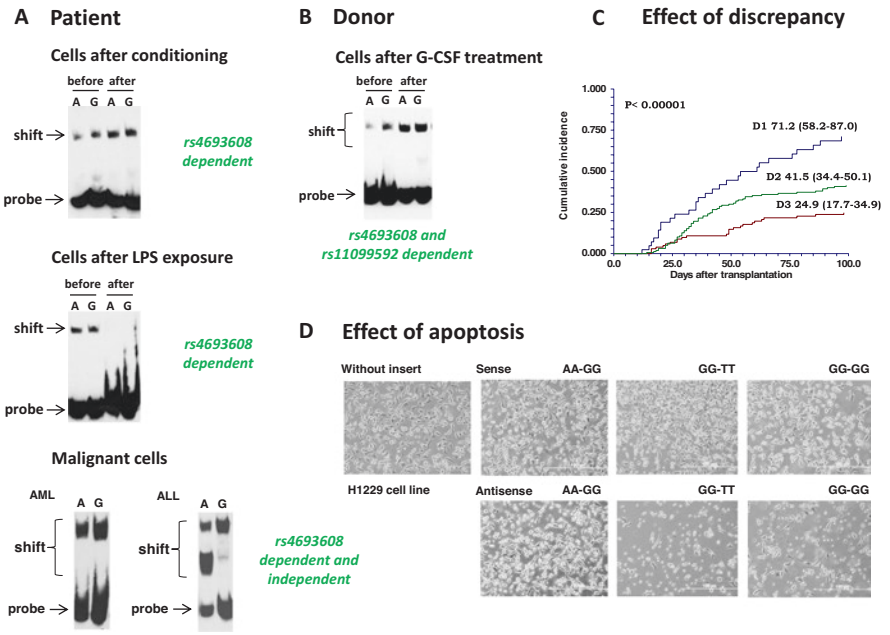

Fig. 8.4 Status of the HPSE gene enhancer following allogeneic hematopoietic stem cell transplantation (HSCT) in various patient and donor cells. (**A**). Electromobility Shift Assay (EMSA) using DNA probes containing rs4693608 in patient cells after conditioning and exposure to pro-inflammatory environment, and malignant patient cells. Three types of cells with different states of the enhancer region interact with donor cells following graft intake. Conditioning led to an increase in affinity of DNA/protein binding to the enhancer region. Pro-inflammatory environment results in obstruction of transcription factor accessibility to the enhancer region. In patient-derived tumor cells, heparanase discontinues its normal self-regulation, and the HLTF binds to the enhancer region resulting in overexpression of cytoplasmatic and extracellular matrix heparanase. (**B**). Electromobility Shift Assay (EMSA) using DNA probes containing rs4693608 in donor cells after 5 days of exposure to G-CSF. G-CSF treatment leads to high-affinity binding of heparanase to the enhancer region with elevation of heparanase level in donor cells. (**C**). Cumulative incidence of acute GVHD grade II-IV after HSCT according to the discrepancy of HPSE gene SNPs rs4693608 and rs4364254 combinations between recipients and donors. The D1 group contains HR-MR and HR-LR recipient-donor genotype combination pairs. The D2 group contains MR-MR, MR-HR, MR-LR, and HR-HR recipient-donor pairs, and the D3 group contains LR-LR, LR-MR, and LR-HR recipient-donor pairs. The incidence was higher in group D1 compared to the D2 and D3 groups ($P < .00001$). (**D**). Apoptotic effect of constructs containing the G allele of rs4693608 SNP in the antisense direction in H1229 cells. Six HPSE enhancer constructs were designed and inserted upstream of the luciferase gene in pGL4.26 vector with minimal promoter. The constructs were transfected using the Ingenio Electroporation Kit (Mirus Bio, Madison, WI) and Nucleofector™ (AMAXA Biosystems, Lonza, Germany). The effect was observed 24 h after transfection

Successful immune reconstitution post-transplant is important for decreasing infection incidence and relapse rate without increasing GVHD. The first recovered cells are neutrophils. Differences in rs4693608 between recipient and donor affect the time of neutrophils recovery. It is conceivable that high heparanase expression level

in the bone marrow microenvironment of AA recipients promotes faster recovery of AG/GG donor neutrophils (AA-AG, AA-GG, and AG-GG recipient-donor pairs) [42].

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-accepted treatment modality for several malignant and non-malignant diseases. The aim of treatment is to replace the damaged hematopoietic cells with normal stem cells. Like other medical treatments, HSCT has clear risks and benefits. The primary benefit is to replace the abnormal hematopoiesis with normal cells in a way that the new immune system will elicit a graft-versus-tumor/leukemia effect. On the other hand, GVHD is also based on the recognition of foreign antigens by T-cells. Prevention of GVHD while preserving graft versus leukemia (GVL) is an elusive goal of allogeneic hematopoietic stem cell transplantation. The holy of HSCT is the ability to induce sufficient GVL to cure the patient without the induction of GVHD. A T-cell therapeutic window between achieving GVL without GVHD is highly desirable [59].

If we focus only on the enhancer region of the HPSE gene after HSCT, at least three types of cells with different states of the enhancer region may interact following graft intake: (i) without bold formatting normal patient cells after conditioning and exposure to pro-inflammatory environment (enhancer obstruction to transcription factors and heparanase overexpression in carriers of AA genotype and low elevation, unresponsiveness or downregulation in possessors of the GG genotype); (ii) tumor patient cells (discontinuation of heparanase normal self-regulation and binding of HLTF to the HPSE gene enhancer region, followed by overexpression of heparanase and involvement in tumor progression) (Fig. 8.4A); and (iii) donor cells after 5 days exposure to G-CSF (high affinity of heparanase to the enhancer, associating with elevation of heparanase level in donor cells) (Fig. 8.4B). We have previously demonstrated that discrepancy of HPSE gene SNPs between recipients and donors was the most prominent factor for risk of acute GVHD, especially in the group receiving reduced toxicity conditioning regimens [41]. We speculate that heparanase-related differences in the level of signals between recipients and donors may lead to activation and subsequent proliferation and differentiation of donor T-cells and NK cells. Since the strongest association was observed in the D1 group, it is conceivable that in D1, as opposed to the D2 and D3 groups, the recipient signal threshold, affected by heparanase, is higher compared to that of their donors. This delicate balance may be further modulated by the recipient and donor inflammatory cytokine polymorphism, leading to hyperactivation of donor T cells and thereby elevate the risk of acute GVHD.

While analyzing the enhancer activity in hematological and non-hematological malignancies, we observed apoptotic effect of constructs that included the G allele of rs4693608 SNP in antisense direction in H229, Panc-1 and SK_N_SH cell lines (Fig. 8.4D) (unpublished data). We suspect that the normal enhancer region with allele G leads to normal eRNA synthesis in malignant cells with subsequent cancer cells apoptosis. Taken together, we hypothesize that the identified effect of discrepancy in HPSE gene SNPs between recipient and donor may lead not only to risk of aGVHD but also improve the potential GVL effect on donor cells. Subsequent studies are needed to clarify how modification in HPSE enhancer activity may decrease the risk of aGVHD and improve the GVL effect of HSCT.

8.6 Summary

Functional SNPs are an excellent tool not only for disease-associated research but also for investigation of actual normal and pathological biological processes, which are difficult to study by other approaches. Research focusing on the HPSE gene rs4693608 SNP, located in the active enhancer, allows us to investigate the complex multiple levels of heparanase regulation, study heparanase-related mechanisms of cancer cell escape from immune-mediated cell death, improve the acute GVHD/GVL balance following HSCT, and elucidate how modification in the HPSE enhancer region may help to develop new approaches for cancer treatment.

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Part III
Tumor Biology

Chapter 9
Heparanase-The Message Comes in Different Flavors

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9.1 Introduction

Activity capable of cleaving macromolecular heparin at a limited number of sites was first reported in 1975 by Ögren and Lindahl [1]. Soon thereafter, Höök et al. reported an endoglycosidase activity that degrades heparan sulfate (HS)-polymers into oligosaccharides [2]. Given the structural role of HS proteoglycans (HSPG) in the assembly of extracellular matrix (ECM) and basement membrane, it was hypothesized that HS-degrading activity would loosen the ECM, thus promoting cell dissemination. Indeed, heparanase activity was found to correlate with the metastatic potential of tumor cells [3–5], a correlation that still direct and guide heparanase research. Subsequent years can be divided to before and after the cloning of the heparanase gene. Until 1999, progress in the field was slow and studies were restricted to measures of heparanase activity in different normal and malignant cells and tissues under various experimental settings [6, 7]. Also, the lack of a simple assay and purified enzyme in sufficient amounts lead to conflicting reports on the biochemical properties and substrate specificity of the enzyme(s) [8]. Heparanase activity was attributed to proteins with a molecular weight ranging from 8 to 130 kDa, raising the possible existence of several HS-degrading endoglycosidic enzymes [8–11]. This confusion was solved when the cloning of a single human heparanase cDNA sequence was independently reported by several groups in 1999 [12–15]. So far, and unlike many other classes of enzymes, no other cDNA sequence encoding an active heparanase enzyme has been identified, indicating

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that mammalian cells express primarily one single dominant heparanase enzyme (historic perspective is presented by Vlodavsky et al., Gaskin et al., and Khanna and Parish, Chaps. 1, 3 and 7 in this volume). With the availability of appropriate tools, heparanase research entered a new era. In the last 20 years, we are experiencing a burst in heparanase research, evident by an average of 100 new citations in PubMed each year. This collective effort has turned heparanase from an obscure enzyme to a valid target for the development of anti-cancer drugs, some of which are under clinical evaluation [16–19].

In 2007, Vreys and David published a comprehensive review article entitled "Mammalian heparanase-What is the message?" [20]. A similar phrase was entitled by Rickles: "If heparanase is the answer, what is the question?" [21]. The 'message', or 'answer', over ten years later, can be summarized as a pleiotropic enzyme that plays an important role in cancer and inflammation, two major facets that are often interconnected. While the pro-tumorigenic properties of heparanase are well taken, some aspects of heparanase biology and its mode of action are still unclear. Here, we review heparanase function in oncology, suggesting a somewhat different interpretation of the results.

9.2 Heparanase and Cancer Progression

9.1.1 Heparanase Induction in Human Cancer

Soon after the cloning of the HPSE gene and the development of anti-heparanase antibodies and probes, many studies examined its expression in human tumors compared with adjacent normal tissue. Immunohistochemistry, *in situ* hybridization, RT-PCR, real time-PCR and enzymatic activity analyses revealed that heparanase is up regulated in essentially all human tumors examined. In most cases, elevated levels of heparanase were detected in about 50% of the tumor specimens, with a higher incidence in pancreatic (78%) and gastric (80%) carcinomas, and in multiple myeloma (86%) [20, 22–24]. Generally, the normal looking tissue adjacent to the malignant lesion expressed little or no detectable levels of heparanase, suggesting that epithelial cells do not normally express the enzyme. This is in agreement with the notion that under normal conditions heparanase expression is restricted primarily to the placenta and platelets, and to lower extent keratinocytes, lymphocytes, neutrophils, and macrophages [9, 10]. In several carcinomas, most intense heparanase staining was localized to the invasive front of the tumor [25–28], supporting a role for heparanase in cell invasion. Furthermore, patients that were diagnosed as heparanase-positive exhibited a significantly higher rate of local and distant metastasis as well as reduced postoperative survival, compared with patients that were diagnosed as heparanase-negative [25, 26, 29–32] (Fig. 9.1). These and more recent studies [33–43] provide strong clinical support for the pro-metastatic function of heparanase. Subsequent studies provided compelling evidence that tie heparanase

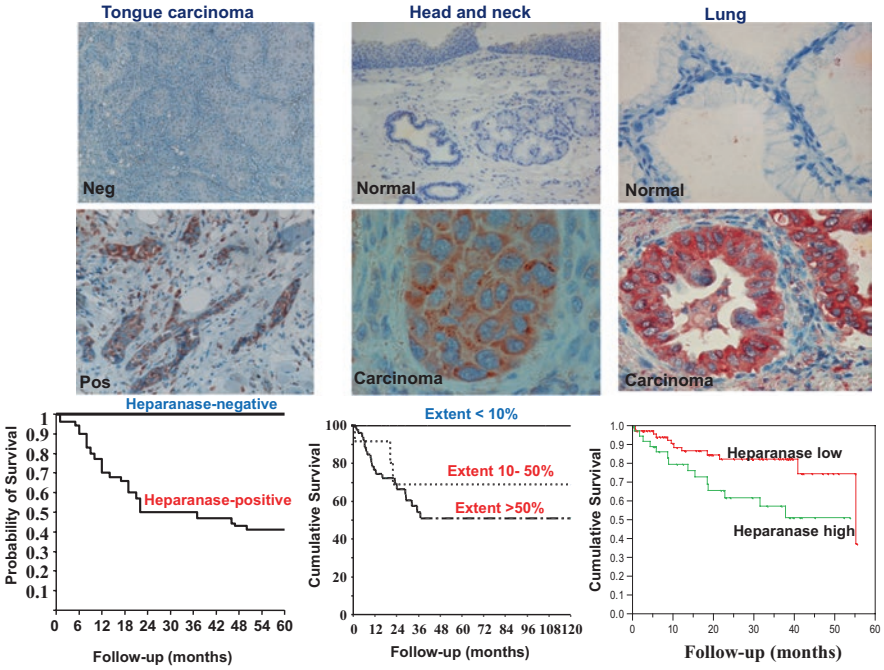

Fig. 9.1 Reduced overall survival curves of patients with tongue (left), head and neck (middle) and lung (right) carcinomas according to heparanase immunostaining intensity. Formalin-fixed paraffin-embedded sections of tumors and adjacent normal head and neck and lung tissues were subjected to immunostaining of heparanase, applying anti-heparanase pAb 733. Staining was graded as 0 (negative), 1 (weak) and 2 (strong). Note that adjacent normal head and neck and lung tissues are stain negative for heparanase. Shown are representative immunostaining at low (upper panels) and high (x100) magnifications (middle panels). Kaplan-Meier analysis showed poor survival of patients with strong (score 1 & 2) heparanase staining or high staining extent (i.e., percent of heparanase-positive cells), compared with patients who were diagnosed as heparanase-negative (score 0) (lower panels)

levels with all steps of tumor formation including tumor initiation, angiogenesis, growth, metastasis, and chemoresistance [40, 44–49]. These and other results (i.e., the critical role of heparanase in the tumor microenvironment) [49, 50] (See also Chapter by Elkin et al., Chap. 17 in this volume) indicate that heparanase is causally involved in cancer progression and collectively provide strong clinical support for the pro-tumorigenic function of heparanase and put forward the concept that heparanase is a valid target in cancer.

The molecular mechanism(s) underlying heparanase induction in tumor cells is not entirely clear, but evidently involves epigenetic alterations (i.e., DNA methylation), hormones, oncogenes, and post-transcriptional regulation [51]. The involvement of SNPs [52, 53] and an enhancer region that activates the promoter [54] is discussed in details by Ostrovsky et al., Chap. 8 in this volume.

9.1.2 Basal and Inducible Heparanase Gene Transcription

A 3.5-kb promoter region of the heparanase gene was cloned by Jiang et al. [55]. Somewhat surprisingly, sequence analysis revealed that the TATA-less, GC-rich promoter of the heparanase gene belongs to the family of housekeeping genes. This may suggest that heparanase is being expressed at low levels by all cells in order to maintain homeostasis (see below). The above-mentioned lack of heparanase detection by immunostaining of normal epithelium adjacent to the tumor lesion may thus result from low levels under the detection of anti-heparanase antibodies. Further analysis revealed three Sp1 sites and four Ets-relevant elements (ERE) within the heparanase promoter [55, 56]. SP1 is a zinc-finger transcription factor that binds GC-rich motif of many gene promoters and is abundantly expressed by all mammalian cell types. Thus, it was long thought to be a regulator of housekeeping genes. Indeed, knockout of Sp1 in mice causes embryonic lethality at an early stage of development (around day 10.5 of gestation) with a broad range of phenotypic abnormalities, suggesting a general function in many cell types [57]. Unlike SP1 and Ets, which are associated with basal heparanase transcription levels [55], Early growth response 1 (Egr1) appears to be related to inducible transcription of heparanase. Egr1 has been shown to bind the human and mouse heparanase gene promoters and to induce heparanase expression in tumor cells [58–60] and in primary T lymphocytes, facilitating their infiltration into the CNS to promote EAE [61]. Egr1 has been shown to bind to the promoters of a range of genes to mediate responses such as wound healing and neo-vascularization, and has been strongly associated with vascular proliferative disorders [60]. Moreover, Egr1 has been implicated in tumor angiogenesis, growth, and metastasis, closely resembling heparanase pro-tumorigenic properties. Egr1 was also noted to inhibit heparanase expression in pancreatic carcinoma cells exposed to radiation [62], suggesting that heparanase gene regulation by Egr1 is cell-type and context-dependent. The clinical significance of heparanase regulation by Egr1 emerged in the study of DNA methylation (Gaskin et al., Chap. 7 in this volume).

9.1.3 Gene Methylation and Egr1

In addition to multiple genetic alterations that govern cell transformation, epigenetic processes, marked by hypermethylation of the promoter region, contribute significantly to gene transcription and cancer progression, likely by down-regulation of tumor suppressor and DNA repair genes. Several studies have convincingly shown that promoter methylation status plays an important role in heparanase gene transcription. By examining a series of tumor-derived cell lines, Shteper et al. found that cells which exhibit heparanase activity also harbor at least one unmethylated allele [63]. In contrast, cell lines which exhibit no heparanase expression or activity, such as C6 rat glioma and JAR human choriocarcinoma, were found to harbor fully methylated alleles [63]. Treating these cells with demethylating agents such as 5-azacytidine restored heparanase activity accompanied by augmented metastatic

capacity [63]. This augmentation was suppressed in mice treated with heparanase inhibitor [63], thus critically relating to heparanase expression and metastasis. Subsequent studies revealed a similar correlation with prostate and bladder cancer-derived cell lines, and, moreover, with prostate and bladder tissue. Hence, significantly higher promoter methylation was found in benign prostatic hyperplasia (BPH) and in normal bladder than in bladder carcinomas, inversely correlating with heparanase expression [64, 65]. Interestingly, Ogishima et al. have noted a correlation between heparanase expression by bladder and prostate carcinomas and the expression levels of Egr1, regulating heparanase expression in a stepwise manner. Hence, heparanase expression was the lowest in methylation-positive and EGR1-negative samples and the highest in methylation-negative and EGR1-positive samples [64, 65]. It should be noted, nonetheless, that while DNA methylation and Egr1 likely play an important role in regulating heparanase transcription, the magnitude of heparanase induction by these factors seems lower than the induction often observed by immunostaining. This may suggest that heparanase expression is also regulated post-transcriptionally.

9.1.4 ARE and Post-Transcriptional Gene Regulation

In mammalian cells, sequence elements rich in adenosine and uridine, called AU-rich elements (ARE), were identified for their ability to target mRNAs for rapid degradation [66–68]. Many ARE-bearing mRNAs encode oncoproteins, cytokines, growth factors and transcription factors [66, 69]. Arvatz et al. have shown that heparanase expression is regulated at the post-transcriptional level by sequences at the 3′ untranslated region (3′UTR) of the gene [51]. Introducing the 3′UTR immediately following the heparanase cDNA reduces heparanase enzymatic activity and protein levels, resulting in decreased cellular invasion capacity. Furthermore, a 185 bp sequence was identified within the 3′UTR that mediates heparanase down-regulation, and an ARE motif has been recognized within this region. Deletion of the entire 185 bp region or the ARE motif eliminated the inhibitory effect of the 3′UTR, resulting in more stable heparanase mRNA, elevated heparanase levels and formation of larger tumor xenografts indistinguishable from those produced by heparanase over-expressing cells in terms of size, vascularization and Akt activation [51]. These results suggest that loss of the ARE is an important regulatory mechanism and driving force contributing to heparanase induction and tumor growth [51].

9.1.5 Heparanase Regulation by Hormones, Tumor Suppressors, Oncogenes and Micro-RNA

Systemic and local mediators are also likely to participate in heparanase gene regulation. The presence of functional estrogen response elements within the heparanase promoter suggests a systemic mechanism by which hormones control

heparanase transcription [70, 71]. Indeed, administration of estrogen markedly enhanced heparanase gene transcription in breast cancer cells, which was completely abolished by estrogen receptor antagonist [72]. Furthermore, a correlation between heparanase and estrogen receptor levels was confirmed by analyzing breast carcinoma tissue array [72], signifying its clinical relevance.

Normal epithelia exhibit relatively low levels of heparanase activity, suggesting that the heparanase promoter may possibly be transcriptionally repressed. This is due, at least in part, to binding of the p53 tumor suppressor and recruitment of histone deacetylases [73]. Mutational inactivation of p53 during cancer development leads to transcriptional activation of heparanase, providing a possible molecular mechanism for the frequent increase in heparanase levels observed in the course of tumorigenesis [73]. Likewise, heparanase expression was found to be under the regulation of oncogenes. For example, overexpression of mutant BRAF (V600E) and mutant Ras (G12 V) resulted in a marked increase in heparanase expression, accompanied by reduced HS on the cell surface [74]. Similarly, knockdown of BRAF expression in a BRAF-mutated KAT-10 tumor cell line led to the suppression of heparanase gene expression, subsequently leading to increased cell surface HS levels [74]. Analyses of the heparanase promoter revealed that the Ets relevant elements are critical for BRAF-induced heparanase expression, in a manner that involves GABPβ (but not Egr1) [74].

More recent studies reported the involvement of several micro-RNAs in heparanase gene regulation. For example, miR-1258 levels inversely correlated with heparanase expression in non-small cell lung cancer and breast cancer cells [75–77]. Functionally, heparanase downregulation by miR-1258 resulted in reduced cell invasiveness *in vitro*, and brain metastasis of breast cancer cells *in vivo* [75–77], emphasizing the relevance of miRNA-dependent heparanase regulation for cancer metastasis. In contrast, Mir-558 was noted to induce heparanase expression, and knockdown of endogenous miR-558 decreased the growth, invasion, metastasis, and angiogenesis of neuroblastoma cells *in vitro* and *in vivo* [78].

Collectively, the results suggest that heparanase expression is tightly regulated [20]; its induction in tumor cells is not mediated by one common cue but rather by complexed mechanisms that can operate in concert [65] but may vary among cells, type of tumor, and patients. Somewhat surprisingly, the detailed experimental results described above are not reflected in many array-type analyses that compare gene signatures in disease states and mainly cancer. We expected that given the common induction of heparanase in human tumors and consequently its bad prognosis (Fig. 9.1), heparanase will be found among the genes increased in tumors vs. normal tissue. This is not commonly observed. The reason is unclear, but may suggest that post-transcriptional mechanisms are more dominant than anticipated, or that mechanisms that were identified in cell lines do not sufficiently mimic tumors in patients. It is also possible that the heterogeneity of human tumors and sampling of a small area of the lesion for RNA extraction does not sufficiently reflect the tumor mass. Common inclusion of heparanase in patients' gene analyses would strengthen the significance of heparanase in cancer, and recruit more investigators to the field.

9.3 Heparanase Signaling-A Message from within

Heparanase up-regulation in primary human tumors correlated in some cases not only with tumor metastasis but also with tumors larger in size [26, 79–83]. The clinical findings have been recapitulated in many pre-clinical models in which overexpression of heparanase promotes tumor growth [20, 22–24, 84, 85]. Likewise, heparanase gene silencing or administration of heparanase inhibitors attenuated tumor growth [20, 22–24, 84, 85] (See chapters 19, 22, 23, 21 by Chhabra and Ferro; Hammond and Dredge; Giannini et al. and Noseda and Barbieri, This book for a detailed discussion on heparanase inhibitors). In addition, recent studies revealed that high levels of heparanase in the tumor metastases predict poor prognosis in stage IVc melanoma patients [39]. This result implies that heparanase not only enhances tumor cells dissemination but also promotes the growth and aggressiveness of the resulting metastases. Notably, larger tumors were produced also by cells engineered to overexpress heparanase mutants and forms (i.e., C-domain, splice variants) that lack heparanase enzymatic activity [45, 46, 86–89] (Fig. 9.2), clearly implying that heparanase function beyond its enzymatic aspect.

The mechanisms underlying the capacity of heparanase to promote tumor growth are not entirely clear. Conceptually, heparanase activity can potentially release a wide range of biological mediators that are sequestered by HSPG and turn on their activity. Among the proteins sequestered by the ECM are typical pro-angiogenic mediators such as PDGF, HGF, bFGF, HB-EGF, and VEGF-A [90, 91]. Indeed, heparanase exerts a strong pro-angiogenic response in pre-clinical models and clinical settings [20, 22, 84, 85, 92, 93]. Experimentally, Elkin et al. have demonstrated that heparanase can release ECM-bound ^{125}I-bFGF in a highly active form that promotes the proliferation of BaF3 cells [93]. Similarly, the addition of recombinant heparanase could release VEGF-A from cardiomyocytes, but this effect was also exerted by latent heparanase, suggesting displacement rather than cleavage of HS [94]. Similar considerations may also hold for the increase of soluble VEGF receptor 1 (sVEGF-R1) in heparanase-transgenic mice [95]. However, the release of ECM-bound angiogenic- and growth- promoting factors was not demonstrated unequivocally in the context of tumor growth, most probably due to the low levels of protein being released and its local nature. Instead, we and others have shown that heparanase induces the expression of VEGF-A [50, 96–98] and VEGF-C [99, 100], leading to increased blood and lymph vessel density. This implies that heparanase not only facilitates tumor cell invasion by loosening the ECM and basement membrane but also increases the density of vessels that mobilize the disseminating cells to distant organs. Subsequent studies revealed that heparanase down-regulates the expression of tumor suppressors [i.e., CXCL10; [45]] and induces the transcription of pro-angiogenic (i.e., COX-2, MMP-9), pro-thrombotic (i.e., tissue factor), pro-inflammatory (i.e., TNFα, IL-1, IL-6, MIP-2), pro-fibrotic (i.e., TGFβ), mitogenic (i.e., HGF), osteolytic (RANKL) and various other genes [22, 50, 99, 101–107], thus significantly expanding its functional repertoire and mode of action in promoting aggressive tumor behavior. The mode by which heparanase regulates gene transcription is

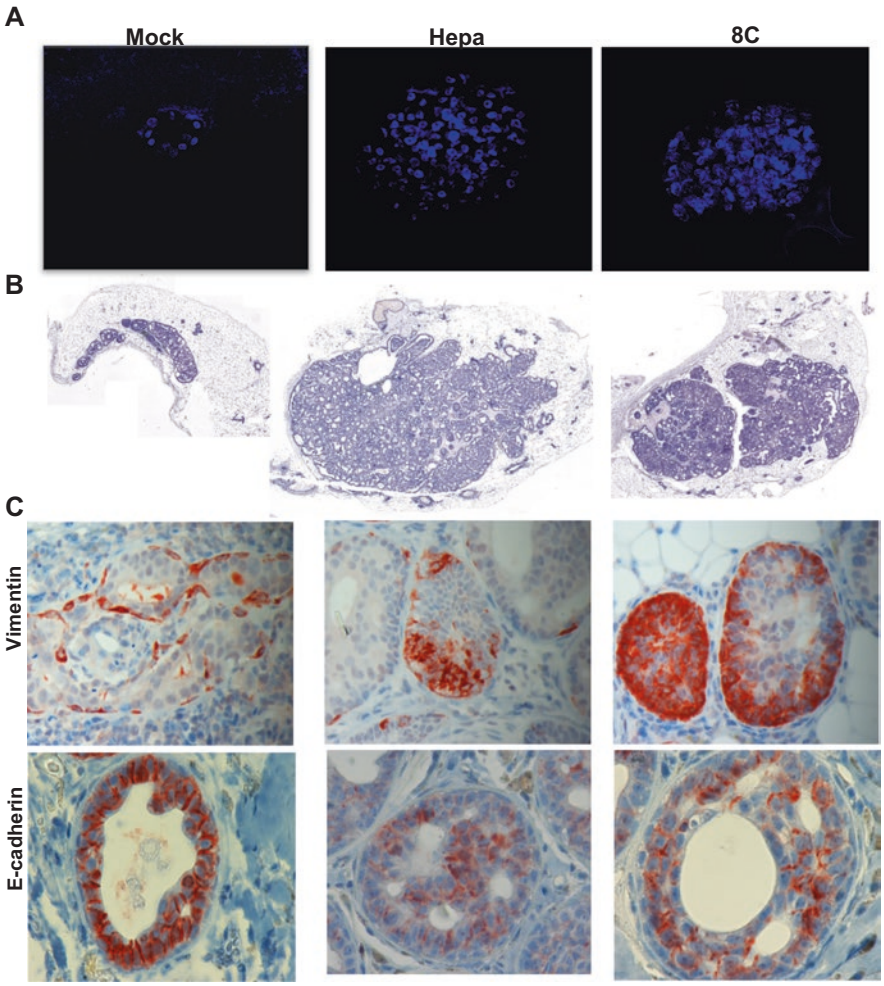

Fig. 9.2 Heparanase promotes the formation of disorganized acinar structures by MCF10A cells and tumorigenicity of MCF10AT1 cells. (**A**). Acinar structures formation. Control (Mock), heparanase (Hepa), and 8C-infected MCF10A cells were plated on, and overlaid with Matrigel for 10 days. Formation of three-dimensional acini-like structures was evaluated by fluorescent confocal imaging applying DAPI nuclei counterstaining. (**B**). Tumorigenicity of MCF10AT1 cells. MCF10AT1 cells were infected with control (Mock), heparanase, or 8C gene constructs and inoculated into SCID/beige mouse mammary fat pad (n = 6). Xenografts were harvested 12 weeks after cell transplantation and formalin-fixed, paraffin-embedded 5-micron sections were subjected to histological analyses. Shown are representative images of whole sections scanned by 3DHISTECH Pannoramic MIDI System attached to HITACHI HV-F22 color camera (3dhistech kft, Budapest, Hungary). (**C**). Immunostaining. Xenografts produced by MCF10AT1 infected cells were stained with anti-vimentin (upper panels) and anti-E-cadherin (second panels) antibodies. Original magnifications: x100

largely unclear but possibly results from its ability to stimulate signal transduction, best exemplified by Src-mediated VEGF-A induction by heparanase [96]. Stimulation of signaling (i.e., increased phosphorylation and activity of protein kinases intrinsic in signal transduction pathways) by heparanase can be indirect, due to the release of HS-bound growth factors. As discussed above, this possibility has not been convincingly proven in preclinical and clinical studies. Alternatively, heparanase was noted to enhance signaling in HS-dependent and -independent manners [89].

9.1.6 HS-Dependent Signaling

Heparanase interacts with syndecans by virtue of their HS content and the typical high affinity that exists between the enzyme and its substrate. This high-affinity interaction directs clustering of syndecans followed by a rapid and efficient uptake of heparanase [108] (see below). Mechanistically, syndecan clustering by heparanase or the KKDC peptide [corresponding to the heparin binding domain of heparanase [109]] enhanced cell spreading and was associated with PKC, Src, and Rac1 activation [110], molecular determinants shown to be induced by syndecans [111–114]. This mode of action likely represents a non-enzymatic signaling function of heparanase in its simplest term [89].

9.1.7 HS-Independent Signaling

Heparanase was noted nonetheless to elicit signaling also in a manner that does not involve HS. Signaling is considered to be HS-independent if it occurs in HS-deficient cells (i.e., CHO 745) or in the presence of heparin, as has been demonstrated for enhanced Akt phosphorylation by heparanase [115]. In fact, heparin, a potent inhibitor of heparanase enzymatic activity, when added together with heparanase, augmented Akt phosphorylation [115], critically implying that heparanase enzymatic activity is not required for Akt activation. In several cases, where tumor xenograft development was examined, heparanase over-expression resulted in tumors bigger in volume and weight [51, 70, 116, 117] coupled with increased Akt phosphorylation [51, 88, 116, 117]. Importantly, heparanase gene silencing was associated with reduced Akt phosphorylation levels [118], further substantiating a role for endogenous heparanase in Akt modulation. Moreover, Akt phosphorylation was markedly attenuated by heparanase inhibitors [46, 119, 120] (also see below). Subsequent studies revealed that heparanase stimulates the phosphorylation of STAT3 and STAT5, Src, EGFR, Erk and the insulin receptor, and moreover activates G-protein receptor signaling [121–124], all function to promote tumorigenesis. Importantly, enhanced EGFR phosphorylation by heparanase was restricted to selected tyrosine residues (i.e., 845, 1173) thought to be direct targets of Src rather than a result of

receptor auto-phosphorylation [125]. Indeed, enhanced EGFR phosphorylation on tyrosine residues 845 and 1173 by heparanase was abrogated in cells treated with Src inhibitors or anti-Src siRNA [121]. Notably, heparanase gene silencing was accompanied by a decrease in cell proliferation, while heparanase overexpression resulted in enhanced cell proliferation and formation of larger colonies in soft agar, in Src- and EGFR-dependent manner [121].

Fux et al. predicted the structure of enzymatically active, single chain, heparanase enzyme, in which the linker segment was replaced by three glycine-serine repeats (GS3), resulting in a constitutively active enzyme [126]. The structure clearly illustrates a TIM-barrel fold, in agreement with previous predictions [109, 127]. Notably, the structure also delineates a C-terminus fold positioned next to the TIM-barrel structure [88]. Fux et al. thus, hypothesized that the seemingly distinct protein domains observed in the three-dimensional model, namely the TIM-barrel and C-domain regions, mediate enzymatic and non-enzymatic functions of heparanase, respectively [88]. Interestingly, cells transfected with the TIM-barrel construct (amino acids 36–417) failed to display heparanase enzymatic activity, suggesting that the C-domain is required for the establishment of an active heparanase enzyme, possibly by stabilizing the TIM-barrel fold [88]. Deletion and site-directed mutagenesis approach further indicated that the C-domain plays a decisive role in heparanase enzymatic activity and secretion [88, 128, 129]. Remarkably, Akt phosphorylation was stimulated by cells overexpressing the C-domain (amino acids 413–543), while the TIM-barrel protein variant yielded no Akt activation compared with control, mock transfected cells [88]. These findings clearly indicate that the non-enzymatic signaling function of heparanase leading to activation of Akt is mediated by the C-domain. Because the C-domain gene construct lacks the 8 kDa segment which, according to the predicted model, contributes one beta strand to the C-domain structure, the resulting protein may exhibit suboptimal Akt activation. Indeed, Akt phosphorylation was markedly enhanced in cells transfected with a mini gene comprising a segment of the 8 kDa subunit, predicted by the model to contribute a beta strand (Gln^{36}-Ser^{55}) to the C-domain structure, linked to the C-domain sequence. These findings further support the predicted three-dimensional model, indicating that the C-domain is indeed a valid functional domain responsible for Akt phosphorylation (see Chapter 5 by Wu and Davies for a detailed discussion of heparanase crystal structure and new insights regarding structural relationships between the latent and active enzyme). The cellular consequences of C-domain overexpression are best revealed by monitoring tumor xenograft growth. Notably, tumor xenografts produced by C-domain-transfected glioma cells appeared comparable to those produced by cells transfected with the full-length heparanase, while the growth of tumors produced by TIM-barrel-transfected cells appeared comparable with control mock-transfected cells [88].

While signaling through HS clustering appears straightforward in its rational, HS-independent signaling by heparanase requires a mediator, possibly in the form of cell surface receptor(s). The existence of cell surface heparanase receptor is supported by binding experiments. Applying iodinated heparanase to HeLa cells revealed the presence of two distinct types of binding sites exhibiting low-affinity

(Kd = 3 mM), high abundant (βmax = $1x10^8$), and high affinity (Kd = 2 nM), low abundant (βmax = $1.7x10^4$) characteristics [130]. Binding studies performed with wild type CHO-KI cells and their HS-deficient CHO-745 counterpart cells have demonstrated that heparanase binding to the high-affinity binding sites is almost identical in both cell types. In contrast, the number of low-affinity binding sites was significantly reduced in CHO-745 vs. CHO-KI cells, and a similar decrease was noted in CHO-KI cells treated with bacterial heparinase III [130]. These studies reinforce the notion that while HSPG serve as low affinity, high abundant binding sites, heparanase also associates with high affinity, low abundant cell surface receptor(s). A first indication for the protein nature of this receptor and its molecular weight emerged from cross-linking experiments, revealing two distinct complexes representing 130 and 170 kDa proteins associated with heparanase [88]. Moreover, Akt phosphorylation by heparanase was found to be mediated by a lipid raft resident protein [118]. Such a receptor has not been isolated and characterized yet. Also, Wood and Hulett have reported that the 300 kDa Cation-independent Mannose 6-Phosphate Receptor (CIMPR; CD222) can bind enzymatically active heparanase [131] and may serve as a heparanase receptor. The affinity of this interaction and the consequences of heparanase binding in term of signaling has not been reported. Alternatively, heparanase may facilitate signaling from within the lysosome.

9.4 Heparanase Uptake – Is the Message within Lysosomes?

A number of studies have shown that secreted or exogenously added latent heparanase rapidly interacts with normal and tumor-derived cells, followed by internalization and processing into a highly active enzyme [108, 115, 130, 132–136], collectively defined as heparanase uptake. Several approaches, including HS-deficient cells, addition of heparin or xylosides, and deletion of HS-binding domains of heparanase, provided compelling evidence for the involvement of HS in heparanase uptake [109, 132]. While syndecans are regarded as the primary receptors for heparanase endocytosis, low-density lipoprotein receptor-related protein (LRP) and the cation-independent mannose-6-phosphate receptor (CIMPR) have been identified as heparanase-binding proteins [131, 136] that contribute to heparanase uptake. Heparanase uptake is regarded as a pre-requisite for the delivery of latent 65 kDa heparanase to lysosomes and its subsequent proteolytic processing and activation into 8 and 50 kDa that compose the active enzyme. Following uptake, heparanase was noted to reside primarily intracellularly within endocytic vesicles, assuming a polar, peri-nuclear localization and co-localizing with lysosomal markers [133, 137] (Fig. 9.3A). Indeed, heparanase processing was blocked by chloroquine and bafilomycin A1 which inhibit lysosomal proteases by raising the lysosome pH [108]. Subsequent studies employing lysosomal preparation, site-directed mutagenesis, gene silencing, and pharmacological inhibitors have identified cathepsin L as the primary lysosomal protease responsible for heparanase processing and activation [138–140]. Moreover, syndecan-1 and 4 are internalized by cells following

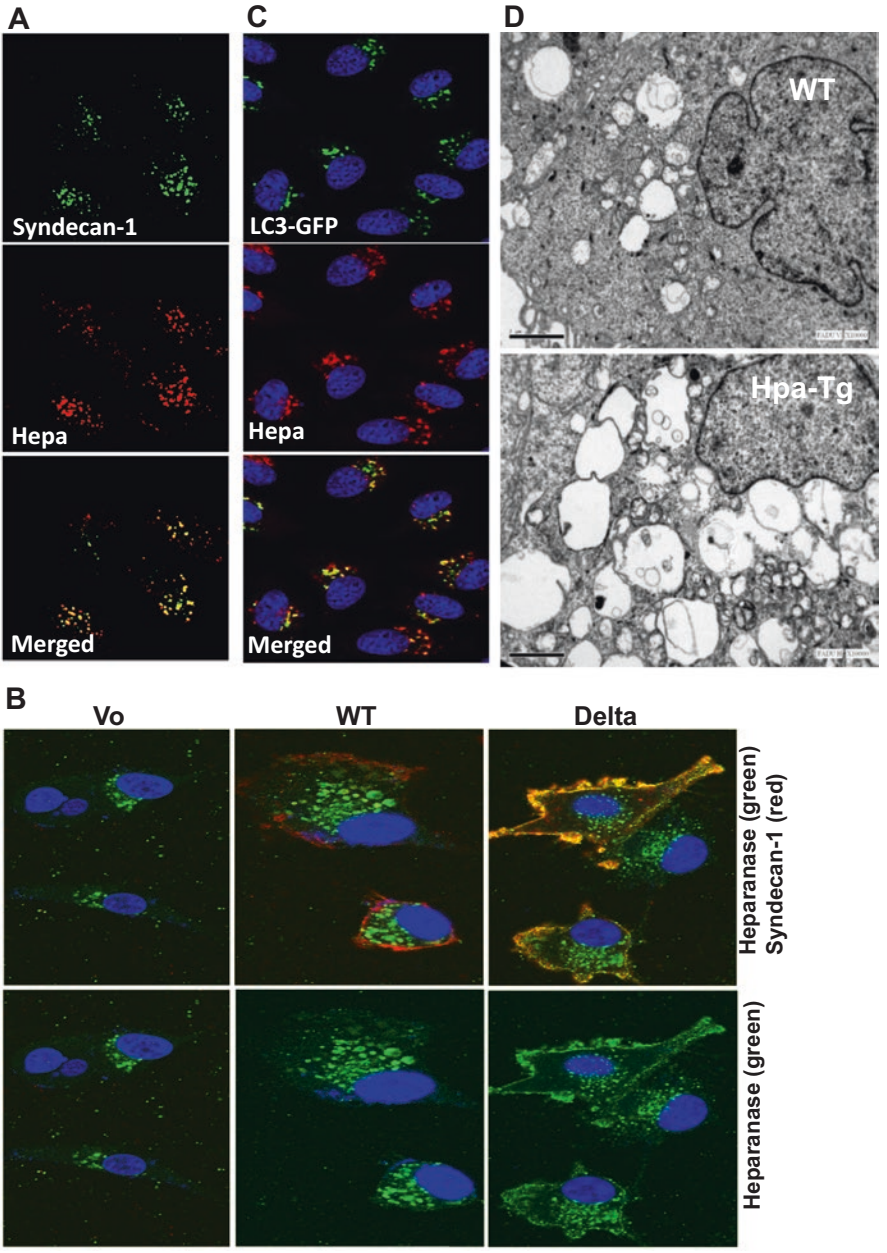

Fig. 9.3 (**A**). Altered syndecan localization in response to heparanase addition. U87 glioma cells were incubated with Myc-tagged latent heparanase (10 μg/ml) for 15 min. Cells were then fixed and stained with anti-syndecan-1 (upper panel, green) monoclonal antibody and with anti-Myc polyclonal antibody (Hepa, second panel, red). Merge images are shown in the lower panel. Note internalization of syndecan into endocytic vesicles upon heparanase addition. (**B**). Heparanase uptake requires the syndecan cytoplasmic tail. Heparanase (1 μg/ml) was added to U87 glioma cells

addition of heparanase, co-localizing with heparanase in endocytic vesicles [132, 141] (Fig. 9.3A). Since syndecans mediate the uptake of a large number of molecules including atherogenic lipoproteins [142, 143] and microorganisms such as bacteria and viruses [144], mechanisms that mediate internalization of syndecan ligands are of interest and clinical significance. Structurally, all syndecans are composed of an extracellular domain, membrane domain, and a conserved short C-terminal cytoplasmic domain divided into the first conserved region (C1), the variable domain (V), and the second conserved region (C2). Each of these cytoplasmic domains has been shown to interact with specific adaptor molecules and to mediate cellular functions [113, 145]. To examine the role of syndecan-1 cytoplasmic domain in heparanase processing, Shteingauz et al. transfected cells with full-length mouse syndecan-1 or deletion constructs lacking the entire cytoplasmic domain (delta), the conserved (C1 or C2) or variable (V) regions [146]. Heparanase uptake was markedly increased following syndecan-1 over expression (Fig. 9.3B, WT), thus challenging the notion that cell surface HS is at saturation and does not limit ligand binding. In contrast, heparanase was retained at the cell membrane, and its processing was impaired in cells overexpressing syndecan-1 deleted for the entire cytoplasmic tail [146] (Fig. 9.3B, Delta). Subsequent studies revealed that the C2 and V regions of syndecan-1 cytoplasmic tail mediate heparanase processing. Furthermore, syntenin, known to interact with syndecan C2 domain, and α actinin were shown to be essential for heparanase processing [146]. These results illustrate the tight regulation of heparanase activation and shed light on syndecan-mediated endocytosis. Interestingly, syndecans and syntenin, via interaction with ALIX, have been implicated in regulating the biogenesis of exosomes [147]. Importantly, heparanase facilitates the production of exosomes and regulates their secretion and composition [148, 149], implying that heparanase-syndecan-syntenin establish a linear axis that regulates exosome formation and the related effects on tumor progression [146] (see Chapters 12, 10 by Sanderson et al., and David and Zimmermann for detailed discussion on heparanase and exosomes).

Fig. 9.3 (continued) over-expressing wt syndecan-1 or syndecan-1 lacking the entire cytoplasmic tail (delta) for 1 hour at 37 °C. Cells were then fixed with cold methanol and subjected to immunofluorescent staining applying anti-heparanase mouse monoclonal antibody (lower panels, green). Merged images with rat anti-syndecan-1 staining (red) are shown in the upper panels. Note increased heparanase-positive endocytic vesicles in cells overexpressing wild type (WT) syndecan-1, but retention of heparanase at the cell membrane, co-localizing with syndecan lacking the entire cytoplasmic tail (Delta). (**C**). Heparanase co-localizes with LC3-II. Heparanase (1 μg/ml) was added exogenously to HeLa cells stably expressing a GFP-LC3 gene construct for 24 hours. Cells were then deprived of amino acids in the presence of chloroquine (50 μg/ml; AA+Chl) for 3 hours or were incubated under serum-free conditions as control (Con). Cells were then fixed with methanol and subjected to immunofluorescent staining applying anti-heparanase (middle panels, red) antibody. Co-localization of heparanase and GFP-LC3 appears yellow (lower panel). (**D**). Electron microscopy. Pancreas tissues from control (Con) and heparanase transgenic mice (Hpa-Tg) were fixed in glutaraldehyde and processed for EM. Shown are representative images at x10,000 magnification. Note a substantial increase in the number and size of autophagosomes in the pancreas of heparanase-transgenic mice

The efficient uptake mechanism and accumulation of heparanase in endocytic vesicles suggest that heparanase is not normally present in association with the ECM, the site of its recognized activity. Active heparanase can get to the cell exterior by one of three mechanisms: 1. Secretion of endocytic vesicles/lysosomes; 2. Processing of latent heparanase into active enzyme outside the cell, and 3. Release by lysosomes/micro-vesicles. The latter, however, is thought to mediate communication between cells and transfer the membrane-enclosed protein and/or its mRNA from one cell to another rather than to release the enzyme. The former mechanisms have been substantiated experimentally [150–152], but the secreted enzyme will be subjected to the same principles of uptake (or re-uptake) described above [20]. The rapid and efficient uptake mechanism of heparanase and its accumulation in lysosomes likely serve as an important regulatory mechanism that limit its extracellular retention, due to the damage that this activity may cause to tissues and cells. The instrumental role of the lysosome in signaling raises the possibility that heparanase accumulation in this organelle not only serves as a reservoir for the enzyme but rather plays an important role in its function.

9.1.8 The Lysosome as a Signaling Organelle

For the past five decades, the lysosome has been characterized as an unglamorous cellular recycling center. This notion has undergone a radical shift in the last 10 years, with new research revealing that this organelle serves as a major hub for metabolic signaling pathways. The discovery that master growth regulators, including the protein kinase mTOR (mechanistic target of rapamycin), make their home at the lysosomal surface has generated intense interest in the lysosome's key role in nutrient sensing and cellular homeostasis [153–156]. The transcriptional networks required for lysosomal maintenance and function are a subject of intense research activity, and their connection to lysosome-based signaling pathways was revealed. Much is now understood about how the lysosome contributes to amino acid sensing by mTORC1, the function of the energy-sensing kinase, AMP-activated protein kinase (AMPK), at the lysosome and how both AMPK and mTORC1 signaling pathways feedback to lysosomal biogenesis and regeneration following autophagy [153–156]. In fact, the lysosome is intimately involved in each of the classic hallmarks of cancer [154], and compounds that impact lysosomal function are under clinical evaluation [154].

In spite of its localization in a highly active protein degradation environment such as the lysosome, heparanase appears stable [108, 137] and exhibits a half-life of about 30 hours [132], relatively long compared with a $t_{1/2}$ of 2–6 h, and 25 min for transmembrane and GPI-anchored HSPG, respectively [157]. Residence and accumulation of heparanase in lysosomes indicate that the enzyme may function in the normal physiology of this organelle. In a search for such function, we revealed a role of heparanase in modulating autophagy [48]. Autophagy is an evolutionarily conserved catabolic pathway through which cytoplasmic components, including macromolecules such as proteins and lipids as well as whole organelles, are sequestered into double-membrane vesicles called autophagosomes. Autophagosomes are subsequently fused with

lysosomes, where the intracellular material is degraded and recycled. This process occurs in every cell at a basal level and is required to remove unfolded proteins and damaged organelles, thus maintaining cellular homeostasis. Autophagy is further induced by starvation and stress, promoting cancer cells survival by providing their metabolic needs [158, 159]. Our results indicate that heparanase is localized within autophagosomes (Fig. 9.3C) and promotes autophagy. Moreover, enhanced tumor growth and chemo-resistance exerted by heparanase are mediated in part by augmenting autophagy [48]. This was concluded because reduced LC3-II (a protein that specifically associates with autophagosomes) levels are found in cells and tissues obtained from heparanase knockout mice as opposed to elevated LC3-II levels found in transgenic mice that overexpress heparanase. Even higher induction of autophagy was evident in head and neck carcinoma and glioma cells overexpressing heparanase [48], in accordance with a strong pre-clinical and clinical significance of heparanase in the progression of these malignancies [36, 51, 81, 88, 99, 103, 121, 122]. Notably, electron microscopy analyses of cells overexpressing heparanase revealed not only a higher number of autophagic vacuoles (Fig. 9.3D), but also abundant release of vesicles, likely exosomes, from the cell surface [48], further supporting the notion that heparanase enhances exosome secretion that contributes to tumor growth [148, 149]. These results imply that heparanase function is not limited to the extracellular milieu but can function inside the cell [48, 160].

The mechanism underlying autophagy induction by heparanase is not entirely clear, but likely involves mTOR1 that plays a pivotal role in nutrient-sensing and autophagy regulation [161]. mTOR1 activity inhibits autophagy, but under starvation, its activity is repressed, leading to autophagy induction. Shteingauz et al. found that heparanase overexpression associates with reduced mTOR1 activity, evident by decreased levels of p70 S6-kinase phosphorylation, an mTOR1 substrate. In contrast, heparanase-knockout cells exhibited increased mTOR1 activity and p70 S6-kinase phosphorylation [48]. Notably, mTOR1 appears more diffusely scattered in control cells, whereas in cells with a high content of heparanase, mTOR1 is found mostly in peri-nuclear regions, co-localizing with heparanase and LysoTracker that labels acidic lysosomal vesicles. This agrees with the notion that activation of mTOR1 by nutrients is associated with peripheral lysosomes, whereas starvation leads to peri-nuclear clustering of lysosomes and decreased mTOR1 activity [162]. These results imply that autophagy induction contributes to the pro-tumorigenic function of heparanase. This emerges from *in vitro* and *in vivo* experiments utilizing inhibitors of autophagy (chloroquine) and heparanase (PG545) alone or in combination [48]. Thus, combining PG545 and chloroquine in a tumor xenograft model resulted in significantly smaller and more differentiated tumors, suggesting that heparanase activity drives cancer cell de-differentiation as part of its pro-tumorigenic properties. Equally important is the ability of heparanase overexpression to confer resistance to stress, chemotherapy and targeted drugs [47], mediated, at least in part, by enhancing autophagy [48]. Indeed, diverse classes of anticancer drugs induce autophagy [163, 164], thus attenuating tumor cell elimination, while autophagy inhibitors overcome chemo-resistance [165, 166]. Based on this concept, chloroquine is currently evaluated in several clinical trials in combination with different classes of chemotherapeutic agents [165, 167].

Modulation of mTOR activity and autophagy by heparanase likely represents only the tip of the iceberg; lysosomal heparanase possibly modulate many other aspects of lysosome function in health and disease, but this notion awaits in-depth validation. Moreover, it is possible that lysosomal heparanase needs to be targeted in order to attenuate tumor growth.

9.5 Heparanase Inhibitors – Are We Targeting Well?

The search for heparanase inhibitors started soon after the appreciation of its pro-metastatic properties [168, 169]. Since then, many heparanase inhibitors have been developed (Discussed in detail by Chhabra & Ferro; Hammond & Dredge; Giannini et al. and Noseda and Barbieri, Chaps. 19, 21, 22 and 23; in this volume). These include, among others, heparin/HS mimetics (i.e., SST0001 = Roneparstat, M402 = Necuparanib, PI-88 = Mupafostat), synthetic, fully sulfated HS mimetic (PG545 = Pixatimod), neutralizing antibodies, small molecules, oligonucleotides (i.e., defibrotide), natural products and their derivatives, and many others [23, 85, 120, 170–173]. Of these, Mupafostat, Roneparstat, Necuparanib, and Pixatimod were examined clinically [16–19]. These compounds as well as the anti-heparanase neutralizing monoclonal antibodies that show some potency in pre-clinical models [49], do not penetrate the cell and their inhibition potential is restricted to the cell exterior. This likely explains the low toxicity of these compounds [16, 17]. Given the above considerations and the seemingly low abundance of heparanase outside the cells, the eminent question would be what exactly are these inhibitors targeting? A possible explanation comes from the observation that the heparin/HS mimetics as well the neutralizing antibodies not only inhibit heparanase activity but also attenuate its uptake, resulting in accumulation of heparanase in the cell culture medium, accompanied with reduced lysosomal content [[49, 50] and our unpublished results]. This may suggest that attenuation of tumor growth results in whole or in part from reduced intracellular content of heparanase. Thus, the ideal inhibitor should target heparanase both inside and outside the cell, attenuating HS cleavage and signaling, in order to neutralize its diverse functions and bring heparanase inhibitors closer to the clinic.

9.6 Is Hpa2 the Answer?

Cloning of a single human heparanase cDNA sequence independently reported by several groups [12–15] implied that one active heparanase enzyme exists in mammals. Further analysis of human genomic DNA led researchers to conclude that the heparanase gene is unique and that the existence of related proteins is unlikely [174]. Based on amino acid sequence, McKenzie and colleagues nonetheless reported the cloning of heparanase homolog termed heparanase 2 (Hpa2) [174]. The full-length *HPSE2* gene consists of 2353 bp encoding a protein of 592 amino acids; Alignment

of the coding region of heparanase and Hpa2 reveals an overall identity of 40% and sequence resemblance of 59%, including conservation of residues critical for heparanase enzymatic activity (Glu_{225} and Glu_{343}) [174]. The segment corresponding to the linker region and cleavage sites of pro-heparanase are not conserved in Hpa2 [20]. Importantly, Hpa2 lacks intrinsic HS-degrading activity, the hallmark of heparanase [141], and seems not to undergo processing in a manner required for heparanase activation. This may be due to differences in the cellular localization of Hpa2 and its sequestration from the lysosome. Wild type Hpa2 (Hpa2c) [141, 174] is secreted and markedly accumulates in the cell conditioned medium following the addition of heparin or HS but not hyaluronic acid, indicating that Hpa2 retains the capacity to interact with HS despite the lack of HS-degrading activity [141]. In fact, Hpa2 exhibits even higher affinity towards heparin and HS than heparanase [141], thus competing for HS binding and thereby inhibiting heparanase enzymatic activity [141]. Moreover, co-immunoprecipitation studies revealed physical association between Hpa2 and heparanase proteins [141], providing an additional route by which Hpa2 can inhibit heparanase enzymatic activity. Immunofluorescent staining illustrates Hpa2 localization on the cell surface following its exogenous addition, co-localizing with and clustering of syndecan-1 and -4 (Fig. 9.5, left image). Unlike heparanase, Hpa2 does not appear to get internalized into endocytic vesicles but rather remains on the cell surface for a relatively long period of time [141]. This result clearly indicates that the rapid and efficient internalization of heparanase together with syndecans (Fig. 9.3A) [108, 109, 175] is unique and not purely a consequence of HS-ligand binding. Moreover, the lack of Hpa2 processing may be due to its sequestration from the lysosome and lysosomal enzymes. Thus, while Hpa2 can inhibit heparanase activity extracellularly, it cannot affect lysosomal heparanase directly. However, Hpa2 attenuate heparanase uptake, possibly due to its high affinity to HS, resulting in depletion of lysosomal heparanase [141]. In this regard, Hpa2 function in a manner similar to HS-mimetic heparanase inhibitors [48]. In addition to the full-length Hpa2 protein (Hpa2c), several variants have been identified as a result of alternative splicing of the *HPSE2* transcript, including Hpa2a (480 aa) and Hpa2b (534 aa) [174]; Another splice variant of Hpa2, composed of only 528 amino acids, was described by Vreys and David [20]. Notably, only wild type Hpa2 is secreted, likely due to extra glycosylation sites that are lost in the splice variants [141]. The biological significance and cellular localization of Hpa2 splice variants are yet to be revealed. Localization of Hpa2 splice variants to the lysosome will imply that whereas wild type Hpa2 can modulate heparanase activity in the cell exterior, its splice variants will modulate heparanase activity inside cells.

9.1.9 Hpa2 in Cancer Progression-an Opposite Answer

Very little attention was given to Hpa2 in general, and only a few studies attempted to reveal its significance in cancer (see Chapters by Roberts and Woolf, and by Mckenzie, Chaps. 34 and 35; in this volume). However, the emerging results clearly

suggest that Hpa2 function in cancer is the exact opposite of heparanase. The notion that Hpa2 function as a tumor suppressor is supported by the following observations. Unlike heparanase, Hpa2 staining is evident in the normal epithelium of the bladder, breast, gastric and ovarian tissues. Notably, Hpa2 levels are reduced substantially in the resulting carcinomas (Fig. 9.4), a staining pattern typical of a tumor suppressor. In other cases, such as head and neck cancer, the opposite is observed [141]. The reason for this behavior is unclear. Importantly, nonetheless, head and neck cancer patients exhibiting high levels of Hpa2 showed prolonged time to disease recurrence (follow-up to failure) and inversely correlated with tumor

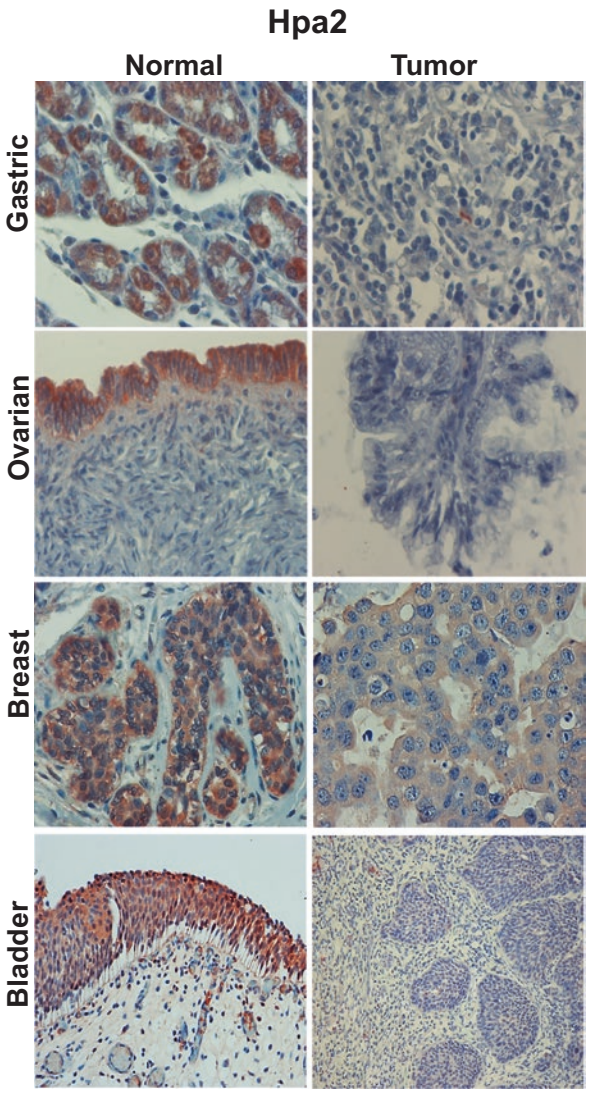

Fig. 9.4 Hpa2 immunostaining appears strong in normal epithelium and is decreased substantially in the resulting carcinomas. Biopsies of gastric (upper panels), ovarian (second panels), breast (third panels), and bladder (lower panels) carcinomas were subjected to immunostaining applying anti-Hpa2 polyclonal antibody. Strong Hpa2 staining is detected in normal epithelium of the gastric, ovarian, breast and bladder tissues adjacent the tumor lesion (left panels) which is decreased substantially in the carcinomas (right panels)

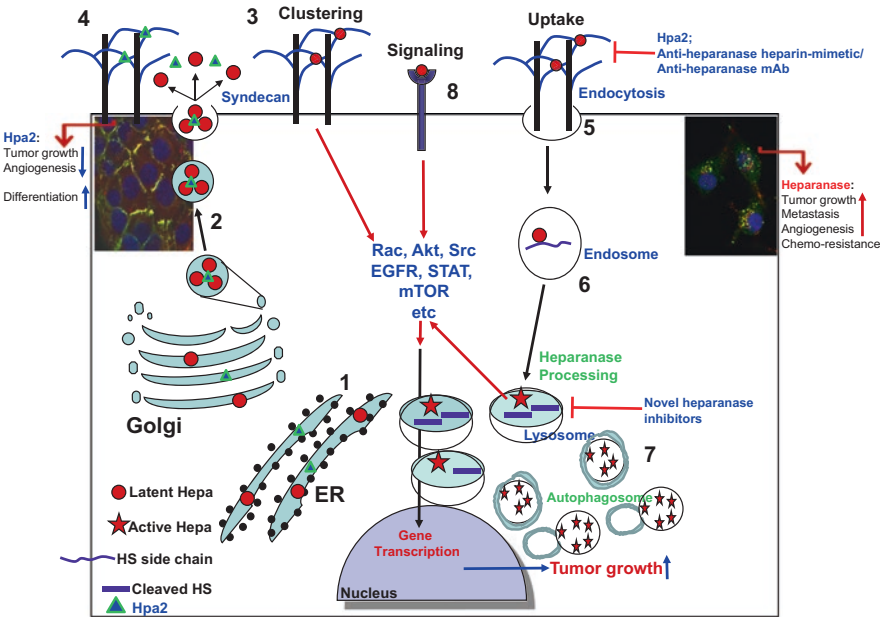

Fig. 9.5 Schematic presentation of heparanase and Hpa2 biosynthesis and trafficking. Pre-pro-heparanase (red circles) and Hpa2 (green triangles) are first targeted to the ER lumen via their own signal peptides (**1**). The proteins are then shuttled to the Golgi apparatus and are subsequently secreted via vesicles that bud from the Golgi (**2**). Once secreted, heparanase rapidly interacts with syndecans, resulting in their clustering and signaling (**3**), followed by rapid endocytosis of the heparanase-syndecan complex (**5**) that accumulates in late endosomes (**6**). Hpa2 interacts with cell membrane HSPG (i.e., syndecans) with higher affinity but unlike heparanase, is not subjected to uptake but rather remains on the cell membrane for a relatively long period of time (**4 & left inset**). Accumulation of Hpa2 in the extracellular compartment is enhanced by heparin or anti-Hpa2 (1c7) monoclonal antibody. Heparanase uptake is inhibited by heparin/heparin mimetics, anti-heparanase monoclonal antibodies, or Hpa2, resulting in extracellular accumulation of the latent enzyme (⊣, **5**). Conversion of endosomes to lysosomes results in heparanase processing and activation (primarily by cathepsin L). Typically, heparanase appears in perinuclear lysosomes (right inset) and is thought to promote autophagy (**7**) and tumor growth, metastasis, angiogenesis, and chemo-resistance due to its enzymatic and signaling (**8**) functions whereas Hpa2 appear to attenuate tumor growth and vascularity. Novel heparanase inhibitors are hoped to target extracellular latent (signaling) and active heparanase as well as the intracellular, lysosomal, enzyme

cell dissemination to regional lymph nodes [141], thus providing clinical relevance for the antitumor properties of Hpa2. Notably, overexpression of Hpa2 in head and neck cancer cells resulted in a marked reduction in tumor growth, associating with a prominent reduction in tumor vascularity (blood and lymph vessels) likely due to reduced Id1 expression [176], a transcription factor highly implicated in VEGF-A and VEGF-C gene regulation [177]. Moreover, growth of tumor xenografts produced by Hpa2 over-expressing cells was not affected by a monoclonal antibody that targets a heparin binding domain of Hpa2 [176], implying that Hpa2 functions

in HS-independent manner. Tumor produced by cells over-expressing Hpa2 were not only smaller but also exhibited a higher degree of cell differentiation (i.e., cytokeratin expression) [176]. Likewise, high levels of Hpa2 in bladder cancer patients correlated inversely with tumor grade and stage [178], further strengthening the significance of Hpa2 as a tumor suppressor and its role in cell differentiation. Thus, heparanase and Hpa2 not only exhibit opposite function in term of tumor growth but also in term of the underlying mechanism. For example, while heparanase induces VEGF-A and VEGF-C expression and promote angiogenesis, Hpa2 attenuate the expression of VEGF-A and VEGF-C and decrease tumor vascularity; whereas heparanase reduce cell differentiation and promote epithelial to mesenchymal transition (EMT) (Fig. 9.2; [179]), Hpa2 increase cell differentiation [176, 178]. This mirrored functionality strongly suggests that Hpa2 exert these properties by modulating heparanase, but we could not demonstrate decreased heparanase activity in cells over-expressing Hpa2 [176], possibly due to the semi-quantitative assay being employed. Given the above considerations, however, it is possible that the main function of Hpa2 is not to inhibit heparanase activity extracellularly, but rather to deplete heparanase from the lysosome.

9.7 Heparanase Message Revisited

Twenty years after cloning the HPSE gene, heparanase research has made substantial progress, clearly revealing the clinical significance of the enzyme and turning heparanase into a valid target for the development of anti-cancer therapeutics. Progress was also made in deciphering the role of heparanase in inflammation, viral infection, diabetes, and other pathologies (see chapters by Elkin et al., Simeonovic et al., Masola et al., and Agelidis and Shukla, Chaps. 17, 24, 27 and 32; in this volume). Disclosing its mode of action and the translation of the knowledge into clinical practice is nonetheless insufficient. In this Review, we challenge some of the concepts that guided the field, hoping that new ideas and thinking will advance basic and translational aspects of heparanase.

Based on the house-keeping nature of its gene promoter, we suggest that heparanase is expressed at low levels by all cells, modulating autophagy and possibly other functions of the lysosome. According to this notion, heparanase function in the lysosome is equally, or possibly more important than its function extracellularly. This may turn most relevant in platelets, neutrophils, lymphocytes and macrophages that show relatively high levels of heparanase expression/activity [13, 50, 60], and also in normal epithelium [180]. Beyond serving as a cellular recycling center, recent evidence suggests that the lysosome is involved in homeostasis, generating building blocks for cell growth, mitogenic signaling, angiogenesis and metastasis, and activation of transcriptional programs [154, 156], repertoire that closely resembles those of heparanase. The PI3-kinase/Akt/mTOR is highly implicated in the regulation of cell metabolism, protein homeostasis, and cell growth due, in part, to the localization of mTOR at the lysosome membrane which is required for its

activation [153, 181]. In fact, Akt is the most common kinase activated by heparanase [46, 51, 88, 101, 115–119, 121, 122, 182–185], and its instrumental role in the regulation of mTOR would likely convey to the lysosome [181]. Clearly, more work is required to critically resolve the significance of heparanase in modulating lysosomal function in normal cells and in tumor growth, metastasis, and chemo-resistance.

As already described above, heparanase inhibitors were solely directed to neutralize its enzymatic activity and most often yielded disappointing results in preclinical models, with the exception of PG545 (Pixatimod). The specificity of this compound is, nonetheless, questionable, because it also exerts heparanase-independent functions and attenuates the growth of tumor xenografts produced by heparanase-negative lymphoma cells [186]. A new generation of heparanase inhibitors, possibly in the form of small molecules, should also target its signaling activity at the cell membrane and inside the lysosome in order to better neutralize all aspects of heparanase function. This will lead to better appreciation of heparanase role in health and disease and, hopefully, will enable improved clinical application of these compounds in cancer, inflammation, and other pathologies.

Conflict of Interest The authors have no potential conflict of interest to declare.

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Chapter 10
Heparanase Involvement in Exosome Formation

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Abbreviations

ALIX	ALG-2-interacting protein X
CD	cluster of differentiation
CTF	C-terminal fragment
ESCRT	endosomal-sorting complex required for transport
EV	extracellular vesicle
ILV	intraluminal vesicle
MVB	multivesicular body
PDZ	postsynaptic density 95/disc-large/zona occludens
SDC	syndecan

10.1 Important Messages, Inserted into an Envelope

Exosomes are small vesicles of endosomal origin, composing part of the complex collection of extracellular vesicles (EVs) that cells secrete. They contain various membrane and cytoplasmic components (i.e. membrane lipids and receptors, small GTPases, mRNAs, and ncRNAs, etc) commonly designated as cargo, with a composition that reflects the state of the cell of origin [87]. Pending on the nature of that cargo, the presentation of these vesicles to 'recipient' cells can sometimes

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'reprogram' the latter [97]. Exosomes are therefore thought to play an important role in intercellular communication [13, 86]. This notion stems mostly from the field of cancer cell biology. For example, tumor-derived exosomes stimulate the formation of a pre-metastatic niche [67], and exosomal communication between cancer-associated fibroblasts and the primary tumor stimulates breast cancer cell motility and metastasis [54]. Yet, the potential impact of exosomes extends far beyond cancer, including maintaining the stemness of progenitor cells and their participation in processes of tissue regeneration, inflammation and neurodegeneration ([21, 39, 71, 80, 82, 93]). Several recent contributions provide an in-depth review on the biology of EVs [95, 98].

10.2 The Making of An Exosome

The mechanisms that control the biogenesis of exosomes and the sorting of specific cargo into these vesicles are only partially understood. Exosome biogenesis begins with the invagination of the plasma membrane leading to the formation of primary endocytic vesicles, and the fusion of these vesicles with each other to create the early endosomal compartment. While early endosomes mature into late endosomes, exchanging RAB5 for RAB7 and retaining a select subset of the endocytosed cargo they contain, a second invagination of the endosomal membrane occurs (but this time away from the cytosol) leading to the formation of so-called intraluminal vesicles (ILVs). Late endosomes that contain multiple (up to 30) ILVs are designated as multivesicular endosomes/bodies (MVE/MVBs). When MVBs fuse with lysosomes, their cargo, including their ILVs, is degraded. However, late endosomes and MVBs can also fuse with the plasma membrane, releasing their ILVs into the extracellular space as exosomes [47, 73] (Fig. 10.1).

Several cellular components that participate in the control of intraluminal budding have been identified. Upon recruitment from the cytosol, the endosomal-sorting complex required for transport (ESCRT) machinery sorts internalized membrane proteins into specific membrane domains, induces the 'inward' budding of these domains (away from the cytosol, into the lumen of the endosome), and mediates membrane abscission to form ILVs [38, 42, 107]. The ESCRT machinery is composed of 4 multi-protein sub-complexes, of which ESCRT-0, -I and -II recognize and sequester membrane cargo at the endosomal delimiting membrane, while ESCRT-III drives membrane budding and actual scission of intraluminal vesicles. It is important to note that originally the ESCRT machinery was found to drive the sorting of ubiquitin-conjugated membrane proteins into vesicles that bud into the lumen of a distinct set of MVEs that ultimately fuse with lysosomes rather than with the plasma membrane, resulting in the degradation of their vesicular contents [70]. Sorting of proteins into exosomes, however, appears to occur independently of cargo ubiquitination [2, 7], and only a selected number of ESCRT components appear involved in exosome formation [4, 12]. It is also important realizing that the mechanisms of cargo-sorting and membrane-budding and abscission are intimately

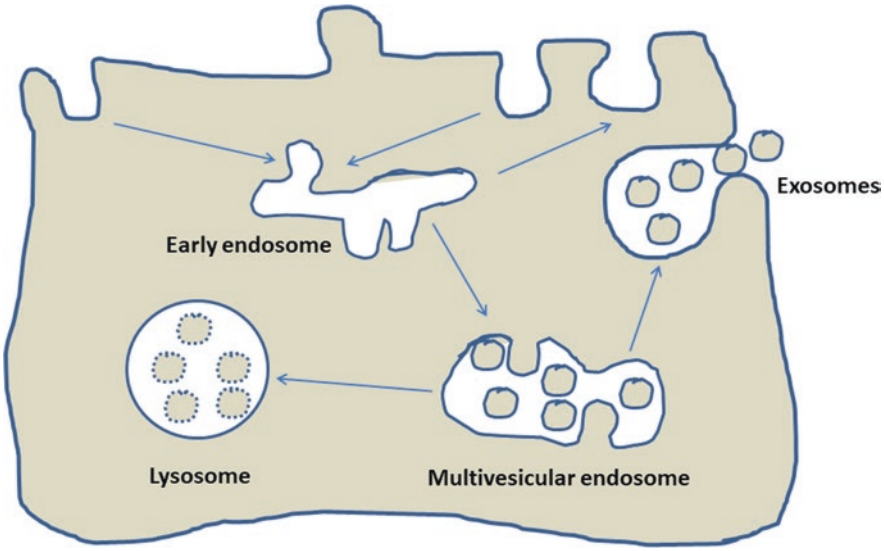

Fig. 10.1 Exosome biogenesis. Intraluminal budding of the limiting membrane of endosomes creates intraluminal vesicles (ILVs). Endosomes that contain ILVs can fuse with lysosomes, but can also fuse with the plasma membrane, releasing their ILVs into the extracellular space as exosomes. Note that, as they result from two consecutive membrane bending events of opposite polarity (the first towards, the second away from the cytosol), ILVs and exosomes have the same surface topology as whole cells (cytosol/inside 'in', cell surface/outside 'out')

intertwined. Clearly, also lipids have important and pleiotropic effects. That includes phosphoinositides and in particular a role of these lipid signaling-intermediates (and thus of signaling processes) in recruiting ESCRT components with sorting functions to endosomal membranes [69]. Other studies have pointed to ceramide [96] and other lipids (i.e. lysobisphosphatidic acid and phosphatidic acid) as mediators of ILV and exosome biogenesis ([30, 52, 59, 18]. Possibly these lipids are driving the lateral segregation of cargo into specialized endosomal membrane regions able to bend inwards. A more extensive and in-depth review of what is known about the mechanisms involved in the biogenesis of these particular membrane domains and organelles can be found in several recent reviews [37, 40, 58, 83, 111].

10.3 The Reception of an Exosome

Once released into the extracellular space, exosomes can reach recipient cells and present or deliver their contents to elicit functional responses. Exosome-mediated intercellular communication requires docking of the vesicles at the plasma membrane, followed by the activation of surface receptors and signaling, or their fusion with target cells, at the cell surface or in endosomal compartments, following internalization [22]. For example, exosomes may remain bound to the cell surface via

integrins and from there activate intracellular signaling pathways, initiating cell migration [68, 90]. Exosomes may also be internalized, by multiple pathways: i.e. receptor-mediated endocytosis, phagocytosis, and macropinocytosis [20, 62, 64]. In general, the ways exosomes interact with cell surfaces and vesicular cargo is transferred to other cells remain poorly understood. These processes are no doubt complex and likely depend on the cargo of the exosomes, and thus the compositions of the exosome-donor cells, and on the identity of the recipient cells, with their particular repertoire of receptors and endo/phagocytic properties. The fate of endocytosed exosomes might be quite different in professional macrophages and in epithelial cells, but the general principles of exosome trafficking are likely shared. It is thought that along some of these paths, under some conditions, internalized exosomes fuse with the limiting membrane of the endosome, delivering their cytosolic cargo (i.e. RNAs) to the cytosol of the recipient cell, in a process that topologically represents the exact reverse of their biogenesis. It would seem that such 'back-fusion' should be of a magnitude sufficient for reaching the stoichiometries that are needed for the delivered cargo having meaningful effects (e.g., for mIRs to effectively target RNAs). Possibly transfers of the required magnitude occur through specific vesicles or only under certain specific conditions (Fig. 10.2).

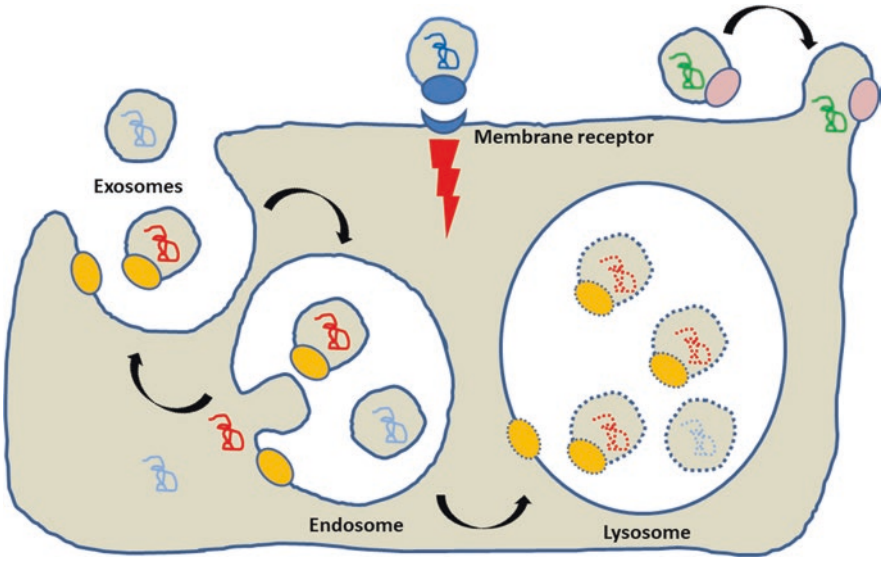

Fig. 10.2 Exosome reception. In the recipient cell (which can be the producing cell itself), exosomes will bind to the cell surface and from there can undergo various fates. Exosomes can activate membrane receptors and initiate signaling directly from the cell surface. Internalization will introduce exosomes into the endosomal pathway. Ultimately, these will reach multivesicular endosomes, where they likely mix with endogenous intraluminal vesicles. Fusion of the multivesicular endosomes with the lysosome will lead to the degradation of these exosomes. Possibly, if not likely this is the major fate of any internalized exosome. Yet, exosomes docked either at the plasma membrane or at the limiting membrane of early and late endosomes can probably also 'back-fuse' with that membrane, releasing their intraluminal contents into the cytoplasm of the recipient. That process is currently poorly understood but is of major importance for the delivery of intraluminal cargoes such as microRNA (miRNA)

10.4 Virus-Like Vesicles, Exosome-Like Viruses?

It should be clear that in many aspects both the biogenesis and secretion of exosomes and the ways by which these vesicles 'transduce' recipient cells is reminiscent of the life cycle of viruses. Moreover, some viruses exploit the host mechanisms of membrane bending and abscission to egress from cells. Human immunodeficiency virus-1 (HIV-1) and Equine Infectious Anemia Virus (EIAV), for example, exploit components of the ESCRT machinery to bud, directly from the plasma membrane or in MVBs [24, 41, 61, 89]. The P(T/S)AP motifs and the $LYPX_nL$ motifs present in the late domain proteins of these retroviruses interact directly with, respectively, TSG101 (an ESCRT-I component) and with ALIX (an auxiliary component of the ESCRT machinery, bridging TSG101 in ESCRT-I and CHMP4 in ESCRT-III), and these interactions are essential for virus release. There is thus even a strong mechanistic analogy between viral budding and ILV/exosome formation. In addition, some viruses use the same mechanisms as extracellular vesicles, and sometimes these vesicles themselves to enter cells. It thus comes as no surprise that exosomes are often considered as 'natural, endogenous' viruses and that the distinction between viruses and extracellular vesicles has even become somewhat 'semantic'. The concept was well formulated and summarized in the 'Trojan exosome' hypothesis, now already 15 years ago [31]. More than ever this conceptual framework remains valid [66, 108]. As we will discuss, also the link of heparanase to exosome biology will underscore this notion (Fig. 10.3).

10.5 Syntenin, Adapting ESCRT Machinery to Endocytosed Syndecans Supports the Biogenesis of Exosomes

Former contributions from our laboratories strongly implicate the syndecan heparan sulfate proteoglycans and their cytoplasmic adaptor syntenin in the biogenesis of exosomes. The syndecans (SDCs) compose a family of type-1 membrane-spanning proteins, exposing heparan sulfate (HS) chains with versatile properties at the cell surface and an evolutionary highly conserved small intracellular domain (ICD) in the cytosol. HS has numerous ligands, including various morphogens, adhesion molecules, and growth factors, e.g. Wnts, fibronectin and fibroblast growth factors (FGFs) to name a few [25, 53]. HS plays an important role in the docking of these factors to cognate signaling receptors, e.g. the binding of FGF2 to FGF-Receptor1 (FGR1), qualifying the cell surface proteoglycans as 'co-receptors'. HS and SDCs, in particular, are also intimately involved in several processes of endocytosis and vesicular trafficking that depend on cellular context and type of ligand [9, 50, 79]. Yet, the biological effects of the SDCs do not solely rely on their HS chains. Direct interactions of their protein cores, in particular of their conserved transmembrane and cytoplasmic domains, but also of their ectodomains,

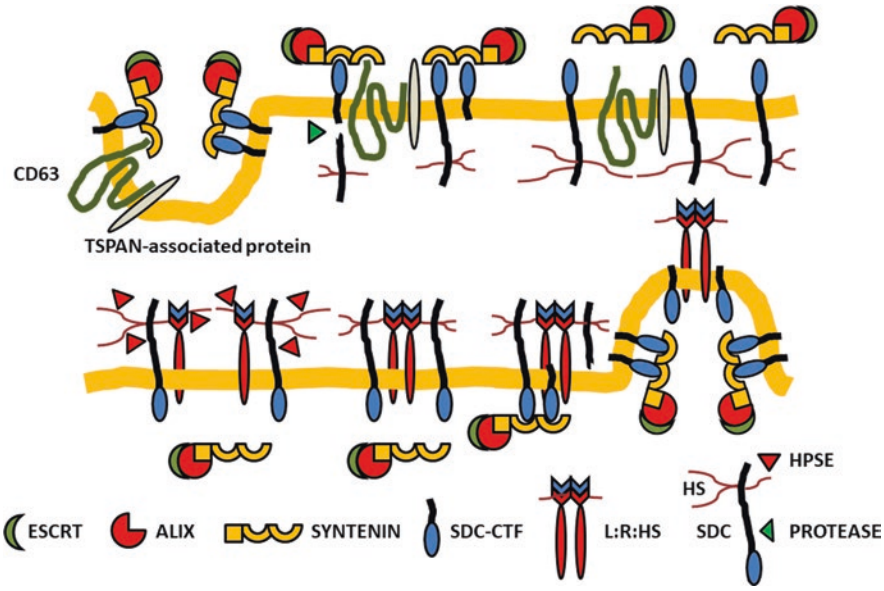

Fig. 10.3 Heparanase 'activates' syndecan for exosome production. Syndecan and cargo bound to the heparan sulfate (HS) of syndecan (e.g., FGF-FGFR complexes assembled on the HS chains of syndecans; Ligand:Receptor:HS) are internalized by endocytosis. In endosomes, syntenin directly interacts with syndecans and the tetraspanin (TSPAN) CD63 via its tandem PDZ domains. During endosome maturation into late endosomes, syndecans (SDC) are trimmed by heparanase (HPSE) and undergo proteolytic cleavage of their extracellular part to generate a membrane-associated syndecan C-terminal fragment (SDC-CTF). These cleavages allow syndecans/syndecan C-terminal fragments to cluster, recruiting syntenin, and stimulate endosomal budding. The syntenin-mediated endosomal budding of syndecan and CD63, and of cargo associated with these proteins, also depends on the direct interaction of the N-terminal domain of syntenin with ALIX (an ESCRT accessory component), and on several ESCRT proteins. Heparanase does not stimulate all types of exosomes; it stimulates the exosomal release of syndecan C-terminal fragment, syntenin, ALIX, and CD63, but has no effect on the release of exosomal flotillin, CD9, or CD81 (two other tetraspanins commonly found in exosomes)

are now well characterized [5, 6]. Of particular importance, in the present context, is that all SDCs feature a strictly conserved EFYA sequence at their cytosolic C-terminus. Syntenin is a protein that binds to the syndecan-ICD, via that C-terminal structure [34]. Syntenin is a small, 298 amino acid cytosolic protein that contains two PSD95/Dlg/zonula occludens 1 (ZO-1) (PDZ) domains in tandem, surrounded by a 100 amino acid N-terminal and a 25 amino acid C-terminal region. The two PDZ domains of syntenin are both necessary and sufficient for syntenin membrane localization and high-affinity interaction with SDCs [33, 119]. Of note, the syntenin PDZ domains also interact with phosphatidylinositol 4,5-bisphosphate (PIP2) [118] and, pending on the activation of ARF6 and PIPK, syntenin-PIP2 interaction controls the endocytic recycling of SDCs and of SDC-associated complexes, i.e. FGF2-FGFR1 complexes, from late recycling endo-

somes back to the cell surface [120]. Syntenin probably occurs in alternative 'open' and 'closed' configurations, likely involving intramolecular interactions between the N-terminal and C-terminal domains of the protein and potentially controlling its further intermolecular interactions and recruitment to cell surfaces. Importantly, the N-terminal domain of syntenin can directly interact with ALIX [4]. This interaction occurs via three $LYPX_nL$ motifs present in the syntenin N-terminal domain that binds the ALIX 'V domain', and is thus reminiscent of the ALIX interaction with late viral domains of HIV-1 and EIAV (see above). *In vitro* BIACore experiments indicate that recombinant SDCs, syntenin and ALIX proteins can assemble in a tripartite complex. In counterpart, *in cellulo* gain- and loss-of-function experiments indicate that SDCs, syntenin, and ALIX work together in exosome formation and composition. The whole of the results suggests a model whereby the clustering of endocytosed SDCs is recruiting syntenin, and syntenin is adapting syndecans and syndecan-associated cargo to ALIX and ESCRT proteins (in particular the CHMP4 proteins of the ESCRT-III complex that bind ALIX), all working together in the budding and abscission of endosomal membranes to form ILVs and sequestering particular cargo in these ILVs [4, 43]. For example, in the presence of FGF2, SDC, syntenin and ALIX control the exosomal release of FGFR1. They also control the exosomal release of CD63, a tetraspanin that is often used as a marker of exosomes, but not that of CD9 or that of flotillin-1 (both also often used as 'exosomal markers'). Of note, like syndecans, CD63 (but not CD9) also binds to syntenin, via a PDZ-BM [51]. Importantly, the production of these SDC-CD63-syntenin exosomes depends on the HS chains of the syndecans and the participation of this HS in the lateral interactions of the syndecans with specific ligands (like FGFs) that lead to their clustering, and from there the recruitment of syntenin. At least in some types of cells and under some conditions, the SDC-syntenin-ALIX connection is an important path that controls up to 50% of the vesicles that are secreted by the cells. Possibly this relates to the versatility of the syndecans as co-receptors and the multitude of signaling processes that require the assistance of HS. Importantly, the participation of SDC-syntenin-ALIX in ILV budding and exosome formation also depends on the activation of ARF6, but in this case on PLD2, an enzyme synthesizing phosphatidic acid, as ARF6 effector [30]. Altogether, these observations poise syntenin as critical 'checkpoint' in the control of the trafficking of syndecan and syndecan-associated endosomal cargo, sending these back to the cell surface or to ILVs and exosomes. Of note, ILV formation, initiated by signaling, ultimately subtracts signaling receptors from the cytosol and terminates their contacts with cytosolic signal transducers and effectors. Thus, the SDC-syntenin connection potentially further extends the role of HSPGs in the control of signaling, far beyond their roles as co-receptors and involvement in signal initiation: sustaining signaling (by recycling) versus terminating signaling (via ILV formation) *in cis*, and transferring signaling cargo (via exosomes) for potential use *in trans*.

10.6 Heparan Sulfate Involvement in Exosome Internalization

Exosome internalization can be studied by confocal microscopy and flow cytometry, using labeled vesicles. For example, using vesicles marked by a fluorescent dye (PKH) with long aliphatic tails that are incorporated into the lipid membrane of the vesicles, in principle labeling all vesicles in the population, or using vesicles that are loaded with specific eGFP-cargo, potentially representing only a specific subset of the exosomes. This way, it can be shown that (in several different types of cells) exosome uptake is dose-dependent and saturable, vesicle accumulation increasing with incubation time and being inhibited by incubation at 4 °C and by the presence of excess, unlabeled exosomes. The group of Matthias Belting has demonstrated that such internalized exosomes co-localize inside cells with HS-epitopes and with cell-surface HSPGs of the syndecan and glypican type. Exosome uptake is significantly inhibited by added HS and heparins, in a dose-dependent way and in a specific manner, closely related chondroitin sulfate having no effect. In addition, multiple mutant cell types, deficient in enzymes involved in HS-synthesis and modification, provide genetic evidence of a receptor function of HSPG in exosome uptake. Similarly, enzymatic depletion of cell-surface HSPG (by treating recipient cells with bacterial heparinases, removing all HS from the cells) or pharmacological inhibition of endogenous PG biosynthesis (by xyloside) significantly attenuate exosome uptake. Although to a certain extent some intact HSPGs are sorted to and associate with exosomes, similar enzyme treatments of the vesicles suggest that exosome-associated HSPGs have no direct role in exosome internalization. Finally, isolated exosomes bind to heparin-substituted beads. Thus, added HS inhibits cellular uptake of exosomes through competition with cell-surface HSPGs for exosome binding. It is important to note that even in the presence of added heparin or HS-depletion from cells, significant uptake activity remained. Yet, on a functional level, exosome-mediated stimulation of cancer cell migration appeared to be significantly reduced in HS-deficient mutant cells, or by treating wild-type cells with heparin, lyase or xyloside. Thus cells use (the assistance of) HSPGs for internalizing exosomes and responding to these vesicles, which significantly extends the role of HSPGs as key receptors of macromolecular cargo [10]. Clearly, these data do not exclude the possibility that exosomes may also exert functional effects through alternative pathways, all or not involving exosome uptake. Given that several viruses have previously been shown to enter cells through HSPGs, these data further implicate HSPG as a convergence point during cellular uptake of endogenous vesicles and virus particles. Of note, specific HS modifications (generating structures based on 3-O-sulfate) have also been implicated in productive viral infection, implying fusion and access of viral contents to the cytosol of the cells [85]. Thus, by extension, possible additional roles for HS in exosome fusion are to be considered.

Interestingly, cells that are syntenin-deficient appear to ‘resist’ transduction by recombinant AAV *in vivo* and by recombinant retrovirus *in vitro* ([19]; Kashyap et al. unpublished results). Compared to controls, syntenin-deficient cells internal-

ize also lesser amounts of PKH-labeled exosomes. Strikingly, syntenin-deficient cells express lower amounts of HS at their cell surfaces, likely at least in part a reflection of the function of syntenin in SDC recycling. Over-expressing SDCs in syntenin-deficient cells markedly enhances the effectiveness of retroviral transduction. So does the re-introduction of wild-type syntenin, but syntenin mutants that are defective in either the recycling-function or budding-function of syntenin do not. The latter is particularly intriguing and remains to be explained, but it might be noted that the PDZ-domains of syntenin bind also avidly to nectin-1 [27], along with 3-O-sulfate-substituted HSPGs, one of the several receptors involved in the entry of HSV-1 into cells. Syntenin controls also the post-endocytic trafficking of oncogenic human Papillomaviruses [32]. Although some of the current evidence remains largely conjectural, all these observations would seem to place syntenin both at the 'sending end' and at the 'receiving end' of exosome biology.

10.7 Heparanase Activates the Syndecan-Syntenin-ALIX Exosomal Pathway

Heparanase is an endoglycosidase, cleaving heparan sulfate chains at internal sites, generating short HS fragments (of 10–20 residues). It is the only mammalian enzyme with such activity [100]. The importance of heparan sulfate for both exosome production and clearance implies that heparanase might influence processes of exosomal exchange. As are exosomes, heparanase is strongly implicated in tumor invasiveness, angiogenesis, and metastasis [16]. The notion receives also support from the emerging evidence for the implication of heparanase in viral infection, as exemplified by herpes simplex virus-1 (HSV-1), one of the first viruses shown to attach to cell surface heparan sulfate (HS) for entry into host cells. During productive infection, the HS moieties on parent cells trap newly exiting viral progenies and inhibit their release. Yet, heparanase expression is upregulated upon HSV-1 infection, modifying the HS present at cell surfaces, facilitating viral release. Thus, heparanase seems to act as a molecular switch for turning a virus-permissive 'attachment mode' of host cells to a virus-deterring 'detachment mode'[36]. Since many human viruses use HS as an attachment receptor, the heparanase-HS interplay may delineate a common mechanism for virus release ([92]; Agelidis and Shukla, Chap. 32 in this volume). By extension, such a scheme might also apply to exosomes.

Consistently, elevating heparanase expression in cells stimulates net exosome production and affects the composition of exosomes, enhancing the loading of these vesicles with cargo that potentially influences angiogenesis ([75, 94]; Sanderson et al., Chap. 12 in this volume). Experimentally, it is fairly easy to increase the levels of heparanase activity within cultured cells. When pro-heparanase is added to cells, the enzyme precursor is rapidly internalized and processed into active heparanase [29, 101]. This conversion occurs in endosomes, where the enzyme normally remains localized [115]. The opposite, fully suppressing endosomal heparanase

activity in cells might be somewhat more complicated (at least with the inhibitors that are currently available), given that sera used for culturing cells contain substantial amounts of platelet heparanase, and given the long half-life of internalized heparanase. An increase in heparanase results in extensive trimming of the heparan sulfate on syndecan and also accelerates the endocytosis of syndecan. Exosomal levels of syntenin and CD63 increase markedly, but most striking is the increase in exosomal syndecan. Of note, most of that syndecan consists of C-terminal fragments (CTFs) that span the membrane but are devoid of any heparan sulfate (or chondroitin sulfate). Conversely, in cells that express high levels of heparanase, stable shRNA-mediated knockdown of the enzyme reduces the amounts of syntenin, CD63, and syndecan-CTFs present in exosomal fractions.

Importantly, the catalytic activity of heparanase is required and heparan sulfate must be provided by syndecan. Indeed, glypicans, heparan sulfate proteoglycans that are linked to the cell surface via glycosylphosphatidylinositol (and thus cannot directly interact with syntenin), cannot substitute for syndecan and restore the effect of heparanase on exosomes. Knockdown of the small GTPase RAB7 abolishes the heparanase-mediated increase in exosomal syntenin, syndecan CTFs, and CD63, confirming that heparanase affects the production of vesicles that are of endosomal origin, the operational definition of exosomes. Furthermore, heparanase stimulates the endosomal budding of syntenin and syndecan and requires ALIX for these effects. Thus, heparanase is an activator of the syndecan-syntenin-ALIX pathway of exosome biogenesis [75]. In contrast, using exosomes loaded with eGFP-syntenin and various recipient cells, added heparanase appears to have little or no effect on (syntenin) exosome uptake. Yet, this aspect has been less investigated, and, as already stated, might also depend on the donor and the repertoire of exosome-associated membrane proteins and cargo. Finally, and also of note, exosomal flotillin-1 and exosomal levels of CD9 and CD81, two tetraspanins also commonly used as exosomal markers, are not affected by heparanase. The specificity of the heparanase effect underpins the hypothesis of multiple different exosomal populations formed through specific biogenesis pathways, one of which is the syndecan-syntenin-ALIX pathway [23, 48].

10.8 Heparanase, Integrating Syndecan Lateral Associations and Spatial Constraints?

How might heparanase influence syndecan-dependent endosomal membrane budding? The initial clustering and introduction of the syndecans in endocytic pathways likely depends on the lateral associations of these molecules with ligand, explaining the need of HS that is present on syndecan. An important second concept is that syntenin is recruited to membranes by clustered ‘bait’, engaging both the PDZ domains of syntenin [33, 119]. Thus, reaching the local concentration of syndecan-ICD required for recruiting syntenin might require the remodeling of the syndecans

by heparanase and ultimately their conversion into a membrane-embedded C-terminal fragment that lacks HS. Finally, membrane budding and endosomal filling likely also imply a reduction of repulsive forces in the endosomal luminal space. Heparanase might have an impact at several levels of such a scheme [14, 75, 88]. A first consideration is that lateral interactions engaging the HS chains of the proteoglycans sometimes depend on HS remodeling. In the case of HS-assisted FGF-FGFR signaling, for example, it is striking that bacterial heparitinase [117], and likewise mammalian heparanase [46, 74], can generate HS-fragments endowed with biological activity, where that activity is rare or even not present in the intact parent HS-chain. At least in the particular case of FGF2-FGFR1, crystal structures reveal that in order for two trimeric FGF-FGFR-HS complexes to assemble and confront one another in hexameric signaling units, the specific HS-structures that foster and stabilize the formation of FGF-FGFR complexes need to occupy a terminal position in each of the two HS chains that are engaged in the formation of a signaling unit [81]. In other words, HS-supported FGF-FGFR interfaces will bring together or cluster two different heparan sulfate chains, in opposing orientations, pending on occupying the 'end-structures' of these chains. In that scheme, pending on the presence of FGF and FGFR, heparanase, converting the HS chains on SDC into shorter chains with the required end-structures, leads to the clustering of SDCs. In short, heparanase potentially 'activates' the lateral HS-mediated associations of the syndecans, inducing the SDC clustering that can recruit syntenin. In that specific context, it might be worth reminding that FGF2 stimulates the production of exosomes that contain FGFR1 (along SDC-CTFs and syntenin), and that the down-regulation of syndecans or syntenin attenuates such effect of FGF2 [4]. A variant on that theme may be provided by lacritin, an epithelial mitogen that activates PLD-mTOR [103] and is linked to autophagy [104]. This mitogen specifically binds to syndecan-1, and not syndecan-4, via heparanase-modified HS and the concomitant exposure of a binding site in the syndecan-1 core protein ([56, 116]; Dias-Teixeira et al., Chap. 4 in this volume). Heparanase-modulated lateral syndecan associations might be initiated in endosomes, where heparanase resides, or, more likely, be initiated on syndecans that recycle from endosomes back to the cell surface (a process that is supported by PIP2-syntenin). Secondly, one might also have to consider the mirror aspects and side effects of these 'fatal attractions'. SDCs are present at cell surfaces in such high copy number, that they probably suffice to cover the entire cell surface [110]. As the HS chains are highly negatively charged, HSPGs will probably tend to repel each other and be 'locked' in their positions unless engaged and 'neutralized' by ligand. Having extended structures, native HS chains also potentially bind multiple ligands at the same time. Potentially such ligands have different mobilities, restricting allover HSPG and ligand mobility: for example, matrix-bound SDC, limiting the mobility and availability of growth factors and growth factor receptor complexes bound and assembled on that same SDC [57]. Heparanase, in contrast, leaves syndecan substituted with small heparan sulfate chains (possibly restricted to a single ligand) and thus more likely free to 'move around'. Consistent with the above notions of potential heparanase effects on SDC engagements and mobility, loading cells with heparanase, markedly shortening the length of the HS

chains on syndecan-1, significantly accelerates the endocytosis of that proteoglycan [75]. Potentially, accelerated endocytosis is helping to increase the concentrations of endosomal syndecan up to levels required for recruiting syntenin. The possible importance of a reduction of the physical dimension of a syndecan becomes also particularly compelling when considering the dimensions of an ILV. The length of an extended native heparan sulfate chain (with a molecular weight of 40 kDa on average) is close to 50 nm. With three chains of heparan sulfate per syndecan, likely projecting outwards and pointing away from each other, the diameter of a syndecan may be close to 100 nm, which is approximately the diameter of an ILV and exosome. Trimming of the heparan sulfate on syndecan may thus substantially increase the number of syndecan molecules that can be packed in defined membrane domains and thereby help create the local concentration of bait (syndecan cytosolic domains) that will allow recruiting syntenin and along with the syntenin also ALIX and ESCRTs. Conceivably, even in ligand-induced SDC complexes supported by trimmed HS (with still a mass of about 7–10 kDa), the SDC-ICDs may remain too far apart for recruiting syntenin. Thus, obtaining the degree of clustering or compaction of syndecan that is sufficient for recruiting syntenin might require the cleavage of the protein, leaving SDC-CTF in association with the endosomal membrane. Possibly, the initially mixed oligomerizations of HS-substituted SDCs and HS-free SDC-CTFs (not subject to self-repulsion), in the end, replaced by the oligomerizations of mainly SDC-CTFs, may stably recruit syntenin and ALIX. In that context, one might surmise that once also ESCRT-III is recruited and that CHMP4 assemblies are surrounding the necks of the buds, HS becomes entirely dispensable for sequestering SDC-CTFs and syndecan-associated cargo in budding membranes. Conceivably, heparanase-mediated trimming of the HS on syndecan might facilitate the access of processing protease generating the SDC-CTF, potentially including metalloproteases and acid proteases. It is noteworthy, in that respect, that upregulation of heparanase also induces the shedding of syndecan-1, by metalloproteinase processing [112], and, inversely, that heparanase-deficient animals show an upregulation of metalloproteinases [113]. It is not clear to what extent cell surface and endosomal activities are involved, but possibly up-regulation of processing protease can compensate for reduced protease-access. Furthermore, the speculative considerations made on molecular HSPG crowding at cell surfaces can also be made for endosomes, where, conceivably, internalized HSPGs might continue repelling each other, in the lateral plane of the limiting membranes, at the level of budding membranes, and between budding and limiting membranes. The most compelling spatial constraints would seem to occur at the level of the neck of the bud, an area of extreme membrane curvature, where ESCRT-III accumulates to mediate membrane fission [83]. HS-persistence on limiting and budding membranes could thus potentially have a vesicle ‘back fusion’ effect. Inversely, HS-removal, possibly initiated at selected parts of the endosomal membrane, creating asymmetric distributions of mass across the bilayer and changes in molecular crowding, might have a ‘permissive’ effect. When endosomes are filling up with multiple ILVs to form MVE, there might also be a general ‘need’ for further reducing the net or effective negative charge of the membranes of these compacting compartments. Such might again be

achieved by neutralizing by ligand, likely enhanced by the acidification of the compartment, increasing the net positive charge of all proteins without affecting the negative charge of the HS, or, more effectively, by reducing the mass of the HS or removing the HS on these membranes altogether. In that respect, heparanase, fragmenting the HS, might also markedly accelerate the process of HS removal by exoglycosidases. Being directed to late endosomes and lysosomes by secretion and recapture, possibly heparanase can allow doing so at a stage during endocytosis, i.e. a time window or compartment, where ILVs can still be diverted from lysosomal degradation and secreted as exosomes (and sole exoglycosidases might come 'too late' to complete the membrane remodeling that is required).

10.9 Heparanase Effects on Exosomal Cargo

It is interesting to note that, upon heparanase addition, the amounts of exosomal cargo that composes the direct 'bait' for the PDZ domains of syntenin (i.e. syndecan CTFs and CD63) continue to increase with increasing heparanase concentrations, but that the effect of heparanase on exosomal syntenin plateaus. These findings are in line with prior observations that the ratio of 'bait' or cargo to syntenin in exosomes is not constant [4]. Indeed, the over-expression of SDCs or CD63 markedly increase the levels of the corresponding SDC-CTFs or CD63 in exosomes but do not affect the levels of syntenin and ALIX in these vesicles. Thus, while intraluminal budding/exosome formation appears to be triggered by the organization of syntenin bait (i.e. syndecan-CTFs and CD63) in structures of higher order, syntenin may adapt only to a part of the bait present in these organizations. This situation would again be similar to the incorporation of viral proteins in budding membranes, where, due to lateral associations between coat proteins, late domains are also functioning *in trans* and not all individual copies of the GAG-proteins that end up in the viral coats need to be provided with direct links to the budding machineries [105]. Similarly, a sizeable fraction, but not all of the syntenin-dependent cargo that ends up in exosomes might need to be directly linked to syntenin. Thus, where the cellular levels of syntenin may become limiting at some point (and exosome numbers might stagnate), heparanase might still increase the 'lateral association'or clustering of syndecans, further stimulating the incorporation of syndecans and syndecan-associated proteins in exosomes. In myeloma cells, for example, heparanase stimulates the accumulation of syndecan-1 and of specific cargo such as hepatocyte growth factor and VEGF in exosomes [94]. Whereas syndecan-1 is a well-known marker of plasma cells and predominates in myeloma cells, more remarkably, also in MCF-7 and other cells the effects of heparanase are very marked for the exosomal levels of SDC1-CTFs. In comparison, effects on the levels of SDC4-CTFs are more moderate [75]. Such differential effect of heparanase on exosomal syndecan might be context-dependent, but suggests differences between the syndecan family members that might relate to their differential subcellular localization, trafficking, heparan sulfate composition, access to heparanase or syndecan-cargo associations.

In particular in terms of that last aspect, it might be important to remind about the increasing evidence for extracellular lateral syndecan associations that involve their core proteins: e.g. syndecan-4 interacting directly with EGFR [5, 6]; the protein tyrosine phosphatase CD148 binding to a region proximal to the transmembrane domain of syndecan-2 [106]; the integrin-assisted syndecan-1 association with IFG1R and HER2 [102]; and finally, in myeloma cells, the heparanase-mediated trimming of the HS on syndecan-1 and the subsequent MMP9-mediated shedding of this syndecan, exposing a juxtamembrane site in syndecan-1 that binds VEGFR2 and VLA-4, thereby coupling VEGFR2 to the integrin [45]. Thus, pending on the receptor combination repertoires activated in the cells, heparanase might engage different syndecans and recruit specific cargo to exosomes.

10.10 Heparanase as Exosomal Cargo

Recently, several enzymes, including membrane-type 1 matrix metalloproteinase (MT1-MP), insulin-degrading enzyme (IDE), sialidase, and also heparanase, were localized on the surface of exosomes secreted by various cell types [65, 77]. For heparanase, such was noted both in myeloma [94] and in epithelial cells [75], where most of the exosome-associated protein is present in enzyme precursor form. It is not clear what exosome component heparanase is bound to, but in epithelial cells exosomal heparanase resists the knockdown of syndecans or heparan sulfate polymerase, suggesting the exosomes are not 'heparanase-syndecan-syntenin-dependent and that binding involves yet to be identified exosomes and alternative receptors [75]. Apparently, these exosomal surface enzymes retain their activity and can degrade their natural substrates present within extracellular spaces. Likewise, heparanase present on the exosome surface can be activated, possibly after exosome uptake, and is capable of degrading heparan sulfate embedded within an extracellular matrix [3]. In that context, it might also be worth reminding of the non-enzymatic function of heparanase, whereby even the catalytically dead enzyme supports mechanisms of cell migration and invasion [26, 28]. Exosomes have well-established functions in polarized, directed cell migration [90]. Whether the presence of heparanase on exosomes may be pertinent in this context is not clear, but might deserve further investigation.

10.11 Conclusion and Prospects

Taken together, the above findings and considerations identify heparanase as a fundamental modulator of exosome biogenesis via the syndecan-syntenin-ALIX pathway, by cleaving and 'activating' the heparan sulfate chains of the syndecans. Thus, heparanase-enhanced tumor growth might in part be mediated by syndecan-syntenin exosomal communication in the tumor-host environment. It is interesting to note

that, more recently, heparanase and syntenin have both also been implicated in processes of auto-phagocytosis. Exosomes and autophagy are linked through the endolysosomal pathway, and a strong interplay exists between both, operating as 'partners in crime' in the context of neurodegeneration and cancer [109]. Heparanase was found to reside within autophagosomes, and to promote autophagy, rendering heparanase-overexpressing cells more resistant to stress and chemotherapy. The mechanism underlying this increase in autophagy is not entirely clear, but likely involves reduced mTOR1 activity [78, 84]. Syntenin is suppressing high levels of autophagy while helping to maintain the protective autophagy that allows tumors stem cells to resist anoikis [91]. A possible relation between heparanase and syntenin in autophagy remains to be explored. Both might have synergic effects on signaling processes that support autophagocytosis. It might be noted that the origin of the membranes that lead to the formation of the phagophore and its elongation, to yield the double membrane that outlines the autophagosomes, remains a matter of debate, but includes the ER, ERGIC, Golgi, plasma membrane and recycling endosomes [60]. Yet, a membrane compartment with a high luminal charge of HSPG would seem improbable. Conceivably, heparanase activity helps insuring a source of such membrane. The sealing of the double membrane around the cargo to be sequestered to form an autophagosome is topologically equivalent to membrane abscission during endosomal ILV and exosome formation and conceivably could depend on syntenin-mediated mechanisms of recruitment. It might be noted that the ATG12-ATG3 complex involved in autophagocytosis binds and recruits ALIX [63] and that ULK1 phosphorylates syntenin, modulating its non-canonical interaction with ubiquitin [72]. Increasingly, attention is now also provided to non-autophagic functions of autophagy-related proteins that include secretion, trafficking of phagocytosed material and egress of viral particles [8]. Directly or indirectly, 'secretory' autophagy and exosome production might have more effectors in common than initially suspected. Possibly, some of the above considerations are also relevant for other modes of 'non-conventional' secretion; e.g., that of pro-inflammatory cytokines such as IL-1β [11, 15]. Much further work is needed, but exciting novel insight can be anticipated here.

It will be of particular interest to delineate the influence of heparanase, i.e., the heparanase-activated syndecan-syntenin-ALIX machinery, on the overall composition of exosomal cargo. Sorting of many membrane proteins into exosomes coincides with their association with tetraspanin membrane proteins [1]. Non-tetraspanin membrane proteins may piggy-back onto tetraspanin webs for their sorting into exosomes. Interestingly, with the help of syndecan, the tetraspanin CD63, which is highly enriched in exosomes, can also be recruited by syntenin [4]. The exosomal levels of CD63 are also modulated by heparanase. The levels of CD9, in contrast, are not. Sorting of tetraspanin webs at endosomes into exosomes could thus, similar to syndecans, be driven by the cytoplasmic adaptor syntenin, and the recruitment by syntenin of tetraspanin webs and syndecan clusters are thus integrated processes. All in all, a complex picture is emerging, in which both CD63 and syndecans, and possibly other membrane proteins that associate with endosomal syndecan and/or

tetraspanin-enriched microdomains, are sorted into exosomes by a shared syntenin-ALIX-ESCRT machinery [88].

Specific exosomal cargo (Wnt11 and c-Met, respectively) has been shown to regulate crucial processes such as cancer cell motility, the onset of metastasis and premetastatic niche formation [54, 67]. Intriguingly, the signaling pathways involved are strongly influenced by heparan sulfate [35, 76], arguing for a potentially pivotal role of the syndecan-syntenin-ALIX machinery and its modulators, like heparanase, in physiological processes linked to exosomes and the transfers of exosomal cargo. Of note, syntenin supports non-canonical Wnt-signalling [17] and directional cell movements in *Xenopus* and zebrafish embryos [49, 55]. In zebrafish embryos, the yolk syncytial layer releases extracellular vesicles with exosome features into the blood circulation. These exosomes are released in a syntenin-dependent manner and are captured, endocytosed and degraded by patrolling macrophages and endothelial cells, affecting the growth of the caudal vein plexus [99]. Heparanase stimulates the migration of vascular endothelial cells, via protein kinase B/Akt activation [28], and is actively involved in the regulation of VEGF gene expression, mediated by activation of Src family members [114]. Recently, c-Src, phosphorylating both syntenin and the ICD of syndecan, was identified as a cytosolic activator of the syntenin-exosome pathway, and syntenin-exosomes as a requirement for non-cell autonomous effects of c-Src on vascular endothelial cell motility [44]. Conceivably, heparanase and c-Src may thus sustain a positive feedback loop in exosomal communication. Yet, in essence, all this remains to be explored. If such proves to be the case, syntenin, heparanase, and c-Src, all often upregulated in cancer, represent interesting targets for modulating exosome effects in cancer therapies.

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Chapter 11
Heparanase in Cancer Metastasis – Heparin as a Potential Inhibitor of Cell Adhesion Molecules

G. Bendas and Lubor Borsig

11.1 Introduction

Cancer progression – metastasis is a process encompassing multiple steps including successful escape of tumor cells from the primary tumor sites, survival in the circulation, evading immune responses, seeding in distant organs, and most importantly initiation and sustained growth at these sites. Even though sustained proliferation of tumor cells is likely the most fundamental trait in tumor cells, the capacity to "modulate" the tumor microenvironment consisting of non-tumorigenic stromal cells significantly contributes to metastasis, virtually at every step of this process [1, 2]. Particularly, the capacity of tumor cells to secrete extracellular matrix-degrading enzymes, such as heparanase or proteases, profoundly contributes to migratory properties of tumor cells, or to the release of factors promoting tumor growth and angiogenesis [3, 4].

Heparanase expression is linked to an invasive phenotype of a variety of cancer types in patients and has been confirmed in numerous animal models [3, 5, 6]. This book covers all known aspects of heparanase biology in great details. Thus the focus of this chapter is on heparanase action in leukocyte recruitment and cell-cell interactions during cancer progression. In addition, the activity of heparin and heparin derivatives on cancer progression will be discussed with respect to their anti-heparanase and anti-adhesive activities; and current developments towards therapeutic applications.

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11.2 Cell Adhesion Promotes Tumor Cell and Leukocyte Migration

The immune system "responds" only when leukocytes are able to cross blood vessels. Circulating leukocytes do not interact with the endothelial cells. Endothelial activation is required to initiate leukocyte adhesion and to enable the migration of leukocytes through the endothelium. The formation of chemokine gradient is a prerequisite for driving the cell recruitment and the capacity of cells to adhere to the vessel wall and to initiate trans-endothelial migration [7]. Selectins are likely the first cell adhesion molecules involved in leukocytes recruitment and transendothelial migration. Inflammatory stimuli activate endothelial cells that increase expression of adhesion molecules including P-selectin, E-selectin and increases vascular permeability [8, 9]. Interactions of vascular selectins with their ligands lead to the leukocyte rolling on activated endothelium, followed by integrin-mediated firm adhesion and finally leukocyte extravasation into the parenchyma, where leukocytes can execute their effector functions [10].

Extravasating leukocytes need to engage the endothelium, and after diapedesis they face the basement membrane surrounding most of postcapillary venules, which is a complex meshwork of collagens interconnected with glycosaminoglycans, such as heparan sulfate. Heparanase is a hydrolytic enzyme enabling degradation of heparan sulfate, thus likely promoting leukocyte migration after extravasation [11, 12]. Interestingly, neither neutrophils nor T cells require heparanase expression for efficient extravasation as has been shown using heparanase deficient mice [11]. In contrast, efficient monocyte extravasation required heparanase expression, in a peritoneal inflammation model. In the pulmonary vasculature, which represents the largest capillary bed in our body, the transendothelial migration of neutrophils also does not require heparanase expression [12]. However, in chronically inflamed lungs, neutrophil accumulation required heparanase, while T cell recruitment remained heparanase independent but was mediated by ICAMs instead. Thus, contrary to tumor cells, where enhanced heparanase expression is linked to invasive behavior, leukocyte transendothelial migration does not require heparanase.

During cancer metastasis, selectin-mediated interactions were shown to be essential for the recruitment of myeloid cells and monocytes that enhances tumor cell extravasation and facilitate metastasis in several mouse models. The metastatic microenvironment further promotes recruitment of monocytes and myeloid cells through enhanced presence of chemokines, e.g., CCL2, CCL5 [9, 13]. These observations show that metastatic tumor cells "highjack" the physiological function of selectins and leukocyte recruitment to promote tumor growth and metastasis.

11.3 Cell Adhesion as Determinant of Metastasis

Cell adhesion defines the physiological function of any cell in the body through contacts to other cells or to extracellular matrix (ECM) component within the tissue environment. Since cell adhesion is connected to signal-transduction pathways affecting cell phenotype, survival, differentiation and migration, alteration in cell adhesion frequently observed in tumor cells directly contributes to cancer progression. During cancer progression, several families of adhesion molecules including cadherins, integrins, junctional-adhesion molecules, and selectins have been studied. The topic of cell adhesion in cancer progression is too extensive to be covered in this chapter and excellently reviewed elsewhere [14, 15]. Thus, we focus on the role of cell adhesion in steps of metastasis in the context of potential involvement of heparanase.

Hematogenous metastasis is a highly orchestrated process describing the ability of tumor cells to enter in the blood circulation, survive the circulation and extravasate in secondary sites, where they can form metastases. Tumor cells in circulation undergo multiple interactions with blood cellular components, such as platelets and leukocytes that are essential for tumor cell survival and further for metastasis. Two major families of cell adhesion molecules, selectins and integrins, have been identified to facilitate cell-cell interactions essential for the metastatic spread (reviewed in [10, 16–19]. Here we will discuss selectins and integrins both as mediators of metastasis and as targets of heparin-based therapeutic approaches.

11.3.1 Selectin as Mediators of Metastasis

Selectins have been identified as key adhesion molecules that mediate adhesive events between leukocytes, endothelial cells, and platelets during leukocyte trafficking and hemostasis [20]. There are three members of the selectin family: P-, L- and E-selectin. Platelets and endothelial cells express P-selectin that is stored in α-granules and Weibel-Palade bodies, respectively; and upon activation rapidly translocates on the cell surface of these cells. E-selectin is expressed in endothelial cells, whereupon activation a *de novo* transcription is initiated, and its cell surface expression lasts longer than of P-selectin. L-selectin is constitutively expressed on cell surfaces of almost all leukocyte subpopulations [21]. Selectins mediate adhesion by heterotypic interactions of their C-type lectin domain with glycan-bearing ligands. The minimal recognition motif for all selectins is the sialyl-Lewis^x sLe^x and its isomer sialyl-Lewis^a (sLe^a) tetrasaccharide that is sequentially synthesized by N-acetyl-glucosaminyltransferases, galactosyltransferases, α1,3-fucosyltransferases IV or VII, and α2,3-sialyltransferases [10, 21]. Due to the relatively low binding affinity towards a single carbohydrate domain, physiological ligands represent a scaffold of clustered domains to increase the avidity of binding. In addition, P- and L-selectin, but not E-selectin, can bind to sulfated glycans including heparin,

heparan sulfate, fucoidan and sulfated glycolipids [22], indicating that selectins recognize rather a carbohydrate "patch" generated in different ways.

The relevance of selectin involvement in hematogenous metastasis has been deduced from the observations that particularly epithelial cancers (carcinomas) undergo profound changes in cell-surface glycosylation [23, 24]. The cancer-induced aberrant glycosylation often goes along with enhanced presence of selectin ligands enabling tumor cells to interact with other blood constituents once they enter the blood circulation [23]. Selectin-mediated interactions of tumor cells with leukocytes, platelets, and endothelial cells provide a mechanistic explanation for the clinical association with poor prognosis of cancer patients [23, 25, 26]. Tumor cells in blood circulation are often covered by platelets, as has been observed in patients and animal models. The first evidence that this platelet-tumor cell interaction is P-selectin dependent has been described in a mouse model with P-selectin deficiency [27]. Platelet's P-selectin binds to carcinoma mucins since glycan removal inhibited platelet-tumor cell interaction and thus also metastasis [28]. The role of platelets in malignancy and metastasis has been thoroughly investigated since then and is excellently reviewed elsewhere [29–31]. Of note, P-selectin expression on endothelial cells also contributes to metastasis as has been shown in bone marrow reconstituted P-selectin-deficient mice [32]. Similarly, E-selectin has been associated with formation of a premetastatic niche, where the recruitment of tumor cells, as well as myeloid-derived cells, facilitates metastasis [33, 34]. Interestingly, endothelial activation is essential for the recruitment of monocytes and tumor cell extravasation [9, 35].

Leukocytes are a key component of the tumor microenvironment, and they exert many activities promoting metastatic dissemination and metastatic niche formation. Particularly, myeloid-derived cells such as monocytes and neutrophils expedite tumor cell extravasation and formation of metastatic niche [36–38]. L-selectin facilitates the recruitment of myeloid cells to tumor cells in the circulation and enables their extravasation through the endothelial cells [13, 39]. The inhibition or the absence of L-selectin resulted in attenuation of metastasis due to the lack of leukocyte-induced endothelial activation [9, 13]. There is little knowledge about the role of L-selectin on lymphocytes in cancer progression, despite the canonical function of L-selectin in facilitating the recruitment of lymphocytes into lymph nodes [21]. In an inflammation model, L-selectin was shown to mediate the recruitment of activated $CD8^+$ T cells to virus-infected organs and thereby confers protective immunity [40]. Whether L-selectin facilitates activated cytotoxic $CD8^+$ T cell recruitment during tumor progression remains unclear.

11.3.2 Selected Aspects of Integrins during Cancer Metastasis

Integrins are ubiquitously expressed trans-membrane glycoproteins with important functions in cellular adhesion and signaling. The structure of integrins comprises non-covalently bound heterodimers with α- and a β-subunit. 18 α- and 8 β-integrin

subunits have been characterized that combine to form 24 unique canonical α/β receptors identified so far. Integrins mediate cell adhesion, primarily to components of the ECM, such as fibronectin, vitronectin, laminin, or collagen, thus contributing to cellular anchorage but also cell motility and invasion. Furthermore, integrins also mediate certain aspects of cell-cell interactions relevant to tumor cell metastasis. Integrins are important mediators of bidirectional cellular signaling due to their anchorage to the cytoskeletal structures (e.g., α-actinin, talin, and vinculin). As a consequence, ligation of extracellular ligands can influence intracellular processes (outside–in signaling) through activation of kinases, GTPases of the Ras/Rho signaling pathways. On the contrary, intracellular signals can induce alterations in the integrin conformation and thus change ligand-binding properties (inside–out signaling).

An immense body of knowledge has been accumulated covering the multiple roles of integrins in oncology and tumor cell metastasis [41]. To name just a few aspects of integrin-triggered interaction during metastasis, integrins mediate growth factor receptor signaling [42], tumor cell chemoresistance [43], epithelial to mesenchymal transition [44] and angiogenesis [45]. For further details, we refer the readers to excellent reviews in this field [41, 46]. In general, the expression pattern of integrins has been associated with the malignant progression of certain tumors and correlated with altered patient's survival. In this context, we will address here only the functions on integrins, which are directly related to cell-cell interaction during metastasis and discuss the potential targets for heparin to interfere in these processes.

The role of platelets in cancer metastasis has been outlined regarding P-selectin. However, platelets also express five different integrins, which contribute to the formation of tumor cell-platelet emboli during metastasis [47]. For instance, α6β1 and αIIbβ3 integrins are directly involved in platelet adhesion to tumor cells, and pharmacological interference with these integrins resulted in attenuated metastasis [48, 49]. The α4β1 integrin, very-late antigen-4 (VLA-4), is found on many cells of hematopoietic origin referring to their role in immune response by binding to the endothelial ligand VCAM-1. In addition, VLA-4 is also aberrantly expressed and active in different tumor types, such as melanoma [50]. The current evidence shows that VLA-4 facilitates melanoma cell binding to activated endothelial cells expressing VCAM-1 at distant sites, and the interference in VLA-4 attenuates melanoma metastasis in different model systems [51–54]. Furthermore, the aberrant expression of the VLA-4 ligand, VCAM-1, by certain tumor cells was shown to mediate trafficking and binding of macrophages into the forming micrometastases and thereby promotes the formation of a permissive microenvironment for tumor cell outgrowth [55]. Integrins have recently been identified to contribute to organ-specific metastasis through their expression on tumor-derived exosomes [56]. Proteomic analyses of tumor-derived exosomes with tropism towards lungs and liver were shown to express predominantly the integrins α6β4 and α6β1 (lungs) and αvβ5 (liver), driving the formation of a pre-metastatic niche in the respective organ.

Taken together, selectin and integrin adhesion receptors promote cancer progression through various mechanisms (reviewed in [16]). As a consequence,

pharmacological interference with adhesion receptor activities remains an attractive option to attenuate metastasis. Although the inhibition of selectins in experimental settings has been efficient in numerous cancer models, there are no clinical studies focusing on selectins. In the case of integrins, the ubiquitous expression of the different integrin subtypes and partly overlapping ligand recognition complicate a specific targeting. Interestingly, heparin and heparin derivatives have been shown to inhibit P- and L-selectin as well as VLA-4 integrin in experimental settings. Since heparins are currently used in the treatment of cancer patients with thrombosis, the question remains to which extent heparin may affect cell adhesion or heparanase activity during metastasis.

11.4 Heparin as an Inhibitor of Cell Adhesion

Heparin, low-molecular-weight heparin (LMWH) and heparin derivatives (further named as heparins) were tested in many different animal models for its potential to attenuate cancer progression (reviewed in [57, 58]). Interestingly, various heparins tested in animal models have shown primarily attenuating effect on metastasis rather than tumor growth. Most of these studies were performed in an experimental metastasis model where tumor cells were directly injected into the blood circulation. Despite many limitations of this experimental approach, the timely defined presence of tumor cells in the circulation has allowed the evaluation of cellular and molecular mechanisms during the hematogenous phase of metastasis. A variety of heparins efficiently reduced cancer progression when applied shortly before or shortly after the intravenous injection of tumor cells [57]. However, the application of heparin twenty-four hours before or after the tumor cell injection had no effect on metastasis [32, 59]. While in the majority of early studies heparins were used at concentrations exceeding the therapeutic dosage rage, later publications have confirmed the efficacy of heparin to inhibit metastasis also at clinically relevant concentrations [60, 61]. The fact that heparins showed anti-metastatic activity only when tumor cells were still in circulation strongly indicate that cellular and molecular events occurring during this phase are potential targets of heparin instead of solely affecting the coagulation pathway [16, 26]. This conclusion is further supported by the observation that half-life of heparin in circulation is rather limited, not exceeding six hours, thus affecting processes like angiogenesis or tumor growth is less likely.

Heparin is a complex natural glycosaminoglycan extracted from porcine intestine, which in clinical preparation is enriched for the ability to inhibit the clotting cascade. Also, heparins have a wide variety of potential biological effects including the ability to interact with integrins, inhibit P- and L-selectin interactions, inhibit heparanase, attenuate angiogenesis, and affect growth factors and chemokines [26, 62]. Despite these many potential effects, experimental data indicate that heparins affect processes during the hematogenous phase of metastasis. Within blood vessels, circulating tumor cells ultimately interact with the endothelium and other

blood constituents (e.g., platelets and leukocytes) that might lead to tumor cell arrest and extravasation also through increased heparanase activity. Thus, the antimetastatic activities of heparins were analyzed by various groups for their potential to affect coagulation, inhibit cell-cell interactions and to block heparanase as depicted in Fig. 11.1 (reviewed in [63]). Although the anticoagulant activity may contribute to reduced metastasis, several studies clearly indicated that heparins without anticoagulant activity attenuated equally well cancer progression in various cancer entities; e.g., lung, colon and breast cancers (reviewed in [64]). The initial finding that P- and L-selectins can effectively bind to heparins [65, 66] initiated a series of studies testing the hypothesis that heparin treatment inhibits selectin-mediated interactions and thereby metastasis [28, 39, 60]. In parallel, it was shown that integrin-mediated interactions during inflammation could be inhibited by heparin [67, 68], suggesting that heparin may also block integrin-mediated interactions during metastasis. Indeed, heparin was shown to block αIIbβ3-integrin-mediated interactions of platelets with melanoma cells [69]. In another study, heparin was shown

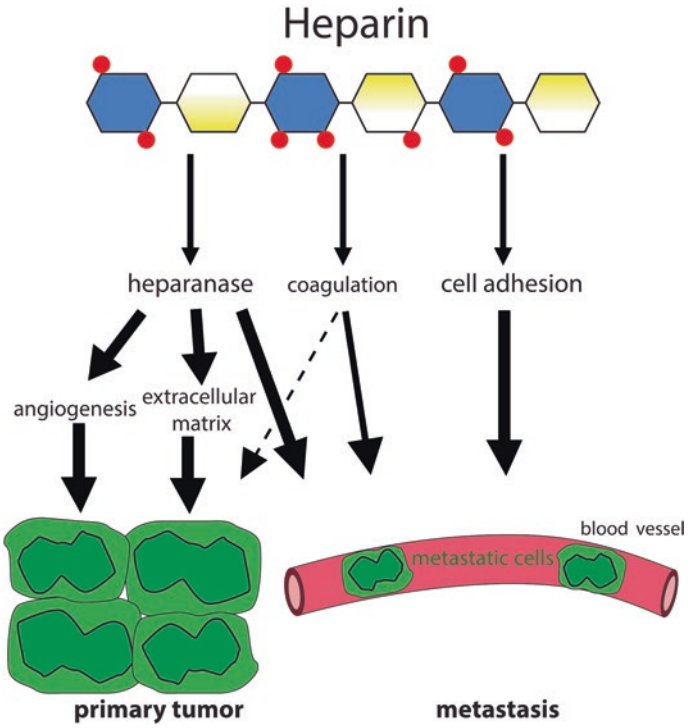

Fig. 11.1 Heparin contains diverse biological activities that affect cancer progression. The main three biological activities affecting heparanase (HPSE), coagulation and cell adhesion as discussed in this chapter. In addition, heparanase positively influences primary tumor growth through the promotion of angiogenesis, and extracellular matrix remodeling also associated with the release of growth factors. Many of these activities have been confirmed *in vitro* and *in vivo*. A direct effect of heparin on metastasis has been confirmed through inhibition of heparanase and cell adhesion

to block α4β1-integrin-mediated adhesion of melanoma cells to the endothelium [70]. Taken together, these studies provided a rationale to explore heparin as an inhibitor of cell adhesion during metastasis, albeit the heparanase-inhibitory activity remained to be resolved.

11.5 The Role of Heparin in Cancer Treatment – Clinical Evidence

The close relationship between cancer and hypercoagulability has been observed already in the nineteenth century by the French physician Armand Trousseau (reviewed in [71, 72]) and is under constant investigation since then (reviewed in [73, 74]). Cancer patients with malignant diseases are at a higher risk of developing thromboembolic complications contributing to morbidity and mortality of the disease when compared to a healthy population. This includes venous thromboembolism (VTE) which encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE); and also arterial thrombosis. Cancer-associated thrombosis in numbers is reflected in six times higher chance to develop VTE compared to non-cancer subjects, that is further increased in patients receiving chemotherapy [75]. Epidemiologically, about 15% of cancer patients will develop VTE, and about 20% of patients with VTE have an unknown neoplasm at the time of diagnosis. Consequently, VTE complications contribute significantly to morbidity and mortality of cancer patients, where VTE is the second leading cause of death. There are several mechanisms being identified to be involved in cancer-triggered thromboembolism enabling the development of specific therapies (reviewed in [72, 75]).

Antithrombotic prophylaxis or treatment is an essential component of clinical therapy especially for patient populations with an increased risk to develop VTE. A multitude of studies and meta-analyses have been performed to define an optimal pharmacological interference with the activated hemostatic system in cancer diseases (reviewed in [63]). The treatment guidelines of first-line therapy for the short- and long-term management of cancer-associated VTE, including those of the European Society of Medical Oncology (ESMO), the National Comprehensive Cancer Network (NCCN), or the American Society of Clinical Oncology (ASCO) currently recommend LMWH as first choice [76, 77]. The clinical handling of LMWH in oncology is experienced for several decades and displays a balanced safety profile, e.g., superiority over vitamin K antagonists. The guidelines recommend the treatment of acute symptomatic VTE by LMWH for longer periods, up to six months. In terms of VTE prophylaxis, LMWH is also guideline-based recommended for certain cancer patients for short term application [78]. Although heparin and particularly LMWH are used for the treatment of cancer patients with thrombotic complication for decades, the question, whether heparin treatment has other anti-cancer activities, going beyond anticoagulation, remains open.

A potential antitumor effect of an antithrombotic treatment using heparin and LMWH has been suggested based on several retrospective and prospective clinical trials of cancer patients at risk of VTE (summarized in [79]). Several controlled prospective studies in the early 2000 showed promising results especially in patients with early, non-metastatic stage of the disease. While there was no effect on overall survival in a variety of solid tumors, the analysis of a subset of patients, who were metastasis-free at the beginning of the trial, demonstrated a significant increase in survival with the LMWH dalteparin in that population [80–83]. An open-label controlled study in small cell lung cancer patients, receiving standard treatment plus/minus LMWH in a therapeutic dose for 18 weeks, showed a significant increase in the overall survival and progression-free survival in the treatment arm [84]. A comprehensive review on all clinical trials conducted by 2007 has been re-evaluated, confirming certain benefits for LMWH-treated cancer patients especially in the early stage of lung cancer [85]. Nonetheless, several further clinical studies could not, or not completely confirm this beneficial LMWH effect on overall survival, while the reduction in VTE as a primary endpoint by LMWH was significant [86–88]. Three of these studies have been performed in non-small cell lung cancer patients using three different LMWH preparations: nadroparin, dalteparin, and tinzaparin, respectively [86, 87, 89].

The reasons for this non-favored outcome in light of LMWH are likely multifaceted due to methodological issues, tumor entities, or treatment regimens; and the use of different LMWH preparations. Furthermore, it remains open to which extent the chemotherapy, which was given in parallel, interferes with any effect of LMWH on patient's survival. Finally, it needs to be considered that all LMWH preparations are tested only for anticoagulant activity, but not for the other biological activities, such as inhibition of cell adhesion or heparanase. There is a considerable difference in the effectiveness of different LMWH preparations on cell adhesion and subsequently on metastasis as tested in preclinical models [60]. Nevertheless, further studies on LMWH are warranted to address the question, how heparins affect cancer progression, beyond coagulation. The design of a clinical study should take into account the biological activity of heparins that has been proven in numerous preclinical studies.

11.6 Heparanase – Another Player in Cancer Progression

Heparanase (HPSE), an endoglycosidase, is the sole enzyme in mammalian organisms able to cleave heparan sulfate (HS) chains into HS fragments [90]. HS macromolecules have an essential role in cell signaling and communication, primarily due to their capacity to bind growth factors and cytokines and thereby create a reservoir of signaling molecules in the ECM and on the surface of cells. Thus, the capacity of HPSE to degrade HS has important implications for remodeling of the cellular microenvironment as has been shown during inflammation and cancer progression [91]. HPSE affects tumor growth and metastasis in various ways (e.g., by fostering

tumor cell extravasation, angiogenesis, bioavailability of HS-bound growth factors, etc.) discussed in other chapters of this book (Vlodavsky et al., Ilan et al.). Many types of cancer show upregulated expression of HPSE that is a marker of poor prognosis in cancer patients [92]. Experimental evidence using transgenic expression of HPSE both in tumor cells or in mice as well as HPSE knock-down strategies convincingly linked HPSE to metastasis [93, 94]. Interestingly most but not all activities of HPSE during tumorigenesis and metastasis were shown to be related to its enzymatic functionality altering the tumor microenvironment. Here we briefly recapitulate the most important aspects of HPSE concerning metastasis, while a thorough discussion of HPSE protumorigenic activities can be found in other chapters of this book and are reviewed elsewhere [5, 95].

The enzymatic function of HPSE was shown to promote tumor angiogenesis and production of exosomes that significantly contribute to the formation of metastases [96, 97]. Recently it was shown that HPSE-mediated shedding of syndecan-1 liberates peptide fragments with VEGF receptor activity [98]. Exosomes are dominant mediators of intercellular communication that drive metastasis by regulating the tumor-host cell interactions both locally within the tumor microenvironment and distally at metastatic sites [99]. The enhanced expression of HPSE in human cancer cells, as well as tumor cell exposure to exogenous HPSE possibly derived from the tumor microenvironment, was shown to dramatically increase exosome secretion through modulation of syndecan-1 signaling [97, 100]. This process relies on HPSE enzymatic cleavage of heparan sulfate and also impacts exosome protein cargo as reflected by higher levels of syndecan-1, VEGF and HGF [97] (David and Zimmermann; Sanderson et al., Chaps. 10 and 12 in this volume). HPSE was identified as a key factor in myeloma cells driving survival and resistance against chemotherapy via activating the ERK signaling pathway [101]. Treatment of colon carcinoma cells with heparanase induced expression of inflammatory cytokines such as CCL2, CCL5, and CXCL1, indicating that heparanase from stromal cells has the capacity to directly activate tumor cells and thereby modulate the tumor microenvironment [102]. Similarly, heparanase activity was shown to activate macrophages in the tumor microenvironment through the Erk, p38 signaling pathway [103]. (Hulett et al., Elkin et al., Chaps. 7 and 17 in this volume).

Concerning the non-enzymatic activity of HPSE, latent HPSE induces adhesion receptor activity for spreading and/or migration of different tumor cell types by inducing a signaling axis via binding and clustering the cellular HSPGs. Latent HPSE facilitates integrin binding and thereby promotes adhesion and metastasis of melanomas, which can be antagonized by LMWH [104]. Interestingly, latent HPSE on endothelial and cancer cells induces tissue factor (TF) expression and thus contributes to coagulation [105]. Furthermore, the latent HPSE binds and displaces tissue factor pathway inhibitor from the endothelial surface, providing another way to modulate thrombosis in cancer [106] (Nadir et al., Chap. 33 in this volume).

The multiple functional mechanisms as to how HPSE facilitates tumor progression and metastasis make it an excellent target for pharmacological inhibition.

Manifold experimental approaches exist at the preclinical and even clinical level to target HPSE from a therapeutic perspective. Not surprisingly, heparins have been one of the first compounds tested in several *in vitro* and *in vivo* models.

11.7 Heparin as an Inhibitor of Heparanase in Metastasis

The role of ECM-bound or cell surface-bound proteoglycans in cancer progression has granted the development of HPSE inhibitors based on heparin structure. Structural evaluations have led to the identification of two high-affinity heparin recognition domains of HPSE, one close to the N-terminus of the enzyme, where blockade of binding to heparin inhibits the enzymatic activity [107]. Meanwhile, in 2015 the crystal structure of HPSE has been solved, providing further insight into the mapping of substrate recognition sites [108].

Heparin has served as a structural scaffold for optimization of the structural requirements for the development of glycosidic inhibitors of HPSE. First structural modifications of heparin identified the requirements for sulfation and N-acetylation to differentiate between anticoagulant and HPSE inhibitory properties of heparin during hemostasis [109, 110]. Later on, the capacity of heparin and heparin derivatives to attenuate lung colonization by melanoma cells has been confirmed using experimental metastasis approach in mice [59]. The effectivity of heparins to attenuate metastasis was restricted to a period shortly before or after tumor cell inoculation, which was considered to inhibit predominantly the tumor cell-derived HPSE during this phase. Another group has confirmed the impact of sulfation degree of heparin derivatives on HPSE inhibition in the same melanoma experimental metastasis model [111]. Notably, in this study, the opening of the iduronic acid ring by oxidative/reductive procedures has been applied and tested for its HPSE inhibitory activity. This approach has been later identified as one of the key methods to optimize heparin for its HPSE inhibitory activity [112] (Naggi et al., Giannini et al., Noseda et al.).

A comprehensive library of heparin derivatives as potential HPSE inhibitors has been assessed *in vitro* [112]. The essential structural requirements of heparin for HPSE inhibition has been linked to its sulfation grade. A partial N-desulfation (replaced by acetylation) is well tolerated and considered as a discriminating factor between anticoagulant and HPSE-inhibiting activity of heparin. However, the preparation of a series of glycol-splitting derivatives (RO-heparins) with various degrees of N-desulfation resulted in the identification of highly efficient compounds [112]. These derivatives were used as scaffolds for the development of HPSE-specific inhibitors such as Roneparstat that has been tested in multiple myeloma patients [113] (Noseda et al., Chap. 21 in this volume). The elimination of conformational restrains by glycol-splitting of the iduronic acid leads to rotational freedom of the heparin molecule, which together with N-acetylation strongly enhanced HPSE binding and thereby inhibitory potential. The structural modifications of heparin discussed with respect to its specific biological activities

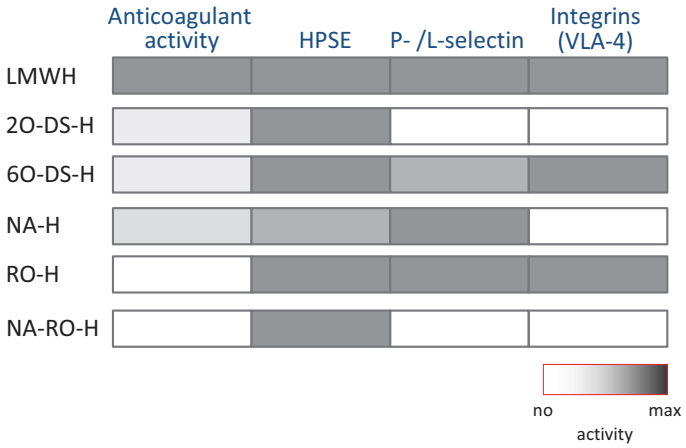

Fig. 11.2 Structural modifications of heparin and their impact on relevant targets during tumorigenesis and metastasis. The anticoagulant activity of heparin is strongly attenuated or minimalized by 2O- or 6O-desulfation; or by opening the iduronic acid ring (glycol-split heparin/RO-H), inhibitory capacity towards HPSE, P- and L-selectin or the integrin VLA-4 are largely preserved. Notably, N-acetylation of glycol-split heparin (NA-RO-H) appears to be a key to differentiate HPSE-inhibitory potential from the other targets

are schematically shown in Fig. 11.2. Further insight into the role of structural flexibility of glycol-split derivatives has recently been provided by an NMR-based conformational analysis and molecular dynamic study [114] (Naggi et al., Chap. 20 in this volume).

Glycol-split heparin derivatives with various degree of N-desulfation/N-acetylation have been tested for their antimetastatic activity [115]. RO-heparin as a specific inhibitor of HPSE inhibited melanoma metastasis, while had little effect on colon carcinoma metastasis. Interestingly, melanoma cells express high levels of HPSE while there was little expression detected in colon carcinoma cells, indicating the efficacy of HPSE inhibition only in cancers associated with high HPSE expression. Further studies investigated heparin-like polymers from mollusk origin for their capacity to inhibit HPSE besides other potential targets and thereby metastasis [116]. Novel strategies to develop HPSE inhibitors based on heparin-like structures resulted in the preparation of a glycopolymer with specific sulfation pattern that appears suitable for attenuating breast cancer metastasis [117].

Several preclinical studies using a variety of animal models confirmed the role of HPSE in metastasis (reviewed in [64, 118]). Based on many studies developing heparin-based inhibitors of HPSE devoid of anticoagulant activities resulted in the development of two drugs: roneparstat and necuparanib, that were subjected to clinical trials as a treatment for cancer with enhanced HPSE activities [113, 119]. The relevance of HPSE inhibition and its impact on cancer progression is well- defined. Nevertheless, heparin-based inhibitors require further analysis to determine the contribution of targeting other biological activities (e.g., cell adhesion) to their antimetastatic activity.

11.8 Dissecting the Role of Heparin in Cancer Progression

Heparin and its derivatives are being studied for their potential to treat cancer in numerous preclinical models, and the results of these studies indicate the capacity of heparin to inhibit cancer progression independently of its anticoagulant activity. Since heparin, particularly LMWH, is still used for the treatment of cancer patients with thrombotic complications, and based on several studies is associated with prolonged survival of some patient groups, heparin remains an attractive option for further development. Many pre-clinical studies have shown that heparin derivatives or non-anticoagulant heparin-based analogs attenuate metastasis irrespective of the animal model or cancer entity [59, 60, 115, 120–122]. As discussed in this chapter, inhibition of cell adhesion and of HPSE are likely the two biological activities contributing most significantly to inhibition of cancer progression. While several studies characterized heparin derivatives for their HPSE-inhibitory activity (e.g., 59, 120), other studies evaluated the selectin inhibitory activity [28, 60]. Later on, heparin derivatives were tested for both HPSE and selectin-inhibitory activity using experimental metastatic mouse models [115, 122]. The selectin-specific heparin derivative (57% N-acetylated-heparin) attenuated metastasis both with colon carcinoma and melanoma cells [115]. On the contrary, HPSE specific heparin derivative (100% N-acetylated, 25% glycol-split heparin) effectively inhibited melanoma metastasis but was ineffective for attenuation of colon carcinoma metastasis. When we used semisynthetic sulfated trimannose C-C dimers, HPSE specific derivative again attenuated only melanoma metastasis, while selectin specific-derivative inhibited both melanoma and colon carcinoma metastasis [122]. Of note, while melanoma cells (B16-BL6) and many other tumor cell types express relatively high levels of HPSE [115, 123, 124], colon carcinoma cells (MC-38) showed low amounts [115]. Taken together, these studies revealed that heparin-based selectin inhibition attenuated metastasis in both B16-BL6 and MC-38 cells, while the inhibition of HPSE affected metastasis of those tumor cells with enhanced HPSE activity.

In recent work, HPSE-neutralizing antibodies were tested as inhibitors of cancer progression using Burkitt's lymphoma and glioma cells [123]. While Raji cells do not produce any detectable HPSE activity, the anti-HPSE antibody significantly reduced tumor growth and metastasis of these cells. These findings indicate that heparanase derived from the tumor microenvironment significantly contributes to tumor progression [123].

11.9 Conclusions

Metastasis is in ninety percent of cancer patients the ultimate cause of death, yet there is no specific anti-metastatic therapy currently available. Based on the current understanding of metastasis, invasiveness, and mechanisms related to tumor cell

survival, HPSE, as the principal modifier of the tumor microenvironment and invasiveness; and cell adhesion mechanisms involving selectins and integrins and enabling tumor cell interactions with other cells during the metastatic process, offer most relevant targets for exploration of future therapies. While specific targeting of HPSE, based on both heparin-glycomimetics and specific antibody-based therapies are ongoing, particularly in hematopoietic malignancies [5, 123, 125], there is little progress on cell adhesion-based approaches. However, heparin derivatives have been extensively studied for their capacity to interfere with cancer progression (reviewed in [10, 16, 26]).

As discussed in this chapter, heparin carries several biological activities that are beneficial for the attenuation of metastasis. In fact, most of heparin preparations used both in preclinical and clinical studies carried out thus far contained various activities such as HPSE and selectin-inhibitory activity. Yet, only the anti-coagulation and HPSE-inhibiting activity of heparin were mostly assessed. Despite many studies, the identification of the critical biological activities of heparin for its anti-metastatic behavior is still not achieved, largely because heparin has not been rigorously tested for all or at least several known activities. Clearly, it is of scientific interest to dissect the role of heparin as an inhibitor of cancer progression, but from the clinical perspective, inhibition of multiple mechanisms involved in cancer progression by heparin might prove beneficial for cancer patients. Thus, further clinical studies designed based on the current knowledge of the potential mode of action of heparins in cancer setting are warranted.

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Chapter 12
Heparanase: A Dynamic Promoter of Myeloma Progression

Anurag Purushothaman and Ralph D. Sanderson

12.1 Introduction

Multiple myeloma is a devastating cancer that is highly dependent on the bone marrow microenvironment for growth and survival [1]. Studies over the past two decades underscore the notion that the heparan sulfate degrading enzyme heparanase plays a major role in modulating the bone marrow microenvironment to support the progression and growth of multiple myeloma. Importantly, high heparanase activity in myeloma cells correlates with enhanced bone marrow angiogenesis, myeloma growth/metastasis and osteolytic bone disease (Table 12.1). A clinical trial testing anti-heparanase therapy in multiple myeloma patients was well-tolerated and showed some potential early signs of efficacy, emphasizing that targeting heparanase is a novel strategy for myeloma therapy [2]. Though heparanase has both enzymatic and non-enzymatic functions, much of its activity in myeloma is dependent on its enzymatic cleavage of heparan sulfate chains of syndecan-1 proteoglycan present on the surface of myeloma cells [3]. Heparanase mediated structural alterations of syndecan-1 leads to enhanced shedding of this proteoglycan from the surface of myeloma cells and high levels of shed syndecan-1 in myeloma patients are associated with poor prognosis and diminished overall survival [4–6]. Shed syndecan-1 plays diverse roles in the myeloma microenvironment, including shuttling of growth factors to both tumor and host cell surfaces and enhancing the formation of signaling complexes at the cell surface [7–9]. In addition, heparanase expression by myeloma cells enhances the activation of signaling pathways (ERK, p–38) and upregulation of multiple genes (VEGF, HGF, MMP-9) associated with enhancing myeloma progression [3, 10]. Recent studies also emphasize the involvement of heparanase in exosome

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Table 12.1 Multi-functional role of heparanase in myeloma progression

Impact	References
Promotes myeloma growth, proliferation and metastasis	[8] [54] [53] [44] [45] [61] [11] [14]
Promotes osteolysis	[54] [52]
Enhance endothelial cell invasion and angiogenesis	[56] [45] [8]
Promotes exosome biogenesis and exosome docking	[66, 71] [67]
Promotes syndecan-1 shedding and myeloma cell migration	[4] [8]
Regulates signaling and gene transcription	[22] [45]
Promotes drug resistance	[74] [13] [69]

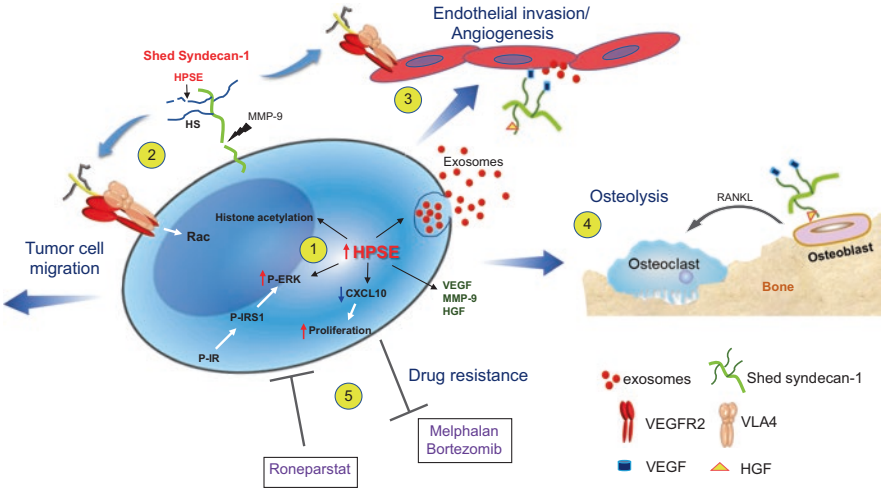

Fig. 12.1 Heparanase triggers multiple pathways that drive myeloma progression. (**1**) Enhanced expression of heparanase by myeloma cells: augments gene transcription by enhancing acetylation of histones, stimulates exosome biogenesis by trimming the heparan sulfate chains of syndecan-1 thereby priming formation of the syndecan-syntenin-ALIX complex, downregulates CXCL10 causing increased tumor cell proliferation and activates ERK via the insulin signaling pathway resulting in enhanced expression of MMP-9 and VEGF. (**2**) Shedding of syndecan-1 from the myeloma surface is driven by the heparanase-mediated trimming of heparan sulfate and by the increase in MMP-9 secretion. The shed syndecan-1 complexes with VLA-4 and VEGFR2 on the tumor cell surface stimulating Rac signaling and resulting in cell migration/invasion. (**3**) Via the same mechanism as in tumor cells, shed syndecan-1 initiates Rac signaling in endothelial cells that promotes angiogenesis. Increased angiogenesis also occurs when angiogenic growth factors (VEGF, HGF) bound to shed syndecan-1 heparan sulfate chains activate receptors on endothelial cells and when exosomes bearing VEGF, HGF and heparanase cargo dock with endothelial cells. (**4**) Similarly, HGF bound to shed syndecan-1 activates the cMet receptor on osteoblasts that via an IL-11 feedback mechanism increases RANKL secretion leading to osteoclast activation and osteolysis. (**5**) Myeloma cells having elevated heparanase expression exhibit resistance to commonly used anti-myeloma drugs, including proteasome inhibitors (bortezomib, carfilzomib) and the alkylating agent melphalan. Conversely, exposure of cells to the heparanase inhibitor Roneparstat blocks the multiple pathways that are stimulated by heparanase (*e.g.,* syndecan shedding, angiogenesis) resulting in decreased drug resistance and inhibition of myeloma growth in vivo

secretion and chemoresistance in myeloma, highlighting its potential in mediating myeloma-host interactions and in dictating the response of myeloma cells to chemotherapeutic anti-myeloma drugs [9, 11, 10, 12] (Fig. 12.1). Importantly, heparanase levels were found to be elevated in myeloma cells from patients after the first and second rounds of chemotherapy, implying that heparanase is a highly desirable and druggable target for myeloma therapy [13]. This chapter focuses primarily on the diverse mechanisms employed by heparanase in the progression of myeloma including upregulation of multiple genes involved in remodeling of the tumor microenvironment, shedding of syndecan-1 from myeloma cell surface and formation of signaling complexes at the cell surface that are involved in tumor cell dissemination, bone damage, and angiogenesis.

12.2 Heparanase Promotes Shedding of Syndecan-1 from the Myeloma Tumor Cell Surface

Shed syndecan-1 represents the soluble form of syndecan-1, containing intact heparan sulfate chains, that is released from the cell surface by proteolytic cleavage of the extracellular domain of its core protein [4]. Shed syndecan can either remain soluble or bind and accumulate on the cell surface or within the extracellular matrix [14–16]. Shed syndecan-1 is detected in a number of tumor types and in myeloma high levels of shed syndecan-1 in patient sera is an independent predictor of poor prognosis [5, 6]. This is consistent with the finding that enhanced expression of a soluble form of syndecan-1 by myeloma cells promotes tumor growth and metastasis in a mouse model [17, 18]. Importantly, heparanase upregulates both the expression of syndecan-1 and its shedding from the tumor cell surface [4, 19]. This is supported by the finding that silencing of heparanase gene expression in myeloma cells is associated with decreased levels of syndecan-1 shedding. Heparanase enzymatic activity is required (i.e., cleavage of heparan sulfate chains) for upregulation of both syndecan-1 expression and shedding because expression of enzymatically inactive form of heparanase failed to stimulate syndecan-1 expression and shedding [4]. Syndecan-1 shedding was also stimulated in myeloma cells after addition of recombinant active heparanase or bacterial heparitinase (heparinase III), indicating that cleavage of heparan sulfate chains by these enzymes renders syndecan-1 core protein more susceptible to proteolysis by proteases that mediate syndecan-1 shedding. Further, it appears that heparanase plays a more direct role in facilitating syndecan-1 shedding by upregulating the expression of proteases (sheddases) that clip the syndecan-1 core protein in the extracellular region near the plasma membrane [20].

12.3 Heparanase Modulates the Expression of Proteases by Myeloma Cells

In myeloma cells, enhanced heparanase activity leads to increased MMP-9 expression and secretion, while silencing heparanase expression results in reduced MMP-9 activity [20]. In addition, levels of molecular determinants involved in the activation of MMP-9 such as urokinase plasminogen activator and urokinase-plasminogen activator receptor are also upregulated by heparanase expression in myeloma cells [20]. These findings have potential relevance in myeloma because inhibiting MMP-9 or uPA/uPAR interactions significantly reduce the shedding of syndecan-1 by myeloma cells. Though shedding of syndecan-1 has been attributed to several metalloproteinase enzymes such as MMP-9, MMP-1, MMP-14, MMP-16 and MMP-7, a role for uPA/uPAR as a sheddase and its correlation with heparanase expression revealed a novel mechanism underlying syndecan-1 shedding. However, it is likely that in myeloma cells, uPA does not directly cleave the syndecan-1 ectodomain at the cell surface, rather it activates the cascade that drives MMP-9 activation and subsequent syndecan-1 shedding. Also, it's interesting that blocking MMP-9 or uPA/uPAR in cells expressing a low level of heparanase did not alter the constitutive level of shedding of syndecan-1 [20]. This suggests that upregulation of heparanase expression activates a shedding mechanism that is distinct from that mediating constitutive shedding. Despite the upregulation of MMP-9 by heparanase in myeloma tumor cells, in heparanase knockout mice, heparanase deficiency was accompanied by a marked elevation of MMP-9, MMP-2, and MMP-14, in an organ-dependent manner [21]. These findings suggest that the onset of heparanase expression marks a key defining event in mediating the induction and/or repression of protease gene depending on the biological setting.

Mechanistically, the upregulation of MMP-9 expression by heparanase in myeloma is mediated through the activation of ERK signaling [20]. In myeloma, heparanase-induced ERK activation is mediated through insulin receptor signaling. Heparanase promotes the phosphorylation of insulin receptors and enhances protein kinase C activity [22]. PKC activity, in turn, upregulates the levels of insulin receptor substrate-1 (IRS-1), the primary intracellular substrate for insulin receptor tyrosine kinase activity. IRS-1 plays a key role in transmitting signals from insulin and insulin-like growth factors. Tyrosine phosphorylation of insulin receptors induces the cytoplasmic binding of IRS-1 to these receptors which then undergo phosphorylation. This enables IRS-1 to activate ERK signaling [22]. Though ERK activation depends on the HS degrading activity of heparanase, the mechanism underlying this phenomenon is not clear. It is possible that trimming of heparan sulfate chains of syndecan-1 on the myeloma cell surface can trigger the clustering and activation of insulin receptors. This notion is supported by the findings that syndecan-1 couples with IGF-1 receptor on myeloma cells, and further the insulin receptor associates with IGF-1 receptors to form an insulin/IGF-1 hybrid receptor at the myeloma cell membrane [23]. This new insight into the mechanism of heparanase induced ERK activation provides a further understanding of how heparanase can impact myeloma

progression. This is particularly relevant given the known effect of ERK activation in promoting myeloma cell proliferation, survival, drug resistance, and angiogenesis [24, 25].

12.4 Heparanase Regulates Gene Expression in Myeloma Cells by Altering Histone Acetylation

Although the tumor-promoting effects of heparanase can in part be attributed to its ability to remodel the extracellular matrix barrier by cleaving heparan sulfate chains, heparanase is also known to influence gene transcription. Elevation of heparanase levels in myeloma cells upregulates expression of multiple genes, including MMP-9, VEGF, and HGF, among others [3]. Heparanase is present and active in the nucleus where it could act locally to regulate gene expression [26]. Studies have shown that translocation of heparanase to the nucleus and degradation of nuclear HS chains regulates esophageal epithelial cell differentiation [27]. Further, the presence of heparanase in the nucleus can also regulate the activity of certain nuclear enzymes. For example, in breast cancer cells, the activity of topoisomerase I, an enzyme involved in DNA replication and transcription, is enhanced by nuclear translocation of heparanase [28]. In T lymphocytes, nuclear heparanase regulates the transcription of a group of inducible immune response genes by associating with euchromatin and controlling the pattern of histone 3 methylation [29]. Heparanase modifies the histone 3 methylation pattern by associating with the demethylase LSD1 and preventing recruitment of methylase MLL. In some cancers, the distinct cellular localization of heparanase (either cytoplasmic or nuclear) may be of prognostic value [30, 31].

Acetylation by histone acetyltransferase (HAT) of N-terminal tails of histones, is a process that correlates with transcriptional activation [32–35]. This process is balanced by selectively removing acetyl groups from histones by histone deacetylase (HDAC). A shift in the imbalance between HAT and HDAC activity modulates transcriptional activity and can lead to cell apoptosis, proliferation and malignancy [34]. Importantly, in addition to heparanase, there are numerous reports showing the presence of heparan sulfate proteoglycans in the nucleus of cells and gene repression due to the reduction in HAT activity mediated by heparan sulfate chains [36–40]. In a cell-free assay, exogenous heparin was demonstrated to block HAT activity, and further, the acetylation of histone H3 is reduced by 50% in pulmonary fibroblasts exposed to heparin [40]. In addition, a decrease in histone H3 acetylation is also observed in tumor cells that take up anti-proliferative glycosaminoglycans [41]. Although the mechanism by which heparan sulfate chains inhibit HAT activity is unknown, the inhibitory activity is dependent upon heparan sulfate chain length and sulfation pattern, indicating that it is not a random inhibition but rather involves some degree of specificity [40, 41]. Regarding mechanisms by which negatively charged heparan sulfate blocks HAT activity, there are multiple possibilities

including direct binding between HS and HAT and HS blocking of the acetylation sites on histones via binding to the positively charged lysine residues (acetylation sites in histones).

Heparanase expression in myeloma cells significantly reduces the amount of syndecan-1 present in the nucleus of these cells [42]. This, in turn, is linked to high HAT activity in myeloma cells and elevated expression of genes that drive an aggressive disease [43]. The molecular mechanism by which heparanase regulates nuclear levels of syndecan-1 is not still clear. However, it's possible that nuclear heparanase degrades syndecan-1 heparan sulfate chains, thereby eliminating syndecan-1 from the nucleus. In addition, it's also possible that heparanase, via unknown mechanisms, can block the transport of syndecan-1 to the nucleus resulting in a significant reduction in total levels of nuclear syndecan-1. Together these findings reveal a novel and important function of heparanase in regulating myeloma cell behavior via upregulating HAT activity and gene transcription.

Heparanase is present and active in plasma isolated from the bone marrow of myeloma patients and studies have shown that both myeloma cells and stromal cells in the bone marrow can express heparanase [44, 19]. The finding that exogenous heparanase can cause upregulation of HAT activity by myeloma cells demonstrates an important and novel mechanism whereby gene expression can be regulated by cross talk between cells within a tumor. Heparanase released from either myeloma cells or host cells could alter HAT activity and gene expression in adjacent tumor or host cells not expressing heparanase. This may be particularly important in cancers such as myeloma, which are highly dependent on the tumor microenvironment for their survival.

12.5 How Does Heparanase Promote, Myeloma Growth, Metastasis, Angiogenesis and Osteolysis?

12.5.1 Down-Regulation of CXCL10 Cytokine

Using a Tet-on system, the mechanisms underlying the pro-tumorigenic activity of heparanase were explored in myeloma. Induction of heparanase expression in myeloma cells by doxycycline increased the colony number and size in soft agar and tumor growth in vivo. As determined by gene array profiling, the induction of heparanase was associated with downregulation of cytokine CXCL10 [45]. Overexpression of CXCL10 in heparanase-high myeloma cells results in significantly fewer and smaller colonies in soft agar compared to control cells, clearly demonstrating that CXCL10 suppresses myeloma proliferation [45]. Silencing of the CXCL10 gene or addition of CXCL10 neutralizing antibody enhanced cell proliferation and colony formation, further supporting the notion that CXCL10 can attenuate myeloma cell proliferation. Importantly, CXCL10 gene silencing resulted in tumor xenografts that were larger than control myeloma tumors, while

overexpression of CXCL10 or its injection into tumor-bearing mice resulted in a marked decrease in tumor development. CXCL10 has multiple functions in inhibiting myeloma progression. It directly inhibits myeloma cell proliferation, endothelial cell proliferation and angiogenesis, and it attracts anti-tumor immune cells [45]. CXCL10 is an interferon-inducible chemokine with potent chemotactic activity on activated effector T cells and other leukocytes that express the CXCL10 G protein-coupled receptor CXCR3. Consistent with this function, an increase in infiltration of cytotoxic NK and T cells was observed in myeloma tumor-bearing mice administered with CXCL10-Ig fusion protein [45].

12.5.2 Upregulation of HGF Expression and Activity

HGF is among the most upregulated genes in multiple myeloma, and elevated levels of HGF in myeloma are associated with poor prognosis [47, 48]. Myeloma cells produce HGF and express its receptor c-met [49]. HGF upregulation in the bone marrow microenvironment of multiple myeloma is associated with lytic bone disease [50]. HGF has a heparin binding domain and thus binds to heparan sulfate proteoglycans, in addition to binding to its c-met receptor [51]. In myeloma, HGF binds to syndecan-1 on the surface of myeloma cells and when syndecan-1 is shed from the surface, the syndecan-1/HGF complex can potentiate signaling via the c-met receptor present on distal cells [51, 52]. Apparently, heparanase expression in myeloma cells fuels this signaling pathway by increasing syndecan-1 shedding and by enhancing the expression of HGF. However, upregulation of HGF expression is not dependent on heparanase enzymatic activity [52].

Heparanase-induced HGF secretion by myeloma cells activates c-met signaling in osteoblasts leading to an increase in IL-11 secretion [52, 50]. IL-11, via a feedback loop, stimulates osteoblasts to produce RANKL, a key factor that drives osteolytic bone disease in myeloma [52]. Studies utilizing SCID-hu and SCID-tibia animal models of myeloma demonstrated that myeloma tumors, growing in bone and expressing high heparanase, increase both local and systemic bone damage as compared to control myeloma tumors expressing much lower levels of heparanase [53, 54]. This enhanced bone damage caused by heparanase appears linked to upregulation of RANKL by myeloma cells or via indirect impact on osteoblasts through the HGF/-IL-11 axis. The latter is supported by the finding that heparanase expression dramatically stimulates osteolysis in distal bones prior to the arrival of tumor cells at those sites [54]. This finding underscores the possibility that heparanase-induced production of HGF and soluble syndecan-1 by myeloma cells act upon distal osteoblasts to produce RANKL and subsequent osteolysis. Another possible impact of heparanase-mediated HGF signaling could be in mechanisms leading to minimal residual disease, a precursor to patient relapse and eventual death. Shed syndecan-1 that is known to accumulate within the bone marrow of myeloma patients likely facilitates the accumulation of a reservoir of HGF that is available for the growth of myeloma cells that escape therapy, thereby contributing

to myeloma relapse [16]. The ability of heparanase to enhance syndecan-1 shedding and the downstream effect of shed syndecan-1 in regulating the activity of HGF and other heparin-binding growth factors are likely crucial promoters of myeloma progression.

12.5.3 Enhanced Angiogenesis and Polarized Migration of Myeloma Cells

Multiple myeloma is a plasma cell dyscrasia characterized by multiple lytic lesions at the time of diagnosis. There is continuous spread or dissemination of tumor cells from the original site of tumor development to multiple sites in the bone marrow niche. Heparanase promotes both bone marrow angiogenesis and metastasis by altering the structure and function of heparan sulfate proteoglycans and contributing to tumor-mediated remodeling of both cell surfaces and the extracellular matrix [44, 55, 53]. These actions dynamically impact multiple regulatory pathways, most notably by augmenting the bioavailability of growth factors and cytokines bound to heparan sulfate chains. Using myeloma and endothelial cell models, the novel roles of heparanase in promoting metastasis and angiogenesis in myeloma have been determined. Surprisingly, key to these mechanisms is heparanase induced shedding of syndecan-1 [4, 8]. Both the heparan sulfate chains and the core protein of shed syndecan-1, through different mechanisms, participate in promoting myeloma cell invasion and endothelial cell angiogenesis.

Upregulation of VEGF Expression and Endothelial Invasion

High heparanase activity in the plasma harvested from the bone marrow of myeloma patients is associated with elevated microvessel density [44]. Heparanase, in addition to enhancing syndecan-1 shedding, upregulates VEGF expression and secretion by myeloma cells [56]. VEGF binds to heparan sulfate chains of shed syndecan-1 present in the conditioned medium of myeloma cells and when incubated with endothelial cells, this complex stimulates ERK signaling leading to enhanced endothelial invasion and angiogenesis [56]. Prior removal of the VEGF/syndecan-1 complex from the conditioned medium, either by treating with heparinase III, a bacterial enzyme that degrades heparan sulfate chains, or by immunoprecipitation using anti-syndecan-1 antibody, abolishes the activation of ERK signaling and subsequent invasion of endothelial cells. It is important to note that immunoprecipitation of shed syndecan-1 from the conditioned medium captures only the intact ectodomain core protein containing heparan sulfate chains, however, the heparan sulfate fragments generated by heparanase action remain in the medium. Surprisingly, these fragments which also have bound VEGF fail to enhance invasion of the endothelial cells [56]. Thus, it appeared in these initial studies that the key mechanism by which

heparanase promoted angiogenesis was by the upregulation of syndecan-1 shedding. The presence of shed syndecan-1 extends the range of proteoglycan function beyond that at the cell surface within the tumor microenvironment. Conceivably, shed syndecan-1 which is known to enter the circulation, could, with its bound VEGF travel to distal sites and initiate angiogenesis that supports the establishment of metastatic lesions [57].

Activation of VEGFR2 Downstream of Heparanase Activity Promotes Polarized Migration of Myeloma Cells and Angiogenesis

Though the above-described roles of shed syndecan-1 are mediated through heparan sulfate binding to VEGF, the role of the syndecan-1 core protein ectodomain in stimulating myeloma migration and angiogenesis was recently discovered. Myeloma cells expressing high levels of heparanase form a highly polarized morphology on fibronectin or VCAM, two ligands enriched in the bone marrow that are recognized by VLA integrin expressed by myeloma cells [8]. However, myeloma cells expressing a low level of heparanase or an enzymatically inactive form of heparanase failed to polarize. Interestingly, it was discovered that when syndecan-1 is shed from the myeloma surface, it exposes a cryptic juxtamembrane sites on the syndecan-1 core protein that bind VEGFR2 and VLA-4 (α4β1 integrin). This coupling of VEGFR2 (that is aberrantly expressed on myeloma tumor cells) to the integrin on the surface of myeloma cells reorients VLA-4 from the uropod (trailing edge of the cell) to the leading edge of the cell and also activates VEGFR2 leading to Rac signaling [8]. These events trigger polarized migration of myeloma cells. Interestingly, it was found that the same molecular mechanisms drive endothelial tube formation, thereby revealing a new mechanism of heparanase activity in driving angiogenesis.

Similar to the interaction of shed syndecan-1 with VEGFR2 and VLA-4, the coupling of syndecan-1 with other integrins also occurs, leading to activation of tyrosine kinases (IGF-1R, HER2, EGFR) in other types of cancer [58–60]. The signaling mechanism involving shed syndecan-1/VGFR2/VLA4 in myeloma cell migration and angiogenesis is highly dependent on heparanase stimulation of syndecan-1 shedding as the initiating step, thereby identifying an important role for this enzyme in myeloma progression. This role of heparanase was confirmed using a heparanase inhibitor Roneparstat (Noseda and Barbieri, Chap. 21 in this volume), a chemically modified anticoagulant heparin derivative, that diminishes syndecan-1 shedding and subsequent myeloma cell invasion and angiogenesis [8, 61]. It is important to note that an active motif in shed syndecan-1 is responsible for promoting the invasive phenotype in myeloma cells by coupling VEGFR2 to VLA4 [8]. This active site, amino acid 210 to 236 of the syndecan-1 ectodomain, is fully functional only when syndecan-1 is shed from the myeloma cell surface. Its binding to VEGFR2 or VLA4 can be mimicked by short peptides, called synstatins (SSTNs), encompassing part of this sequence that acts to competitively inhibit this mechanism. Peptides that bind only VLA integrin (SSTN 210–233) or VEGFR2 (214–240)

block myeloma cell invasion and angiogenesis due to their inhibition of the coupling of VGFR2 and VLA-4 [8]. The inhibitory synstatins are likely to show promise against myeloma extravasation and spread [62].

It is well documented that heparanase enhances both tumor metastasis and angiogenesis. Many of the known mechanisms of heparanase activity likely contribute to these processes in various ways. However, to our knowledge, the finding that heparanase induces shedding of syndecan-1 leading to the downstream activation of Rac is the first demonstration that, by this single mechanism, both metastasis and angiogenesis are stimulated.

12.6 Impact of Heparanase on Exosome Biogenesis by Myeloma Cells and on Exosome Docking with Target Cells

12.6.1 Exosome Biogenesis

Exosomes are best defined as extracellular vesicles that are released from cells upon fusion of endocytic multivesicular bodies with the plasma membrane [63, 64]. This liberates the vesicles contained within the multivesicular body into the extracellular milieu. Once released these vesicles are referred to as exosomes. Exosomes are composed of a vast array of cellular molecules, most prominently proteins and nucleic acids. Once exosomes dock with either adjacent or distal cells they can reprogram these recipient cells. Although essentially, all cells can secrete exosomes, cancer cell secretion of exosomes is elevated, and these exosomes can play important roles in promoting tumor progression and metastasis [65]. Heparanase plays an important role in exosome biogenesis by participating in activation of the syndecan-syntenin-ALIX complex [66–68]. Briefly, trimming of heparan sulfate chains of syndecan-1 by heparanase facilitates the binding of the syndecan cytoplasmic domain via syntenin to the syntenin-ALIX complex. This leads to recruitment of a larger complex of proteins known as the endosomal sorting complex required for transport (ESCRT). This complex activates the budding and scission process that generates the intraluminal vesicles (David and Zimmermann, Chap. 10 in this volume). Utilizing myeloma cells, it was discovered that high heparanase expression dramatically enhances cellular production of exosomes [66]. Heparanase enzymatic activity is required for enhanced exosome biogenesis because enzymatically inactive heparanase, even when present in high levels, does not substantially increase exosome biogenesis. Addition of recombinant heparanase to myeloma cells expressing a low level of the enzyme also enhances exosome biogenesis, indicating that heparanase released into the tumor microenvironment can aid in driving exosome secretion within the bone marrow [66]. Importantly, heparanase has been shown to be present in its soluble and active form within plasma harvested from the bone marrow of myeloma patients [44].

Heparanase also regulates the protein cargo of myeloma-derived exosomes which is evident from the fact that exosomes from heparanase-high myeloma cells contain high levels of syndecan-1, VEGF and HGF in their cargo compared to exosomes from heparanase-low cells [66]. The difference in exosome cargo due to heparanase expression, in fact, reflects on the impact of exosomes on tumor and host cell behavior. For example, exosomes from myeloma cells expressing high levels of heparanase stimulated myeloma cell spreading on fibronectin, and endothelial cell invasion through Matrigel matrix better than exosomes from myeloma cells expressing low levels of heparanase [66]. Further analysis of exosome secreted by myeloma cells revealed that heparanase is present as cargo on the surface of these exosomes and is available to degrade heparan sulfate within the extracellular matrix [66, 69]. Moreover, this exosomal heparanase can be delivered to cells within the tumor microenvironment and perhaps distally to other parts of the body [69]. Because of the known role of heparanase in promoting angiogenesis and metastasis, exosomes bearing heparanase may play a role in establishing niches to which tumor cells eventually home and grow.

12.6.2 Docking of Exosomes with Target Cells

The functional effects of exosomes rely on their interaction with, and subsequent delivery of cargo to, target cells. Heparan sulfate chains on the surface of target cells function as receptors for exosomes and can also assist in the internalization of exosomes [70]. A role for heparanase in exosome uptake remained unknown until recently when it was revealed that in cells expressing a high level of heparanase, the exosomes secreted contained abundant fibronectin on the exosome surface [71]. Fibronectin binds to heparan sulfate via a strong and well-characterized heparin/heparan sulfate binding domain. This region of the fibronectin protein, designated as the Hep-II domain, is located within the C-terminal repeat units 12–14 of fibronectin [72]. Exosomes from heparanase-high cells interacted with target cells much better than did the exosomes from heparanase-low cells pointing to the fact that the levels of fibronectin on exosomes correlates with exosome ability to interact with target cells [71]. Mechanistically, it was demonstrated that fibronectin is bound to heparan sulfate on the surface of exosomes and facilitates exosome interaction with heparan sulfate chains present on the surface of target cells. Therefore, cell surface heparan sulfate proteoglycans such as syndecan-1 play a dual role in the interaction between exosomes and cells. Heparan sulfate on the exosome surface binds fibronectin, and subsequently, when the exosome encounters a target cell, it binds via fibronectin to heparan sulfate on that cell. Because heparan sulfate is ubiquitously expressed on cell surfaces, the mechanism described here may be a general mechanism of exosomes binding to most cells and is likely not mediating targeting of exosomes to specific cell types. However, some specificity of exosome binding to cells could be conferred through the structure of cell surface heparan sulfate chains. For example, cells lacking heparan sulfate 2-*O*- or -*N*-sulfation exhibited reduced

exosome binding compared to cells containing these structures [70]. Cell surface heparan sulfate proteoglycans, after binding to exosomes, are internalized by cells and thus function as internalizing receptors for exosomes, rather than just cell surface attachment sites [70]. Even though the mechanism by which exosomes release their cargo within cells is not clear, once internalized, exosomes can fuse with the delimiting membrane of the endocytic compartment of target cells to deliver the cargo [73].

Together, these studies underscore the importance of the heparanase/syndecan axis in regulating the biology of exosomes and show that their impact is not restricted solely to exosome biogenesis and cargo content but also impact exosome-target cell interaction, a key step in the ability of exosomes to regulate cell behavior. These findings not only support a role for heparanase in regulating exosome action in myeloma but also expose multiple ways by which exosome-cell interactions can be therapeutically targeted in patients. Importantly, a fully sulfated 12-mer heparin mimetic, and heparin-derived heparanase inhibitor, Roneparstat, are both capable of inhibiting exosome binding to cells [71]. Since both the 12-mer mimetic and Roneparstat lack anti-coagulant activity, these compounds could potentially be delivered to patients to block exosome uptake by target cells, thereby diminishing the biological impact of exosomes in disease settings.

12.7 Heparanase Modulates Sensitivity of Myeloma Cells to Therapy

Gene expression profiling of myeloma cells from patients demonstrated that following high dose chemotherapy, the cells present upon tumor relapse exhibited a high level of heparanase expression [74, 13]. These data are clinically relevant because heparanase expression within the bone marrow microenvironment of newly diagnosed myeloma patients treated with chemotherapy and stem cell transplantation is associated with shorter survival [19]. Also, elevated heparanase level is associated with myeloma cell resistance to bortezomib and melphalan, two drugs widely-utilized for anti-myeloma therapy [74]. Mechanistically, heparanase promotes drug resistance by activating ERK signaling, and this signaling pathway requires enzyme activity of heparanase [74]. Heparanase thus plays an important role in determining the outcome of anti-myeloma therapy. Usage of inhibitors of heparanase such as Roneparstat, in combination with drugs like melphalan, can, therefore, enhance the efficacy of melphalan even against highly aggressive myeloma [74]. Moreover, in a model of dexamethasone resistant multiple myeloma, the combination of Roneparstat with dexamethasone inhibited tumor growth [61]. All these findings point to the fact that heparanase inhibitors can be potential drugs to target minimal residual disease in myeloma patients, because inhibition of heparanase may interfere with the reestablishment of a tumor-promoting microenvironment, thereby preventing relapse. Combining anti-heparanase therapy with standard chemotherapy drugs may prevent myeloma relapse and improve patient outcome.

12.8 Heparanase Inhibitor for Myeloma Therapy

Heparanase impacts multiple regulatory pathways within the myeloma microenvironment that together drive myeloma growth, dissemination, angiogenesis, osteolysis, and chemoresistance (Table 12.1, Fig. 12.1). Heparanase, therefore, plays an important role in the pathogenesis of multiple myeloma, and its inhibition will disrupt the myeloma microenvironment leading to diminished myeloma growth. These results prompted a first in man, multicenter phase I clinical study of Roneparstat in advanced heavily pretreated refractory myeloma patients who had exhausted currently available anti-myeloma therapies [2]. The drug was well tolerated and in some patients showed early signs of efficacy. Roneparstat is composed of 100% N-acetylated and glycol-split heparin. It is a potent inhibitor of heparanase enzyme activity (IC_{50} = 3 nM) that is devoid of any significant anticoagulant activity [75, 76] (Noseda & Barbieri; Cassinelli, Torri and Naggi, Chaps. 20 and 21 in this volume). The impact of Roneparstat has been tested in vivo using different models of myeloma where human myeloma tumor cells were injected either subcutaneously, into fragments of human bone implanted in mice, or intravenously into the mouse tail vein [74, 61]. Roneparstat significantly inhibited growth, angiogenesis and bone metastasis of myeloma tumors in these models. Analysis of myeloma tumors from animals treated with Roneparstat demonstrated that these tumors have diminished levels of VEGF, HGF and MMP-9, reduced angiogenesis and reduced levels of shed syndecan-1 compared to animals treated with vehicle [61]. This highlights that the mechanism of action of Roneparstat is consistent with it having anti-heparanase activity in vivo. Further, using an in vivo model of disseminated myeloma, where myeloma cells expressing a high level of heparanase home and grow exclusively in bone, Roneparstat in combination with either bortezomib or melphalan, significantly decreased both the number of animals with detectable tumors and tumor burden compared to animals treated with either of these drugs alone [74]. The ability of Roneparstat to dramatically reduce tumor growth in bone when used in combination with either bortezomib or melphalan indicates that blocking heparanase diminishes drug resistance in myeloma.

12.9 Concluding Remarks

Over the last two decades, heparanase has been shown to be involved in many important steps necessary for the progression of multiple myeloma (Fig. 12.1). Based on the copious evidence demonstrating the role of heparanase in myeloma growth, metastasis, angiogenesis, exosome biogenesis, and chemoresistance, heparanase can be defined as a multifunctional protein whose activity fuels the aggressive progression of myeloma. Surprisingly, many of the downstream impacts of heparanase are dependent on its ability to enhance the shedding of syndecan-1. Of note is the novel role of shed syndecan-1 in activating VEGFR2, by coupling

VEGFR2 with VLA-4 thereby initiating downstream signaling pathways that trigger polarized migration of myeloma cells and endothelial cells. It will be important to determine if this mechanism is at play in other types of cancer. Considering the fact that much of the heparanase function in myeloma progression is dependent on its enzymatic cleavage of heparan sulfate chains, and that heparanase is the only known mammalian endoglycosidase that cleaves heparan sulfate chains and is not expressed abundantly in normal tissue, this enzyme presents an ideal pharmaceutical target for myeloma and other cancers.

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Chapter 13
Involvement of Heparanase in Gastric Cancer Progression and Immunotherapy

Bo Tang and Shiming Yang

13.1 Introduction

Heparanase is a 61.2 kDa protein with 543 amino acids encoded by its mRNA, and then this pro-enzyme is post-translationally cleaved into 8 and 50 kDa subunits that non-covalently associate to form the active heparanase [1, 2]. Heparanase is an endo-β-glucuronidase that cleaves heparan sulfate (HS) side chains, regulating the structure and function of heparan sulfate proteoglycans (HSPG) and remodeling cell surfaces and the extracellular matrix [3–6]. HSPGs mainly inhibit cellular invasion through promoting tight cell-cell, cell-ECM interactions, and self-assembly of the ECM [7, 8], which facilitate the biological activity of bound ligands (i.e., FGF, HGF, VEGF). Cleavage of HSPGs by heparanase could release these bound ligands and convert them into bioactive mediators, ensuring rapid tissue response.

In normal cells and tissues, heparanase is kept tightly regulated at transcriptional and post-translational levels as well [9], and the gene promoter of which is constitutively inhibited and the gene is not transcribed, largely due to promoter methylation [10–12]. Nevertheless, heparanase expression is enhanced in almost all cancers including ovarian, stomach, pancreas, colon, bladder, brain, prostate, breast, liver, myeloma and rhabdomyosarcoma and so on [13–19]. Multiple evidence indicate that heparanase could not only promote the breakdown of ECM but is also is involved in regulating the bioavailability and activity of growth factors and cytokines. Briefly, cleavage of HS by heparanase could promotes tumor progression via disassembly of extracellular barriers for cell invasion, release of HS-bound angiogenic and growth promoting factors, and induction of signal transduction pathways bound to heparan sulfate to promote growth and metastasis signaling [20–22] (see chapters xyz). Various studies showed that enhanced heparanase expression correlates

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with increased tumor size, tumor progression, advanced metastasis and poor prognosis [23–25]. Moreover, knockdown or inhibition of heparanase markedly impairs tumor progression further highlighting the role of heparanase in cancer progression and the potential role of anti-heparanase therapy for multiple types of cancer [26–33]. Recent studies demonstrate that a major function of heparanase in various cancers. This review will mainly focus on the role of heparanase in gastric cancer progression and immunotherapy.

13.2 Heparanase in Gastric Cancer Progression

13.2.1 Heparanase Expression in Gastric Cancer

The role of heparanase in gastric cancer has not been well elucidated. To date, several studies investigated the expression levels of heparanase in gastric cancers. Chen et al. investigated the expression of heparanase in gastric cancer tissues and non-cancerous gastric tissues and found that the expression of heparanase mRNA was positive in 29 cases of gastric cancer with a positive rate of 67.4%, while its expression rate was only 10% in non-cancerous gastric tissues [34]. And the Expression was correlated with the tumor size, serosal infiltration, lymph node metastasis, distant metastasis and TNM staging of gastric carcinomas [32]. Tang et al. [35] investigated the expression levels of heparanase mRNA of heparanase in 116 cases of gastric carcinomas and showed that heparanase mRNA was positive in 83% of cases, while no positive labeling was identified in normal gastric epithelium. Endo et al. [36] evaluated the heparanase mRNA expression in gastric cancer tissues and normal gastric tissues using qPCR analysis, and the positive rate in gastric cancer tissues was significantly higher than that in normal tissues. Our research group also examined the expression levels of heparanase by immunohistochemistry and found that the expression levels of heparanase were significantly higher in gastric cancer tissues than those in adjacent normal tissues [37]. Thus, various studies validated the higher expression of heparanase in gastric cancer tissues.

13.2.2 Heparanase in Gastric Cancer Metastasis and Progression

The prognosis of gastric cancer is poor due to the early invasion and metastasis which are the most common causes of death in gastric cancer [38]. It is generally known that the invasion of the basement membrane and extracellular matrix is one of the critical steps for cancer cell metastasis [39]. As an important component of the extracellular matrix (ECM), heparan sulfate proteoglycans (HSPGs) can serve as extracellular barrier and functional receptor coupling with various

growth factors. Thus, degradation of HSPGs may plays a critical role in cancer cell invasion and metastasis. Heparanase is an endo-β-glucuronidase that cleaves heparan sulfate (HS) side chains, thus facilitating disassembly of the ECM and enhancing cell invasion, suggesting heparanase plays an important role in tumor invasion and metastasis. So far, The role of heparanase in tumor development and progression is well documented. Wang et al. [40, 41] reported that heparanase expression in primary gastric carcinoma cells was related to the metastatic behavior of gastric cancer. Similarly, Xie et al. [42] found that heparanase mRNA expression was significantly correlated with invasion and TNM stage of gastric cancer. Our research group also reported that heparanase is weakly expressed in the normal gastric tissues but is significantly increased in gastric cancer tissues. and the Higher expression of heparanase was associated with advanced TNM stage and depth of invasion [43] and, importantly, shorter survival time post-operation [43]. Nevertheless Notably, specific silencing of the heparanase gene significantly suppressed the adhesion, invasion, and metastasis of gastric cancer cells *in vitro* [44, 45]. Taken together, heparanase is overexpressed in gastric cancer and greatly associated with the invasion and metastasis of gastri cancer metastasis and poor prognosis. While inhibition of heparanase could suppress gastric cancer invasion.

13.2.3 Regulation of Heparanase Expression in Gastric Cancer

The vast majority of studies showed that heparanase was overexpressed in gastric cancer cells and played a key role in tumor invasion and metastasis. Various studies also focused on the regulatory mechanisms of heparanase expression in gastric cancer. Cao et al. [46] found that NF-κB signaling was significantly activated in gastric cancer tissues, and the activation was related to increased heparanase gene expression and correlated with poor clinicopathological characteristics such as lymphatic invasion, pathological stage, and depth of invasion, suggesting that NF-κB signaling is a major controller for regulator of heparanase expression in gastric cancer. Moreover, our research group further reported that HGF, a growth factor that binds HSPGs, could significantly increase heparanase expression at both mRNA and protein levels through PI3K/Akt/NF-κB signaling pathway and finally promote gastric cancer metastasis [43]. We also found that telomerase reverse transcriptase (TERT) could act as a co-activator of c-Myc to transactivate heparanase promoter activity, upregulate heparanase expression in gastric cancer cells, and promote invasion and metastasis of gastric cancer [37]. MicroRNA (miRNA) as small non-coding RNA molecules with 18–25 nucleotides post-transcriptionally regulate gene expression in various cancer types [47, 48]. Shi et al. [49] reported that miR-1258 could act as a tumor suppressor to inhibit invasion and metastasis by inhibiting heparanase expression. miR-429 also acts as a tumor-suppressor gene to inhibit transcription

and translation of the heparanase gene, and reduce the invasion ability of gastric cancer cells by downregulating heparanase expression [50]. On the other hand, miR-558 recognizes its complementary site within the heparanase promoter to decrease the binding of Smad4, and hence activate the transcription and expression of heparanase in gastric cancer cell lines [51]. These findings demonstrate that different genes facilitate the progression of gastric cancer through directly regulating heparanase expression.

13.3 Heparanase as an Immunotherapeutic Target in Gastric Cancer

Nowadays, immunotherapy has emerged as a novel strategy for cancer therapy because of its weak side effects and targeting characteristics. One of the key components of immunotherapy is using the immune cells to be loaded with the tumor-associated antigens (TAAs) to induce antigen-specific anti-tumor immunity [52–54]. An ideal TAA is supposed to be uniquely expressed in tumors, which can induce not only antitumor immunity but also have crucial functional roles in tumor development [55, 56]. It is well documented that heparanase is overexpressed in almost all malignant tumors, and higher expression is associated with tumor progression and poor prognosis [57, 58]. Therefore, heparanase has been recognized as a suitable universal TAA in because of its crucial role in progression and metastasis of multiple tumors.

13.3.1 Heparanase Gene-Based Immunotherapy

Dendritic cells (DCs), the most efficient antigen-presenting cells (APCs), have an important role in the initiation and regulation of tumor-specific immune responses, leading to the rapid development of DC-based cancer immunotherapy. Genetic modification of DCs with TAA genes is an effective strategy for activating DCs in tumor immunotherapy [59, 60]. Our research group firstly used the recombinant adenovirus vector containing the full-length cDNA of heparanase (rAd-Hpa) to transfect DCs from peripheral blood mononuclear cells of healthy HLA-A2-positive donors to generate heparanase gene-modified DC vaccine [61]. Then, this genetically modified DC vaccine was used to activate T lymphocytes from the same donors to generate heparanase- specific cytotoxic T lymphocytes (CTLs). The study showed that the modified DCs activate heparanase-specific CTLs, resulting in specific lysis of human gastric cancer KATO-III cells that were heparanase positive and HLA-A2 matched, while there was no killing effect on SGC-7901 cells that were heparanase positive but not HLA-A2 matched. Meanwhile, the studies revealed that the modified DCs can increase interferon IFN-γ secretion by the CTL cells to enhance non-specific immunological killing [61].

13.3.2 Heparanase Peptide-Based Immunotherapy

CTLs play key roles in tumor immunosurveillance through recognition of TAAs expressed on the surface of tumor cells [62, 63]. It is well known that CTL epitopes binding to MHC, rather than integral TAA, induce CTL reactions [64]. These epitope peptides usually are comprised of eight to ten amino acids, with two to three primary anchor residues that interact with the MHC-I molecules and two to three amino acid residues that bind to the T cell receptor (TCR) [64–66]. Therefore, identification of suitable CTL epitopes from TAA is extremely important for targeted immunotherapy.

Heparanase, as a suitable universal TAA, was firstly predicted Three epitopes derived from the human heparanase amino acid sequence were first predicted by Sommerfeldt et al. [67]. Their results showed that DCs loaded with the three predicted peptides of human heparanase (hHpa) could promote heparanase-specific CTLs to lyse cancer cells [67]. Our research group used super motif and quantitative motif methods to predict another three HLA-A2-restricted heparanase epitopes including hHpa277 (277–285, KMLKSFLKA), hHpa405 (405–413, WLSLLFKKL), and hHpa525 (525–533, PAFSYSFFV), which were found to elicit HLA-A2-restricted CTL responses specific for KATO-III gastric cancer cells, SW480 colorectal cancer cells and U2OS osteogenic sarcoma cells [68]. To investigate the *in vivo* immune response elicited by heparanase CTL epitopes, we further predicted candidate CTL epitopes derived from the mouse heparanase protein (mHpa) [69]. *In vitro* experiments showed that the predicted peptides could activate heparanase-specific CTLs to lyse three kinds of carcinoma cells expressing both heparanase and H-2Kb. *In vivo* experiments further indicated that the predicted peptides could immunize against tumors and successfully treat tumor-bearing hosts [69]. We further evaluated the *in vivo* immune response elicited by the above human heparanase CTL epitopes including hHpa277, hHpa405 and hHpa525 using HLA-A2 transgenic C57BL/6 mice and showed that these peptides could be presented naturally in vivo and also elicited heparanase-specific lysis of various gastric cancer cells [70]. These results suggest that the predicted heparanase peptides are novel CTL epitopes capable of inducing heparanase-specific CTLs *in vitro* and *in vivo*, serving as valuable targets for immunotherapy.

13.3.3 Multiple Antigen Peptide (MAP)-Based Immunotherapy

Synthetic epitope peptides are inadequate to clinical use due to their small molecular weight, single structure, weak immunogenicity, and rapid degradation, which cannot elicit an ideal immune response in the body [71, 72]. Nowadays, the multiple antigen peptides (MAP) can increase the molecular weight of the peptide, elevate immunogenicity and boost its activity against tumors [73, 74]. Yang et al. [75] firstly designed 3 MAP vaccines of human heparanase based on B-cell epitopes and found

that these MAP vaccines could inhibit the invasiveness of tumor cells *in vitro*. We then designed three 4-branched MAPs based on the human leukocyte antigen (HLA)-A2-restricted CTL epitopes of human heparanase. The results showed that the MAP vaccines could induce heparanase-specific CTL and much stronger lysis of gastric cancer cells compared with their corresponding linear peptides without killing effect on heparanase-expressing autologous lymphocytes and dendritic cells [72]. Zhang et al. [76] designed 8-branched MAPs comprising FLNPDVLDI and found that it could induce specific CTLs for human heparanase *in vitro*, which effectively secreted IFN-γ and potently lysed human tumor cells. These findings indicate that MAP vaccines based on CTL epitopes of human heparanase might be valuable for cancer immunotherapy.

13.3.4 Heparanase in CAR T-Cell Therapy

The generation of chimeric antigen receptor (CAR) T-cell therapy has revolutionized T cell-based immunotherapy for the treatment of some cancers [77–79]. CAR T-cells (which are genetically engineered T cells expressing CARs on their surface) therapy is a form of adoptive cell therapy which has recently gained attention due to success in clinical trials and FDA approval [80–82]. CAR T-cell therapy is mainly used in treating hematological malignancies particularly in infants, achieving up to 90% clinical response rates in acute lymphoblastic leukemia [83], which leads to numerous clinical trials of CAR T-cell therapy against multiple hematological antigens such as CD19, CD20 and CD22 [84, 85]. However, the clinical efficacy of CAR T-cell therapy in solid tumors has been greatly limited with side effects and lack of therapeutic response [86–88]. Multiple factors are responsible for limiting the efficacy of CAR T-cell therapy in solid tumors such as gastric cancer, colorectal cancer and breast cancer. Among these factors, the extracellular matrix (ECM) around solid tumors may hinder T-cell penetration [89]. Heparanase is the only known mammalian β-D-endoglycosidase capable of cleaving the heparan sulfate chains of HSPGs, thereby degrading ECM. Based on this, Ignazio et al. [90] firstly engineered CAR-T cells to express heparanase and showed improved capacity to degrade ECM, which promoted tumor T-cell infiltration and antitumor activity. The results suggest that modification of CAR T-cells to express high levels of heparanase may be of benefit for the application of CAR-T cells in individuals with stroma-rich solid tumors.

13.4 Conclusions and Perspectives

Since the cloning of the heparanase gene, its effect was mainly associated with tumor metastasis and angiogenesis, both major aspects of tumor progression [91–93]. In addition to its enzymatic HS-degrading activity, heparanase also acts *via*

non-enzymatic mechanisms that regulate various signal transduction, exosome formation [94–96], autophagy [97, 98], inflammation [99, 24, 100] and chemoresistance [101]. Nevertheless, the role and mode of heparanase action in gastric cancer remain to be better elucidated. Increasing studies focusing on the expression and role of heparanase in gastric cancer demonstrated that heparanase is overexpressed in gastric cancer and closely associated with cancer metastasis and poor prognosis. It is therefore essential to elucidate the mechanisms regulating heparanase expression in gastric cancer, including the involvement of TERT, HGF, and miRNAs. Our understanding of how heparanase is upregulated in gastric cancer is still incomplete. Another challenge in the field is the development of clinically effective heparanase-targeted therapy to treat cancer. Nowadays, immunotherapy has emerged as a novel strategy for cancer therapy because of its weak side effects, specificity and targeting characteristics. Heparanase has been recognized as a suitable universal TAA because of its crucial role in progression of multiple tumors. Several heparanase-based CTL epitopes have been shown to elicit specific antitumor immunity *in vitro* and *in vivo* against various tumors, which could be further enhanced by creating MAP vaccines. Moreover, generation of CAR-T cells with high expression levels of heparanase was found to promote tumor T-cell infiltration and antitumor activity. Further studies are needed to unravel the mechanisms of heparanase action in gastric cancer and optimize heparanase-targeted immunotherapy of gastric cancer.

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Chapter 14
Involvement of Heparan Sulfate and Heparanase in Neural Development and Pathogenesis of Brain Tumors

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14.1 Malignant Brain Tumors

14.1.1 Incidence and Symptoms of Brain Tumors

Primary malignant central nervous system (CNS) tumors represent about 2% of all cancer types, although it accounts for high mortality rates [19, 102]. Brain malignancies are the leading cause of death from solid tumors in children and the third cause of death from cancer in adolescents and adults aged from 15 to 34 years [84]. Even though some tumors may be classified as benign, it can be deadly as a result of continuous growth and invasion into the confined space of the brain. Common symptoms and treatment options may vary depending on the tumor type, location, size and the age of the patient. Characteristic symptoms of brain tumors are headaches, vision problems, seizures, memory loss, and poor coordination. Meningiomas are the most common benign brain tumors, and gliomas that encompass the highly aggressive, grade IV GBM, are the most prevalent malignant brain tumors [19]. Primary brain tumors present a bimodal distribution, exhibiting a smaller peak in the pediatric population, at 5–9 years old, and a significantly higher number of affected individuals in the 60–69 age group [22]. The majority of the CNS tumors thus appear after 50 years of life, but also, they are the second most frequent cancer type in children between 5–15 years [22].

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14.1.2 Glioma and Glioblastoma

Malignant gliomas are the most common primary brain tumor. They often exhibit an infiltrative nature and resistance to radio/chemotherapy, as well as destruction of peri-tumor normal brain tissue. Grade IV glioma also called glioblastoma (GBM), is the most frequent and most malignant form of glioma, with only a 15-month median survival time for patients receiving standard therapy, i.e., maximal safe resection, followed by radiation and chemotherapy [172]. GBM is histologically characterized by diffuse infiltration, high cellular density, microvascular proliferation, and areas of necrosis surrounded by pseudopallasiding cells. GBMs can be divided into primary and secondary GBM, where primary GBMs account for 90% and where the disease occurs without the existence of low-grade gliomas. Secondary GBMs, on the contrary, develop from lower grade glioma.

14.1.3 Genetic and Epigenetic Alterations in Glioblastoma

Development of GBM involves the accumulation of a large number of genetic and epigenetic alterations, such as point mutations, chromosome rearrangements, insertions, deletions, copy number alterations, and aberrations in DNA methylation as well as histone modifications. The loss of heterozygosity (LOH) in GBM is frequently found in chromosomes 1p, 10p, 10q, 13q, 19q, and 22q. LOH in 10q (47–70%), 10p (47%), *EGFR* amplification (36%), $p16^{INK4a}$ (31%), *TP53* (28%), and *PTEN* (25%) are most frequently present in primary GBM. In secondary GBM, the most common alterations are LOH in 22q, *TP53* mutations (65%), and LOH in 19q (54%) and 10q (54–63%) [127]. Moreover, mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene have drawn attention as a novel paradigm in prognosis and is nowadays included in the diagnosis according to the WHO criteria [108]. This mutation was initially identified in integrated genomic sequencing, where it was found that recurrent mutations occur in the active site of *IDH1* in 12% of all GBM patients. *IDH1* mutations preferentially occur in younger patients, and in most patients with secondary GBM. Furthermore, patients with an *IDH1* mutation display a significantly favorable prognosis [130]. The cytosolic isocitrate dehydrogenase 1 protein encoded by *IDH1* is responsible for the reduction of NADPH, and the production of NADPH is essential for the regeneration of reduced glutathione, which eventually leads to resistance to apoptosis and protection against oxidative damages [95]. This may explain the elevated sensitivity to therapies in mutant *IDH1* cells.

Generally, GBM cells display global hypomethylation, but also regional hypermethylation at selected gene-associated CpG islands, that are un-methylated under normal conditions. Promoter hypermethylation frequently occurs in the MGMT (O^6-methylguanine-DNA methyl-transferase) gene, TIMP-3, and RB1 [127]. Histone modifications greatly influence transcription, and they are less stable than

DNA methylation and balanced by activities of histone-modifying enzymes, such as histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs). Recurrent *H3F3A* mutations are prevalent in pediatric and young GBM patients [155], and different *H3F3A* mutations may suggest different cellular origins [173]. As an example of the relevance for histone modifications, the lysine-specific demethylase 1 (LSD1) was found to regulate the tumorigenicity of GBM by influencing the expression of Olig2, Sox2, and POU3F2 [91].

14.1.4 Aberrant Signaling Pathways in Glioblastoma

Studies of human GBM genomes and large-scale gene expression profiling have identified several signaling pathways commonly mutated in GBM. Primarily they are as follows: the PI3K-PTEN-AKT-mTOR pathway, the RAS/MAPK pathway, the TP53 pathway, and the RB pathway. These are key pathways controlling cell proliferation, survival, apoptosis, tumor angiogenesis, and metastasis.

The PI3K-PTEN-AKT and RAS/MAPK pathways are both induced by receptor tyrosine kinases (RTKs). Receptors of EGF and PDGF, two growth factors important in GBM tumorigenesis and CNS development, belong to RTKs and the amplification of the EGFR and PDGFR genes has been found in 13% and 45% of GBMs, respectively [1]. The activation of the RTK pathways subsequently initiates downstream effectors, such as PI3K and RAS. PTEN can antagonize the function of PI3K, and the mutation or deletion of PTEN has been reported in 38% of GBM [1]. Neurofibromin 1 (NF1) is a negative regulator of RAS [111], and NF1 mutations and deletions have been found in over 20% of GBM [1].

TP53 and RB are crucial regulators in cell cycle progression and tumor suppressor genes, frequently silenced in GBM. Normally, in the G_1 phase, pRB is inactivated by cyclin D/cyclin-dependent kinase 4/6 (CDK4/6)-induced phosphorylation, which leads to a release of E2F and the subsequent entry into the S phase. $p16^{INK4a}$ is an inhibitor of cyclin D/CDK4/6, and $p14^{INK4a}$ neutralizes MDM2, an E3 ubiquitin ligase negatively regulating TP53. They are both encoded by *CDKN2A* and are frequently homozygously deleted in GBM (2008). TP53 can bind to the promoters of over 1000 downstream genes, including p21, which subsequently blocks the cell cycle and initiates programmed cell death.

14.1.5 Molecular Classification of Glioblastoma

Glioblastoma is characterized by an extreme inter- and intra-tumoral heterogeneity [25]. The Cancer Genome Atlas research network (TCGA) has provided molecular classification of adult GBMs based on their gene expression and mutational profiles. Hence, defined molecular GBM subgroups have been identified [181] [189]. The Proneural (PN) subtype is associated with PDGFRA, IDH1, and TP53 mutations.

High expression of PDGFRA, NKX2–2, and OLIG2, i.e., genes associated with oligodendrocyte development is a typical feature of PN GBM. A subset of younger GBM patients with PN tumors exhibits global hypermethylation (termed glioma-cytosine-phosphate-guanine-CpG island methylator phenotype, G-CIMP) [16] and have better prognosis than G-CIMP negative tumors. However, comprehensive DNA methylation profiling in a large cohort of glioma patients shows that the relation of G-CIMP and IDH1 status is not always correlated [16].

The Classical subtype (CL) is characterized by amplification of EGFR homozygous, deletion of the Ink4a/ARF locus and lack of TP53 mutations. In addition, neural stem and progenitor cell markers were highly expressed in CL GBM. As mentioned above, loss of MGMT and incapacity of this enzyme to repair the mismatch increases the sensitivity to TMZ. MGMT-methylated tumors of the CL subgroup respond better to temozolomide as compared with non-MGMT-methylated CL GBMs [124].

The mesenchymal (MES) subtype is characterized by hemizygous NF1 deletion and low levels of NF1 mRNA. Mesenchymal markers (CHI3L1/YKL40, MET) and astrocytic markers (CD44, MERTK) are high in the MES subtype. Several markers of the MES subtype are shared with the EMT process [203]. There is a high degree of necrosis and inflammation in MES GBMs and an expression signature of wound healing, and NF-κB target genes [181].

14.1.6 Medulloblastoma (MB)

Medulloblastoma is the most common form of malignant embryonal pediatric brain tumor. It is believed to arise from granule neuron progenitors (GNPs) or other undifferentiated stem or progenitor cells in the cerebellum, in or near the brain stem [55, 199]. MB have traditionally been divided by histopathology; classical MB (> 70% of cases) characterized by dense small round cells with large nuclei and small cytoplasm. The desmoplastic/nodular MB (D/N) (~20% of cases) were named due to the high connective tissue content in the internodular regions. The anaplastic/large cell MB (LC/A) (~5% of cases) with a poorer prognosis has been defined by round cells with prominent nucleoli, numerous mitotic figures and apoptotic bodies [38, 72]. MB with extensive nodularity (MBEN) shows a better prognosis [138].

14.1.7 Molecular Subtypes of Medulloblastoma

Studies over the last ten years have revealed, distinct molecular MB subtypes that are related to clinical outcome. This has, at least to some extent, enabled patient-specific treatment options, sparing children with the most benign variants from extensive treatment, thus reducing some treatment-related side effects. MB has been recently further categorized into subtypes of the already existing subgroups that

reveal intertumoral heterogeneity, by combining expression and methylation data analysis of patient samples [23]. This allows further categorization of MB tumors and provides prognostic information and risk stratification. Very recent single-cell transcriptomic studies demonstrate different cellular origins for the different molecular subtypes, mirroring the different cell populations in the developing cerebellum [182].

The WNT subgroup has the most favorable prognosis among all the subgroups and represents 10% of all MBs [188]. Approximately all the WNT MBs are identified by activation of the WNT signaling pathway, usually caused by activating mutations in the beta-catenin (CTNNB1) gene resulting in stabilization of the protein [40]. WNT MBs have been further classified into WNT α and β [23].

The SHH subgroup has an intermediate prognosis and SHH MBs represent 30% of all MBs; with 5-year survival ranging between 60–80% [166]. The most frequent alterations in SHH MBs appear in the SHH pathway components, mainly patched1 (PTCH1), suppressor of fused (SUFU) but also focal amplifications of MYCN and GLI2 [188]. Also, mutations of TP53 are found in childhood SHH MBs and are in more than half of these cases associated with germline TP53 mutations from the Li-Fraumeni syndrome and have poor outcome. Additionally, SHH MB has recently been further classified into 4 different subgroups. The SHH α subtype is defined by TP53 mutations, MYCN and GLI2 amplification associated with a very poor prognosis in children, while SHH β MBs are relatively metastatic, thus resulting in poor outcomes in infants. The SHHγ subtype is characterized by better outcomes in infants without signature mutations in comparison to SHHδ subtype, which frequently contains TERT promoter mutations and defines mostly adult patients [23].

The Group 3 subtype of MB is the most aggressive and invasive of the four subgroups and represent approximately 25% of all MBs [166]. Group 3 tumors are often located in the fourth ventricle near the brainstem, but as they show a very invasive phenotype, almost 50% of Group 3 patients display metastasis at diagnosis [143]. There is amplification of MYC in approximately 20% of cases in this subgroup. Notch and transforming growth factor beta (TGF-β) signaling pathways have also been found altered in Group 3 MBs [89, 125]. Recent integrative analysis suggests 3 subtypes in Group 3 MB: Group 3α tumors which exhibit metastasis at diagnosis; Group 3β have a high frequency of GFI1 activation and orthodenticle homeobox 2 (OTX2) amplification and Group 3γ, often exhibiting MYC amplification with an invasive phenotype at diagnosis [23].

The Group 4 subtype has an intermediate prognosis and comprises 35% of all MBs [166]. They are often located into the fourth ventricle near the brainstem and are commonly metastatic at diagnosis. Frequent changes in Group 4 MBs include inactivating mutations in the histone lysine demethylase gene KDM6A, gene duplication of synuclein-alpha interacting protein (SNCAIP) as well as MYCN and CDK6 gene amplification [116]. Group 4 tumors can be further subdivided into 3 subtypes: Group 4α usually has focal CDK6 amplification, chromosome 7q gain, 8p loss and MYCN amplification. Despite the fact that Group 4γ exhibits similar mutation profile, it does not have MYCN gene amplification. Finally, Group 4β is augmented in SNCAIP and PRDM6 overexpression [23].

14.2 Cancer Stem Cells

14.2.1 The Concept of Cancer Stem Cells

The regulation of stem cell number in tissues needs to be highly controlled since mutations affecting stem cells may result in uncontrolled proliferation, and ultimately the development of cancer. The concept of cancer stem cells (CSCs) describes the stem cell-like cell of origin, which is believed to initiate tumor formation (reviewed in [28]). At the same time, the term cancer stem cell is also used for those rare self-sustaining cells in a tumor that have properties such as the specific ability to resist irradiation and chemotherapies, not shared by the bulk of tumor cells [109]. This preserves dormant cancer stem cells, that can seed a new tumor and are believed to be responsible for relapse after therapy. Cancer stem cells thus possess a unique capacity for growth and progression and are probably responsible for relapse. The cancer stem cell hypothesis predicts that solid tumors have a hierarchical organization, where CSCs drive tumor maintenance and recurrence. Tumor expansion would thus be the result of unlimited ability for self-renewal by CSCs that are more resistant to chemotherapy and irradiation, than the majority of tumor cells. Therefore, unless the CSCs are targeted, cancer treatment will not be successful. Multiple studies have described this concept for malignant brain tumors, both those affecting the adult population [164] and pediatric brain tumors [68]. Over the last decade, attempts have been made to define characteristics and markers of brain tumor stem cells (reviewed in [99]) but the hypothesis has been challenged by the concept of intrinsic plasticity driving tumor potential in a non-hierarchical manner [34].

14.2.2 Models of Cancer Stem Cells from Brain Tumors

Building on the view that CSCs constitute only a marginal part of the total tumor, studies of whole tumors are not the best model of the CSC niche, and therefore a reason to culture CSCs separately. Furthermore, if the hierarchical structure of CSCs is correct, new CSC clones with different genetic alterations may emerge over time due to selection and genomic instability, giving rise to tumor heterogeneity. This underscores the need for large numbers of cell lines for each tumor type. Finding new drugs and drug combinations that target the CSCs remains an unmet medical need. Development of drugs for this purpose has been hampered by the lack of valid cell models [101] and cancer drug screens have relied on serum-cultured cell lines. GBM cells can be expanded using neural stem cell culture conditions [139], and we have established a panel of clinically annotated and experimentally validated cancer stem cell lines from GBM [195]. This resource, termed the Human Glioma Cell Cultures (HGCC), is a collaborative effort to provide newly established and well-characterized cell lines derived from GBM patient tumor tissue. The

HGCC cell lines which have been established and cultured under stem cell conditions are available as an open resource (http://www.hgcc.se) along with accompanying data for research and drug discovery.

Serum-free culture conditions are presumed to preserve characteristics of the original tumor, but it has proven more challenging to propagate medulloblastoma than GBM, using neural stem cell culture conditions. This is illustrated by the observation that classical medulloblastoma cell lines, established in the 1980s are still the most prevalent cell culture methods for this disease (reviewed in [80]). The difficulty in establishing patient-derived medulloblastoma cell lines also skews in vitro studies because the WNT and Group 4 subtypes, while comprising half of the cases, are very scarcely represented when it comes to cell lines. A recent serum-free protocol that employs high-adherence plastic for monolayer culture, rather than sphere formation, showed improvement in success rate [152]. Alternatively, unmanipulated, human medulloblastoma cells can be propagated as xenografts, retaining stem cell-like properties [32].

14.3 Heparan Sulfate and Heparanase in Neural Development

14.3.1 Heparan Sulfate and Heparanase in Development

Heparan sulfate proteoglycans (HSPGs) are composed of a core protein onto which highly charged sulfated saccharide chains are attached. They interact with a large number of physiologically important molecules. The major enzyme that degrades HS is heparanase (HPSE), an important regulator of ECM remodeling that has been shown to promote the growth and invasion of several cancer types. The crucial role of heparan sulfate (HS) in mouse development has been demonstrated by a number of mutational studies on HS biosynthesis and modification enzymes. The deletion or deficiency in enzymes required for biosynthesis initiation and elongation leads to almost a complete lack of HS that causes severe phenotypes. GlcAT-1 knockout mice showed embryonic lethality before the 8-cell stage because of failed cytokinesis [82]. Mice deficient in EXT1 failed to gastrulate and generated smaller embryos due to defects in forming organized mesoderm and extra-embryonic tissues [106]. Mice with the complete depletion of EXT2 exhibited phenotypes similar to EXT1-deficient mice. Although, the heterozygotes had a normal life span and were fertile, they displayed multiple abnormalities in cartilage differentiation [171] and failed to respond properly to FGF signaling [159]. NDST enzymes define the basic sulfation state of HS chains and NDST1 deficiency resulted in neonatal lethality due to a condition resembling respiratory distress syndrome [146]. Disruption of C5 epimerase led to perinatal lethality, with renal agenesis, lung defects, and skeletal malformations [104]. Depletion of uronyl 2-O-sulfotransferase/glucosaminyl 6-O-sulfotransferase-1/glucosaminyl 3-O-sulfotransferase-1 led to perinatal,

embryonic, and partial lethality, respectively [20, 62, 162], while depletion of XylT2, NDST2, SULF1, and SULF2 in mice only caused mild phenotypes [4, 29, 45, 96]. However, SULF1/SULF2 double mutant mice exhibited delays in myogenic differentiation and regeneration after skeletal muscle injury [97], and NDST1/NDST2 double-knockout embryos died as early as E3.5 [71].

The importance of HSPGs in neural development is well established, for example, in axon guidance. Mice with the conditional knockout of EXT1 in nestin-positive cells showed severe guidance errors in major commissural tracts [77]. Complete loss of HS2ST or HS6ST1 led to axonal navigation errors in retinal ganglion cells [140] and severe corpus callosum phenotypes via the alteration of ERK signaling [27]. In addition, HS has also been shown as a requirement for neural progenitor cell proliferation via modulating cell signaling. For instance, the loss of HS2ST resulted in a significant proliferation reduction in cerebral cortical precursors [113]. Ablation of perlecan in the developing mouse brain led to decreased delayed cell cycle progression in neural progenitors due to altered SHH and FGF2 signaling [56]. Furthermore, syndecan-1 knockdown reduced neural proliferation via modulating response to WNT ligands [190].

Heparanase (HPSE) is the predominant degradation enzyme for HSPGs. It is an endo-ß-glucuronidase that cleaves the ß-1,4-glycosidic bond between D-glucuronate and D-glucosamine, liberating fragments between 4 to 7 kDa [185]. The active form of HPSE is secreted, and it acts on the cell surface and ECM, releasing HS-binding molecules and dissembling the ECM in association with cell migration and tissue remodeling. There are also reports suggesting the nuclear translocation of HPSE during cell differentiation [88] and in tumor cells [123]. Under normal conditions, HPSE is expressed in platelets, mast cells, placental trophoblasts, keratinocytes, and leukocytes. In pathological conditions, such as inflammation, atherosclerosis, and cancer a marked elevation of HPSE expression is frequently observed [183, 184] (Vlodavsky et al., Gaskin et al., Ilan et al., Chaps. 1, 7 and 9 in this volume).

Mouse strains overexpressing HPSE or that are devoid of HPSE have been generated. Somewhat surprisingly, neither of these mouse strains exhibits severe phenotypes; they are fertile and have a normal life span, without prominent functional or pathological alterations. HPSE knockout mice exhibited an accumulation of long HS chains and showed a marked elevation in matrix metalloproteinase (MMP) [201]. HPSE-overexpressing mice had a profound decrease in HS chain length and exhibited a reduction in food consumption and an accelerated hair growth rate. Also, they showed increased levels of urinary proteins, enhanced neovascularization, and disruption in epithelial basement membranes [202]. There is very little information about HPSE in brain development, but the levels have been reported to be highest during early postnatal development, especially in the neocortex [121]. The same authors found that there is differential expression between different regions of the brain, and in the neocortex, the amount of enzymatically active HPSE decreases sharply after the first two weeks after birth.

14.3.2 *Heparan Sulfate-Dependent Signaling in the Neural Stem Cell Niche*

The neurogenic and tumorigenic niches are similar and we have reported that the composition of the extracellular matrix (ECM) of the former undergoes developmental changes [13]. Heparan sulfate proteoglycans (HSPGs), main components of the niche modulate the activities of other factors, e.g. growth factors (reviewed in [197]). A vital role for HS biosynthesis in neural stem cell differentiation has been reported [46]. One crucial role of HS is to function as a co-receptor for growth factors on the cell surface. The mechanism of HS-dependent signaling was first found and has since been extensively studied, with regard to FGF2. HS chains increase the binding affinity of FGF to its receptor [142, 200] and play an essential role in ligand-receptor binding kinetics [47]. A similar signaling model was later described in other pathways, including BMP, WNT, SHH, PDGF, and VEGF signaling [2, 42, 54, 114, 144, 150, 177].

Besides regulating cell signaling, HSPGs have multiple functions in cell physiology. They transport chemokines across cells and present them on the cell surface. Serving as a component of the ECM, HS chains facilitate cell-ECM interaction and cell adhesion via cooperation with integrins and adhesion receptors. As receptors for proteases and their inhibitors, HS chains regulate their activity and spatial distribution. Altogether, HSPGs have the potential to manipulate major processes in the body and therefore have important implications in normal stem cell differentiation, development, and pathological conditions. HPSE, by virtue of cleaving HS, can modulate these signaling cascades, for example, HPSE is necessary to sustain FGF-2 signaling in epithelial-mesenchymal transition of proximal tubular epithelial cells to form myofibroblasts [112]. Moreover, FGF2-signaling in melanoma cells is modified by HPSE [145].

14.3.3 *Heparan Sulfate and Heparanase in Stem Cell* In Vitro *Differentiation*

Although the crucial role of HSPGs and their modification enzymes in embryonic development has been demonstrated in a series of mouse models, the severe phenotypes of the animals prevent further study on their functions in mouse nervous system development (see above). Instead, using ES cell differentiation *in vitro*, the function of HSPGs in stem cell commitment and differentiation can readily be evaluated. Moreover, in normal ES cell differentiation, the regulations of N-, 3-O-, and 6-O-sulfation have been observed [86]. ES cells exhibit a low level of N-sulfation and increased expression of NDST4, HS3STs, and HS6STs during differentiation to NSPCs [120]. During differentiation from neuroepithelial precursors to neurons, the cells distinctly changed their 6-O-sulfation pattern and HS chain length [18]. These discoveries suggested a role of sulfated HS in stem cell differentiation. When ES

cells are differentiated using a monolayer differentiation protocol [30] they first go through an expansion of NSPCs, followed by differentiation of NSPCs into mature neural lineages, i.e., neurons and glia. In our own studies, we observed that during the expansion phase, HPSE mRNA increases dramatically, followed by a gradual decrease during final differentiation. The latter coincides with a rise in the amount of HS during a phase when NSPCs are maturing into neurons and glia. Thus, the expression of HSPE is reduced, while the quantity of HS increases during neural differentiation [196].

To understand the role of HS and HPSE in neural differentiation, ES cells with deletions or overexpression of biosynthetic enzymes and modifying genes have been used. The complete knockout of EXT1 causes absence of HS chain synthesis, which has severe consequences for neural differentiation. EXT1-knockout ES cells had phenotypically normal colonies and a high expression of pluripotent markers, but depletion of EXT1 led to a differentiation arrest when subjected to monolayer differentiation [86, 92]. Although EXT1-knockout ES cells could form embryonic bodies, they could not generate terminally differentiated cells [70]. When directing these ES cells to neural differentiation, the addition of soluble heparin could partially rescue differentiation to mature neurons [86]. In another ES cell line, using the knockdown of EXT1 with short hairpin RNA, soluble GAGs were capable of inducing neural differentiation via influencing various RTK pathways [137]. NDST1/NDST2 double-knockout ES cells were completely devoid of N-sulfation but retained a very low level of 6-O-sulfation [71]. Similar to EXT1-knockout ES cells, NDST1/NDST2 double-knockout ES cells maintained a normal phenotype and pluripotency in a culture. However, they generally failed to differentiate upon embryoid body formation [98]. Angiogenic sprouting could occur in NDST1 / NDST2-deficient embryoid bodies, but the adhesion of pericytes to nascent sprouts was reduced, owing to the dysregulation of transforming growth factor beta and PDGFB signaling [100].

When using stepwise protocols, by allowing the ES cells to first differentiate to multipotent progenitors, surprisingly, the NDST1/NDST2 ES cells were able to give rise to osteoblasts, albeit with lower efficacy than wild-type ES cells, but no adipocytes were generated [46]. Under conditions inducing neural differentiation, these ES cells appeared to be blocked at a primitive ectoderm-like state, expressing the early ectodermal marker FGF5 without proceeding to neural progenitors. However, the differentiation to neural precursors could be restored by a combination of heparin and FGF2 or FGF4, but this only succeeded in a very narrow concentration range [46].

Studies of ES cells overexpressing HPSE have shown that they possess a faster proliferation potential, and they also formed larger teratomas in vivo, than their wild type counterparts. This faster growth rate was kept during differentiation, as monitored by the monolayer protocol for neural induction, and they also show enhanced activation of ERK and AKT pathways [196]. Interestingly, neural progenitors overexpressing HPSE differentiated to a larger extent into oligodendrocytes, than wild type ES cells that hardly generated oligodendrocytes at all, and this increase was at the expense of neurons that were reduced, while the proportion of astrocytes did not

change [196]. This shows that alterations in HS levels and composition can change how the stem cells use various signaling pathways and consequently alter their differentiation potential.

14.4 Heparan Sulfate and Heparanase in Cancer Stem Cells

14.4.1 HS, HPSE and Cancer Stem Cells

Cancer stem cells recapitulate many characteristics of normal stem cells. Early studies showed that several stem cell differentiation programs depend critically on an adequately modified HS, for example in myoblast differentiation [142] and hematopoietic stem cells [60]. As already mentioned, loss of function studies for HS biosynthetic genes have shown their critical role in vertebrate development since HS2ST or NDST-1 knockout mice die in the neonatal period [20, 146]. As described above, intriguingly, HPSE knockout mice do not display any major phenotypic disturbance, and there are no reports of affected stem cell pools when HPSE gene is lacking, although their mammary glands displayed a more abundant branching compared with glands from wild type mice [201]. In addition, in vivo neovascularization in a matrigel plug was pronounced in HPSE knockout mice, and ex vivo sprouting assays revealed an increased sprouting [201]. It has also been reported that the function and activation of macrophages are hampered in HPSE null mice [61] as they express lower levels of cytokines and exhibit reduced mobility. If, and how, any of the above alterations relate to effects on stem cell pools, or their progeny, remains to be investigated.

Several studies have shown that properly sulfated HS is required for ES cells to switch from self-renewal to initiation of differentiation of specific cell lineages [92], for instance, to capillary structures [83], or neural progenitors [86]. During ES cell differentiation, HS of the more differentiated progeny become more complex and increasingly sulfated [137]. Stem cell differentiation thus relies on correctly sulfated proteoglycans, and cancer stem cells, in contrast, would carry HS with a lower degree of sulfation, which endows them with survival advantages. This is corroborated by the higher expression of HPSE in cancer stem cells, e.g., in breast cancer [76], and glioblastoma [93]. Another example of how deregulated proteoglycans influence cancer stem cells is that serglycin, normally found in the secretory granule of hematopoietic cells, when overexpressed, serves as a marker of poor prognostic in lung cancer. Here, serglycin was reported to enhance stemness properties by induction of NANOG expression in NSCLC [59].

14.4.2 HPSE in GBM Stem Cells

Using patient-derived glioblastoma stem cell cultures [195], we have shown that HPSE is highly expressed, compared to normal brain, and that both the latent 65-kDa and the enzymatically active 50-kDa forms can be detected [93]. That HPSE produced by GBM stem-like cells was functional could be determined by reduced cell numbers upon either shRNA downregulation of HPSE, or by treatment with the HPSE inhibitor PG545. In an attempt to determine if HPSE expression could be associated with specific features of GBM, we found that Mesenchymal GBM cells express the highest levels of HPSE when compared to primarily the Classical subtype [93]. This was confirmed in tumor tissue when we analyzed the TCGA dataset, where expression of HPSE was highest in the Mesenchymal subtype and, therefore, it seems plausible that HPSE expression reflects GBM heterogeneity. Heparanase-overexpressing glioma cells were also more resistant to stress and chemotherapy [161], a well-described feature of cancer stem cells.

14.5 Heparan Sulfate and Other Proteoglycans in Brain Tumors

14.5.1 ECM Remodeling as Part of the Brain Tumor-Supporting Microenvironment

Less attention has been paid to the brain tumor ECM compartment, than to the cancer cells and non-tumor cells of the tumor microenvironment. The ECM of the normal brain is distinct from other organs and consequently, the brain tumor matrix is different from that of other solid tumors. Any tumor stroma outside the brain is usually rich in fibrillar collagens, while in the CNS, glucosaminoglycans, proteoglycans and glycoproteins are predominant constituents. ECM molecules are highly functional entities in almost every aspect of brain tumor biology, in addition to their anchoring and organizing functions. Taking up between 10 and 20% of the volume of the brain [15], the ECM molecules thus not only provide structural support but are also part of signaling systems that can be co-opted by the brain tumor to enhance cancer cell proliferation, invasion, vascularization, immune infiltration, etc. There are many ways by which proteoglycans can support malignancy of the brain tumor microenvironment and thereby contribute to the failure of clinical trials. For example, HPSE is increased in glioblastoma stem cells [93], and abnormal receptor tyrosine kinase activity is a common denominator of GBM [181]. Since extracellular availability of growth factors is orchestrated by e.g. HSPGs, excess HS degradation by HPSE is a way by which brain tumors could modify the microenvironment to drive oncogenic signaling.

14.5.2 Characteristics of the Extracellular Matrix in the Brain

The adult brain ECM can be described in three compartments: that of the neural interstitial matrix, i.e., (i) ECM molecules in the parenchyma, (ii) the basement membrane ECM, and (iii) the perineuronal nets. The ECM of the brain parenchyma consists mainly of networks of hyaluronan and proteoglycans, which are produced intracellularly and then secreted into the extracellular space [15] where it surrounds cells and attaches to the cell membrane [11]. Other components are glycoproteins such as tenascins and to a smaller extent, collagens, laminin, and fibronectin. The basement membrane surrounds the pial surface and forms a barrier between the vasculature and the parenchyma. It mainly contains collagen IV, laminins, fibronectin, dystroglycan, and heparan sulfate proteoglycans, e.g., in the form of perlecan [79]. Finally, the perineuronal nets are mesh-like structures of proteoglycans, tenascin R, and link proteins around neuronal cell bodies [94]. The role of perineuronal nets is to stabilize synapses and therefore, they are important in regulating CNS plasticity [187].

For normal development to proceed, and to prevent aberrant remodeling in the adult brain, ECM components are strictly regulated during neurogenesis, differentiation, neural migration and axonal outgrowth [9]. ECM molecules that are abundant during embryogenesis and early postnatal development regain expression levels in glioma, for example, tenascin-C [52, 53, 69]. It is well established that ECM molecules contribute to the extrinsic regulation of the local microenvironment of neural stem cell niches in the brain [43]. This regulation occurs at several levels including adhesion to other cells of the niche. Mechanical properties of the ECM results in different matrix stiffness which influences stem cell fate, and stem cell-ECM interactions mediate different signaling events.

14.5.3 Analyzing Proteoglycans in Brain Tumors

An early study showed that high-grade glioma cells in culture, to a larger extent than normal cells, produce HS and release GAG chains into the cell culture medium [169]. The same authors demonstrated this as a diffuse and intense staining of HS which was localized to the surface of the cell, in contrast to normal cells or low-grade astrocytoma that displayed punctate HS staining. Bertolotto et al. [14] investigated surgical specimens of human glioma and normal brain, and found very high glucosaminoglycan levels, particularly heparan sulfate and dermatan sulfate in GBM, compared to normal brain.

A multidimensional mapping of specific proteoglycans of brain tumors remains to be presented. A GBM cohort has been analyzed (The Cancer Genome Atlas, TCGA) for RNA expression of proteoglycan core proteins, biosynthetic and modifying enzymes [186]. The authors found several of these genes to be differently

expressed, both when comparing tumors to non-neoplastic tissue controls, and also between GBM subtypes.

Recent approaches for analyzing proteoglycans in brain tumors include mass spectrometry and Raman microspectroscopy. The latter was used in a recent study [90] and presents identification of proteoglycans based on their vibrational signatures. For this proof of principle paper, a medulloblastoma specimen was investigated and proteoglycans were found to be deregulated. Liquid chromatography-mass spectrometry analysis was employed in a study by Tran et al. [178] to profile HS disaccharide content and structure across patient-derived sphere cultures of GBM cells. The authors found significant heterogeneity in the HS content and structure between patients, and suggested that the intertumoral differences in proteoglycan expression could be analyzed to determine which tumors would more likely respond to HSPG modification.

14.5.4 Examining the Cancer Genome Atlas for Proteoglycans with Deregulated Expression in Glioblastoma Patients

Overall, many genes that had previously been reported to promote cancer progression, such as those involved in metastasis, were among the most highly regulated genes revealed upon examination of the TCGA cohort [186]. Both membrane-bound and secreted proteoglycans are, in general, more highly expressed in GBM than normal brain tissue, which could suggest proteoglycans and their synthesizing and degradation enzymes as new cancer biomarkers for GBM. CSPG4, also denoted NG2, was first identified as a marker of oligodendrocyte precursor cells [122] and its overexpression has been detected in glioma [160]. Furthermore, oligodendrocyte precursors have been identified as one type of glioma-initiating cells [107], and due to its overexpression in a vast majority of GBM cases, NG2 may have prognostic value [175]. Protein tyrosine phosphatase receptor β/ζ (PTPRZ1) is also highly expressed in the TCGA cohort. It has been associated with glioma formation and recently, a small molecule inhibitor of PTPRZ1 was found to inhibit glioma formation in vivo [50]. A very high expression of CD44 was also noted [186], which is in line with CD44 being reported as overexpressed in glioma, especially in the mesenchymal subtype [134] and commonly used to enrich for cancer stem cells [5].

Out of the modular proteoglycans, versican showed the highest expression in GBM [186], and other studies confirm high levels in mouse and human glioma of this secreted proteoglycan [75]. Another study showed that antibodies to versican could reverse the migration-promoting effect of TGF-beta2 on glioma [7]. Furthermore, versican was found, among other ECM genes, to be part of a signature for invasiveness of low-grade astrocytoma [151].

Among HSPG core proteins that have been reported to be altered in glioma, are glypican-1 [174] and syndecan-1, the latter shown to be upregulated via NFkB activation [191]. Syndecans 2, 3 and 4 are ubiquitously expressed in normal brain and

glioma [191], whereas syndecan-1 is not detected in the normal brain. In a study of over 100 glioma samples, high syndecan-1 expression correlated to shorter survival, and grade IV patients had the highest expression [198]. Several small leucine-rich proteoglycans are highly expressed in GBM, among them fibromodulin. Fibromodulin was identified in a screen of epigenetically regulated genes in GBM and found to be an essential regulator of glioma cells [115].

14.5.5 Heparan Sulfate and Chondroitin Sulfate Biosynthetic Enzymes in Glioblastoma

During HS biosynthesis, the nascent HS chain is modified by several enzymes, in the order as follows: *N*-deacetylase/*N*-sulfotransferase, C_5-epimerase, 2-*O*-sulfotransferase, 6-*O*-sulfotransferase, and 3-*O*-sulfotransferase. The NDSTs define the design of sulfation patterns, which in turn dictate the affinity for different ligands [31]. For the enzymes that synthesize HS and CS, there is a wide variation in mRNA expression in GBM. Five of the CS biosynthetic enzymes are upregulated, and the other four downregulated [186]. No comprehensive public data set link specific CS biosynthesis genes to brain tumor development or progression, but the role for CSPG in glioma has been studied [163]. Silver et al. report that intense staining of CSPG was seen around non-invasive gliomas, similar to that described for brain injury, where CSPG has repulsive actions [153]. Furthermore, there was very little glycosylated CSPG in xenografts of diffusively infiltrative glioblastoma [163]. This may seem somewhat in disagreement to some reports of upregulated core proteins in brain tumors, but when the level of glycosylation, rather than the expression of core proteins, was analyzed the former seems to be the determining factor as to whether CSPG promotes or inhibits invasion. In another study, "underglycosylated" brevican was associated with late stages of glioma progression, such as invasion, whereas it did not affect glioma stem cells [37]. To date, there is not enough data to conclude precisely how CS biosynthetic genes contribute to malignant brain tumors.

Understanding if the biosynthetic mechanism of HS is altered in glioblastoma is critical for determining the roles for HSPG in brain tumors. Therefore, it is interesting to note that according to TCGA data, all four NDST genes were downregulated in GBM, thereby suggesting less elaborate sulfation of HSPG in GBM [186]. A vast majority of the HS biosynthetic genes are down-regulated in GBM (12 out of 15 genes in TCGA). HS3ST3a1 was among the three upregulated genes and is highly expressed in glioma. HS3ST3a1 was the predominant sulfotransferase in glioma cells, which is not the case in normal human astrocytes [174]. TCGA revealed low expression of all 6O-sulfotransferases, and higher expression of two out of four 3O-sulfotransferases, which could indicate that low 6O-sulfation is a feature of GBM. This is supported by RT-PCR on a cohort of glioma patients of different

grades, where both grade III and grade IV gliomas had lower expression of HS6STI and HS6ST2 than non-tumor tissue from the same patient [180].

14.5.6 Heparan Sulfate Modifying Enzymes in Glioblastoma

Once the HS chain has been completed it can be further edited by sulfotransferases, SULF1 and SULF2, both of which are up-regulated in GBM [186]. SULF2 removes 6-O-sulfate moieties and thereby activates several signaling pathways. As mentioned, a common denominator of glioma is the abnormal tyrosine kinase activation, and because HS-GAGs have a negative charge, they can bind many growth factors and thus play a key role in RTK activation. A typical example hereof is PDGFRA, which is often amplified in GBM, and PDGF ligands are frequently expressed at high levels in this tumor. PDGF is considered a driver gene in glioma and has been shown to cause glioma in mice (reviewed in [158]). Phillips et al. [135] described SULF2 overexpression in human GBM and cell lines derived from GBM patients, and showed that knockdown of SULF2 led to smaller tumors in mice. This corresponded to HSPG-dependent signaling by PDGFRA, presumably through increasing the availability of growth factors in the tumor microenvironment. The effect was most notable in the proneural subclass of GBM, which is primarily driven by PDGF signaling, but not in classical GBM where perturbed EGFR signaling is a key feature. Furthermore, the importance of SULF2 in glioma is supported by the gene being identified through insertional mutagenesis in retrovirus-driven PDGF-induced mouse glioma [85].

14.5.7 Heparanase in Glioma and Medulloblastoma

HPSE, as described, is the main HS degrading enzyme, which releases HS-bound bioactive molecules and thus primes the tumor microenvironment to support cancer spread. HPSE is implicated in metastasis and invasion of many types of cancers [183] and has also been reported to be overexpressed in GBM [74, 93] and medulloblastoma [165, 168]. Fig. 14.1 summarizes the current knowledge about HPSE in brain tumors, and its complex route of activation. First, the pre-pro-HPSE is directed to the ER lumen via its signal peptide. The 65 kDa, latent form of HPSE is then transferred to the Golgi apparatus, and then into secretory vesicles that bud off from the Golgi. When HPSE reaches the outside of the cell, it interacts with HSPGs and quickly taken up again as a complex with HSPG into early and late endosomes. Next, the endosomes fuse with lysosomes and HPSE is activated, upon cleavage by Cathepsin L, to the 50 kDa enzymatically active form. The lysosomal HPSE can take different routes, either secreted to perform extracellular functions, but also translocate to the nucleus (Ilan et al., Chap. 9 in this volume). Our own recent finding shows that HPSE can stimulate signaling pathways by interacting with CD24 [10].

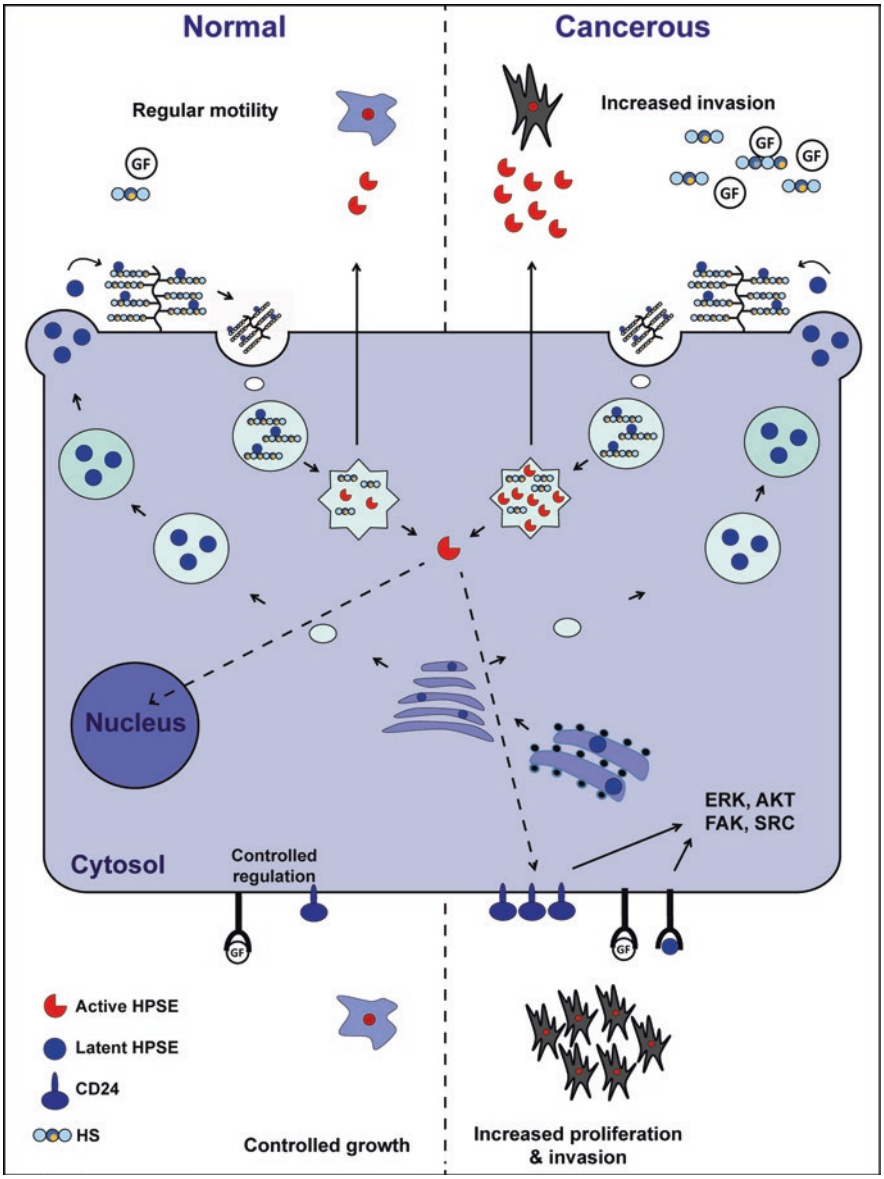

Fig. 14.1 HPSE in normal and cancerous tissues, e.g. brain tumors, and the complex process of HPSE activation. Upon synthesis, the pre-pro-HPSE is first directed to the ER lumen via it's signal peptide. The 65 kDa latent form of HPSE is then transferred to the Golgi apparatus, and then into secretory vesicles (light blue) that bud off from the Golgi. When HPSE reaches the outside of the cell, it interacts with HSPGs and rapidly taken back again into early and late endosomes as a complex with HSPG. Next, the endosomes fuse with lysosomes (star-shaped vesicle) where HPSE is activated, upon cleavage by Cathepsin L, to the 50 kDa enzymatically active form. The lysosomal HPSE can take different routes, either secreted to perform extracellular functions and/or also translocate to the nucleus. Our recent finding shows that HPSE can also stimulate signaling pathways by interacting with CD24

Hong et al. described increased levels of HPSE mRNA in glioma, compared to normal brain, but could find no correlation to the WHO malignancy grade, when comparing oligodendroglioma, anaplastic astrocytoma and GBM [74]. The expression data were confirmed by western blotting and immunostaining, and by transplanting human GBM-derived spheres to immune-deficient mice. In contrast, in another report, HPSE was not detected in human GBM, and following injection of U87 GBM cells into the brain of immunologically compromised mice, HPSE expression in these cells disappeared [179]. The histological staining of glioma patient tissue was performed with two different HPSE antibodies for these two studies, something that might explain the contradictory data. Using the U87 cell line, other investigators showed that modest over-expression of HPSE in U87 cells enhanced tumor size after xenografting, but not the level of HPSE expression [204]. Possible explanations for the above discrepancies could be the use of different cell lines, i.e., patient-derived cells cultured using serum-free stem cell conditions [74] versus U87 [179], which is a classical serum-cultured glioblastoma cell line. Another study revealed that when HPSE was overexpressed in U251 GBM cells, it led to increased invasion, colony formation, and AKT phosphorylation [73].

We found strong overexpression of HPSE in glioma patients, using a cohort of 182 glioma patients with different WHO-grade tumors, and report low-grade gliomas to be less intensely stained by anti-HPSE antibodies than high-grade gliomas in the neuropil [93]. Also, we reported that down-regulation of HPSE reduced GBM proliferation in vitro, while the addition of HPSE enhanced cell growth, and activated ERK and AKT signaling [93]. Based on our data obtained using HPSE transgenic or knockout mice, we reported that the HPSE host brain level affects tumor size. We suggest that surface-associated or secreted HPSE promotes the invasive properties of high-grade gliomas and, consequently, enhance tumor progression by HPSE residing in the microenvironment. Also, in pediatric brain tumors, we detected high HPSE levels compared to non-tumor brain, and when treating pediatric brain tumor cells (medulloblastoma and other embryonal tumors) with HPSE their growth was stimulated [168], as was the case with glioblastoma.

Furthermore, we found for both glioma and medulloblastoma that the latent 65 kDa form of HPSE which requires intracellular processing to become active, rapidly activates the ERK and AKT signaling pathways, before we could detect any enzymatically active HPSE [93, 168]. Therefore, the mechanisms for HPSE action in brain tumors could be both enzymatic and non-enzymatic. To study the underlying mechanisms of HPSE in brain tumors, we used inducible U87 glioblastoma cells for overexpression of HPSE. Differential expression analysis identified CD24, a mucin-like cell adhesion protein, as upregulated by both active and enzymatically inactive HPSE [10]. Patients who express high HPSE and CD24 had a shorter survival time than those who had high HPSE and low CD24 levels. When overexpressing CD24, it stimulated glioma aggressiveness in vitro, and tumor growth as xenotransplants, which could be blocked by anti-CD24 or anti-HPSE antibodies. Furthermore, antibodies to L1CAM, one of the CD24 ligands, also attenuated the

tumors in vivo [10]. Our results thus describe a new HPSE-CD24-L1CAM axis at work in glioma tumorigenesis.

When we used an inhibitor of HPSE, PG545, it efficiently killed pediatric brain tumor cells, but not normal human astrocytes, suggesting specificity to cancer cells that express high levels of HPSE [168]. The compound PG545 also inhibited tumor cell invasion in vitro and very potently reduced flank tumors in mice. Taken together, findings from several laboratories, including our own, indicate that HPSE in malignant brain tumors affects both the tumor cells themselves and their microenvironment. Thus, HPSE plays a substantial role in the progression of brain tumors and may represent a therapeutic target.

14.6 Heparanase Inhibition as a Novel Brain Tumor Therapeutics?

14.6.1 Rationale for Heparanase Inhibition

Based on a vast literature, it is clear that HPSE contributes to tumor progression, which has led to an interest in targeting the enzyme for therapeutic purposes. Mostly, HS mimetics have been suggested as potential inhibitors of the enzyme, although many different aspects have to be considered. HS mimetics vary in size and kinetics and have different efficiencies against HPSE [64]. As HPSE is typically not highly expressed in normal tissue, side effects to inhibition should be manageable.

14.6.2 Low Molecular-Weight Heparin

Heparin or heparin-derivatives such as low molecular-weight heparin (LMWH) have been suggested for cancer treatment, but its contribution to survival improvement is not clear. [8, 48]. Enoxaparin decreased the growth of non-small cell lung cancer [3] and, similar to tinzaparin and dalteparin, has been shown to reduce FGF-induced mitogenesis through ERK kinase inhibition in endothelial cells [167]. Tinzaparin and unfractionated heparin (UFH) decreased metastases in colon adenocarcinoma and melanoma cell lines [170] and inhibited endothelial tube formation, VEGF expression, and angiogenesis [117, 118]. Most of the antitumor effects of heparin-like derivatives are the result of sequestering and blocking growth- and angiogenic- promoting factors [48, 110]. There are clinical studies where LMWH has been given to GBM patients, but without significant prolonged survival, although a trend was noted (reviewed in [154]). In the Eastern Cooperative Oncology Group clinical trial, dalteparin was tested for potential overall survival benefits, in combination with radiation therapy, but this study was closed early, due to the introduction

of temozolamide [148]. Another randomized placebo-controlled trial included newly diagnosed WHO grade 3 or 4 glioma patients, who were given long term dalteparin, but this trial was also closed early, partly due to bleeding [132]. In a third study, GBM patients were treated with enoxaparin using 1 and 2-year overall survival as main endpoints, and progression-free survival as an additional endpoint. Here, there was a significant benefit for the 13 patients on LWMH compared to the control 17 patients at one year, but not at two years follow-up [207].

14.6.3 PI-88 (Mupafostat)

PI-88 (Mupafostat) is a mix of highly sulfonated mannan oligosaccharides [44]. PI-88 exerts its anti-angiogenic and anti-metastatic properties by inhibiting HPSE and blocking interactions of FGF-1/2 and VEGF with their receptors [129]. In preclinical studies, PI-88 reduced the invasion and metastasis of rat adenocarcinoma cells [129] and decreased leukemic cell burden in mouse models [81]. It also inhibited late-stage tumor growth and early progenitor lesions in a pancreatic mouse model, and that was linked to a decrease in cell proliferation, angiogenesis, and increased tumor apoptosis [87]. PI-88 is the most well-studied heparan sulfate mimetic in clinical trials to date, through several phase I and II trials for patients with hepatocellular carcinoma (HCC) [12, 26, 103]. A phase III trial as adjuvant therapy was initiated for patients with HCC, but this trial was lately canceled upon interim analysis [157] (Chhabra and Ferro, Chap. 19 in this volume). No clinical trials for PI-88 have, to date, been registered in clinicaltrials.gov for brain tumors.

14.6.4 SST0001 (Roneparstat)

SST0001 (Roneparstat) is a modified glycol-split heparin, which is fully N-acetylated and hence exert little or no anticoagulant activity [147]. SST0001 inhibits HPSE enzymatic activity and displays a decreased capacity to release ECM-bound FGF-2 in comparison to unmodified heparin. In multiple myeloma cells, SST0001 inhibited HPSE and expression of HGF, VEGF, and MMP-9, resulting in decreased angiogenesis. It also inhibited HPSE-mediated degradation of syndecan-1, which enhances myeloma cell proliferation [147]. SST0001 (=Roneparstat) reduced the growth of disseminated myeloma tumors in vivo when combined with conventional chemotherapy [141]. SST0001 has been examined in clinical trial for multiple myeloma and is documented to be safe at a dose of 200 mg/day [51] (Noseda and Barbieri, Giannini et al., Cassinelli et al., Purushothaman and Sanderson, Chaps. 12, 15, 21 and 23 in this volume). No trials on brain tumor patients have been initiated.

14.6.5 M402 (Necuparanib)

M402 (necuparanib) is an N-sulfated glycol-split (GS) modified heparin. It has the advantageous properties of a heparan sulfate-like molecule but was specially engineered to considerably decrease anticoagulant activity. Hence, it has been used alone and/or combined with standard chemotherapy, and it showed substantial anti-metastatic activity in preclinical models [205]. A clinical phase I/II intervention trial was started for M402, combined with nab-paclitaxel and gemcitabine for the treatment of metastatic pancreatic cancer but terminated due to insufficient efficacy. It has not been tested against brain tumors.

14.6.6 PG545 (Pixatimod)

PG545 (Pixatimod) is a synthetic, single molecular entity fully-sulfated tetrasaccharide [36]. In comparison to many other HS-mimetics, its structure allows enhanced pharmacokinetic properties and decreased anticoagulant properties [35]. PG545 is a highly effective inhibitor of HPSE compared to the other HS mimetics used to date [64]. In pancreatic cancer cell lines, it inhibited Wnt/b-catenin signaling and reduced the proliferation of tumor cells by the proangiogenic growth factors VEGF, FGF-1, and FGF-2 [36]. PG545 has been studied in multiple preclinical models in various tumor subtypes, exhibiting potent antitumor, anti-metastatic, and anti-angiogenic effects [36, 63, 128, 193]. Importantly, PG545 is the only HS mimetic investigated so far that has an immune-stimulatory effect and exerts its major anti-lymphoma effects through activation of the immune system via natural killer (NK) cells [17] (Bendersky, Yang and Brennan, Chap. 18 in this volume). PG545 was tested in a Phase 1a study to establish the maximum tolerated dose for patients with advanced solid tumors, and although it gave some adverse effects, such as fever and elevated triglycerides, PG545 is considered to have a safety and pharmacokinetic profile that merits further development [65] (Hammond and Dredge, Chap. 22 in this volume). We have found a very good inhibitory effect of PG545 both in vitro and in vivo (mouse) in GBM [93] and pediatric brain tumors [168], but due to lack of ability to cross the BBB, PG545 in its present form would be difficult to develop for brain tumors.

14.6.7 Small Molecule Approaches to HPSE Inhibition

Low molecular-weight inhibitors against HPSE are still lacking, but with the 3D structure of HPSE being resolved [194] (Wu and Davies, Chap. 5 in this volume), hopefully, future efforts of designing new inhibitors as therapeutic agents will be more successful (Giannini et al., Chap. 23 in this volume). Another approach could be metallo-shielding of HS by polynuclear platinum complexes that are positively charged, as these would mask the ECM-resident HS from HPSE action [133].

14.7 Challenges to Brain Tumor Treatment

14.7.1 Invasiveness

CNS tumors are different from other malignancies due to their location, and they rarely metastasize outside of the brain, even though they rapidly invade the surrounding brain parenchyma. An invasive and aggressive growth pattern is a feature of malignant brain tumors, but their invasiveness is different from other malignant solid tumors that commonly extravasate into the blood and lymphatic vessels. In the brain, motile glioma cells can take several routes, along blood vessels, following white matter tracts, or, diffusively in the brain parenchyma [57]. A recent study of human GBM using radiology, suggests that white matter tracts are the preferred direction for human GBM invasion, possibly due to their anatomical features [41]. Invasive tumor cells cannot be removed at initial surgery, and therefore contributes to the fatal outcome by seeding new tumors.

14.7.2 Heterogeneity

Brain tumors exhibit extensive inter- and intra-tumoral heterogeneity [49, 78, 116] which arises from expansion of clones carrying different mutations [37]. Intratumoral heterogeneity leads to selective pressure, either by clonal evolution or by chemo- and/or radiotherapy. Hence, the resistant clones remaining after therapy will be the ones forming the recurrence, thus creating a secondary tumor [126], which is further diversified by treatment-induced mutations. Indeed, GBMs constantly evolve so that within one GBM tumor several subtypes can co-exist and individual cells within the same patient exhibit a spectrum of expression profiles, which leads to selection of tumor subclones [131]. This extreme heterogeneity is challenging for GBM drug discovery because a candidate drug response may vary widely between cell lines from different GBM patients, and even in clones from the same patient due to plasticity [156].

14.7.3 The Blood-Brain Barrier

One of the most challenging aspects for brain tumor treatment is the blood-brain barrier (BBB) which limits the entry of therapeutic molecules into the brain [39]. It consists of tight junctions that seal off the brain endothelial cells in order to protect the brain from the crossing of unwanted endogenous and exogenous particles. The complex vasculature of the BBB, compared with blood vessels in the rest of the body, serves as a major obstacle to successful therapeutic agent delivery to the

brain [39, 119]. Though advanced stage brain tumor may compromise the BBB to a certain degree, it is still not possible for most drugs to penetrate [136]. Attempts are progressing to be able to successfully target brain tumors, and invent novel CNS delivery systems for future clinical application, such as pulsed ultrasound [21]. As described earlier in this chapter, our efforts to deliver PG545 to block HPSE action in orthotopic brain tumors yielded no detectable drug in the brain tissue, despite the advanced stage glioma [168].

14.7.4 Drug Penetration in Brain Tumor Tissue

Chemotherapeutic agents against malignant brain tumors have been disappointingly inefficient, partially due to their unsuccessful accumulation across the tumor mass [58]. This is not only due to the inability to cross the BBB. Another challenge is the existence of a blood-tumor barrier (BTB) [24]. The BTB is considered a barrier because it is composed of small microvessel populations that block the access of large-drug molecules into the tumor [58] and is different from the BBB. BTB microvessels have high expression of drug efflux transporters [6, 176] and ABC transporters [105] compared to normal brain. Furthermore, GBM blood vessels have several features that compromise their functionality, including a high degree of microvascular proliferation and thus, even if a drug is delivered to the brain tumor, distribution across the tissue is hampered by the highly abnormal GBM vasculature [33].

14.8 Summarizing the Role of Heparanase for Brain Tumor Hallmarks

Systematic description of the so-called hallmarks of cancer provides a conceptual overview of principle ways for cancer cells to overcome the protective functions of the host organism [67]. This includes the specific capacities of the tumor microenvironment, as outlined by Hanahan and Coussens [66]. In this chapter, we have reviewed how HSPG and HPSE can contribute to the malignant brain tumor phenotype. Below, and in Fig. 14.2, we summarize the specific role of HPSE for some brain tumor hallmarks.

14.8.1 Promoting Proliferation

Several studies across many cancer types have shown that an increase in HPSE correlates to an increase in cell number, but the mechanisms behind augmented amounts of cells could be either increased cell proliferation, decreased cell death, or

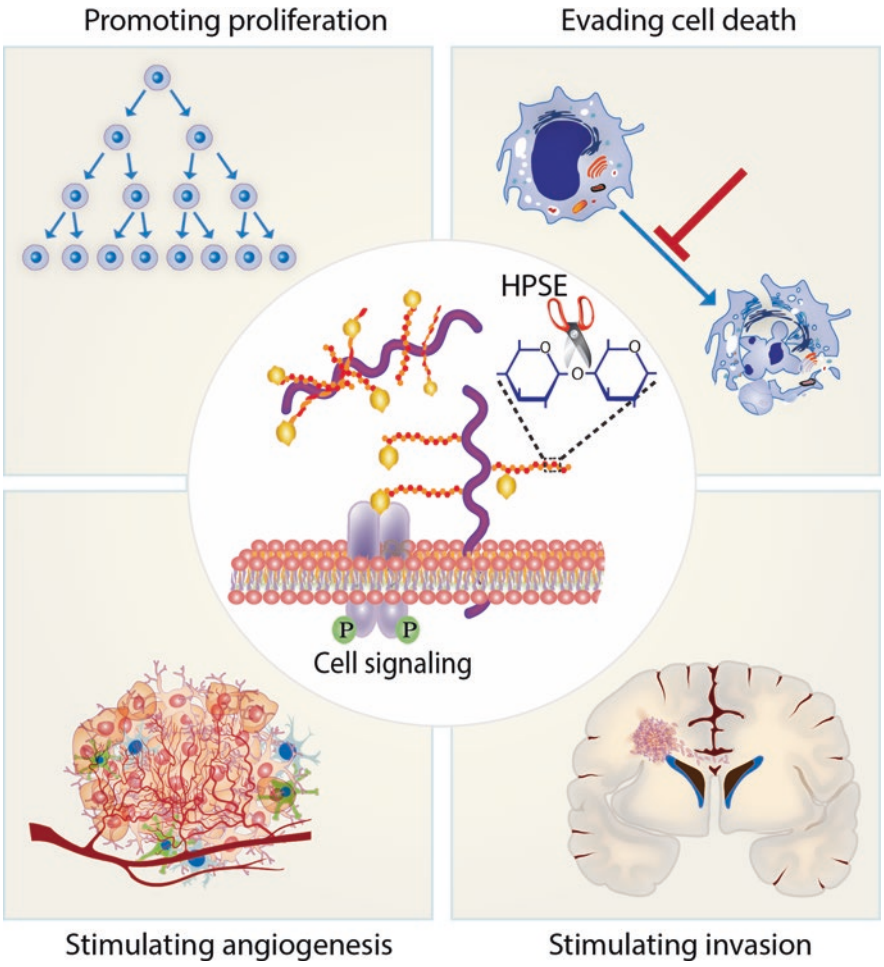

Fig. 14.2 Role of HPSE for brain tumor hallmarks
Promoting proliferation: In many cancer types, including brain tumors, overexpression of HPSE correlates with increased cell number, and this could be due to augmented cell proliferation, a decrease in cell death, or combination of both. Ki67 staining and BrdU incorporation have shown that proliferation is among the effects by HPSE on brain tumor cells. Evading cell death: The finding that HPSE inhibition induced massive expression of cleaved caspase 3 in flank tumors of medulloblastoma indicates that HPSE is involved in suppressing apoptosis. Stimulating angiogenesis: HPSE has been shown to stimulate brain tumor angiogenesis. One way to exert this function may be through the release of angiogenic factors, such as VEGF that are bound to HSPG in the ECM. Stimulating invasion: Several studies show that HPSE increases brain tumor cell motility as revealed by scratch assays, chemotaxis assays, and invasion assays through Matrigel, or collagen gels. These studies also revealed activation of signaling pathways that are commonly associated with cell migration and invasion

a combination of both. In their study, Hong et al. show that overexpression of HPSE in U251 cells leads to an increase in cell growth compared to control cells, as measured by the MTT assay [73]. On the other hand, Zetser et al. reported a decrease in proliferation, as measured by BrdU incorporation in U87 cells overexpressing HPSE, compared to the parental cell line [204]. In GL261 mouse glioma, addition of recombinant HPSE or conditioned medium from HPSE-expressing cells increased the cell number and activated ERK and AKT pathways, and inhibition of HPSE by shRNA or HPSE inhibitor PG545 reduced cell numbers both in GL261 cells and patient-derived GBM cells [93]. The same results were obtained for medulloblastoma and another embryonal tumor [168]. Blocking HPSE was also found to reduce pediatric brain tumor cell proliferation in vivo [168] and HPSE overexpressing GBM cells had higher numbers of Ki67-positive cells than non-HPSE expressing tumor cells [74].

14.8.2 Evading Cell Death

Cancer cells have developed several mechanisms to overcome cell death, such as avoiding apoptosis or the ability to adapt to hypoxia. That HPSE is involved in suppressing apoptosis was suggested by the finding that HPSE inhibition induced massive expression of cleaved caspase 3 in xenografts of medulloblastoma [168]. Albeit a different brain tumor, pituitary tumor cell culture viability was decreased when HPSE was inhibited [149] further underscoring the role of HPSE in tumor cell survival. In order to withstand stress, caused for example by cytotoxic agents such as chemotherapy, cancer cells may also induce autophagy, an evolutionary conserved and important homeostatic cellular recycling mechanism [192]. HPSE can enhance the stress-resistance of GBM cells by increased autophagy in HPSE-overexpressing cells [161].

14.8.3 Stimulating Angiogenesis

Several studies show that HPSE stimulates brain tumor angiogenesis. For example, a GLI splice variant, TGLI1, has been shown to support glioma primarily through neovascularization, and that this effect is mediated by HPSE and VEGF-A [206]. In orthotopic glioma, tumor vascularization, as measured by CD31 staining in the peritumoral area, was enhanced in HPSE-Tg mouse brain compared to HPSE-KO brain [93] and in xenografts of medulloblastoma HPSE inhibition greatly reduced CD31 staining [168].

14.8.4 Stimulating Migration and Invasion

Most of the studies addressing HPSE in glioma report an increased migration and invasion as a consequence of overexpressed HPSE. In U87 cells, overexpression of HPSE leads to faster migration that covered the empty area in a scratch assay and to a more pronounced invasion through Matrigel [204]. Likewise, an increase in the number of U251 cells towards a chemotaxis gradient and increased invasion was reported for U251 cells overexpressing HPSE [73]. Similarly, a massive reduction of invasion in collagen gels, and attenuated migration in scratch assays were noted with pediatric brain tumor cells in response to HPSE inhibition [168].

14.8.5 Concluding Remark

In conclusion, our review of the current literature suggests that an improved understanding of the biology HSPG biosynthesis and degradation, particularly the involvement of HPSE, should have implications on designing therapeutic approaches towards treating GBM and medulloblastoma. Up to date, few drug targets have been directed towards ECM molecules in the brain tumor microenvironment, and therefore, validating the efficacy of inhibiting HS turnover as a potential therapy to brain tumors is highly warranted.

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Chapter 15
Heparanase: A Potential Therapeutic Target in Sarcomas

Giuliana Cassinelli and Cinzia Lanzi

Abbreviations

BMP	Bone Morphogenic Protein
ECM	Extracellular Matrix
EXT	Exostosin
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
GAG	Glycosaminoglycan
GIST	Gastrointestinal Stromal Tumor
Hh	Hedgehog Ligand
HIF1α	Hypoxia-inducible Factor 1α
HS	Heparan Sulfate
HSPG	HS Proteoglycan
IGF1R	Insulin-like Growth Factor 1 Receptor
InsR	Insulin Receptor
MO	Multiple Osteochondroma
NDST	N-deacetylase/N-sulfotransferase
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
Ptc1	Patched 1
VEGF	Vascular Endothelial Growth Factor

15.1 Sarcomas

Sarcomas are rare mesenchymal tumors accounting for about 1% of all cancers in adults and 15–20% of pediatric tumors. They constitute a heterogeneous family of bone and soft tissue malignancies that comprises more than 70 subtypes [1].

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Molecular classification distinguishes two categories:(1) genetically simple sarcomas characterized by a tumor-specific chromosomal translocation or point mutation and a near-diploid karyotype; and (2) genetically complex sarcomas that lack consistent specific genetic changes but present unbalanced translocations, changes in chromosome number, genetic deletions and amplifications characteristic of unstable genome. The current view is that sarcomas should be considered as a collection of histologically and genetically distinct malignancies, a feature that, together with rarity, makes treatment and diagnosis in several cases, particularly challenging.

A huge variety of genetic alterations has been described in sarcomas. Nevertheless, a few general events driving sarcomagenesis can be recognized [2]. Most genetically simple sarcomas harbor pathognomonic chromosomal translocations. The resulting fusion genes, encoding chimeric transcription factors (e.g., EWS-FLI1 in Ewing's sarcoma, PAX3/7-FOXO1A in alveolar rhabdomyosarcomas) or chromatin remodeling proteins (e.g., SS18-SSX1/2 in synovial sarcoma), induce transcriptional dysregulation of target genes. Epigenetic control of the transcriptome can also be subverted by genetic alterations that change the composition of chromatin remodeling complexes (e.g., loss of SMARCB1 in rhabdoid tumors). Other genetic changes directly alter cell signaling components [e.g., COL1A1-PDGFBB fusion gene in dermatofibrosarcoma protuberans, KIT or PDGFR mutations in gastrointestinal stromal tumors (GIST)]. Among sarcomas with complex genetic profiles, a category characterized by intermediate complexity harbors few recurrent amplifications leading to oncogene co-amplifications (e.g., CDK4 and MDM2 co-amplified with 12q chromosome in well-differentiated/dedifferentiated liposarcomas). Highly complex sarcomas, including, among others, osteosarcomas and embryonal rhabdomyosarcomas, harbor multiple numerical and structural chromosomal aberrations with no specific pattern. In these tumors, recurrent genomic alterations identified with some frequency include inactivating mutations of tumor suppressor genes (e.g., TP53, NF1, RB1, PTEN) [3].

Traditionally, the different types of sarcomas have been treated in the same manner despite differences in histology and biology. Surgery, with or without radio- and chemo-therapy, is the critical management for local control. Treatment of metastatic disease, which develops in 40–50% of patients, remains a challenge. Systemic doxorubicin-based cytotoxic regimens have been the gold standard since early seminal observations by Bonadonna et al. of the anthracycline clinical activity in sarcomas in the late 1960s [4–6]. First-line treatment of GIST and dermatofibrosarcoma protuberans represents an exception as they have shown a peculiar sensitivity to the tyrosine kinase inhibitor imatinib which is able to block the oncogenic activation of KIT and PDGFRβ, characteristic of these tumors [7]. Although treatment response varies among the different histologies, a substantial proportion of patients derives no benefit from first-line chemotherapy or experiences recurrence. Over the last years, the treatment options in second-line and beyond have expanded for soft tissue sarcomas, being increasingly subtype-directed [8, 9]. In fact, recent clinical trials evidenced a selected activity of various drugs in specific histotypes with progression-free survival benefit. From these studies, a few drugs received approval for use in sarcoma subtypes. These included the tyrosine kinase inhibitors sunitinib,

regorafenib (GIST, dermatofibrosarcoma protuberans) and pazopanib (soft tissue sarcomas), the DNA binding and multi-tasking trabectedin (liposarcoma and leiomyosarcoma) and the microtubule-targeting eribulin (liposarcoma). Several other histology-driven therapies are currently under investigation [5, 10, 11].

Next-generation sequencing technologies are increasingly applied in sarcoma translational research. These potent tools offer now the opportunity to discover molecular abnormalities in the different sarcomas subtypes improving our knowledge of the biology of these challenging diseases and potentially identifying new actionable alterations and genome-based drug targets [3, 12]. A few studies reported targetable pathways in genetically complex sarcomas. For instance, gain of function of IGF1R and PI3K/mTOR signaling pathways have been described in a subset of patients with osteosarcomas [13, 14] and mutations along the receptor tyrosine kinase/RAS/PI3K pathway have been identified as frequent in embryonal rhabdomyosarcomas [15]. Although clinical validation will be needed to assess safety and efficacy of new treatments derived from these studies, there is great hope that implementation of next-generation sequencing to guide therapeutic treatments will improve the outcome of patients with bone and soft tissue tumors in the next future [16].

Advances in understanding the pathogenesis of sarcomas have evidenced a crucial role for the tumor microenvironment. As in other solid tumors, the complex interactions between tumor cells and components of the microenvironment are essential for sarcoma growth and dissemination and influence the response to therapies [17]. Vascular invasion by tumor cells, as well as VEGF expression and circulating VEGF levels, have been identified as prognostic factors in several studies [18]. Elevated expression of other pro-angiogenic factors, such as PDGFB and FGF2, has been associated with a worse prognosis [19]. Mechanisms underlying the enhanced expression of angiogenic factors are tumor-type specific. In addition to the increased expression of hypoxia-inducible factor (HIF1α) that activates the transcription of VEGF during the angiogenic switch [20], specific genetic alterations have been associated with elevated expression of growth factors. In osteosarcomas, VEGF pathway genes have been found amplified [21] and high levels of the growth factor correlated with progression and poor survival [22, 23]. In Ewing's sarcomas, VEGF-A and PDGF-C have been shown to be upregulated by the specific fusion oncoproteins EWS-ETS and EWS-FLI, respectively [24, 25]. Growth factors such as VEGF, PDGF, and FGF2 activate receptor tyrosine kinase pathways in sarcoma and stromal cells driving proliferation, survival, motility, and angiogenesis through paracrine/autocrine loops. In fact, most receptor tyrosine kinase inhibitors approved and under investigation in sarcomas are thought to exert their effects by acting on the stroma and directly on tumor cells [18].

Effects of sarcoma therapies on the innate immune system have also been described. For instance, imatinib was shown to induce NK cell response in GIST patients, and trabectedin was found to induce depletion of monocytes, including tumor-associated macrophages, in soft tissue sarcoma patients [26, 27]. The sarcoma immune microenvironment is still poorly characterized and, not surprisingly, appears to be highly variable. Inflammation, T cell infiltration, and checkpoint

proteins expression are dependent on the tumor histotype [28, 29]. Immunotherapy, an area of intense investigation that has already revolutionized the standard of care in other tumors, is still in an early phase of clinical development for sarcomas. The main approaches that are being investigated involve checkpoint inhibitors and adoptive T cell therapy. Encouraging responses have been observed only in selected sarcoma sub-groups so far. However, studies exploring new immune system enhancing approaches are ongoing. In addition, a variety of combination strategies, aimed at improving efficacy and assessing the safety of immune-modulating therapies in different sarcoma subtypes are under clinical evaluation [11, 30].

15.2 Heparanase in Sarcomas

Heparanase enzymatic activity was first described in murine sarcoma cell lines, before its gene cloning, in the late 1980s. Early papers reporting heparan sulfate (HS) endoglycosidase activity in sarcoma cells also described a relationship with the cell metastatic potential (Table 15.1). In highly spontaneously metastasizing mouse cell lines of Rous sarcoma virus-induced fibrosarcoma, the enzyme activity was found 20 fold higher compared to non-metastasizing or normal counterparts [31]. Similarly, extracellular matrix (ECM) degradation by tumor cell lines derived by nickel-induced rat rhabdomyosarcomas was characterized by partial hydrolysis of HS. The ECM degrading activity of subclones representative of various metastatic degrees correlated with the ability to spontaneously metastasize to the lung from the primary s.c. tumor site, but not after i.v. injection. These findings suggested that additional tumor cell capabilities, such as adhesion to biologic supports, are relevant in determining lung homing and colonization [32].

Accumulating evidence indicates that secreted heparanase can exert local effects in the tumor microenvironment as well as systemic effects. Thanks to the latter feature, Shafat and colleagues demonstrated the possibility to quantify heparanase protein in human biological fluids by an ELISA method [33]. Elevated levels of heparanase were found in the plasma of 64 pediatric patients with hematological and solid tumors, including 15 sarcomas (7 osteosarcomas, 4 rhabdomyosarcomas, 4 Ewing's sarcomas), compared with healthy controls. Evaluation of plasma levels after chemotherapy showed a correlation with response to treatment, although with a trend not statistically significant in the heterogeneous sarcoma subgroup, suggesting that heparanase could represent a potential tumor marker. The positive immunostaining in 5 out of 8 Ewing's sarcoma biopsy specimens showed for the first time heparanase expression in human sarcoma [34]. The same group subsequently extended the immunohistochemical analysis of heparanase to a cohort of 69 Ewing's sarcoma patients. Positive staining was found in all specimens. Notably, the intensity of staining, which was scored as strong in 51% of cases, correlated with patient age and tumor size, two parameters associated with worse prognosis in Ewing's sarcoma. Correlation with metastasis, the main disease prognostic factor, could not be analyzed in this cohort due to low case number [35].

Table 12.1 Expression of heparanase in sarcomas

Histological type/cell lines	Evidence	Ref.
Rous sarcoma virus-transformed mouse fibroblasts with various metastatic power	Fibrosarcoma cell lines degraded HS. Enzyme activity higher in metastasizing cell lines.	[31]
Rat rhabdomyosarcoma cell lines with various metastatic power	Cell lines degraded ECM by partially hydrolyzing HS. ECM degrading activity correlated with in vivo ability to spontaneously metastasize to the lung from sc primary tumor.	[32]
Pediatric sarcomas (osteosarcoma, rhabdomyosarcoma, Ewing's sarcoma)	High enzyme activity in patients' plasma. Positive by immunohistochemistry 5/8 Ewing's sarcoma specimens.	[34]
Ewing's sarcoma	Positive by immunohistochemistry; intensity (high in 51% of cases) correlated with patient age and tumor size prognostic factors.	[35]
Alveolar and embryonal rhabdomyosarcoma	mRNA and protein expression in cell lines of both subtypes. High enzyme activity in plasma from patients. High levels of HPSE mRNA in tumor biopsies.	[36]
Human pediatric sarcoma cell lines (Ewing's sarcoma, alveolar and embryonal rhabdomyosarcomas, rhabdoid tumor, osteosarcomas)	Protein expression	[37]
Human synovial sarcoma cell lines and tumor xenografts	Protein expression	[38]
Adult soft tissue sarcomas (malignant fibrous histiocytoma, liposarcoma, leiomyosarcoma, angiosarcoma, chondrosarcoma synovial sarcoma, not defined subtype)	Positive by immunohistochemistry; overexpressed in nearly 50% of cases.	[39]
Alveolar orbital rhabdomyosarcomas	High mRNA and immunostaining in tumor specimens compared to normal tissue	[56]
Osteosarcomas	Overexpression detected in 37/51 osteosarcoma tissues by immunohistochemistry. Heparanase expression correlated with a poor chemotherapeutic response, metastasis and poor survival rate. Enzyme expression levels as an independent prognostic factor.	[118]
Osteosarcoma specimens and human cell line	Positive expression in 51% of cases by immunohistochemistry. Heparanase silencing by shRNA decreased expression of HIF-1α and reduced U2OS cell proliferation and migration/invasion	[119]
Osteosarcoma cell line	mRNA and protein expression in MG63 cell line. Heparanase silencing significantly inhibited cell adhesiveness and invasiveness	[120]
Murine osteosarcoma cell lines	In cell lines derived from FBJ virus-induced mouse osteosarcoma, heparanase mRNA correlated with high metastatic potential.	[121]

Heparanase expression was confirmed for the first time in rhabdomyosarcomas by Masola and colleagues. Human cell lines of both the alveolar and embryonal subtypes were found to express heparanase mRNA and protein, while enzyme activity was assessed in conditioned media. On the other hand, real-time PCR revealed a higher heparanase expression in 12 rhabdomyosarcoma biopsies compared to fetal skeletal muscle, and enzyme activity in plasma from 15 patients was significantly higher compared to healthy controls. The involvement of heparanase in rhabdomyosarcoma cell invasiveness was shown by gene silencing [36]. Subsequently, heparanase expression was confirmed in several cell lines from both soft tissue- and bone-sarcomas [37, 38].

Kazarin et al. [39] examined the expression of heparanase in biopsies from a heterogeneous cohort of 101 adult soft tissue sarcoma patients. Samples from primary tumors and metastases included malignant fibrous histiocytomas and sarcomas with no defined subtype histology, which together represented 50% of cases. Other histologies included liposarcomas, leiomyosarcoma, angiosarcomas, chondrosarcomas, and synovial sarcomas. Heparanase immunohistochemical staining indicated a large extent (> 50% of cells) in more than 95% of samples and overexpression in nearly 50% of cases including all subgroups. No correlation was found, however, with the risk of disease recurrence evaluated in 55 patients, or between the primary tumor and metastasis from the same patient evaluated in 10 cases. Unfortunately, the sample size was too small for any statistical analysis related to specific sarcoma sub-types. These findings highlighted the need to address the clinical significance of heparanase, and likely any tumor biomarker, in homogeneous sarcoma subtypes due to the high histological and molecular heterogeneity of these tumors [39].

As widely described in other sections of this Book, heparanase has multiple functions. Through its HS degradation activity, heparanase modulates structural and biochemical functions of HS proteoglycans (HSPGs) working in concert with them so that together they have been referred to as the heparanase/HSPG axis [40, 41]. As an endo-β-glucuronidase, heparanase participates in the complex biosynthetic/catabolic machinery, also including glycosyltransferases, sulfotransferases, and endosulfatases, which allow cells to finely control HS composition and sequence. Alterations of these HS modifying enzymes may profoundly affect the ability of HSPGs to interact with hundreds of growth factors, cytokines, chemokines, and several other structural and regulatory proteins, thereby influencing their multiple functions [42, 43]. Indeed, HS deregulation and alterations in HSPGs expression have been reported in several pathological conditions [44]. In cancer, they have been shown to influence both initiation and progression, regulating growth and survival, differentiation, angiogenesis, immune response, metastasis and response/resistance to a given drug treatment [reviewed in 45–48]. Several preclinical studies, focusing on cell-surface associated HSPGs, i.e., glypicans and syndecans, or HS metabolizing enzymes, evidenced subtype-specific roles in sarcoma pathobiology in keeping with the high histological and molecular heterogeneity of these tumors [41]. In most cases, however, the connection with heparanase expression has not yet been elucidated. For instance, glypican-5, overexpressed in rhabdo-

myosarcomas, was found to promote cell proliferation by enhancing signaling of heparin-binding growth factors such as FGF2, HGF, Wnt and Hedgehog (Hh) ligands [49, 50]. Hh signaling is thought to play an oncogenic role in rhabdomyosarcomas [51, 52]. Li and collaborators [50] demonstrated that glypican-5 participates in activation of Hh signaling by promoting the interaction of the Sonic Hh ligand with its receptor Patched (Ptc1). By using a non glycanated glypican-5 mutant, the authors demonstrated that the HS chains are essential for binding of both the ligand and receptor [50]. Interestingly, another member of the glypican family, glypican-3, also expressed in rhabdomyosarcomas [53], exerts an opposite role in the regulation of Hh signaling by competing with Ptc1 for Sonic Hh binding. Differently, from glypican-5, glypican-3 binds only the Hh ligand, mostly at the core protein [54]. The cooperation between glypican-5 and the Hh signaling in supporting sarcomagenesis also emerged in a comprehensive transcriptome analysis of a human mesenchymal stem cell line performed at various stages during the gradual transformation to sarcoma upon prolonged culture. At late stages, both glypican-5 and Ptc1 were found significantly overexpressed and co-localized. Moreover, silencing of the HSPG by RNA interference reduced cell proliferation [55]. In another study, heparanase and Hh pathway components, Ptc1, Smoothened, and glioma-associated oncogene homolog-1, were analyzed in a series of 23 alveolar orbital rhabdomyosarcomas by immunohistochemistry and nested RT-PCR. Consistent results with both techniques showed elevated expression of either heparanase or the Hh signaling components compared with normal muscle. In contrast, expression levels in samples from patients that underwent preoperative chemoradiotherapy were not significantly different from the normal tissue [56]. Although a role for heparanase in regulating Hh signaling has already been proposed in another tumor, i.e., medulloblastoma [57], mechanistic links with deregulated expression and functions of Hh components and glypican-5 have yet to be elucidated in rhabdomyosarcomas.

In the next sections, we review the literature reporting on the biological significance of heparanase expression and function in specific sarcoma sub-types.

15.3 Bone Sarcomas

Bone-forming tumors are benign or malignant neoplasms defined by neoplastic cells that differentiate along the lines of osteoblasts, and able to secrete the organic components of bone, which in turn may or may not mineralize [58]. They are heterogeneous tumors characterized by a broad spectrum of biological behaviors ranging from indolent to very aggressive with a rapidly fatal outcome. The three most common forms of primary bone tumors are osteosarcoma, Ewing's sarcoma, and chondrosarcoma. Whereas osteosarcoma and Ewing's sarcoma, mainly affecting adolescents and young adults, exhibit a high propensity to metastasize to the lungs, chondrosarcoma, more frequently observed after the age of 40, is characterized by a high frequency of local recurrence. The combination of chemotherapy,

surgical resection and radiotherapy have contributed to improving patients' outcome. Nonetheless, refractory and metastatic bone sarcomas remain lethal.

The occurrence of bone sarcomas in the context of rare hereditary disorders has provided unequivocal evidence of the relevance of mutations of genes coding for HSPGs (e.g., Glypican 3 in Simpson-Golabi-Behmel syndrome) or HS biosynthetic enzymes (e.g., exostins (EXTs) in Multiple Osteochondroma (MO) syndrome) in promoting and sustaining neoplastic growth. Emerging evidence indicates that the machinery involved in bone development and homeostatic processes, including angiogenesis which is intimately coupled with osteogenesis through reciprocal crosstalk [59], can be recruited and hijacked by neoplastic cells. Here, we summarize studies addressing the involvement of HSPGs and their synthesizing and modifying enzymes, with particular reference to heparanase, in bone physiology and disorders, focusing on the pathobiology of chondrosarcoma and osteosarcoma.

15.3.1 HSPGs and Heparanase in Bone Development and Biology

Bone is a specialized connective tissue composed of bone forming cells, the osteoblasts, deriving from mesenchymal stem cells, and bone resorbing osteolytic cells, the osteoclasts, considered as highly specialized macrophages derived from the monocyte lineage [60–62]. The formation of bone proceeds broadly via two types of processes. The intramembranous ossification, characteristic of flat bones, occurs through the differentiation of mesenchymal progenitor cells that proliferate and then differentiate into osteoblasts producing an osteoid matrix which undergoes calcification. The endochondral ossification, characteristic of appendicular skeleton and vertebral column, develops through an intermediate cartilaginous process. The progenitor cells in the growth plate, a highly organized structure driving long bone elongation, differentiate into chondrocytes that secrete a cartilaginous matrix. Then, the chondrocytes undergo hypertrophy and secrete proangiogenic factors to promote blood vessel formation and influx of mesenchymal progenitors which differentiate into chondroclasts, osteoclasts and osteoblasts. The cartilage template is then degraded by chondroclasts and replaced by a mineralized matrix synthesized by osteoblasts. The development of bone requires coordination between cell-cell, cell-matrix, and growth factor-mediated signaling to achieve ossification and mineralization [63]. In particular, osteoblastic differentiation requires ordered presentation and balance of several growth promoting elements including circulating molecules (e.g. growth factors, cytokines) and tissue architecture-related signals (cell-cell contact and cell adhesion) which share HS as a major co-factor [63–65]. In turn, osteoblasts produce many crucial mitogenic and adhesion factors that bind extracellular HS chains. The bone presents a highly specialized microenvironment and, although collagen is the prevalent organic component, HSPGs represent the most bioactive elements of the developing matrix. Actually, during osteogenesis,

expression and temporal changes in HSPG structure (e.g. HS sequence and length variation, critical positioning of sulfate groups) are instrumental in the concerted signaling flow of molecules coordinating mesenchymal stem cells growth/commitment and, ultimately, the osteoblast phenotype [63, 66]. In fact, HSPGs interact with a wide number of bioactive molecules with a central role in osteogenesis including Hhs, FGFs and their receptors, bone morphogenic proteins (BMPs), as well as collagens, laminins, and fibronectins. As HS interacting abilities "follow HS structure", the activity HSPG biosynthetic and modifying enzymes, including heparanase, may critically influence the signaling triggered by HS-binding molecules [63, 65–67].

Several lines of evidence support a relevant, although not yet fully elucidated, role of heparanase in bone formation and remodeling. Depending on the cellular context, the cell differentiation status and the surrounding microenvironment, heparanase has been associated with the osteogenic or osteolytic process. In the bone microenvironment, it has emerged as a relevant endogenous factor playing crucial functions in cell-cell communication and cell differentiation through modification of HSPGs and modulation of gene expression.

Saijo et al. [68] described sequential changes of heparanase and VEGF expression during endochondrial ossification in a model of fracture repair in mice. Heparanase, highly expressed in osteo(chondro)clasts at the chondro-osseous junction in the growth plate (physiological condition) and in the fracture callus (pathological condition), was suggested to promote fracture repair by recruiting VEGF into the local microenvironment and then osteoclast precursors and osteoprogenitors. Kram et al. [69] described the expression of heparanase in osteoblastic cells and its ability to stimulate bone formation and mass. Progressive increasing expression of heparanase mRNA was observed in murine bone marrow stromal preosteoblast MC3T3-E1 cells undergoing osteoblastic differentiation in osteogenic medium, whereas heparanase was undetectable in MC3T3-E1 cells incubated in non-osteogenic medium. In contrast, heparanase transcript, abundantly present at the monocytic stage of osteoclastogenic cultures, was found markedly decreased in cultures at an advanced stage of differentiation, suggesting downregulation of the enzyme during osteoclastogenesis. Notably, ex vivo bone marrow stromal cells derived from transgenic mice overexpressing human heparanase (hpa-tg mice), or MC3T3-E1 cells exposed to soluble human heparanase, spontaneously underwent osteogenic differentiation even in absence of osteogenic medium. These findings demonstrated the ability of heparanase to directly induce osteogenic differentiation and stimulate osteoblast activity. Moreover, observation of the skeletal phenotype of wt vs hpa-tg mice supported a positive regulation of bone formation by the heparanase-HSPG system as the transgene caused a marked increase of trabecular bone mass and cortical thickness. In this model, stimulation of bone formation was independent of the proangiogenic function of heparanase but likely related to its ability to regulate availability and activity of HS-binding proteins (e.g. VEGFs, FGFs) directly implicated in the control of osteoblast number and functions. Conversely, hpa-tg mice-derived bone marrow cells that underwent osteoclastic differentiation following stimulation with M-CSF and RANK, displayed an increased

osteolytic activity with respect to the cells derived from wt animals [70]. These findings highlighted the relevance of the microenvironment in influencing heparanase functions and bone marrow cell behavior. Studies examining the expression of HSPGs and related enzymes in MC3T3-E1 cells undergoing osteoblastic differentiation provided insights into the temporal, structural and functional changes in HSPGs during osteogenesis [66, 71]. Proliferating cells (day 5) displayed a high level of HSPGs, mainly glypican-3 known to promote FGF- and BMP-mediated mitogenic signaling [62]. In this experimental model, the active production of HSPGs was associated with increased expression of HS synthetic enzymes (i.e. glycosyltransferases EXTs, HS N-deacetylase/N-sulfotransferases NDSTs, 2- and 6-O sulfotransferases), highlighting the need for longer, more sulfated and complex HS chain bound to a variety of HSPG core proteins to sustain the growth process. During the shift of MC3T3-E1 cells from a proliferative to a differentiated status (day 14), a progressive reduction of HS chain complexity was observed. Indeed, in cells fully committed to osteogenic differentiation, the production of short and highly sulfated HS chains correlated with increased expression of NDST-1 and glypican-3 protein core. Thus, osteogenically committed cells likely need the production of fewer, short and homogeneous, but more highly sulfated HS side chains to mediate specific growth factor signals to switch from proliferation to differentiation [71]. Mineralizing MC3T3-E1 cells (day 20) were characterized by the presence of short and less sulfated HS and high expression levels of heparanase. Moreover, these cells exhibited increased expression of syndecan-2, a HSPG involved in bone ECM deposition and tissue consolidation [66, 72]. Overall, these findings indicated a different HSPG profile and a systematic HS variability with more complex sugars made during the MC3T3-E1 cell growth process compared to the subsequent phases of osteogenic differentiation characterized by intense HSPG turnover likely bolstered by higher levels of heparanase.

Glypican-3 was demonstrated to mediate MC3T3-E1 cell commitment toward osteogenesis by inducing the osteogenic transcription factor Runx2 [71]. Complex crosstalk has been described between Runx2 and the FGF2/HSPG axis which forms an ECM-regulated feedback loop controlling osteoblast proliferation and differentiation [73]. Signaling mediated by FGFs is fundamental for bone development [74]. Indeed, disruption of the FGF2 gene in knock-out mice resulted in decreased bone mass whereas mutations in FGFRs are responsible for several clinically distinct craniosynostosis syndromes in humans [75, 76]. Reintroduction of Runx2 in mouse calvaria Runx2-null osteoprogenitor cells was reported to markedly increase expression of genes related to FGF2/HSPGs axis (e.g. FGFR2 and FGFR3, syndecan −1, −2, −3, glypican-1) [73]. In addition, the transcription factor increased expression of EXT1 and heparanase and altered the relative expression of NDSTs and O-sulfotransferases. As HS structural diversities determined by saccharide sequence, sulfation degree and pattern are known to affect FGF/FGFR signaling outcome [67, 77], Runx2 indirectly changed osteoprogenitor responsiveness to FGF2 during the transition from active proliferation to growth arrest. In turn, FGF2 and HS from differentiating MC3T3-E1 cells stimulated Runx2 expression [78].

By a combination of ex vivo and in vitro approaches, along with pharmacological inhibition of heparanase by the phospho-sulfo-mannan PI88, Brown et al. [79] investigated the contribution of the enzyme in long bone formation in developing mice. High expression levels of heparanase mRNA and protein were detected in perichondrium, periosteum and at the chondroosseous junction, sites of crucial signaling events regulating bone length and width. Moreover, experiments performed in the murine chondrogenic cell line ATDC5, suggested that heparanase activity was strictly titrated at the transition from chondrogenesis to osteogenesis. A biphasic pattern of heparanase expression was also observed during the osteogenic differentiation timeframe (0–21 days) of rat marrow stromal cells [80]. Protein and mRNA expression levels reached a peak on days 10 and 14, respectively, followed by a gradual decline. Notably, consistent with a declined osteogenic differentiation ability over the age, heparanase expression in osteogenic differentiated marrow stromal cells from aged rats was weaker compared with that from young rats.

15.3.2 HSPGs and Heparanase in Bone Disorders

Smith et al. [81] described for the first time the expression and function of heparanase in human primary osteoblasts and found lower levels of expression and activity in human osteoporotic osteoblasts from bone fragments compared to the cells from healthy subjects. The significant correlation found between the decrease in heparanase mRNA expression and the activity of the bone turnover marker alkaline phosphatase in osteoporotic osteoblasts was consistent with the downregulation of several osteogenic genes (e.g. VEGFA, FGFR2, COL15A1, BMP3). Osteoblasts exposed to exogenous heparanase displayed increased levels of histone H3 phosphorylation at Ser 28, a modification coupled with the induction of transcription of immediate-early genes [82]. These findings suggested a direct involvement of HPSE in human osteoblastogenesis through histone H3 modulation and epigenetic regulation of osteogenic gene expression.

Heparanase has also been implicated in cartilage disruption and subchondral bone remodeling occurring in human osteoarthritis, a characteristic adult disease state of cartilage. Gibor et al. [83] described heparanase expression and enzymatic activity in adult human osteoarthritic cartilage and suggested a contribution of the enzyme in the pathologic interactions between the chondrocytes and their pericellular matrix. In fact, the addition of exogenous heparanase to cultured human primary chondrocytes induced the expression of the metalloproteinases MMP13 and ADMTS4, acting as ECM catabolic enzymes, and downregulated anabolic genes (i.e., aggrecan core ACAN and COL2A1). The effect on catabolic gene products, partially mediated by FGF2 signaling, was reverted by treatment with the heparanase inhibitor PG545. This observation is consistent with findings in multiple myeloma models evidencing that secretion of heparanase, along with other molecules promoting matrix degradation, enhances bone destruction within the tumor microenvironment [84] (Sanderson et al., Chap. 12 in this volume).

Recently, in apparent contrast with previous findings, Chanalaris et al. [85] did not find differential expression of heparanase mRNA in human knee cartilage from osteoarthritic donors with respect to specimens from normal subjects. Nonetheless, osteoarthritic cartilage samples showed a marked dysregulation of the expression of HS biosynthetic and modifying enzymes with increased expression of the EXT glycosyltransferases, the glucuronyl epimerase GLCE, and the sulfotransferase HS6ST1. Also, HS6ST1 was demonstrated to boost FGF2-ERK signaling in human chondrocytes. Overall, these findings support the involvement of highly dynamic modulation of HS structure and function in the regulation of bone formation under both physiological and pathological conditions.

15.3.3 HSPGs and Heparanase in Osteochondromas and Chondrosarcomas

Chondrosarcomas constitute a heterogeneous group of malignant bone tumors, characterized by the production of cartilage matrix and displaying different histopathology and clinical behaviors. Following osteosarcoma, chondrosarcoma is the second most frequent primary malignancy of the bone [86]. Conventional chondrosarcomas are typically low or intermediate grade and are characterized by indolent clinical behavior and low metastatic potential, whereas high-grade chondrosarcomas (5–10%) are associated with high metastatic potential and poor prognosis. Localized chondrosarcomas are generally well managed by surgery. Chondrosarcomas are inherently resistant to chemo- and radio-therapy due to low mitotic fraction, activation of multidrug resistance pumps, and limited drug penetration into the tumor microenvironment characterized by poor vascularity and abundant hyaline-dense ECM. Conventional chondrosarcomas occur either de novo in the bone medulla or arise, as secondary tumors, from preexisting benign cartilage lesions, named enchondromas and osteochondromas, during periods of bone growth in a site adjacent to the growth plate [87, 88]. Enchondromas can develop central chondrosarcoma whereas osteochondromas can be precursors of peripheral chondrosarcomas. Enchondromas arise within the metaphyseal portion of the bone. Osteochondromas that appear as cartilage-capped bony neoplasms on the outer surface of bones, can occur as sporadic/solitary or as multiple lesions in the context of hereditary Multiple Osteochondroma (MO) syndrome. MO is an autosomal dominant disorder characterized by short stature, skeletal deformities and the formation of osteochondromas (exostoses). This syndrome represents an interesting model of oncogenesis driven by complex deregulation of HSPG synthesis and metabolism [65, 87, 89, 90]. Loss-of-function mutations of the tumor suppressors EXT genes have been identified in both sporadic and MO osteochondromas, although associated with different gene alterations and mechanisms [87, 91, 92]. EXT1/2 glycosyltransferases function in hetero-oligomeric complexes to polymerize HS chain. Loss of either enzyme causes a total deficit of HS chains resulting in embryonic lethality.

Specific EXT mutations are considered early-stage molecular alterations able to increase the proliferative capacity of normal chondrocytes. In fact, by causing reduction/lack of HS, or HSPG mislocation, EXT mutations produce a deep perturbation of signaling pathways tightly implicated in the regulation of chondrocyte proliferation/differentiation, such as Indian Hh, BMP, and FGF pathways. McCormick et al. [93] demonstrated that EXT1 mutation caused aberrant processing and cytoplasmic accumulation of HSPG resulting in abnormal diffusion of Hh ligands in the extracellular environment at the growth plate. Absence of HS and intracellular accumulation of syndecan-2 and CD44v3 HSPGs were also observed in the osteochondroma and peripheral chondrosarcoma cartilage [94]. In mice carrying EXT1 mutation, a reduced amount of HS potentiated Indian Hh signaling resulting in delayed hypertrophic differentiation and increased chondrocyte proliferation [95]. Moreover, an increased diffusion area of Indian Hh was supposed to produce a loss of polar organization allowing chondrocytes to growth in the wrong direction. Additional molecular insights revealed that a somatic "second hit", likely complementing germline EXT mutations to further decrease HS production, is required for osteochondroma development. Actually, loss-of-heterozygosity, aneuploidy, and other large genomic changes can render local resident cells EXT1- or EXT2-null [96]. Further clinical observations and experimental data from mouse models add levels of complexity in the scenario of osteochondroma formation and its potential evolution towards peripheral chondrosarcoma. The observed heterogeneous distribution of HS-positive and -negative cells in murine and human osteochondromas paved the basis for a "niche-based" model of oncogenesis implicating both cells with homozygous inactivation of EXT genes and wild type cells in shaping osteochondroma [92, 97, 98]. The EXT-negative cells present in the osteochondromas would create an extracellular mutation-promoting environment favoring the acquisition of late-stage mutations (e.g., p53, Rb) in EXT-positive cells retaining one or both copies of EXT genes. Such alterations, occurring in EXT-positive cells likely endowed with stem-like genotype, would provide a proliferative advantage over the osteochondroma EXT-null cells [92, 99]. Thus, osteochondromas would serve as a niche which facilitates the committed stem cells/EXT wild type chondrocytes to acquire genetic changes to develop malignant secondary peripheral chondrosarcomas.

Heparanase has been recently defined as an important culprit coupled with EXT loss in Multiple Osteochondroma (MO) [92]. Early studies by Trebicz-Geffen et al. [100] provided the first evidence of higher levels of heparanase in specimens and cell cultures from MO patients compared with solitary exostoses and healthy subjects, suggesting that increased HS degradation, in addition to reduced synthesis by EXT loss of function, could contribute to HS low levels in MO. Increased expression of heparanase was also described in tumor cartilage from MO by Yang and colleagues [101]. Huegel et al. [102] evidenced, by immunohistochemical staining, the presence of heparanase in all chondrocytes within the exostoses and hypothesized that, in the MO syndrome context, the endoglycosidase upregulation results from a feedback mechanism triggered by EXT inactivation-induced modulations in HS levels [102]. Consistently, treatment of primary mesenchymal cells with the HS

antagonist Surfen significantly increased heparanase level. Although somewhat paradoxical and counterintuitive, heparanase plays a concurrent role in stimulating chondrogenesis by further decreasing the levels of HS. Incubation of ATDC5 chondrogenic cells with human recombinant heparanase was found to promote cell migration, proliferation, and differentiation. Coherently, in vitro chondrogenesis was significantly counteracted by the heparanase inhibitor, glycol-split heparin, roneparstat (= SST0001) [102] (Noseda and Barbieri, Chap. 21 in this volume). These findings are in accordance with the inverse relationship between EXT and heparanase expression reported in several types of cancer cells [92]. Overexpression of heparanase was detected in 5/7 specimens from human chondrosarcoma patients although the tumor subtype was not reported [39].

An additional study from Presto et al. [103] showed that NDST1 directly interact with EXT2 during HS chain formation and that EXT mutations can modulate expression/function of NDST1 thus affecting both HS polymerization and sulfation. By combining different analytic methods, Veraldi and colleagues [104] investigated the structural complexity of HS from human EXT-mutant MO and peripheral chondrosarcoma specimens compared with HS from prepubescent growth plate and fetal cartilagineous samples. Most pathologic samples of both osteochondromas and peripheral chondrosarcomas displayed HS characterized by higher sulfation degree compared with other samples. In line with this observation, a positive correlation was found between enhanced expression of the 6-O sulfotransferases HS6ST1 and HS6ST2 and histological grade of chondrosarcoma, pointing to a relevant role for HS 6-O sulfation in disease progression [105, 106].

Interestingly, central chondrosarcomas, devoid of EXT alterations, are distinct genetic entities with respect to peripheral chondrosarcomas; they were shown to exhibit aberrant cytoplasmic accumulation of HSPG (e.g., CD44v3 and syndecan-2) and deregulated Indian Hh signaling [107]. Aberrant localization of HSPGs was also observed in low-grade variant of clear cell chondrosarcoma as well as in aggressive mesenchymal and dedifferentiated subtypes [108]. These observations highlight deregulation of HSPGs as a common feature in bone cartilage tumors although the underlying molecular mechanisms have not yet been elucidated.

15.3.4 HSPGs and Heparanase in Osteosarcomas

Osteosarcoma, the predominant form of bone cancer primarily occurring in children and adolescents, preferentially arises in the long bones near the metaphyseal growth plates [60, 109]. Osteosarcoma is mostly sporadic but a greater incidence is observed in subjects with Page's disease of bone, after therapeutic radiation, and in certain cancer predisposition syndromes (e.g., Li-Fraumeni). Current therapies integrate surgery and combinatorial chemotherapy resulting in cures in about 70% of non-metastatic patients. Unfortunately, an overall 5-year survival rate of about 20% is reported for patients with metastatic or relapsed disease [60]. Etiological factors and pathogenic mechanisms underlying osteosarcoma development are very complex

and not yet fully elucidated. The challenging genomic complexity and instability, along with intratumoral and intertumoral heterogeneity makes very hard the identification of drivers as well as vulnerabilities for novel effective therapeutic approaches. The "multiple drivers" hypothesis pointed out the contribution of both first drivers (e.g., p53, Notch1, Ptc1) and synergistic drivers (e.g., Rb1, PTEN) in osteosarcomagenesis. According to this hypothesis, the nature and the number of alterations deeply impact the onset, the latency, and progression of this malignancy [109]. Recently, novel types of genetic abnormalities were described including chromothripsis (i.e., a phenomenon in which a single catastrophic event results in massive genomic rearrangements and remodeling of chromosomes) and kataegis (i.e., a pattern of localized hypermutation colocalized with regions of somatic genome rearrangements) [109].

Osteosarcomas are composed of malignant osteoblasts producing immature bone and osteoid tissue, an organic mineralized matrix primarily composed of collagen I [60]. Various hypotheses have implicated among osteosarcoma originating cells, mutation-harboring mesenchymal stem cells, osteoblast-committed cells undergoing defective differentiation, and/or osteocytes [110]. In mesenchymal stem cells, the inactivation of p53, frequently disrupted in these tumors, was shown to promote early osteogenesis by accelerating osteoblastic differentiation while impairing osteocyte terminal maturation [111]. On the other hand, osteoblasts from pluripotent stem cells derived from Li-Fraumeni patients, harboring mutant p53, were able to recapitulate in vivo osteosarcoma features [112]. Also, murine osteocytes immortalized by SV-40, inactivating p53, were shown to originate osteosarcomas [113]. Taking into consideration the osteosarcoma high heterogeneity, it is likely that all three cell types can contribute to osteosarcomagenesis.

Defective osteogenic differentiation resulting from deregulation of Hh, Notch, Wnt, and BMP signaling pathways and overactivation of several growth factors/receptor tyrosine kinase axes (e.g. VEGF/VEGFR, IGF1/IGF1R, PDGF/PDGFR), have been involved in osteosarcoma development [60]. Deregulation of HSPGs and related enzymes that could greatly affect these signaling pathways contributing to osteosarcomagenesis, were described in several reports. For instance, a strong expression of syndecan-2 was found in mature osteoblasts, whereas low levels were observed in osteosarcoma cell lines [72, 114]. Syndecan-2 exogenous expression in U2OS osteosarcoma cells decreased migration/invasion and chemoresistance suggesting an oncosuppressive role for this HSPG. Consistently, syndecan-2 levels were found higher in bone tumors of patients responding to chemotherapy with respect to non-responders [114]. Conversely, increased expression of syndecan-4 in high-grade osteosarcomas was associated with large tumor size and distant metastases [115]. The HS 6-O-sulfatase SULF2 was shown to be a direct transcriptional target of p53 in several cancer cell lines including U2OS cells [116]. Importantly, p53 has been shown to directly bind heparanase promoter inhibiting its activity, whereas mutant p53 variants failed to exert an inhibitory effect [117]. Several preclinical and clinical studies have correlated heparanase expression with aggressive tumor phenotype [see Chap. 1]. High heparanase expression was detected by immunohistochemistry in 37/51 osteosarcoma specimens with protein expression levels

correlating with poor response to chemotherapy, metastasis occurrence, and poor survival rate. Moreover, multivariate analyses revealed the protein overexpression as a significant independent risk factor for distant metastasis [118]. High levels of heparanase were also detected in plasma samples from pediatric cancer patients including 7 patients suffering from osteosarcomas [34]. Zeng et al. [119] confirmed the expression of heparanase in 51% of human osteosarcoma biopsies and found a significant correlation with tumor size. Moreover, these authors noted that 40% of the samples were positive for both heparanase and HIF1α. The expression of both proteins correlated with the presence of lung metastasis and poorer patients' survival, suggesting functional cooperation in promoting angiogenesis and tumor progression. Actually, in U2OS cells, heparanase silencing by shRNA decreased expression of HIF1α and reduced cell proliferation and migration/invasion. Likewise, proliferation, adhesiveness, and invasiveness of the human osteosarcoma cell line MG63 were significantly inhibited by heparanase silencing [120]. In cell lines derived from FBJ virus-induced mouse osteosarcoma, heparanase expression was found associated with a high metastatic potential [121]. Cell surface expression of HS was found significantly higher in poorly metastatic FBJ-S1 cells with respect to the FBJ-LL highly metastatic cells consistently with lower levels of both heparanase and EXT1 expression. Moreover, the authors demonstrated by molecular approaches that FBJ-S1 cell motility was regulated by heparanase, under EXT1 control. In U2OS and SAOS osteosarcoma preclinical models, treatment of mice harboring tumor xenografts with the heparanase inhibitor Roneparstat induced a significant antitumor activity providing preclinical proof of principle that targeting heparanase could represent a valuable therapeutic approach in this malignancy [37].

15.4 Targeting Heparanase in Sarcomas

Several lines of evidence, discussed in details in other sections of this Book, highlight the implication of heparanase in critical processes of tumor biology (e.g., growth, angiogenesis, metastasis, drug resistance) and its upregulation in the vast majority of malignancies examined, including carcinomas and hematological tumors as well as sarcomas. Such evidence, and the favorable feature of being the only HS degrading endoglycosidase, not substitutable with other enzymes, has supported the idea that heparanase could be a suitable target and promoted the development of heparanase inhibitors as anticancer therapeutics [46, 122] (Chhabra and Ferro; Hammond and Dredge; Noseda and Barbieri, Naggi and Torri; Giannini et al., Chaps. 19, 20, 21, 22 and 23 in this volume). A few studies tested the potent heparanase inhibitor roneparstat (^{100}NA,RO-H, ST0001) (Noseda and Barbieri, Naggi and Torri, Chaps. 20 and 21; in this volume), a chemically modified non-anticoagulant heparin, in preclinical models of human sarcomas [41]. The first sarcoma model applied in these studies was Ewing's sarcoma [35], a natural choice as roneparstat had previously been shown effective in multiple myeloma models [123, 124]. Ewing's sarcoma is an aggressive tumor that mainly develops in bones, shar-

ing with multiple myeloma a functional microenvironment characterized by complex interactions between cellular components (tumor cells, osteoclasts and other stromal cells), humoral factors (growth factors and cytokines) and the ECM which provides a favorable "niche" for tumor growth and progression [125, 126]. The biological phenotype of both tumors has been shown to be influenced by signaling pathways mediated by growth factors (e.g. IGF-1, PDGF, bFGF, VEGF) some of which are transcriptional targets of the oncogenic fusion protein EWS-FLI1 which drives tumorigenesis in Ewing's sarcoma [127, 128]. In studies on multiple myeloma, the cooperation between heparanase and the HSPG syndecan-1 was demonstrated to regulate the functions of several growth factors in the bone niche, promoting myeloma cell growth, angiogenesis, and metastasis, effects that were counteracted by roneparstat [123, 124, 129]. Similarly, the glycol-split heparin effectively inhibited TC71 Ewing's sarcoma cell invasion stimulated by VEGF and bFGF through Matrigel, a reconstituted basement membrane highly rich in HSPGs. Moreover, roneparstat induced a strong antitumor effect in mice harboring TC71 tumor xenografts with 25% of cures noted in treated animals [35].

In a subsequent report, investigation of the effects of roneparstat was extended to a panel of six human pediatric sarcoma models including bone (osteosarcoma, Ewing's sarcoma) and soft tissue (rhabdomyosarcomas, rhabdoid tumor) histotypes with simple or complex genotype [37]. The study confirmed the ability of the heparin derivative to abrogate cell invasion induced by heparin/HS-binding growth factors (PDGF, bFGF, VEGF, HGF). Moreover, a marked drug inhibitory effect on the release/secretion of several angiogenesis-related molecules was reported (e.g., VEGF, MMP-9). All sarcoma cell lines growing in mice as tumor xenografts were responsive to roneparstat antitumor effect with maximum tumor growth inhibition (around 90%) obtained in the genetically simple sarcoma models (i.e., TC71 Ewing's sarcoma, RH30 alveolar rhabdomyosarcoma, and the A204 rhabdoid tumor previously misclassified as a rhabdomyosarcoma). Combination treatments with roneparstat and antiangiogenic agents, the anti-VEGF antibody bevacizumab and the tyrosine kinase inhibitor sunitinib, were shown to significantly increase the antitumor efficacy compared to single-agent therapies in the Ewing's sarcoma model.

An additional study by Cassinelli and colleagues addressed the impact of roneparstat treatment on sarcoma cell signaling [130]. As HS mimetic, roneparstat can act as a multi-target agent inhibiting heparanase and competing with HS in their broad regulatory functions. Overall, these effects are expected to influence growth factor signaling in both tumor and stromal cells. Focusing on RTKs-mediated signaling, the authors applied a multiplexed phosphoproteomic approach to investigate the effects of drug treatment on receptor activation in sarcoma cells comprising various histotypes. Roneparstat was found to inhibit in a context-dependent manner growth factor/receptor tyrosine kinase axes implicated in sarcoma pathobiology, and inhibition was further validated by cellular functional assays. In vivo, reduced activation of EGFR, ERBB4, InsR, and IGF1R in tumor xenografts from treated mice confirmed the drug pharmacodynamic effect. The good tolerability of roneparstat evidenced in preclinical tumor models suggested that it could be used in combination with conventional cytotoxic drugs. The combination with the camptothecin

irinotecan, a drug of clinical interest in pediatric sarcoma patients, was well tolerated and highly effective in the A204 rhabdoid tumor xenograft significantly enhancing tumor growth inhibition, complete responses, and cures as compared to single drugs administration. A204 cells are characterized by constitutive high activation of PDGFRα which support rhabdoid tumor growth but is not directly implicated as a driver of malignant transformation. Early characterization of roneparstat activity in tumor models evidenced its antimetastatic potential against experimental metastases induced by intravenously injected B16 murine melanoma cells [131]. Using the orthotopic A204 rhabdoid tumor that metastasizes from the primary xenograft site to the lung, Lanzi and colleagues confirmed the antimetastatic activity of the heparin derivative in a human sarcoma model of spontaneous dissemination (unpublished, Fig. 15.1). Notably, the heparin derivative was also able to counteract malignant transformation driven by the COL1A1/PDGFB fusion oncogene generated by chromosomal translocation in dermatofibrosarcoma protuberans [130]. In this sarcoma, the constitutive activation of PDGFRβ is induced through an autocrine loop supported by the functional PDGFBB produced by processing of the chimeric oncoprotein [132].

Another heparin derivative, the supersulfated low molecular weight heparin ssLMWH with high anti-heparanase activity, was tested in human synovial sarcoma models [38]. ssLMWH inhibited anchorage-independent growth in soft agar and invasion in Matrigel of synovial sarcoma cells. Moreover, it downregulated the activation of several receptor tyrosine kinases. In cells with elevated constitutive activation of IGF1R, a strong synergistic effect was shown with the dual IGF1R/InsR tyrosine kinase inhibitor BMS754807. Previous studies have associated IGF1R expression with a high incidence of metastases in synovial sarcoma [133] while its activation has been shown to be promoted by the IGF2 ligand whose transcription is epigenetically induced by the SS18-SSX fusion oncoproteins peculiar of these sarcomas [134]. Despite a complete inhibition of IGF1R and InsR, BMS754807 did not achieve effective inhibition of downstream signaling pathways in synovial sarcoma cells, likely due to bypass resistance pathways. In contrast, the combined treatment with BMS754807 and ssLMWH enhanced inhibition of both AKT and ERK signaling which resulted in apoptosis induction and suppression of cell motility in vitro. An impressive effect was also obtained in vivo by the drug combination that abrogated the orthotopic growth of synovial sarcoma xenografts and their spontaneous dissemination to the lungs.

Similarly to heparin derivatives, DMBO, designed as mimetic of the pyranosidic ring structure of HS, was found to bind growth factors and cytokines (i.e. VEGF, HB-EGF, TNF-α) and to inhibit heparanase catalytic activity. In in vitro assays, the oxazine inhibited osteosarcoma cell proliferation, migration, and invasion. In vivo, it was able to inhibit liver experimental metastases induced by intravenously injected cells [135].

The dual nature of heparin derivatives and the oxazine DMBO, as heparanase inhibitors and HS mimetics, hampers a precise mechanistic interpretation of their biological effects [136]. Two recent reports described new small molecule inhibitors of heparanase enzymatic activity [137, 138] (Giannini et al., Chap. 23 in this volume). The best compounds in these series showed inhibitory effects similar to those observed with roneparstat and ssLMWH on invasion of rhabdoid tumor and synovial

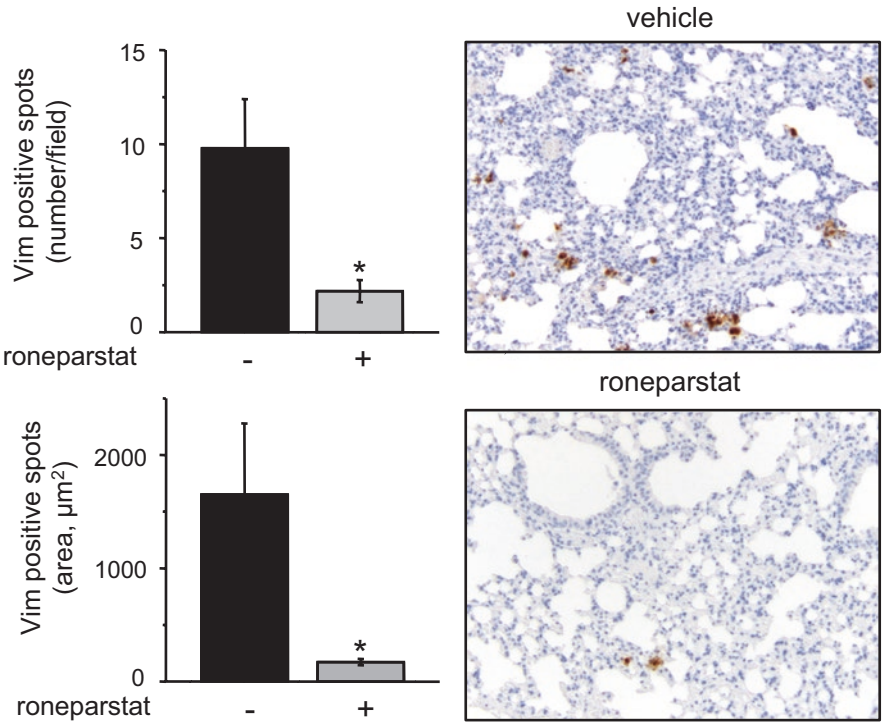

Fig. 15.1 Inhibition of spontaneous lung micrometastases from orthotopic A204 rhabdoid tumor by the heparanase inhibitor roneparstat. A204 cells were injected i.m. in SCID mice. Roneparstat was administered s.c at 60 mg/kg (twice/day, 5 days/week) for 6 weeks. After treatment interruption, mice were sacrificed when primary tumors had similar volumes. Lungs were formalin fixed and paraffin embedded. Sections were subjected to immunohistochemistry with anti-human vimentin antibodies and positive spots were quantified. ∗, $P < 0.05$ by Student't-test

sarcoma cells and the expression of proangiogenic factors in osteosarcoma cells. These findings suggest that inhibition of heparanase endoglycosidase activity is shared by agents belonging to different chemical classes.

15.5 Concluding Remarks

Sarcomas are characterized by an aggressive phenotype, angiogenesis, and propensity to metastasize primarily to the lung. Identification of specific vulnerabilities has been successful only in a few histologies. For most patients with advanced disease, survival rates with available systemic therapies (i.e., conventional cytotoxic, new targeted and histology-driven) remain low, while immunotherapy is still in early clinical phases. New therapeutic approaches able to counteract sarcoma progression and improve patients' outcome are highly desirable.

Since early reports describing the detection of an endoglycosidase able to produce biologically active HS fragments in sarcoma cells, heparanase was associated with the cell metastatic potential. The emerging role of heparanase in bone formation and remodeling during development suggests that its multiple functions in cooperation with HSPGs can be hijacked by bone sarcoma cells and exploited to promote cell signaling, angiogenesis, and dissemination. Likewise, aberrant cooperation of heparanase with other HS modifying enzymes appears to participate in the pathogenesis of cartilaginous tumors through a complex and still incompletely understood interconnection between HS, heparanase, the heparanome, and the transcription machinery. Several aspects of heparanase deregulation and pathological functions in sarcomas remain to be elucidated including the relationship with oncogenic players and molecular pathogenesis in the various histological subtypes.

Whereas most studies examining the clinical significance of heparanase in human malignancies have been carried out in hematological or epithelial tumors, investigation applying homogeneous cohorts of sarcoma patients is challenging because of the rarity and high heterogeneity of these malignancies. Only a few studies have correlated heparanase expression with poor prognosis in patients with Ewing's sarcoma and osteosarcoma to date.

Nevertheless, studies addressing the effects of heparanase inhibitors in sarcoma models have provided preclinical proof-of-concept that heparanase represents a druggable vulnerability in either bone or soft tissue sarcomas. The potential of HS mimetics to improve current therapies was confirmed by the enhanced inhibition of sarcoma xenograft growth and spontaneous metastatic dissemination as well as the high rate of cures in combination regimens with cytotoxic and targeted agents. These findings provide a rational basis for including sarcomas in the evaluation of HS mimetics undergoing clinical development. It will be also interesting in future studies to target the heparanase/HSPG system with new heparanase targeting approaches by specific inhibitors (e.g., small molecules, antibodies) currently undergoing preclinical development.

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Part IV
Immune Cells

Chapter 16
Heparanase is Involved in Leukocyte Migration

Nobuaki Higashi, Tatsuro Irimura, and Motowo Nakajima

Abbreviations

BM	Basement Membrane
CAR	Chimeric Antigen Receptor
fMLP	formyl Methionyl Leucyl Phenylalanine
Hpse	Heparanase
HSPG	Heparan Sulfate Proteoglycan
MMP	Matrix Metalloproteinase

16.1 Introduction

Cell migration is essential for exerting self-defense mechanisms in order to capture and kill pathogens invading into the body and to transfer information on to the acquired immune system. Although some resident immune cells are present in peripheral tissues for this purpose, a large number of cells can be recruited into infected or injured sites in the event of an emergency via extravasation.

Circulating immune cells transmigrate through blood vessels into extravascular regions in response to inflammatory stimuli. This process includes a series of heterocellular interactions with endothelial cells. In the case of neutrophils, migration through the endothelial cell layer can be rapid (< 2–5 min), while that through the

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basement membrane (BM) underneath can take much longer (> 5–15 min) [8], suggesting that passage through the subendothelial BM is a rate-limiting step to achieve transmigration.

In analogy to metastatic cancer cells that can pass through subendothelial BMs, the action of heparanase in the invasive step of immune cells caught the attention of immunologists. Basement membranes, sheet-like structures with a thickness of approximately 30–100 nm depending on the sites, are composed of type IV collagen, laminin, entactin/nidogen, and heparan sulfate proteoglycan (HSPG) with a core protein, perlecan. Heparanase (Hpse) degrades the heparan sulfate (HS) chains in BM and thereby is involved in the invasion of melanoma cells through the BM. In addition, the expression level of Hpse is correlated to metastatic efficiency *in vivo* [13]. Immune cells express a relatively higher level of Hpse than other types of cells, implying a functional role of Hpse in extravasation.

This section focuses on the regulatory roles of Hpse enzyme in immune cell migration, a physiological event necessary to sustain immune reactions. We will summarize recent evidence showing that engagement of this enzyme occurs quickly, can be triggered by intracellular trafficking and does not necessarily require the induction of Hpse gene transcription.

16.2 Early Findings on the Expression of Hpse in Immune Cells

Classical studies surveying heparan sulfate degradation activity in different types of cells revealed relatively high expression of Hpse especially in activated T cells, macrophages, granulocytes, platelets and mast cells [22]. More recently, comprehensive expression analysis in different cells and organs demonstrated particularly high expression of Hpse transcripts in whole blood cells, especially CD14+ monocytes and CD33+ myeloid cells, when compared to other organs except for placenta [20].

16.3 Involvement of Heparan Sulfate Proteoglycans in Transmigration

Transmigration through blood vessels into extravascular regions includes a series of heterocellular interactions with endothelial cells. In the case of neutrophil extravasation, these interactions are: (a) selectin-dependent interaction or tethering, (b) firm adhesion with integrins, (c) transmigration by stimulation with chemokines and other migratory agents, and (d) passing through the endothelial cell layer and subendothelial BM (Fig. 16.1). In steps (c) and (d), migrating cells which pass through postcapillary cremasteric venules exert lateral migration to seek “gaps” or

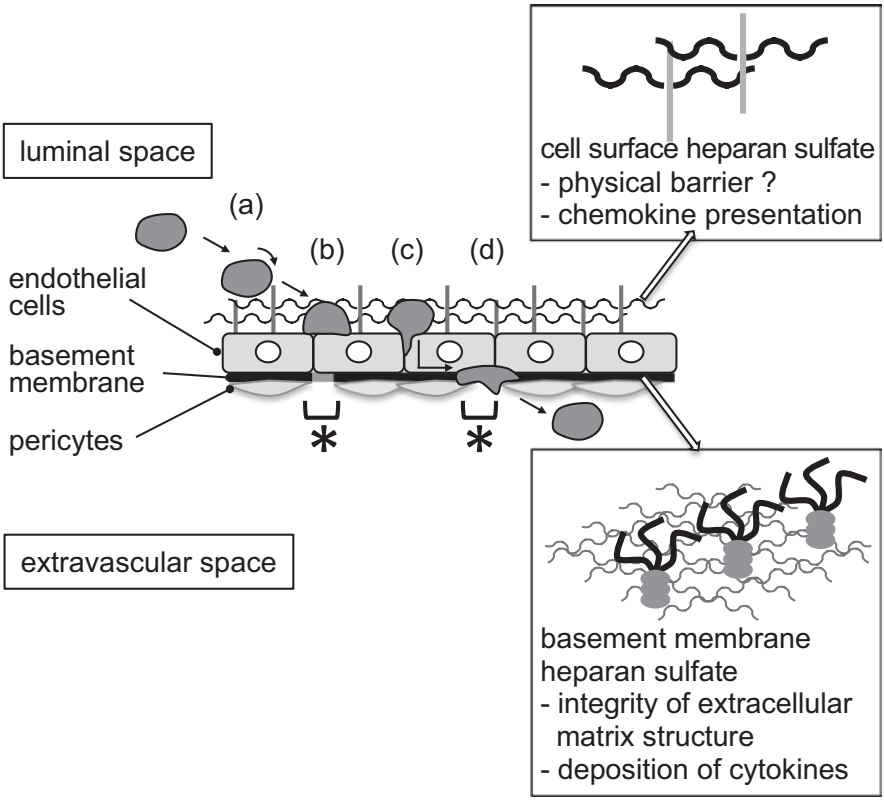

Fig. 16.1 Extravasation of circulating immune cells and heparan sulfate**.** Vascular vessels are enwrapped by subendothelial BM and pericytes. Extravasation is a reaction of serial cell-cell interaction: (**a**) tethering, (**b**) firm adhesion, (**c**) transendothelial migration, (**d**) passing through the subendothelial BM. In (d), the migrating cells migrate in the subendothelial space to seek "gaps" or low expression region covered with a lower amount of collagen IV and laminin (asterisk in the figure)

"low expression regions" in the BM, which are characterized by relatively lower amounts of collagen IV and laminin and also a lack of pericytes wrapped around the outside of the BM [24].

As HSPG is a main constituent of the BM, cellular locomotion is hindered or may be supported by HSPG that distributes homogeneously in the BM including "low expression regions" [24]. Furthermore, expression of Hpse may influence the formation of chemokine gradients on extracellular matrices, which are essential for directional migration. It has been shown that HSPG controls the extravasation of circulating immune cells [9]. Collectively, it is reasonable to speculate that both Hpse-mediated cleavage of HS and HS-mediated adhesion of immune cells to the vasculature influences the extravasation process, especially in step (d). Both immune cells and activated endothelial cells are possible sources of Hpse.

16.4 Engagement of Hpse Triggered by Intracellular Trafficking of This Enzyme in Monocytes

We examined whether Hpse is involved in transmigration through the BM using an in vitro invasion model. Significantly larger numbers of differentiated than undifferentiated U937 cells invaded the model BM, and this invasion was inhibited by addition of anti-Hpse neutralizing monoclonal antibody. Concomitantly, differentiated but not undifferentiated U937 cells exerted degradation activity of radiolabeled HS in a BM-like extracellular matrix. Hpse expression levels were not significantly different in differentiated and undifferentiated U937 cells, which led us to conclude that the elevated HS degradation activity in differentiated U937 cells was not due to transcriptional upregulation or enhanced processing of Hpse [16].

We then focused on the question of how the Hpse action is triggered during the extravasation process. Considering that the extravasation process is initiated during a short period of stimulation, the triggering event is expected to be rapid and not to require new transcripts. To assess the mechanism of this quick "turn-on", the cellular distribution of Hpse was examined. In differentiated U937 cells, Hpse was distributed pericellularly, forming a patch-like structure (Fig. 16.2a). When the cells were allowed to adhere, the patches redistributed to form a spot on the rim of each

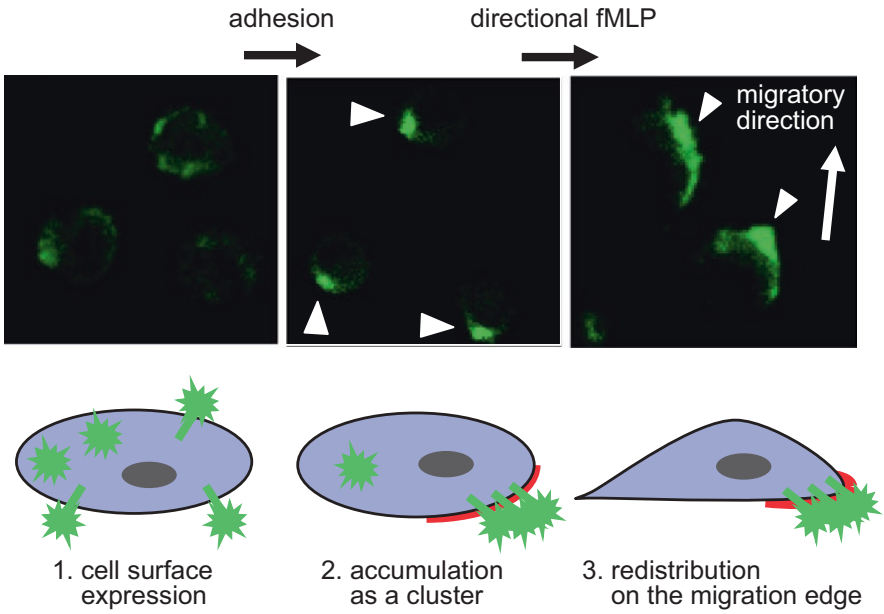

Fig. 16.2 Regulation of intracellular heparanase distribution during macrophage migration. (**a**) Hpse is shown as patch-like distribution pericellularly in differentiated U937 cells. (**b**) The patches are capped to form a spot on the rim of the cells during adhesion of macrophages. (**c**) Directional migratory stimuli redistribute the Hpse at the leading edge of migration

cell (Fig. 16.2b). Directional migratory stimuli apparently triggered the relocalization of Hpse to the leading edge of migration (Fig. 16.2c). Such condensed Hpse accumulation also occurred in peripheral blood-derived monocytes stimulated with fMLP [16], and a similar cell surface expression of Hpse was reported in mouse dendritic cells [2]. Therefore, Hpse redistribution to the migratory edge is likely to be functionally relevant for migration of cells into the draining lymph nodes upon topical antigen administration as well as extravasation.

16.5 Appearance of a "Drilling Device" on Migrating Macrophages

Another interesting aspect is that the spot of Hpse accumulation functions like an invadosome. Invadosomes are protrusions of the plasma membrane that are implicated in degradation of the extracellular matrix in cancer. Just like invadosomes, spots of Hpse include another matrix degradation enzyme, MT1-MMP, and adhesion molecules (integrins and CD44, our unpublished data). Actin dynamics possibly regulate the relocalization of these molecules, as expected from observation of similar capping of adhesion molecules in neutrophils and lymphocytes that are abrogated by disruption of the cytoskeleton. Pretreatment with cytochalasin D, which inhibits actin polymerization, actually abrogated relocalization of Hpse on the leading edge. In the cells that are ready to migrate, a putative scaffold structure on or beneath the cell surface permits the accumulation of molecules involved in the invasion process. To identify the scaffold molecules that associate with Hpse, lysates of cell surface-labeled neutrophils were immunoprecipitated with anti-Hpse antibody. A 43 kDa protein was detected as a cell surface membrane protein (unpublished).

A similar "drilling device" structure on macrophages, has been recently reported as assembled podosomes, which accumulate the matrix degradation enzyme MT1-MMP and adhesion molecules on the cell surface and are involved in cell migration [6]. Ordinary podosomes distribute as many independent spots along the attachment surfaces in macrophages, neutrophils, dendritic cells, and osteoclasts (2D adhesion). Such podosomes in macrophages and osteoclasts assemble together inside of the stiff extracellular matrix in three dimensions [25]. This assembly is triggered by phosphorylation of guanine nucleotide exchange factor Sos1 by Src kinases [1] . The Hpse-condensed invasive units identified in human macrophages are similar to podosomes because functional molecules for adhesion and matrix degradation are accumulated for cell invasion. However, the Hpse-condensed invasive units are generated on a 2D structure without any stiff extracellular matrices. The relationship between the invasive units and podosomes should be further clarified. It has been reported that monocytes transmigrate through the BM via the formation of membrane protrusions and deformation of their cell body *in vivo* [23]. The distribution of the 'drilling device' on the membrane protrusions is going to be explored in the future.

16.6 Neutrophil Migration and Invasion Associated With Intracellular Trafficking of Hpse

Under resting conditions, granulocytes and mast cells store Hpse in granules. Immunocytochemistry showed that Hpse in unstimulated neutrophils distributed especially in tertiary granules together with gelatinase [11]. Because neutrophil invasion is suppressed in the presence of a heparanase inhibitor, heparastatin (SF4), it is suggested that HS degradation by Hpse is involved in the process. It was shown that the cell lysate of mouse bone marrow neutrophils degrades basement membrane HS. This degradation activity was abolished when the lysate was pretreated with anti-Hpse mAb conjugated resin, suggesting that Hpse is responsible for the degradation of basement membrane HS by neutrophils [21]. To further explore the molecular mechanism underlying Hpse accumulation, the cellular redistribution of Hpse in fMLP-stimulated neutrophils derived from human peripheral blood was examined. fMLP induced cell surface Hpse expression as detected by flow cytometry (our unpublished data).

To shut down the migratory activity, Hpse accumulation may be dispersed, or alternatively, Hpse may be simply released out of the cell body. In a histological study of dermal tissue under inflammation, it has been indicated that neutrophils residing along the vessels expressed Hpse, whereas neutrophils residing further apart from the vessels and infiltrating the inflamed tissue were negative for Hpse expression [7]. Although termination of the migratory process has not been studied, an interpretation is that the migrating neutrophils have lost Hpse upon degranulation of stored enzymes. We detected the release of Hpse into the supernatant when bone marrow-derived neutrophils were stimulated with TNF α (unpublished).

16.7 Evidence Provided by the Use of Hpse Gene-Deficient Mice

Migration of immune cells during inflammation has been recently examined using mice deficient in the Hpse gene. Involvement of Hpse in the process is not simple (Table 16.1); mice deficient in the Hpse gene significantly decreased migration of dendritic cells to lymph nodes [3, 15], invasion of monocytes into inflammatory peritoneum [19], invasion of eosinophils in the lung in an allergic asthma model together with lower concentration of IgE release in blood [12], and pulmonary neutrophil adhesion in a sepsis model [17]. The last paper focuses on adhesion of circulating neutrophils onto the endothelial layer, which is hindered under non-inflammatory conditions by the glycocalyx covering the endothelium. Schmidt et al. [17] concluded that Hpse is involved in cleavage of the glycocalyx layer to promote adhesion. Other inflammatory reactions were not affected by a deficiency in Hpse expression. Therefore the involvement of Hpse in migration is neither cell-type-specific nor organ-specific. The following points should be considered to inter-

Table 16.1 Involvement of heparanase in leukocyte migration

Cell type	Events	Involvement	Reference
DC	Lymph node migration (cell injection, FITC paint)	Yes	[3]
DC	Lymph node migration (FITC paint)	Yes	[15]
Neutrophils	Adhesion to lung endothelial cells	Yes	[17]
Lymphocytes	Skin inflammation	No	[19]
Neutrophils	Skin inflammation	No	
Neutrophils	Zymosan peritonitis	No	
Monocytes	Zymosan peritonitis	Yes	
Neutrophils	Lung inflammation	No	[12]
Eosinophils	Allergic lung inflammation	Yes	

pret the experimental results. (1) whether the inflammation is induced by direct administration of inflammatory reagents or by an indirect effects as a result of systemic inflammation, (2) whether the early phase (6 h) or relatively late phase (24 h) of inflammation is examined, (3) whether the reaction is allergen-specific or inflammatory, and (4) whether the mode of migration is mesenchymal or amoeboid under the experimental conditions because matrix degradation is not required during amoeboid migration. Another question is whether HS in basement membrane or on the surface of endothelium is the substrate of Hpse during the entire process of extravasation. Dermal inflammation resulted in a massive deposition of HS around the basolateral side of postcapillary venules of inflamed skin. Because such deposition did not occur in Hpse gene-deficient mice, it is suggested that Hpse is involved in the HS deposition process [18]. Although it is conceivable that HS deposition might stabilize chemokine gradients to achieve directional migration of immune cells, the involvement of HS deposition in cell migration remains unclear.

16.8 Therapeutic Use of Hpse Inhibitors

To examine whether or not experimental inflammation is pharmacologically controllable, heparastatin(SF4) that mimics the putative transition state of enzymatic glycosidic hydrolysis [14] was administrated into dorsal air pouches in a mouse model where inflammation was induced with fMLP or carrageenan injection. In heparastatin(SF4)-treated mice, the number of infiltrated neutrophils and monocytes into the dorsal air pouch regions was significantly reduced concomitantly with reduced TNFα production in the pouch [21]. Administration of other Hpse-specific inhibitors is also effective for suppression of inflammation [5, 12].

Another therapeutic approach has been tried in a mouse model of type 1 diabetes. Mouse pancreatic islets are abundant in HS, a critical molecule required for β cell survival. In the NOD mice model, infiltration of mononuclear cells that produce enzymatically active Hpse caused degradation of islet HS and β cell death. Administration

of the heparanase inhibitor PI-88 preserved HS in the islet and protected NOD mice from type 1 diabetes, which may provide a novel therapeutic cue [26].

16.9 Perspectives

Hpse is involved in migration of dendritic cells, monocytes, eosinophils, and neutrophils in some inflammatory disease models. To turn "on" the function of Hpse during the migratory process of monocytes and neutrophils, Hpse is redistributed intracellularly and accumulates in a spot on the leading edge of migration. Although candidate molecules involved in the spot formation are likely to be identified, the mechanism underlying the turn "on" of Hpse function is still obscure. While it will take time to gain more mechanistic insights, Hpse enzyme inhibitors might be used to interfere with the accumulation process of Hpse and to further advance the treatment of various disease conditions. Increasing the migratory capacity of immune cells may also be of therapeutic use in certain types of pathological conditions. For example, the endowment of CAR-T cells with invasive capacities for advanced immunotherapy [4] and designed neovascularization with branching structures guided by drilling macrophages [10] are promising outcomes of the current research.

Some unanswered questions are listed below:

1. Spots of Hpse accumulation on the leading edge of migration represent transient structures that appear in a limited time period. What cellular structure makes it possible to compose such spots? Does this structure easily collapse after the completion of cell migration?
2. Hpse is localized on the cell surface during migration but released extracellularly in the case of degranulation of neutrophils. What are the mechanisms underlying Hpse (re)localization and release, in particular which associating molecules determine these processes?
3. How can we optimize the route of administration of Hpse enzyme inhibitors for therapeutic purposes? Is condensed accumulation of Hpse in inflammatory cells controllable by drug intervention?

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Chapter 17
Role of Heparanase in Macrophage Activation

Michael Elkin

17.1 Introduction

Macrophages represent an important component of the innate immune system, constitute up to 20% of the cells in tissues throughout the body, and being critical regulators of tissue development, homeostasis and inflammation [1, 2]. Macrophages also contribute to a broad spectrum of inflammatory pathologies, atherosclerosis, diabetes and its complications, as well as cancer [3, 4], therefore representing attractive therapeutic targets. Yet, translation of macrophage-targeting approach into the clinic remains extremely challenging, in part due to the highly heterogeneous and dynamic nature of macrophage phenotypes [5, 6]. Thus, a better understanding of the mechanisms enforcing macrophage activation, as well as definition of the specific functions/phenotypes in a tissue- and stimulus-dependent manner, is required for therapeutically manipulating macrophages in various clinical settings [4, 5].

A key feature of macrophages is their ability to 'tailor' their responses according to environmental stimuli [4]. Older concepts of macrophage responses relied mainly on a binary model, i.e., classical versus alternative activation. Macrophages were proposed to induce an "M1" or "M2" response, analogous to the T_H1 or T_H2 response; within this dichotomous framework of activation/polarization, macrophages treated with LPS and IFN-γ were referred to as M1 macrophages, while those stimulated with anti-inflammatory cytokines (i.e., IL-4, IL-13, IL-10) were referred to as M2 macrophages [7]. In recent years, however, the binary M1/M2 polarization model is often regarded as oversimplistic, as it has been shown that macrophages can adopt overlapping M1-like and M2-like phenotypes [5, 6]. A more complex scenario of macrophage polarization was proposed, based on observations

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that transition between the functional states of macrophages occurs along a continuum of phenotypes, which are tightly regulated by the microenvironment [2–5, 7].

Important regulators/mediators of macrophage polarization were characterized, including Signal Transducer and Activator of Transcription (STAT) pathway family members, Peroxisome Proliferator-Activated Receptor gamma (PPARγ), several members of cAMP-responsive element-binding protein (CREB), CCAAT-enhancer-binding proteins (C/EBP) transcription factors, as well as interferon regulatory factors (reviewed in [8]). Additional types of bioactive molecules, including products of lipid metabolism, microRNAs, and long non-coding RNAs, were also implicated in regulating macrophage polarization [8].

Heparan sulfate (HS) and, more recently, heparanase have been shown to contribute to the altered macrophage phenotypes as well. Several studies linked heparanase expressed by macrophages [9] (as well as by other types of immune cells) [10–13] to their ability to penetrate basement membranes, extravasate, and accumulate in target organs. The notion that immunocytes represent the major source of the enzyme in inflammation was more recently transformed, due to appreciation that heparanase expression often occurs in epithelial and/or endothelial compartment in specific inflammatory settings, including human disorders [i.e., rheumatoid arthritis [14], diabetes-related cardiovascular conditions [15, 16], inflammatory lung disease [17], inflammatory bowel disease [18], psoriasis [19]], as well as in mouse models of delayed-type hypersensitivity [20], chronic colitis [18], sepsis-associated lung injury [17] and diabetic nephropathy [21, 22].

While effects of heparanase on macrophage activation/polarization are likely multifactorial, triggering of toll-like receptor (TLR) signaling (described in detail below) has emerged as a key mechanism. Collectively, this chapter summarizes the role of heparanase in the regulation of macrophage function in the pathogenesis of various disorders and provides information that may help to establish the rationale for heparanase-targeting interventions aimed at preventing adverse macrophage activation, as a novel opportunity to improve outcomes of some of the most challenging diseases.

17.2 The Core

Heparanase profoundly influences the biological activity of innate immunocytes [17, 18, 23–28], with macrophages being the most extensively studied target cell population of the enzyme. Modulation of toll-like receptor (TLR) signaling provides an attractive explanation for heparanase-mediated change in macrophage phenotype. TLRs recognize highly conserved microbial structures and are best-known for their role in host defense from infection. Intact extracellular heparan sulfate was shown to inhibit TLR4 responses and macrophage activation, while its removal relieves this inhibition [29, 30]. Indeed, heparanase-mediated reduction of intact

heparan sulfate at the macrophage cell surface by 50% was shown to increase significantly binding of TLR4 ligand to its receptor *in vitro* [18, 31], demonstrating that degradation of cell-surface heparan sulfate by the enzyme increases the accessibility of TLR. This notion is also consistent with the recently reported ability of heparanase to increase innate immunocyte binding to adhesion molecules presented on the blood vessel wall [17]. On the other hand, soluble heparan sulfate fragments released by heparanase degradation [32], were found to stimulate TLR (in particular TLR4) signaling *in vitro* [29, 30, 33] and *in vivo* [34]. Of note, some heparanase inhibitors (i.e., HS mimicking compounds) may reportedly act as TLR ligands as well, due to their structural similarity to soluble HS, and thus modulate macrophage responses regardless of their heparanase-inhibiting properties [22].

Thus, heparanase represents a highly relevant but equally challenging therapeutic target in numerous macrophage-driven inflammatory/malignant disorders. Observations described in this chapter and elsewhere warrant further systematic analysis and continuous searching for the effective heparanase- inhibiting compounds, toward future translation to the clinical setting. In parallel, dissection of the exact molecular mechanisms underscoring heparanase action in shaping macrophage responses will help to better define target patient populations in which future anti-heparanase therapies could be particularly beneficial.

17.3 The Details

Macrophages represent one of the most diverse immunocyte populations, constantly shifting between various phenotypes/functional states. Both tissue residing macrophages and those recruited from the circulation, represent a key contributing factor in the pathogenesis of some of the most challenging diseases, such as cancer, inflammatory disorders, diabetes and its complications. Elucidation of heparanase role in shaping macrophage responses in the aforementioned conditions is therefore of high importance in the attempts to better characterize the precise molecular mechanism underlying these pathologies.

During the last decade, studies utilizing specific factor/condition that initiates adverse macrophage responses enabled to gain better insight into the role of heparanase in inflammatory reactions associated with various disorders. These studies were furthered by the ability to set up *in vitro/ex vivo* experimental systems incorporating recombinant enzyme, along with utilization of heparanase-deficient and -overexpressing mouse models, to dissect the macrophage-sensitizing action of heparanase. With these advances in mind, in the next sections, we describe heparanase effects on macrophage activation induced by disease-specific stimuli, including LPS, circulating diabetic/obesogenic melieu components, as well as HS degradation fragments generated by the enzyme.

17.3.1 Macrophages Polarization toward Non-resolving Inflammation in the Presence of Microbial Products

When acute inflammation is not properly resolved, the composition of the infiltrating leukocytes changes from neutrophils to macrophages, dominant cellular players in chronic inflammation. The ability of heparanase enzyme to modulate the inflammatory phenotype of macrophages, preventing inflammation resolution and switching macrophage responses to a chronic inflammation pattern was first demonstrated in studies focusing on inflammatory bowel disease (IBD). Ulcerative colitis (UC) and Crohn's disease (CD) represent the two major forms of IBD - a chronic condition of the gastrointestinal tract resulting from inappropriate and exaggerated mucosal immune response believed to arise from a multitude of factors including genetic alterations, epithelial defects and luminal flora composition [35, 36].

Macrophages were shown to be paramount for IBD development, as well as its progression to colonic cancer (the most feared long-term complication of IBD) [37–43]. The gastrointestinal mucosa represents the largest reservoir of macrophages in the body [43]. Intestinal macrophages are derived from blood monocytes that are recruited to the lamina propria by endogenous chemoattractants in the non-inflamed mucosa and by inflammatory chemokines and bacterial products during inflammation. In active inflammatory bowel disease, there is an increase in the mucosal macrophage population, derived from circulating monocytes [38, 41, 42]. These recruited macrophages are phenotypically different from the resident population and play a major role in mediating the chronic mucosal inflammation seen in IBD patients [37, 41, 42]. Unlike resident macrophages, they express Nod-like receptors (NLRs), TLRs, and release numerous cytokines, such as TNF-alpha, IL-1, IL-6, IL-8, IL-12, IL-18, along with reactive metabolites of oxygen and nitrogen [37, 42, 44, 45]. Direct support for macrophage involvement in the regulation of IBD has been obtained from analysis of mice with selective disruption of STAT3 in their macrophages [40]. Moreover, depletion of macrophages in the $IL10^{-/-}$ mouse prevents development of colitis, which otherwise occurs owing to unregulated production of inflammatory cytokines by macrophages [39]. In particular, TNF-alpha, produced by non-lymphoid cells, mostly macrophages, was found to be essential for the development of colitis using the adoptive T-cell model of colitis induction.

The major advances in understanding components of IBD physiopathology in the past two decades led to increased therapeutic options, including development of small molecule drugs, as well as introduction to clinical practice of several anti-TNFα monoclonal antibodies, and antibodies against additional proinflammatory molecules [46, 47].

Yet, despite the improved surveillance/therapy, no curative treatments are available for IBD at present [47], emphasizing the need for characterization of new molecular targets. The role of inflammatory cytokines (i.e., TNF-alpha, IL-1, IL-6) in IBD is well documented for more than a decade [48–50], while the involvement of ECM-degrading enzymes (i.e., heparanase) only recently came to appreciation. Of note, heparanase is constantly overexpressed by the colonic epithelium in both

clinical and experimental colitis [18, 51, 52], but it is not expressed in healthy colon epithelium [51, 53]. Moreover, the data from experiments utilizing heparanase-overexpressing transgenic mice in a model of chemically induced colitis indicate that heparanase activity preserves abnormal activation of the resident macrophages. It appears that pathogenesis of chronic colitis and the associated colon tumorigenesis may involve a vicious cycle through which heparanase of epithelial origin, acting synergistically with the local flora and cytokine milieu, facilitates abnormal activation of macrophages, which in turn stimulate further production/activation of the enzyme in the inflamed colon.

Indeed, augmented recruitment and continuous activation of macrophages occur in heparanase-overexpressing colons. As stated above, macrophages are known to have a dual role in inflammation. In the scenario of inflammation resolution (an active process leading to normal structural and functional state), macrophages perform phagocytosis and produce anti-inflammatory cytokines [54, 55], thereby preventing inflammatory responses from lasting too long [2]. However, if inflammation resolution is deregulated, macrophage response switches to the pattern of chronic inflammation [56]. Macrophages dominate in chronic inflammatory foci and generate significant amounts of growth factors, cytokines and reactive oxygen species [48, 57]. The increase in mucosal macrophage population is well documented in UC patients [37]. Recruitment and activation of macrophages within the intestinal mucosa play a key role in the pathogenesis of both human UC [37, 58] and experimental colitis [38]. Furthermore, macrophages are linking cells between inflammation and cancer [57, 59, 60]. For instance, the tumor-promoting cytokines IL-1, IL-6 and TNF-alpha [49, 61] are produced mainly by activated macrophages and, along with macrophage-derived growth factors and reactive oxygen species, foster tumor initiation and progression [57, 58]. Elevated levels of heparanase are found in chronically-inflamed mouse colon (similar to colonic tissue of UC patients [51]). Further research revealed exacerbated chronic inflammatory phenotype in the colon of heparanase-overexpressing transgenic mice in the course of chemically-induced colitis [18]. Although having little effect on the acute phase of colitis, heparanase overexpression profoundly affected the chronic phase of chemically-induced colitis, as demonstrated by microscopic and biochemical analyses of inflammatory phenotypes preserved in heparanase-transgenic but not *wild-type* mouse colon four weeks after cessation of DSS treatment [18]. In particular, augmented recruitment and continuous activation of macrophages were detected in heparanase overexpressing transgenic colons, leading to the hypothesis that heparanase over-expression directly affects macrophage activation. This mode of action was further supported by *in vitro* observations; thus when mouse macrophages were stimulated with lipopolysaccharide (LPS, a canonic ligand of TLR4) in the absence or presence of active heparanase (recapitulating UC-conditions, i.e., heparanase-rich environment and abundant microbial flora), heparanase strongly sensitized macrophages to TLR4-dependent activation *in vitro*, as indicated by a marked increase in TNF-alpha, IL-6 and IL-12p35, cytokines known to be induced by TLR4 signaling and tightly involved in the pathogenesis of UC [58]. Given that one of the key aspects of IBD pathogenesis is the involvement of lumenal flora and TLR signaling [62, 63], the ability of

heparanase to sensitize macrophages to LPS activation is of particular significance in light of the increased epithelial permeability to lumenal microbial products, characteristic of IBD. Thus, in the setting of chronic intestinal inflammation, heparanase may preserve inflammatory conditions by reprogramming macrophage response from resolution of inflammation to unresolved colitis, bringing about continuous activation of TNF-alpha-producing macrophages, enhanced NFκB signaling and increased expression of NFκB regulated inflammatory cytokines [18] and Fig. 17.1.

Interestingly, macrophages not only represent a cellular target for heparanase action but also decisively regulate heparanase in chronic colitis, both at the transcriptional and posttranslational levels (Fig. 17.1). Activated macrophages are capable of inducing heparanase expression in intestinal epithelium, most likely through TNF-alpha-mediated stimulation of transcription factor EGR1 [64], a powerful inducer of heparanase transcription in colonic cells [18, 65]. Furthermore, macrophages appear to be involved in post-translational activation of the heparanase proenzyme in the inflamed colon through cathepsin L (CatL)-dependent mechanism. Cathepsin L is the only known protease capable of proper proteolytic activation of

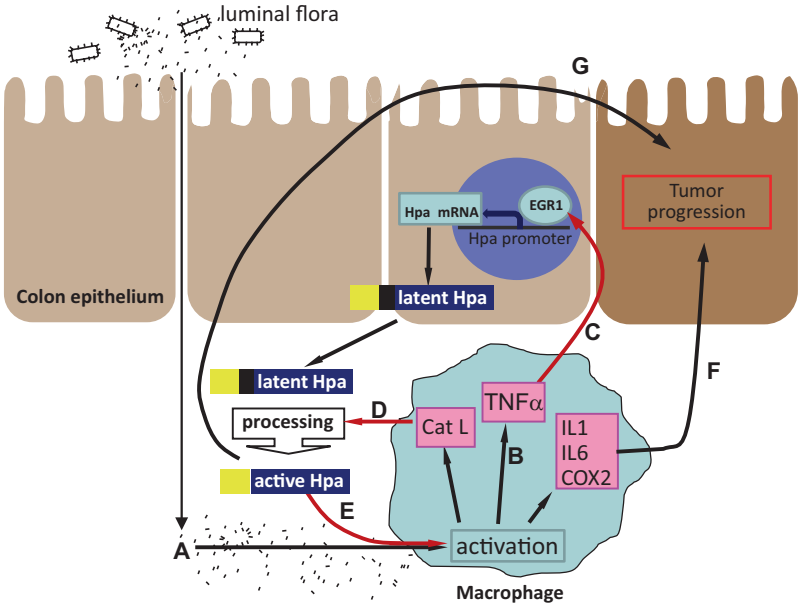

Fig. 17.1 A model of chronic inflammatory circuit driven by heparanase, that promotes colitis and the associated tumorigenesis. Adverse activation of macrophages by the luminal flora due to epithelial barrier function defects (**A**) results in increased levels of TNFα (**B**) and induces heparanase expression in colon epithelium via an EGR1-dependent mechanism **(C)**. **D:** The latent pro-heparanase is processed into its enzymatically-active form by Cath L (which is also supplied by the activated macrophages), and in turn sensitizes macrophages to further activation by luminal flora **(E)**, thus preventing inflammation resolution and creating pro-cancerous inflammatory environment **(F)**. In addition, heparanase promotes tumor take and progression via stimulation of angiogenesis, release of ECM-bound growth factors and removal of extracellular barriers for invasion **(G)**

65 kDa latent heparanase [66, 67]. It also plays an important pathophysiological role in colonic inflammation and macrophages are the primary cellular source of inducible CatL expression in the inflamed colon of UC patients and in DSS-induced mouse colitis [44]. Moreover, macrophages are unique in their ability to secrete mature CatL and allow extracellular accumulation of the active enzyme [68]. Thus, macrophages contribute to proteolytic processing of proheparanase in colitis, providing a pool of extracellular active CatL [18].

17.3.2 Heparanase Effects on Macrophage Responses in the Setting of Non-infectious "Aseptic" Inflammation

Augmented macrophage activation in the presence of elevated heparanase levels was detected not only in response to the highly conserved microbial structures (i.e., LPS), but also in the various settings of non-resolving "sterile" inflammation, including arterial wall remodeling following injury, atherosclerotic plaque progression toward vulnerability, kidney disorders and cancer [19, 22, 25, 28, 31, 69]. Two examples - sterile solid tumors and kidney disease, exemplifying effects of heparanase on macrophage-driven malignant and non-malignant conditions - are described in more detail below.

Heparanase Shapes the Cancer-Promoting Phenotype of Tumor-Associated Macrophages in Pancreatic Carcinoma

The microenvironment of solid tumors, including pancreatic carcinoma, and in particular infiltrating macrophages, represent an important contributing factor to tumor aggressiveness and resistance to treatment [6, 70–75]. Tumor-associated macrophages (TAM) are known to supply bioactive molecules (i.e., cytokines, growth factors, anti-apoptotic proteins) and activate tumor-stimulating signaling pathways (e.g., STAT3), thus promoting tumorigenesis in several anatomic sites (including pancreas) [59, 70, 74, 76–80]. A key role of TAM in numerous inflammation-related cancer progression scenarios, along with reports suggesting that under certain conditions macrophages may oppose malignancy [59, 81], highlight occurrence of dynamic changes in the TAM phenotype during tumor development. In the past, it was proposed that once the tumor is initiated and progresses toward malignancy, the macrophage phenotype changes from the "classically" activated (M1) to the "alternatively" activated M2 type. However, more recent data suggest that the dichotomous M1-M2 model insufficiently describe macrophage activation, as TAM often share features of both classically and alternatively activated populations, generally oriented toward promoting tumor growth [59]. In contrast to the dualistic scheme of macrophage polarization, a current multidimensional model of activation involves integration of the signals to which macrophages are exposed in their specific microenvironment [4].

In the majority of inflammation-associated sterile tumor sites, non-microbial environmental signals and endogenous substances (i.e., constituents released by damaged/necrotic cells, products of altered metabolism) may contribute to TAM stimulation through pattern recognition receptors (i.e., TLR) [82–84]. Yet, these signals alone are often not sufficient to elicit the tumor-promoting activity of macrophages [85]. The possible role of heparanase in enforcing TAM polarization toward tumor-promoting phenotype was highlighted in studies on pancreatic carcinoma [31]. Clinical studies revealed that heparanase is not expressed in normal pancreas, but its expression is induced already at the early stages of pancreatic tumorigenesis [86]. In fact, overexpression of heparanase is a characteristic feature of pancreatic tumors [86–90]. Increased levels of the enzyme correlate with aggressiveness/poor prognosis of pancreatic carcinoma [86–90]. Interestingly, overexpression of heparanase also correlates with increased TAM infiltration in both experimental and human pancreatic cancer [31]. Moreover, macrophages derived from heparanase-rich pancreatic tumors (which grew faster in mouse host), display pronounced pro-cancerous phenotype, evidenced by overexpression of MSR-2, IL-10, CCL2, VEGF and increased production of IL-6, an important player in PDAC pathogenesis [31]. Furthermore, *in vitro* presence of active heparanase enzyme rendered macrophages (stimulated by necrotic cells which are often present in pancreatic tumor tissue) pro-cancerous phenotype, as exemplified by their enhanced production of IL-6 and additional key cytokines implicated in pancreatic tumorigenesis, as well as their ability to augment pancreatic carcinoma cell proliferation (via IL-6/STAT3 dependent mechanism [31]).

Heparanase Fosters Macrophage Activation in Kidney Disease

Several kidney disorders provide an opportunity to examine heparanase contribution to abnormal macrophage activation in the setting of non-malignant chronic inflammation [22, 28, 91–93]. A good example is diabetic nephropathy (DN). The kidneys represent primary targets of diabetes [94] and DN is the leading cause of end-stage renal disease in the western world [95–98]. Clinically, DN is characterized by the development of proteinuria (pathological quantities of urine albumin excretion), glomerular lesions, and consequent decline in glomerular filtration rate, which often progresses over 10–20 years, and if left untreated could be fatal [98, 99].

DN occurs as a result of a complex interplay between hemodynamic and metabolic events, including increased systemic/intraglomerular pressure, activation of vasoactive hormone pathways and intracellular second messengers, and induction of various nuclear factors (i.e., NFκB), growth factors and cytokines (i.e., VEGF, TGF-β) [100, 101]. The classical view on DN as a consequence of solely metabolic and hemodynamic alterations is being recently updated, with clear evidence indicating that activation of innate immunity and chronic low-grade inflammation play a significant role in the pathogenesis of both diabetes mellitus and its complications, including diabetic nephropathy [102, 103]. Immunocytes [primarily macrophages [104–107]] and numerous inflammatory molecules, such as chemokines, adhesion

molecules, nuclear factors (i.e., NFκB) and cytokines (i.e., TNF-α, IL-6, IL-1), have been implicated in diverse pathogenic pathways related to diabetic nephropathy (reviewed in [102]).

In particular, many clinical studies in DN patients have reported elevated serum and urinary concentrations of TNF-α as compared to non-diabetic individuals. Moreover, their concentrations increase in tandem with DN progression. TNF-α protein and expression levels are induced in renal glomeruli and tubules in animal models of diabetes. The effects of TNF-α on DN progression may be due to the fact that TNF-α is directly cytotoxic to renal cells, inducing apoptosis and necrotic renal cell death. Additionally, TNF-α can alter intraglomerular blood flow and reduce glomerular filtration as a result of disproportion between factors promoting vasoconstriction and vasodilation as well as due to changes in endothelial cell permeability. TNF-α can also directly induce the formation of reactive oxygen species by renal cells [101].

Macrophages are considered the major immune cells infiltrating the kidney in type 1 and type 2 diabetes and critically contribute to the development of renal damage [108]. In a diabetic kidney, macrophages, activated by various elements of the diabetic milieu [i.e., high glucose [109, 110], AGEs [111–113], albumin [114]], release ROS and proinflammatory cytokines (e.g., TNF-α, IL-1, IL- 6) which cause injury to podocytes and tubular cells [100–102, 108]. These macrophages also secrete pro-fibrotic cytokines (PDGF, TGF-β) that induce mesangial and fibroblast proliferation and the development of sclerosis, fostering DN progression [104, 108, 113].

Involvement of heparanase in DN had been addressed in several reports (reviewed in [115]). The findings linking heparanase to DN include elevated levels of heparanase in the kidneys and urine of DN patients [116, 117], induction of renal heparanase expression in murine models of type 1 diabetes [117], as well as *in vitro* studies demonstrating that hyperglycemic conditions enhance heparanase expression in rat and human glomerular epithelial cells [118]. Moreover, failure of heparanase-knock out mice to develop DN in the SZT-induced diabetes model [21] suggested a causal involvement of heparanase in DN pathogenesis.

In parallel, the occurrence of heparanase-driven inflammatory circuit and its importance in DN pathophysiology was demonstrated [22]. Heparanase (overexpressed in diabetic kidney) appears to sensitizes macrophages to stimulation by DM components (high glucose, AGEs, albumin), as manifested by a significant increase in their production of TNF-alpha, a key inflammatory mediator in the pathogenesis of DN [22]. In line with these findings, TNF-alpha expression in response to *in vitro* stimulation by DM components was impaired in heparanase-deficient macrophages. Importantly, elevated levels of TNF-alpha were detected in renal tissue of diabetic *wt* mice (which developed albuminuria and renal damage following 16 weeks of STZ-induced diabetes) and correlated with increased expression of heparanase, while no increase in TNF-alpha was detected in renal tissue of heparanase-deficient mice which failed to develop DN in response to STZ-induced diabetes. In agreement, increased numbers of TNF-alpha-producing macrophages were found in diabetic kidneys of *wild type* but not heparanase-deficient mice [22].

Concluding Remarks The data pointing on the heparanase effects on macrophage polarization toward pro-inflammatory and/or pro-tumorigenic phenotype, although still incomplete, provides several interesting opportunities for further studies. Previously known and newly described functions of heparanase in modulating the inflammatory phenotype of macrophages in various pathologies necessitate systematic analysis of heparanase-inhibiting compounds which are currently under intensive investigation, toward future translation to the clinical setting. Therapeutic benefits are expected to be gained in both inflammation and malignancy by strategies designed to disrupt heparanase-driven heterotypic interactions between epithelial, endothelial, and immune cells.

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Chapter 18
Immunomodulatory Activities of the Heparan Sulfate Mimetic PG545

Victoria Bendersky, Yiping Yang, and Todd V. Brennan

18.1 Diversity of Mechanism of PG545

Heparanase regulates multiple biological activities that enhance tumor growth and metastatic spread [1–7]. Heparanase is the only mammalian enzyme that is able to cleave and degrade heparan sulfate (HS), a key structural component of the extracellular matrix (ECM) that serves as a barrier to cell invasion and also as a reservoir for cytokines and growth factors critical for tumor growth and metastasis [1, 8–10]. For this reason, heparanase is an attractive target for the development of novel anticancer therapies [11, 12]. Thus far, heparanase inhibiting therapies have followed two broad approaches: small molecules heparanase inhibitors, and HS mimetics that compete with HS as heparanase substrates [11, 13–16].

Multiple small molecule heparanase inhibitors have been developed, however, none have successfully progressed to clinical trials [11]. In contrast, HS mimetics have demonstrated encouraging results and have entered or completed phases of clinical trials for various cancers [11]. For example, muparfostat (PI-88) has been tested for the treatment of post-resection hepatitis virus-related hepatocellular carcinoma in phase III clinical trial focusing on its anti-microvascular invasion properties [17]. Roneparstat (SST0001), a non-anticoagulant heparin with anti-heparanase activity, has completed phase I trial with promising safety and tolerability profile in multiple myeloma [18, 19]. Necuparanib (M402) has completed phase I/II trials

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focusing, among other activities, on the anticoagulant properties of this HS mimetic in pancreatic cancer [20] (Chhabra and Ferro; Noseda and Barbieri; Giannini et al., Chaps. 19, 21 and 23 in this volume).

Recently, another HS mimetic, pixatimod (PG545, Fig. 18.1), has shown promising utility in several cancer scenarios. It has completed a phase I trial and lends itself to thorough investigation as a single entity molecular therapy [21] (Hammond and Dredge, Chap. 22 in this volume). PG545 is a synthetically manufactured tetrasaccharide with a lipophilic cholestenol modification that significantly increases its *in vivo* half-life [12, 22–25]. PG545 also has minimal anticoagulant activity, a common side effect encountered with other HS mimetics [22, 26–28]. PG545 offers a diverse variety of proposed mechanisms of action in cancer therapy, including angiogenic inhibition, inhibition of growth factor release, cell migration inhibition, apoptosis of tumor cells, induction of endoplasmic reticulum (ER) stress response, dysregulation of autophagy, and natural killer (NK) cell activation through TLR9 pathways (Fig. 18.2).

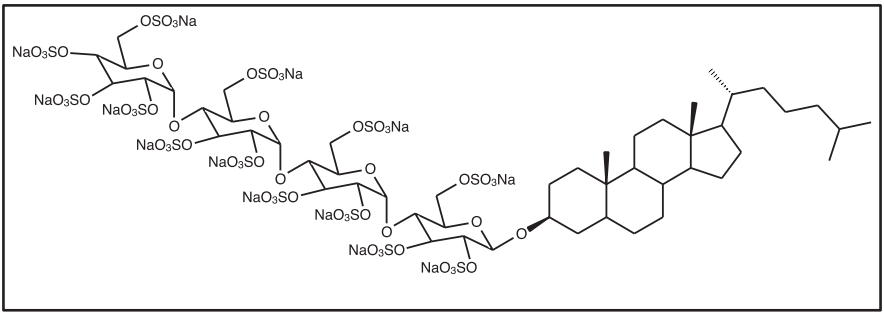

Fig. 18.1 Chemical structure of PG545, based on Dredge et al. 2011 [12]

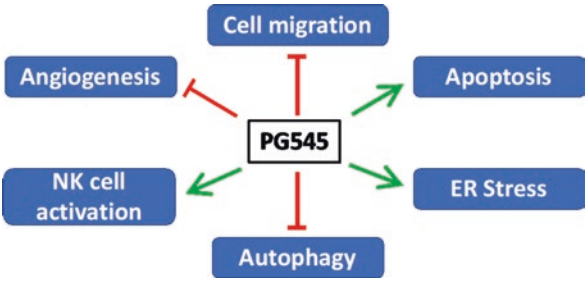

Fig. 18.2 Proposed anti-tumor mechanisms of PG545. Green arrows indicate stimulation and red bars indicate inhibition of processes involved in regulating tumor growth and metastasis

18.2 Inhibition of Angiogenesis

Heparanase plays an important role in tissue remodeling and growth factor signaling required for angiogenesis and growth of primary and metastatic tumors [25]. A number of anti-angiogenic agents have been tested and implemented in various cancers [25, 29–33]. However, agents that slow down the growth of the primary tumor can also lead to more aggressive tumor metastasis [25, 34–36]. Hence, an agent that can combat both blood vessel growth and metastasis is needed [25].

Apart from its role in allowing ECM invasion by endothelial cells (ECs), heparanase also accelerates the proangiogenic response by releasing sequestered HS-bound vascular growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [37–39]. PG545's ability to block ECM dissolution prevents the release of HS-bound vascular growth factors, thereby inhibiting angiogenesis required for tumor growth [13, 25, 37, 40]. For example, a study by Ostapoff et al. on pancreatic ductal adenocarcinoma in mouse models confirmed that PG545 reduced ECM deposition, angiogenesis, and metastasis [25]. The reduction in microvascular density of tumors was confirmed by significantly reduced expression of endomucin and by a decreased number of ECs expressing phospho-histone H3, a marker of cell mitosis [25].

An earlier study by Dredge et al. similarly found that PG545 inhibited angiogenesis *in vivo* and produced anti-tumor and anti-metastatic effects in murine models of breast, prostate, liver, lung, colon, melanoma and head and neck cancers [12]. Using subcutaneously implanted AngioSponge™, angiogenesis inhibition was determined by quantifying the number of vessels with identifiable lumen that stained positive for CD31/PECAM-1, an EC cell-surface adhesion molecule [12]. Significant inhibition of angiogenesis in this model was observed after treatment by PG545 [12].

18.3 Inhibition of Tumor Cell Migration

In addition to preventing the physical movement of tumor cells by preventing ECM breakdown, heparanase inhibition also regulates tumor cell migration and metastasis. Giri and colleagues studied the antitumor activity of PG545 *in vitro* using migration and invasion assays with the ovarian cancer cell line, SKOV-3 [41]. Cells stimulated with HS-binding growth factors, including heparin-binding EGF-like growth factor (HB-EGF), hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF-2), VEGF, and stromal cell-derived factor 1 (SDF-1) demonstrated tumor cell migration and invasion that was inhibited by PG545. The study concluded that PG545 significantly inhibited growth factor-mediated cell migration and attenuated growth factor-induced activation of epidermal growth factor receptor (EGFR), extracellular-signal-regulated kinase (ERK), and protein kinase B (PKB, also known as AKT). These data demonstrate that PG545 inhibits cell migration and

invasion. *In vivo* experiments revealed a marked reduction in tumor volume in response to treatment with PG545 [41].

18.4 Tumor Cell Apoptosis

Although considerable attention has been given to PG545 in solid tumor carcinoma scenarios, comparatively few studies have concentrated on the efficacy of PG545 in the treatment of lymphomas [8, 42, 43]. A broad study by Weissmann et al. determined that PG545 is highly effective as an anti-lymphoma drug both *in-vitro* and *in-vivo* [8]. Mechanistically, this decrease was supported by post-treatment findings of elevated levels of cell-cycle inhibitor p21, decreased levels of pro-proliferation protein kinases (including Akt, c-Src, and c-Myc), and decreased levels of the pro-survival protein, Bcl-6 [8].

Importantly, the study confirmed that PG545 has a direct pro-apoptotic effect on lymphoma cells as early as 6 hours after application. This was not the case with human prostate, lung, and other cancer cell lines that were part of the study, suggesting an anti-tumor mechanism that is unique to lymphoma [8, 44, 45]. Tumor necrosis factor alpha (TNF-α), a cytokine that can induce cell death, demonstrated a 2- to three-fold increase following the addition of PG545 to lymphoma cells. Also, PG545 caused elevated levels of phosphorylated IκBα (NF-κB inhibitor alpha), a major regulator of the NF-κB pathway that controls cell proliferation and survival [8]. Interestingly, this study also found that PG545 elicits cell apoptosis in lymphoma cells devoid of heparanase activity, thus indicating that heparanase-independent functions of PG545 may exist [8].

18.5 Prolonged ER Stress Response

The Weissmann study further identified a novel connection with cell apoptosis through a persistent endoplasmic reticulum (ER) stress response [8, 46, 47]. The ER performs highly complex functions for survival and maintenance of cellular homeostasis [8, 48, 49]. Any alteration in the ER microenvironment, such as misfolded or unfolded protein, activates adaptive responses controlled by the unfolded protein response and NF-κB signaling pathway [8, 46–51]. These systems can be triggered by chemical compounds, cytokines, Toll-like receptor (TLR) ligands, nucleic acids, certain lipids, bacteria, and viruses [46]. When PG545 elicits a persistent ER stress that is severe or prolonged, the unfolded protein response is insufficient to restore homeostasis and turns into a toxic signal leading to cell death [8, 50–53]. This was supported by findings of a 3- to six-fold increase of BiP, cleavage of caspase 8, increased phosphorylation of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) and eukaryotic translation initiation factor 2 subunit 1 (elF2α), increased levels of activating transcription factor 4 (ATF4), and increased expres-

sion of C/EBP homologous protein (CHOP) [8]. Thus, these data indicate that PG545 can induce ER stress response, supporting additional studies to further define its mechanistic involvement in ER stress and anti-tumor therapy.

18.6 Cell Autophagy

Cell autophagy is an evolutionarily conserved mechanism through which intracellular material, such as misfolded proteins or damaged organelles, are sequestered into double-membrane vesicles called autophagosomes that subsequently fuse with lysosomes to be degraded and to recycle their components. A study by Shteingauz et al. demonstrated that overexpression of heparanase augments autophagy and leads to enhanced tumor growth [54]. Mechanistically, autophagy induced by heparanase appears to involve the mammalian target of rapamycin (mTOR) pathway. This nutrition sensing kinase acts as a master negative regulator of autophagy. For example, during starvation, mTOR is inhibited and this induces autophagy [54]. mTOR1 activity can be assessed by the phosphorylation status of ribosomal protein S6 kinase beta-1 (S6K1), also known as p70S6 kinase, a specific downstream substrate of mTOR1. In cells overexpressing heparanase, reduced phospho-p70S6 kinase levels, as well as increased intensity of the autophagy marker LC3-II, were observed, indicating increased autophagy. Correspondingly, treatment with PG545 attenuated autophagy in human and murine cancer cells, implying that it acts as an autophagy inhibitor [54].

Theoretically, PG545's function as an autophagy inhibitor is pharmacologically appealing since the blockade of autophagy may increase sensitization of tumor cells to drug-induced cell death and enhance the efficacy of cancer therapies [55]. However, the involvement of PG545 in autophagy is still controversial and context dependent, as Weissmann et al. found that PG545 actually increased autophagy in two lymphoma cell lines (Daudi/Burkitt's lymphoma and SU-DHL-6/human B lymphoma) [8]. Thus, further investigation is needed to better understand the effect of PG545 and other heparanase inhibitors on the regulation of autophagy.

18.7 NK Activation Through TLR9-MyD88 Pathway

NK cells are critical to the host's immunological defense against malignancies. Enhancing NK activation and cytotoxicity are promising antitumor therapies. The role of PG545 in NK cell activation has been studied in murine models of B cell and T cell lymphomas [22]. This study showed that PG545 activates NK cells *in vivo* as shown by their production of activation markers, proliferation, and effector cytokine secretion [22]. Applying methods of NK cell depletion, the anti-lymphoma activity of PG545 was demonstrated to be NK cell-dependent. Interestingly, it was found that NK activation by PG545 was completely dependent on the TLR9/MyD88 path-

way. The activation of NK cells by PG545 in wild-type mice was profound but entirely abrogated when administered to TLR9 and MyD88 knockout mice, while preserved in TLR2, TLR4 and TRIF knockout mice. This study also demonstrated that PG545 enhances TLR9-mediated activation of DCs by promoting the accumulation of unmethylated CpG containing DNA in the lysosomal compartment, that in turn leads to enhanced production of IL-12, a proinflammatory and NK cell activating cytokine [22]. In support of these findings, the activation of NK cells in humans has recently been shown in a phase I study examining the use of PG545 in patients with advanced solid tumors [21].

18.8 Summary

In summary, PG545 offers a diversity of mechanisms of action in tumor therapy that include angiogenic inhibition, inhibition of growth factor release, cell migration inhibition, cell apoptosis, activation of ER stress response, dysregulation of autophagy, and NK cell activation. It appears, however, that PG545 have anti-tumor effects that are both heparanase dependent and heparanase independent. Further investigation into the role that heparanase and its inhibitors play in tumor progression will advance our understanding of how these mechanisms can be leveraged to the development of novel effective cancer therapies.

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Part V
Heparanase Inhibitors and Clinical Considerations

Chapter 19
PI-88 and Related Heparan Sulfate Mimetics

Mohit Chhabra and Vito Ferro

19.1 Introduction

As discussed in detail throughout this volume, heparanase is an endo-β-D-glucuronidase that degrades heparan sulfate (HS) in the extracellular matrix (ECM) and basement membranes and plays a crucial role in numerous pathological processes such as inflammation, metastasis, and angiogenesis. The development of heparanase inhibitors has therefore long been an attractive strategy for drug discovery, especially for cancer and inflammatory diseases (for reviews see refs. [1–6]). Early attempts to inhibit heparanase enzymatic activity focused on high molecular weight HS mimetics such as heparin or sulfated polysaccharides [7]. However, none of these inhibitors were suitable as cancer therapeutics because of their potent anticoagulant activity. These early studies were also hampered by the lack of availability of pure enzyme, which was only cloned for the first time in 1999 by Vlodavsky [8] and Parish [9], and of suitable assays (for a recent review see ref. [10]). The Parish group subsequently screened a library of sulfated oligosaccharides for inhibition of human platelet heparanase [11] (shown to be identical to the tumor enzyme [12]) using a newly developed heparanase assay [13]. The sulfated oligosaccharides, obtained by chemical sulfonation of natural oligosaccharides, were also screened for antiangiogenic activity [14]. It was found that inhibitory activity in both assays was critically dependent on chain length and degree of sulfation, with highly sulfated linear oligosaccharides of five or more monosaccharides in length being the most active. Maltohexaose sulfate and PI-88 ("phosphomannopentaose

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sulfate") were the most promising compounds with the latter studied in more detail. PI-88 potently inhibited heparanase and in vitro angiogenesis, both with an IC_{50} of 2 μg/mL. PI-88 was then shown to inhibit in vivo tumor growth in the rat mammary adenocarcinoma 13762 MAT model, to reduce tumor vascularity, and to inhibit metastasis [14].

These and other preclinical studies described in more detail below, led to the development (by Progen Pharmaceuticals and later Medigen Biotechnology Corporation) of PI-88, also known as muparfostat, as an anticancer drug candidate and the first heparanase inhibitor to progress to clinical trials [15]. PI-88 was ultimately evaluated in a Phase III clinical trial in post-resection hepatocellular carcinoma (HCC) [16], however, despite encouraging signs, it did not meet its primary endpoint (disease-free survival) and has not been approved for use. Over the years since its discovery, PI-88 has been evaluated in many nonclinical studies in support of its clinical development for cancer. In addition, given the role of heparanase and various HS-binding proteins in numerous other pathological processes, it is not surprising that PI-88 has been studied extensively in many non-cancer indications, both as a heparanase inhibitor or as a general HS-mimetic. These studies also spawned drug discovery efforts to prepare next generation HS mimetic heparanase inhibitors with improved properties [17, 18], ultimately resulting in the discovery of PG545 (pixatimod) [19], which is currently in Phase I clinical trials in cancer patients [20]. In this chapter, we present a summary of the preparation, physico-chemical and biological properties of PI-88, including preclinical/clinical and structure-activity relationship studies. In addition, we discuss the development of related HS mimetic heparanase inhibitors for cancer therapy, leading to the discovery of PG545.

19.2 Synthesis and Structural Characterization of PI-88

PI-88 is a complex mixture of monophosphorylated, polysulfated mannose oligosaccharides prepared by sulfonation [14, 21] of the oligosaccharide phosphate fraction (OPF) from *Pichia holstii* NRRL Y-2448 phosphomannan [22] and has been represented in most publications as having the structure **1** (Fig. 19.1A). The major

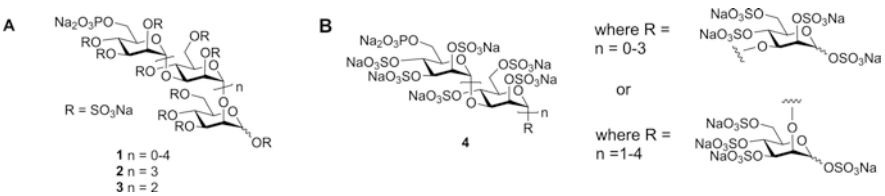

Fig. 19.1 (**A**). Depiction of the structure of PI-88 (1) showing the major components, pentasaccharide (2) and tetrasaccharide (3). (**B**). More complete depiction of the structure of PI-88 (4) including the presence of minor isomers

component (~60%) of this mixture is the sulfated α(1 → 3)/α(1 → 2)-linked pentasaccharide **2**, which led to the early name for PI-88, "phoshomannopentaose sulfate". PI-88 also contains a significant amount of the α(1 → 3)/α(1 → 2)-linked tetrasaccharide **3**, which together with **2** accounts for approximately 90% of the total oligosaccharide content, with the remaining 10% composed of di-, tri- and hexasaccharides [23, 24]. Recently published studies [25, 26] have provided a more complete picture of PI-88's composition, revealing the presence of minor amounts of all-α(1 → 3)-linked oligosaccharide isomers and demonstrating that only α(1 → 3)-linked disaccharides are present. The structure of PI-88 is thus best represented by structure **4** (Fig. 19.1B) as this more accurately reflects its composition. These studies also confirmed that the α-anomers are the dominant species (α/β ~9:1), most oligosaccharides are fully sulfated, and any undersulfated species present in PI-88 samples are due to degradation via anomeric desulfation. Molecular dynamics simulations combined with NOE measurements of pentasaccharide **2** predict a linear and rigid backbone conformation [26].

PI-88 has been radiolabelled [27] with either [^{35}S] or [^{14}C] for pharmacokinetic [28] and tissue distribution studies [29] (discussed below) by sulfonation of the OPF with [^{35}S]-sulfur trioxide pyridine complex, or by utilizing [^{14}C]-D-glucose in the *Pichia* fermentation to produce [^{14}C]-labelled OPF.

19.3 Inhibition of Heparanase

PI-88 is a potent competitive inhibitor of heparanase with a K_i = 7.9 nM [30]. It acts essentially as a substrate mimic, binding to the active site of the enzyme via electrostatic interactions and blocking HS substrate access. A molecular docking study of the major pentasaccharide component of PI-88 (i.e., **2**) with heparanase was conducted using a heparanase homology model [31] (prior to the publication of the X-ray crystal structure for heparanase [32]). This study indicated that the pentasaccharide is able to bridge two of the three previously identified heparin/HS-binding domains, HBD-1 and HBD-2. The docked poses could be clustered into two binding modes. In the majority of binding modes, the sulfates make electrostatic interactions with the side chains of Lys159, Lys161, and Arg272, whereas hydrogen bonds were made with the side chains of Gln270, Tyr298, and Ser228. No interactions were observed with the phosphate group of PI-88 [31]. Interestingly, previous structure-activity relationship studies have shown that the phosphate group of PI-88 also has little, if any, effect on binding to angiogenic growth factors [33]. PI-88 has been shown to inhibit heparanase activity in serum samples from pancreatic carcinoma patients [34], and to block heparanase-mediated cell surface HS cleavage in human pancreatic cancer (PANC-1) cells [35], human endometrial cells [36], and human embryonic kidney (HEK293) cells transfected with the mutant *BRAF* or *RAS* gene [37].

19.4 Nonclinical/Preclinical Studies

Apart from its potent inhibition of heparanase, PI-88 is a potent antagonist of HS-protein interactions and its anticancer and other biological activities, including unwanted side effects, are due to its polypharmacology. PI-88 blocks angiogenesis [14, 17, 38–40] partly through its inhibition of heparanase, which affects vascular remodeling and release of angiogenic growth factors from the ECM. In addition, PI-88 also blocks the interactions of angiogenic growth factors such as FGF-1, FGF-2, and VEGF with HS [33, 41] and their receptors, and it stimulates the release of the endogenous antiangiogenic protein, tissue factor pathway inhibitor [42]. It has been shown to inhibit angiogenesis in various in vitro assays, e.g., the human placental vessel [14], the rat aorta [18, 43] and the chick chorioallantoic membrane (CAM) assays [38], as well as the endothelial cell tube formation (Matrigel) assay [17] and growth factor-induced cell (HUVECs and dHUMVECs) proliferation assays [17]. PI-88 has also been shown to inhibit the endosulfatases HSulf-1 and HSulf-2 [44] which play roles in angiogenesis and cancer progression [45].

Early in vivo studies of PI-88 in cancer models confirmed its capacity to inhibit tumor growth, metastasis, and angiogenesis and supported its progression into the clinic. In the invasive rat mammary adenocarcinoma 13762 MAT model, PI-88 showed a 50% inhibition of primary tumor growth, reduction of metastases to the lymph node by ~ 40%, inhibition of blood-borne metastasis by more than 90%, and reduction of tumor vascularity by ~30% [14]. PI-88 also inhibited tumor growth in the BC1 and DAMA syngeneic mammary adenocarcinoma models in female dark agouti rats [46] and reduced the malignant cell load in mice with juvenile myelomonocytic leukemia and rats with acute myeloid leukemia [47]. In the RIP1-Tag2 transgenic mouse model of pancreatic islet β-cell carcinoma, PI-88 suppressed tumorigenesis at the early stage and inhibited tumor growth at later stages [39]. In murine orthotopic HCC models, PI-88 blocked up-regulation of heparanase and inhibited tumor recurrence and intrahepatic metastasis following partial liver resection, with recurrent HCC tumors showing enhanced sensitivity to PI-88 [48].

PI-88 has also shown antimetastatic activity in the B16 experimental mouse metastasis model [18] and in vivo antiangiogenic activity in the mouse AngioChamber and AngioSponge assays [17], and in the oxygen-induced retinal neovascularization mouse model [40]. Pharmacokinetic studies in animals demonstrated that PI-88 was not orally available, but had high subcutaneous bioavailability (100% in rats), was excreted primarily via the kidneys [29, 49] and had a half-life of 0.83 ± 0.09 h in the rat [28]. Pharmacodynamically, PI-88 has anticoagulant activity (see below), causing a linear and dose-dependent increase in the activated partial thromboplastin time (APTT) which correlates with the AUC and C_{max}. In early studies, including clinical studies, PI-88 concentration in plasma was determined by using the APTT as a surrogate [50].

Anticoagulant activity is a common side effect of HS mimetics that has limited the development of unmodified heparins and sulfated polysaccharides as heparanase inhibitors. One approach to minimizing this side effect is to modify (low

molecular weight) heparin chains by lowering the degree of sulfation and/or glycol-splitting [51], such as in Roneparstat (Naggi et al., Giannini et al., Noseda et al., Chaps. 20, 21 and 23 in this volume). Another approach is to minimize the chain length, i.e., use sulfated oligosaccharides rather than polysaccharides, as in PI-88. PI-88 was found to be of sufficient chain length for potent heparanase inhibition [14], but short enough that its anticoagulant activity did not preclude its clinical use [52]. The anticoagulant activity of PI-88 has been thoroughly profiled [21, 42, 52, 53] and found to be primarily due to enhancing the ability of heparin cofactor II to inhibit thrombin. At one time PI-88 was under consideration for development as a drug for vascular diseases such as thromboembolism and restenosis [54] due to its anticoagulant activity and inhibition of smooth muscle cell proliferation [41].

Heparanase plays a key role in inflammation, as reviewed in other chapters of this book (i.e., Elkin, Gaskin et al.). Heparanase inhibitors, therefore, have potential as therapeutics for a range of diseases characterized by inflammation [55], and not surprisingly, PI-88 displays anti-inflammatory activity in several animal models. For example, it was active in a rat model of multiple sclerosis (experimental autoimmune encephalomyelitis) and significantly inhibited thioglycollate-induced air pouch inflammation in mice [56]. PI-88 also inhibited eosinophil recruitment [57] and accumulation [56] in mouse lungs in models of lung inflammation and chronic asthma, respectively. In addition, PI-88 caused a marked attenuation of acute colitis and prevented body weight loss in a mouse model of inflammatory bowel disease [56], and reduced proteinuria in a model of passive Heymann nephritis [29]. In diabetes models, PI-88 dramatically reduced Type 1 diabetes incidence and reduced islet inflammation in diabetes-prone NOD mice [58], and significantly inhibited retinal leukostasis and reversed retinal dysfunction in diabetic rats [59]. Recently, human β-cells cultured with PI-88 showed significantly improved survival and protection against hydrogen peroxide-induced death [60] (Simeonovic et al., Chap. 24 in this volume).

PI-88 displays antiviral activity against viruses that utilize HS as an entry receptor or co-receptor. This activity is likely to be due, in part, to its inhibition of heparanase, which has recently emerged as an important player in viral infection, spread and pathogenesis [61, 62] (Agelidis and Shukla, Chap. 32 in this volume). PI-88 has been shown in vitro to inhibit herpes simplex virus (HSV) infection of cells and cell-to-cell spread of HSV-1 and HSV-2 [63], and to inhibit infection of cells by the poxvirus vaccinia virus (VACV) [64]. It has also shown antiviral activity in vivo against dengue virus and the encephalitic flaviviruses, Japanese encephalitis virus, West Nile virus, and Murray Valley encephalitis virus [65].

19.5 Clinical Studies

PI-88 has been evaluated in a number of clinical trials with several hundred patients having been administered the drug, either alone or in combination with chemotherapy, as summarized in Table 19.1. The early clinical studies of PI-88 have been

Table 19.1 Summary of PI-88 Clinical Trials[a]

Identifier	Phase	Indication	Patients	Ref.
	I	Healthy volunteers (IV)	24	
	I	Healthy volunteers (SC crossover)	22	
	I	Advanced cancers (IV)	14	[70]
	I	Advanced cancers (IV), Asian population	9	
	I	Advanced cancers	42	[67]
	Ib	Advanced cancers (docetaxel combination)	16	[68]
	II	Multiple myeloma	19	
NCT00073892	I/II	Melanoma	44	[71]
NCT00068172	I/II	Melanoma	88	
NCT00130442	II	Melanoma (dacarbazine combination)	131	
NCT00103389	II	Lung cancer (docetaxel combination)	98	
NCT00097851	II	Lung cancer (docetaxel combination)	100	
NCT00268593	II	Prostate cancer (docetaxel combination)	48	[72]
NCT00247728	II	Liver cancer (post resection)	172	[73, 74]
NCT00568308	III	Liver cancer (post resection)	600	
NCT01402908	III	Liver cancer (post resection)	520	[16]

[a]From https://ClinicalTrials.gov and reference [17]. PI-88 administered as SC injection unless otherwise indicated. IV = intravenous

thoroughly reviewed [15, 66] and are only summarized herein. PI-88 was initially administered by intravenous (IV) infusion and then in subsequent trials by subcutaneous (SC) injection. Phase I/II clinical studies demonstrated that PI-88 had an acceptable pharmacokinetic profile [67–69] and was generally safe and well tolerated, with few serious adverse events reported. Mild anticoagulant effects were reported in all patients, however, the dose-limiting toxicity was immune-mediated thrombocytopenia, observed in ~5% of patients, which was inferred due to the development of anti-heparin platelet factor (PF4) antibodies [67, 70]. The thrombocytopenia was dose-related, but platelet counts returned to normal upon cessation of drug treatment.

In these early studies, PI-88 showed some promising signs of patient benefit. For example, in a Phase I trial in patients with advanced solid tumors, one patient with melanoma showed a partial response for over 50 months and nine patients, including five with melanoma, had stable disease for over six months [67]. These positive outcomes supported the progression of PI-88 into Phase II trials, either alone or in combination, for melanoma [71], multiple myeloma, prostate cancer [72] and lung cancer. These studies only provided modest results and so these indications were not pursued further. However, a Phase II trial of PI-88 as an adjunct therapy in post-resection HCC showed that, at a dose of 160 mg/day, PI-88 was safe and showed promise in reducing recurrence for up to one year following curative resection [73]. Compared with the control group, PI-88 treatment resulted in an increase in the recurrence-free rate from 50% to 63%, and the time to recurrence at the 36th percentile was postponed by 78%. An observational follow-up study extended the follow-up period to three years [74] and provided further support to these findings.

These encouraging outcomes led to the evaluation of PI-88 in a Phase III clinical trial as adjuvant therapy for post-resection HCC with the primary end-point being to assess disease-free survival [16]. Secondary end-points were overall survival, recurrence time and safety. The study did not meet the primary end-point but did reveal a possible positive protective effect of PI-88 in a subgroup (40%) of patients with microvascular invasion, showing a significant prolongation of disease-free time after completion of the one-year treatment. PI-88 was also shown to have a good safety profile, with only one confirmed case of immune-mediated thrombocytopenia [16].

19.6 Synthetic Studies

PI-88 is a complex mixture readily manufactured from *Pichia holstii* NRRL Y-2448 phosphomannan. However, there has been significant interest in accessing homogeneous α(1 → 3)/α(1 → 2)-linked oligomannosides to facilitate structure-activity relationship (SAR) studies and for the development of analogs as next-generation heparanase inhibitors/HS mimetics. Early SAR studies utilized individually purified α(1 → 3)/α(1 → 2)-linked oligomannosides without terminal phosphate groups isolated from the PI-88 manufacturing process. Upon sulfonation, these gave compounds **5**–**8** (Fig. 19.2) which correspond to the major oligosaccharide components of PI-88 but where the phosphate has been replaced by sulfate. In angiogenic growth factor binding studies [33, 75], it was shown that replacement of the phosphate with

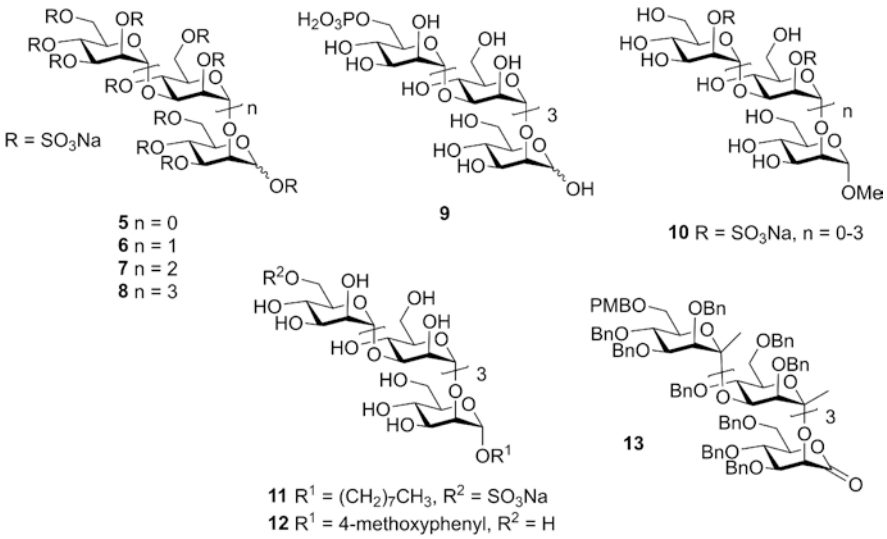

Fig. 19.2 Structures of synthetic oligomannosides containing the α(1 → 3)/α(1 → 2)-linked pentasaccharide backbone of the major component of PI-88

sulfate had little or no effect on binding. These studies also confirmed that stronger binding correlated with longer chain length. Subsequently, these same compounds, along with their all (1 → 3)-linked congeners, were prepared by total synthesis from monosaccharide building blocks using a "1 + 1" iterative strategy [76]. Heparanase inhibition studies with these oligosaccharides showed that while the penta- and tetrasaccharides were potent competitive inhibitors, the shorter di- and trisaccharides were only partial competitive inhibitors and did not completely inhibit the enzyme, even at very high concentrations. This suggests that short oligosaccharides incompletely block access of substrate HS to the active site of heparanase [76].

Several other synthetic approaches to the α(1 → 3)/α(1 → 2)-linked pentasaccharide backbone of PI-88 and analogs have been reported, although in most cases without subsequent biological evaluation of the products. Hu and coworkers synthesized the pentasaccharide **9**, complete with terminal 6^{V}-phosphate, via a "1 + 1" iterative strategy using thioglycoside donors [77]. Mong and coworkers also utilized thioglycoside donors to access methyl di- to pentamannosides **10** selectively sulfated at C-2 of each non-reducing end mannose unit. Du and coworkers used trichloroacetimidate donors in a "3 + 2" building block strategy to prepare the octyl pentamannoside **11** with a terminal 6^{V}-sulfate [78]. Interestingly, this compound showed mild anti-angiogenic activity in the CAM assay. Iadonisi and coworkers also used a "3 + 2" building block strategy to prepare 4-methoxyphenyl pentamannoside **12**, but they assembled both their tri- and disaccharide building blocks and the pentasaccharide from three monosaccharide building blocks using a sequential one-pot glycosylation strategy [79]. Ikegami and coworkers prepared an interesting methyl substituted analog **13** of the PI-88 pentasaccharide backbone using *exo*-methylene sugars as donors [80].

19.7 PI-88 Analogs and Next-Generation Heparanase Inhibitors

While PI-88 was a promising first-in-class heparanase inhibitor, the fact that it is a complex mixture was problematic for its clinical development, particularly with respect to its structural characterization, manufacturing process and assessment of SAR. In addition, the pharmacokinetics of PI-88 were less than ideal with a relatively short half-life necessitating frequent daily or twice daily dosing in preclinical models. A series of PI-88 analogs, termed the "PG500 series" were synthesized and evaluated. The aim was to prepare simple, easy to characterize analogs as single chemical entities and to determine if the modifications had any impact on the pharmacokinetics and biological activity. The initial compound design was based on the major PI-88 component and featured a single α(1 → 3)/α(1 → 2)-linked pentasaccharide backbone, replacement of the terminal 6^{V}-phosphate with sulfate, and the installation of an alkyl glycoside (e.g., benzyl, octyl) as a single, α-anomer (Fig. 19.3) [28]. The compounds were synthesized from the individually purified

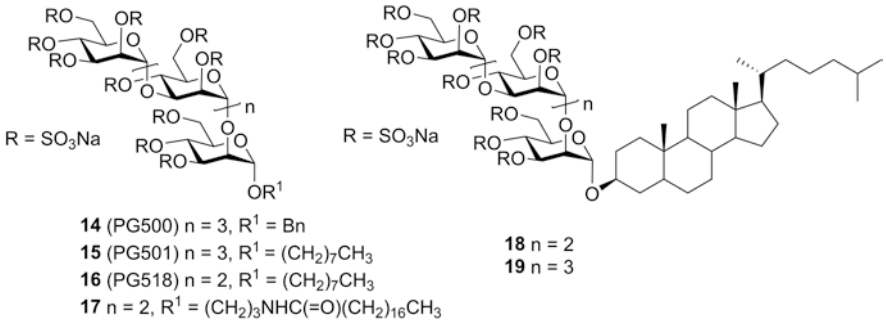

Fig. 19.3 Structures of selected "PG500 Series" compounds: homogeneous PI-88 analogs as next-generation HS mimetics and heparanase inhibitors

oligomannosides isolated from the PI-88 manufacturing process via a simple glycosylation/deprotection/sulfonation process. Alternatively, the oligosaccharides could be synthesized from monosaccharide building blocks [81] via a "1 + 1" iterative strategy [76]. These initial studies showed that the compounds maintained similar in vitro heparanase inhibition and angiogenic growth factor binding activity to PI-88. Of particular note were preliminary in vivo studies in rats using [^{35}S]-labelled compounds which showed improved pharmacokinetic properties [28]. For example, systemic clearance of octyl glycoside **15** (PG501) was three times slower than PI-88.

Following these positive findings, additional compounds were synthesized and tested, including tetrasaccharide analogs, with the testing expanded to include various cell-based assays indicative of angiogenesis (e.g., growth factor-induced cell proliferation, tube formation (Matrigel) and rat aorta assays) [17, 18, 81, 82]. The new analogs generally had similar or slightly better in vitro activity to PI-88 but displayed superior activity in the Matrigel assay. The lipophilic modifications at the aglycone also attenuated the anticoagulant activity, a common side effect of HS mimetics [82]. Two compounds, **14** (PG500) and **15** (PG501), were also tested in vivo alongside PI-88 in the AngioChamber and AngioSponge mouse angiogenesis models, with all compounds showing significant inhibition of angiogenesis [17]. Compound **15** and its tetrasaccharide homolog **16** (PG518) were then tested in the B16 mouse melanoma model, an aggressive tumor model *resistant* to PI-88 [82]. Compounds were administered twice daily for 7 days at a dose of 30 mg/kg to C57BL mice three days after tumor challenge. Treatment with both compounds significantly inhibited tumor growth (94 and 86%, respectively) up to day 10. Tetrasaccharide **16** also exhibited an improved pharmacokinetic profile in rats compared with pentasaccharide homolog **15**.

The above promising results prompted further investigations into this class of compounds to investigate the effects of significantly increasing the lipophilicity of the aglycone substituent and of modifying the oligosaccharide backbone. It was found that the attachment of long alkyl chains or a steroid such as cholestanol resulted in compounds, e.g., **17**–**19** (Fig. 19.3) and **20**–**25** (Fig. 19.4) with signifi-

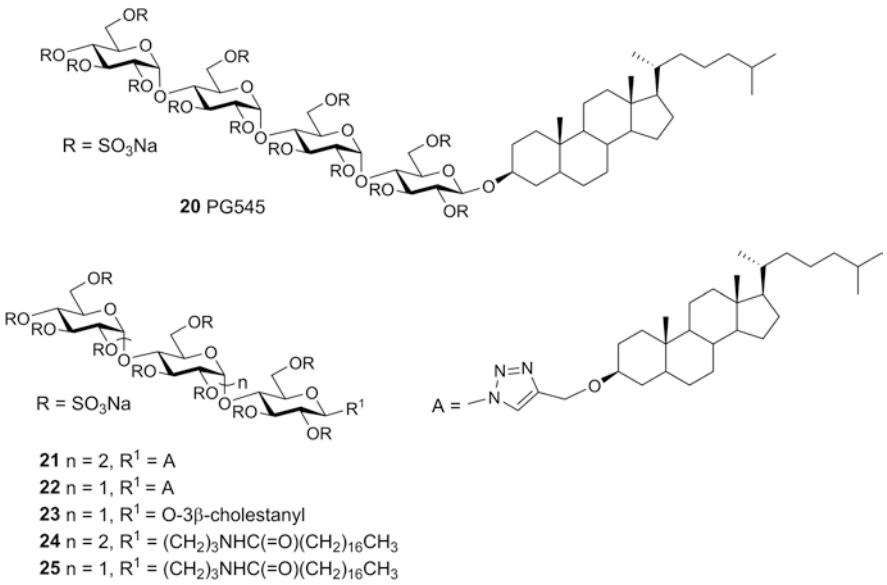

Fig. 19.4 Structures of selected "PG500 Series" compounds with a malto-oligosaccharide backbone

cantly improved in vitro and ex vivo antiangiogenic activity compared with both PI-88 and less lipophilic compounds in the series (e.g., **14**–**16**) [18, 19]. For example, the IC_{50} values of some compounds in growth factor-induced HUVEC proliferation assays were up to approximately 28-fold (for FGF-2) or 90-fold (for FGF-1 and VEGF) lower compared with PI-88 [18]. Interestingly, the highly lipophilic aglycones conferred potent heparanase inhibitory activity even on shorter oligosaccharides that previously were only partial competitive inhibitors. For example, sulfated mono- and disaccharides with cholestanol aglycones (structures not shown) still inhibited heparanase with nM K_i values. In addition, the aglycones further attenuated the anticoagulant activity, to the point where some APTT and Heptest values were barely above normal levels [19]. The nature of the oligosaccharide chain was also observed to only have a limited effect on activity. As observed in Parish's original screening studies, HS mimetics based on malto-oligosaccharides, i.e., α(1 → 4)-linked glucose residues (Fig. 19.4), possessed similar activity to manno-oligosaccharides as found with PI-88. This observation has important consequences for manufacturing of potential drug candidates because the α(1 → 3)/α(1 → 2)-linked mannopentaose backbone must either be isolated by tedious chromatography from phosphomannan hydrolysates, or prepared by multistep total synthesis. On the other hand, maltotetraose, which provided a suitable oligosaccharide backbone for potent compounds, is commercially available and inexpensive.

19.8 Discovery of PG545 (Pixatimod)

The improvements in in vitro and ex vivo activity conferred by these highly lipophilic modifications also translated into significantly improved in vivo efficacy. Several compounds were evaluated in the B16 murine melanoma and experimental metastasis models and were shown to be far superior to **14** and **15**. In the metastasis model, when administered once daily for 12 consecutive days at a lower dose of 10 mg/kg, compounds **20**–**22** potently inhibited metastatic nodules. In the solid tumor model, which is resistant to PI-88 treatment, daily administration at 15 mg/kg for 12 days, 3 days after tumor challenge, resulted in potent inhibition of tumor growth with no evidence of palpable tumors until the cessation of drug treatment [18]. This compared favorably with **14** and **15** where palpable tumors were evident after several days of treatment at 30 mg/kg (b.i.d.). Compound **20** (PG545) was also shown to significantly inhibit tumor growth in the HT29 colon xenograft model when administered daily at 5 mg/kg [18]. Preliminary pharmacokinetic evaluation of these compounds indicated that the lipophilic aglycones had significantly increased the apparent half-lives in the rat. Subsequent, more detailed studies with PG545 revealed that it had a long half-life (>50 h) enabling once or twice weekly dosing in preclinical models while maintaining substantial efficacy [83].

The above studies led to the selection of PG545 (pixatimod) as a clinical candidate, supported by extensive pharmacological evaluation in multiple preclinical cancer models [83, 84], as summarized below and detailed by Hammond and Dredge, Chap. 22 in this volume. PG545 is a potent inhibitor of heparanase with a reported K_i of 4.4 nM [85] to 6 nM [19]. Detailed kinetic analyses indicate that PG545 inhibits heparanase with parabolic competitive kinetics, suggestive of multiple modes of binding and inhibition [85]. PG545, either alone or in combination with chemotherapy agents, has been shown to potently inhibit tumor progression and metastasis in several syngeneic, orthotopic and xenograft murine models of cancer [86–97]. A number of studies have recently revealed that PG545 also possesses immunomodulatory activity [98]. It inhibits the infiltration of tumor-associated macrophages [86, 87], possibly via inhibition of heparanase, and also strongly stimulates $CD11c^+$ dendritic cells, via toll-like receptor 9 (TLR9) and IL-12 leading to activation of IFN-γ producing natural killer cells [92]. An initial Phase I trial using subcutaneous administration (ClinicalTrials.gov Identifier: NCT01252095) was halted due to unexpected injection site reactions. Subsequently, the route of administration was changed to an intravenous infusion and PG545 was administered in this fashion in a Phase Ia monotherapy study in patients with advanced solid tumors (ClinicalTrials.gov Identifier: NCT02042781) [20], where it demonstrated a tolerable safety profile and evidence of immune cell stimulation and disease control in some patients. PG545 has recently been shown to also enhance T cell infiltration in combination with anti-PD-1 therapy. It is currently undergoing a Phase Ib study in combination with the checkpoint inhibitor nivolumab (Opdivo®) in patients with advanced cancer/pancreatic adenocarcinoma [98].

Similarly to PI-88, HS mimetics of the PG500 series display other biological activities such as antiviral activity [28, 99–102]. Interestingly, unlike PI-88 and simple PG500 series compounds, the more highly lipophilic compounds such as PG545 not only inhibit infection and cell-to-cell spread, they also possess virucidal activity [99–101] due to the ability of the cholestanol group to disrupt viral lipid envelopes [103]. In addition, in in vivo studies, inhibition of heparanase by PG545 has been shown to have nephroprotective effects in a murine model of ischemic reperfusion acute kidney injury [104], and to attenuate atherosclerosis and liver steatosis in a murine model [105].

19.9 Conclusions

The HS mimetic PI-88 (muparfostat) is a potent heparanase inhibitor that has demonstrated the potential that this compound class has as therapeutics for cancer, as well as other indications mediated by heparanase. PI-88 was the first heparanase inhibitor to be evaluated in human clinical trials in cancer patients, ultimately progressing to Phase III trials in post-resection HCC. Despite early promise, it failed to meet its primary endpoint and has not been approved for use. However, its early clinical and preclinical success inspired the search for next-generation HS mimetic heparanase inhibitors with improved properties and greater potency. These efforts led to compounds (the "PG500 Series") with improved physicochemical and drug-like properties, including pharmacokinetics, which translated into improved potency in various assays and preclinical cancer models. These efforts led to the discovery and selection of PG545 (pixatimod) for clinical development [19]. PG545 is not only a potent heparanase inhibitor, but it has recently been shown to possess immunomodulatory activity, which may be a critical component of its mechanism of action. PG545 recently completed a Phase Ia study in patients with advanced solid tumors [20]. The positive data from this study supported the continued development of PG545 and it is currently in a Phase Ib trial in combination with the checkpoint inhibitor nivolumab. Meanwhile, this class of heparanase inhibitor continues to show promise in a range of other indications mediated by heparanase.

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Chapter 20
Non-Anticoagulant Heparins as Heparanase Inhibitors

Giuseppe Cassinelli, Giangiacomo Torri, and Annamaria Naggi

In memory of Benito Casu

The editors and authors of Chapter 20 dedicate this chapter to the memory of Benito Casu (1927–2016), remembering his pioneering contributions in Glycoscience starting from his early studies on the structure and conformation of glucopolymers up to seminal papers of SARs of heparins and their "non-anticoagulant derivatives inhibiting heparanase, endowed with potential anticancer and anti-inflammatory therapeutic applications".

Benito Casu joined the "G. Ronzoni Institute" in Milan, with an 'organic chemistry' degree of the University of Pavia, in 1951 pioneering chemo-physical studies of natural glucopolymers, using innovative spectroscopic methods including NMR.

In 1968 he obtained the professorship in Chemical Spectroscopy and the nomination. The next year he joined, as visiting scientist, the Chemistry Department of McGill University at Montreal (Canada) directed by prof. A. Perlin. In a fruitful sabbatical year, he contributed, primarily through early NMR studies of heparin, to the notion that the flexibility of its chains, fundamental for binding proteins and biological activities, are mediated by different conformations of L-iduronic acid residues (H). These studies earned him an international reputation, the direction of the Institute and an invitation to join a French Academic/Industrial research project succeeding in identifying the heparin antithrombin binding region (ATBR) fundamental for its anticoagulant activity. This discovery paved also the way to the present important clinical applications of Low Molecular Weight Heparin (LMWH). Under his direction the Ronzoni Institute promoted a number of important translational projects, including projects supported by EC grants: BRIGHS: Biotechnological Routes In Generating Heparin-like Saccharides; BANG: Biologically Active Novel Glycosaminoglycans, focusing on the preparation of "bioheparin" through chemo enzymatic processes starting from a bacterial biosynthetic precursor; and "Heparanase inhibitors for cancer therapy", joining the teams and editors of this book.

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In the framework of this preface it is impossible to assess the content and the breath of the contributions (articles, book chapters, invited plenary lectures) of prof. Casu and his coworkers, all had also the merit of sharing time and knowledge with visiting scientists and young students. Prof. Casu was the Coordinator of the carbohydrate group of the Italian Chemical Society, National member of the International Advisory Board of the most important carbohydrate chemistry and Biochemistry Journals and books. He received many distinctions and Awards most remarkable, in 1998, the "Honorary Doctorate in Medicine" from the University of Uppsala (Sweden) for his studies in glycoscience.

Let's conclude this preface remembering an almost unknown creative talent of Benito Casu in sculpturing rocky stones, and his eclectic performance in dosing and shaking "gin-fizz" for friends and in the drink-mill of Villa Vigoni as an evening closing event of participants at the annual GAG Symposium in Loveno, organized by Benito from 1991 together with prof. Job Harenberg and Ronzoni coworkers.

Abbreviations

2OdesH or ODSH	2-O,3-O-desulfated heparin
AT	antithrombin
ATBR	(heparin) antithrombin binding region
CHS	chondroitin sulfate
DCoxH	dicarboxylated oxy heparin
DeS	dermatan sulfate
D-Gal	galactose
D-GlcA	glucuronic acid
D-GlcN	glucosamine
DS	sulfation degree
ECM	extracellular matrix
GAG	glycosaminoglycan
gs	glycol split
HBP	heparin binding protein
HMW	high molecular weight
HS	heparan sulfate
HSBP	heparan sulfate binding protein
HSPG	heparan sulfate proteoglycan
IdoA-L	iduronic acid
K5PS	*E.coli* K5 polysaccharide
LMWH	low molecular weight heparin
NA	N-acetylated domain
NAH	N-acetyl heparin
NS	highly sulfated domain
NS-K5	N-sulfated K5PS
NS-OSK5	N,O sulfated K5PS

OS-K5	O-sulfated K5PS
oxyH	oxyheparin
PS	polysaccharide
PST	pentasulfated trisaccharide
ROH	reduced oxy-heparin
SAHS	sulfoamino heparosan sulfate
ssLMW	supersulfated LMWH
TSD	trisulfated disaccharide
UFH	unfractioned heparin

20.1 Introduction

Heparan sulfate proteoglycan (HSPG) physiological functions, fundamental for development, homeostasis and signaling, are dependent on the integrity of heparan sulfate (HS). Heparanase is an endo-β-D-glucuronidase which cleaves chains of HS present at both cell surfaces and the extracellular matrix (ECM). Early on, its deregulation appeared to be involved in tumor cell growth, migration and metastasis, and later on in other pathologies such as inflammation, diabetes, atherosclerosis, and nephropathy [1–4].

Heparin, exclusively produced by mast cells, is a highly sulfated form of HS. Early studies evidenced the HS higher charged congener unfractionated heparin (UFH) as an efficient heparanase inhibitor as well as substrate [5, 6]. Then, investigations were mainly oriented to identify non-anticoagulant heparin derivatives to overcome the UFH anticoagulation and bleeding side effects as well as to improve its pharmacokinetics and bioavailability. This chapter describes how heparin-derivative heparanase inhibitor research has evolved referring to landmark [7] and more recent contributions focusing on structure-activity relationship. Perspectives for the development of new agents of this class as potential drugs in cancer and other pathologies are also illustrated.

20.2 Heparan Sulfate

Heparanase targets are the HS chains covalently linked to core proteins of the ubiquitous and multifunctional HSPGs, present at cell surfaces and ECM and involved in cell signaling, survival, proliferation, migration, and invasion. The HSPG biological functions are mostly carried out through electrostatic binding/interaction of the negatively charged HS chains with a multitude of proteins including chemokines and cytokines, growth factors and their receptors as well as enzymes. HS is expressed in almost all cells of mammalian species as linear polyanionic chains, differing in size (20 up to 100 kDa) and sulfated domain distribution among species and tissues of the same species. The composition of natural physiological HS chains

can be altered in several pathological conditions [8–11]. Heparin and HS-like glycosaminoglycans (GAGs) have been isolated from avian intestinal mucosa [12], terrestrial invertebrates [13, 14], marine crustacean and mollusk species [15–17] as well as bacterial and virus species [12]. In the Golgi compartment of all animal cells and mast cells of connective tissue, biosynthesis of HS and heparin chains, respectively are completed through common steps leading to the formation of a specific tetrasaccharidic linkage region (D-GlcA β-1-3-O-D-Gal β-1-3-O-D-Gal1-β-3-O-D-Gal-1-β-4-O-D-xylopyranosyl-1-α) to join L-serine of the HSPG core protein. The following elongation steps lead to a common linear high molecular weight (HMW) homogeneous GAG, N-acetyl-heparosan, constituted by 4-O-β-D-glucopyranosyl 1–4-O-N-acetyl- α D-glucosamine.

A sequence of incomplete enzymatic reactions induces modifications and heterogeneity in terms of composition and size distinct for HS and heparin chains. The highly sulfated domains (NS), constituted by sequences of the trisulfated disaccharide 4-O-L-IdoA2S 1α-4-O-D-GlcNS6S are prevalent in heparin chains, while in the N-acetylated domains (NA), the disaccharide 4-O-α-D-GlcA-1β-4-O-D-GlcNAc) is more abundant in HS chains. NS and NA domains in both HS and heparin are disseminated among mixed transition regions (NS/NA) [9–18].

Experimental studies showed that the heparin chains can interact with a great number of proteins, classified as heparin binding proteins (HBPs). Taking into consideration the ubiquitous presence of HSPGs and their multifunctional interactions in physiological media with a great variety of proteins, these should be better classified as HS binding proteins (HSBPs) [10]. However, functional studies are hampered by the fact that samples of pure HS are very expensive and difficult to isolate. The first HS rich preparation was isolated in 1948 as by-product of heparin manufacturing process and named “heparin monosulfate” for its low sulfate content [19]. On the other hand, the presently available pharmaceutical heparin is mainly derived from the porcine intestinal mucosa extracts which contain other linear GAGs, [dermatan- (DeS) and chondroitin-sulfate (ChS)] which are difficult to separate from HS [12]. In fact, DeS and ChS are present as minor components of an antithrombotic drug (Danaparoid sodium) containing 80% of low molecular weight (LMW) HS, and known in the EC market as Orgaran [20].

20.3 Heparanase: Discovery and Characterization

The term “heparanase” appeared for the first time in a paper of 1978 [21] to define a “heparitinase”-like endoglucuronidase, present in guinea-pigs basophil leukocytes, able to cleave GAGs resistant to chondroitinase ABC, such as HS but not heparin. However, three years earlier in 1975, a heparanase-like endoglucuronidase from murine mast cells was found able to cleave macromolecular heparin to functional heparin [22] and in 1983 lymphoma cells were found able to degrade HS chains of HSPGs of subendothelial ECM [23]. Finally, in 1984 other authors reported for the first time in the title of their paper the term heparanase to design an

endoglucuronidase, able to degrade HS, produced by the highly invasive lung metastatic murine B16-BL6 melanoma cells [24]. Endoglucuronidase activities were previously described in other mammalian tissues and cells such as rat liver tissues [25], human skin fibroblasts and placenta [26], human platelets [27] and activated T lymphocytes [28]. A partially purified human platelet enzyme was found able to cleave both HS and heparin [29]. In the following years, the increasing interest in heparanase function and role in several pathological contexts has been documented by an ever-growing number of reports summarized and commented in [3] and in Vlodavsky et al., Chap. 1 in this volume. Since 1984 heparanase-like activity was detected in several human normal and malignant cells and tissues. However, the lack of selective activity assays along with the low concentration and instability of crude enzyme have hindered heparanase isolation and characterization until 1999 when five teams have independently reported the cloning and functional expression of the human heparanase gene in mammalian and insect cells [30–34] (Vlodavsky et al., Ilan et al., Gaskin et al., Pinhal et al., Chaps. 1, 7, 9 and 36 in this volume). Heparanase, at present the only known mammalian endo β-D-glucuronidase, is physiologically expressed primarily in platelets, activated white blood cells and placenta. The heparanase human gene encodes for a pre-proenzyme which undergoes removal of the N-terminal signal peptide in the endoplasmic reticulum to give rise to the pro-enzyme of 65 kDa. Cleavage of a 6 kDa linker peptide by cathepsins in the lysosomes leads to an active enzyme constituted of two subunits of 50 and 8 kDa that are not covalently linked, as also confirmed by X-ray crystal structures [35–39] and (Vlodavsky et al., Gaskin et al., Chaps. 1 and 7 in this volume).

Modeling investigations on heparanase interactions with HSPGs, HS/heparin, and related oligosaccharides have also been reported along with molecular model of human heparanase proposing the binding mode of HS oligosaccharide to catalytic amino acids [40]. Early studies identified the minimal HS sequences recognized by heparanase in a GlcA flanked by two NS,6S D-GlcN units, the second one can be also 3-O-sulfated [41, 42] (Fig. 20.1a).

The disaccharide sequence GlcAβ1–4-GlcNS,3,6S [42], is a constituent of the pentasaccharide heparin-antithrombin binding region (ATBR) and of the synthetic mimic α-methyl glycoside of N-sulfated pentasaccharide Fondaparinux (Fig. 20.1b) clinically used as antithrombotic agent [43]. As a good heparanase substrate, it has been included as a component of heparanase activity assay kit particularly useful for kinetic analysis and screening of enzyme inhibitors [44, 45]. The heparanase cleavage site was described to be also dependent on the sulfation pattern of the neighboring sequences [46]. The same team identified the most suitable cleavage site in the pentasaccharide GlcNAc6S-GlcA-GlcNS-Ido2S-GlcNS6S [46]. Other common cleavage sites have been identified by the analytical profiling of heparanase digests of HS of different origin and sulfation degree [47].

HS cleavage is affected by heparanase expression levels and proteolytic activation but can also be influenced by the activity of other HS biosynthetic and modifying enzymes. Notably, the multiple substrate recognition allows heparanase to degrade HS chains independently of cell specificity and environment [9]. Hence, heparanase localization and activating processes are relevant in determining its

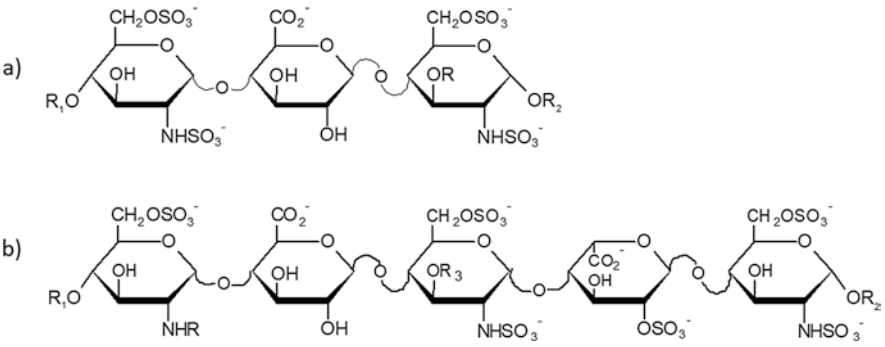

Fig. 20.1 (**a**) minimal heparanase recognized sequence: R = H; SO_3^-; R^1 and R^2 uronic acids of Hep/HS chain. (**b**) Antithrombin binding site: R = SO_3^-; NAc R^1 and R^2 = uronic acids of Hep/HS chain; R^3 = SO_3; Fondaparinux: R = SO_3^-; R^1 = H; R^2 = Me; R^3 = SO_3^-

biological function in a variety of healthy and malignant cells and tissues [35]. Moreover, heparanase upregulation has been described in several malignancies and pathological conditions including acute and chronic inflammation, fibrosis, amyloidosis, diabetes and related nephropathies, osteoarthritis, atherosclerosis and other vessel wall pathologies [3, 6] and (Vlodavsky et al., Ilan et al., Elkin; Simeonovic et al., Masola et al. Li and Zhang, Chaps. 1, 9, 17, 24, 25 and 27; in this volume).

The first evidence of heparanase activity in the murine melanoma B16-BL6 [24] and T-lymphoma [23] experimental models, were provided by Nakajima et al. [24] and Vlodavsky et al. [23] associating the in vivo metastatic potential of these cells with HS degradation. Excellent reviews [2, 3, 6, 7, 11] reported findings on heparanase overexpression in several malignancies and functional studies in cancer models highlighting its causal relevant role in sustaining tumor growth and progression.

Of note, heparanase overexpression was shown to accelerate HSPG turnover along with upregulation of their HS N- and O-sulfation degree suggesting a functional correlation between the endo-β-D-glucuronidase expression level and HS oversulfation [48, 49]. Interestingly, the N-unsubstituted D-glucosamine ($GlcNH_3^+$) in the disaccharide GlcA-β1–4-$GlcNH_3^+$6S, very uncommon (0.7 to 4% of total GlcN) in natural HS chains [50], was quite abundant in HS of mammary carcinoma cells [51, 52]. More recently, the same disaccharide was also found in a significant amount in HS chains of highly invasive breast cancer cell lines expressing heparanase. These findings suggest a possible role of $GlcNH_3^+$6S in imparting heparanase degradation resistance to HS chains in these malignant cells. Indeed, the synthetic tetrasaccharide (TD 4–143,1): GlcAβ1–4-O-$GlcNH_3^+$6S-1α-4-O-GlcAβ1–4-O-$GlcNH_3^+$6S α-octyl glycoside (Fig. 20.2 a) inhibited heparanase and suppressed cancer cell invasion *in vitro* [53].

Also, the synthetic pseudopentasaccharide [ED 80061] (Fig. 20.2b), bearing at the reducing end a 2-deoxy-1 N-imido D-glucuronic acid moiety, was shown to be a potent heparanase inhibitor (IC_{50} 11 nM) with antimetastatic activity in the B16-F10 and MAT 13702 experimental models [54].

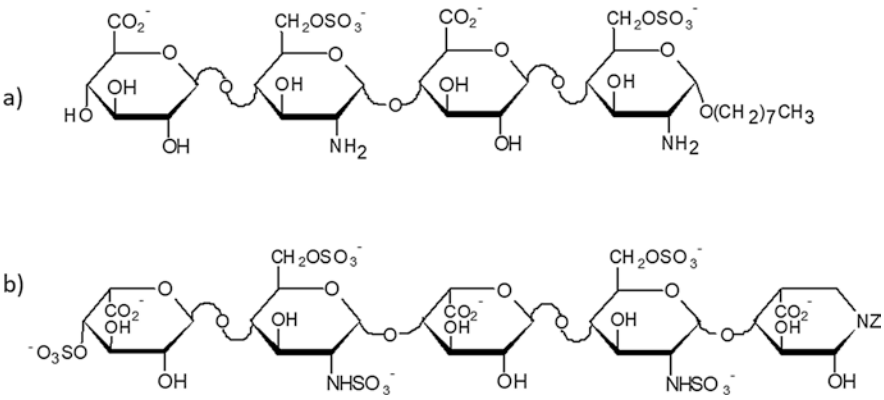

Fig. 20.2 Structures of two synthetic oligosaccharide heparanase inhibitors: (**a**) TD 4–143,1 [53]; (**b**) pseudopentasaccharide [ED 80061] [53]

Overall, these findings provide useful information concerning the heparanase-HS interaction and structural determinants to be exploited for the design of efficient heparanase inhibitors devoid of side effects.

20.4 Heparosan-Related Heparanase Inhibitors

20.4.1 Natural and Semi-Synthetic Derivatives

Invertebrate and bacterial N-acetyl heparosan derivatives endowed with a peculiar structural chemo-diversity have provided the opportunity to perform in-depth SAR study and to define structural determinants responsible for different biological activities.

A heterogeneous HS (Mw ~ 27 KDa), mainly constituted of N-acetyl heparosan sequences $(GlcA\text{-}GlcNAc)_n$ was isolated from viscera of the bivalve mollusk *Nodipecten nodosus*. NMR analysis indicated that the major disaccharide 4-O-D-GlcAβ1–4-D-GlcNAc showed a low sulfation degree due to partial and random 2- and/or 3-O-sulfation of D-GlcA along with partial N- and 6-O sulfation of GlcNAc (Fig. 20.3). Endowed with heparanase and P-selectin inhibitory activity and a low anticoagulant activity (five-fold lower than porcine heparin), the mollusk HS showed anti-metastatic and anti-inflammatory effects *in vivo* without bleeding effect [55].

A capsular polymeric (Mw 35–49 kDa) GAG of the *Escherichia coli* strain K5 showed the same structure of the HS/heparin natural biosynthetic precursor N-acetyl heparosan constituted by a regular sequence of $[GlcA\beta1–4\text{-}GlcNAc\ \alpha1–4]_n$ [56]. This discovery was extremely useful in the search for new anticoagulant and anti-thrombotic heparins endowed with better pharmacokinetic and fewer side effects

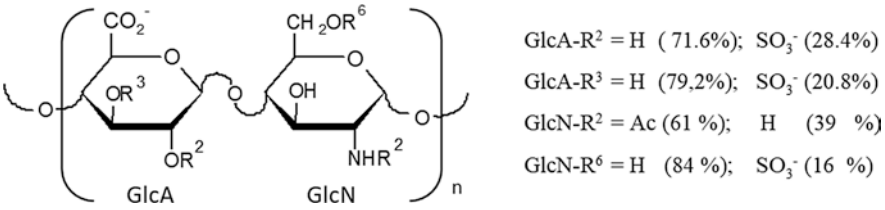

Fig. 20.3 Major disaccharide units of *Nodipecten nodosus* HS. D-GlcA:glucuronic acid; D-GlcN: glucosamine

and analogously, in the identification of non-anticoagulant congeners to be evaluated in other therapeutic fields. The progress in the knowledge of the HS/heparin biosynthetic pathway [57, 58] has opened the way for chemo-enzymatic synthesis of polymers called "bioheparin" [59] and "bioengineered heparins" [60]. These novel approaches were stimulated, at the end of the nineties, by the "mad-cow crisis", which urged the search for new animal sources of heparin or semisynthetic derivatives to compensate the withdrawal of bovine heparin from the market. Looking for heparin-like GAGs, several N-deacetylated N-sulfated sulfoamino heparosans were firstly obtained and then subjected to O-sulfation at the 6-O position of GlcNS and 2-O, 3-O sulfation of GlcA [59]. A number of semisynthetic O-sulfated sulfamino heparosans (SAHSs), differing in degree and pattern of O-sulfation as well as molecular size, were tested in the mouse B16-BL6 melanoma model. Among these compounds, the two high Mw SAHS-2 (Mw 25,7 kDa) and SAHS-4 (Mw 22.7 kDa) and a low molecular weight derivative SAHS-5 (Mw 3.2 KDa), showed a remarkable anti-metastatic activity, with the sole SAHS-4 displaying a modest anticoagulant activity [61]. Highly N,O-sulfated heparosans were found to bind FGF-2 and inhibit FGF-2-induced endothelial cell proliferation and angiogenesis likely interfering with the formation of FGF-2/FGFR/HS complexes [62–64]. Various species of O-sulfated N-acetyl heparosan (OSK5) (Fig. 20.4) were reported to bind FGF-1, −2 and − 8 with different FGF signaling antagonist activity influenced by the type of FGF and FGFR expressed and by the cellular context [65]. This class of derivatives (OSK5), along with new preparations of O-sulfated sulfamino heparosans (NSOSK5), were also tested as heparanase inhibitors in a translational project "Heparanase" supported by the EC, which recognized the enzyme as a potential therapeutic target for cancer. New powerful analytical tools, such as 2-D NMR spectroscopy, have allowed a better characterization of the component profile and sequence of heparosan derivatives and complex GAGs [66]. Focusing on their biological activities, the most representative are the HMW derivatives NSOS-K5 and OS-K5 (11–15 kDa) which displayed a stronger heparanase inhibitory *in vitro* in comparison with the corresponding ultra LMWH (2–3 kDa) [67].

The anticoagulant activity of LMW NS,OS and OS derivatives was found negligible and lower than that of HMW NS,OS congener. The HMW OS-K5 and NS,OS-K5 preparations were shown to inhibit metastatic dissemination of human breast cancer MDA-MB-231 cells [65]. Interestingly, the same K5 derivatives,

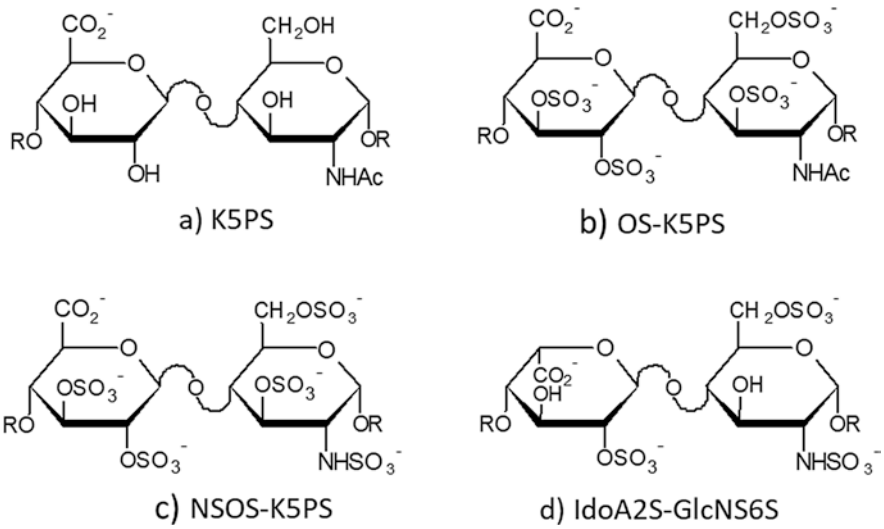

Fig. 20.4 Predominant disaccharide units of capsular polysaccharide from *E. coli* K5 (K5PS) (**a**), its sulfated variants (**b, c**) and typical heparin trisulfated disaccharide (**d**)

endowed with heparanase inhibitory activity, inhibited HIV replication in T cells and macrophages, likely preventing the virus attachment to the host cells [63], an event that implicates interaction of the virus with cell surface HS (Agelidis and Shukla, Chap. 32 in this volume). A very HMW (35 kDa) NS,OS-K5, endowed with moderate anticoagulant activity, was reported to inhibit *in vivo* bone osteolysis and tumor growth of the highly metastatic human breast cancer MDA-MB-231 (SA) cell line [68].

The project "bioheparin" by chemoenzymatic processes led to derivatives endowed with modest or low anticoagulant activity, tested as potential antiangiogenic, antiviral, and anti-inflammatory agents [69]. The other project of chemoenzymatic synthesis of GAGs starting from N-acetyl heparosan led to a "bioengineered heparin" [70] and to some intermediate oligosaccharides which have allowed to define the substrate specificity of heparanase [46, 70–72].

20.5 Heparin Derivatives

Early studies showed an effective heparanase inhibition by UFH, even if some of UFH sequences can be recognized and cleaved by heparanase [4, 5, 41]. However, its unwanted anticoagulant activity hampered its safe use as an inhibitor of heparanase. The elimination or at least the reduction of the anticoagulant activity can be obtained through different types of chemical modifications of the structure of heparin such as oversulfation, partial desulfation, reduction of Mw or selective modifications of the residues at the antithrombin binding site representing the major determinant

of anticoagulant activity. Among heparins endowed with heparanase inhibitory activity, N-acetylated and O-desulfated species of non-anticoagulant heparins were the first to be tested and found active *in vivo* in the metastatic B16 melanoma model whereas carboxy reduced heparin was almost inactive [4, 5]. Moreover, UFH fragments, consisting of at least 16 units, were found active both *in vitro* and *in vivo* [5].

20.5.1 LMWs, Ultra LMWHs and Derivatives

The clinical use of LMWHs in oncology has been approved for preventing venous thromboembolism as well as for their better pharmacokinetic and pharmacodynamic properties compared with UFH [73]. Results of randomized studies concerning the benefits of UFH and LMWHs in cancer patients were published since the early 1980s without a clear conclusion on their real impact on cancer patients' response to therapy. Heparanase and selectins are inhibited by LMWHs, albeit with a somewhat lower efficacy than UFH. Taking into consideration the heterogeneity of the starting UFH chains in term of size, beyond the anticoagulant activity, their depolymerization can offer other compositional and interaction differences affecting the pharmacological properties of LMWH. For example, early *in vivo* evaluation of antimetastatic effects evidenced a significantly higher activity of dalteparin (Fragmin) in comparison with that of nadroparin or enoxaparin [5]. Size fractionation of tinzaparin allowed separation of HMW fractions whose components, not present in the other two LMWHs, are endowed with high selectin inhibitory activity but low antiXa activity [74]. Preclinical *in vivo* evaluation in tumor xenografts models evidenced that tinzaparin was able to sensitize cis-platin resistant ovarian cancer [75]. A non-anticoagulant ultra LMWH (2.5 kDa), obtained by hydrogen peroxide catalyzed radical heparin hydrolysis assisted by ultrasonic waves, exhibited antiheparanase activity intermediate compared with those of tinzaparin (Mw 7 kDa), dalteparin (6.3 kDa) and enoxaparin (5.5 kDa) [76]. A glycopolymer constituted by a N-sulfated poly2-aminoethyl methacrylate carrying the heparin disaccharide ΔU2S-GlcNS,6S, was reported to inhibit heparanase, B16 melanoma cell migration, and adhesion to platelets and microvascular endothelial cells [77]. Of note, the old orally active Sulodexide, constituted by LMWH and DeS in a 80:20 mixture, isolated from porcine intestinal mucosa, has been used since 1974 as antithrombotic drug, displayed low anticoagulant activity and bleeding effects [78]. More recent studies evidenced that Sulodexide provided benefits in patients with diabetic nephropathies through inhibiting heparanase [79].

20.5.2 Supersulfated Heparins

A supersulfated LMWH (ssLMWH), prepared by controlled depolymerization of UFH and endowed with low anticoagulant activity was demonstrated to inhibit heparanase, proinflammatory molecules such as leukocyte elastase, cathepsin G and

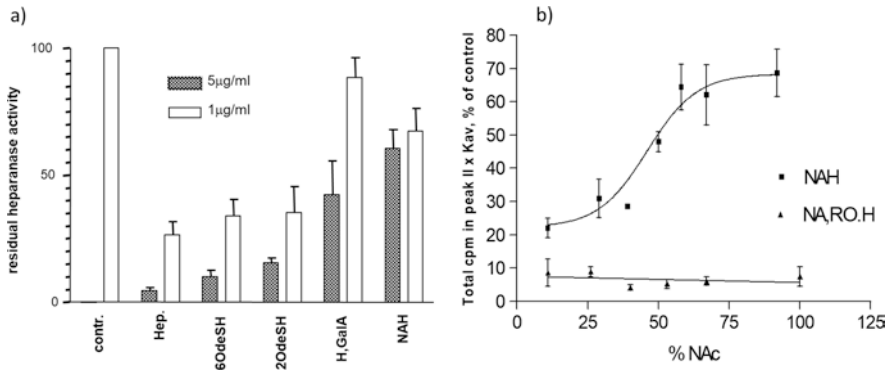

Fig. 20.5 Heparanase inhibitory activity of heparin (Hep) and desulfated heparin derivatives: (**a**) heparin (Hep), 6-O desulfated heparin (6OdeSH), 2,3-O desulfated heparin (2O-deSH), 2,3-O desulfated heparin with change of configuration (L,GalA), N-acetyl heparin (NAH); (**b**) Inhibition of heparanase by N-acetyl heparins with different acetylation degree and corresponding 25% glycol-split derivatives

hepcidin [80–82]. Tested *in vivo* in the metastatic B16 melanoma model, it exhibited antimetastatic activity similar to that of UFH [61]. Recently, a remarkable antitumor activity of ssLMWH has been demonstrated both *in vitro* and *in vivo* in synovial sarcoma experimental models [83]. Inhibition of synovial sarcoma cell growth and invasion was associated with downregulation of the activity of receptor tyrosine kinases of the EGFR, PDGFR and IGFIR families and heparanase inhibition. The combination of ssLMWH with an inhibitor of IGF receptors, synergistically inhibited cell proliferation and motility and promoted apoptosis. *In vivo* ssLMWH synergized with the receptor tyrosine kinase inhibitor to suppress orthotopic synovial sarcoma growth and spontaneous lung metastatic dissemination [83]. However, it is necessary to consider the risk that derivatives with a high degree of sulfation, may stimulate other competitive biological mechanism or cause unwanted reactions such as the activation of prekallikrein as observed with oversulfated chondroitin sulfate, known as OSCS [84, 85].

20.5.3 O-Desulfated Heparins

The presence of 2-O and 6-O sulfation, with at least one of the two positions retaining a high sulfation degree, was found to be essential for inhibition of heparanase. As shown in Fig. 20.5, effective heparanase inhibition was exhibited by fully 2-O-desulfated heparin whereas two intermediates bearing modified 2-O-desulfated IdoA units, namely 2,3 epoxy L-uronic acid and GalA, were practically inactive [86, 87].

Basic conditions needed for 2-O-desulfation can cleave also the 3-O-sulfate of GlcN, fundamental for the AT binding thus, producing a further decrease in the anticoagulant activity of the 2,3-O-desulfated heparin (2OdesH / ODSH). Along with a high potency in inhibiting heparanase, 2OdesH showed *in vivo* antitumor activity in CaPan-2 pancreatic adenocarcinoma xenografts and antimetastatic activity in the B16-F10 mouse melanoma experimental model [88, 89]. Recently, 2OdesH has been described to block the release of the inflammation mediator, high mobility group box 1, by inhibition of p300 acetyltransferase activity [90]. A 2OdesH is currently under clinical investigation as CX01 in combination treatment of acute myeloid leukemia [Sect. 20.7.5].

6-O-desulfated heparin (6OdesH) also showed selectin inhibitory activity [86, 87] along with low anticoagulant activity. Interestingly, its LMW congener inhibited the aggregation of *Plasmodium falciparum*-infected red blood cells with uninfected erythrocytes to form rosettes [91].

Regarding other glycoconjugates and their applications, a 6-O-desulfated nadroparin conjugate with deoxycholic acid was orally active and able to suppress neovascularization and bone destruction in murine arthritis experimental models [92].

20.5.4 N-Acyl-N-Desulfated Heparins

Heparin N-desulfation can be modulated from 10 up to 100% and the products used as intermediates to obtain N-acyl heparins. When the remaining N-sulfation degree is low, compounds were non-anticoagulant and generally endowed with low heparanase inhibitory activity (Fig. 20.5b). *In vivo* antimetastatic activity was reported for fully N-acetyl [4, 5], N-hexanoyl [5], low and ultra low N-succinyl heparins [93]. Beside of being almost non-anticoagulant, the advantage of N-acetyl derivatives over UFH is their incapacity of releasing active bFGF from cells and ECM [94], an event that promotes tumor growth and angiogenesis [95].

A SAR study on N-acetyl heparins with N-acetylation degree ranging from 29 up to 100% showed a drastic decrease of heparanase inhibitory activity with degree of N-acetylation higher than 50%. These findings suggest that the interaction with the enzyme needs at least one N-sulfated glucosamine per tetrasaccharide [87]. Surprisingly, the formation of flexible joints inside the chain obtained by periodate oxidation of the non-sulfate uronic acid residues showed increased anti-heparanase activity (Fig. 20.5, 5b). This result has highlighted the glycol split (gs) derivative of fully N-desulfo-N acetyl heparin (G4000, ^{100}NA-ROH, SST0001) for the development of a potential drug (Roneparstat) which will be discussed in more detail in the next section. In a follow-up study, N-acetyl heparins, ranging from 39 up to 100% N-acetylation were tested as P- and L-selectin inhibitors in comparison with UFH. The 58% N-acetylated heparin displayed a good selectin inhibitory activity as well as anti-metastatic activity when tested *in vivo* in murine MC38 colon carcinoma and B16 melanoma experimental models [95].

20.5.5 Glycol-Split Heparins: Semisynthesis and Activities

Controlled depolymerization of UFH by periodate oxidation which cleaves the linkage between the hydroxylated C(2)-C(3) of non-sulfated hexuronic acid gives oxyheparin (oxyH), susceptible to be reduced with $NaBH_4$ leading to reduced oxyheparin (ROH). This approach incorporates the gs-residues while preserving the sulfation pattern and degree with a low reduction of Mw [96]. Structural characterization of ROHs obtained from UFH from different animal sources are reported by Alekseeva et al. [97]. They displayed a significant reduction of anticoagulant activity mainly related to the periodate oxidation of the GlcA residues linked to tri-O-sulfated GlcN of the ATBR, whose integrity is essential for the anticoagulant activity of heparin [98, 99]. Early investigations suggested that, other than temperature and pH values, neighboring residues could influence and differentiate the periodate oxidation rate of IdoA versus GlcA [100, 101]. Other studies evidenced that mild acid hydrolysis of ROH gave oligomers bearing at the non-reducing end of non-anticoagulant LMWH, the N,3,6 trisulfated glucosamine residues [102]. In a recent kinetics study of enoxaparin periodate oxidation, NMR-HSQC showed that the complete IdoA oxidation occurred in 2 hr. while GlcA was only partially oxidized after 8 hr. This difference may be explained by the higher conformational flexibility of gs IdoA suitable for the periodate cyclic complex intermediates whose formation could be partially hindered by the GlcA neighboring residues [103]. A number of non-anticoagulant ROH were tested in a variety of therapeutic areas where UFH was active. The residual anticoagulant activity of ROH as well as of N- and O-desulfated heparins may result from interactions outside the ATBR, mediated by heparin cofactor II and the release of vascular tissue factor pathway inhibitor [104]. Investigators of Glycomed (Alameda) and the University of Boston observed severe bleeding in mice harboring human pancreatic adenocarcinoma Ca Pan-2 xenografts and murine B16-F10 melanoma administered with s.c. ROH, an effect likely due to antiplatelet and heparin cofactor II activity [88]. A modified preparation of ROH (Mw 11 kDa), with a cofactor II activity comparable to that of heparin, but lower (10–15%) anti-Xa activity was developed by Glycomed as an adjuvant in cardiovascular intervention to prevent vascular restenosis [105].

A non-anticoagulant oxy-heparin fragment carrying a hydrophobic polystyrene chain (NAC-HCPS) exhibited *in vitro* and *in vivo* antiangiogenic and antimetastatic activities in murine B16 melanoma and Lewis lung cancer (3LL) models. NAC-HCPS also inhibited 3LL tumor growth and vascularization, likely by means of inhibiting endothelial cell proliferation stimulated by VEGF165, FGF-2, or HGF [106]. ROH prepared according to the method of Casu et al. [96] inhibited P-selectin-mediated cell adhesion of human colon carcinoma cells to immobilized platelets [107]. ROH preparation, designated as low anticoagulant heparin (LAC), showed good tolerability without bleeding complication when given s.c., i.p. and i.v. to mice at dosages able to inhibit tumor cell dissemination in several murine metastatic models. LAC activity was associated with inhibition of cancer cell adhesion and extravasation in lung capillary by competing with cell-surface HS interaction [108].

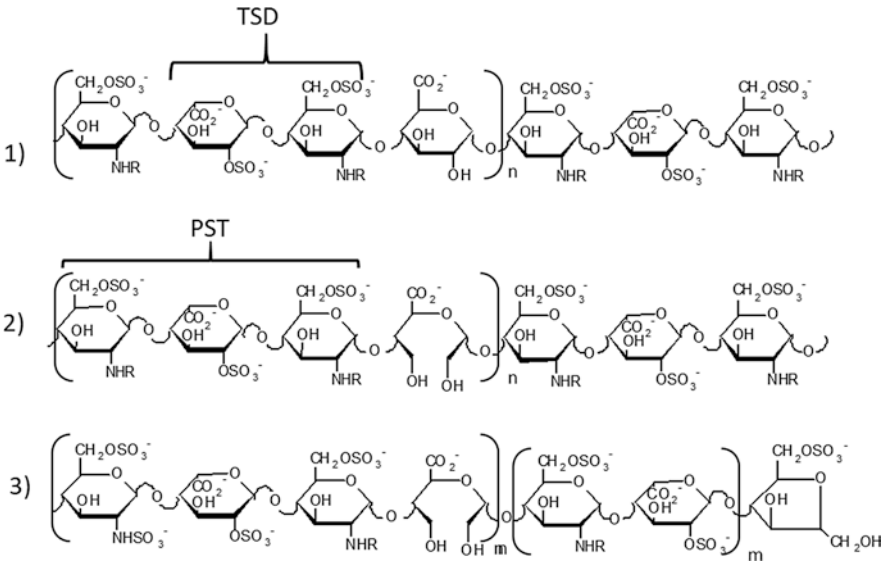

Fig. 20.6 Prevalent sequences in regular regions of heparin and chemically modified heparins. (**1**) heparin; (**2**) 50% 2-O-desulfated heparin (prevalently PST.U sequences), ST1514; (**3**) 50% 2-O-desulfated and glycol-split LMW heparin (prevalently PST.sU sequences); ST2184. R = SO_3^- or Ac; TDS trisulfated disaccharide; PTS pentasulfated trisaccharide

The seminal report of Folkman et al. 1983, disclosing the inhibition of angiogenesis and tumor growth by heparin and its fragments, was the start-up for investigating the molecular mechanisms underlying tumor neovascularization (neoangiogenesis) [109]. Heparin chains bind with high-affinity FGFs and in particular, FGF-2, recognized as one of the major angiogenesis promoting factors. The heparin minimum FGF binding fragment was identified in the pentasulfated trisaccharide GlcNS6S-Ido2S-GlcNS6S (PST) followed by IdoA2S (Fig. 20.6 (2) mainly present in the high sulfated region of UFHs [110]. With the aim of generating 2-O-sulfation gaps along heparin chains, alkaline treatment of UFH led to heparin derivatives bearing epoxy uronic acid units that were then hydrolyzed to L-galacturonic acid units. These heparin derivatives were converted by periodate oxidation/$NaBH_4$ reduction to gs-derivatives. Graded 2-O-desulfation led to a heparin derivative characterized by a 1:1 ratio IdoA2S: (GlcA+IdoA) residues. The following periodate oxidation/$NaBH_4$ reduction gave a heparin chains bearing 50% gs uronic acids which showed a low anticoagulant activity due to glycol-splitting of GlcA essential for the binding to ATBR. The gs-residues generating flexible joints along the chains improved the FGF-2 antagonist and angiostatic effect as well as anti-heparanase activity [111, 112].

Derivate **2** in Fig. 20.6 named ST1514 (Mw 11 kDa), was further investigated as along with its LMW derivatives **3** ST2184 (Mw 5.8 kDa). ST2184 displayed *in vitro* antiangiogenic and VEGF165 antagonist activity by interfering with the binding of the growth factor to its receptors [112]. ST1514 and ST2184 inhibited *in vivo* metastatic

lung dissemination of B16-BL6 melanoma cells in mice. ST2184 was also reported to reduce angiogenesis in human MeVo melanoma xenografts and to potentiate the antitumor activity of a camptothecin derivative [113]. In addition, ST1514, being a potent heparanase inhibitor, was able to reduce wound vascular density and inflammation in heparanase overexpressing transgenic mouse model of wound healing and delayed-type hypersensitivity [114, 115].

To evaluate new potential heparin applications, N-acetyl and gs-heparin and their LMW derivatives were assessed as HS competitors and anti-inflammatory agents in chronic airway diseases caused by *Pseudomonas aeruginosa*. Indeed, HS has been recognized as cellular receptor for *Pseudomonas aeruginosa* and binding site for its flagella. HS competitions by heparin derivatives can reduce bacterial burden acting as adjuvants with clinically used antibiotics. N-acetyl heparin (C23, Mw 17.2 kDa), its LMW derivative (8 kDa), ROH (C3gs20, Mw 16,5) and three LMW derivatives (8,12.6, 9.6 kDa, respectively) were tested in a mouse model of chronic *Pseudomonas aeruginosa* air way inflammation. Only the HMW products were able to reduce the inflammatory response, an effect mediated by reduction of cyto−/chemo-kine levels and of neutrophil elastase activity, and by inhibition of neutrophil recruitment [116] that correlated with anti-heparanase activity.

To dissect structural determinants for effective heparanase inhibition, a library of non-anticoagulant heparins was prepared by graded or fully O-desulfation, N-acetylation of N-desulfated of UFH. Periodate oxidation and borohydride reduction were also applied to give the corresponding gs ROHs. Preliminary tests showed that some derivatives from this library, including the gs derivative of fully N-desulfo-N acetyl heparin (^{100}NA-ROH), were effective in inhibiting lung metastasis from B16-BL6 mouse melanoma cells [117]. Further experiments showed that both the heparanase and selectin inhibitors ^{58}NAH (58% N-acetyl heparin) and ROH were able to inhibit lung metastasis formation in MC38 colon carcinoma mouse model expressing selectin ligands [72].

The choice of fully N-acetyl ROH as the lead compound was based on its inability to release FGF-2 from ECM, its low anticoagulant activity, its remarkable inhibition of heparanase enzymatic activity and its *in vivo* antimetastatic activity in the B16-BL6 melanoma model. Conversely, it was found inactive *in vivo* on the MC38 colon carcinoma model correlating with a lack of P- and L-selectin inhibitory activity [72]. Likewise, the low anticoagulant and weak heparanase inhibitor NAH was inactive as a selectin inhibitor. Nevertheless, it displayed a higher antimetastatic activity than UFH, in the MC38 colon carcinoma model. These findings suggested that its antimetastatic effects could be independent of inhibition of coagulation, heparanase, and selectins [72]. Indeed, other studies evidenced an anti-inflammatory activity of NAH which inhibited the function of inflammatory mediators such as human neutrophil elastase, IL-8 and TNFα [118–120]. Two non-anticoagulant LMW-ROH (8 and 10 kDa) obtained by heparinase I depolymerization of UFH followed by glycol-splitting, showed *in vivo* antimetastatic activity in the B16-F10 metastatic model. Conversely, only the 8 kDa LMW-ROH was able to inhibit spontaneous lung dissemination when B16-F10 cells were inoculated s.c. without affecting the primary tumor growth [74].

The increase of heparanase inhibitory activity produced by glycol splitting of UFH, LMWH and ultra LMWH [76, 87] was confirmed by assessing the effect of the heparin-related synthetic trisaccharide 4-OMeGlcNS6S-GlcA-α1,6 anhydro GlcNS. Strikingly, its gs derivative showed an increase of one order of magnitude in inhibiting the enzyme (IC_{50} = 30 μg/ml versus IC_{50} = 2 μg/ml for the gs derivative). Both trisaccharides were used for molecular modeling studies validated by NOESY-NMR data, the first evidencing the gs-GlcA conformation [122].

Of note, in comparison with the previously described LMW-ROHs, the structural peculiarities of ^{100}NA-ROH reside in a semisynthetic process based on reactions which preserve the UFH natural 2,3,6-O sulfation as well as the Mw range. The structural characterization showed the presence of both gs-GlcA within the ATBR sequence and the gs-uronic acid residues mainly interspersed within 6-O-N-sulfated disaccharides. Indeed, the reduced (~30%) overall sulfation degree lowered protein unspecific interactions and the chain higher flexibility, induced by the gs-residues, conferred to ^{100}NA-ROH an enhanced heparanase inhibitory activity and a more selective proteins interaction [123]. A recent study reported kinetic analysis and modeling of the heparanase-inhibiting mechanism of Roneparstat. Dose-inhibition kinetics confirmed its high potency in inhibiting heparanase enzymatic activity (IC_{50} = 3 nM) and suggested different interaction features implicating a complex binding mechanism, involving one or multiple ^{100}NA-ROH molecules depending on concentration ratios. Analysis of docking solutions indicated that a single chain of the inhibitor (e.g., Roneparstat) could interact with both heparin-binding domains of the enzyme or two different sequences of Roneparstat can interact with each of the heparin-binding domains, depending on the inhibitor/enzyme binding stoichiometry [124].

20.6 New Glycol-Split Non-anticoagulant Heparin as Heparanase Inhibitors

A follow-up translational project entitled “Novel heparanase inhibitors for cancer therapy” proposed and developed by teams of the Ronzoni Institute (Milan, Italy), the Technion University (Israel Institute of Technology, Haifa, Israel) and the University of Alabama at Birmingham (USA), was supported by the National Institutes of Health (NIH). This project was mainly devoted to optimizing ROHs inhibiting heparanase and multiple myeloma growth in experimental models [125]. Some compounds that emerged in these studies are described below.

20.6.1 N-Desulfated ROHs

Periodate oxidation of GlcN residues of N-deacetylated heparin to obtain non anticoagulant heparin was reported in a patent [126]. For the preparation of new heparanase inhibitors, periodate oxidation of N-desulfated heparins was performed in

Table 20.1 Comparison of physical and biological properties of RO-N desulfated heparins

Code	description	% gs/ monomers	Mw kDa	Anti-Heparanase activity IC_{50} ng/mL	*In vivo* % CAG tumor inhibition*
G8340	RO-N-desulfated heparin	62	8.4	20	75
G8438	RO-N-desulfated heparin	44	6.8	60	n.d.
G9578	RO-N-desulfated heparin	47	6.3	75	63
G4000	Roneparstat	25	16.0	3	62

**in vivo* human CAG multiple myeloma growth inhibition after 14 days treatment with drugs administered at 60 mg/Kg/day by subcutaneous continuous delivery [128]

aqueous neutral media to split C2-C3 linkages of both N-desulfo GlcN units and of non-sulfated uronic acid residues. A previous study has reported a graded N-desulfation of UFH (from 20 up to 100%) by known modification methods [87, 127]. The N-desulfated gs-compounds were obtained by a final borohydride reduction [128]. Physical and biological properties of three representatives of this new class of ROHs are shown in Table 20.1.

The significant reduction of Mw values can be explained by the instability induced by depolymerization due to the formation of two adjacent gs residues generated by nonsulfated uronic acids and N-desulfated glucosamines naturally present in UFH. When compared with Roneparstat, the newly generated compounds exhibited a somewhat lower but still significant heparanase inhibitory effect, which can be explained by their lower Mw. Regardless, the new compounds displayed a comparable antimyeloma activity. As previously observed with substitution of N-sulfate groups with nonpolar N-acetyl groups [87], the addition to gs-uronic acid residues of further flexible joints, randomly generated from the gs-glucosamines, maintained the heparanase inhibitory activity, suggesting that the unmodified sequences can still bind and inhibit the enzyme.

20.6.2 Dicarboxylated Oxy-Heparins (DCoxyHs)

Periodate oxidation of UFH nonsulfated uronic acid residues led to oxy-heparins (oxyHs), characterized by the split of the C2-C3 linkage and the formation of two aldehyde groups which were further oxidized to carboxy groups yielding dicarboxylated oxy heparins (DCoxHs). A number of oxy-heparins obtained by periodate oxidation of UFH or of its derivatives, such as partially or fully 2-O-desulfated fully-acetyl-N-desulfated and partially N-desulfated heparins [87], were used as intermediates. The oxidation of aldehyde to carboxyl was performed using sodium chlorite ($NaClO_2$) in aqueous media, pH 4, 0 °C for 24 h or at a neutral pH in the presence of oxidation catalysts [129]. The data in Table 20.2 show that the majority of the reported DCoxHs exhibited high efficacy in inhibiting *in vitro* heparanase and CAG multiple myeloma growth *in vivo,* independently of the Mw. It is noteworthy

Table 20.2 ROHs and DCoxHs. Comparison of physical and biological properties

code	description	%RO/ UA	Mw KDa	Anti-Heparanase activity IC_{50} ng/mL	*In vivo* % CAG tumor inhibition
G8223	RO heparin	25	17	2–8	n.d.
G8249	50%RO heparin	50	9.8	18	60
G4000	Roneparstat	25	16.0	3	62
		%DC/ UA			
G10810	DCoxy heparin		15.0	10	50
G9685	DCoxy heparin50%2Odes	38	11.7	10	68
G8767	DCoxy heparin50%2Odes	40	9.1	n.d	52
G7927	DCoxy heparin50%2Odes	47	6.4	10	n.d.
G8733	DCoxy heparin100%2Odes	14	5.5	n.d.	53
G10847	DCoxy heparin100%2Odes	63	5.5	n.d.	25

*UA = uronic acid

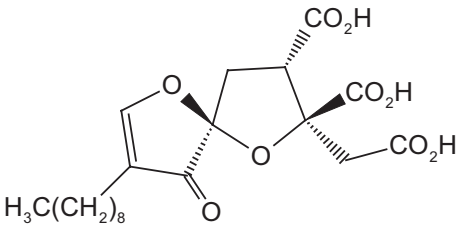

Fig. 20.7 Structure of trachyspic acid

that the random presence of about 40% of dicarboxylated gs-uronic acid residues, instead of both 2-O-sulfated and non-sulfated uronic acid, along the heparin sequences, did not affect the interaction with the enzyme or its inhibition. Accordingly, these modifications did not significantly change the *in vivo* tumor growth inhibition in comparison with Roneparstat.

The interaction with heparanase and its inhibition by a tricarboxylate moiety was first demonstrated with the natural enzyme inhibitor trachyspic acid, a metabolite of *Talaromyces trachyspermus* [130], and was later confirmed by its synthetic (+) enantiomer (Fig. 20.7) [131].

20.6.3 New Biotin-Conjugated N-Acetyl-Glycol Split Heparins

Recently, different classes of biotinylated N-acetyl gs-heparins were obtained by several approaches. Taking advantage of diverse reactive functions of oxy-N-acetyl heparins, intermediate spacers with different size have been used for coupling and keeping

the biotin moiety at different distances from the heparin chains. The advantages of the biotin coupling are related to its chemical moiety and its easy detection by chemico-physical methods useful for pharmacokinetic studies. As biotin receptors are overexpressed in several cancer cell lines and solid tumors, biotin-conjugation not only improves bioavailability but can also contribute to tumor targeting and drug delivery [132]. All the biotin conjugates prepared showed heparanase inhibiting IC_{50} in the nM range similar to Roneparstat [133]. Likewise, these compounds displayed a similar efficacy comparable to that of Roneparstat in inhibiting CAG myeloma tumor growth and metastatic dissemination of B16-F10 melanoma cells.

20.7 Clinical Candidates and New Applications

20.7.1 Heparin Derivatives and Oligomers Interacting with Viral Envelope

The role of heparanase enzymatic activity in supporting viral infection has recently emerged, suggesting new potential applications of heparanase inhibitors [134] (Angelidis and Shukla, Chap. 32 in this volume). As reported by Skidmore [135], UFH inhibited the interaction of dengue, Herpes simplex, yellow fever and T lymphocyte viruses with HS, known to favor viral entry. UFH and heparosan derivatives, including N-acetyl and de-O-sulfated derivatives were assayed upon H5N1 virus infection. 2-O-desulfated and ssLMWH showed an anti-viral activity comparable to that of UFH whereas N-acetylation was detrimental [91]. In a recent study investigating the interaction of GAGs with the Zika virus envelop protein (Zikave), porcine intestinal UFH was shown to bind Zikave more efficiently than other GAGs (ChS, DeS). Among the UFH oligomers, the binding with Zikave was inhibited starting from the 18-mer [136]. A number of derivatives, including heparin and N-acetyl heparin and their 2-O, 6-O, 2,6 di O desulfated derivatives, were assayed for effect on H5N1 influenza virus invasion comprising a H5 pseudo typed HIV system. In comparison with UHF, 2-O-desulfation increased the activity, supersulfation led to a comparable activity, and N-desulfation-N-acetylation exhibited somewhat lower activity [135].

20.7.2 Sevuparin (DF F01) and Tafoxiparin (DFX232)

Early studies evidenced that strains of *Plasmodium falciparum* associated with severe forms of malaria use HS as a host adhesion receptor. Taking into consideration the structural and functional analogies between HS and UFH, the latter was used to treat severe malaria with overall positive outcomes. However, UFH administration was discontinued due to severe intracranial bleeding [137]. It was demonstrated that inhibition of interactions between parasite and erythrocytes can be achieved by heparin fragments

sizing more than 3.5 kDa (dodecasaccharide) bearing natural N,6-O and 2-O sulfation [138]. A nonanticoagulant LMW ROH, Sevuparin (Mw 7.4 kDa), obtained by mild acid hydrolysis of ROH, showed the same activity of UFH both *in vitro* and *in vivo* in severe malaria models [139]. Clinical trials are evaluating the adjuvant activity of Sevuparin both in malaria patients and subjects with sickle cell disease, an inherited form of anemia [140, 141]. Another LMW ROH, Tafoxiparin (Mw 6.0 kDa) obtained by mild alkaline β-elimination, was found to disrupt rosettes, especially in the majority of fresh blood isolated from children with complicated malaria. Tafoxiparin represents potential adjuvant agent in the treatment of severe malaria [139]. As previous *in vitro* studies have demonstrated that Tafoxiparin increased both myometrial smooth muscle cell contractility and IL-8 activity in cervical fibroblasts [142], it is currently being evaluated in Phase II trial in pregnant women with slow progressing labor or labor arrest. In addition, with humanitarian approval, two pediatric patients suffering from Gorham-Stout syndrome, a rare bone disorder characterized by progressive bone loss and lymphatic vessel leakage, were successfully treated with Tafoxiparin [143].

20.7.3 Necuparanib (M 402)

A rationally designed LMW ROH (M 402, Mw 5.5–6.0 kDa) obtained by nitrous acid controlled depolymerization of UFH followed by glycol splitting, showed a reduced anticoagulant activity. Through its high-affinity binding, it displayed the ability to interfere with the function of several HS-binding proteins, such as chemokines, pro-angiogenic factors, P-selectin and heparanase ($IC_{50} = 5$ μg/mL). It showed an efficient *in vivo* antitumor, antiangiogenic, and antimetastatic activity in preclinical models [144, 145]. It underwent clinical evaluation in breast and pancreatic cancer, but the subsequent Phase 2 trial was discontinued after interim futility analysis for insufficient efficacy [146].

20.7.4 Roneparstat (G4000, ^{100}NA-ROH, SST0001)

Preclinical studies evidenced the pleiotropic effects of this N-acetyl ROH heparin including interference with heparanase-syndecan-1 axis relevant in multiple myeloma development, [125, 147, 148]. Roneparstat was also shown to interfere with the function of several HS-binding proteins other than heparanase. Indeed, other studies indicated its ability to interfere with receptor tyrosine kinase signaling and heparanase-induced expression of genes associated with aggressive tumor phenotypes [149, 150]. This NA-ROH (Mw 16 kDa) demonstrated a remarkable antitumor, antiangiogenic, immunomodulatory and antimetastatic activity in several preclinical models of both hematological (e.g., multiple myeloma, lymphoma) and solid tumors (e.g., sarcomas, pancreatic and breast carcinoma). It was safely administered in mice in prolonged treatment schedules both alone and in combination

with other antitumor agents [151–159]. An excellent safety profile was also emerged by recent results of Phase I clinical trial in advanced relapsed/refractory multiple myeloma [160] (Noseda and Barbieri, Chap. 21 in this volume). A recent study has assessed the role of heparanase in developing renal fibrosis arising in transplanted organ as a consequence of ischemia/reperfusion damage. *In vivo* tests showed that active doses of Roneparstat were well tolerated in animal models. A recent study evidenced that Roneparstat, by inhibiting heparanase, almost restored renal function, plasma creatinine and albuminuria. These results opened the way to further investigations on the potential efficacy of Roneparstat in reducing acute kidney injury and preventing chronic pro-fibrotic damage induced by ischemia/reperfusion injury [161].

20.7.5 CX-O1 (ODSH)

The low anticoagulant CX-01 retaining most of the anti-inflammatory properties of heparin is under clinical evaluation as adjuvant in acute myeloid leukemia and in refractory myelodysplastic syndrome [https://clinicaltrials.gov/ct2/show/NCT02995655]. It has recently received Orphan Drug and Fast Track Designations from the FDA for the treatment of acute myeloid leukemia [162]. It has been shown to interfere with CLC12/CXCR4 axis, inhibiting leukemia stem cell homing in the marrow stromal niches by competitive interaction with HS. Moreover, a randomized phase II trial in untreated metastatic pancreatic cancer has assessed that the combination of CX-01 with gemcitabine/nab-paclitaxel appears beneficial in term of disease control [163]. Worthy of note, local treatment with CX-01, significantly reduced neutrophil elastase in cystic fibrosis patients in combination with dornase [164].

20.8 Concluding Remarks

UFH remains the main source of semisynthetic efficient heparanase inhibitors. Non-anticoagulant heparin derivatives endowed with heparanase inhibitory activity reported in this chapter, retain part of the pleiotropic pharmacological effects of the starting heparin. This class of heparin derivatives interferes with the emerging role of heparanase in inflammatory diseases and other pathologies. All show good safety and tolerability along with hints of efficacy on several pathologies. On the other hand, the anticancer agents under clinical trials, namely Roneparstat and CX-01, given their HMW and low sulfation degrees, compositionally better mimic HS. These peculiarities allow these compounds to better interfere and inhibit the interactions between HSPGs and heparanase overexpressed by tumor cells and present in their environment. Given the need for long-term treatments, the development of orally active agents of this class represents an attractive research field.

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Chapter 21
Roneparstat: Development, Preclinical and Clinical Studies

Alessandro Noseda and Paola Barbieri

21.1 Introduction

Heparanase (HPSE) is a mammalian endo-β-D-glucuronidase that cleaves Heparan Sulfate (HS) chains and participates in microenvironment modulation. In fact, HS cleavage results in remodeling of the extracellular matrix (ECM) as well as in regulating the release of many HS-linked molecules such as growth factors, cytokines, and enzymes involved in inflammation, wound healing and tumor invasion.

The attention of the scientific community has been initially mostly attracted by the role of HPSE in cancer which has been extensively investigated. HPSE upregulation has been documented in a variety of human tumors correlating in some cases to an increased vascular density and poor postoperative survival [1, 2] (Ilan et al., Vlodavsky et al., Gaskin and Hulett, Chaps. 1, 7 and 9 in this volume).

A pro-metastatic and pro-angiogenic role for HPSE has been demonstrated in many primary human tumors since high levels of HPSE correlate with lymph node and distant metastases, elevated microvessel density and reduced survival of cancer patients. Data have also been reported that HPSE regulates heparan sulfate proteoglycan syndecan-1 and promotes its shedding from the cell surface. Shed syndecan-1, in turn, controls tumor growth, metastasis, and neo-angiogenesis by promoting growth-factor signaling in the tumor's milieu and by binding to integrins and growth factor receptors on the cell surface thereby driving rac signaling [3]. In addition to its intimate involvement in the egress of cells from the bloodstream, HPSE activity

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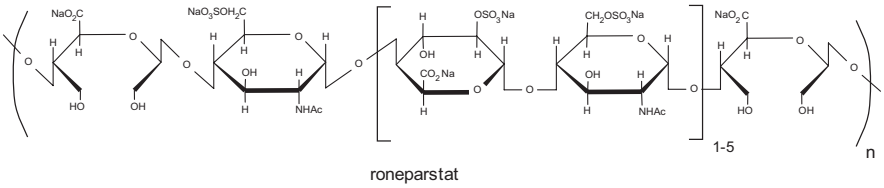
releases from the ECM and tumor microenvironment a multitude of HS-bound growth factors, cytokines, chemokines, and enzymes that affect cells and tissue functions [3–6]. Moreover, increased HPSE after exposure of tumor to chemo and radiation therapy implies the involvement of HPSE in drug resistance [7–10]. Noteworthy, HPSE has also been reported to play a fundamental role in other pathologies beside cancer, thus making this pharmacological target of great interest since it provides a broad range of potential applications. Particularly, the involvement of HPSE has been documented in various inflammatory disorders, such as rheumatoid arthritis, hepatitis C infection, chronic and acute pancreatitis, Barrett's esophagus, Crohn's disease and ulcerative colitis, sepsis, and others [4, 5]. Some of these diseases are typically implicated in the initiation of several cancer types in the GI tract, pancreas, liver, and other tissues, thus further highlighting the role of HPSE as a molecule linking between inflammation and cancer.

The precise mode of HPSE action in inflammatory reactions is complex, and its involvement with various inflammation mediators/players has been nicely reviewed by several authors [2, 4, 5, 11, 12]. A significant increase in the expression and enzymatic activity of HPSE has been reported in several inflammatory conditions, typically associated with degradation of HS and extensive remodeling of the ECM and consequently with activation of inflammatory cells and inflammation mediators, endothelial-mesenchymal transition (EMT), and fibrotic processes. In fact, HS enzymatic remodeling by HPSE can affect the architecture of the ECM and thereby facilitate recruitment, extravasation, and migration of lymphocytes toward inflammation sites, leading to activation of innate immune cells [4, 5, 13]. A change of the cellular microenvironment may result from this complex interaction when HPSE is overexpressed.

Considering that once inactivated there are no other molecules capable of performing the same function(s) of HPSE, it is evident that this enzyme may be an effective and attractive target for the development of new drugs [5]. However, despite the growing interest in the scientific community and the fact that several different pharmacological structures endowed with anti-HPSE activity have been identified as having anti-HPSE activity [4] (Chhabra and Ferro; Hammond and Dredge; Giannini et al., Chaps. 19, 22 and 23 in this volume), this target is however still largely unexploited. In fact, only 4 HS mimetic compounds, all inspired by a heparin-based scaffold, have successfully made their way to the clinic. Two of them, Pixatimod (PG545, Zucero Therapeutics, Australia) and Roneparstat (Leadiant Biosciences S.p.A), are still in active development.

21.2 Ronepartstat

Roneparstat (lab codes G4000, SST0001), a chemically modified 100% N-desulphated, N-reacetylated and 25% glycol-split heparin with very low anticoagulant activity,is a competitive HPSE Inhibitor with a molecular weight between 15,000 and 25,000 Da [14, 15].



roneparstat

Among anti-HPSE inhibitors belonging to this class of compounds, Roneparstat is the only example where a simple oxidation-reduction (redox) reaction allowed the introduction of biologically functional groups without altering the natural heparin-based scaffold and resulting in a prominent reduction of anticoagulant properties associated to a strong inhibition of HPSE activity. A study aimed at investigating the kinetics of HPSE inhibition, through dose-inhibition curves, confirmed the high potency of Roneparstat ($IC_{50} \approx 3$ nM) and highlighted a different behavior of the inhibitor depending upon its concentration, suggesting the existence of multiple protein-ligand interaction modes [16]. To the best of our knowledge, this is a unique and distinctive mechanism among this class of inhibitors; such peculiarity may explain the rather high potency of Roneparstat in inhibiting HPSE and, at the same time, multifaceted vs. a single target drug interaction modality may prospectively make resistance more unlikely to occur.

Roneparstat was shown to possess anti-proliferative and anti-angiogenic properties in vitro and in vivo resulting from HPSE inhibition and ECM rearrangement. Early findings suggest the disruption of the HPSE/syndecan 1 axis in Multiple Myeloma (MM) [17], but because the role of HPSE is complex, the molecular mechanisms involved in determining the effect of Roneparstat can be multiple. For example, Roneparstat role in counteracting the expression of certain EMT markers has been demonstrated in different types of diseases, either cancerous [18] or non-cancerous [13]. Additionally, Roneparstat was also shown to inhibit HPSE enhanced histone deacetylase activity in U266, MM.1S, and CAG human myeloma cells [19]. Furthermore, a pleiotropic role of Roneparstat relating its biological activity to multi-target inhibition of tyrosine kinases was also suggested [20]. These same authors speculated that Roneparstat activity might not be solely related to its potent heparanase inhibiting properties. In fact, glycol-split heparins may function as HS mimetics and inhibit the interaction between basic fibroblast growth factors (bFGF) and vascular endothelial growth factor (VEGF) with related receptors, exerting an antiangiogenic and antitumor effect as displayed by this class of compounds [21–24]. Biotinylated Roneparstat derivatives have been recently designed with the purpose of improving tumor targeting [25]. Roneparstat has been extensively studied in cancer both in preclinical models and in humans as well as in other preclinical settings of non-oncological indications. Here below, the most significant studies are reviewed.

21.3 Roneparstat Preclinical Studies in Multiple Myeloma (MM)

HPSE role in MM has been extensively characterized [26–29]. This prompted extensive investigations of HPSE inhibition via Roneparstat in this tumor type. Roneparstat showed a significant anti-myeloma effect in various murine models of MM, with a reduction of subcutaneous growth of different MM cell lines, when administered either alone or in combination with dexamethasone; additionally, a significant effect on tumor burden was observed when combined with Bortezomib or Melphalan. Specifically, six different models have been used in different experimental settings as detailed and referenced below (Tables 21.1a, 21.1b and 2). Five of these models (RPMI-8226, MM.1S, KMS-11, MM.1R, CAG HPSE) employed human cell lines in SCID mice, while MPC-11 is a murine cell line used in syngeneic Balb/c mice.

CAG HPSE cells are very aggressive human myeloma cells, transfected with the human HPSE gene resulting in cells expressing high levels of HPSE. These cells exhibit a highly aggressive phenotype. Following tail vein injectioin in mice, they home to and grow rapidly within bones, thereby mimicking the late stages of MM. In this model, tumor burden was evaluated by measurements of human immunoglobulin kappa protein levels in the mouse serum and by bioluminescence imaging [9]. Tables 21.1a and 21.1b summarize the findings of Roneparstat as a single agent in MM models.

Roneparstat at doses of 60 mg/kg/twice daily (subcutaneous injections) or 30/mg/kg/day (via osmotic pumps) in various MM in vivo models was able to inhibit tumor growth by 50% to 98%, assessed as tumor volume inhibition (TVI). In two models, human immunoglobulin light chain (K chain) production, a hallmark of MM this being a disease affecting plasma cells having increased immunoglobulin generation, was also very significantly inhibited in Roneparstat treated mice.

In all cell lines and models examined, Roneparstat inhibited tumor angiogenesis as determined by CD34 staining for vasculature. In the SCID-hu mouse model, Roneparstat delivered by osmotic pump at 30 mg/kg/day also inhibited the growth of CAG HPSE tumors in human fetal bone [17]. Roneparstat delivered subcutaneously (sc) twice a day (bid) at 60 mg/kg/day was also able to potently inhibit myeloma KMS-11 or RPMI-8226 when tumor fragments were implanted subcutaneously (Table 21.1a).

In addition to this direct anti-tumor effect, Roneparstat at a dose of 30 mg/kg/day given via osmotic pump for 28 days, significantly inhibited bone homing of highly aggressive CAG- HPSE homing to bone in SCID mice [17]. Li et al. [18] also showed that MM cell lines and primary myeloma cells from MM patients express several EMT markers (e.g., E-cadherin, Vimentin, Fibronectin, and RANKL) and that the expression of these markers is regulated by HSPE, thus associating these events to remodeling of the microenvironment that facilitates MM progression. By inhibiting HPSE, Roneparstat blocks HPSE induced mesenchymal features. A similar pattern has been described in the kidney, where HPSE is overexpressed after hypoxia and reoxygenation, leading to renal EMT activation [13], as well as in peritoneal [30] and liver [31] fibrosis.

Table 21.1a Roneparstat single agent (SCID mice – xenografts)

Model	Dose	Resuts		Reference
		TVI%	K chains % inhibition	
RPMI-8226 model, tumour cells injected sc	30 mg/kg/day	56	-	17
RPMI-8226 model, tumour cells injected sc	60 mg/kg/day twice daily	50	-	Leadiant Biosciences unpublished results
RPMI-8226 model, tumour fragments implanted sc	60 mg/kg/day twice daily	98	-	Leadiant Biosciences unpublished results
RPMI-8226, tumour fragments implanted sc	60 mg/kg twice daily	99	-	17
MM.IS model, tumour cells injected sc	30 mg/kg/day	50	-	17
CAG HPSE high model tumour cells injected sc	60 mg/kg/day twice daily	77	70	Leadiant Biosciences unpublished results
KMS-11 model, tumour cells injected sc	60 mg/kg twice daily	98	-	17
SCID hu model CAG HPSE, tumour cells injected directly in human fetal femora sc implant	30 mg/kg/day	Tumour burden reduction by bioluminescence assay	85	17

TVI: tumor volume inhibition; SCID: Severe Combined Immunodeficiency; sc: subcutaneous; Roneparstat was used at doses of 60 mg/kg/twice daily (sub cutaneous injections) or 30/mg/kg/day (via osmotic pumps); k: kappa protein serum levels

Table 21.1b Roneparstat single agent (immunocompetent mice)

		Results		**Reference**
Model	**Dose**	**TVI %**	**K chains % inhibition**	
Syngeneic (MPC-11) model cells injected sc	**30 mg/kg/day**	**61**	-	**17**

TVI: tumor volume inhibition; sc: subcutaneous; Roneparstat was applied at doses of 30/mg/kg/day delivered via mini-osmotic pumps

Table 21.2 Roneparstat in combination regimens

Model	Dose	Results		Reference
		TVI%	K chains % inhibition	
MM.1R model, tumour cells injected sc SCID mice	60 mg/kg/day + dexamethasone 1 mg/kg/day	80	-	17
Syngeneic (MPC-11) model cells injected sc Balb/c mice	60 mg/kg/day + dexamethasone 1 mg/kg/day	97	-	17
CAG HPSE high cells model Cells intravenously injected in mouse tail veins SCID mice	120 mg/kg/day + bortezomib 0.5 mg/kg/twice a week	75%-80% (Tumour burden reduction by bioluminescence assay)	70 (only 3/10 animals had detectable levels of serum κ)	9
CAG HPSE high cells model Cells intravenously injected in mouse tail veins SCID mice	60 mg/kg/day + melphalan 1 mg/kg/week	90%-95% (Tumour burden reduction by bioluminescence assay)	100	9

TVI: tumor volume inhibition; SCID: Severe Combined Immunodeficiency; sc: subcutaneous; Roneparstat was used at doses of 60 and 120 mg/kg/ daily by subcutaneous injections; k: kappa protein serum levels

Table 21.2 summarizes the findings of Roneparstat in MM models in combination with other anti-myeloma drugs. In fact, further to its efficacy when administered as a single agent, Roneparstat did show a very good activity when combined with dexamethasone [17]. Roneparstat (60 mg/kg/day for 14 days) and dexamethasone (1 mg/kg/day for 14 days) combination therapy was tested against subcutaneous myeloma tumor growth in SCID mice (using human dexamethasone resistant MM.1R Myeloma cells) and in Balb/c mice (using murine MPC-11 Myeloma cells), thereby representing drug-resistant and immuno-competent models of myeloma, respectively. In both settings, the combination therapy significantly inhibited tumor growth more effectively than single-agent therapy alone. In the drug-resistant MM.1R model, combination therapy inhibited tumor growth by 80% and in the syngeneic model, combination therapy inhibited tumor growth by 97%. In both cases, assessment of the combination of Roneparstat and dexamethasone revealed a synergistic effect in inhibiting myeloma tumor growth [17].

A preclinical in vivo combination experiment was conducted in CAG HPSE xenografts [9]. Tumor burden was evaluated by measurements of human kappa-levels and bioluminescence after combination treatment with Roneparstat (120 mg/kg/day for 14 days) plus bortezomib (0.5 mg/kg/twice a week for 14 days) or Roneparstat (60 mg/kg/day for 14 days) plus melphalan (1 mg/kg/week for 14 days). Results showed that Bortezomib and Melphalan efficacy in tumor inhibition was increased when they were combined with Roneparstat. More specifically, Bortezomib and Roneparstat combination therapy resulted in 70% of the treated animals exhibiting no detectable tumor, while only 30% of the mice had no detect-

able levels of K chains when the agents were administered separately [9]. Similarly, Melphalan and Roneparstat combination therapy resulted in 100% of the animals exhibiting no detectable tumor. While only 30% and 11% of the animals, respectively, had no detectable tumor when treated with Melphalan or Roneparstat alone [9]. This was evident both from kappa-levels and bioluminescence imaging data; a decrease of the latter always parallelled a decrease in the former.

Notably, this increased efficacy was also shown when Roneparstat was given as sequential therapy after Melphalan (Fig. 21.1) [9]. In fact, after establishing tumor dissemination one week after HPSE-high cell injection in SCID mice, treatment with Melphalan alone (2.5 mg/kg/week) led to 4/11 cancer-free animals, while the sequential treatment of Melphalan (2.5 mg/kg/week) for two weeks followed by one week of by Roneparstat (120 mg/kg/day) yielded 7/11 cancer-free animals, a cure rate not too dissimilar from the 10/11 cancer-free mice obtained with the simultaneous administration of Melphalan (2.5 mg/kg/week) and Roneparstat (60 mg/kg/day) for two weeks.

This opens several potential options, as the possibility to apply a microenvironment modulating anti-HPSE drug in between cycles of treatment to stabilize and enforce the effect of anti-cancer drugs or to use Roneparstat in maintenance therapy regimens. Moreover, positive data of Roneparstat efficacy when combined with Bortezomib or Melphalan are supported by the fact that chemotherapies are known to increase HPSE expression [8].

Finally, the synergistic effect of Roneparstat with bortezomib and melphalan also indicates their potential use in amyloidosis, even if the role of HPSE is disputed [32, 33]. Very preliminary findings suggest that Roneparstat does not worsen amyloid fibril deposition, as one would expect if HPSE exerts a protective role in

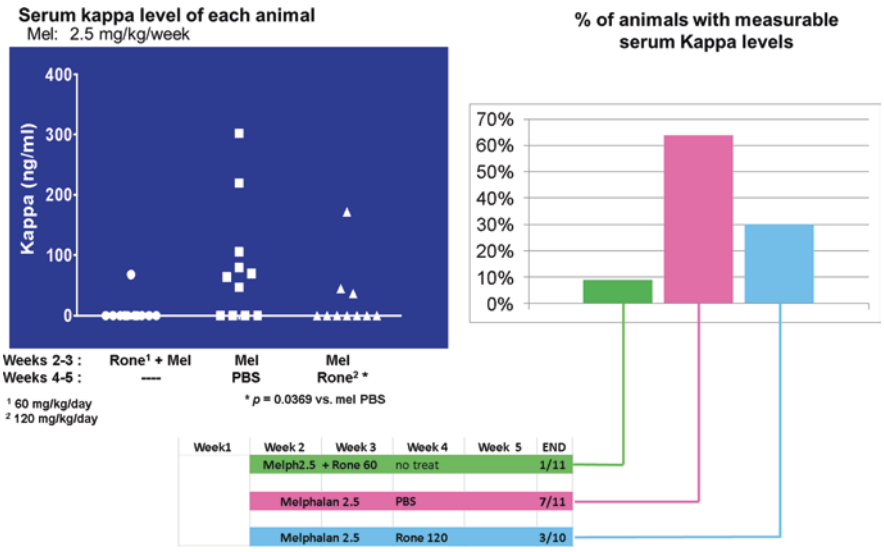


Fig. 21.1 Anti-myeloma effect of combined and sequential use of Roneparstat with Melphalan

amyloidogenesis (Li and Zhang, Chap. 25 in this volume). Therefore, the use of Roneparstat in combination with bortezomib and melphalan, that together with dexamethasone are normally used in the treatment of primary AL-amyloidosis [34, 35] is conceivable.

21.4 Roneparstat Preclinical Studies in Other Cancers

Lymphomas Roneparstat showed activity in tumors other than MM. An antitumor effect was reported in lymphomas, more precisely in the SU-DHL preclinical model of aggressive diffuse-large B cell lymphoma (DLBCL), when given alone (60 mg/kg/twice daily, 5 days/week for 3–4 weeks) or in combination with Cyclophosphamide, Rituximab or Bevacizumab [36]. Similar to what was documented in MM, the effect seems to be mediated via modulation of the tumor microenvironment.

Sarcomas Sarcomas have been extensively investigated. Roneparstat (0.5 and 1 mg/mL) inhibited TC71 Ewing sarcoma cell invasion in a Matrigel assay. Similar inhibition was obtained with rhabdomyosarcoma RD, A204, and RH30 sarcoma cells [20]. This finding was paralleled by a 90% tumor weight inhibition by Roneparstat at 60 mg/kg, s.c., twice daily for 23 consecutive days starting on day 1 after tumor inoculation [37]. Roneparstat (1 mg/mL) reduced the release in vitro of factors involved in angiogenesis and tumor progression by various sarcoma cell lines such as TC71 (Ewing Sarcoma), U20S (osteosarcoma), RD (embryonal rhabdomyosarcoma), and 204 (rhabdomyosarcoma). Among these factors are VEGF, VEGF-C, PDGF-AA, ribonuclease angiogenin (ANG), endothelin-1 (ET-1), endocrine gland derived-VEGF (EG-VEGF), and proteins directly involved in organization and remodeling of the ECM such as MMP-9, pentraxin (PTX3) and uPA [22]. Roneparstat (60 mg/kg twice daily sc for 5 days/week for 4 weeks) exerted a TVI ranging between 64% and 95% in the same sarcoma models implanted in nude or SCID mice. Ewing sarcoma TC71 xenografts were particularly sensitive to Roneparstat, with almost 20% of the animals being free of detectable cancer. This same model was used for combination studies which demonstreated that Roneparstat (60 mg/kg twice daily sc for 5 days/week for 5–6 weeks) combined with bevacizumab (4 mg/kg i.v. every 4 days for 8 total injections) or sunitinib (40 mg/kg, oral, daily for 5 days/week for 4 weeks) strongly improved the efficacy vs. single agent activity [23].

A strong inhibitory effect of Roneparstat (60 mg/kg twice daily sc for 6 days/week for 4–6 weeks), was reported in human A204 rhabdoid sarcoma xenograft, especially when combined with 50 mg/kg/day Irinotecan [20]. With this well-tolerated combination, TVI reached 100%, with 8/8 animals experiencing complete remission. Only three tumors regrew over a 50-day observation period following the last administration. Interestingly, these authors expanded their observations on the mechanistic aspects and demonstrated an effect of Roneparstat and supersulfated low molecular weight heparin [21] in vitro and in vivo on the

function of co-expressed receptor tyrosine kinases (FGF, ERBB, PDGF, IGF) in several sarcoma models (human A204 rhabdoid sarcoma, U20S osteosarcoma, SK-N-MC and TC71 Ewing sarcoma). This suggests that the anti-HPSE activity may cooperate with RTK expression inhibition in generating an anti-tumor activity [21, 23] (see also Cassinelli and Lanzi, Chap. 15 in this volume).

Other Solid Tumors An antimetastatic activity was shown in pancreatic and breast cancer as well as in melanoma models. More specifically, Roneparstat antimetastatic activity was observed, when delivered intraperitoneally twice a day at 30 mg/kg/day for 3 weeks, to significantly inhibit Panc02 pancreas primary orthotopic tumor growth in C57BL/6 J immunocompetent mice and to decrease the number of mesenteric lymph node metastases (Leadiant Biosciences, unpublished results). Panc02 expressed elevated levels of endogenous HPSE as compared to normal pancreatic tissue (Leadiant Biosciences, unpublished results). The antimetastatic activity of Roneparstat was observed in B16/BL6 murine melanoma cells injected iv in syngeneic C57BL/6 mice. A single dose of Roneparstat at 60 mg/kg/day sc inhibited the number of lung metastases by 63% [38]. Moreover, Roneparstat (60 mg/kg/day bid sc for 28 days) was shown to inhibit bone metastases induced by an intracardiac injection of MDA-MB231 breast carcinoma in BALB/c mice (Leadiant Biosciences, unpublished results). Also, continuous administration of 30 mg/kg/day Roneparstat via osmotic pumps has shown activity in Lapatinib resistant breast cancer brain metastasis [39].

On the assumption that irradiation stimulates HPSE expression by Egr1 transcription factor, thus counteracting the benefits of radiotherapy, Roneparstat (2 μg/mL) abolished the invasion of pancreatic cancer PANC1 cells, stimulated by irradiation with 10Gy. This paralleled in vivo results, where 0.6 mg/day/mouse Roneparstat given i.p for seven days prevented the HPSE mediated reaction to irradiation and enhanced the effect of 10Gy irradiation in SCID mice orthotopically injected with PANC1-LUC pancreatic cancer cells [7].

21.5 Roneparstat in Other Disease Models

Roneparstat has been studied in several other preclinical models of different diseases, which are discussed here below.

Kidney Disease and Failure Masola et al. [13] showed that Roneparstat prevented hypoxia/re-oxygenation induced EMT, thus showing a potential effect on acute renal failure of which ischemia and reperfusion (I/R) can be a major cause. More specifically, I/R induces HPSE overexpression and consequent EMT activation. Notably, HK2 human tubular cells showed an increase in α smooth muscle actin, vimentin, and fibronectin in response to HPSE overexpression, while cells in which HPSE is silenced and where HPSE is not induced after I/R, failed to exhibit this effect [13]. Similarly, in vivo, HPSE overexpressing Balb/c mice (HPA-tg mice) show an increased expression of HPSE after I/R while wild type (WT) mice do not. This was paralleled by augmented expression of α smooth muscle actin and vimentin in HPA-tg mice only. I/R caused histopathological damage in all animals, but to

a much greater extent in HPA-tg mice. Exposure of HK-2 cells to 200 μg/mL of Roneparstat either before 24 h hypoxia or prior to 24 h reoxygenation down-regulated HPSE expression and counteracted EMT marker activation [13, 40] (Masola et al., Abassi and Goligorsky, Chaps. 27 and 28 in this volume). In more recent papers the same authors further explored this from a mechanistic point of view and extending it to chronic kidney dysfunction. They elegantly showed that HPSE plays a pivotal role in the regulation of renal inflammation by modulating macrophage polarization, the crosstalk between renal tubular cells and macrophages, and tissue damage after I/R. Moreover, they showed that Roneparstat reduced the expression of M1 macrophage markers without influencing M2 macrophage polarization in U 937 cells [41]. This observation was confirmed in vivo in a model of I/R using C57BL mice, following a 30-minute clamping of the left renal artery. Roneparstat (0.6 mg/day/mouse) significantly reduced the infiltration of M1 macrophages, the production of proinflammatory and profibrotic cytokines, while maintaining basal TLR expression levels, and preventing I/R-induced tubular cell apoptosis after 48 hours and seven days [40]. These findings were confirmed by the ameliorated renal functions (BUN, creatinine, and histology) in Roneparstat treated I/R mice, thus suggesting that better organ recovery and the prevention of fibrosis could be achieved. The authors highlighted that the specific Roneparstat reduction of the M1 but not M2 component is of great interest because it could provide a new strategy to control the initial inflammation that causes tissue damage, without inhibiting M2 macrophages that facilitate repair [41] (Masola et al., Abassi and Goligorsky, Chaps. 27 and 28 in this volume). This was confirmed by the same authors [40] assessing Roneparstat impact on the expression of specific genes associated with M1 polarization in human renal proximal tubular HK-2 cells and U 937 monocytes as well as in an in vivo I/R model. Evidence demonstrating the ability of Roneparstat to protect the kidney from chronic damage and fibrosis induced by I/R was also produced. Roneparstat (0.6 mg/day and 1.2 mg/day for 8 weeks) administered after 30 min renal left artery clamping in C57BL mice was found to reduce tubular atrophy and interstitial fibrosis. It also ameliorated renal function, EMT, inflammation and oxidative stress [40]. Notably, these results were obtained at doses comparable to those proven to be active in several other models as well as to the range tested in humans, yielding measurable drug exposure with good tolerability (Masola et al., Chap. 27 in this volume).

Gil et al. [42] reported that Roneparstat markedly decreased the extent of albuminuria and renal damage in animals affected by diabetic nephropathy. In streptozocin induced diabetic Balb/c mice, treatment with 0.6 mg/day/mouse for 12 weeks, halved the 24 h albumin excretion increase, and prevented the increase of serum creatinine and blood urea levels. The effect on 24 h albumin increase was also confirmed in another experimental setting using streptozocin induced diabetic DBA2 mice receiving 0.6 mg/day/mouse of Roneparstat [42].

Fibrotic Diseases Masola et al. reviewed the role of HPSE in fibrosis [30, 31]. These authors showed that glucose-induced gene and protein upregulation of VEGF and EMT and mesothelial-mesenchymal- transition (MMT) markers were prevented by Roneparstat, restoring the normal trans-epithelial resistance and

permeability [30]. More specifically, exposure of rat peritoneal mesothelial cells (RPMC) to 50–200 mmol glucose for six days causes an increase in MMT markers as α smooth muscle actin, vimentin, fibronectin, E-cadherin and filamentous actin. Treatment with 10–200 μg/mL Roneparstat on day 3 reverted these effects without inducing any cytotoxic effect. Moreover, mesothelial cells exposed to a peritoneal dialysis solution, that is known to induce long term peritoneal fibrosis and lower permeability, suffered from the same MMT marker alteration, which was completely reverted by Roneparstat (Masola et al., Chap. 27 in this volume).

Secchi et al. [31] discussed the interplay between HPSE and liver fibrosis and used hepatic stellate cells LX-2, exposed to conditioned medium obtained from the U937 macrophages incubated with or without TNF-α. In this model, Roneparstat reduced the expression of α smooth muscle actin and fibronectin without affecting VGEF and collagen expression, thus suggesting a role of HPSE inhibition and Roneparstat in counteracting profibrotic events. These data are consistent with those reported in MM for the same markers [18].

Acute Pancreatitis In a BALB/c mouse model of cerulein-induced acute pancreatitis, HPSE has been shown to influence a number of key deleterious determinants, namely the induction of lipase and amylase, increased tissue edema, recruitment of neutrophils, induction of cytokines (i.e. TNFα, IL-6), activation of NFκB and STAT3 signaling as well as the pancreatic index [43]. Notably, all were significantly diminished by the treatment with 1 mg/mouse of Roneparstat given 30 minutes and 24 h prior to cerulein administration. The same paper reports a comparable protective effect with another HPSE inhibitor, Pixatomid, given at 0.4 mg/mouse 2 and 24 hours before cerulein (Khamaysi et al., Chap. 29 in this volume).

Bone diseases Roneparstat (2–200 mg/mL) reduced cartilage nodule formation in micromass culture as well as the micromass diameter after 4 and 6 days of incubation [44]. Micromass cultures were prepared from the mesenchymal cells of embryonic limb buds. Expression level analysis of the chondrogenic genes collagen II, aggrecan, and Runx2 showed a significant reduction in Roneparstat treated micromasses at the same time points. A dose-dependent effect was observed in almost all parameters.

HPSE is thus supposed to play a role in mobilizing chondrogenic factors and enhancing their bioavailability and diffusion among condensed prechondrogenic cells. Its effective suppression via Roneparstat would hamper this process and elicit a strong anti-chondrogenic effect, whereas chondrogenesis may be altered as in human exostosis [44].

21.6 Clinical Experience with Roneparstat

Roneparstat has completed a phase I, multicenter, international trial in patients with advanced MM which has been fully reported recently [45]. Nineteen patients with advanced relapsed/refractory MM were enrolled into the study and completed a

total of 57 cycles (514 doses), with a median of 2 cycles (range 1–11) and four patients receiving >5 cycles. Roneparstat was well tolerated and safe at all doses tested. Reproducible plasma levels of Roneparstat were measurable at the two highest dose levels, as shown in Fig. 21.2 depicting the mean patient plasma concentrations at day 1 and day 12 (single and repeat dosing, respectively) of the first therapy cycle. There was a dose-related increase in mean Cmax between 200 mg/day and 400 mg/day doses, both on day 1 (1.67 μg/mL vs 2.45 μg/mL) and day 12 (2.07 μg/mL vs. 5.95 μg/mL). The mean exposure (AUC0-t) at day 1 after repeated dosing was 16.2 μg.h/mL and 37.25 μg.h/mL, while at day 12 it was 15.4 μg.h/mL and 133 μg.h/mL for 200 mg and 400 mg/day doses, respectively.

Upon repeated dosing, tmax was achieved at approximately 3 h post-dose. On Day 12 and at 400 mg/day, the estimated T1/2 was approximately 14–20 h (2 patients).

Seventeen patients that received at least one cycle of Roneparstat were evaluated for overall response assessment. One partial response (PR) (5.9%) and 9 stabilizations of disease (SD) (52.9%) were observed. The remaining patients (41.2%) presented disease progression (PD). The PR occurred in a patient receiving 50 mg Roneparstat, who relapsed after three prior therapy lines with a continuous increase of the monoclonal component. The response was characterized by a rapid decrease of the monoclonal component, from 1.75 g/dL at baseline to 0.99 g/dL at cycle 1, and 0.71 g/dL at cycle 6. The patient remained on therapy until cycle 9 with sustained clinical benefit. Two of the 9 SDs were sustained with significant clinical benefit (10 and 7 months) following 200 mg and 400 mg of Roneparstat. Notably, the PR and one prolonged SD patients received a low dose of concomitant dexamethasone (up to 40 mg/week), while the other prolonged SD did not receive any dexamethasone.

The clinical data show that Roneparstat presents an excellent safety profile, without clinically relevant systemic reactions, and an excellent tolerability profile. Systemic exposure appears measurable in a reproducible and linear fashion at 200 and 400 mg. This study allowed identification of doses within the range from 300 to 400 mg/day as suitable for further development of the drug [45]. Far from being conclusive, because efficacy was not among the primary scope of this phase I trial, these data combined with the extensive preclinical evidence on the ability of HPSE inhibition to influence the bone marrow microenvironment in myeloma patients, and the synergistic effect of Roneparstat when combined with Bortezomib or Melphalan, suggest the possibility to capitalize and improve the role of HPSE inhibition in myeloma treatment.

21.7 Conclusions

HPSE has been extensively studied, and the interest in this important enzyme continues to increase. The interplay between HPSE and several molecular mechanisms involved in microenvironment modulation and affecting cell growth and

progression, inflammation and fibrosis have become quite clear. Given the complex fine-tuning and the modulatory/adaptative role that HPSE may exert, HPSE inhibition may not be resolutive. Therefore, HPSE inhibition may well be perceived as a complementary target that could be associated with other drug therapies. Thus, proper combination regimens with anti-HPSE agents have been and still should be considered. All this contributed to the increasingly growing interest on the discovery and speculative scientific side, which however has not yet been paralleled by a similar push in the medical and drug development community. The development of anti-HPSE agents looks fascinating but difficult, considering the multifaceted cascade that HPSE inhibition can lead to. However, HPSE inhibition is associated to solid efficacy data in different indications, even though some at a preclinical level, optimal combination regimens, and good clinical safety and Tolerability profile. This should endorse further exploitation of anti-HPSE agents and the validation of this target in the clinic.

Furthermore, the molecular interaction between the enzyme and possible inhibitors has been further characterized [4, 23] leading to the design of new HPSE inhibitors [25, 46–50] including non-HS mimetics (natural, semi-synthetic, and rationally designed small molecules) (Giannini et al., Chap. 23 in this volume). This may expand the number of prospective candidates and options to further explore this target.

Roneparstat is a very potent and probably the most widely studied anti-HPSE agent. Studies have been conducted in several disease settings and experimental models suggesting a potential therapeutic role in various indications either alone or in combination regimens. An important share of the data generated is focused on its potential in treating cancer, particularly in MM, possibly via the modulation of the microenvironment, mediated by Roneparstat anti-HPSE activity. However, several authors have collected sound evidence about its efficacy in many disorders, with kidney failure as probably the largest non-cancer indication explored. Moreover, Roneparstat role in modulating immunological response, fibrosis, EMT and MMT has also emerged quite clearly, thus enlarging the potential therapeutic applications worth being considered. The clinical experience conducted so far does not allow any definite conclusion on the efficacy of Roneparstat. However, the phase I data suggest that Roneparstat is extremely safe and well tolerated and that provides reproducible and linear drug exposure at levels that are consistent with activities seen in preclinical models. These are fundamental pre-requisites for further clinical development, independently of the indication.

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Chapter 22
Heparanase Inhibition by Pixatimod (PG545): Basic Aspects and Future Perspectives

Edward Hammond and Keith Dredge

22.1 Introduction

Pixatimod is a high-affinity inhibitor of heparanase, the master regulator of the extracellular environment through its enzymatic cleavage of heparan sulfate (HS) and its non-enzymatic signaling functions. Pixatimod, formerly known as PG545, became the lead clinical candidate from the PG500 series, first developed by Progen Pharmaceuticals a decade ago. Many of these compounds showed potent anti-cancer activity in vitro and in vivo but pixatimod was selected for further development due to its enhanced activity and improved pharmacokinetics. In addition, pixatimod also targets other proteins, such as HS-binding growth factors. Numerous studies have been published describing the biological effects of pixatimod. Here, a brief summary is presented of its activity demonstrated using in vitro systems, prior to a discussion about the in vivo data supporting the utility of pixatimod against cancer. Finally, we will provide an update of the compound's clinical status and future directions.

22.2 Targets of Pixatimod

There has been a wealth of data published about the biological activities of pixatimod. With its sulfated oligosaccharide moiety, the molecule has HS-like properties (Fig. 22.1) and is a potent heparanase inhibitor with a K_i of 6 nM [1]. Detailed examination of the inhibition kinetics of heparanase revealed that pixatimod has a

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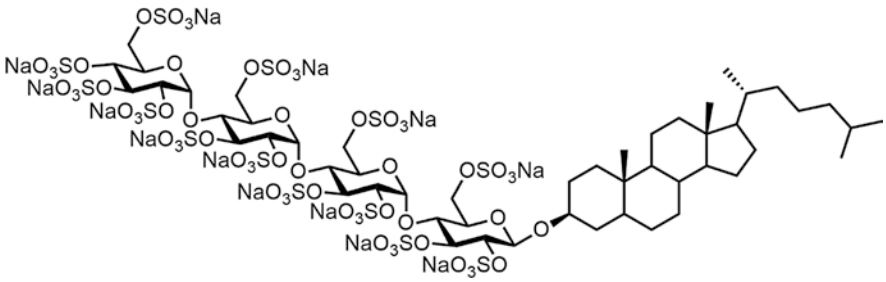

Fig. 22.1 Structure of pixatimod

higher affinity for heparanase than an analog comprising just the sulfated oligosaccharide minus the cholestanol moiety [2]. Further analysis showed that pixatimod is a parabolic competitive inhibitor of this enzyme: such an inhibitor binds at multiple inhibitory sites and the initial interaction increases affinity for subsequent binding [2]. This increased affinity and unusual binding mode is conferred on pixatimod by the cholestanol moiety because these traits are absent from the oligosaccharide analog without cholestanol.

Pixatimod, because it possesses HS-mimetic properties, is also a potent inhibitor of HS interactions with a number of HS-binding growth factors and signaling proteins. These proteins include VEGF, FGF2, FGF1, HB-EGF and Wnt proteins [1, 3, 4]. Because many of these proteins require or benefit from, HS-binding as part of their signaling interaction [5–8], by inhibiting HS-binding pixatimod blocks the relevant signaling pathway [4]. Nevertheless, there is compelling evidence that much of pixatimod's anti-cancer activity is related to heparanase inhibition [9–11].

22.3 In Vitro Activity

Studies of the biological properties of pixatimod in cellular systems have shown it to have a range of anti-cancer effects. Pixatimod reduced the proliferation rates of a number of cell lines in vitro including human cancer cell lines [4, 12], mouse cancer cell lines [12, 13] and human umbilical vein endothelial cells [1]. It has also demonstrated an ability to inhibit the invasion and migration of various cell types through artificial matrices resembling the extracellular matrix (ECM, [4, 14–16]). These activities are likely related to inhibition of heparanase, but also the inhibition of growth factor signaling. It should be remembered that heparanase, through its enzymatic activity, can significantly promote the signaling of growth factors and heparanase itself has also been shown to have signaling functionality which is independent of its enzymatic activity [17, 18] making it difficult to resolve individual roles in some studies.

Several studies have also made the connection between pixatimod treatment and induction of apoptosis in cancer cells [3, 19, 20]. Typically, induction of apoptosis was assessed by either (1) staining with Annexin V (for phosphatidylserine) and propidium iodide and quantifying with flow cytometry, or (2) measuring intracellular markers of apoptosis, such as the cleaved versions of caspase 3, caspase 8 and PARP. Heparanase has been shown to promote active cell growth and apoptosis avoidance by reducing nuclear syndecan-1 and, thus, modulating histone acetyltransferase (HAT) activity [21, 22]. Therefore, the apoptotic activity of pixatimod could be dependent upon its inhibition of heparanase. Indeed, other heparanase inhibitors have also been shown to induce apoptosis [23]. However, it is also possible that pixatimod is exerting this activity by inhibiting Wnt signaling which can also suppress apoptosis [24–26]. In support of this latter mechanism, Weissmann et al. [20] demonstrated that pixatimod-driven apoptosis appears to be independent of heparanase, at least in the lymphoma cell lines assessed.

Pixatimod treatment of cancer cells in vitro and tumor-bearing mice also induces metabolic changes, including the suppression of the glycolytic phenotype commonly found in cancer cells by reducing expression of glycolytic enzymes and impairing glucose uptake in vivo [27]. Additionally, it has been linked to autophagy although its role remains unclear with conflicting reports [10, 20, 28].

22.4 In Vivo Activity

Pixatimod has been tested for anti-cancer activity using a large number of mouse cancer models. Summary tables have been prepared of the published xenograft models (Table 22.1), syngeneic models (Table 22.2) and models treated with pixatimod and a clinically relevant agent in combination (Table 22.3). This body of work comprises approximately 50 individual models – about 30 xenografts and about 20 syngeneic models – across 13 cancer disease types (Fig. 22.2). These range from hematological malignancies (lymphoma) to cancers heavily driven by environmental factors (lung, mesothelioma, skin) to those with a genetic driver (ovarian, breast) and cancers linked to infectious agents (liver). There are models of most of the major organs, including some of the most difficult to treat cancers such as pancreatic and glioma, and the six types responsible for the most cancer deaths in the USA: lung, colon, pancreatic, breast, liver and prostate [29].

During early in vivo studies, pixatimod was administered daily at relatively low doses (for example 5 mg/kg in mice). Once pharmacokinetic data became available and it became apparent that at this dosing frequency pixatimod would be accumulating, administration regimes were changed to either weekly or twice weekly, typically at dosing levels around 20 mg/kg. After the first clinical trial of pixatimod, administration for patients was changed from subcutaneous (SC) injection to intravenous (IV) infusion prompting the use of systemic modes of delivery, such as IV and intraperitoneally (IP) injections, in pre-clinical studies. See the Pixatimod Dose column of Tables 22.1–22.3 for specific details.

Table 22.1 Xenograft cancer models in which pixatimod has shown activity

Study	Description	Tumor model	Pixatimod dose	Significant findings
[1]	Xen (SC)	Colon (HT-29)	SC, 5 mg/kg/d 5-FU 75 mg/kg/Wk	↓ tumor growth
[30]	Xen (SC)	Breast (MDA-MB-231)	SC, 20 mg/kg/Wk and 2xWk 30 mg/kg/Wk and 2xWk	↓ tumor growth
[30]	Xen (SC)	Prostate (PC3)	SC, 20 mg/kg/Wk 20 mg/kg/2xWk	↓ tumor growth
[30]	Xen (SC)	Liver (HepG2)	SC, 20 mg/kg/Wk 20 mg/kg/2xWk	↓ tumor growth
[30]	Xen (SC)	Head and neck (Cal27)	SC, 20 mg/kg/Wk	↓ tumor growth
[30]	Xen (spl)	Colon (HT-29)	SC, 20 mg/kg/Wk	↓ tumor growth and metastases
[12]	Xen (IP)	Pancreatic (AsPC-1)	IP, 10 (5) mg/kg/Wk	↑ survival
[12]	Xen (orth)	Pancreatic (MiaPaca-2)	IP, 5 mg/kg/2xWk	↓ tumor growth and metastases
[4]	Xen (SC)	Ovarian (A2780)	SC, 20 mg/kg/Wk IV, 15 mg/kg/Wk IV, 7.5 mg/kg/2xWk	↓ tumor growth at all doses
[10]	Xen (SC)	Glioma (U87, OX heparanase)	SC, 20 mg/kg/Wk	↓ tumor growth
[27]	Xen (IP)	Ovarian (OV202, UX HSulf-1)	IP, 20 mg/kg/2xWk	↓ tumor growth
[11]	Xen (SC)	Lymphoma (Ramos, Raji, Daudi, SU-DHL-6, OCI-LY-19)	IP, 20 mg/kg/Wk	↓ tumor growth in all models
[11]	Xen (IV)	Lymphoma (Raji)	IP, 20 mg/kg/Wk	↓ bone marrow colonisation
[31]	Xen (SC)	Colon (SW480)	IP, 20 mg/kg/Wk	↓ tumor growth
[19]	Xen (SC)	sPNET (PFSK-1) Medulloblastoma (D283)	IP, 20 mg/kg/Wk	↓ tumor growth
[16]	Xen (SC)	Lung (HCC-827)	IP, 20 mg/kg/Wk	↓ tumor growth
[16]	Xen (SC)	Lung (A549)	IP, 20 mg/kg/Wk	↓ tumor growth
[16]	Xen (SC)	Lung (HTB-182)	IP, 20 mg/kg/Wk	↓ tumor growth
[16]	Xen (SC)	Lung (nine PDX)	IP, 20 mg/kg/Wk	↓ tumors in 8 of the 9 PDX
[16]	Xen (SC)	Lung (two PDX)	IP, 20 mg/kg/Wk	↓ metastasis in 2 PDX that spontaneously metastasised
[15]	Xen (SC)	Mesothelioma (MSTO-211H)	IP, 0.4 mg/mouse/Wk cisplatin 3 mg/kg/2Wk	↓ tumor growth versus cisplatin
[15]	Xen (IP)	Mesothelioma (MSTO-211H)	IP, 0.4 mg/mouse/Wk cisplatin 3 mg/kg/2Wk	↓ tumor growth and ↑ survival versus cisplatin
[15]	Xen (IP)	Mesothelioma (CD487)	IP, 0.4 mg/mouse/Wk cisplatin 3 mg/kg/2Wk	↓ tumor growth versus cisplatin
[15]	Xen (IP)	Mesothelioma (NCI-H2052)	IP, 0.4 mg/mouse/Wk	↓ tumor growth

IP, intraperitoneal; IV, intravenous; mg/kg/2xWk, mg per kg dosed twice weekly; mg/kg/2Wk, mg per kg dosed every two weeks; orth, orthotopic inoculation; OX, over expressing; PDX, patient-derived xenograft; SC, subcutaneous; spl, splenic inoculation; sPNET, supratentorial primitive neuroectodermal tumor; UX, under expressing; Xen, xenograft model. Where a second dose is given in brackets, this was administered later in the study, usually as a lower maintenance dose.

Table 22.2 Syngeneic cancer models in which pixatimod has shown activity

Study	Description	Tumor model	Pixatimod dose	Significant findings
[1]	Syn (met)	Melanoma (B16)	SC, 10 mg/kg/d	↓ metastases
[1]	Syn (SC)	Melanoma (B16)	SC, 15 mg/kg/d	↓ tumor growth
[30]	Syn (met)	Melanoma (B16)	SC, 20 mg/kg/2xWk	↓ metastases
[30]	Syn (SC)	Lung (LL/2)	SC, 20 mg/kg/Wk 40 mg/kg/Wk sorafenib 60 mg/kg/d	↓ tumor growth and metastases ↓ metastases versus sorafenib
[32]	Syn (orth)	Breast (4T1)	SC, 20 mg/kg/2xWk 25 mg/kg/2xWk sorafenib 60 mg/kg/d	↓ tumor growth ↓ metastases versus sorafenib
[32]	Syn (mast)	Breast (4T1)	SC, 20 (10) mg/kg/Wk sorafenib 60 mg/kg/d	↑ survival versus sorafenib ↓ metastases
[32]	Syn (mast)	Breast (4T1)	SC, 20 mg/kg/Wk	↑ survival
[12]	Syn (orth)	Pancreatic (Pan02)	IP, 5 mg/kg/2xWk	↓ tumor growth and metastases
[12]	Transgenic	Pancreatic (mPDAC)	IP, 5 mg/kg/2xWk	↑ survival, ↓ tumor growth and metastases
[9]	Chemical	Transgenic mice OX heparanase	IP, 0.4 mg/mouse/Wk	↓ the number of lesions, by approximately 12 fold
[4]	Syn (IP)	Ovarian (ID8)	IP, 20 mg/kg/Wk	↓ ascites formation and tumor growth
[33]	Syn (IV)	Lymphoma (A20)	SC, 20 mg/kg/Wk	↑ survival
[33]	Syn (SC)	Lymphoma (EL-4)	SC, 20 mg/kg/Wk	↑ survival
[11]	Syn (SC)	Lymphoma (ESb in NOD/SCID)	IP, 20 mg/kg/Wk	↑ survival
[13]	Syn (SC)	Glioma (GL261)	SC and IP, 20 mg/kg/Wk	↓ tumor growth (either SC or IP)
[31]	Syn (SC)	Colon (CT26)	IP, 20 mg/kg/Wk	↓ tumor growth
[31]	Genetic	Colon ($Apc^{Min/+}$)	IP, 20 mg/kg/Wk	↓ polyp number and size
[15]	Syn (SC)	Mesothelioma (AE17, AK7, RN5)	IP, 0.4 mg/mouse/Wk	↓ tumor growth in all models

IP, intraperitoneal; IV, intravenous; mast, mastectomy model; met, metastatic model; mg/kg/2xWk, mg per kg dosed twice weekly; orth, orthotopic inoculation; OX, over expressing; SC, subcutaneous; Syn, syngeneic model. Where a second dose is given in brackets, this was administered later in the study, usually as a lower maintenance dose.

Table 22.3 Cancer models in which pixatimod combined with a therapeutic agent has shown activity

Study	Description	Tumor model	Pixatimod dose	Significant findings
[30]	Xen (orth)	Liver (Hep3b2.1–7)	SC, 20 mg/kg/Wk sorafenib 30 mg/kg/d	Combination inhibited tumor growth. (Monotherapies reduced but significant.)
[3]	Xen (orth)	Pancreatic (AsPC-1)	IP, 20 mg/kg/2xWk gemcitabine combination	Pixatimod and combination inhibited tumor growth compared to control. Combination inhibited tumor growth compared to gemcitabine. Pixatimod and combination inhibited metastases compared to control.

(continued)

Table 22.3 (continued)

[4]	Xen (SC)	Ovarian (A2780)	SC, 20 mg/kg/2xWk paclitaxel combination	Pixatimod inhibited tumor growth versus control. Combination improved over paclitaxel and control.
[4]	Xen (SC)	Ovarian (SKOV3)	SC, 20 (10) mg/kg/Wk carboplatin combination	Pixatimod inhibited tumor growth versus control. Negligible benefit of carboplatin combination.
[4]	Xen (IP)	Ovarian (A2780)	SC, 20 mg/kg/2xWk cisplatin+paclitaxel combination	Pixatimod increased survival. Combination increased survival more than pixatimod or cis + pac.
[33]	Syn (IV)	Lymphoma (A20)	SC, 20 mg/kg/Wk cyclophosphamide combination	Combination led to 100% survival at 60 d in established disease (3 Wk).
[16]	Xen (SC)	Lung (PDX)	IP, 20 mg/kg/Wk cisplatin combination	Pixatimod overcame cisplatin resistance to reduce tumor growth but combination was no better than pixatimod alone.
[34]	Syn (orth)	Breast (4T1.2)	IP, 15 mg/kg/Wk anti-PD1 combination	Pixatimod and combination inhibited tumor growth.

IP, intraperitoneal; IV, intravenous; mg/kg/2xWk, mg per kg dosed twice weekly; orth, orthotopic inoculation; PDX, patient-derived xenograft; SC, subcutaneous; Syn, syngeneic model; Xen, xenograft model. Where a second dose is given in brackets, this was administered later in the study, usually as a lower maintenance dose.

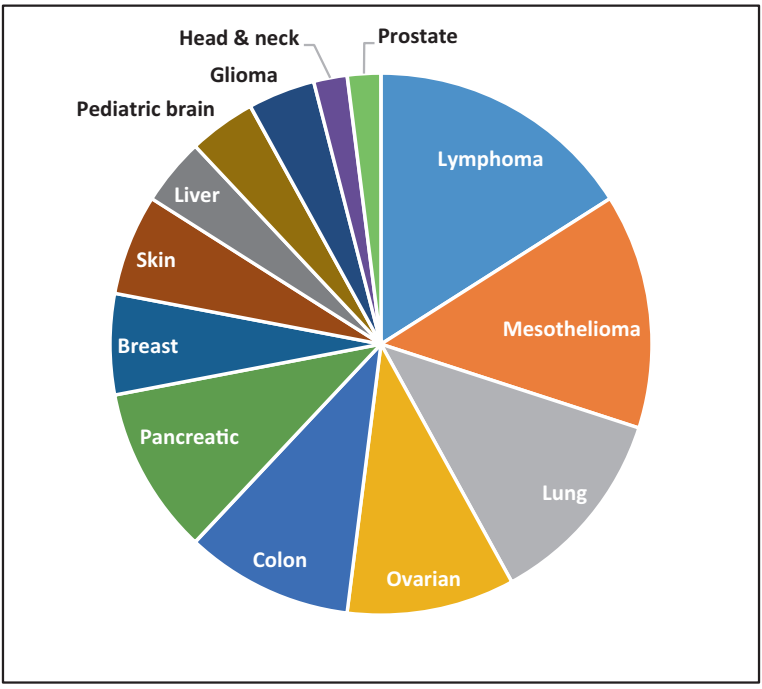

Fig. 22.2 Breakdown of pixatimod evaluation in mouse cancer models by cancer type. All studies, both xenograft and syngeneic, are included

22.4.1 Colorectal Cancer

Colorectal cancer (CRC) is one of the most important cancers facing healthcare services with an estimated 50,600 deaths in the USA resulting from it in 2018 ranking it as the second most deadly cancer [29]. Globally, the situation is similar with an estimated 861,700 deaths making it the second most deadly [35]. Despite a relatively high 5 year survival rate of 64.5%, ultimately prognosis is poor with most cases of metastatic CRC remaining incurable [36]. Given anti-angiogenic agents, such as bevacizumab, aflibercept and ramucirumab, have shown some effectiveness in CRC, the anti-angiogenic activity of pixatimod may also benefit these patients.

Pixatimod has been tested against five models of colorectal cancer, including three xenografts, one syngeneic model and a genetic model. Using models where human HT-29 cells were inoculated either subcutaneously [1] or into the spleen [30], pixatimod reduced the growth of primary tumors. The splenic HT-29 study takes advantage of a well-characterized model where HT-29 cells spontaneously metastasize from the spleen to the liver and colon [37, 38]. In this experiment, pixatimod dramatically reduced the number of colon metastases and abolished liver metastases, demonstrating the capacity of this compound to inhibit metastatic spread, most likely through its inhibition of heparanase.

A later study of colorectal and intestinal cancer studied the effects of pixatimod in an SW480 xenograft, a CT26 syngeneic model and the Apc Min/+ genetic model [31]. This group demonstrated that heparanase suppresses the cyclin-dependent kinase inhibitors p21 and p27 allowing cancer cell proliferation. Inhibition of tumor growth in the SW480 and CT26 models by pixatimod was associated with increased levels of p21 and p27 in the tumors resulting from heparanase blockade. The well characterized Apc min/+ mouse strain has a truncation of the Apc gene at codon 850 which results in the mice forming polyps in the small intestine [39, 40]. These polyps are rich in heparanase and, consequently, when these mice are treated with pixatimod the resulting number and size of polyps are significantly reduced. Analysis of the polyps revealed increased levels of p21 and p27 in polyps from pixatimod-treated mice compared to controls, further emphasizing the relationship between heparanase suppression of p21/p27 and subsequent reversal by pixatimod.

22.4.2 Pancreatic Cancer

Pancreatic cancer, typically, has very poor prognosis with a 5-year survival rate of only 8.5% in the USA, which is the lowest of the cancers monitored by the SEER Cancer Statistics Review [29]. This, combined with a relatively high incidence rate translates into an estimated 44,300 deaths in 2018, making it the third most deadly cancer in the USA [29]. Worldwide, the situation is similar with an estimated 458,900 cases and 432,200 deaths last year substantiating the magnitude of this disease [35]. Pancreatic ductal adenocarcinoma (PDAC) is the most prevalent form of pancreatic cancer, comprising 85% of cases [41], and is very difficult to treat with a 5-year survival rate of less than 5% [42].

The efficacy of pixatimod against a range of PDAC models has been examined by two research groups [3, 12]. These models range from a SC xenograft to orthotopically inoculated xenografts, an orthotopic syngeneic model and a transgenic model. Much of the focus of this work was to compare the effect of pixatimod alone or in combination with gemcitabine, which is one of the few effective treatments available for PDAC patients [42, 43].

Pixatimod increased survival of mice in an IP inoculated human AsPC-1 xenograft model compared to both the untreated control and the gemcitabine group [12]. In the transgenic mPDAC model, pixatimod significantly increased survival, reduced tumor size and reduced metastasis to the liver compared to controls but there was no significance compared to gemcitabine. This model uses a well-characterized genetically transformed mouse strain that has two modifications: (1) a mutated form of KRAS (G12D) which promotes intracellular signaling and cell proliferation and (2) deletion of the CDKN2A gene (coding for two proteins: Ink4a and Arf) which is involved in cyclin-dependent kinase regulation and has tumor-suppressing activity [44, 45]. Functionally similar mutations, to activate KRAS and inactivate CDKN2A, are very common in PDAC [43, 46]. The resulting mice produce pancreatic tumors at about 4 weeks which have pronounced similarities to PDAC in humans [44].

Given the clinical relevance of this model, a detailed analysis of the effects of pixatimod on the tumor and ECM was conducted. Pixatimod-treated mPDAC tumors showed signs of reduced cell proliferation, as demonstrated by reduced staining of phosphorylated histone H3, and increased apoptosis, shown by increased cleaved caspase-3 [12]. It also affected vascular function in mPDAC tumors with reduced microvessel density (endomucin staining) and reduced levels of phosphorylated histone H3 in endothelial cells. Furthermore, pixatimod treatment also inhibited both ECM remodeling (collagen deposition) and epithelial-mesenchymal transition (EMT) as demonstrated by increased Ecad and reduced vimentin in tumors. These responses, including EMT [47, 48], are consistent with pixatimod inhibition of heparanase.

Analysis of the tumors in other PDAC models, such as the xenograft MiaPaca-2 and syngeneic Pan02, showed similar responses to those observed in the mPDAC model. Pixatimod had similar anti-tumor and anti-metastatic efficacy in these two models. Importantly, in addition to showing significant reductions in tumor weight and liver metastases compared to untreated controls, these reductions were also significantly more pronounced compared to gemcitabine's effects.

Another group examined the efficacy of pixatimod alone and in combination with gemcitabine in an orthotopic AsPC-1 xenograft model [3]. Treatment of the three experimental groups, all delivered twice weekly IP, consisted of 20 mg/kg (pixatimod); 25 mg/kg (gemcitabine); 10 mg/kg pixatimod and 12.5 mg/kg gemcitabine (combination). Pixatimod reduced the growth of primary tumors in this model compared to both the control and gemcitabine alone. There was no significant difference between the pixatimod and combination groups, although it should be remembered that the combination received half the doses of the monotherapies. Pixatimod alone and the combination were also the most effective at preventing metastasis to the lung, although neither was significantly better than gemcitabine.

Histological analysis of the tumors revealed that the combination and pixatimod treatments reduced levels of the proliferation marker PCNA and increased those of the apoptosis marker cleaved caspase-3. Pixatimod exposure was also associated with reductions in VEGF, cyclin D1, MMP-7 and β-catenin levels in tumors. The authors discuss these results in relation to inhibition of Wnt signaling by pixatimod, which they demonstrate in vitro. However, many of these changes are also consistent with heparanase inhibition.

22.4.3 Ovarian Cancer

The estimated number of people diagnosed last year with ovarian cancer was 22,200 in the USA and 295,400 worldwide, which is comparatively high considering that only half the population is susceptible [29, 35]. Despite a relatively high 5-year survival rate of 47%, most patients will eventually develop resistance to treatment and succumb to their illness [29]. This is borne out by the ratio of estimated deaths from this disease in the USA for 2018 (14,070) compared to the estimated number of new cases (22,240). At 63% this ratio is markedly higher than the same calculations for colorectal (36%), breast (15%), prostate (18%), leukemia (40%), kidney (23%) and bladder (21%) cancer, for example. Of the major neoplasms, only pancreas (80%), liver (72%) and lung (66%) are higher. The standard of care for ovarian cancer remains a combination of carboplatin and paclitaxel which, although often initially effective against ovarian tumors, usually leads to resistance and progression [49, 50]. There is, therefore, a clear need for additional therapy modes to treat this disease.

Pixatimod has been tested in four xenograft models and one syngeneic model of ovarian cancer with promising activity either in comparison to, or in combination with, standard treatments for this disease. It inhibited tumor growth and increased survival in an A2780 xenograft model when dosed SC and IV [4]. This mode of dosing has clinical significance because pixatimod was delivered SC in its first clinical trial, but this was changed to an IV infusion after injection site reactions resulted from SC administration. In the same model, pixatimod showed additive activity when administered with paclitaxel (15 mg/kg once weekly). Likewise, in a SKOV3 xenograft model, it showed activity alone and additive activity in combination with carboplatin (40 mg/kg once weekly).

An IP syngeneic model (ID8) was employed to explore pixatimod activity in a site-appropriate immunocompetent system. In this experiment, pixatimod prevented the growth of tumors and reduced ascites formation, which is a major clinical complication associated with ovarian cancer [4]. Additionally, plasma from some of these models (A2780 and ID8) was analyzed and elevated levels of heparanase and other HS-binding proteins were observed, indicating that pixatimod was binding to the HS-binding sites of these proteins and, thus, flushing them out of the ECM and into the plasma. This would not only inactivate heparanase by preventing it from binding to substrate HS, but it also removes it from the peri-tumoral locations where it can promote the proliferation and spread of cancer cells.

This group [4] next examined the possibility of using pixatimod as maintenance therapy following cisplatin and paclitaxel. The objective of such an approach in the clinic would be to reinforce the efficacy of this cytotoxic combination and prevent or reduce resistance from developing. Treatment with cisplatin (6 mg/kg) and paclitaxel (15 mg/kg) occurred on days 3, 6 and 9. Pixatimod administration commenced on either day 3 or day 10 for the combination groups and day 3 only for the monotherapy. All treatment groups showed significantly prolonged survival compared to control, with the triplet combination groups showing the greatest enhancement, however, these were not significant compared to the pixatimod alone or the cytotoxic doublet [4]. This anti-tumor activity was associated with reduced cell proliferation, as indicated by Ki67 staining, and reduced microvessel density, as measured by CD31 staining, in the tumors.

A separate study used a genetically modified OV202 human ovarian cancer cell line transformed to under-express HSulf-1, which is a sulfatase that has tumor suppression properties [27]. This enzyme, which de-sulfates HS at 6-O positions of glucosamine residues, is commonly downregulated in ovarian cancers [51, 52]. HSulf-1-catalyzed modification of HS chains reduces the activity of a range of HS-binding signaling proteins and pathways including Akt and ERK, reducing cancer cell proliferation and spread [53–55]. While the effect that HSulf-1 catalysis has on heparanase affinity for substrate HS has not been directly assessed, 6-O sulfation is considered to be essential for heparanase cleavage of HS chains [56] implying that tumors downregulating HSulf-1 would also be promoting heparanase activity. When pixatimod was tested in the xenograft model of OV202 under-expressing HSulf-1 cells IP inoculated into nude mice, it was found to significantly reduce tumor growth [27]. The pixatimod-treated tumors were found to have considerably higher levels of the apoptosis markers cleaved caspase-3 and PARP, and lower levels of phosphorylated ERK and c-Myc in addition to reduced levels of cancer-induced glycolytic enzymes. These tumoral responses are consistent with pixatimod inhibition of HS-binding growth factors and/or heparanase.

22.4.4 Lung Cancer

Successful efforts in reducing tobacco smoking have led to a decline in the incidence of lung cancer in the USA over recent decades. However, this disease is still the largest cause of cancer deaths in the USA (154,100) by a factor of approximately three-fold over the next deadliest cancer and globally, where antismoking efforts have not been as successful, lung cancer is the most common and also by far the most deadly cancer with an estimated 1,761,000 deaths in 2018 [29, 35]. Most lung cancer patients have non-small cell lung cancer (NSCLC) which can be further divided into adenocarcinoma or squamous cell carcinoma (SCC). The prognosis for these patients is not promising with few curative treatments available apart from surgery for early-stage patients [57].

Pixatimod has shown activity in several lung cancer models including three xenografts, a syngeneic model and a panel of nine patient-derived xenografts (PDX). A recent study of the efficacy of pixatimod in this disease setting began by testing it

in three NSCLC xenograft models: HCC-827 (adenocarcinoma), A549 (adenocarcinoma) and HTB-182 (SCC; [16]). This group found that pixatimod inhibited the growth of the tumors in these models. Overexpression of heparanase by the HCC-827 cell line enhanced cell invasion in vitro and tumor growth in vivo, suggesting that heparanase is an important driver of these cancers. To more closely mimic the tumor characteristics of the human disease, a panel of PDX models were established, comprising six SCC and three adenocarcinoma cancers. PDX models are thought to accurately reflect the original parent tumor and its response to treatment although, because each is unique, comparisons with the literature are difficult [58, 59]. Pixatimod was active, inhibiting tumor growth by >50%, in eight of these nine PDX [16]. Spontaneous metastasis to lymph nodes was observed in two of the PDX: one each of SCC and adenocarcinoma. When treated with pixatimod, this metastasis was effectively abolished.

Platinum-based chemotherapy is an important treatment option for patients with advanced NSCLC [60] so the efficacy of pixatimod against a PDX model that was resistant to cisplatin was examined [16]. Pixatimod was effective at overcoming cisplatin resistance and reduced both primary tumor growth and metastasis, but the combination with cisplatin was no more effective than pixatimod alone. Because surgery is often used clinically if the disease is not advanced, pixatimod treatment as a surgical neoadjuvant/adjuvant was also investigated. Pixatimod proved effective as a neoadjuvant treatment (administered both before and after surgery) but was not when given as an adjuvant treatment (administered only after surgery; [16]).

Analysis of the untreated PDX primary tumors and metastatic lesions, where they occurred, showed them to be rich in heparanase. Tumors treated with pixatimod showed reduced blood vessel formation (CD31 staining) and impaired function, as indicated by their collapsed structure [16]. Pixatimod exposure was also associated with reduced tumoral ERK activation and exclusion of macrophages from the tumor interior leading to their accumulation at the periphery in a fashion previously seen with heparanase knockout mice [61]. These observations are all consistent with pixatimod inhibition of heparanase resulting in reduced tumor growth and metastasis.

Pixatimod has also been compared to sorafenib in a syngeneic Lewis lung carcinoma model, LL/2 [30]. Sorafenib has shown evidence of anti-tumor activity in NSCLC, but this was not sufficient in phase III clinical trials to be approved for the treatment of this disease [62, 63]. Nevertheless, this anti-angiogenic agent is a good comparator molecule for pixatimod because it also possesses anti-angiogenic properties. Both compounds reduced the growth of the primary tumor in the LL/2 model, but only pixatimod inhibited the metastatic spread to the lungs which is a typical feature of this model [64].

22.4.5 Mesothelioma

Mesothelioma occurs relatively rarely in most populations, but its incidence has been rising steadily since the industrial use of asbestos, exposure to which is the cause of about 80% of cases [65]. The disease was little known before the twentieth

century [66], but because of increasing occurrence and a long latency period, the projected caseloads for developed countries like Australia, USA, UK and Italy persist well into the twenty-first century even with improved health and safety standards concerning asbestos [67–70]. In developing countries, the situation is less clear with under-reporting and widespread use of asbestos strongly suggesting that a significant and increasing number of cases will arise in countries such as China, India, Kazakhstan and Thailand [71]. Even in developed countries like the USA, the 5-year survival rate is only 10.2%, which is the second lowest of the cancers monitored by the SEER Cancer Statistics Review [29]. Given these statistics, there is a clear need for more treatments for this disease.

The most common form of mesothelioma is that arising in the mesothelial lining of the pleura, accounting for about 90% of cases [72]. Surgery is an option for a minority of patients but generally, chemotherapy with cisplatin and pemetrexed or raltitrexed is used [73, 74]. It has been hypothesised that targeted therapy based on genetic profiling to identify activated oncogenes, whilst showing some success in other cancers, has largely failed in mesothelioma because malignancy in this disease is generally driven by the inactivation of tumor suppressor genes, such as CDKN2A, NF2 and BAP1, rather than by oncogene activation [74]. Pixatimod, with its alternate sets of cellular and molecular targets compared to targeted therapies, may prove to be more successful in targeting such cancer.

A study of the efficacy of pixatimod against mesothelioma conducted experiments using four xenograft models and three syngeneic mouse models of pleural mesothelioma and compared the activity of pixatimod with cisplatin [15]. In a SC inoculated MSTO-211H xenograft model, pixatimod significantly inhibited tumor growth compared to both control and cisplatin-treated groups (3 mg/kg every 2 weeks). Using three luciferase-labeled IP models (MSTO-211H, CD487 and NCI-H2052) pixatimod significantly increased survival compared to control and cisplatin (MSTO-211H) and significantly reduced tumor size as measured by luciferase-dependant luminosity compared to control and cisplatin (MSTO-211H, CD487 and NCI-H2052). Pixatimod also inhibited tumor growth in three SC inoculated syngeneic models: AE17, AK7 and RN5 [15].

A heparanase knockout study, using the AE17 cell line and syngeneic model, showed that tumor growth in this model is heavily dependent upon heparanase content [15]. The knockout tumors, compared to the wildtype, showed impaired vasculature, reduced proliferation (Ki67), higher apoptosis (cleaved caspase-3), reduced Akt activation (as demonstrated by reduced phosphorylation and increased FoxO1) and increased p21 and p27. When examining tumors from the MSTO-211H xenograft model that had been treated with pixatimod, similar results were seen compared to the heparanase knockout tumors, confirming the heparanase-inhibition mechanism of action for this compound.

Pixatimod also altered the localization of macrophages within AE17 syngeneic and MSTO-211H xenograft tumors: macrophages were arrested in the tumor periphery compared to control mice where macrophages were found throughout the tumor mass. Similar reductions in intra-tumoral macrophages have been seen in other studies using different models of varying cancer types [9, 12, 16]. These observations

are likely the direct result of heparanase inhibition by pixatimod given the importance of this enzyme for macrophage invasion and function [61, 75]. Further examination of the macrophages associated with tumors in this mesothelioma study revealed that pixatimod did not alter the polarization of these cells between M1 and M2 types despite there being some evidence that heparanase is involved in the transition of macrophages into a pro-cancerous cell type [76].

22.4.6 Liver Cancer

Liver cancer is the only major cancer type that is increasing in prevalence in the developed world. In the USA for example, the number of new cases per year per 100,000 people has risen from 6.04 to 9.08 in the last twenty years [29]. Similar trends are being observed in other developed countries such as Australia, New Zealand, UK, Germany and France [77, 78]. While the reason behind this increase is not certain, the risk factors for liver cancer include hepatitis B and C infection, alcohol consumption, aflatoxin exposure and obesity, of which hepatitis infection and obesity are thought to be likely candidates [79, 80]. The number of new cases during 2018 for the USA and worldwide are estimated to have been 42,200 and 841,100, respectively [29, 35].

The majority of liver cancers are hepatocellular carcinoma (HCC) accounting for 75–85% of cases [35, 77]. Pixatimod has been evaluated in two xenograft models of HCC, one in which the cancer cells were inoculated SC (HepG2) and the other was an orthotopic model (Hep3b2.1–7). In the HepG2 study, pixatimod significantly reduced tumor growth, with a tumor growth inhibition (TGI) of 55%, at a dose of 20 mg/kg once weekly [30]. However, when the same dose was administered twice weekly the effect was not significant (TGI 45%). The reason for this is not clear because in most studies that use different dose levels of pixatimod there is a positive dose to response relationship. General health considerations with the experimental mice also do not appear to be a factor because body weight loss was less than 5% and consistent between the groups.

Pixatimod also showed efficacy in the orthotopic Hep3b2.1–7 model, which more closely reflects HCC than SC models [81], inhibiting tumor growth with a TGI of 52% when administered at 20 mg/kg twice weekly [30]. The combination of pixatimod with the approved HCC drug sorafenib was also assessed using this model. There was little difference between pixatimod and sorafenib (30 mg/kg daily) as monotherapies with TGI of 55% and 58% respectively, but the combination showed significantly reduced tumor growth (TGI 85%) and was reasonably well tolerated. This model was also used to examine radiolabelled pixatimod distribution through the circulation, liver, kidney and tumor tissues. The data indicate good distribution of pixatimod into tumor tissue which, when expressed as concentration ratios between tumor: liver or tumor: kidney, are comparable to data for the approved drug gefitinib [82].

22.4.7 Lymphoma

The term lymphoma encompasses a range of cancers derived from lymphocytes which are usually grouped into the Hodgkin lymphoma (HL) and Non-Hodgkin lymphoma (NHL) categories. The majority of cases are NHL, amounting to 74,700 in the USA and 509,600 worldwide last year, which is 90% and 86% of total lymphomas, respectively [29, 35]. In developed countries, prognosis is generally reasonably good with 5-year survival in NHL at 71.4% in the USA translating into a ratio of estimated deaths in 2018 to new cases of 27% [29]. In contrast, the outcomes for patients in developing regions of the world are poorer, with the equivalent ratios at 57% in Asia, 45% in Latin America and the Caribbean and 67% in Africa [83].

Two groups have examined the efficacy of pixatimod in lymphoma models, conducting experiments in eight different types, five of which were xenografts and the other three were syngeneic models. One group focused upon a panel of five human SC xenograft models covering some of the major types of NHL including: diffuse large cell lymphoma (OCI-LY-19) one of the most common NHL in adults; follicular B cell lymphoma (SU-DHL-6) a common indolent NHL; and several strains of Burkitt's lymphoma which is a type of NHL common in developing regions where it is typically associated with Epstein-Barr virus (EBV) infection (Raji and Daudi, [84]) and they also included a non-EBV Burkitt's lymphoma (Ramos). Pixatimod significantly inhibited tumor growth in all of these models [11].

The hypothesis that pixatimod's activity was due to heparanase inhibition was tested by examining heparanase expression by the NHL cell lines. Interestingly, not all of these cell lines express high levels of heparanase when cultured in vitro, including Raji, SU-DHL-6 and OCI-LY-19. However, an examination of the tumors produced by the Raji cell line showed that they contained high levels of heparanase which was probably produced by host cells and recruited by the tumors. Even NHL cell lines that were heparanase negative relied upon heparanase expression, albeit by host cells, for tumor growth and were, therefore, susceptible to pixatimod blockade of heparanase. This was further demonstrated by an EL-4 study, a model that has both a heparanase negative cell line but also fails to recruit heparanase to its tumors, in which pixatimod did not inhibit the growth of the heparanase-independent tumors [11]. Curiously, another group showed efficacy of pixatimod in an EL-4 model but they did not examine heparanase levels in the tumors [33]. There are a number of possible reasons for this difference including different genotypes of the EL-4 cell lines; phenotype differences due to culturing practices; pixatimod administration was IP in Weissmann et al. [11] but SC in Brennan et al. [33].

Additional analysis of tumors and organs from these NHL models provided more evidence supporting the hypothesis that heparanase inhibition was the mechanism for pixatimod's activity [11]. For example, SU-DHL-6 tumors showed reduced blood vessel formation (CD31) and signs of apoptosis induction (cleaved caspase-3). Pixatimod also had strong activity against metastasis, which is frequently promoted by heparanase [85, 86], in an IV Raji bone marrow colonization model and in a syngeneic ESb NOD/SCID model.

The Brennan group used syngeneic models in immunocompetent mice to examine how pixatimod modulates components of the immune system to target tumors [33]. These studies showed that pixatimod inhibited tumor growth in A20 and EL-4 syngeneic models of lymphoma and that this inhibition involved natural killer (NK) cell activation in a TLR9-dependent mechanism [33]. The proposed mechanism involves pixatimod promoting the accumulation of CpG oligonucleotides in the lysosomal compartment of dendritic cells leading to TLR9-mediated activation of these cells and thence NK cell mobilization. Though a recent publication has shown that NK cell anti-tumor activity is dependent upon heparanase [87] results from Brennan et al. [33] show that pixatimod enhanced NK cell infiltration into tumors in vivo. It is not clear from the Brennan study whether heparanase inhibition by pixatimod is associated with this pixatimod activity, but given the multifaceted roles of heparanase in cancer, inflammation and tissue re-organization, further investigation is required to elucidate the respective activities of pixatimod in in vivo studies.

Another finding of interest from this study is the observation that pixatimod in combination with cyclophosphamide caused tumor remission in an A20 established tumor model. This has clinical significance because cyclophosphamide is used to treat lymphoma, as part of the R-CHOP regimen, and has also been investigated as a metronomic (low dose) treatment in this and other cancers [88–90]. As previously noted, a separate study showed that pixatimod was not effective in a similar SC-inoculated EL-4 model which was attributed to a lack of EL-4-produced or host-derived tumor-associated heparanase [11]. The Weissmann study did not examine pixatimod efficacy in the A20 model so there are no other possible comparisons between these studies.

22.4.8 Breast Cancer

Breast cancer is one of the most prevalent cancers with an estimated 2,088,800 cases being diagnosed in 2018 worldwide [35]. For women, it is the most common cause of cancer mortality with 626,680 deaths in 2018 worldwide [83]. In the USA, breast cancer is the most common cancer diagnosed, although with a 5-year survival rate of nearly 90% the outcomes for patients are relatively positive [29]. However, not all types of breast cancer are amenable to treatment with triple-negative genotypes (negative for estrogen receptor [ER], progesterone receptor [PR] and HER2) typically having a much poorer prognosis [91, 92].

Pixatimod activity in breast cancer models, both syngeneic and xenograft, has been assessed in several studies. It showed activity in an MDA-MB-231 triple negative model [93], inhibiting the growth of SC implanted tumors versus untreated controls [30]. Demonstrating efficacy in a triple negative model has positive implications for the clinical use of pixatimod in this population of breast cancer, which although only 15–20% of the total [91], is in need of treatment options because these patients do not respond to HER2 inhibitors (such as trastuzumab) nor hormonal treatments (such as tamoxifen).

In addition to the triple negative xenograft model, pixatimod has also been tested in two versions of the 4T1 syngeneic orthotopic model. One version of this model involves the orthotopic inoculation of 4T1 cells into the mammary fat pads of experimental mice and during the experiment primary tumor growth is measured. At the conclusion of the study, the lungs can be examined, macroscopically and microscopically, to assess metastatic spread. In the second version, the primary tumor is removed along with the mammary fat pad (mastectomy) 5 days after inoculation, which promotes metastasis, particularly to the lungs [94, 95]. The location and frequency of the metastatic lesions in this model are consistent with human breast cancer [96]. Moreover, because the 4T1 models are conducted using immunocompetent mice (BALB/c), they allow examination of the interactions between pixatimod and the entire immune system and how these affect cancer progression.

Pixatimod inhibited primary tumor growth in the orthotopic 4T1 breast cancer model, as did the angiogenesis inhibitor sorafenib [32]. However, when the lungs from this study were examined for metastases, it was discovered that pixatimod had inhibited metastasis in a dose-dependent manner but that sorafenib had increased the number of lung metastasis. Such exacerbation of metastatic spread has been observed before with members of this class of anti-angiogenesis VEGFR inhibitors including sorafenib and sunitinib [30, 97, 98].

When pixatimod and sorafenib were tested in the mastectomy model, pixatimod significantly increased survival compared to both the untreated control and the sorafenib group [32]. In contrast, sorafenib showed no improvement which is not surprising given that survival in this model is inversely linked with metastasis. In another 4T1 mastectomy experiment, treatment was withheld until 1 day before mastectomy to prevent pixatimod from affecting the growth of the primary tumor and exert its effects only on metastases. Again, pixatimod significantly prolonged survival in this metastasis focused study [32].

Pixatimod has been evaluated in mouse cancer models in combination with a number of cytotoxic and targeted chemotherapeutics. However, one of the most promising recent approaches to cancer treatment has been the development of immunomodulatory agents, particularly the T cell checkpoint inhibitors [99–102]. Pixatimod's mechanism of action – blocking TAM, suppressing metastasis, inhibiting angiogenesis, activating NK cells – was considered complementary to a checkpoint inhibitor so this combination was investigated using the 4T1 orthotopic model [34]. Using an anti-mouse PD-1 antibody, because the approved therapeutic antibodies target human PD-1 and would not work in a syngeneic mouse model, the combination treatment demonstrated potent activity against 4T1 primary tumors [34]. Pixatimod alone also significantly reduced tumor growth compared to controls but was not as effective as the combination. Analysis of the tumors of satellite mice revealed that the combination significantly boosted the numbers of $CD4^+$ and $CD8^+$ T cells infiltrating into the tumors compared to control and anti-PD-1 alone. The combination also boosted the total number and activation status of NK cells in the tumors indicating that this treatment promoted both innate and adaptive responses against the tumor, highlighting the potential that combining pixatimod with a checkpoint inhibitor would have for treating cancer.

22.4.9 Other Cancers

Models of several other cancer types have been used to examine pixatimod activity in vivo, including glioma and pediatric brain cancer, head and neck, prostate, melanoma and a skin carcinogenesis model. Heparanase is highly expressed in glioma and its subtype glioblastoma, the most malignant form of this group of brain cancers [13]. In a syngeneic model of glioma (GL261) that was demonstrated to be heparanase dependent, pixatimod inhibited tumor growth whether administered SC or IP [13]. Using a SC xenograft model with U87 glioma cells transformed to overexpress heparanase, pixatimod potently inhibited tumor growth [10]. This model, incorporating U87 cells overexpressing heparanase, has previously been shown to be heparanase dependent for tumor growth with mock-transfected U87 cells producing much slower growing tumors [103, 104]. A separate study of pediatric brain cancers used two SC implanted xenograft models: a central nervous system embryonal tumor model (PFSK-1) and a medulloblastoma model (D283) both of which express higher levels of heparanase than comparable non-cancerous cells [19]. Pixatimod was effective in both models at inhibiting primary tumor growth. Tumor analysis revealed that pixatimod treatment led to reduced tumoral expression of the proliferation marker Ki67, increased expression of the apoptosis marker cleaved caspase-3 and impeded the development of tumor vasculature both in terms of vessel quantity and size [19]. All of these outcomes are consistent with heparanase inhibition.

Pixatimod has been tested in SC tumor growth and lung colonization models using the mouse B16 melanoma cell line and in a chemically induced mouse skin carcinoma model. It was effective at blocking lung colonization by B16 cells when injected IV at both daily dosing (10 mg/kg, [1]) and at a more pharmacokinetically appropriate twice weekly (20 mg/kg, [30]). Pixatimod was also effective at reducing the growth of SC inoculated tumors of B16 [1]. The B16 model is regarded as poorly immunogenic [105, 106] and produces tumors with relatively little infiltration by host immune cells [107, 108] suggesting that such activity from an agent that does not target a specific B16 mutation or over-expressed protein, has particular significance. A chemically induced skin carcinogenesis model in transgenic mice overexpressing heparanase was used to examine pixatimod activity in a cancer setting in which progression was heparanase dependant [9]. Pixatimod potently suppressed tumor progression in this model and, interestingly, also blocked macrophage infiltration into the tumors, consistent with other studies ([12, 15, 16] Fig. 22.3).

In xenograft models of prostate (PC3) and head and neck (Cal27) cancers, pixatimod also significantly inhibited the growth of SC inoculated tumors [30]. The PC3 model is hormone insensitive so this model is representative of the clinical setting after prostate patients have evolved resistance to the hormonal drugs which are typically used to treat stage III and IV prostate cancer [109, 110]. Therefore, there is a need for drugs that are active against this castrate-resistant form of prostate cancer. The head and neck cancer model (Cal27) is an SCC which is the most common type of this cancer. Malignancy of these cancers is thought to be driven, at least in part,

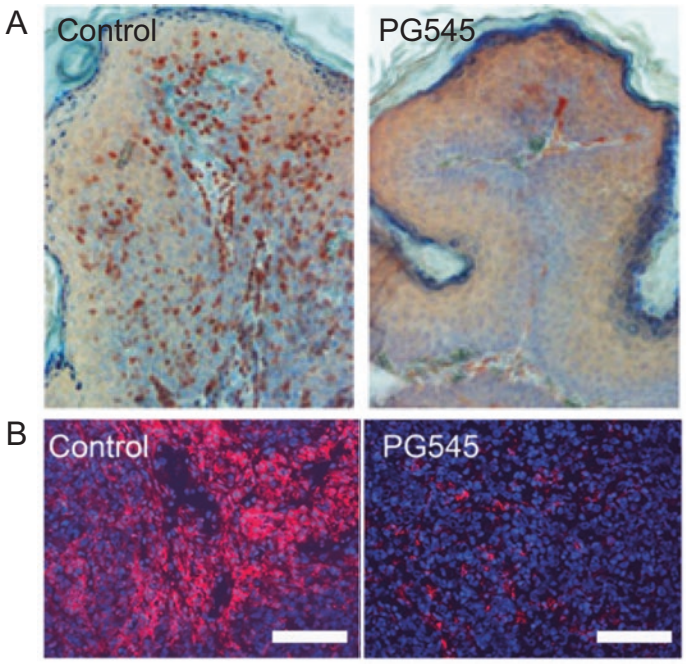

Fig. 22.3 Pixatimod (PG545) blocks the invasion of macrophages into tumors in mouse cancer models. (**A**) Tumors from a chemically induced skin carcinogenesis model (in heparanase overexpressing mice) were stained for F4/80 (brown) to show macrophages (Boyango et al. [9]). (**B**) MiaPaca-2 xenograft tumors were stained for F4/80 (red) to show macrophages (Ostapoff et al. [12]). Scale bars, 100 μm

by the polycomb family member Bmi1 which promotes EMT, cell proliferation and invasion [111, 112] and the Cal27 cell line strongly expresses this protein [113]. Although there is no demonstrated connection between heparanase and Bmi1, these cancer-promoting processes have all been shown to be suppressed by pixatimod inhibition of heparanase in other studies, suggesting a likely explanation for activity in the Cal27 model and justification for clinical investigation.

22.5 Clinical Development

Pixatimod monotherapy has been shown to be well tolerated in patients when administered IV up to doses of 100 mg once-weekly and is currently being assessed in combination with the PD-1 antibody nivolumab. In the first trial of pixatimod in humans, the drug was given SC and led to injection site reactions in four patients at

25 and 50 mg which halted the trial (NCT01252095). Nevertheless, the compound appeared to cause no systemic safety concerns and analysis of plasma samples from these four patients provided evidence that pixatimod was, indeed, binding to HS-binding proteins including heparanase [4].

Administration of pixatimod as an IV infusion alleviated the skin reactions associated with SC injection and allowed the maximum tolerated dose (MTD) to be determined as 100 mg in a subsequent phase Ia trial which enrolled 23 solid tumor patients (NCT02042781). While no objective patient responses according to RECIST criteria were reported with pixatimod treatment, clinical benefit was reported in some patients with a disease control rate of 38% [114]. Moreover, there was evidence of innate immune cell activation and modulation of HS-binding proteins, although the data for heparanase in patient plasma showed no clear trends [114]. However, given the heterogeneity of the cancer types and patient populations, it is not surprising that the heparanase response was not definitive. This trial also reported an assessment of the pharmacokinetics of pixatimod in patients, with a long half-life of 141 h, supporting weekly dosing and approximately linear exposure with dose. Given the positive results of the pre-clinical 4T1 data for the combination with an anti PD-1 agent, a phase Ib trial of pixatimod with the approved PD-1 antibody nivolumab was initiated with a dose escalation cohort of advanced cancer patients and expansion arms recruiting mPDAC or MSS mCRC patients (ACTRN12617001573347).

22.6 Conclusion

Since pixatimod, then known as PG545, was first published in 2010, there have been numerous studies detailing the biological activities of this anti-cancer agent. It is a potent inhibitor of heparanase, although the mechanism of this interaction was discovered to be different from that expected for a conventional HS mimetic. Pixatimod has demonstrated activity in vitro and in vivo against numerous cancer-promoting processes including cell proliferation, invasion, metastasis, angiogenesis and EMT. It has also shown activity in about 50 syngeneic and xenograft mouse models of cancer covering numerous cancer types including colon, pancreas, lung, ovary, liver and breast. Some of these studies have favorably compared pixatimod activity with clinically relevant agents and others have described combinations of pixatimod with such agents, all highlighting the potential therapeutic opportunities that this compound presents. Clinical investigation of pixatimod has identified a safety and tolerability profile as a monotherapy leading to the recent exploration of an exciting combination with an immunotherapeutic. Based on the preclinical data and initial clinical findings, future studies in combination with immunotherapy and chemotherapy are warranted.

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Chapter 23
The Control of Heparanase Through the Use of Small Molecules

Giuseppe Giannini, Gianfranco Battistuzzi, and Silvia Rivara

23.1 Introduction

Heparanase is a multifaceted protein endowed with enzymatic activity – it is the only known mammalian *endo*-β-D-glucuronidase – and non-enzymatic functions. For its functional properties and biological role, as hitherto known, this enzyme could be considered as an "animal with two tails" or a "double-edged sword". In fact, under normal physiological conditions heparanase is present in a few tissues and expressed at high levels in placenta and some bloodborne cells including platelets, mast cells, lymphocytes, and neutrophils, contributing to wound healing, tissue remodeling and embryo development [1]. Differently, in a variety of human pathological processes, such as cancer and inflammation, where it is overexpressed, researchers are unveiling its increasingly important role [1]. In several major diseases heparanase is considered a negative marker, contributing to establishing those conditions that, in turn, support severe pathological scenarios such as tumor growth and metastases, tissue inflammation, glomerular diseases and other diseases that continue to be identified. Given its multiple functions, and potential for agents affecting its activity, we described heparanase as a "rainbow pharmacological target" [3], and other researchers as a "multitasking protein" [4].

Heparanase hydrolyzes glycosaminoglycan heparan sulfate (HS) side chains of cellular heparan sulfate proteoglycans (HSPGs) [5]. HSPGs are mainly expressed in the basement membrane (BM) (e.g., perlecan, collagen XVIII), on the cell surface (e.g., syndecans 1–4, glypicans 1–6) and in the extracellular matrix (ECM). HSPGs bind to ECM components such as laminin, collagen IV and fibronectin, participating

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in the structural integrity and insolubility of ECM and BM and affecting cell-cell and cell-ECM interactions. Heparanase regulates the bioavailability and activity of several bioactive molecules interacting with HS, such as growth factors, lipoproteins, chemokines, and enzymes. HS degradation into smaller fractions allows the release of bioactive saccharide fragments and bound factors, activating signaling processes in response to local environmental alterations. For these reasons, HSPGs function both as a barrier to cell migration and as a reservoir of HS-bound growth factors and cytokines. Additionally, non-enzymatic functions have been characterized for heparanase, such as promotion of cell adhesion and signaling, stimulation of phosphatidylinositol 3-kinase-dependent endothelial cell migration and invasion, and phosphorylation of protein Akt [1].

Heparanase is a well-established anticancer target, being involved in tumor growth, vascularization, and metastasis. On the other end, several other therapeutic applications have been proposed for its inhibitors, spanning from inflammatory disorders, renal disease, and systemic sclerosis to some rare diseases such as fibrodysplasia ossificans progressiva and hereditary multiple exostoses. Recently, heparanase has also been proposed as a novel approach to control HS-dependent viral infections [6 and Agelidis and Shukla, Chap. 32 in this volume] and virucidal activity against herpes simplex has been reported for an oligosaccharide inhibitor [7].

The crystal structure of human heparanase was disclosed only in 2015 [8 and Wu and Davies, Chap. 5 in this volume], but attempts to identify inhibitors of this enzyme began many years before the full characterization of its structure and biological role. Given the similarity of heparin with heparanase substrate, HS, early attempts to identify heparanase inhibitors were based on the chemical modification of heparin structure. Considerable efforts were thus expended in the development of modified heparins and related polysulfated compounds with reduced anticoagulant activity. In particular, modified heparins or sulfated oligosaccharides, such as muparfostat (PI-88), necuparanib (M-402) and roneparstat (SST0001), or fully synthetic compounds such as pixatimod (PG545) are potent heparanase inhibitors and the only ones that reached so far the clinical evaluation, in some cases in advanced clinical trials [Chhabra and Ferro; Hammond and Dredge; Noseda and Barbieri, Chaps. 19, 21 and 22 in this volume]. In fact, although in the last 20 years several antibodies, vaccines and antisense oligonucleotides behaving as effective and specific inhibitors of heparanase have been identified, no one has been evaluated clinically [3].

Some of the first small-molecule heparanase inhibitors were discovered from natural sources. In 1992, trachyspic acid was isolated from the culture broth of *Talaromyces trachyspermus* SANK 12191 and, in 1994, a screening program for inhibitors of HIV-1 protease performed in Ciba-Geigy laboratories led to the isolation of tetronic acid derivatives from an *Actinomycete* strain, which were later optimized in a medchem project by the Japanese RIKEN Discovery Research Institute, providing potent and selective heparanase inhibitors endowed with antimetastatic activity. Starting from the nineties of the last century, the design and synthesis of novel, structurally varied heparanase inhibitors was pursued by pharmaceutical companies, such as InSight Biopharmaceuticals (then Celltech R&D Ltd) and Oxford GlycoSciences that, between 1999 and 2003, undertook a program aimed at developing small-molecule heparanase inhibitors. In the last 20 years an enormous

effort in the development of heparanase inhibitors has been accomplished by Sigma-Tau IFR, then Leadiant Biosciences, which has brought a molecule in the clinical phase and deposited several patents on both polysulfated oligosaccharides and small-molecule inhibitors.

This chapter presents an overview of the most relevant small-molecule heparanase inhibitors, starting from the natural ones and moving to those obtained by synthesis, up to the oligo/polysaccharide derivatives that mimic natural heparanase substrates. A detailed description will also be dedicated to the results obtained from a medchem project carried out in the last years by our group, which allowed the identification of small molecules endowed with an anti-heparanase activity comparable to that of the sulfated oligo- and polysaccharides evaluated in clinical trials. A brief mention to compounds that have reached the clinical trial phase will be made in the last part of the chapter.

Quantitative measures of inhibitory potency (e.g., IC_{50}, K_i values) will be reported only for select compounds, as they are heavily influenced by the assay used for their determination. The reader is referred to the cited references for this information and for fine structure-activity relationships (SARs). Heparanase inhibitors, their SARs and biological activity have also been described in several excellent reviews [3, 9, 10, 11, 12].

23.2 Heparanase Inhibitors

23.2.1 Natural Products

Several natural products possess anti-heparanase activity and some of them, heparin derivatives and polysulfated oligo- and polysaccharides, have been investigated until the advanced clinical trials for cancer treatment. These compounds are extensively described in other chapters of this book [Chhabra and Ferro; Hammond and Dredge; Noseda and Barbieri, Chaps. 19, 21 and 22 in this volume], and we will also mention them in a dedicated paragraph, later in this chapter. Focusing on small-molecule natural products endowed with anti-heparanase activity, the first compound well characterized and claimed for this activity was trachyspic acid (Fig. 23.1), a metabolite produced by *Talaromyces trachyspermus* SANK 12191. Trachyspic acid was first patented in Japan by Sankyo Co. [13], and published by Shiozawa H. et al. in 1995 [14]. Attempts to prepare trachyspic acid by total synthesis led first to a racemic mixture [15, 16], and then to an enantiomerically pure product [17, 18].

In 1996, the three heparanase inhibitors, A-72363 A-1, A-2, and C were isolated from the culture filtrate of *Streptomyces nobilis* SANK 60192. Spectroscopic

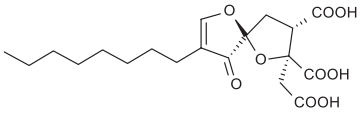


Fig. 23.1 Structure of (+)-trachyspic acid

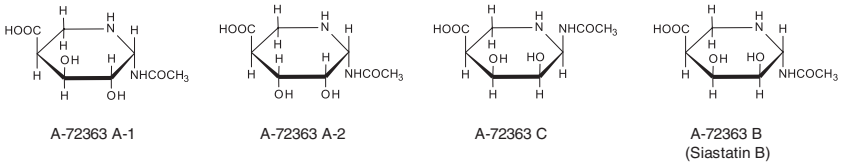


Fig. 23.2 Iminosugars isolated from *Streptomyces nobilis*; derivative A-72363 C is endowed with anti-heparanase activity

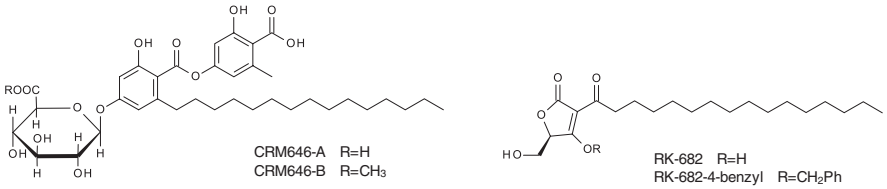


Fig. 23.3 Glucuronides CRM646-A and B, and tetronic acids (RK-682 and 4-benzyl analog)

studies revealed that they are diastereomers of siastatin B, a neuraminidase inhibitor devoid of anti-heparanase activity [19]. These iminosugars differ only in their configuration, yet each compound showed strikingly different specificities toward the various glycosidases tested. The only derivative with an anti-heparanase activity, in the micromolar range, was A-72363 C [20] (Fig. 23.2).

In 2000, Ko H.R. et al. isolated two fungal metabolites, CRM646-A and CRM646-B (Fig. 23.3), with anti-heparanase activity from *Acremonium* sp. MT70646 (KCTC 8973P) [21, 22]. The two inhibitors are glucuronides with a dimeric 2,4-dihydroxy-6-alkylbenzoic acid aglycone, which were prepared by total synthesis for the first time in 2005 [23]. These conjugates strongly inhibited the migration of B16-F10 melanoma cells and showed no cytotoxicity up to 100 μM concentration.

Another natural compound endowed with potent anti-heparanase activity is the 3-acyl-5-hydroxymethyltetronic acid RK-682 (Fig. 23.3), isolated from Actinomycete strain DSM 7357 and *Streptomyces* sp. 88–682 [24, 25]. Rational drug design performed by the Japanese RIKEN Discovery Research Institute led to 4-benzyl-RK-682 with improved selectivity for heparanase and able to inhibit invasion and migration of human fibrosarcoma HT1080 cells [26].

In 2014, the effect of kiwi essence on the occurrence of metastasis of Lewis lung adenocarcinoma was tested in mice. Supplementation of kiwi essence to chemotherapy allowed a reduction in the number of metastases, and expression of heparanase protein and mRNA was significantly reduced [27]. More recently, a few studies evaluated the anti-heparanase activity of a natural isoquinoline alkaloid with proven antiangiogenic and anticancer activities, berberine (Fig. 23.4), found in several plants including European barberry, goldenseal, goldthread, Oregon grape, phellodendron, and tree turmeric. Berberine is most commonly used against diabetes, high cholesterol and high blood pressure, or applied directly to the skin to treat burns and cancer sores. In 2013, Yan L. et al. tested berberine in bladder cancer T24 cells.

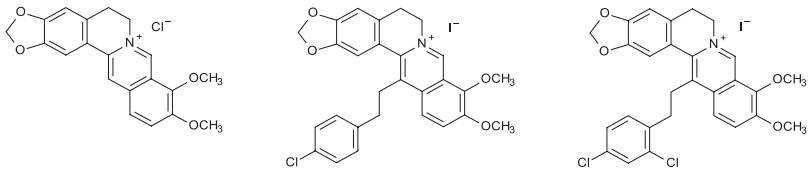


Fig. 23.4 Berberine and its phenylethyl derivatives NAX014 and NAX060 endowed with anticancer activity

They showed that heparanase mRNA and protein levels were highly expressed in human bladder cancer and markedly down-regulated by both heparanase-specific siRNA and berberine. Treatment with berberine attenuated migration and invasion ability of T24 cells [28]. In 2015, Pierpaoli E. et al, demonstrated that berberine and its synthetic derivative, 13-(4-chlorophenylethyl)berberine iodide NAX014, exert antiproliferative activity against HER2-overexpressing breast cancer cells, inducing apoptosis, modulating the expression of cell cycle checkpoint molecules involved in cell senescence, and reducing both HER2 expression and phosphorylation on tumor cells. Besides, NAX014 reduced expression of heparanase in tumors compared to control animals. The same effect was also observed with another synthetic dichlorophenylethyl derivative of berberine, NAX060 [29, 30].

Yang Y. et al. tested the effect of another natural substance, curcumin, on proliferation and heparanase expression in cultured blood vessel endothelium cells from ovarian cancer. They found that curcumin significantly inhibits cell proliferation and heparanase expression in a dose-dependent and time-dependent manner [31].

In 2017, a team of researchers from Chinese and Sweden Universities investigated the mechanism of elemene, a natural plant drug extracted from *Curcuma wenyujin*, widely used for cancer treatment in China for more than 20 years. Elemenes are a group of natural sesquiterpenes found in a variety of plants. They include α-, β-, γ-, and δ-elemene which are structural isomers (Fig. 23.5). Elemenes contribute to floral aromas of some plants, and are used as pheromones by some insects. In the past, elemene was investigated for inhibition of cancer cell growth, regulation of tumor microenvironment, inhibition of epithelial-mesenchymal transition, downregulation of neoangiogenesis and inhibition of ECM degradation by matrix metalloproteinases. Authors showed that the antiproliferative and antimetastatic effects of elemene were associated with downregulation of heparanase expression, as well as with a decrease in phosphorylation of the associated extracellular signal-regulated kinase (ERK) and AKT [32].

Carrageenans are a family of high molecular weight, sulfated galactans that are extracted from red seaweed and extensively used in the food industry for their rheological properties. They are characterized by long homogeneous linear chains of repeated disaccharide units consisting of a 1,3-linked β-D-galactopyranose (G unit) alternating with a 1,4-linked α-D-galactopyranose (D unit), differently sulfated depending on the species. The G2S-D2S,6S disaccharide unit (S = sulfated), bearing three sulfate groups, forms γ-carrageenans, which are known as the most sulfated plant-based polysaccharides with an ester sulfate content of about 35% in

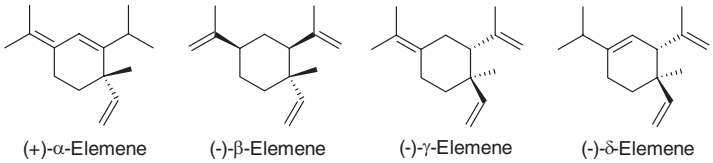


Fig. 23.5 Representation of elemene isomers

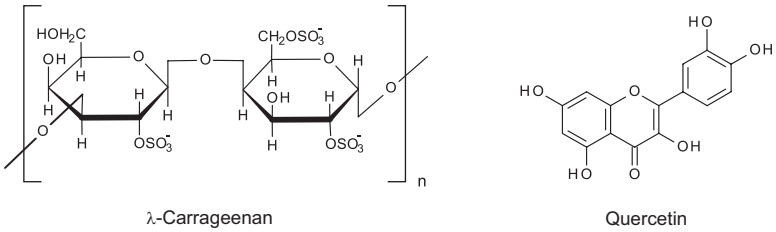


Fig. 23.6 Structure of γ-carrageenan and quercetin

weight (Fig. 23.6). Like other polysaccharides, carrageenans have many pharmacological properties, including anticoagulant, antiviral, antioxidant and anticancer activities [33]. In 2019, a study on the anti-heparanase activity of γ-carrageenan oligosaccharides (γ-CO) was reported [34]. Through a depolymerization method for producing γ-CO with progressive desulfation, Groult H. et al. investigated the influence of polymeric chain length and degree of sulfation (DS) on anti-heparanase activity. A γ-carrageenan oligosaccharide of 5.9 kDa was identified as a suitable anticancer candidate as it displayed one of the lowest anticoagulant properties among the γ-CO produced while having a remarkable inhibitory effect on MDA-MB-231 breast cancer cell migration. Previously, compounds obtained through depolymerization associated to glycol spitting of λ-carrageenan were reported and investigated for their anti-heparanase activity [35].

The polyphenol derivative quercetin is a flavonoid found in a variety of fruits, vegetables, leaves, and grains, and is used as an ingredient in dietary supplements, beverages, and foods. Quercetin was assayed for its inhibitory activity on cervical carcinoma in nude mice and for the relationship between this in vivo effect and heparanase expression. Researchers found that tumor growth was reduced by quercetin treatment, which also produced a decrease in heparanase protein levels [36]. Reduction of heparanase mRNA and protein expression in cervical cancer cells following treatment with quercetin was also observed in vitro [37].

23.2.2 Synthetic Small Molecule Compounds

Parallel to SAR investigations on natural substances, a commitment toward the design and chemical synthesis of new compounds endowed with anti-heparanase activity has begun. Activity went on in a pioneering way until the end of 2015 when

the crystallographic structure of human heparanase was released [Wu and Davies, Chap. 5 in this volume]. Synthetic small molecule heparanase inhibitors were developed according to classical ligand-based drug design approaches, often by structural optimization of some lead compounds. For this reason, it is possible to group most of the known inhibitors in a few structural classes, characterized by urea, benzazole, and quinoline scaffolds.

Urea Derivatives

Symmetric and asymmetric urea-based low-molecular-weight compounds have been extensively investigated. An attempt to rationalize information on inhibitory activity of urea derivatives was made by Bathini R. et al. in 2013 who reported a three-dimensional quantitative structure-activity relationship (3D-QSAR) study on a set of 43 1,3-bis[4-(1*H*-benzimidazol-2-yl)-phenyl ureas, furanyl-1,3-thiazol-2-yl and benzoxazol-5-yl acetic acid derivatives with the aim to validate a pharmacophore model for the design of heparanase inhibitors [38]. The first example of symmetrical urea is represented by suramin (Fig. 23.7), a synthetic polysulfonated naphthylurea initially developed as an antitrypanosomal drug (Chagas' disease), and more recently reported as a heparanase inhibitor through a primarily non-competitive mechanism of inhibition [39]. Its ability to downregulate heparanase expression has been related to its efficacy in reducing cancer cell proliferation [40, 41]. Suramin reached the clinical trial phase as an anticancer agent, but clinical application was hampered by severe side effects, likely related to its ability to interact with multiple cellular pathways. However, the naphthylurea scaffold stimulated the synthesis of suramin analogs like NF127, NF145, and NF171which resulted endowed with higher enzyme inhibitory potency and lower toxicity than the parent compound [42].

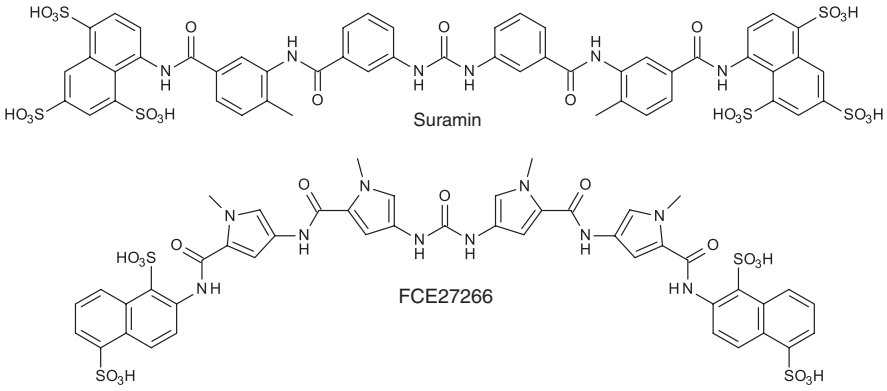


Fig. 23.7 Structures of suramin and FCE27266

Besides suramin, related sulfonated distamycin-A derivatives, sometimes reported as suradista, were synthesized [43]. In this series, compound FCE27266 (Fig. 23.7) and analogs demonstrated the same promising combination of antimetastatic and antiangiogenic activity as suramin, with lower cytotoxic effects [44, 45]. FCE27266 is a hybrid structure between distamycin and suramin, obtained by replacement of suramin benzene rings with N-methylpyrroles characteristic of distamycins. Twenty years later, our group proved that the antiangiogenic and antimetastatic activities of suradista (FCE27266) are associated with anti-heparanase activity. In 2017, we described our findings about FCE27266, as well as the synthesis of a series of structurally related compounds. FCE27266 and some derivatives (e.g., SST0548NA1, SST0613NA1, and SST0546NA1 in Fig. 23.8) proved to be potent heparanase inhibitors [46, 47].

To conclude with urea derivatives, we cite a patent filed in 2005 by Imclone Systems Inc. on the preparation of [(benzimidazol-2-yl)phenyl](phenyl)urea derivatives. This patent describes symmetric and asymmetric urea-based low molecular-weight compounds (e.g., compounds **1** and **2** in Fig. 23.9) with anti-heparanase activity in the micromolar range [48].

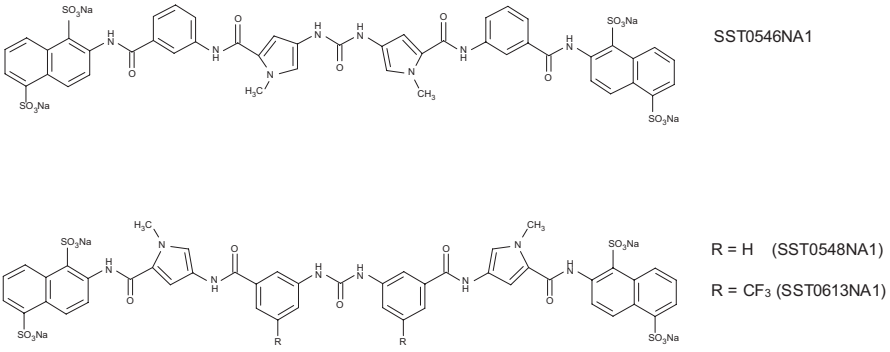


Fig. 23.8 Mixed suramin-distamycin A derivatives with potent anti-heparanase activity

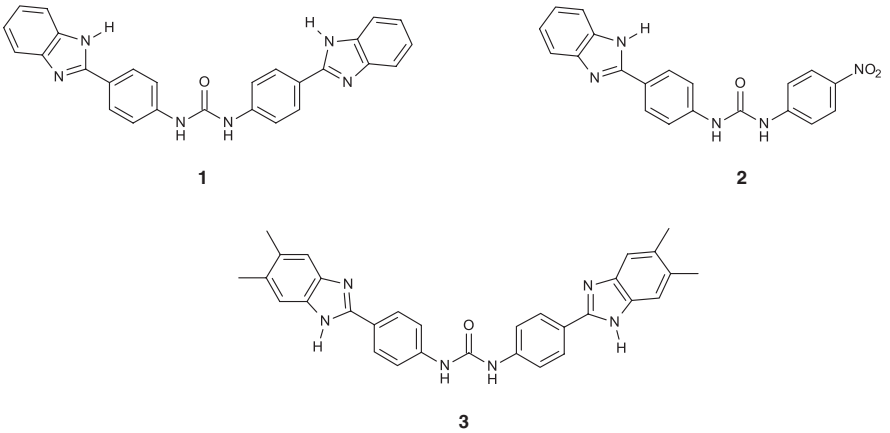


Fig. 23.9 Examples of benzimidazol-2-yl urea derivatives reported by ImClone Systems Inc.

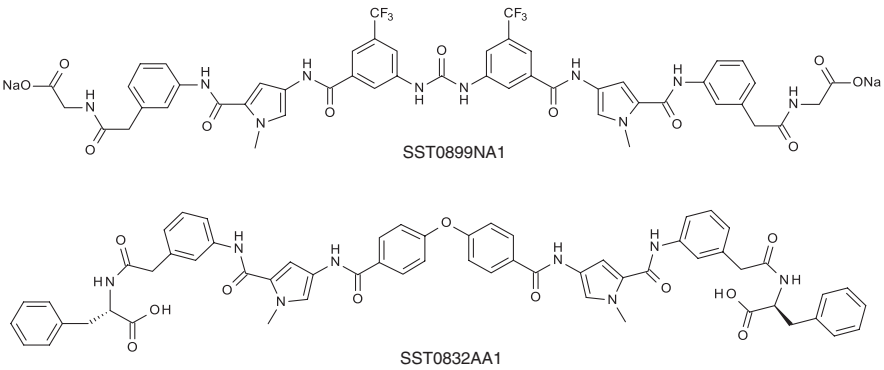


Fig. 23.10 Examples of potent anti-heparanase symmetrical tris-aryl-amide derivatives

In 2006, ImClone Systems Inc. reported the synthesis and anti-heparanase activity of a series of 1,3-bis-[4-(1*H*-benzoimidazol-2-yl)-phenyl]-urea derivatives, variously substituted on the benzene portion of the benzimidazole rings. Insertion of methyl groups in positions 4, 5 and 6 had a favorable effect, leading to the 5,6-tetramethyl derivative **3** (Fig. 23.9) with IC_{50} = 75 nM, which is one of the most potent heparanase inhibitors reported to date. These compounds showed good efficacy in a B16 lung metastasis model [49].

In continuation with the study carried out on the aryl-amido-naphthalene sulfonate derivatives described above (FCE27266 and SST analogs, Fig. 23.8), we investigated the anti-heparanase activity of symmetrical tris-aryl-amide analogs, lacking the naphthalene sulfonate portions. Some derivatives, e.g., SST0899NA1, SST0832AA1 in Fig. 23.10, proved to be potent agents, able to inhibit not only heparanase activity, but also the transcription of genes encoding for pro-angiogenic factors (e.g., FGF1 and 2, VEGF, MMP9) in tumor cell lines [50].

Benzazoles

During the past decade, several series of heparanase inhibitors based on a variously substituted benzazolyl scaffold, more often benzimidazol-2-yl and 2- or 5-substituted benzoxazolyl derivatives, were reported. The benzoxazole OGT2115 has been included in commercial catalogs as a reference heparanase inhibitor [51] (Fig. 23.11).

In 2002, Insight Biopharmaceuticals Ltd. issued a patent application on the preparation of benz-1,3-azole (benzimidazole, benzoxazole and benzothiazole) derivatives as heparanase inhibitors [52]. Compounds **4** and **5** (Fig. 23.12) were among the most potent, micromolar, heparanase inhibitors. Compound **6** was able to reduce mouse melanoma tumor growth and metastasis.

In 2004, Celltech R&D Ltd reported the results of a screening campaign which led to the identification of the 2,3-dihydro-1,3-dioxo-1*H*-isoindole-5-carboxylic acid derivative **7** (Fig. 23.13) as a new micromolar heparanase inhibitor with modest anti-angiogenic activity. Structural optimization was performed inserting substituents on the phenyl and the benzoxazole rings, leading to the submicromolar inhibitors **8**

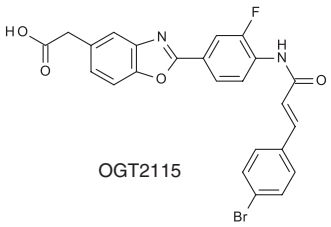
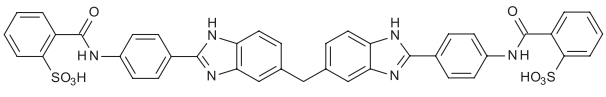


Fig. 23.11 Structure of commercially available heparanase inhibitor OGT2115



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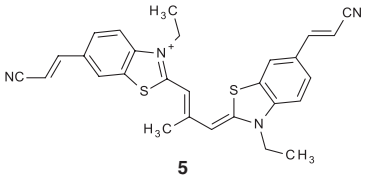

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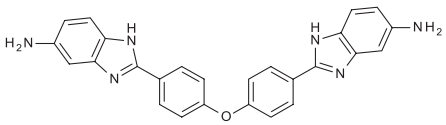

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Fig. 23.12 Benzazole inhibitors claimed by Insight Biopharmaceuticals Ltd

and **9** characterized by high selectivity (> 100 fold) towards human β-glucuronidase and with appreciable activity in the angiogenesis assay. This series, which included 2- or 4-methoxy and 4-propylamino substitution on the central phenyl ring, led to the selection of the 5-phenyl derivative OGT2492 and of inhibitor **9** as lead compounds for further optimization [53, 54].

One year later, in 2005, Celltech R&D Ltd identified a class of furanyl-1,3-thiazol-2-yl-acetic acid derivatives, exemplified by compound **10** in Fig. 23.13, as potent heparanase and angiogenesis inhibitors. Given the synthetic limitations experienced with furanyl-thiazoles and the pharmacokinetic liabilities observed for compound **10**, a scaffold hopping approach was applied which led to a series of benzoxazol-5-yl acetic acid derivatives [55]. Several compounds possessed submicromolar IC_{50} values against heparanase, as observed for cinnamic acid amide **11** and the reversed cinnamic acid derivative **12** in Fig. 23.13. SAR indicated that the linkage between the amide group and the pendant aromatic ring was critical for the observed activity. Interestingly, introduction of a fluorine atom on the central phenyl ring led to an increase in anti-heparanase activity, with the greatest effect observed for position 2 (e.g., compound **12**). The positive role of the fluorine atom has been

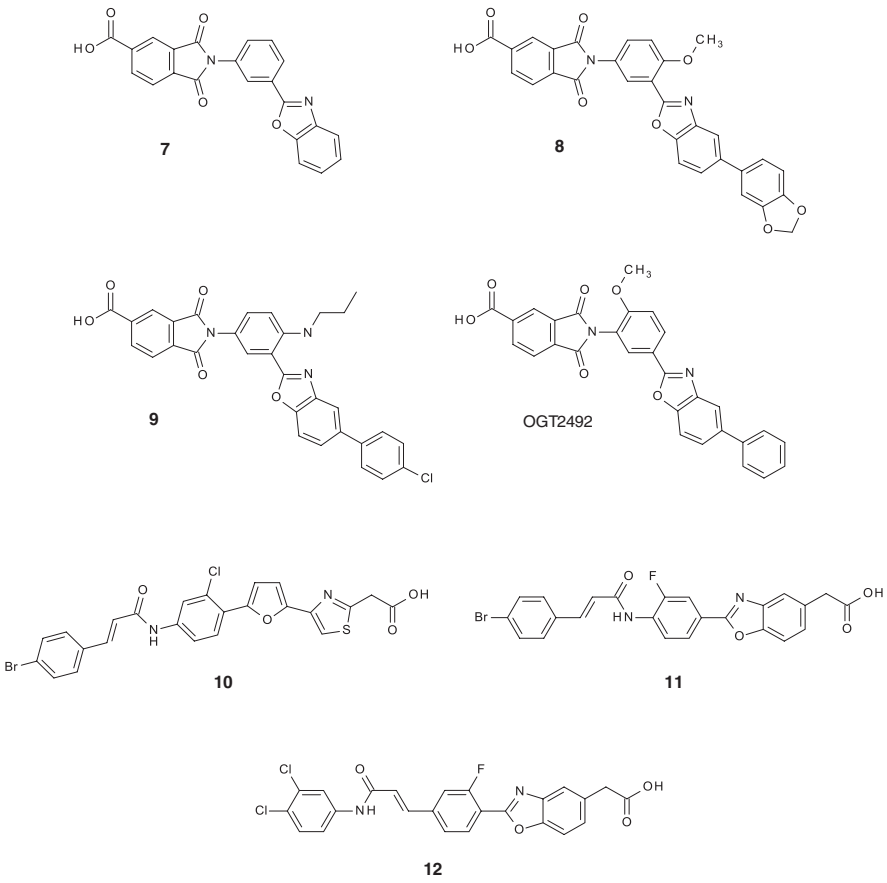

Fig. 23.13 Benzoxazole derivatives developed by Celltech R&D Ltd

recently confirmed by our studies, as later discussed. The synthesis of the benzoxazol-5-yl acetic acid derivative **11** with strong heparanase and angiogenesis inhibitory activities, and thus of possible commercial interest, was described in detail [56]. Several variously substituted 2-phenyl-benzoxazol-5-yl acetic acid derivatives were also reported in patent applications [57].

The benzoxazole nucleus is present in another series of heparanase inhibitors from Oxford Glycosciences Ltd. in which an acidic chain is bound to a 2-phenyl-benzoxazole through an urea linker. The benzoxazole ring is usually substituted in position 5 or 6, and an alkylamino chain can be present on the phenyl ring [58]. Compound **13** in Fig. 23.14 is a micromolar heparanase inhibitor.

In addition to the work mentioned above on urea derivatives [49], again in 2006 ImClone Systems Inc., starting from a hit compound identified by high throughput

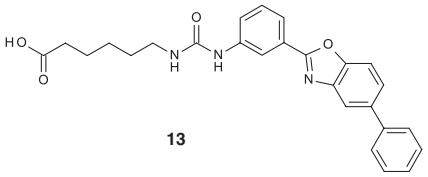

Fig. 23.14 An example of 2-phenyl-benzoxazoles from Oxford Glycosciences Ltd.

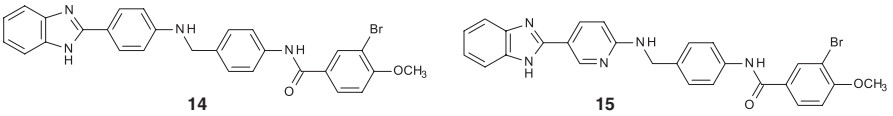

Fig. 23.15 (Benzimidazol-2-yl)-arylamino derivatives reported by ImClone Systems Inc.

screening, described the synthesis and anti-heparanase activity of N-(4-{[4-(1*H*-benzoimidazol-2-yl)-arylamino]-methyl}-phenyl)-benzamides. The potent and flexible inhibitor **14** (Fig. 23.15) was further optimized providing the pyridine derivative **15** with similar submicromolar heparanase inhibitory activity and oral exposure in mice [59, 60, 61].

Starting from this prior art, we designed, synthesized, and evaluated new benzoxazole and benzimidazole derivatives, combining the benzazolyl acetic acid moiety [55] with a [(4-substituted)-benzylamino]-phenyl side chain [59] in position 2 of the benzazolyl nucleus. We identified some very potent compounds characterized by the presence of a fluorine atom and/or an amino acid residue, e.g., derivatives **16**, **17**, and **18** (Fig. 23.16) that showed submicromolar IC_{50} values as heparanase inhibitors. Molecular docking studies were performed to rationalize their interaction with the enzyme. Importantly, cell invasion assay confirmed the antimetastatic potential of compounds **17** and **18**. Consistently with its ability to inhibit heparanase, compound **18** decreased expression of genes encoding for pro-angiogenic factors such as MMP-9, VEGF, and FGFs in tumor cells [62, 63]. The results of this study represent another step forward in the knowledge of the SARs for benzazolyl heparanase inhibitors. Further optimization might lead to a more effective modulation of heparanase enzymatic activity, thus actively contributing to the development of therapeutic tools for those clinical indications in which heparanase proved to be a relevant pharmacological target, including cancer metastasis.

More recently, our group reported the design, synthesis and evaluation of new symmetrical 2-phenyl-benzazol-5-yl-acetic acid derivatives, in which the two moieties were connected through several functional groups (e.g., urea, thiourea, guanidine, 2-propanol). Compound **21** (Fig. 23.17) is one of the most potent heparanase

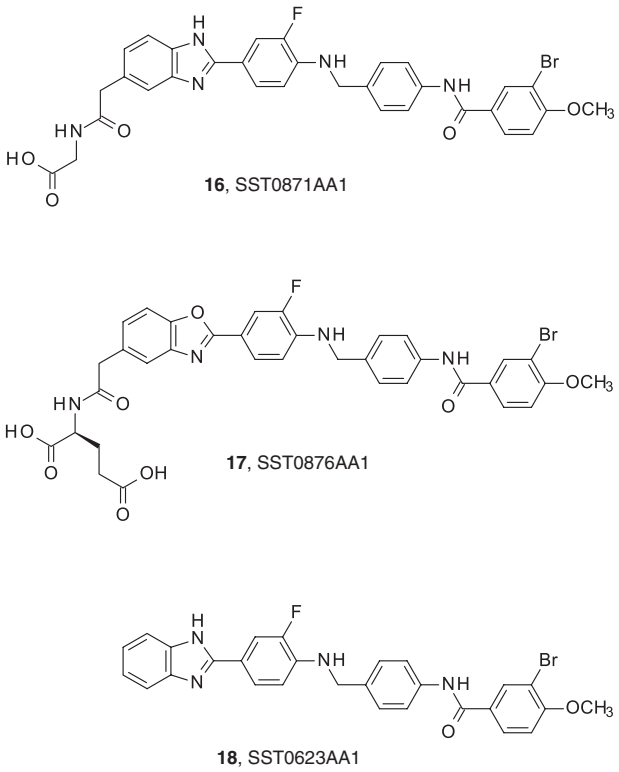

Fig. 23.16 Substituted 2-phenyl-benzoxazoles or -benzimidazoles with potent anti-heparanase activity

inhibitors reported to date with an IC_{50} = 0.08 μM and is characterized by fluorine substituents on the phenyl rings and a thiourea linker. The terminal benzazol-5-yl-acetic acids were further functionalized with the amino acid glycine. Molecular docking studies were performed on the best compounds **19** and **21** to rationalize their interaction with the enzyme. Invasion assay confirmed the anti-metastatic potential of compounds **19**, **20**, and **21**. Moreover, representative compounds, such as **19**, **22** and **24** proved to inhibit heparanase without interfering with tumor cell proliferation, suggesting useful applications in non-oncology fields [64, 65].

As previously reported, the introduction of a fluorine atom in ortho position to the urea group remarkably improved heparanase inhibitory activity, as seen for urea (**19**) or thiourea (**24**) derivatives compared to their non-fluorinated analogs **22** and **23**, respectively (Fig. 23.18). Moreover, introduction of thiourea as the central core appeared to be a successful approach, leading to increased potency compared to the urea counterpart both in the fluorinated and non-fluorinated series. Acidic side

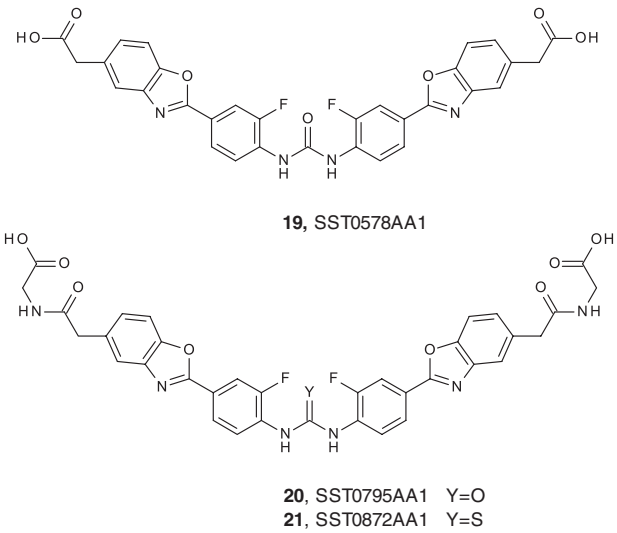

Fig. 23.17 Symmetrical 2-phenyl-benzoxazol-5-yl-acetic acid derivatives

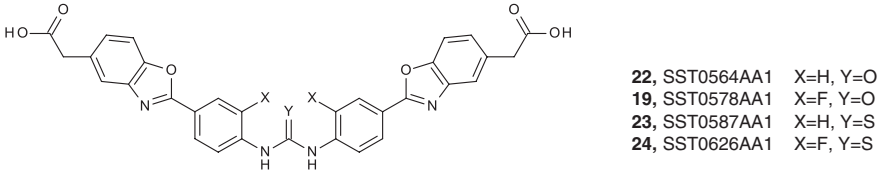

Fig. 23.18 Symmetrical 2-phenyl-benzoxazol-5-yl-acetic acid derivatives with urea and thiourea central core and fluorine substitution

chains, such as acetic acid and/or acetic groups functionalized with amino acids, positively affected inhibitory potencies, suggesting that side chains endowed with increased degrees of freedom could allow a better fitting to the enzyme binding pocket. In silico studies confirmed that the acidic portions of the flexible side chain of compounds **19** and **21** could efficiently interact with heparanase residues within the substrate binding cleft, which might explain the high inhibitory potency observed for these compounds.

Indoles, Carbazoles and Fluorenes

In 2002, InSight Biopharmaceuticals Ltd. claimed a set of compounds endowed with anti-heparanase activity having indole (**25**), carbazole (**26**) or fluorene (**27**) scaffold [66, 67]. Derivatives **25** and **26** are micromolar heparanase inhibitors evaluated in colorimetric assays and, following i.p. administration, they were able to reduce primary tumor growth and tumor metastasis in a mouse melanoma model (Fig. 23.19).

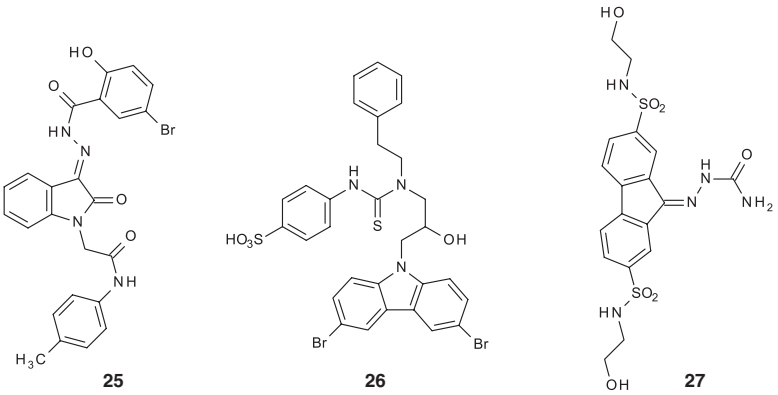

Fig. 23.19 Select indole, carbazole and fluorene derivatives claimed as heparanase inhibitors

Diphenylethers

In the same year, 2002, InSight Biopharmaceuticals Ltd. filed a patent application claiming anti-heparanase activity for diphenylether derivatives (e.g., compounds **28** and **29** in Fig. 23.20) Compound **29** is described as a micromolar heparanase inhibitor able to reduce tumor growth in a mouse model of melanoma following intraperitoneal administration [68].

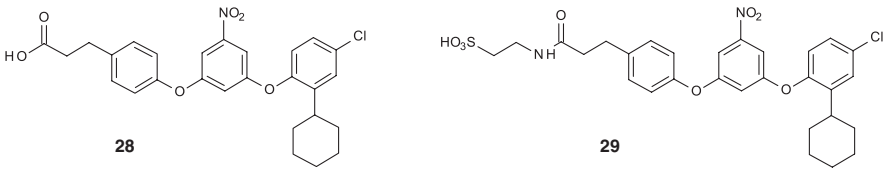

Fig. 23.20 Diphenylether derivatives with anti-heparanase activity

Rhodanines

Rhodanine is a 5-membered heterocyclic compound possessing a thiazolidine core. Some rhodanine derivatives have shown pharmacological properties, although their poor selectivity limits their clinical application. In 2006, InSight Biopharmaceuticals filed a patent application on novel rigidified compounds having a rhodanine-like residue. Acidic derivatives, e.g., compound **30** in Fig. 23.21, were able to inhibit heparanase activity and binding of VEGF and bFGF to heparin, with IC_{50} values in the micromolar range [69].

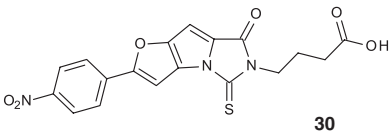

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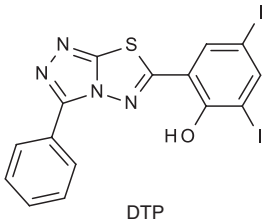
Fig. 23.21 Rigidified rhodanine derivative claimed as a heparanase inhibitor

Triazolo-Thiadiazoles

In 2017, Baburajeev C.P. et al. reported the results of the in vitro screening of a library of small molecules characterized by a variety of scaffolds. 150 compounds were tested for their ability to inhibit the enzymatic activity of human heparanase, identifying [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives as active substances. The most potent anti-heparanase derivative, DTP represented in Fig. 23.22, was able to inhibit proliferation, migration, and invasion of hepatocellular carcinoma and Lewis lung carcinoma cells with IC_{50} values in the micromolar range. Docking studies into the crystal structure of human heparanase provided a hypothesis for the interaction with the substrate binding site of the enzyme [70].



DTP

Fig. 23.22 The [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivative DTP recently identified as a heparanase inhibitor in a screening campaign

Furanthiazole

In 2003, Courtney S.M. et al. filed a patent application on furanthiazole derivatives as heparanase inhibitors. These compounds are furanylthiazole acetic acid derivatives with a 2-chloro-phenyl substituent on the furan ring. Compounds having a benzamide or cinnamamide substituent on the para position of the phenyl ring (e.g., compound **10** in Fig. 23.13) are micromolar/submicromolar heparanase inhibitors, able to inhibit angiogenesis [71]. Some derivatives were also published in 2005 and were further developed into potent benzoxazolyl acetic acid analogs (see dedicated paragraph "Benzazoles") [55].

Quinolines and Quinazolines

Virtual screening of a commercial collection of drugs and drug-like compounds through docking into a homology model of human heparanase and fitting to a ligand-based pharmacophore model identified potential new ligands which were

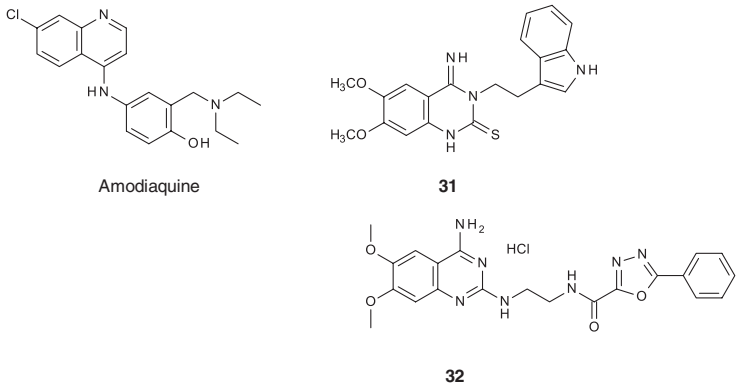


Fig. 23.23 The antimalarial drug amodiaquine and quinazoline derivatives identified as micromolar heparanase inhibitors

then experimentally evaluated for their ability to inhibit the enzyme catalytic activity. This approach highlighted the antimalarial drug amodiaquine (Fig. 23.23) as a micromolar heparanase inhibitor. SAR investigation around the amodiaquine scaffold failed to identify more potent inhibitors [72].

Albeit amino-quinazoline derivatives were mainly investigated as antitumor agents, some functionalized quinazoline compounds are reported as heparanase inhibitors. Two recent patents disclose functionalized dihydro- and tetrahydroquinazoline derivatives with anti-heparanase activity in the single/double digit micromolar range. Most of the claimed compounds are (3-substituted)-3,4-dihydro-4-imino-6,7-dimethoxy-quinazoline-2(1*H*)-thione derivatives, as compound **31** in Fig. 23.23 with micromolar heparanase inhibitory potency, which was evaluated for its ability to prevent/delay the onset of diabetes in mice. In fact, insulin-producing islet beta cells require HS for their survival, and it has been found that normal HS content of beta cells is severely compromised and ultimately ablated during type 1 diabetes onset/progression [73 and Simeonovic et al., Chap. 24 in this book]. Other claimed heparanase inhibitors are 2,4-diaminoquinazoline derivatives, variously substituted on one or both amino groups. Compound **32** (Fig. 23.23) is a micromolar heparanase inhibitor tested in an in vivo mouse model of age-related macular degeneration induced by photo-oxidative damage. The compound, delivered by intravitreal injection, was able to maintain retinal function. Compound **32** and its structural derivatives were therefore claimed as agents for inhibiting the development and progression of ocular inflammatory disorders, such as age-related macular degeneration, diabetic retinopathy, macular edema, etc. [74, 75].

DMBO and Related Spiroheterocyclic Compounds

In 2010, Basappa M.S. et al. simulated the structure of HS monosaccharide pyranose ring with an oxazine ring and reported a novel oxazine derivative, a sugar/pyranoside mimetic compound, DMBO or 2-(2,6-difluorophenyl)-5-(4-

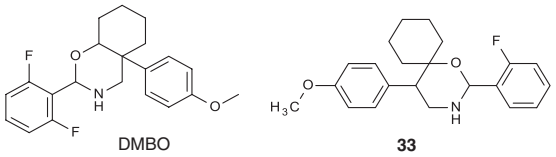


Fig. 23.24 Heparanase inhibitor DMBO and a spiroheterocyclic oxazine derivative

methoxyphenyl)-1-oxa-3-azaspiro[5.5]undecane (Fig. 23.24). DMBO inhibits heparanase activity possibly acting as a substrate mimetic, and was able to target multiple pathways involved in cancer progression, metastasis, and angiogenic events. DMBO was also able to bind several growth factors and cytokines (e.g., TNF-α, HB-EGF, VEGF) and exhibited strong anti-proliferative activity in vitro against osteosarcoma and ovarian tumor cell lines [76]. In 2016, based on the scaffold of DMBO, novel spiroheterocyclic heparanase inhibitors were synthesized with anti-heparanase activity and inhibition of HeLa cells growth. In this series, compound **33** was reported as very active and able to dose- and time-dependently inhibit cervical cancer cell growth and to induce apoptosis [77].

Azasugars and Related Glycopolymers

Azasugars (or iminosugars) are nitrogen derivatives of sugars in which the nitrogen atom replaces the oxygen of the tetrahydropyran ring. Azasugars received considerable attention as carbohydrate mimetics, and both natural and synthetic glycosidase inhibitors carrying an azasugar portion have been reported [78, 79]. Natural azasugars obtained from *Streptomyces nobilis* SANK 60192 with anti-heparanase activity are described in Sect. 23.2.1 – Natural products. Starting from azasugar units, larger molecules were synthesized, to better mimic the natural substrates of heparanase and improve selectivity for the enzyme. Thus, several oligosaccharides containing an azasugar component were investigated as heparanase inhibitors.

The research group directed by Dr. Petitou was involved for many years in the development of heparin derivatives and azasugars. During his long career in Endotis Pharma and Sanofi-Aventis, Dr. Petitou invented fraxiparin, the first Low Molecular Weight Heparin (LMWH) which was tested in clinics and eventually reached the market. Moreover, the first chemical synthesis of the drug fondaparinux was carried out by his team, and he was strongly involved in the development of the drug (it was the first synthetic complex oligosaccharide to be marketed). Overall, six new chemical entities (including idraparinux) issued from Dr. Petitou's research entered development during the last 10 years. His research activities also include azasugars with anti-heparanase activity (e.g., compound **34** in Fig. 23.25) [80].

Disaccharides mimicking the structure of heparin and heparan sulfate were synthesized by Csíki and Fügedi and evaluated as heparanase inhibitors. They contain a D-glucosamine unit α-(1 → 4)-linked to an azasugar analog of L-iduronic acid or D-glucuronic acid (compounds **35** in Fig. 23.26) [81]. In 2001, Takahashi S. et al. [82] reported the disaccharide heparanase inhibitor **36** (Fig. 23.26), which includes a

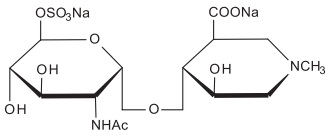


Fig. 23.25 An example of aza-uronic acid with anti-heparanase activity reported in a patent by Sanofi-Aventis

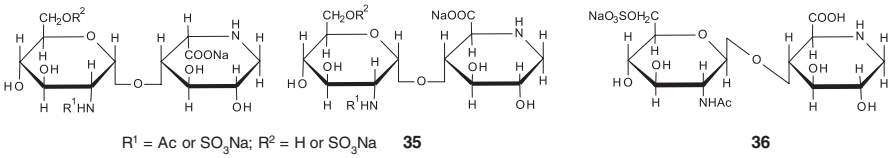


Fig. 23.26 Disaccharide inhibitors of heparanase of containing an azasugar moiety

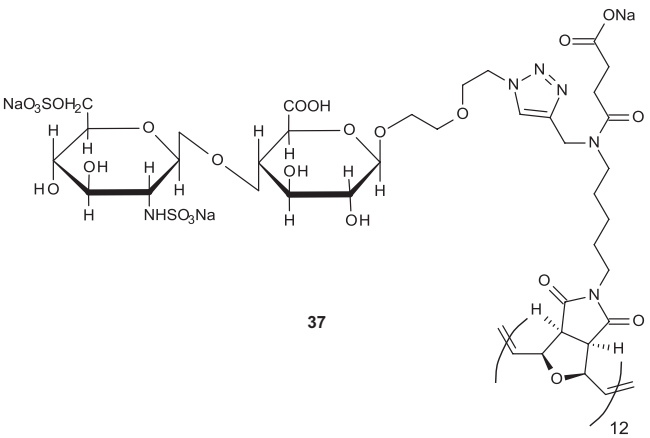


Fig. 23.27 Heparan sulfate-mimicking glycopolymer endowed with picomolar heparanase inhibitory activity

2,6-dideoxy-2,6-imino-L-gulonic acid unit, with micromolar inhibitory potency against cell-extracted heparanase, similar to that of siastatin B analogs described in Sect. 23.2.1.

In a systematic study based on a disaccharide portion linked to polymeric residues, the compound with 12 repeating units of GlcNS(6S)α(1,4)GlcA (compound **37** in Fig. 23.27) was the most potent heparanase inhibitor, with an IC_{50} value in the subnanomolar range. Computational studies were carried out to identify relevant HS-heparanase interactions as a template for the design of HS-mimicking monomers containing the essential disulfated disaccharide component for maximal heparanase inhibition and minimal cross-bioactivity. This synthetic glycopolymer showed minimal interaction with serine proteases in the coagulation cascade and with

several HS-binding proteins, such as angiogenic growth factors and platelet factor 4. Compound **37** possessed no proliferative effects on human umbilical endothelial cells (HUVECs) and exhibited potent antimetastatic activity against 4T1 mammary carcinoma cells [83, 84, 85].

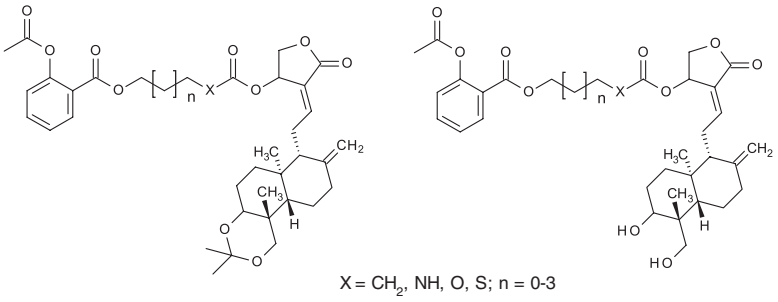


Fig. 23.28 Acetylsalicylic acid derivatives conjugated with andrographolide, claimed as heparanase inhibitor with antitumor activity

Acetylsalicylic Acid and Derivatives

Epidemiological, clinical, and experimental studies testify the impact of long-term use of acetylsalicylic acid in reducing cancer incidence, delaying malignant processes, and decreasing the risk of tumor metastasis and cancer mortality. Investigation on the antitumor mechanism of acetylsalicylic acid led to the discovery of its anti-heparanase activity, shared with salicylic acid, with IC_{50} values in the millimolar range. Acetylsalicylic acid was found to inhibit heparanase-promoted cell migration and invasion of murine melanoma cell lines, and also inhibited VEGF release. In vivo it demonstrated antimetastatic and anti-angiogenic activity, as well as tumor growth inhibition in murine models of cancer [86]. More recently, a patent application claims acetylsalicylic acid derivatives as able to strongly inhibit the activity of heparanase in various tumor cells. In these compounds, acetylsalicylic acid is conjugated with andrographolide (a labdane diterpenoid). The structure of such derivatives is depicted in Fig. 23.28 [87].

Miscellanea

Shiseido Co. Ltd. deposited several patent applications claiming the anti-aging and anti-wrinkle activity of compounds endowed with anti-heparanase activity, useful for amelioration or prevention of skin aging. In fact, decomposition of proteoglycan HS in the basal membrane by aging or photo-aging improves the activity of HS-bound growth factors, such as VEGF-A, which lead to dermis angiogenesis, lymphangiectasis, and elastin breakdown. Inhibition of heparanase activity suppresses

the release of growth factors that accompanies decomposition of HS and allows migration of growth factors between the epidermis and dermis to be controlled, thereby aiding in anti-aging of the skin. Compounds **38–40** (Fig. 23.29) were tested in a simulated skin model to evaluate the permeability and angiogenesis of VEGF in the presence or absence of HS [88]. Also, anti-heparanase and wrinkle improving activities were claimed for cinnamic acid derivatives (e.g., compound **41**) and 4-isobutylresorcinol **42** (Fig. 23.29) [89, 90]. The cyclic carboxamide derivative **43** is reported as a heparanase inhibitor that can be used to prevent skin aging and as a skin whitener, preventing or suppressing skin pigmentation [91].

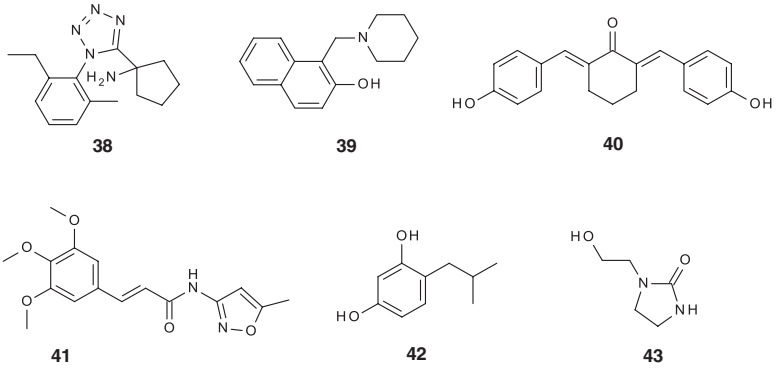
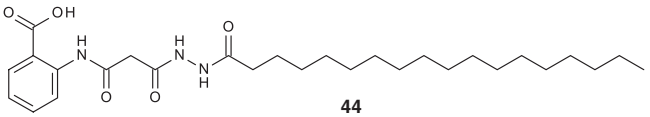


Fig. 23.29 Heparanase inhibitors claimed as wrinkle ameliorating agents by Shiseido Co. Ltd

In 2005, Insight Biopharmaceuticals Ltd. claimed more than one hundred compounds belonging to four different classes as heparanase inhibitors useful in diseases and disorders caused by or associated with heparanase catalytic activity, such as cancer, inflammatory disorders, and autoimmune diseases. Compounds were micromolar or submicromolar heparanase inhibitors, and they were assayed for cytotoxicity on human sarcoma HT1080 cells, inhibition of cell invasion and in vivo antitumor activity. Examples of heparanase inhibitors reported in the patent are compounds **44–47** in Fig. 23.30 [92].

A recent patent application by Leadiant Biosciences SA reports the anti-heparanase activity of several commercially available compounds (e.g., SST0856AA1 and SST0859AA1 in Fig. 23.31) which were identified applying computational techniques. Compounds **48** (SST0856AA1) and **49** (SST0859AA1) were able to block both invasion and adhesion of human cancer cell lines (fibrosarcoma, glioblastoma astrocytoma and osteosarcoma) and significantly inhibited the transcription of genes encoding for pro-angiogenic factors (e.g., FGF1 and 2, VEGF, MMP9) in tumor cell lines [93].



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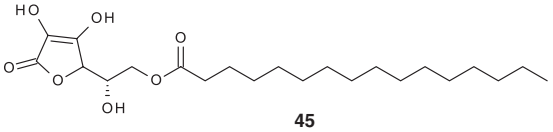

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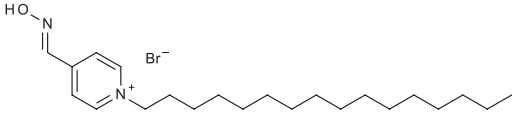

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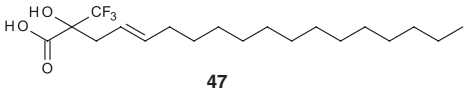

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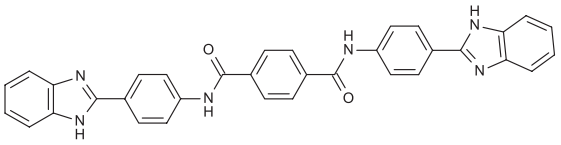
Fig. 23.30 Heparanase inhibitors claimed by Insight Biopharmaceuticals Ltd



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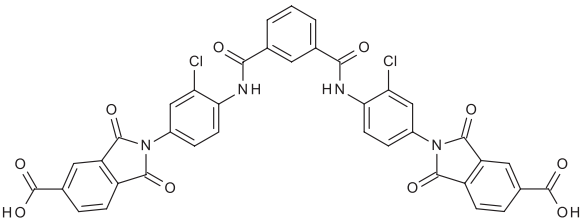

49 (SST0859AA1)

Fig. 23.31 Commercially available heparanase inhibitors active on cancer cell lines described by Leadiant Biosciences SA

23.3 Heparanase-Inhibitor Complex Models

The release of the crystal structure coordinates in 2015 [8 and Wu and Davies, Chap. 5 in this volume] allowed to investigate the putative binding mode of inhibitors to heparanase. Indeed, previous docking studies were based on homology models of the enzyme [72, 86, 94, 95]. The mature form of heparanase is composed of two chains (8 kDa N-terminal chain and 50 kDa C-terminal chain, obtained from proteolytic activation of proheparanase) noncovalently assembled into a $(\beta/\alpha)_8$-TIM barrel domain in which the catalytic site is located, and a carboxy-terminal β-sandwich domain. The substrate binding cleft has an elongated shape, with the catalytic acidic amino acids Glu225 and Glu343 placed in the middle of a narrow channel. At the two extremities, the binding site is surrounded by heparin binding domains HBD-1 and HBD-2 which are two short amino acid sequences rich in polar and basic residues involved in interaction with heparan sulfate substrate [96]. Close to the catalytic residues, a glycine loop acts as the recognition site for the carboxylate group of the substrate glucuronic acid (Fig. 23.32, left). Docking of benzimidazolyl- and benzoxazolyl-acetic acid derivatives (described in Sect. 23.2.2.2) identified a common binding scheme in which the carboxylate interacts with residues mainly from HBD-2. The 2-substituted benzazolyl nucleus is accommodated within the substrate binding site, interacting with relevant amino acids (i.e., the catalytic glutamates and the glycine loop). Conjugation of the terminal acetic group with polar amino acids (e.g., glycine, glutamic acid) led to an increase of potency, likely due to the possibility to undertake additional interactions with amino acids from HBD-2 and its surroundings, while maintaining proper interactions with residues of the substrate binding site. In Fig. 23.32 (right) the docking pose obtained for the glutamic conjugate **17** is represented [62].

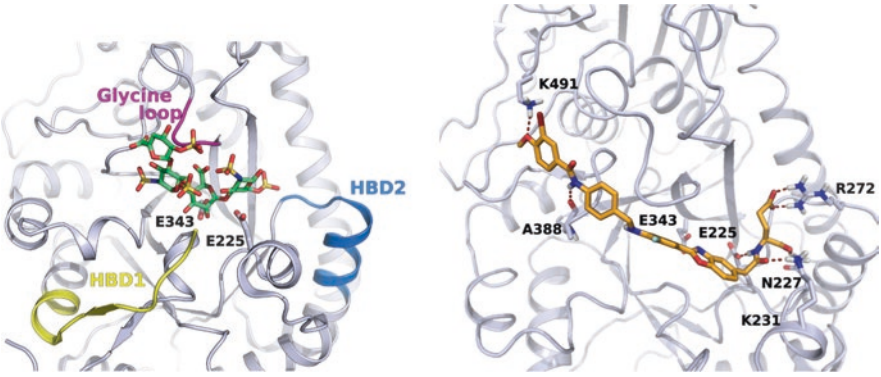

Fig. 23.32 Left: crystal structure of heparanase co-crystallized with a tetrasaccharide inhibitor (pdb: 5E9C). Right: docking pose obtained for glutamic acid-conjugated benzoxazolyl derivative **17** [62]

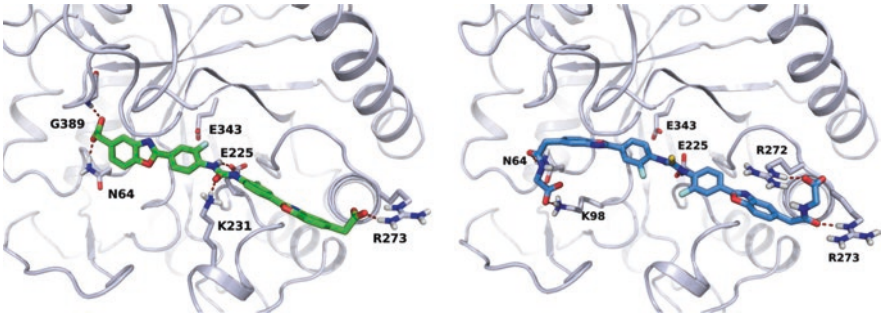

Fig. 23.33 Docking poses of urea (**19**) and thiourea (**21**) symmetrical 2-phenyl-benzazol-5-yl-acetic acid derivatives into the crystal structure of human heparanase [64]

The most potent symmetrical 2-phenyl-benzazol-5-yl-acetate derivatives, carrying a central urea or thiourea fragment, were also docked into the crystal structure of human heparanase to gain insights into their inhibitory mechanism. The compounds were accommodated within the substrate binding cleft, with the (thio)urea portion undertaking hydrogen bonds with catalytic residue E225. Additional interactions were formed between the terminal acetic acid groups (Fig. 23.33, left) or the conjugated amino acids (Fig. 23.33, right) with polar residues from HBD-2 and residues close to HBD-1 [64], likely contributing to the higher inhibitory potency of these compounds compared to the neutral 5,6-dimethyl-benzimidazolyl derivative **3**.

Triazolo-thiadiazole derivatives were also docked into the crystal structure of human heparanase. Compound DTP (described in Sect. 23.2.2.6) interacts with Asn224 and Asp62 in the enzyme catalytic site [70]. Disaccharide-functionalized monomeric precursors of HS-mimicking neo-glycopolymers (described in Sect. 23.2.2.10 and Fig. 23.27) were docked in the crystal structure of human heparanase highlighting direct interactions of the triazole ring with the active site of the enzyme and of the terminal carboxylate with HBD-2 [97].

23.4 Heparin Mimetics

Heparin is a natural medication used worldwide as anticoagulant or blood thinner. It finds multiple clinical applications, from prevention of deep vein thrombosis to pulmonary embolism. Fractionated versions of heparin, known as low molecular weight heparin (LMWH) and ultra-LMWH (ULMWH) are also available. Heparins are used in the treatment of heart attacks and unstable angina, but applications can also be found in oncology. In this field, a major limitation is associated with the anticoagulant activity. For this reason, over the years, efforts have been made to

prepare heparin mimetics, through semi-synthetic or total synthesis approaches, to increase the antitumor activity – as anti-heparanase, anti-angiogenic, anti-inflammatory – and to reduce or eliminate the blood-thinning activity. These mimetics are often designed to increase potency and binding selectivity towards specific proteins involved in disease manifestations [98, 99].

Heparin is a close structural analog of HS and an inhibitor of heparanase. It competes with HS to be accommodated into the substrate binding site, and it is also hydrolyzed by heparanase [100]. However, its anticoagulant activity and the ability to displace growth factors from HSPGs in the ECM and on cell membranes promoted the investigation on poly- and oligosaccharide heparin derivatives devoid of these side effects, while retaining the heparanase inhibitory activity. Beside unfractionated heparins, anti-heparanase activity was also observed for LMWHs. In particular, in 2016, heparin-derived ULMWHs showed anti-heparanase and anti-angiogenic efficacy coupled to a moderate anticoagulant activity [101].

In 2006, Zhao H. et al. reported the anti-heparanase activity of the sulfated oligomannuranate JG3, a semi-synthetic marine-derived oligosaccharide (Fig. 23.34). JG3 inhibited heparanase by binding to known HS binding domains (KKDC and QPLK [96]), and its activity was competitively inhibited by heparin. Moreover, JG3 was able to block the release of basic fibroblast growth factor (bFGF) from its deposit sites in the ECM and to inhibit activation and signaling of bFGF receptor [102].

In 2010, polysulfated penta- and tetrasaccharide glycosides containing α(1 → 3)/α(1 → 2)-linked mannose residues were synthesized as HS mimetics and evaluated for their ability to inhibit heparanase, angiogenesis and tumor growth. They showed anti-heparanase IC_{50} values in the high nanomolar range, similar to that measured for the structurally-related oligosaccharide muparfostat (described in paragraph X.4.1). The tetrasaccharide **50** (Fig. 23.34) showed potent interference with angiogenic growth factors FGF-1, FGF-2, and VEGF and antitumor activity in vivo in a mouse melanoma model resistant to muparfostat. Compound **50** is also characterized by reduced anticoagulant activity and by a good pharmacokinetic profile in rats [103].

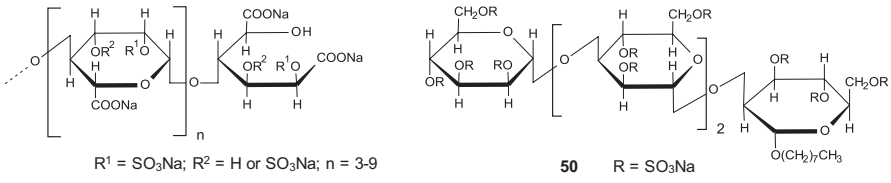

Fig. 23.34 Structure of mannuranate oligosaccharide derivative JG3 (left) and tetrasaccharide sulfate mannose derivative

23.4.1 Heparanase Inhibitors Advanced to Clinical Trials

In the last 20 years, considerable efforts by pharmaceutical companies have led to the development of heparanase inhibitors based on the polysaccharide scaffold of heparin characterized by reduced anticoagulant activity. This is the case of muparfostat, pixatimod, roneparstat, and necuparanib (Fig. 23.35) developed as potent heparanase inhibitors and discussed in detail in other chapters of this book [Chhabra and Ferro; Hammond and Dredge; Noseda and Barbieri, Chaps. 19, 21 and 22 in this volume]. Below is a concise profile of these inhibitors, which so far are the only heparanase-inhibiting compounds that have reached the clinical evaluation stage. Two of them are semisynthetic derivatives of heparin (roneparstat and necuparanib), one is a heterogeneous mixture of sulfomannan oligosaccharides (muparfostat), and the fourth is a synthetic tetrasaccharide conjugated to a steroid moiety (pixatimod).

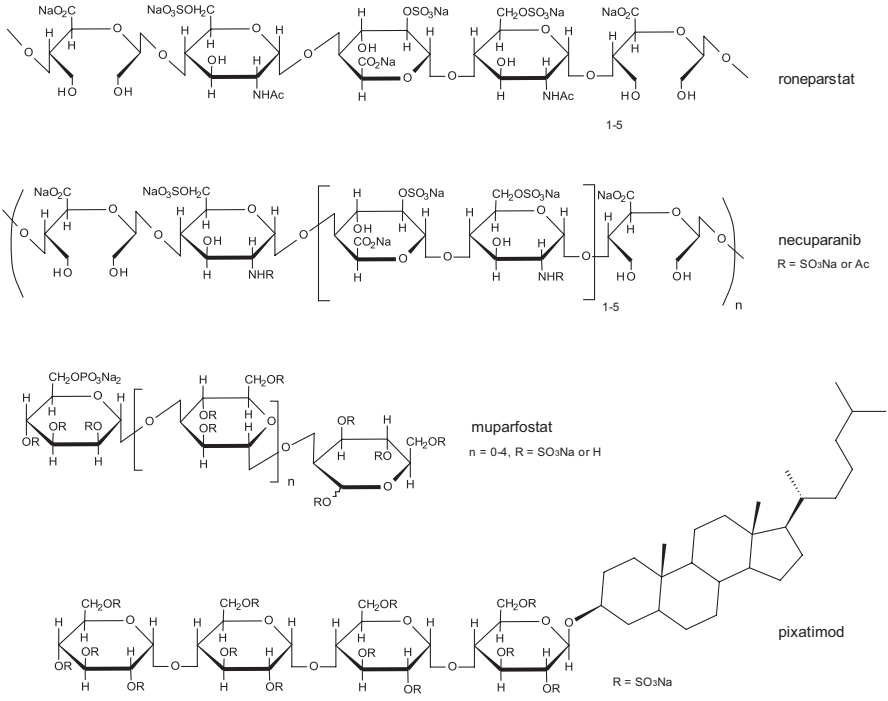

Fig. 23.35 Heparanase inhibitors evaluated in clinical trials

Roneparstat, SST0001

Originally patented by Sigma-Tau IFR, now Alfasigma SpA, roneparstat was then developed by Leadiant Biosciences as part of a comprehensive international collaborative effort where our group was deeply involved in the medicinal chemistry program. Roneparstat is an oxidized-reduced 100% N-acetyl and 25% glycol-split

heparin derivative (Fig. 23.35) [100, 104]. Such chemical modifications on the structure of heparin abolish or reduce to a minimum level its anticoagulant effects, but retain or enhance affinity and inhibitory activity toward heparanase [105]. Detailed analysis of the kinetics of heparanase inhibition by roneparstat and molecular modeling simulations revealed a complex mechanism of interaction between the enzyme and the flexible inhibitor. In fact, inhibition curves can be fitted by a biphasic interaction model consisting of a bimolecular one-to-one binding process at lower concentrations of roneparstat and a multiple interaction, ideally involving two molecules of the inhibitor and one of the enzyme, at higher concentrations. This complex behavior, confirmed by the analysis of a data matrix with variable concentrations of substrate and inhibitor, is consistent with the existence of multiple interaction modes. Molecular models built by docking procedures and molecular dynamics simulations showed that roneparstat can occupy the whole substrate-binding site of heparanase, composed of two heparin-binding domains (HBDs) on both sides of the catalytic spot, but, alternatively, the two HBDs can bind different portions of roneparstat, which may belong to the same or different inhibitor molecules. This can be related to the complexity of the inhibition curve, which shows a deviation from the ideal bimolecular interaction at higher concentrations, where crowding effects can favor multi-molecular interactions [106].

Combination of high inhibition of heparanase, low release/potentiation of ECM-bound growth factors (e.g., FGF-2) and lack of anticoagulant activity points to roneparstat as an antiangiogenic and antimetastatic agent. Roneparstat has been investigated as a potential treatment for cancer, including multiple myeloma (MM) [107, 108, 109], as well as in other non-cancer inflammatory diseases, as reviewed elsewhere [105 and Noseda and Barbieri, Chap. 21 in this volume]. In March 2015, the US FDA granted the product orphan designation for the treatment of MM, followed in Europe, in April 2015, by EMA that granted the product orphan designation for the treatment of plasma cell myeloma. A phase I clinical study has been recently completed successfully [110]. To improve the pharmacokinetic/pharmacodynamic profile of roneparstat, our group has recently developed a new class of biotin-conjugated roneparstat analogs. These derivatives, designed with the purpose of improving tumor targeting, are characterized by the introduction of a biotin moiety in the N-acetyl glycol-split polysaccharide scaffold. The biotin portion allows to maintain the anti-heparanase activity of the parent compound without inducing toxicity [111].

Necuparanib, M402

Developed by Momenta Pharmaceuticals, necuparanib (Fig. 23.35) is a semisynthetic glycol-split heparan sulfate glycosaminoglycan mimetic with reduced anticoagulant activity, produced from unfractionated heparin through sequential oxidation and reduction. Necuparanib binds to and inhibits, apart of heparanase, the activity of multiple growth factors, chemokines, and adhesion molecules, and it has been proposed for the treatment of cancer and metastasis. It interferes with matrix metalloprotease (MMP) family members; reduces metalloproteinase 1 (MMP1), increases tissue inhibitor of metalloproteinase 3 (TIMP3) protein levels, and elevates RNA

expression of TIMP3 [112]. In preclinical studies, necuparanib was investigated both in in vitro and in vivo models of pancreatic cancer [113]. In May 2012, a proof-of-concept phase I/II trial was initiated in advanced metastatic pancreatic cancer which was discontinued in 2016 [114]. Further studies have been conducted until August 2018. No further development has since been reported.

Muparfostat, PI-88

Developed by Progen Pharmaceuticals Inc. more than two decades ago, muparfostat (Fig. 23.35) is a complex mixture of chemically highly sulfated monophosphorylated mannose oligosaccharides, ranging from di- to hexasaccharide, with the major components of pentasaccharide (60%) and tetrasaccharide (30%), derived from the extracellular phosphomannan hydrolysate of the yeast *Pichia holstii* NRRL Y-2488. Muparfostat is a potent heparanase inhibitor, and it inhibits angiogenesis directly by antagonizing the interactions of angiogenic growth factors, such as FGF-2 and VEGF, and their receptors with HS [115, 116]. Muparfostat is associated with decreased cancer cell proliferation, increased apoptosis, inhibition of angiogenesis and with a significant reduction in the number of metastatic tumor lesions. Its administration is associated with side effects, such as anticoagulant activity and immune-mediated thrombocytopenia. In 1988, muparfostat began phase I clinical trials in cancer patients in the UK and Australia and had progressed until Phase III clinical trials for postresection hepatocellular carcinoma [117, 118]. More recently, the conformational equilibrium in solution of the most abundant pentasaccharide component of muparfostat has been investigated by a combination of computational and experimental techniques [119]. Several derivatives of muparfostat were synthesized, comprising sulfated pentasaccharides devoid of the terminal 6-*O*-phosphate group and with modifications at the reducing end of the carbohydrate chain. These compounds were able to inhibit heparanase activity and to tightly bind to proangiogenic growth factors, showing longer in vivo half-lives [120]. Other polysulfated penta- and tetrasaccharide glycosides containing $\alpha(1 \rightarrow 3)/\alpha(1 \rightarrow 2)$-linked mannose residues substituted with lipophilic groups at the reducing end of the molecule were characterized by strong heparanase inhibitory activity, potent anti-angiogenic activity in cell-based and ex-vivo assays, with some derivatives active in melanoma mouse models resistant to muparfostat [103]. Synthetic efforts to prepare sulfated oligosaccharide mannose derivatives of muparfostat, with both $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 2)$ or only $\alpha(1 \rightarrow 3)$ glycosidic bonds led to potent competitive inhibitors of heparanase [121 and Chhabra and Ferro, Chap. 19 in this volume].

Pixatimod, PG545

Zucero Therapeutics (formerly Progen Pharmaceuticals) developed tridecasodium pixatimod (Fig. 23.35), the lead from the PG-500 series of oligosaccharide HS mimetics [122]. Structurally, it is a single entity oligosaccharide, an $\alpha(1 \rightarrow 4)$-linked

tetramaltoside derivative with a lipophilic portion (cholestanol aglycon) at the reducing end of the molecule [123]. Pixatimod interferes with two important processes in tumor development, namely angiogenesis via inhibition of VEGF, FGF-1 and FGF-2, and metastasis via inhibition of heparanase activity [124, 125, 126]. The drug is being investigated primarily for the treatment of cancer, but also explored in non-oncology indications (i.e., inflammatory disease, ocular disease). Kinetics of heparanase inhibition by pixatimod was investigated, and its parabolic competitive behavior was rationalized through a bimolecular model of interaction with the heparanase substrate binding site [127]. A phase I trial of pixatimod was initiated in November 2010 for patients with advanced solid tumors; in September 2011, Progen halted recruitment and was to close the trial after patients showed unexpected local injection site reactions. The company believed the adverse site reactions were specific to humans and were not seen in preclinical studies. Progen reviewed a change from subcutaneous (sc) to intravenous (iv) administration of tridecasodium pixatimod to address the issue and conducted additional pharmacokinetic, safety and efficacy studies. Administration of pixatimod as an iv infusion alleviated the skin reactions associated with sc injection and allowed to determine the maximum tolerated dose in a subsequent phase Ia trial which enrolled patients with solid tumors [128]. Clinical testing has shown pixatimod to be well tolerated as a monotherapy, and it is currently being investigated in combination with the anti-PD-1 drug nivolumab in a pancreatic cancer phase I trial [Hammond and Dredge, Chap. 22 in this volume].

23.5 Conclusions and Prospects

Heparanase is a multifaceted protein endowed with endo-β-D-glucuronidase enzymatic activity and non-enzymatic functions. It plays an important role in regulating critical biological and pathological processes by releasing HS/heparin-bound bioactive molecules, which makes it a promising pharmacological target. However, its role is often debated and not unique. This is why we have recently defined it as a "rainbow pharmacological target" [3]. Preclinical studies on heparanase explored its potential as a biomarker [129, 130, 131] and pharmacological target in several diseases, primarily cancer, but also chronic inflammation, diabetic nephropathy, viral infection, bone osteolysis, thrombosis, and atherosclerosis, apart of more recent investigations on some rare diseases. The challenge for researchers all over the world committed to this target is to find potent, selective inhibitors with favorable pharmacokinetics and acceptable side effects, in order to ensure control of its overexpression and hyperactivity in pathological conditions. To this aim, many approaches have been explored so far, including nucleic acids, proteins, monoclonal antibodies, polysulfated saccharides, and small molecules. This chapter summarizes, to the best of our knowledge, the most relevant small-molecule single compounds and/or chemical classes hitherto investigated as inhibitors of heparanase activity.

Heparanase inhibitors are extremely variegated, comprising natural and synthetic compounds, small molecules and macromolecules, with different chemical and physicochemical properties (e.g., neutral and negatively-charged small molecules, polysulfated oligosaccharides). Inhibitors identified, designed and developed so far have been obtained applying traditional medchem strategies. In the future, given the recent disclosure of the X-rays structure of human heparanase, it is conceivable that new, improved inhibitors will be available which may take advantage of the knowledge of the stereo-electronic requirements for efficient binding interaction with the protein. The interest of the scientific community and pharmaceutical companies in heparanase functions and activity modulation has progressively increased in the last two decades. Figure 23.36 shows the number of documents focusing on heparanase or dealing with its inhibitors and their clinical trials, as well as the number of patent applications and review articles in the last 30 years. While in the last 3–4 years clinical studies are being reduced, the interest towards heparanase and its inhibitors remains constant, at high levels. It is the opinion of the authors that this trend is suggestive of a change of pace that might be recorded in the coming years, from a major interest for substrate mimetic macromolecules/heparin derivatives to small molecules, especially, as mentioned above, after the publication of the X-rays structure of human heparanase [8]. On the other hand, it is difficult to imagine a successful result with biological inhibitors (antibody, antisense oligonucleotide, saccharides), at least in a short time, given that the attempts pursued so far have not led to results worthy of interest for clinical application.

Overall, the scientific and medical interest, the biological information available, the strategies to design and develop heparanase inhibitors are mature enough to support the investigation and approval of heparanase inhibitors.

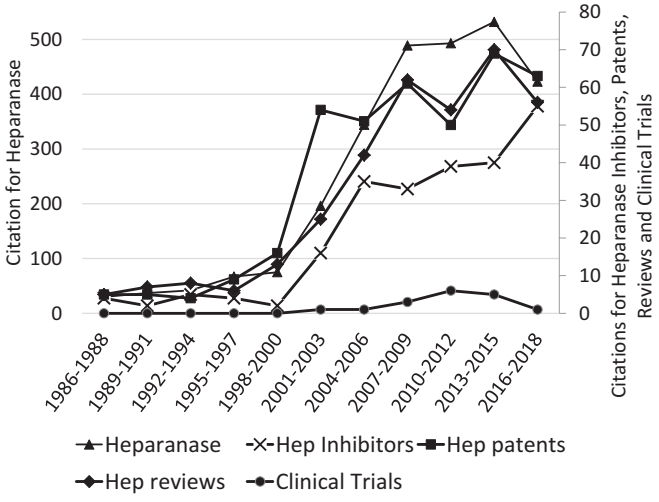

Fig. 23.36 Chemical Abstract (SciFinder®) data analysis. Distribution of topic "Heparanase" (left Y axis) and "Heparanase inhibitors", "Heparanase Patents", Heparanase reviews", and "Heparanase clinical trials" (right Y axis) from 1986 to 2018

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Part VI
Other Diseases and Indications

Chapter 24
Heparanase and Type 1 Diabetes

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24.1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which autoreactive T cells and other inflammatory leukocytes destroy insulin-producing cells (beta cells) in the pancreas. The beta cells are localized in numerous small spherical-like endocrine structures called the islets of Langerhans. The islets are distributed throughout the pancreas but in the human pancreas, the islet density is higher in the tail region of the pancreas [1]. In addition to beta cells, the islets also contain alpha cells that produce glucagon, somatostatin-producing delta cells and other minor cell types (pancreatic polypeptide (PP)-producing cells and ghrelin-secreting cells). Collectively, the hormones produced by the different islet endocrine cell populations regulate glucose metabolism. The clinical onset of T1D is marked by a chronic loss of beta cell mass which ultimately leads to loss of insulin production and elevated blood glucose levels (hyperglycemia). In both non-obese diabetic mice (a model of spontaneous autoimmune T1D that resembles human T1D) and in man, inflammatory leukocytes migrate into the pancreas, infiltrate the islets and selectively destroy the beta cells [2–6].

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From an immunological standpoint, the basis for the specificity of T1D disease for beta cells is attributed to defective negative selection during T cell development in the thymus, loss of tolerance to beta cell-specific autoantigens and export of beta cell-specific autoreactive T cells to the circulation and peripheral lymphoid tissue (i.e., loss of central tolerance to beta cell autoantigen). In addition, impaired peripheral tolerance regulating the expansion of autoreactive T cell clones and/or the emergence of neoepitopes in the periphery which fail to be presented in the thymus also contribute to the development of beta cell-specific autoimmunity [5, 7, 8]. Recent studies of islet and beta cell biology have revealed critical roles for heparan sulfate (HS) proteoglycans (HSPGs) in shaping the architecture of islets, islet differentiation as well as beta cell survival and function. In this review we will summarise the evidence for: (i) the unusually high content of highly sulfated HS in beta cells, a property which renders them exquisitely vulnerable to damage by heparanase, the only mammalian HS-degrading endo-β-D-glycosidase [9–11], (ii) heparanase as a key destructive mechanism for islets/beta cells, cell surface phenotypic marker of inflammatory leukocytes and therapeutic target for T1D, (iii) heparanase as an intracellular regulator of gene expression, influencing the activity or function of immune cells and possibly beta cells, and (iv) heparanase as a mediator of vascular damage and remodeling in long-term established diabetes, resulting in serious secondary complications.

24.2 Heparan Sulfate (HS) Is Highly Expressed in Normal Pancreatic Islets

Pancreatic islets are uniquely enriched in HS. In normal healthy islets, HS is localized in the peri-islet basement membrane (BM) in the form of the HSPG perlecan [12, 13] and is highly expressed, albeit with different sulfation patterns, in islet beta and alpha cells [14–17]. HS exists as linear chains of a repeating disaccharide composed of uronic acid (glucuronic acid or iduronic acid) and *N*-acetylated glucosamine. HS chains are chemically modified primarily by *O*- and *N*-sulfation, and epimerization of glucuronic acid to iduronic acid. However, these modifications are variable along the chain length, resulting in enormous heterogeneity with regions that are more highly sulfated interspersed with other regions that are relatively less-sulfated [18–21]. Significantly, these patterns can vary in a tissue- or cell- specific manner [16, 19, 22].

24.2.1 Peri-Islet Basement Membrane

Both mouse and human islets are surrounded by a BM that consists of collagen type IV, laminin, nidogen and the HSPG perlecan [12, 13, 23]. Based on this composition, the boundary of normal islets in situ in the pancreas conforms to a conventional BM

[21, 24]. Perlecan is a large HSPG (400 kDa) and the associated HS chains (up to ~ 400 residues in length) interact with other matrix proteins to form a densely-packed matrix network [10], a property which would be expected to stabilize the islet architecture. In addition, the high negative charge of HS (due largely to sulfate groups) allows the chains to bind growth factors, cytokines and chemokines, establishing a local depot for these bioactive proteins within BMs [10, 21, 25]. Importantly HS in the islet BM, as in sub-endothelial BMs, contribute to a barrier structure or sieve for protecting the islets against invading or migrating cells [10, 12, 21, 26], a property that is highly relevant to the pathogenesis of T1D (see Sect. 24.4.). The origin of the matrix proteins that form the islet BM during islet differentiation is difficult to decipher. However, perlecan in the mouse islet BM was found to colocalize with von Willibrand factor, suggesting that the BM matrix proteins may be derived from endothelial cells [23]. Studies of isolated islets and beta cell lines have provided further support for this concept. During the process of islet isolation by collagenase-mediated digestion of mouse and human pancreas, the BMs of islets are destroyed and are not repaired during culture [27, 28]. Subsequent islet isotransplantation studies demonstrated that the reconstitution of islet BMs occurs early after transplantation from matrix proteins secreted by vascular endothelial cells which transiently migrate around the periphery of the engrafted islets [28]. RT-PCR analyses have also demonstrated that several beta cell lines lack perlecan mRNA, further indicating that this islet BM HSPG is unlikely to be produced by beta cells [16].

24.2.2 Beta Cells

In addition to the conventional localization of HS in the extracellular matrix (ECM), BMs and on cell surfaces [21, 25], histochemical staining with Alcian blue showed that beta cells have unusually high levels of intracellular HS [17, 29]. Immunohistochemical and immunofluorescence staining of human pancreas sections and intracellular flow cytometry using 10E4 anti-HS monoclonal antibody (mAb) further revealed that human and rodent beta cells contain highly sulfated HS [14, 16, 17]. Specifically, 2-*O* and 6-*O* sulfation, as well as N-acetylation/sulfation, were observed in rat beta cells, confirming the high sulfation status of beta cell HS [16]. Discrepancies in immunohistochemical or immunofluorescence detection of beta cell HS by 10E4 mAb exist in the literature and can be attributed to mAb dilution and differences in antigen retrieval methodology, with pronase digestion being a critical preparatory step [14, 23]. Intracellular HS in mouse and human beta cells correlated with intra-islet staining for the core proteins of several HSPGs, including collagen type XVIII, syndecan-1 and the part-time HSPG, CD44 [14, 29]. RT-PCR analyses of sorted beta cells and the MIN6 beta cell line identified a more extensive panel of HSPGs, including other syndecans (2–4) as well as glypicans (1 and 4) [15, 30, 31]; furthermore, immunostaining of rodent pancreas detected syndecan 4 core protein in beta cells [23].

Multiple functions have been identified for HS in beta cells. The fortuitous discovery that islets lose much of their HS content but retain their HSPG core proteins after their isolation revealed a critical role for the HS chains of HSPGs in maintaining beta cell survival [14, 17, 29]. Staining with vital fluorescent dyes and flow cytometry analyses unquestionably demonstrated that after culture with highly sulfated HS but not under-sulfated HS, the viability of beta cells was significantly restored when compared to untreated control cells [14, 17]. Protection by exogenous HS mimetics (i.e., HS replacers) was dose-dependent [17]. Subsequent screening of HS-like compounds identified a highly restricted repertoire of HS replacers for beta cells, dependent on sulfation status, oligo- or poly- saccharide length and/or chain flexibility (Table 24.1). Recent studies using a fluorescently labeled HS

Table 24.1 Ability of compounds to rescue beta cell viability and induce ROS resistance

Compound*	(kDa)	Viability (%)	Resistance to H_2O_2 (ROS)
Heparins			
Porcine mucosal heparin	12.5	**94**	+
-glycol split,	10	**97**	+
-glycol split, deNS, reNA	10	**97**	+
Low Mol Wt Heparin (Enoxaparin)	3	38	-
-peroxidolysis	3	27	-
-peroxidolysis-glycol split	3	**92**	+
-nitrous acid-glycol split	3	30	-
Sulfated oligosaccharides			
PI-88 (20%tet / 70%pent)	2-2.5	**92**	+
Maltohexaose sulfate	3	**96**	+
Maltopentaose sulfate	2.5	**93**	+
Maltotetraose sulfate (75%tet/25%pent)	2	39	-
Bis-lactobionic acid amide (C12 link)	2	64	-
Other polysaccharides			
Dextran sulfate	5.5	**90**	+
Pentosan PS	5	**91**	+
HS High S	12-15	**87**	+
HS Low S	15	29	-
Chondroitin sulfate A	20	50	-
Chondroitin sulfate B	30	46	-
Chondroitin sulfate C	60	32	-
Chondroitin sulfate D	~60	32	-
Hyaluronic acid (HA) low MW	80	75	-
HA decasaccharide	2	66	-
HA	>1mDa	31	-
Chitosan	100	30	-
Fucoidin (F. vesiculosis)	20	80	±

* Shaded region identifies compounds with both protective properties.

replacer (FITC-heparin) and correlative light and electron microscopy (CLEM) demonstrated rapid uptake of heparin by isolated mouse beta cells and sequestration of the HS replacer within mitochondria and insulin granules (unpublished data). Significantly, the capacity of exogenous HS to protect beta cell viability correlates invariably with the establishment of resistance to hydrogen peroxide-induced beta cell death, i.e., oxidative damage (Table 24.1). Thus, experimental evidence using isolated mouse and human beta cells and the INS1 beta cell line strongly support the idea that HS acts as a non-enzymatic anti-oxidant or quencher of reactive oxygen species (ROS) [14, 16, 17]. This property is consistent with the site-specific accumulation of HS replacer in intracellular compartments that generate high levels of ROS (mitochondria and insulin granules). Due to their high metabolic and biosynthetic activities, beta cells produce high levels of ROS via oxidative phosphorylation (in mitochondria), disulfide bond formation (during insulin biosynthesis/folding) and exocytosis of insulin (from insulin granules) [32–34]. The ROS-quenching mechanism of HS may involve a structural framework for supporting the oxidation/reduction of metal ions (i.e., forming a redox cycle), similar to that found in certain bacteria [35, 36] and for anti-oxidant enzymes (e.g., superoxide dismutase) [37]. The levels of anti-oxidant enzymes are low in beta cells [38, 39] and although their gene expression can be induced [40, 41], the delayed effect could be disadvantageous to beta cell viability and the demand for insulin secretion. Highly sulfated HS acting as a non-enzymatic ROS quencher in beta cells, on the other hand, would offer the major advantage of a constitutive mechanism for immediately neutralizing locally generated ROS to prevent cellular toxicity and thus maintain beta cell survival (Fig. 24.1a).

HS also plays an important role in insulin secretion. HS-deficiency due to blockade of HS synthesis in Exostosin-like 3 knockout (Extl3KO) beta cells decreased insulin secretion in response to high glucose [15]. In particular, Syndecan-4 and 3-*O* sulfated HS (3-*O*-sulfated, *N*-sulfated ±6-*O* sulfated glucosamine residues) are essential for normal insulin secretion in MIN6 beta cells [30, 31]. Importantly, the treatment of isolated islets with heparanase or bacterial heparitinase was found to degrade islet HS and induce beta cell apoptosis [15, 17]. Thus, the parallel dampening of glucose-stimulated insulin secretion could potentially have been impacted by impaired beta cell viability [17]. Nevertheless, these early studies demonstrated the sensitivity of beta cell HS to enzymatic cleavage.

24.2.3 Alpha Cells

Glucagon-producing alpha cells in islets contain HS in intracellular granules and at the cell surface. However, in contrast to beta cells, alpha cell HS contains more regions that are unsulfated or less sulfated (containing only *N*- and 2-*O* sulfation) and show a relative lack of 6-*O* sulfation [16]. While speculative, negatively charged HS chains in alpha cell granules may bind to positive charges carried by glucagon protein [42] potentially aiding their storage in the secretory granules, as previously

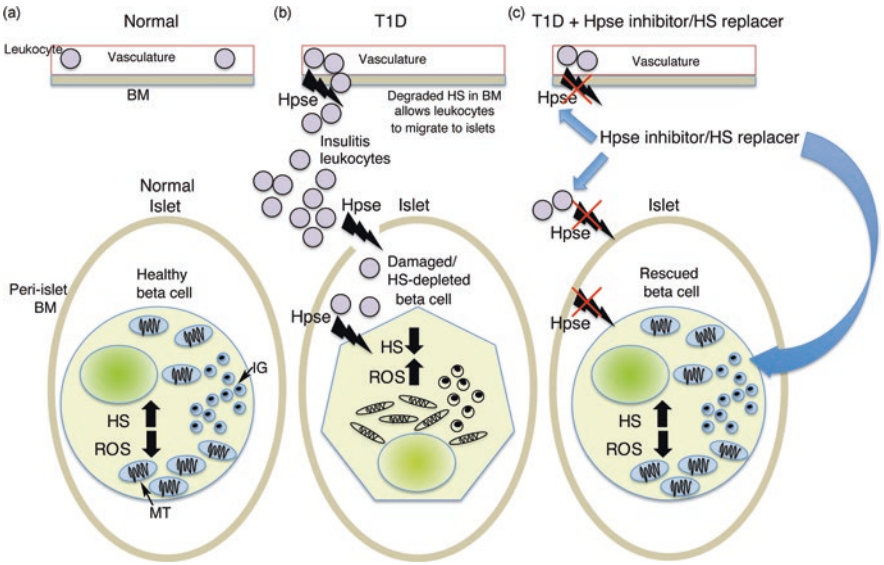

Fig. 24.1 HS status of normal and T1D beta cells, contribution of leukocyte heparanase (Hpse) to T1D development, and protection from T1D progression using heparanase inhibitor/HS replacer drug therapy. (a) High levels of HS (blue) inside normal beta cells [in mitochondria (MT) and insulin granules (IG)] neutralise reactive oxygen species (ROS) and preserve beta cell survival; (b) Hpse-mediated migration of leukocytes across pancreatic sub-endothelial basement membrane (BM) and peri-islet BM in T1D, and beta cell death due to loss of intracellular HS and increased oxidative damage; (c) Dual activity Hpse inhibitor/HS replacer drugs impede leukocyte migration, rescue beta cells by HS replacement and halt T1D progression

suggested for mast cells in the immune system [43]; such ionic interactions could potentially also occur in the insulin granules of beta cells. Alpha cell HS and fibroblast growth factor (FGF) may be involved in FGFR-mediated signaling in beta cells, an interaction which could mitigate toxicity due to high exogenous levels of glucose, lipid, and cytokines, particularly in the context of Type 2 diabetes (T2D) [44].

24.3 Islet Cell Heparanase

Heparanase is synthesized as a pre-proenzyme which undergoes post-translational processing to form a 65 kDa proenzyme. The cysteine protease Cathepsin L cleaves the proenzyme to form catalytically active heparanase, a heterodimer consisting of two polypeptides (48 kDa (mouse) - 50 kDa (human) and 8 kDa) [10, 45, 46]. Heparanase is expressed endogenously in pancreatic islets. Quantitative RT-PCR analyses revealed ~20-fold higher levels of heparanase transcripts in isolated islets from normal (non-autoimmune) and immunoincompetent NODscid donors,

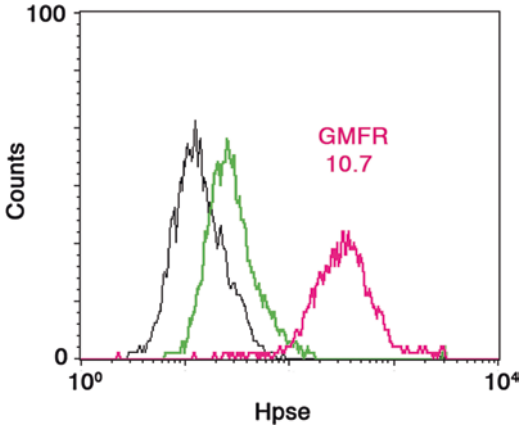

Fig. 24.2 Heparanase is expressed intracellularly in beta cells. Detection by flow cytometry of intracellular heparanase in freshly isolated islet beta cells using HP3/17 anti-heparanase (Hpse) mAb (pink histogram). Mouse $IgG2_{bk}$ was used as the isotype control (green histogram). Black histogram shows islet cell autofluorescence. GMFR, geometric mean fluorescence ratio versus the isotype control. Data are representative of n = 3 independent experiments

compared to normal mouse kidney [17]; heparanase mRNA was also detected in isolated rat islets [16]. Immunodetection of heparanase protein in islets/islet cells is influenced by the choice of anti-Hpse antibody and the method of detection. Weak cell surface heparanase immunostaining was found in the islets of mouse and human pancreas using HP130 anti-heparanase mAb and a polyclonal anti-HPA1 antibody (Insight Biopharmaceuticals) [14, 17]. In contrast, heparanase can be easily detected intracellularly in isolated primary mouse and human beta cells by flow cytometry using HP3/17 anti-heparanase mAb (Insight Biopharmaceuticals) (Fig. 24.2); furthermore, Western blotting identified both inactive and active forms of heparanase in islets from non-autoimmune NODscid mice [17]. Islet heparanase, however, is probably not beta cell-specific since *Hpse* transcripts have also been reported in the aTC1–6 alpha cell line [16].

Endogenous heparanase in islet beta and alpha cells is likely to function in the homeostatic turnover of intracellular and cell surface HS [18, 47, 48]. As with other cell types, HS degradation would be expected to take place in cytoplasmic endosomes or lysosomes [49–51], consistent with the observed intracellular localization of beta cell heparanase (Fig. 24.2). During heparanase synthesis, latent heparanase is exocytosed and then taken up into endosomes/lysosomes where it becomes activated by Cathepsin L to cleave HS [45, 47, 52, 53]. It is possible that the weak staining of heparanase on the surface of beta cells reflects transient membrane expression (anchored possibly by binding to syndecan-1) prior to its internalization into endosomal compartments and lysosomes, as reported in other models [47]. In addition, lysosomes can fuse with autophagosomes, regulating the turnover of cellular components [54]. It is conceivable that lysosomal heparanase could contribute to the

turnover of HS-containing intracellular organelles via homeostatic autophagy, a process required for normal beta cell function [55]. In addition to heparanase, other degradative enzymes including exoglycosidases and sulfatases could also contribute to the breakdown of HS fragments [49–51, 56]. Iduronate-2-sulfatase protein was identified in the lysosomes of both beta- and alpha- cell lines and could contribute to the turnover of intracellular HS [57]. Heparanase in beta cells could also have non-catalytic roles in directly or indirectly regulating gene expression (see Sects. 24.6.1 and 24.6.2). Importantly, the multiple functions of endogenous heparanase are likely to support the survival and function of normal beta cells rather than contribute to beta cell destruction in T1D.

24.4 Exogenous Heparanase: A Novel Destructive Mechanism in the Pathogenesis of T1D

The hallmark pathological features of T1D in NOD mice include (i) initial peri-islet accumulation of inflammatory leukocytes (insulitis) and (ii) their subsequent invasion into the islet tissue [4, 6], with the latter culminating in beta cell destruction, progressive loss of insulin production and hyperglycemia (clinical onset of T1D). In contrast, gross non-invasive peri-islet insulitis is less frequently observed in human T1D [3, 4]. T1D autoimmune disease is a chronic process marked by an asynchronised attack on the islet population, with distinct regions of the pancreas being targeted progressively [58, 59]. However, compared to human T1D pancreas, the inflammatory response is more pronounced in the NOD mouse [4]. Islet-infiltrating leukocytes can deploy a panel of destructive agents to damage beta cells. Importantly, autoreactive CD8 T cells, recognizing beta cell autoantigens in the context of Class I Major Histocompatibility Complex (MHC) provide fundamental specificity for damage to beta cells and not other islet endocrine cells. In the islet microenvironment cell-cell (T cell-beta cell) contact is essential for perforin/granzyme-mediated cytotoxicity and/or Fas-FasL death receptor signaling [60, 61]. The local production of cytokines, particularly IFNγ and TNFα, by autoreactive CD4 T cells can induce the production of chemokines by beta cells (particularly CXCLl0) and exacerbate leukocyte recruitment [62, 63]. Activation of the NLRP3 inflammasome in islet cells (presumably beta cells) is critical for the expression of islet-derived CCL5 and CXCL10 chemokines in NOD mice [64]. Cytokine-mediated induction of iNOS expression in beta cells increases intracellular reactive nitrogen species (RNS) and ROS levels which are directly toxic to beta cells [65]. In addition, ROS produced by cytokine-activated antigen-presenting cells (APCs) such as macrophages can damage beta cells and together with T cell-derived ROS are essential for the diabetogenic effector function of autoreactive T cells [62, 66]. How non-beta islet endocrine cells escape damage due to this non-specific inflammation in the islet microenvironment is perplexing. While beta cells are highly sensitive to oxidative damage, this property alone may not fully account for beta cell-specific damage in T1D.

We have previously discovered that heparanase plays a critical role in the development of T1D in NOD mice [17, 26]. We found that heparanase transcripts were upregulated in isolated islets (with insulitis) from prediabetic adult donors and correlated with a significant increase in CD45 mRNA expression, suggesting that the major increase in heparanase gene expression was attributable to the insulitis-associated leukocytes. In parallel, (i) the active 48 kDa form of heparanase protein, as detected by Western blotting analyses of isolated NOD islets (with insulitis), also increased with the progression of T1D disease, and (ii) immunohistochemical staining of pancreas sections confirmed cell surface expression of heparanase on insulitis leukocytes, particularly at the interface between the insulitis and the islet tissue [17, 26]. Likewise, we have reported that heparanase is expressed at the cell surface of insulitis leukocytes in human pancreas from recently diagnosed T1D cadaver donors [14], reflecting the clinical importance of the enzyme in T1D disease.

Catalytically active heparanase is presented on the surface of leukocytes during T1D disease progression and critically impacts the disease process at multiple levels including leukocyte migration, islet invasion and beta cell damage/death [17, 26]. As in other models of inflammation, heparanase (with optimal activity at pH 5.5–6.0) cleaves HS in the sub-endothelial BM to facilitate the infiltration of leukocytes into nearby tissue [10, 21, 67], including pancreas tissue during T1D development. In the NOD mouse, leukocytes are then recruited around the periphery of the islets forming a characteristic non-destructive or benign insulitis [68, 69]. Eventually, the insulitis leukocytes in both mouse and human T1D produce matrix-degrading enzymes, including heparanase and proteases, that convert the status of the insulitis to a destructive phenotype [13, 17, 26]. This shift is marked by activation of leukocyte-derived heparanase either by the local production of Cathepsin L [70] or due to the moderately acidic pH of inflammatory sites [71]. Active heparanase then solubilizes HS in the islet BMs, facilitating the infiltration of immune cells into the islet cell mass [12, 14, 17, 26]. Ongoing production of active heparanase by intra-islet leukocytes results in degradation of the highly sulfated HS in islet beta cells, as observed by our histochemical, immunohistochemical and immunofluorescence studies of NOD and T1D human pancreas sections [14, 17, 26, 72]. Based on our in vitro studies, the depletion of islet HS renders beta cells highly sensitive to oxidative (ROS-mediated) damage and death (Fig. 24.1b) [14, 17, 72]. Unlike beta cell destruction by autoreactive T cells, a process which is autoantigen-specific, heparanase-mediated damage represents an important supplementary antigen-independent mechanism of beta cell demise. Additionally, elevated endogenous levels of ROS/RNS in beta cells due to cytokine-induced iNOS expression could exceed the protective capacity of beta cell HS which normally functions to quench moderate levels of ROS generated during metabolism. Excessive intracellular levels of ROS/RNS could possibly depolymerize or solubilize intracellular HS chains [73, 74]; however, this process, requiring *de novo* iNOS gene expression, would be less efficient than the degradation of HS by leukocyte-derived heparanase. The proportion of islets succumbing to damage by heparanase and other destructive immune mechanisms increases during the progression of T1D autoimmune disease, ultimately

reaching a point at which insufficient production of insulin results in hyperglycemia and the clinical onset of T1D. Although islet alpha cells also contain HS, the alpha cell mass is not destroyed in T1D and can even undergo expansion [14, 75]. This paradox could be explained by the presence of less-sulfated HS (2-*O* and *N*-sulfated or non-sulfated sugars) and absence of 6-*O*-sulfated glucosamine in alpha cells and the expected selective cleavage of glycosidic bonds between glucuronic acid and *N*-sulfated or *N*-acetylated/6-*O* sulfated glucosamine by leukocyte-derived heparanase [16, 50, 76, 77]. Importantly, alpha cells could also compensate for any partial loss of intracellular HS during T1D via anti-oxidant enzymes which are expressed at much higher levels than in beta cells [78].

Although T1D is regarded as a T cell-dependent autoimmune disease, surprisingly we have found that heparanase expression is significantly increased on myeloid cells but not lymphoid cells in the peripheral blood of pre-T1D and T1D-onset NOD mice (Fig. 24.3). Moreover, peak levels of cell-surface heparanase were observed on myeloid leukocytes in corresponding insulitis lesions (Fig. 24.3a). These findings suggest that (i) heparanase levels on myeloid leukocytes in peripheral blood act as a sensitive biomarker of islet pathology and damage prior to the clinical onset of T1D, (ii) myeloid leukocytes may function as heparanase-dependent path-makers enabling other immune cells (e.g., T cells) to also invade islets and destroy beta cells, and (iii) during the progression of chronic T1D autoimmune disease, myeloid leukocytes and lymphoid cells (T cells) may directly contribute to beta cell damage by both heparanase -dependent and -independent mechanisms. Consistent with our findings in NOD mice, only circulating monocytes showed significantly enhanced

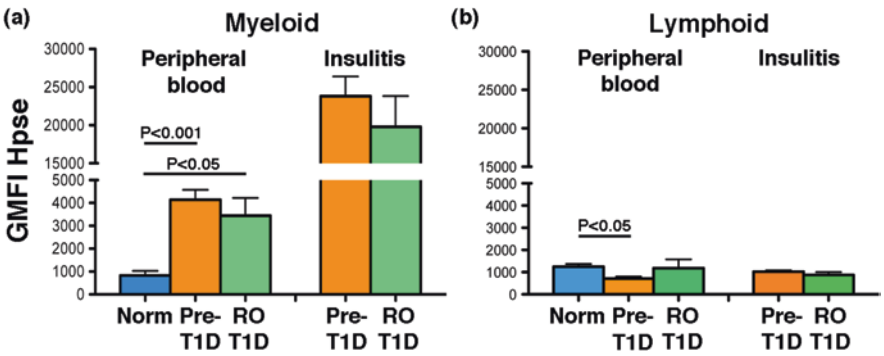

Fig. 24.3 Expression of heparanase (Hpse) on peripheral blood and insulitis leukocytes in prediabetic and recent T1D-onset NOD female mice. Flow cytometry analyses show that Hpse expressed on $CD45^{+}CD11c^{-}CD11b^{+}$ myeloid cells (a) but not $CD45^{+}CD11c^{-}CD11b^{-}$ lymphoid cells (b) is significantly upregulated 4-5 fold in NOD peripheral blood compared to normal B6.SJL controls (Norm); Hpse levels are further amplified by myeloid cells in NOD insulitis. B6.SJL islets lack insulitis and were not tested. B6.SJL (Norm; blue bar; n=14), prediabetic (pre-T1D) NOD/Lt at 12-18 weeks of age (orange bar; n=10) and recent-T1D onset (RO) NOD/Lt (green bar; n=6). GMFI, geometric mean fluorescence intensity. P<0.001, P<0.05, non-parametric ANOVA with Dunn's multiple comparisons test

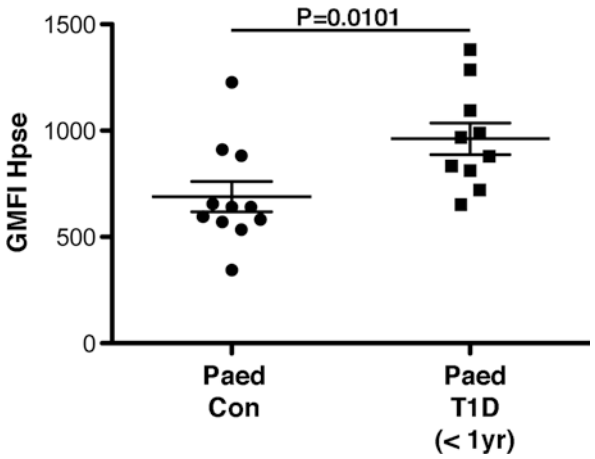

Fig. 24.4 Heparanase expression on human peripheral blood monocytes from healthy controls and recent-T1D onset (RO) donors. Flow cytometry data show mean Hpse GMFI ± SEM for $CD45^{+}CD14^{+}$ monocytes from paediatric donors < 1 yr post-T1D onset (Paed T1D; n=10), compared to paediatric controls (Paed Con; n=11). Hpse staining on circulating T1D monocytes is significantly increased 1.4-fold compared to healthy controls. GMFI, geometric mean fluorescence intensity. P=0.0101, Mann-Whitney Test

heparanase levels in children recently diagnosed with T1D (< 1 year) (Fig. 24.4). Myeloid cells may also provide an exogenous source of Cathepsin L to activate heparanase on nearby cells in T1D, as previously reported for a mouse model of colitis [70]. The low incidence of T1D in Cathepsin L-knockout NOD mice, however, could be due to sub-optimal antigen presentation and/or diminished heparanase activation [79].

24.5 Dual Activity Heparanase Inhibitors/HS Replacers Represent a New Class of Therapeutic for T1D

We confirmed a critical role for heparanase in the development of T1D by demonstrating a significant ~50% reduction in the incidence of T1D in NOD mice treated long-term with PI-88, a potent heparanase inhibitor [17] (for more information see Chapter 3). Subsequent studies demonstrated comparable protection against T1D onset after treatment with chemically modified heparins lacking anticoagulant activity (unpublished data). PI-88 therapy significantly reduced islet invasion by insulitis leukocytes and better preserved the HS levels in islets with destructive insulitis. These phenotypic modifications are best explained by inhibition of leukocyte migration across the peri-islet BM as well as the ability of PI-88 to also act as HS replacer, respectively [17]. In fact, PI-88 was as effective as heparin in replacing

lost HS in isolated beta cells and providing protection against oxidative damage (Table 24.1) [17]. Long-established hyperglycemia in rats was not ameliorated by 14 day-treatment with PI-88, clearly indicating that the drug does not directly modulate glycemia [80]. Our studies, therefore, identified PI-88 as a first-in-class dual activity heparanase inhibitor/HS replacer therapeutic for mediating protection against T1D (Fig. 24.1c).

Currently, there is no cure for T1D. Although T cells are considered to be the major players in beta cell destruction [2, 3], recent clinical trials testing the blockade of T cell activation and function (anti-CD3 mAb, CTLA4Ig), transient depletion of B lymphocytes (antigen-presenting cells), as well as cytokine-based strategies for immunomodulation in new-onset T1D patients have resulted in only a transient reduction in insulin requirements and a limited delay in T1D progression [81–86]. These disappointing outcomes have identified a critical need to develop more effective therapeutics as well as combinations of therapies for impeding disease progression and for rescuing beta cells that still remain at T1D diagnosis. While PI-88 uniquely targets inflammation in T1D and protects beta cell survival, possible safety issues relating to its residual anti-coagulant activity and potential to induce heparin-like thrombocytopaenia preclude its translation to the clinic for preventing the progression of T1D [87, 88].

24.6 Intracellular Roles for Heparanase in Modulating Gene Transcription, Cell Differentiation/Function and Disease

Catalytically-active heparanase produced by insulitis leukocytes has a destructive role in facilitating islet infiltration and beta cell damage/death during the pathogenesis of T1D in NOD mice and humans (see Sect. 24.4.) [14, 17, 26, 72]. In contrast, other studies have identified an additional function for heparanase in regulating gene transcription.

Long-term intraperitoneal administration of exogenous heparanase to 6 week-old NOD mice induced *protection* against diabetes onset [89]. Whilst at first glance this outcome argues against a pathogenic role for heparanase, Bitan et al. argued that the enzyme was probably inactivated by the local pH in the peritoneal cavity [89]. The same study demonstrated that both active and inactive heparanase preferentially skewed the responsiveness of NOD T cells towards an anti-inflammatory Th2 phenotype (characterized by IL-4, IL-10 production) in vitro. In parallel, the reduced production of IFNγ and TNFα by NOD peritoneal macrophages also indicated down-regulation of the M1 phenotype. The amelioration of T1D disease was therefore consistent with altered expression of immune genes, as also demonstrated for experimental autoimmune encephalomyelitis (EAE) [89, 90]. Evidence subsequently emerged for the intra-nuclear localization of heparanase in a range of cell types, impacts of heparanase on intracellular signaling pathways and the enzyme's direct or indirect effects on gene expression [72].

24.6.1 Direct Role for Heparanase in Regulating a Gene Transcription Complex

The transfer of endogenous heparanase to the nucleus in a variety of cells is chaperoned by Heat shock protein 90 (Hsp90). This process is further aided by fatty acid (e.g., palmitate) which promotes lysosomal permeabilization, the intracellular release of heparanase and the enzyme's access to nuclear entry routes [91]. In immortalized human Jurkat T cells, endogenous heparanase (50 kDa) has a direct role (albeit independent of its catalytic activity) in the transcription of a specific set of immune response genes. Heparanase binds to an active transcription complex with demethylase LSD1 and RNA polymerase II (RNAPII), promoting the demethylation of histone H3 and subsequent expression of IL-2 and IFNγ genes [72, 92]. The discrepancy between this report and the induction of Th2 gene expression following treatment of murine T cells with exogenous heparanase [89, 90] strongly suggests that intracellular and extracellular heparanase could exert different effects on gene expression in T cells.

In the retina, glucose-enhanced levels of the catalytically active form of heparanase correlate with increased expression of vascular endothelial growth factor (VEGF) protein, consistent with the disease process of neovascularization in diabetic retinopathy [80] (see Sect. 24.7.). Similar to the transcription of immune response genes [92], heparanase has also recently been reported to interact with RNA Pol II, bind to the VEGF gene promoter and initiate VEGF gene transcription in high glucose-treated human retinal microvascular endothelial cells. A reduction in VEGF expression by treatment with PI-88 further suggested a role for active heparanase; the latter could function by removing HS-inhibition of topoisomerase-1 to allow relaxation of supercoiled DNA and active transcription [93].

24.6.2 Indirect Intracellular Functions of Heparanase

Signalling

A number of different cell surface receptors, including mannose 6-phosphate receptor, low-density lipoprotein receptor, and HSPGs, bind extracellular heparanase. However, it is unclear whether these individual receptors operate in a cell-specific manner and promote different intracellular processes [47, 94]. Heparanase has been reported to stimulate intracellular signaling in a number of cell types and to indirectly alter gene expression. Thioglycolate-stimulated heparanase-knockout (Hpse-KO) mouse peritoneal macrophages treated in vitro with exogenous heparanase demonstrated increased intracellular signaling (phosphorylation of p38 and p-JNK), increased expression of TNFα, IL-1β and enhanced levels of the transcription factor c-Fos [95]. In the absence of treatment, Hpse-KO macrophages showed reduced cytokine gene expression, implicating an intracellular role for endogenous

heparanase in regulating the transcription of cytokine genes [95]. Heparanase produced by NOD myeloid cells and human monocytes in T1D (Figs. 24.3 and 24.4) could therefore also affect the profile of cytokines produced. Heparanase-mediated cell signaling and gene transcription (see Sect. 24.6.1) can, therefore, impact the function of immune cells.

Effects of High Glucose on Heparanase Levels and Gene Transcription

Although intracellular signaling generally correlates with non-enzymatic properties of heparanase [96], other roles for heparanase were found to depend on the enzyme's catalytic activity. During oesophageal keratinocyte differentiation heparanase is translocated to the nucleus to degrade intra-nuclear HS [97]. Significantly, heparanase was reported to degrade HS chains (e.g., attached to Syndecan-1 core protein) in the nucleus of myeloma cells and endothelial cells, providing a plausible mechanism for reversing HS-mediated inhibition of histone acetyltransferase (HAT) to initiate gene transcription [98–101].

Elevated levels of glucose in the circulation and in culture upregulate heparanase protein in vascular endothelial cells [80, 102, 103]. HSP90-mediated transfer of heparanase to the nucleus of coronary artery endothelial cells cleaves nuclear HS, raises HAT activity and increases the expression of genes associated with both glucose metabolism/glycolysis [lactate dehydrogenase 2 (LDHA); pyruvate dehydrogenase kinase 2 (PDK2)] and with inflammation [e.g., vascular cell adhesion factor 1 (VCAM1), VEGF] [91]. In addition, high glucose-induced latent or active heparanase produced by endothelial cells can be subsequently taken up by nearby cardiomyocytes to cleave intra-nuclear HS and upregulate MMP-9 expression. Extracellular MMP-9 degrades HSPG core proteins, enabling bound lipoprotein lipase to be transferred to the vascular lumen to enhance triglyceride breakdown and provide fatty acid for cardiomyocyte metabolism [104] (see Sect. 24.7 and Shang et al., Chap. 30 in this volume). These findings demonstrate that high glucose-stimulated production of heparanase can directly impact gene transcription endogenously as well as indirectly modify gene expression in neighboring cells.

In contrast, hyperglycemia per se appears not to increase heparanase levels in islet cells, based on comparative studies between wild-type and heparanase-transgenic (hep-tg) mice. Nevertheless, the global overexpression of latent heparanase in hep-tg mice increased the expression of a broad range of pancreatic islet genes, enhanced hormone secretion and altered the intra-islet distribution of beta cells and alpha cells [105]. Of more than 2000 genes affected, HS-glucosamine 3-sulfotransferase (required for HS synthesis) and the HSPG core protein Sdc-1 were upregulated, suggesting that endogenously produced latent heparanase is important for maintaining HS in islet cells. This role would be expected to be offset by the participation of active heparanase in the homeostatic turnover of islet cell HS [17, 47, 91]. Hep-tg islets also showed increased glucagon gene transcription and decreased glucagon receptor expression, resulting in hyperglucagonemia; insulin gene expression remained unchanged, but insulin secretion was enhanced in response to streptozotocin-induced mild hyperglycemia. Interestingly, heparanase overexpression

and hyperglycemia in hep-tg mice also impacted other tissues, increasing the production of FGF21 (in liver) and GLP-1 (in the gastrointestinal tract) to lower blood glucose levels. Overall, these studies suggest that endogenous heparanase influences gene transcription in islet cells and helps to regulate the HS content in beta cells, insulin secretion, as well as beta cell-endocrine cell interactions. During T1D development/progression, the actions of endogenous beta cell heparanase are likely to be over-ridden by the catalytic activity of heparanase produced by invading insulitis leukocytes (see Sects. 24.4 and 24.5.).

24.7 Heparanase, a Contributor to the Secondary Complications of Diabetes

Long-term or established T1D can result in micro- and macro- vascular disorders, largely due to the imperfect regulation of blood glucose levels by exogenous insulin therapy. Diabetic nephropathy is characterized by a complex interplay between hyperglycemia, glycated serum proteins and heparanase [77]. The endothelial glycocalyx (extracellular layer of cell-surface and associated unanchored proteoglycans) and sub-endothelial BMs are characterized by a high HS content and act as targets for heparanase activity. Notably, under normal conditions, the negative charge carried by HSPGs in the glomerular glycocalyx binds positively charged regions of plasma proteins such as albumin (which is amphoteric and carries both negative and positive charges) [106], limiting the access of plasma proteins to the endothelium. This process contributes to the selective permeability of the glomerular filtration system [107].

Heparanase is produced by endothelial cells in response to hyperglycemia and ROS, while elevated levels of ROS can induce heparanase in renal epithelial cells [77, 108]. Latent heparanase produced by glomerular endothelial cells can be transiently bound to HSPGs in the glycocalyx. In nephropathy, inflammatory macrophages produce and secrete heparanase as well as Cathepsin L that can activate glycocalyx-bound heparanase; the resulting breakdown of extracellular HS reduces the glycocalyx layer and increases vascular permeability and inflammation [77]. Advanced glycation end-products (AGEs) generated in diabetes and endothelin-1 produced by endothelial cells enhance heparanase expression in podocytes [77, 109]. The local production of active heparanase also degrades HS in the glomerular BM (GBM). However, the relative contribution of GBM HS to charge-dependent filtration has become the subject of debate, fuelled by the absence of proteinuria in mice lacking GBM HS. Instead, GBM HS is thought to function in the size selectivity of macromolecules [110]. Overall, heparanase activity impairs size- and charge-selective filtration by glomeruli, resulting in microalbuminuria/proteinuria i.e., excretion of albumin in urine [107]. Evidence underpinning a key role for heparanase in diabetic nephropathy include the up-regulation of heparanase transcripts in parallel with loss of glomerular HS [111, 112], detection of heparanase in the urine of diabetic patients [113], absence of proteinuria in streptozotocin-diabetic Hpse-KO mice and reduced albuminuria following treatment with a heparanase inhibitor

(SST0001) [114]. Interestingly, reduction of microalbuminuria in T1D patients has been reported after treatment with enoxaparin (low molecular weight heparin, LMWH), sulodexide (80% LMWH, 20% dermatan sulfate) or danaparoid (mainly sulfated HS), with a subsequent study correlating sulodexide-mediated protection with inhibition of heparanase [115, 116].

Proliferative diabetic retinopathy is another example of micro-vascular disease associated with long-term diabetes and is marked by endothelial cell damage, local adhesion or arrest of inflammatory cells, angiogenesis and associated disruption of the retinal subendothelial BM. Heparanase plays both enzymatic and non-enzymatic roles in damaging the retinal BM and enhancing the local production of VEGF to drive neovascularization [117] (see Sect. 24.6.1). Detection of heparanase activity, syndecan-1 and Cathepsin L in the vitreous fluid of patients with proliferative diabetic retinopathy is consistent with an important function for heparanase in the disease process [117, 118]. Furthermore, in streptozotocin-treated rats with long-term diabetes, PI-88 therapy ameliorated retinal inflammation and endothelial dysfunction, further implicating heparanase in the development of retinopathy and diabetes-associated blindness [119].

Heparanase plays a complex role in diabetes-associated cardiovascular disease resulting from atherosclerosis in large blood vessels (macro-vascular disease) and a switch to fatty acid metabolism in cardiomyocytes (see Sect. 24.6.2.2 and Shang et al., Chap. 30 in this volume). Endothelial-derived heparanase promotes the uptake and metabolism of fatty acids (due to glucose unavailability; see Sect. 24.6.2.2), triggering a potential setting for abnormal lipid accumulation, a risk factor for cardiomyopathy [120]. Inflammation is an early feature of atherosclerosis and heparanase-assisted degradation of HS in the subendothelial BM permits the leakage of heparanase-expressing inflammatory monocytes and low-density lipoproteins (cholesterol) into the blood vessel wall, promoting the formation of atherosclerotic plaque [120]. The local production of inactive heparanase exacerbates cytokine secretion and macrophage activation, enhancing intra-plaque inflammation and plaque progression [121, 122]. Further growth of plaque deposits can lead to advanced macro-vascular disease typified by the narrowing and occlusion of major blood vessels, plaque rupture and thrombosis [120, 122].

24.8 Concluding Remarks

Heparanase, via its catalytic role in degrading HS, significantly contributes to the pathogenesis of T1D disease at the level of leukocyte migration, islet invasion and importantly, beta cell damage and death. These features highlight heparanase as a key therapeutic target for arresting T1D progression. Studies of T1D human pancreas specimens have identified that beta cell destruction continues to progress beyond the time of clinical diagnosis and that a residual beta cell mass (~20% of original mass) can still exist at T1D onset [3]. Clinical trials using conventional

immune intervention therapies have been unsuccessful in halting the disease process. We propose that a dual-acting heparanase inhibitor/HS replacer offers a highly novel approach to both inhibiting islet inflammation and rescuing beta cells from oxidative damage. Indeed the capacity to directly preserve beta cell mass is unmatched by other immune-modulating strategies. Currently, the main challenge is to develop a safe, synthetic heparanase inhibitor/HS replacer for clinical translation and the treatment of recent-onset T1D. In the future, this new therapeutic could partner with other interventions that target autoreactive T cells, antigen-presenting cells (e.g., B lymphocytes) or that expand regulatory T cells. We propose that this combined approach could offer the best potential for preserving the survival and function of the beta cells that remain at T1D onset, and for reducing or eliminating the need for exogenous insulin therapy. In the setting of a heparanase-targeted therapy, maintenance of the normal house-keeping and regulatory functions of active and inactive heparanase inside cells may require the restricted localization of the enzyme within subcellular compartments. Importantly, heparanase inhibitors (± HS replacer activity) could also potentially offer a therapeutic application for impairing the development of secondary T1D-associated vascular disease.

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Chapter 25
Implications of Heparan Sulfate and Heparanase in Amyloid Diseases

Jin-Ping Li and Xiao Zhang

25.1 Amyloidosis and Amyloids

Amyloidosis (Pl. amyloidoses) is a group of diseases characterized by abnormal deposition of endogenous proteins in different organs. The diseases are clinically categorized into systemic and localized amyloidosis. In systemic amyloidosis, plasma proteins are deposited in various organs, whereas localized amyloidosis involves proteins mainly produced by cells near the deposition site [1]. So far, more than 30 different proteins were identified as amyloid proteins [2]. The common features of amyloid proteins are: (1) molecularly, most of them are derived from a specific precursor protein following certain post-translational modifications, usually proteolytic cleavage; (2) biochemically, these proteins misfold and aggregate to form insoluble fibrils; (3) pathologically, different proteins target specific organs, and (4) histologically, the amyloid deposits are stained by Congo red, an aniline dye, showing apple-green birefringence under polarized light. In addition to the specific amyloid protein component, the amyloid fibrils are co-deposited with several other substances including heparan sulfate proteoglycan (HSPG), regardless of the amyloid protein species [3, 4].

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25.1.1 Amyloid Production and Aggregation

The term “amyloid” denotes a protein in its insoluble fibrillar form present in pathological deposits of organs [1]. It was initially used to describe starchy constituents of plants, and the term was adopted to replace the expressions “lardaceous” or “waxy”, used to describe tissue changes in several pathological human conditions believed to be due to starch-like substances. Later analyses revealed that the major component of “amyloid” is protein rather than sugar. In fact, sugars (glycosaminoglycans, GAGs) indeed occur in the deposits, though not starch neither as a major constituent.

The fibrillar structure of amyloid is attributed to defective posttranslational conformational changes of an otherwise soluble protein, i.e., “protein misfolding”, leading to conversion into insoluble highly ordered fibrillar aggregates [5]. The amyloid protein is characterized by its secondary cross β-pleated sheet structure that allows the binding to Congo red dye. Fibril formation of amyloid proteins is often preceded by the formation of various intermediate oligomer states lacking typical fibrillary appearance, or protofibrils with β-sheet structure [6]. Although fibrillar amyloid deposits in tissues give rise to pathological conditions, oligomers and protofibrils are considered, at least in some cases, to have more pronounced toxic effects.

25.1.2 Aβ-Associated Amyloidosis (Alzheimer’s Disease)

The incidence of Alzheimer’s disease (AD) is the highest among the known amyloid diseases. It is a major central nervous system disease, affecting neuronal activities, characterized by progressive neurodegeneration with a clinical phenotype of cognitive impairment. The classification of AD as an amyloid disease is based on the histopathological hallmark of extracellular deposition of the beta-amyloid peptide, Aβ [7, 8]. Aβ is generated by proteolytic cleavage of the amyloid β precursor protein (AβPP), a transmembrane glycoprotein expressed abundantly in the brain. Clinically, AD can be classified into sporadic and familial forms. The familial AD cases are associated with or caused by genetic mutations, primarily in the AβPP gene, often leading to an early onset of the disease [8]. However, about 90% of the clinical AD cases are sporadic and have no genetic correlation. The causes of this group of patients remain unclear and most likely involve multiple factors. Clearly, overproduction or impaired clearance of the Aβ peptide are principal causes for deposition of the amyloid peptide. The imbalanced metabolism can be affected by neuroinflammation, the implications of which, however, remain unclear as to the effects on disease progression. Overall, the majority of AD cases are age-dependent.

25.1.3 IAPP-Associated Amyloidosis (Type 2 Diabetes)

Islet amyloid polypeptide (IAPP), also known as amylin, is a 37-amino acid peptide [9] derived from the precursor protein, pro-IAPP [10]. Pro-IAPP is produced by β cells in the islets of Langerhans in the pancreas and stored in the secretory granules along with insulin. Deposits of IAPP in the pancreas are associated with two disease conditions: insulinoma, a rare neuroendocrine tumor, and type 2 diabetes [11]. Most of the type 2 diabetes patients (90%) are complicated with IAPP amyloidosis, having IAPP deposition at various degrees in the islets (predominantly in β-cells). Such IAPP deposition constitutes a hallmark of type 2 diabetes [12], the extent of the amyloid burden apparently correlating with the severity of the disease [13].

25.1.4 AA Amyloidosis (Inflammatory-Associated Amyloid Disease)

AA amyloidosis is primarily associated with various chronic inflammatory and persistent infectious diseases such as rheumatoid arthritis, tuberculosis, and leprosy [14, 15]. The protein fibrils of AA originate from cleavage of serum amyloid A protein (SAA), an apolipoprotein constituent of high-density lipoprotein (HDL). SAA is an acute phase reactant synthesized in the liver. During acute-phase of inflammation, the plasma level of SAA can be dramatically elevated, even by several thousand-fold [16]. In most cases, with the control of infection/inflammation, SAA in the plasma decreases and returns to a normal level. However, under certain circumstances, such as repeated infection and inflammation, the SAA becomes denatured/misfolded and deposited in organs, most commonly in the spleen, kidney, and liver. This type of amyloidosis termed AA amyloidosis leads to irreversible degeneration of the affected organs, being one of the rare but high mortality diseases.

25.1.5 TTR-Associated Amyloidosis (Cardiomyopathy and Polyneuropathy)

ATTR (Amyloid **T**rans**t**hy**r**etin) amyloidosis is a multisystemic disease affecting several organs, primarily the nervous system and the heart [17]. Transthyretin (TTR) is a plasma transport protein carrying thyroid hormone T_4 and the proteins binding to retinol. Native TTR, mainly produced in the liver, is a tetramer of 4 identical subunits each of which is composed of 127 amino-acid residues. The native tetramer form is stable and functional; however, due to mutations or unknown causes, the tetramer can become dissociated, resulting in unstable monomers that form the ATTR fibrils by aggregation [18]. Up to date, more than 100 variations in the TTR gene have been reported, the majority of which is linked to ATTR amyloi-

dosis [19, 20]. The mutation-associated ATTR causes the diseases of familial amyloid polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC). FAP is due to accumulation of ATTR around peripheral nerves, and FAC is characterized by deposition of ATTR in cardiac tissues [21]. Similar to the pathology of Aβ in AD, cases of non-genetic associated ATTR have been diagnosed, caused by the dissociation of wildtype TTR tetramer into monomers that become aggregated. The resultant cardiac deposition of amyloid in aging patients is characteristic of sporadic senile systemic amyloidosis (SSA) [22]. Notably, though less frequent than AD, ATTR amyloidosis still affects almost 25% of the population > 80 years of age and is one of the most common systemic amyloidosis [23].

25.2 Detection of HSPG and HS in Amyloid Plaques

Histological and biochemical analyses of biopsy and autopsy tissues from various amyloidosis diseases consistently show HSPGs along with the major species of amyloid peptides in the lesions. The pioneer studies by Kisilevsky and co-workers have identified GAGs (mainly HS) in the neuritic plaques of AD brain [24]. In vitro studies have demonstrated the interaction of HS with several amyloid peptides, which may even accelerate fibrillization of the amyloid peptides [25–28], where indications of selective binding of amyloid to certain structural motifs along the HS chain have been described.

Altered, mostly, elevated expression of heparanase has been observed in several pathological conditions which affect the structural properties and functions of HS. In comparison, heparanase expression in amyloid diseases remains uninvestigated. Notably, a few recent studies applying the heparanase transgenic mouse model show a correlation between heparanase expression, HS structure and amyloid deposition [27, 29–32]. However, analysis of AD brain tissues detected elevated heparanase expression in comparison to age-matched brain [33]. Systemic examination of heparanase expression in the lesion tissues from other amyloid diseases should be encouraged.

25.2.1 HS in the Brain of AD Patients and Mouse Models

Association of HS and HSPG with amyloid pathology is in particular based on studies of AD. The presence of GAGs in Aβ deposits was first detected using staining of brain sections from AD patients with Congo red for Aβ and Alcian blue for sulfated GAGs [34]. With development of more specific antibodies against different species of Aβ and HSPG, several subtypes of HSPG isoforms, including syndecans 1–3, glypican-1 and agrin, were immunolocalized not only in the Aβ deposits of plaques and vasculature but also in neural fibrillary tangles (NFT) of AD brains and in transgenic animal models of Aβ amyloidosis [35]. Recent studies using advanced

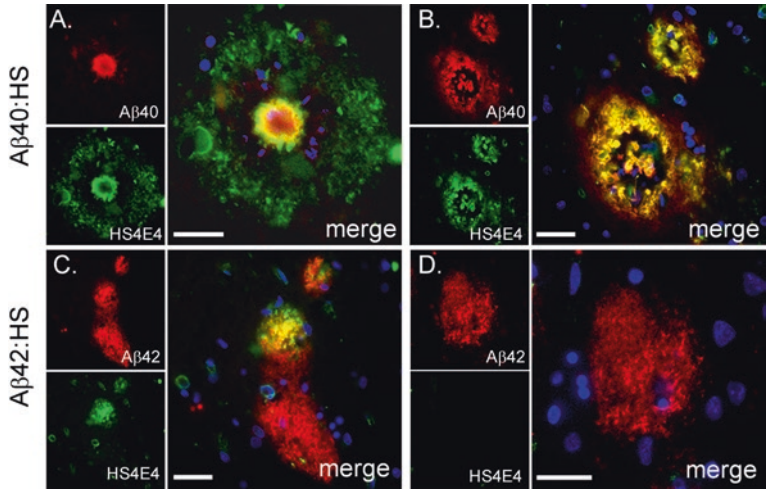

Fig. 25.1 *Heparan sulfate (HS) accumulated with amyloid β-peptide 40 (Aβ40) in neuritic plaques but was absent from Aβ42-rich diffuse plaques in the brain of Alzheimer's disease (AD).* Paraffin-embedded hippocampal sections (15 μm thick) from sporadic and Swedish β-amyloid precursor protein (βAPP) 670/671 cases were double immunostained for Aβ40 or Aβ42 and HS (HS4E4) and counterstained with DAPI for nuclei (blue). *Sporadic AD*: The neuritic plaques with a wagon-wheel morphology had essentially three components: an outer halo, which is HS positive, but Aβ40 negative (**A**: Aβ40); an inner core, which is Aβ40 positive and colocalized with a superficial layer of HS (**A**: HS4E4); and a void between the halo and the core, which is positive for nuclei (**A**: merge). Diffuse Aβ42-rich plaques occasionally presented with areas of HS accumulation, however, the distribution was uneven (**C**). *Swedish βAPP 670/671 AD*: These neuritic plaques appeared as thick-ring constructs and are composed similarly to the wagon-wheel plaques of sporadic AD (**B**); however, the outer halo, as well as the core, stained strongly for Aβ40 and HS (B: Aβ40, HS4E4, merge). Diffuse Aβ42-rich plaques (**D**:Aβ42) showed no colocalization with HS (**C** merge). (The figure is part of Fig. 25.1 taken from O'Callaghan et al., Brain Pathol. 2008; 18(4): 548–561)

antibodies have shown pattern-selective co-localization of HS with different types of Aβ deposits, such as neuritic plaques with dense cores selectively stained by anti-Aβ40 antibody and anti-HS antibody, or diffuse plaques selectively stained by anti-Aβ42 antibody, but essentially lacking HS immunosignals (Fig. 25.1) [36, 37].

25.2.2 HS in Cardiomyopathy (ATTR)

In comparison to Aβ amyloidosis in AD, information on the composition of ATTR deposits with regard to HSPG is limited. An early study reported the detection of GAG in isolated TTR amyloid fibrils [38]. A more recent report using immunohistological staining revealed cardiac co-deposition of HS with TTR in a case of SSA cardiomyopathy (Fig. 25.2) [26]. Due to lack of proper diagnostic means, heart disease due to ATTR amyloidosis has been underestimated such that even fatal cardiac dysfunction has remained without a clear diagnosis. Given the progress in the

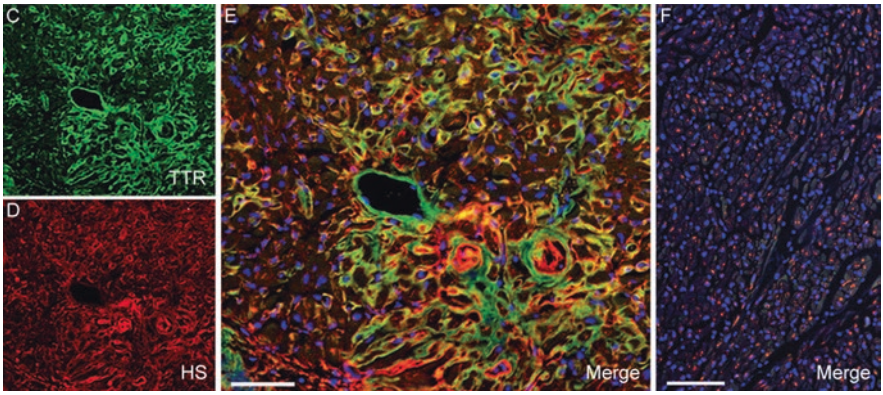

Fig. 25.2 *Colocalization of TTR amyloid and HS in the heart of cardiomyopathy*. Myocardial sections (15 μm thick) from a 70-y-old patient with reported cardiomyopathy were double immunostained with antibodies against TTR (**C**, green) and HS (**D**, red). Merging the red and green fluorescent channels show overlapped positive signals for TTR and HS in the patient specimen (**E**). A normal heart from age-matched control subject shows negative staining of TTR in (**F**). DAPI counterstaining for nuclei (blue). Original magnification, **A–F,** 200. Scale bar, 50 μM. (The figure is part of Fig. 25.1 taken from Noborn et al., PNAS 2011, 108 [14] 5584–5589)

development of novel diagnostic and therapeutic methods, the disease is now drawing attention by both researchers and clinicians [17, 39]. More specimens should be systematically examined to firmly establish HSPG-ATTR co-deposition in ATTR-amyloidosis.

25.2.3 HS in the Islets of Type 2 Diabetes (AIAPP)

Perlecan, a HSPG present in the basement membrane, has been identified as a component of AIAPP deposits in the islets of type 2 diabetes patients using histochemical and immunohistochemical methods [40]. Accumulation of perlecan in islet amyloid deposits has also been demonstrated in transgenic mice that overexpress amylin (IAPP) [41]. However, as in ATTR amyloidosis, reports on the correlation of GAGs or HS with AIAPP deposition in the lesion tissues of patients are circumstantial. With the development of specific antibodies against different types of GAGs and proteoglycans, immunohistochemical examination of specimens from type 2 diabetes patients will enable a more systematic characterization of AIAPP deposits, and a better understanding of the role of HS and possibly other GAGs in the pathogenesis of type 2 diabetes.

25.2.4 HS in the Organs of AA Amyloidosis

The cases of SAA are relatively rare, so most of the information on AA amyloidosis derives from studies on animal models. Amyloid fibrils from experimental AA amyloidosis in mice induced by amyloid enhancing factor (AEF) and an inflammatory stimulus (subcutaneous injection of $AgNO_3$) were examined by high-resolution ultrastructural analysis and the results revealed structures of AA fibrils composed of basement membrane type HSPG [34]. The co-deposition of HS with AA amyloids was also detected in the mouse model of AA amyloidosis [29]. It was proposed that perlecan might have a primary role in the formation of SAA amyloids [42].

25.3 In Vitro Studies on the Interaction of HS/HSPG With Amyloid Proteins

HS is involved in ionic interactions with a multitude of biological ligands including amyloid proteins, largely owing to its overall negative charge or in some cases specific sequences of sulfated sugar residues. Conversely, some amyloidogenic proteins contain HS-binding peptide domains [43].

AβPP and Aβ were found to bind to both the core protein of glypican 1 and free HS chains [25, 44]. Heparin binds AβPP with a higher affinity than HS [45], likely due to its higher sulfation degree. Analysis of the Aβ42 peptide sequence revealed a consensus "heparin-binding domain" at residues 12–17 (VHHQKL), likely to be involved in the interaction with HS [46]. Efforts have been made to define the fine structure of HS that interacts with Aβ by incubation of HS/heparin oligosaccharides with the amyloid peptide. The results showed that a hexasaccharide domain of HS identified in human cerebral cortex containing critical 2-*O*-sulfated iduronic acid residue binds to fibrillar Aβ [25].

Human IAPP has been shown to bind to heparin and perlecan resulting in acceleration of IAPP fibril formation *in vitro* [46, 47]. A heparin-binding domain was first identified in the N-terminal cleavage site of pro-IAPP [48], Arg-22 and His-29 being critical for the interaction with HS [49]. Both the N-terminal flanking region of pro-IAPP and HSPG are found in islet amyloid deposits and are assumed to play roles in IAPP amyloidogenesis [50]. Studies using cell culture models have shown that HSPG synthesized in β cells are capable of binding amyloidogenic human IAPP [51]. Inhibition of HSPG synthesis by WAS-406 and Azaserine in explant cultures of islets isolated from transgenic mice overexpressing human IAPP was found to reduce amyloid formation [52]. A recent study showed that heparin promoted IAPP aggregation in a size-dependent manner [27].

In contrast to the extensive studies on the binding of Aβ and IAPP to HS/heparin, information about the interactions of other amyloid peptides with HS is limited. Early evidence for the interaction of SAA with heparin was obtained by affinity chromatography on heparin-Sepharose [53]. Functional evidence of heparin-SAA

binding was demonstrated in a recent study where the addition of heparin to SAA or HDL preparations promoted SAA aggregation in a dose and size dependent manner [28]. HS was found to dissociate SAA from HDL particles isolated from inflamed mouse plasma, resulting in free SAA peptide prone to aggregation [54]. A peptide corresponding to the proposed HS-binding site of SAA was demonstrated to block amyloid deposition in a cell AA amyloidosis model [55]. Moreover, analogs of N-acetylglucosamine shown to inhibit HS biosynthesis also precluded the development of AA amyloidosis in cell and mouse models [56]. Additionally, HS isolated from organs of AA amyloidosis differed in structure from that of healthy control organs, suggesting an association of a specifically modified HS with the amyloid deposits in the affected organs [57].

Information about HS/heparin-TTR interaction is increasing. Sulfated GAGs such as heparin were found to accelerate the conversion of preformed TTR oligomers into larger aggregates [58]. An independent study on the interactions of recombinant wild type TTR with HS/heparin showed that the ability of GAGs to promote fibril formation is dependent on the length and overall charge of oligosaccharides [26]. Subsequent biochemical studies implicated a reactive histidine residue in TTR in binding to HS, which is influenced by pH [59].

Collectively, the available information indicates a common feature of HS-amyloid interaction, i.e., binding requires a certain degree of sulfation and chain length of HS, though different amyloid proteins, due to variations of peptide sequences, may prefer a specific sulfation pattern or sequence of the HS expressed in a defined organ (Fig. 25.3).

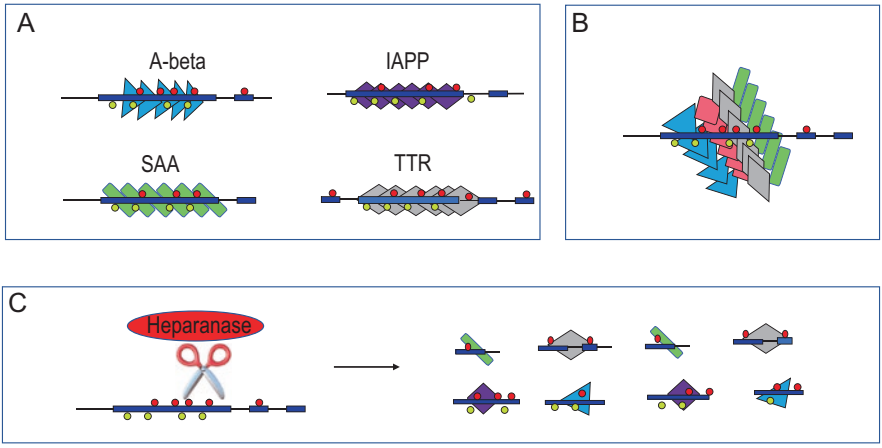

Fig. 25.3 *Hypothetical interaction models of HS with different amyloid peptides and the potential functions of heparanase.* It is still unclear whether different amyloid peptides bind to different HS structures expressed in specific organs (**A**) or to a common structure of HS ubiquitously present in all HS (**B**). Heparanase cleavage of HS results in shorter fragments of HS that are still able to bind amyloid proteins but lost the ability to aggregate the proteins (**C**). The blue ribbons and lines represent the long HS chains, and the yellow and red dots indicate sulfate groups

25.4 In Vivo Observations of HS and Heparanase on Amyloid Deposition

25.4.1 Effect of HS/HSPG-Heparanase on Amyloid Deposition

The in *vivo* effects of HSPG on amyloid deposition have been studied in animal models. A pioneering study employed infusion of synthetic human Aβ40 peptide, either alone or along with perlecan, into the hippocampus of rats [60]. Significant enhancement of amyloid deposition was observed in rats receiving both Aβ and perlecan in comparison to rats receiving Aβ alone, as judged by Congo red staining. It was proposed that perlecan stabilized the plaque-like Aβ deposits and protected the Aβ aggregate from proteolytic degradation. The core protein of perlecan alone failed to induce the AD-like pathology, indicating a critical role for HS [61]. This conclusion was strengthened by a recent study of endogenous Aβ deposition. Transgenic mice expressing human AβPP containing the Swedish mutation develop Aβ deposition with aging and this pathology was greatly attenuated in the double transgenic mice expressing both the mutated AβPP and human heparanase [31]. This beneficial effect of heparanase is due to degradation of HS, the truncated HS chains having reduced scaffold capacity required to bind Aβ and promote its aggregation (Fig. 25.3). The shorter HS fragments generated by heparanase may even have a protective effect by binding to amyloid monomers or oligomers, preventing them from aggregation. This clarification could explain how Enoxaparin (low-molecular-weight heparin) treatment reduced Aβ burden in brains of transgenic mice expressing mutated human AβPP [62].

In a different approach, aggregated Aβ was injected into the mouse brain, followed by examination of aggregate clearance [32]. Immunohistological analysis of brain tissues revealed that the injected Aβ was rapidly cleared in wildtype mice but persisted in mice overexpressing human heparanase. Clearance of aggregated Aβ was part of a neuroinflammatory response involving infiltration of monocytes. Attenuation of this process in response to heparanase overexpression is due to impaired function of chemokines carried by vascular endothelial HSPG. Heparanase overexpression similarly attenuated neuroinflammation induced by LPS injection.

The role of HS in SAA amyloidogenesis was strikingly illustrated by the effect of heparanase overexpression in mice subjected to inflammatory challenge [29]. Histochemical analysis showed strong Congo red staining of SAA deposits in spleen and liver of wild-type mice one week after induction. In contrast, in the heparanase transgenic mice, SAA deposition was inversely related to expression levels of the enzyme. Liver, with strong heparanase expression, was thus virtually devoid of amyloid, whereas spleen showed low heparanase expression but abundant SAA deposition. This inverse correlation between heparanase expression and susceptibility to AA amyloid formation provides evidence for a scaffold function of HS in the process. This conclusion is in accordance with the observation that subcutaneous administration of enoxaparin and dalteparin (low-molecular-weight heparins) precluded AA amyloid deposition in the same type of AA amyloidosis mouse model [63], presumably by competitively interacting with amyloid peptides (Fig. 25.3).

25.4.2 Implications of HS in Amyloid Toxicity

In general terms, the solid amyloid deposition will impair organ function. However, in AD patients the extent of Aβ plaque accumulation does not always correlate well with severity scores of the disease, and a significant number of non-demented individuals are found to have considerable Aβ plaque loads in their brains. Conversely, in some AβPP transgenic animal model functional impairment is frequently observed prior to the onset of Aβ deposition. It was proposed that intermediate states, rather than mature fibrils, of the amyloid peptide, are cytotoxic species because the levels of soluble Aβ fibrils correlated better with the degree of dementia than insoluble fibrillar deposits. Accordingly, soluble Aβ oligomers were found to adversely affect synaptic structure and plasticity [64]. A CHO cell model study demonstrated a role for HS in uptake and cellular toxicity of the Aβ40 peptide [65]. This toxicity was attenuated in cells overexpressing heparanase. Moreover, the addition of heparin to human umbilical vein endothelial cells (HUVEC) prevented internalization of added Aβ40 and protected against its toxicity. Taken together, these findings suggested that cell-surface HS mediate Aβ internalization and toxicity in a chain length-dependent manner, which is modulated by heparanase. An earlier study reported internalization of TTR through an unidentified cell surface receptor [66]. The potential involvement of cell-surface HS in such internalization needs to be investigated.

25.5 Heparanase in Amyloid Diseases

Information linking heparanase to amyloidosis is overall limited. However, since HS/HSPGs appear to be functionally involved in various types of amyloid deposition through similar mechanisms, the degradation and modification of HS chains by heparanase action are likely to affect disease patterns. Notably, the effects of heparanase are often multifaceted and at times seemingly contradictory. Thus, whereas heparanase overexpression in murine tissues was found to prevent AA amyloid deposition [29], heparanase knockout resulted in accelerated clearance of the same deposits, an effect ascribed to upregulation of matrix metalloproteases [30]. Likewise, the pathophysiological significance of elevated heparanase expression in brains of AD patients [33] remains unclear. Explant culture of islet under high glucose developed IAPP amyloid in samples isolated from mice overexpressing human IAPP, but not in samples isolated from mice that also overexpress human heparanase [27]. Similarly, overexpression of heparanase in AβPP transgenic mice reduced deposition of endogenous Aβ and increased solubility of the Aβ amyloid [31]. On the other hand, heparanase overexpression led to attenuation of neuroinflammatory responses that promote clearance of injected aggregated Aβ [32]. Both of these effects could be ascribed to degradation of HS by overexpressed heparanase, which

affects the functions of HS under different amyloidosis stages. Notably, the inhibitory effect of heparanase on neuroinflammatory processes [32, 67] stands out as an exception while in general, heparanase appears to promote inflammatory conditions (Elkin, Chap. 17 in this volume).

25.6 Concluding Remarks

Virtually, all known amyloid proteins interact with HS/heparin *in vitro*. Refined experimental techniques, as well as increasing availability to *in vivo* models, have gradually revealed the involvement of HS/HSPG in various phases of amyloidosis pathology [68]. While some of the proposed mechanisms may be common, others are distinct to different amyloidoses. HS chains serving as scaffolds for the aggregation of amyloid mono- or oligomers appear to be a common feature. Identification of minimal HS structures that can bind to amyloid but not to promote aggregation may potentially lead toward the development of anti-amyloid drugs. However, there are also more complex scenarios for consideration, such as the interaction between HS and HDL particles leading to release of amyloid-producing SAA or between vascular endothelial HS and chemokines required to induce neuroinflammation (see above). In principle, all amyloid conditions involving HS polysaccharide may potentially be modulated by heparanase, although not necessarily in a predictable manner. Analysis of more lesion tissues of various amyloid diseases for heparanase expression and HS molecular structure would be an important step toward a better understanding of the pathophysiological roles of heparanase in amyloid diseases.

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Chapter 26
Heparanase in Kidney Disease

Johan van der Vlag and Baranca Buijsers

The main function of the kidney is filtration of blood and excretion of metabolic waste products into urine. The primary filtration of blood occurs in the glomerulus, a network of microcapillaries. The primary urine flows to the tubules, which are crucial for fluid resorption, maintenance of electrolyte balance, and resorption of glucose. The combination of a glomerulus and tubules is called a nephron, which is the functional unit of the kidney. Each kidney contains about one million nephrons. Within the glomerulus, the fenestrated endothelial cells covered with a thick glycocalyx, line the glomerular capillaries, whereas visceral epithelial cells, the podocytes, are covering the microcapillaries at the outside. There is an extracellular matrix layer between the glomerular endothelial cells and podocytes, which is called glomerular basement membrane (GBM). All together, endothelial cells with glycocalyx, GBM and podocytes form the glomerular filtration barrier (GFB) (Fig. 26.1). The GFB filters the blood and prevents leakage of blood proteins in size and charge-dependent manner [1, 2]. Dysfunction of any of the components of the GFB contributes to the development of glomerular diseases, including diabetic nephropathy (DN) and glomerulonephritis (GN), thereby leading to proteinuria, i.e., leakage of protein into the urine [3].

26.1 Glomerular Filtration Barrier in Healthy Situation

Glomerular endothelial cells are highly specialized cells that cover the inner side of glomerular capillaries. Furthermore, the endothelial surface constitutes up to 50% out of the fenestrated area under normal conditions. The high degree of fenestration

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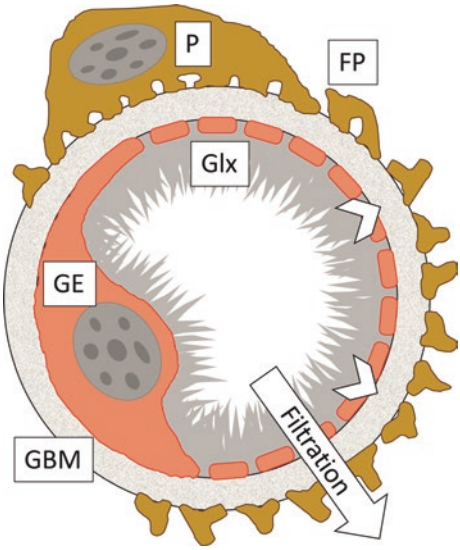

Fig. 26.1 Representation of the glomerular filtration barrier (GFB). The GFB consists of three components; the specialized epithelial cells called podocytes (P), which form tight slit diaphragms using their foot processes (FP), the highly fenestrated glomerular endothelial cells (GE) covered by the glomerular glycocalyx (Glx) and the glomerular basement membrane (GBM) connecting the podocytes and endothelial cells

of the endothelial cells is thought to facilitate the passage of water and small solutes. The size of albumin, the most predominant serum protein that is restricted to the glomerular barrier, is only 3.6 nm, which seems relatively small in relation to the 50–80 nm large fenestrae of the glomerular endothelium [3]. However, the endothelial cells are covered with a glycocalyx that also fills the fenestrae, and which facilitates in large part the charge and size dependent filtration of blood proteins, including albumin [1]. The endothelial glycocalyx is composed of a network of various membrane-bound glycoproteins and proteoglycans, such as perlecans, versicans, glypicans, and syndecans. The proteoglycans form the structural and functional backbone of the glycocalyx. Furthermore, proteoglycans are the main contributors to the glomerular charge barrier due to their highly negatively charged glycosaminoglycan (GAG) side chains. Moreover, GAGs are able to bind and regulate the activity of a myriad of bioactive molecules such as chemokines and growth factors and therefore contribute to cellular and tissue homeostasis [3–7]. One of the most predominant sulfated GAGs in the glomerular endothelial glycocalyx is heparan sulfate (HS). The importance of the endothelial glycocalyx has been shown by several experiments, for example, removal of non-covalently bound components of the endothelial glycocalyx caused a 12-fold increase in the fractional clearance of albumin without any observable changes regarding endothelial morphology [8]. Moreover, glycocalyx importance has been indicated by enzymatic destruction of the glycocalyx resulting in elevated albumin excretion [1].

The epithelial cells of the glomerulus, also called podocytes, are specialized, highly differentiated cells covering the outer side of the glomerular capillaries, thus facing the bowman's capsule, the beginning of the tubular component of the nephron [3]. The complex cellular architecture of podocytes consists of a cell body, major processes that extend outward from their cell body and form interdigitated foot processes (FPs) that enwrap the glomerular capillaries [9]. FPs harbor actin filaments as cytoskeletal elements that are anchored to the GBM via focal adhesions [10].

The FPs of podocytes play an important role in maintaining podocyte structure and supporting the glomerular capillary wall, which is necessary as the glomerular capillary requires high hydrostatic pressure to maintain glomerular filtration [1, 10, 11]. Furthermore, FPs also have a thick, negatively charged coat, the podocyte glycocalyx, facing the urinary space [9]. Notably, it appears that the thickness of the podocyte glycocalyx is also affected by diabetic conditions [12]. However, it remains elusive how the glycocalyx on podocytes contributes to barrier function of the GFB.

Besides glomerular endothelial cells and podocytes being key players in glomerular function individually, the interplay between glomerular endothelial cells and podocytes also plays a crucial role in glomerular function. For instance, the formation of the glomerular endothelial fenestrae depends on vascular endothelial growth factor (VEGF) that is produced by podocytes [13, 14].

26.2 Heparan Sulfate in Charge-Selective Filtration

For decades it was thought that negatively charged HS in the GBM was crucial for charge selective permeability of the GFB. HS in the GBM is namely decreased in many glomerular diseases, and this decreased HS expression is inversely correlated with the degree of proteinuria [15]. The primary role of HS in the GBM charge-selective filtration was questioned by studies in which genetically engineered mouse models with disturbed HS and HSPG expression in the GBM were used. No proteinuria or glomerular abnormalities were observed in mice lacking the most abundantly expressed proteoglycan core protein agrin or both agrin and perlecan [16, 17]. Furthermore, mice lacking EXT1, an essential HS polymerizing enzyme, in podocytes only showed mild albuminuria, although glomerular ultrastructural abnormalities such as foot process effacement could be observed [18]. Finally, degradation of HS by transgenic overexpression of heparanase in mice displayed a five fold reduction in GAG-associated anionic sites in the GBM, but no severe albuminuria or ultrastructural abnormalities were observed [19]. In summary, several mouse models targeting HS expression in GBM do not show proteinuria. These observations may suggest that HS in the glomerular endothelial glycocalyx is the main determinant for charge and size selectivity of the GFB and thus not HS in the GBM. Nevertheless, aforementioned studies were performed in healthy mice and therefore do not exclude a possible role of reduced HS in the GBM under pathological conditions.

26.3 Activation of Heparanase

Heparanase is the only known human endo-β (1-4)- D-glucuronidase capable of degrading HS [20, 21]. Heparanase is a member of the Carbohydrate Active Enzymes (CAZy) Glycoside Hydrolase (GH)79 family of carbohydrate processing enzymes. The recognized cleavage site of HS by heparanase is a trisaccharide accommodated into the heparanase binding cleft. Furthermore, specific sulfation of HS is key for heparanase interaction with HS [22] (Wu and Davies, Chap. 5 in this volume). The enzymatic activity of heparanase relies on an acidic environment as

the optimal activity of heparanase is between pH 5 and pH 6. Raising the lysosomal pH by administration of substances like bafilomycin A1 and chloroquine can, therefore, block the heparanase enzyme activity. Under normal conditions, heparanase acts mainly intracellular. Generally, secreted proheparanase is quickly bound to the cells and internalized, mediated by HS, low-density lipoprotein receptor-related proteins and mannose-6 phosphate receptors. After internalization proheparanase is transferred into late endosomes and lysosomes [23]. Cathepsin-L subsequently cleaves proheparanase into its active form consisting of one subunit of 50 kDa and another subunit of 8 kDa (Fig. 26.2) [24]. After activation of heparanase in the endo-

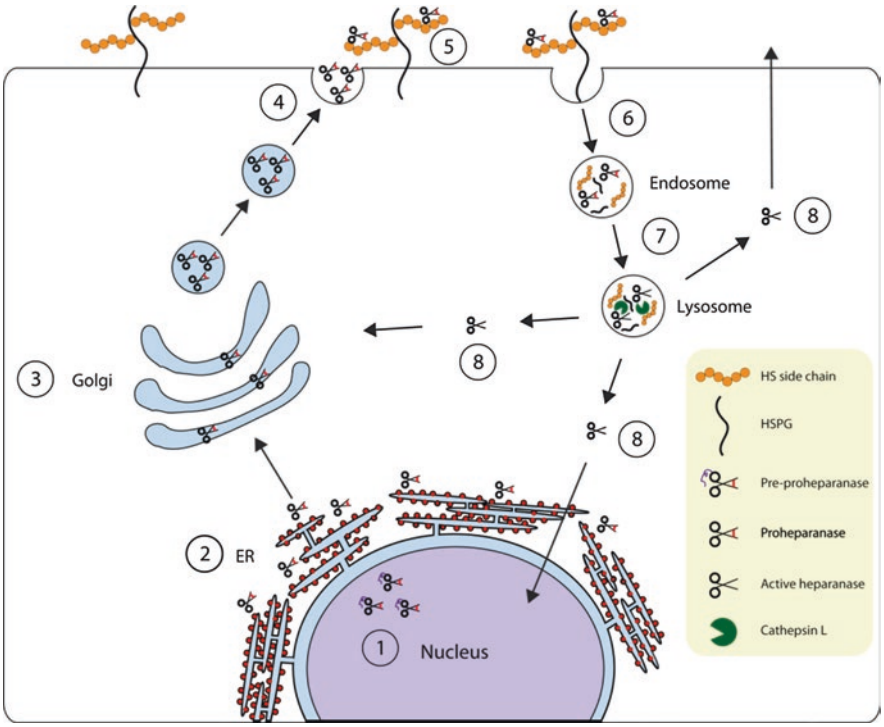

Fig. 26.2 Schematic overview of heparanase biosynthesis and trafficking. (1) Heparanase is synthesized in the nucleus as pre-proheparanase and subsequently trafficks to the endoplasmatic reticulum (ER). (2) In the ER, the signal peptide of pre-proheparanase is cleaved off, resulting in proheparanase. (3) Proheparanase trafficks to the Golgi apparatus, where proheparanase is packaged into vesicles. (4) Subsequently, proheparanase is secreted into the extracellular matrix. (5) Once proheparanase is located in the extracellular matrix, proheparanase can bind to cell-associated HSPGs (in particular to syndecan). (6) Binding of proheparanase to HSPGs then causes internalization of the complex consisting of HSPGs and proheparanase by endocytosis. (7) As endosomal maturation takes place, the endosomes will become more acidic and thus convert into lysosomes, which will activate cathepsin L. Cathepsin L will cleave out an internal linker domain of proheparanase thereby processing proheparanase into the active heparanase heterodimer. (8) Upon activation, heparanase can be transported back to the Golgi apparatus, where heparanase will cause further remodeling of the intracellular HS structures. Furthermore, heparanase can be transported to the nucleus, where it is involved in the process of chromatin remodeling, probably by affecting histone acetyltranferase activity. Finally, heparanase can be transported back to the cell surface where it will be secreted into the ECM and degrade HS of the glycocalyx

some, heparanase can be taken up by the Golgi system again (Fig. 26.2). However, heparanase activity in the Golgi system will be relatively low due to the neutral pH. Heparanase can also be transported from the endosome to the nucleus where heparanase is involved in facilitating chromatin remodeling to increase transcription of specific genes (Fig. 26.2) (Khanna and Parish, Chap. 3 in this volume). The mechanism behind the transcriptional activation by heparanase is still poorly understood. It is known that HS can inhibit histone acetylation in the nucleus [25, 26], whereas nuclear heparanase was shown to promote cleavage of HS, thus relieving HS-mediated inhibition [27]. Besides transportation to the Golgi system or the nucleus, heparanase can also be transported from the endosome back to the cell surface after activation (Fig. 26.2). Whereupon heparanase can degrade HS in the ECM, including HS in the glycocalyx of endothelial cells and podocytes. Degradation of HS in the glycocalyx disrupts glomerular barrier function and causes release of HS-bound chemokines and growth factors. Furthermore, cleavage of HS by heparanase in the glycocalyx generates potentially bioactive HS fragments [28]. Under physiological healthy conditions transport of activated heparanase from the endosome back to the cell surface does not frequently occur, except in placental trophoblasts and blood-borne immune cells as those cells require extracellular heparanase for physiological tissue remodeling and cell invasion [29]. However, under inflammatory conditions, such as diabetic nephropathy, extracellular heparanase activity is increased [23, 29].

26.4 Heparanase in Proteinuric Diseases

As all components of the GFB are important for proper glomerular filtration, proteinuria will occur if any of the GFB layers (Fig. 26.1), glomerular endothelial cells covered with glycocalyx, GBM, or podocytes, is affected by disease [3]. As mentioned previously, HS is one of the most predominant sulfated GAGs in the glycocalyx and the only known human endo-β (1-4)- D-glucuronidase capable of degrading HS is heparanase. Enzymatic degradation of HS by heparanase is known to affect several physiological and pathological processes such as morphogenesis, neovascularization, and tumorigenesis, but more important for the focus of this chapter glomerular barrier function, immune reactivity and inflammation [7, 30–32]. The role of heparanase in development of proteinuria was for the first time suggested in rats with puromycin aminonucleoside (PAN)-induced nephrosis, a model for minimal change disease, due to elevated levels of heparanase correlating with the loss of glomerular HS [33, 34]. Similar results were found in rats suffering from passive Heymann nephritis (PHN), a model for membranous glomerulopathy. Importantly, in the PHN rat model, it was shown that administration of a polyclonal antibody against heparanase reduced the level of proteinuria [35]. The importance of active heparanase in the development of experimental glomerular disease has also been shown by the use of heparanase inhibitor PI-88, since PI-88 administration to rats with PHN reduced proteinuria and preserved glomerular HS expression [36]. Furthermore, inhibition of heparanase by PI-88 was also effective in other animal models for glomerular diseases, such as type 1 diabetes [37] (Simeonovic

et al., Chap. 24 in this volume). Inhibition of heparanase by the specific heparanase inhibitor SST0001 (= Roneparstat) in type 1 diabetic wild type mice also resulted in less albuminuria and better renal function compared to vehicle-treated diabetic mice [33, 38], thereby confirming results obtained by heparanase inhibition using PI-88.

In general, an increased heparanase expression can be observed both in glomerular endothelial cells and podocytes in various experimental glomerular diseases, such as STZ-induced diabetic nephropathy and adriamycin nephropathy, a model for focal segmental glomerulosclerosis (FSGS), and human glomerular diseases, such as diabetic nephropathy, IgA nephropathy, minimal change disease, dense deposit diseases and membranous glomerulopathy, whereas in tubular cells high heparanase expression levels can be observed in both proteinuric conditions and healthy conditions [33, 39]. The elevated heparanase expression in glomerular cells in patients with glomerular diseases is associated with a decreased expression of HS in the glycocalyx. Furthermore, reduced HS expression has been found to inversely correlate with the degree of proteinuria. Finally, heparanase knockout mice are resistant to develop experimental diabetic nephropathy and proteinuria [38]. Similarly, the development of experimental glomerulonephritis is largely prevented in mice deficient for heparanase. Although renal function was decreased in both wt and heparanase deficient mice after induction of experimental glomerulonephritis with LPS or anti-GBM, heparanase deficient mice showed better renal function and less renal damage compared to wt mice. Furthermore, heparanase deficiency preserved glomerular HS expression in experimental glomerulonephritis [40]. Taken together, heparanase is in large part responsible for the glomerular degradation of HS and thereby for the development of glomerular diseases associated with proteinuria [39] (Table 26.1).

Table 26.1 Involvement of HS and HPSE in proteinuric diseases

Disease/animal model	Species	Glomerular HS expression	Heparanase expression	Proteinuria	Reference
Diabetic nephropathy	Human	Reduced	Increased	+	(42)
Systemic lupus erythematosus	Human	Reduced	Increased	+	(42)
Minimal change disease	Human	Reduced	Increased	+	(42)
Membranous glomerulonephritis	Human	Reduced	Increased	+	(42)
Dense deposit disease	Human	Reduced	Increased	+	(43)
IgA nephropathy	Human	Reduced	Increased	+	(44)
Experimental diabetic nephropathy	Mouse	Reduced	Increased	+	(38)
Experimental glomerulonephritis	Mouse	Reduced	Increased	+	(40)
Focal segmental glomerulosclerosis	Mouse	Reduced	Increased	+	(45)
Puromycin amino nucleoside-induced nephrosis	Mouse	Reduced	Increased	+	(44)
Minimal change disease	Rat	Reduced	Increased	+	(45)
Membranous glomerulopathy	Rat	Reduced	Increased	+	(45)

A cellular process linking heparanase and proteinuric diseases is autophagy. Autophagy is a cellular defense mechanism that clears cell debris and misfolded proteins and generates metabolic precursors and ATP. Notably, autophagy is important for cell survival under stressful conditions. Both HS and heparanase are involved in autophagy. HS constitutively inhibits autophagy, while heparanase positively stimulates the autophagy process through a non-enzymatic mechanism [23, 31, 41].

26.5 Regulatory Factors of Heparanase in Proteinuric Diseases

There are multiple factors involved in the regulation of glomerular heparanase expression and activity in diseases, including glomerular diseases. Cathepsin L, lysosomal cysteine protease, is involved in cleavage of proheparanase into active heparanase [24]. Furthermore, cathepsin L has three other substrates, namely the CD2-associated protein (CD2AP), synaptopodin and dynamin, in the podocyte that are known to be crucial for maintaining normal architecture of the podocyte cytoskeleton [42–46]. Notably, degradation of one of these three substrates results in development of podocyte foot process effacement, proteinuria and renal failure [42, 44, 47–49]. Elevated cathepsin L expression can be observed in several glomerular/proteinuric diseases including diabetic nephropathy, membranous glomerulopathy, minimal change disease and segmental glomerulosclerosis [42]. Inhibition of elevated cathepsin L expression with an irreversible inhibitor of cysteine proteases results in reduction of proteinuria [42, 50, 51]. Importantly, compared to wild type mice, cathepsin L deficient mice with streptozotocin-induced diabetes preserve their renal function and do not develop albuminuria, mesangial matrix expansion, tubulointerstitial fibrosis, podocyte injury nor display renal macrophage influx, which is most likely caused by the inability of cathepsin L-deficient mice to activate heparanase [50]. Notably, the latter study revealed that heparanase mediated effects on glomerular HS loss in experimental diabetic nephropathy, precede the effects of cathepsin mediated synaptopodin loss.

Another important factor that regulates heparanase expression in glomerular diseases is endothelin-1, which is a powerful vasoconstrictor that is released upon activation of endothelial cells. Endothelin-1 signals via the two G-protein coupled receptors available in the kidney, the endothelin receptor type A and the endothelin receptor type B [52–54]. Endothelin-1 induction is involved in various intracellular signaling pathways, resulting in vasoconstriction, proliferation, inflammation, extracellular matrix production, and fibrosis [55–58]. Furthermore, elevated expression of endothelin-1 has been observed in several human and experimental glomerular diseases, including diabetic nephropathy, FSGS and glomerulonephritis [59–61]. Treatment with endothelin receptor antagonist reduced proteinuria and improved renal function in several human and experimental glomerular diseases, which indicates that endothelin-1 plays an important role in the development of

proteinuria and renal damage in several glomerular diseases [62–67]. Recently, a study showed that endothelin-1 signaling, as occurs in endothelial activation, induces heparanase expression in the podocyte both *in vitro* and *in vivo*. Endothelin-1 stimulated podocytes did show an increased transendothelial albumin passage *in vitro*, while endothelin-1 had no direct effect on endothelial cells, suggesting crosstalk between podocytes and endothelial cells *in vivo*. Furthermore, mice with a podocyte-specific knockout of endothelin receptor type A and endothelin receptor type B (podETRKO) showed reduced albuminuria compared to wild type (wt) mice after induction of experimental diabetic nephropathy. Moreover, heparanase and HS expression were normal in the diabetic podETRKO mice compared to wt mice and glycocalyx thickness was reduced by 50–60% on both the endothelium and podocytes of wt mice after induction of experimental diabetic nephropathy, whereas endothelial glycocalyx thickness was preserved in the diabetic podETRKO mice and podocyte glycocalyx was still 25% reduced in diabetic podETRKO mice. These observations show that endothelin-1 induced heparanase expression in podocytes might ultimately lead to damage of the endothelial and podocyte glycocalyx, thereby leading to proteinuria and renal failure in experimental diabetic nephropathy [12].

Endothelial nitric oxide synthase (eNOS) is an enzyme, which is present in endothelial cells, that is responsible for production of nitric oxide (NO). Previous studies have shown that NO is important in maintaining a healthy endothelium. Furthermore, reduced NO production contributes to endothelial dysfunction [68, 69]. Several studies indicated that eNOS deficiency exacerbates renal injury in experimental FSGS, accelerated anti-GBM glomerulonephritis, and diabetic nephropathy [70–75]. Moreover, eNOS gene delivery reduced proteinuria and renal failure in a rat model for FSGS [76]. Furthermore, a relation between eNOS and heparanase has been shown in a model for FSGS as eNOS seems to prevent adriamycin-induced heparanase induction and the development of proteinuria in mice. Normally adriamycin-induced nephropathy (AN) resistant strain failed to develop proteinuria after AN induction, whereas proteinuria and impaired renal function occurred in eNOS-deficient mice. Notably, heparanase expression was increased in the eNOS deficient AN mice. Additionally, glomerular HS expression was normal in wt AN mice but reduced in eNOS deficient AN mice [77].

Vitamin D is a steroid hormone that is important in regulation of calcium and phosphate homeostasis. The availability of the enzyme that is responsible for the activation of vitamin D is reduced in chronic kidney disease (CKD) patients [78]. The regulation of heparanase expression through vitamin D signaling was demonstrated by an increased heparanase expression and the development of proteinuria in knockout mice incapable of converting vitamin D into its active form. Furthermore, heparanase expression was reduced after treatment with the active form of vitamin D in rats with adriamycin-induced nephropathy [23, 79]. These results show that the protective effect of vitamin D on proteinuria could be mediated by reduction of the increased glomerular heparanase expression associated with proteinuric diseases.

26.6 Heparanase as Key Player in Diabetic Nephropathy

DN is a life-threatening complication of diabetes and dysfunction of the GBM plays an important role in the pathogenesis of DN [38]. To date, abnormalities of the glomerular endothelium in DN have been reported in various studies. One study showed a reduced amount of fenestrated glomerular endothelium in diabetic patients, i.e., healthy controls in this study contained about 41% fenestrated glomerular endothelium compared to 32% in microalbuminuric patients and 25% in macroalbuminuric patients. In the same study, it has been shown that podocyte detachment and thus loss of podocyte foot processes occurs in diabetic patients [80, 81]. Moreover, it has been revealed that endothelial damage and podocyte damage occur simultaneously [81]. A role of heparanase in DN was suggested due to reported elevated levels of heparanase both in kidney and urine of DN patients compared to healthy controls. Furthermore, up-regulation of heparanase has been shown both *in vitro* and *in vivo* in hyperglycemic conditions [7, 82]. However, the essential role of heparanase in DN has been illustrated using heparanase knockout mice. These mice, incapable of producing heparanase, did not show any sign of albuminuria in response to STZ induced diabetes, while a five-fold increase in urinary excretion rate was observed in wild type mice after STZ induced diabetes. Importantly, the expression of 3-O-sulfated HS domains was reduced in glomeruli of diabetic wild type mice, whereas no change in HS expression was detected in glomeruli of diabetic heparanase knockout mice. Additionally, less albuminuria could be observed in type 1 and type 2 diabetic mice treated with heparanase inhibitor compared to untreated control [38].

Another potential role of heparanase in the development of diabetes and diabetes complications is associated with pancreatic β-cell failure as a result of increased extracellular heparanase activity. Islet-specific autoreactive T cells can produce heparanase that promotes the migration of leukocytes going from the pancreatic blood vessels into the islet, which causes an immune response while simultaneously depleting islet β-cells of intracellular HS, which is necessary for β-cell survival [30].

The diabetic milieu is one of the strongest inducers of heparanase expression; urinary heparanase levels correlate with reduction in systemic glycocalyx volume and albuminuria in patients with type 1 and type 2 diabetes [33]. Additionally, it has been demonstrated that high glucose levels alter the biosynthesis of sulfated GAG domains, in particular that of HS [13, 83]. Aforementioned glucose-dependent alteration of GAGs indicates a possible role of hyperglycaemia in systemic glycocalyx reduction. Furthermore, hyperglycaemia and glycated serum proteins as well as other factors such as ROS, aldosterone, and angiotensin II upregulate heparanase expression in various cell types including endothelial cells and podocytes [33, 84, 85].

In addition to the role of heparanase in loss of HS in the GFB as outlined above, another role of heparanase in DN pathology has been suggested. HS-bound growth factors, cytokines and bioactive HS fragments can be released in the glomeruli due to heparanase [7, 86]. Furthermore, heparanase has been implicated in coupling macrophage activation, chronic inflammation and renal injury under diabetic conditions. The view of DN being solely caused by metabolic or hemodynamic alterations

has shifted as clear evidence indicated that activation of innate immunity and chronic inflammation play a substantial role in the pathogenesis of diabetes and its complications, thus also DN. Various inflammatory molecules such as cytokines as well as immunocytes have been implicated in diverse pathogenic pathways related to DN [87–89]. Macrophages are the primary immunocytes infiltrating the diabetic kidney, thereby contributing to the development of renal damage [90, 91]. It has been shown that under diabetic conditions latent heparanase produced by glomerular cells and post-translationally activated by cathepsin-L of tubular origin, sustains continuous activation of kidney-damaging macrophages by diabetic milieu components, thereby creating chronic inflammatory conditions and fostering macrophage-mediated renal injury [7]. After infiltration of the kidneys, macrophages can be activated by various triggers of the diabetic milieu, such as high glucose [92], free fatty acids [93], and advanced glycation end products (AGE) [94], to release reactive oxygen species (ROS) and proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-1 and IL-6, which cause injury to podocyte and tubular cells [7, 89, 95, 96]. It has been demonstrated that diabetic WT mice showed elevated TNF-α levels, whereas no increase in TNF-α could be observed in diabetic heparanase knockout mice, which also failed to develop DN in response to STZ-induced diabetes [38]. Moreover, increased numbers of TNF-α producing macrophages were found in diabetic kidneys of WT mice, but not heparanase knockout mice [7] (Elkin, Chap. 17 in this volume).

26.7 Immune Cells in Glomerular Diseases and Heparanase Mediated Sensitization

Several mechanisms can be proposed that could be involved in the pathogenesis of proteinuria with respect to the role of heparanase-mediated loss of HS; (1) loss of barrier function due to reduced presence of HS in glomerular endothelial glycocalyx, (2) changes in glomerular cell-GBM or cell-cell interactions due to the loss of HS, (3) the induction of signaling cascades resulting in changed cell properties, (4) release of HS-bound growth factors, cytokines, chemokines and bioactive HS fragments, (5) contribution to influx of immune cells and a proinflammatory cytokine milieu, and/or (6) increased cellular activation due to heparanase-mediated shaving of cells, which may, for instance, improve the accessibility of cytokine receptors to their specific ligands [15, 33, 40].

As heparanase facilitates both turnover and recycling of HS, it is not surprising that heparanase plays a crucial role in remodeling of HS in the glycocalyx, thereby altering barrier function and/or mediating inflammatory processes. Furthermore, it has been shown that modulation of HS in the glycocalyx by heparanase can affect the binding of chemokines and growth factors and leukocytes recruitment. Leukocytes and macrophages are important mediators of proteinuria, glomerular damage, and the local cytokine milieu. Heparanase plays a substantial role in leukocyte and macrophage glomerular influx as indicated by a study determining the leukocyte and mac-

rophage glomerular influx in heparanase knockout mice compared to wt mice after induction of anti-GBM and LPS-induced glomerulonephritis. Macrophage influx was reduced in heparanase knockout mice in both models for glomerulonephrites and leukocyte influx in anti-GBM glomerulonephritis. Consistently, several inflammatory HS domains on the glomerular endothelium associated with leukocyte trafficking were reduced in heparanase deficient mice compared to wt mice. Furthermore, mRNA expression of types 1 and 2 T helper cell cytokines such as TNF-α, interferon-γ, IL-12α, IL-12β, IL-10 and IL-6 was lower in heparanase deficiënt mice compared to wt mice in both anti-GBM and LPS-induced glomerulonephritis, thereby illustrating that heparanase deficiency in experimental glomerulonephritis leads to a less proinflammatory cytokine milieu in the kidney [40] (Elkin, Chap. 17 in this volume).

Patients with diabetic nephropathy show increased levels of monocyte chemoattractant protein-1 (MCP-1) in their renal tissue and urine, suggesting that macrophages have a pathogenic role in the development of proteinuria and glomerular damage, and the progression of renal disease in humans [97]. Inhibition of MCP-1 by the spiegelmer Emapticap pegol mNOX-E36 resulted in decreased albuminuria in patients with type 2 diabetic nephropathy. Furthermore, MCP-1 inhibition by the Emapticap decreases albuminuria in diabetic Apoe knockout mice and is associated with a shift in tissue macrophage phenotype towards alternatively activated anti-inflammatory M2 macrophages, resulting in reduced levels of TNF-α and expression of glomerular cathepsin L and heparanase. Moreover, inhibition of MCP-1 by Emapticap caused restoration of the glomerular glycocalyx and barrier function [23, 98]. Notably, the enzyme that cleaves proheparanase into its active form, cathepsin L, can be secreted by macrophages [37, 50]. The secretion of cathepsin-L by macrophages together with their ability to produce heparanase provides a link between infiltration of macrophages in the diabetic kidney and heparanase. Moreover, it was shown that macrophages are more prone to activation by for instance INF- γ, after they have been pre-treated with heparanase, resulting in, amongst others, increased TNF-α production (Fig. 26.3) [7, 99]. The mechanism behind this sensitization of macrophages by heparanase is only poorly understood, but it has been suggested that toll-like receptors (TLRs) 2 and 4 mediate cellular hyper-activation by binding of heparanase-generated HS fragments [100–102]. Enhanced accessibility of receptors for their specific ligands has been suggested as a second possible mechanism in heparanase-mediated sensitization of cells. Finally, highly sulfated HS expressed by macrophages normally sequesters IFN-β, thereby maintaining type I IFN reception in a quiescent state. Reduction of cell-associated HSPG or alteration of HS composition, either genetically or enzymatically, increases the bioavailability of IFN-β, which might result in macrophage activation [103, 104].

It can thus be hypothesised that increased levels of heparanase in the glomerulus may sustain leukocyte and macrophage influx, macrophage inflammatory responses, and renal damage in proteinuric diseases. A recent study even showed that heparanase-mediated sensitization is not limited to macrophages, because heparanase-mediated hypersensitivity for insulin was shown in breast cancer cells [105], thereby postulating the possibility of sensitization of other cell types involved in the development of diabetic nephropathy.

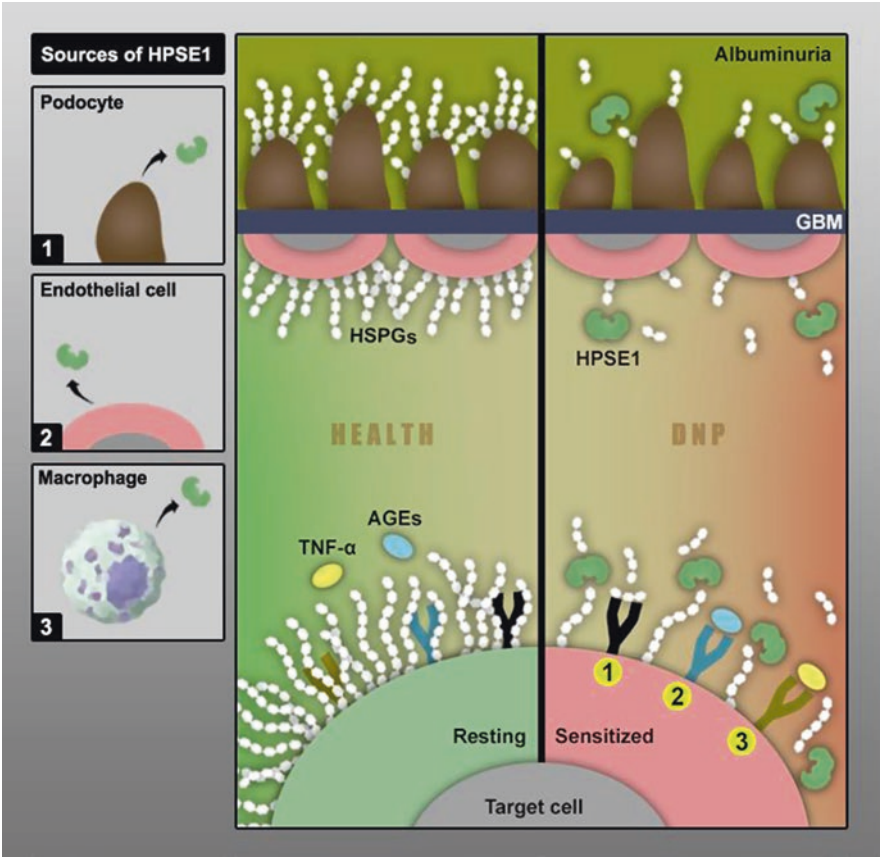

Fig. 26.3 The interplay between glomerular and immune cells leading to increased glomerular heparanase activity and albuminuria. During the development of diabetic nephropathy (DNP) glomerular heparanase activity is increased, thereby degrading heparan sulfate (HS) in the glomerular filtration barrier, which leads to proteinuria. Heparanase may be derived from immune cells such as macrophages and glomerular cells, such as podocytes and glomerular endothelial cells (left panel). The local inflammatory cytokine milieu acts on both immune and glomerular cells, thereby further enhancing heparanase and cathepsin L expression (not shown), which is required to activate pro-heparanase. Exciting recent data show that active heparanase can sensitize cells by degrading HS at the cell surface, as depicted in the lower part of the figure. Heparanase-generated HS then binds to TLR2 and TLR4 (depicted by 1), thereby increasing cellular activation. Additionally, heparanase mediated shaving of cells may improve the accessibility of cytokine receptors (depicted by 2 and 3) for their specific ligands, which also leads to increased cellular activation. Overall, the increased glomerular heparanase activity shaves the glycocalyx from both endothelial cells and podocytes, thereby facilitating albuminuria

26.8 Heparanase As a Pharmacological Target

Due to its prominent role in glomerular disease, extracellular heparanase would serve a promising pharmacological target. Intracellular heparanase expression and activity plays a key role in cell survival and communication and should therefore not

be targeted for therapy, except in cases where cells have to be killed like in cancer. Various compounds aiming to inhibit heparanase expression or reduce heparanase activation are under development and/or tested for their therapeutic benefit. However, most of these compounds are directed towards cancer therapy and might target both intracellular and extracellular heparanase.

There are several possible approaches to inhibit heparanase expression or reduce heparanase activation. One such approach makes use of so-called HS-mimetics, which are drugs that compete with natural HS substrate by binding to the HS substrate-binding domain of heparanase [23, 106, 107]. One such HS-mimetic is SST0001, a polymer with a heparin-like structure. As an analogue of the natural substrate of heparanase HS, heparin is considered to be a potent inhibitor of heparanase [106]. One disadvantage of HS-mimetics relies on their structural resemblance to natural HS, thereby increasing the possibility of off-target effects. There are hundreds of proteins known to interact with HS, together called the heparan sulfate interactome, or heparanome. Among the heparan sulfate interactome are proteins that are involved in various cellular and biological processes such as cell attachment, migration, invasion and differentiation, morphogenesis, organogenesis, blood coagulation, lipid metabolism, inflammation, and responses to injury [108]. One possible off-target effect of HS-mimetics is that they can influence coagulation by modulation of HS-mediated interactions between thrombin, antithrombin III, and protein C inhibitor [108]. Furthermore, HS-mimetics can be taken up by cells and modify the intracellular regulatory function of heparanase, as outlined. Another down-side of HS-mimetics is their possibility to provoke an inflammatory response since HS can serve as a ligand for TLR2 and TLR4 on macrophages and other cells, thereby provoking an inflammatory response [109].

The second class of HS-mimetics is synthetic HS tetrasaccharides containing unsubstituted glucosamine residues, like GP545. These synthetic HS structures are made to be resistant to heparanase activity and can, therefore, be applied as a heparanase inhibitor [110]. Furthermore, multiple oligosaccharides that are derived from marine algae are currently tested for their possible ability to modify HS-heparanase interactions. Sulfated polysaccharides that resemble glycosaminoglycans are present in different algae species in the marine environment. One such compound that is currently being explored is λ-carraheptaose, which is a highly sulfated polysaccharide derived from red algae. The λ-carraheptaose seems to act simultaneously as competitive inhibitor of heparanase, and thus as HS-mimetic, and as inhibitor of FGF-2 signaling [111]. A second compound is a fucosylated form of chondroitin sulfate, which is extracted from marine echinoderm. This fucosylated form of chondroitin sulfate was shown to reduce heparanase expression in the glomerulus thereby protecting rats from streptozotocin-induced diabetic nephropathy [23].

Administration of heparanase substrates such as soluble HS, heparin, low-molecular-weight heparin or the heparinoid danaparoid all are, in potential, able to reduce albuminuria in patients with diabetes mellitus, but these compounds can have some off-target effects as previously mentioned. One example of such class of drugs is sulodexide, a highly purified mixture consisting of 80% of low-molecular-weight heparin and of 20% of dermatan sulfate [112]. Conflicting results have been obtained regarding sulodexide. Sulodexide was shown to be effective in restoring the glycocalyx thickness and showed a trend towards normalization

of systemic albumin clearance in a study of type 2 diabetes mellitus patients However, in two other studies no such effect was observed in type 2 diabetes mellitus and diabetic nephropathy patients [23]. These contradicting findings can possibly be explained by the presence of different biological active structures as various sulodexide preparation were used obtained from different animal sources. Due to the lack of insight into the specific structures within different sulodexide preparations that are responsible for heparanase inhibition and subsequent anti-proteinuric effects, wrong conclusions may have been drawn from aforementioned studies.

A second strategy to inhibit heparanase mediated HS breakdown makes use of inhibitors that directly block the HS-binding site on heparanase. There are three potential HS-binding domains in heparanase identified that could serve as a target for heparanase inhibition. A peptide directed against the Lys158-Asp171 domain of heparanase physically interacts with HS and heparin and thereby inhibits heparanase activity [113]. Moreover, a polyclonal antibody and two monoclonal antibodies raised against this region are currently being developed and may provide a new class of drugs leading to a reduced heparanase activity [113, 114].

Some drugs that are currently used in renal medicine have been shown to suppress glomerular heparanase expression and activity, such as drugs targeting regulators of heparanase as outlined, i.e. endothelin-1, eNOS and vitamine D, angiotensin-converting-enzyme (ACE) inhibitors and angiotensin-II-receptor blockers (ARBs) [79, 85]. These drugs have been associated with reduced albuminuria in clinical trials, possibly due to their ability to reduce heparanase activity. Endothelin-A (ETa)-receptor blockade is currently under exploration in randomized clinical trials for its renoprotective potential as it has been shown to reduce albuminuria in clinical studies. Selective ETa-receptor blockade facilitates preferential ETb-receptor stimulation by endothelin-1 and increased nitric oxide production by the endothelium [23].

Several drugs currently tested or used for treatment of renal dysfunction target pathways that reduce heparanase activity both in immunocytes, and endothelial cells and podocytes, and might therefore be adequate to resolve residual albuminuria. Clinical studies that have therapeutically targeted monocytes by blocking their chemokine receptor CCR2 (also known as CD192) or by blocking the CCR2 ligand, MCP-1, support the idea that albuminuria can be reduced by targeting immunocyte activation as both targeting of immunocytes and kidney cells have shown a positive effect on albuminuria in patients with diabetic nephropathy [115, 116]. The obtained reduction in albuminuria due to blockage of the CCR2 ligand was associated with a reduced cathepsin L release by tissue macrophages [23]. As outlined, cathepsin L is important in heparanase activation, and reduction of cathepsin L expression/activity was therefore further associated with reduced heparanase activity and restoration of the endothelial glycocalyx and barrier function. Due to its key role in heparanase activation, cathepsin L could also be considered a potential therapeutic target. Only several non-specific cathepsin L inhibitors have been tested and showed reduction of proteinuria in experimental models of anti-glomerular basement membrane glomerulonephritis [23, 51, 117]. To date, more specific cathepsin L inhibitors have been developed, however, their therapeutic effects in glomerular diseases have not been explored yet.

Another promising treatment strategy includes the use of heparanase 2, which is an inactive heparanase variant as it shares 44% identity and 59% similarity with heparanase, but lacks enzymatic activity [118]. It has been shown that heparanase 2 inhibits heparanase activity [118, 119]. Heparanase 2 possibly acts via its higher affinity to HS compared to heparanase, thereby blocking the binding of heparanase to HS. As heparanase cannot bind to HS on the cell surface, it fails to get internalized and will, therefore, remain inactive. In addition, heparanase 2 may physically interact with heparanase, thereby preventing the cleavage of HS chains. An advantage of the use of heparanase 2 to inhibit heparanase compared to HS-mimetics is that heparanase 2 is not likely to activate macrophages, which is a problem in the case of HS-mimetics as outlined above. However, the potential of heparanase 2 as an inhibitor of heparanase activity in glomerular diseases remains to be explored in experimental models.

Besides the application of heparanase as a target for therapy, heparanase activity might also serve as a suitable biomarker for risk stratification and treatment titration as urinary heparanase excretion is increased in patients with diabetes, especially in case of albuminuria, whereas no urinary heparanase activity is present in healthy individuals.

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Chapter 27
Impact of Heparanse on Organ Fibrosis

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27.1 Introduction

Fibrosis, or the formation of fibrous scar tissue in response to a lesion, is part of a normal repair and healing process. In young individuals and in cases where the damage is only momentary, the scars resolve over time and are replaced by new tissues that restore the initial functionality of the affected tissue or organ. In older subjects and in the situation of a permanent lesion or damage, the scars do not resolve and the fibrotic tissue tends to accumulate. In organs such as the heart, lungs, kidneys or liver, the accumulation of fibrous tissue can progressively alter their normal architecture and function with potentially devastating results. It is estimated that fibrotic disease is the third leading cause of death, after cardiovascular disease and cancer, in western, developed countries [1, 2]. Although there is considerable heterogeneity in the etiological mechanisms underlying the development of fibrotic diseases and their clinical manifestations, numerous studies have identified the common molecular alterations responsible for an uncontrolled accumulation of extracellular (ECM) in affected tissues and the replacing of normal tissues with fibrotic ones. The first step is the initiation of the response, driven primarily by injuries directed to parenchymal cells and consequent apoptotic/necrotic cell death. The second one is the activation of a chronic inflammatory response triggered by injured cells and sustained by recruited inflammatory cells (e.g., leukocytes, macrophages,

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and T-cells) and secreted cytokines (interleukins and TNF-α). The third step involves fibrogenic cells which are activated by inflammatory cells and their pro-fibrotic factors (primarily TGF-β, PDGF and FGF-2) and drive the scarring process by the secretion of abundant ECM proteins (predominantly collagens type I and III, fibronectin and laminin). Finally, the persistence of chronic injury together with the fact that the synthesis of ECM is not counterbalanced by its degradation and resorption progressively promote fibrotic progression which leads to end-organ failure [3].

At the cellular level, it is widely accepted that fibroblasts and myofibroblasts are the key effectors responsible for the synthesis of ECM proteins in fibrotic disorders of many organs. Myofibroblasts are actively proliferating cells with contractile characteristics due to the expression of the alpha isoform of smooth muscle actin (α-SMA). The initial conviction that myofibroblasts could derive from the activation and proliferation of resident fibroblasts has been gradually revised based on the observation that the origin of myofibroblasts is variable. Indeed, it has been shown that bone marrow-derived fibrocytes, epithelial cells undergoing epithelial-to-mesenchymal transition (EMT) and endothelial cells undergoing endothelial-to-mesenchymal transition (EndMT) as well as pericytes can contribute to forming a reserve of myofibroblasts [3]. In addition to being involved in the synthesis and deposition of the extracellular matrix, these cells, thanks to their contractile capacity, contribute to the deformation of the tissue parenchyma which in turn supports the pathogenesis and failure of the affected organ. The molecular processes that drive fibrosis are triggered at the cellular level by different signaling pathways. The main one involves TGF-β and requires its binding to a serine-threonine kinase type II receptor which recruits and phosphorylates a type I receptor. This receptor complex once activated phosphorylates the SMAD proteins which enter the nucleus and act as transcription factors modulating the expression of target genes, including those codifying fibrous proteins of the extracellular matrix in most of the fibrogenic cells. Alternative signaling pathways can be activated by other growth factors, or by cytokines or vasoactive factors, which trigger other pathways such as those involving MAPKs (Mitogen-activated Protein Kinases) or Rho-associated protein kinases (ROCKs). It should also be emphasized that some ECM molecules can also stimulate fibrosis through the link with integrins and activation of the related signaling pathways [4, 5]. Hand in hand with the excessive deposition of ECM, the fibrosis is also dependent on a reduced degradative capacity of enzymes responsible for remodeling various components (fibrous proteins and proteoglycans) of the matrix. These enzymes include Matrix Metalloproteinases (MMPs) and heparanase (HPSE) produced by a broad spectrum of cells [6, 7]. Regarding HPSE, several pieces of experimental evidence produced by our group and by others have shown an active involvement of this endoglycosidase in the development of fibrosis of some organs including the kidney, the liver, and the mesothelium. Rather than by the catalytic activity responsible for cutting the side chains of heparan sulfate proteoglycans (HS), the involvement of HPSE in fibrosis appears to be dependent on the fact that HPSE also promotes the release and diffusion of various HS-linked molecules such as growth factors, cytokines, and enzymes. The present chapter intends to summarize

all the notions and experimental evidence so far produced concerning the involvement of HPSE in the development of organ fibrosis.

27.2 Kidney Fibrosis

Renal fibrosis is a dynamic event that culminates in the accumulation of excessive connective tissue in the kidney [8, 9]. Clinical and molecular studies have proved that tubulo-interstitial fibrosis can be considered a consistent marker of chronic kidney disease (CKD) progression independently of the original nephropathy [10, 11]. Although new therapeutical strategies have reduced the progression of CKD-to-End-Stage Renal Disease (ESRD) [9], CKD is a condition that affects more than 10% [12] of the global population, and thus it represents a major public health issue. It is important to observe that the principal causes of renal fibrosis are type 2 diabetes and ischemic/hypertensive nephropathy [13], while CKD with the associated fibrosis also represents a significant risk factor for cardiovascular diseases [14].

Over the last few years, many efforts have been made to understand the molecular mechanisms at the basis of renal fibrosis [15]. However, the validated treatments to prevent CKD progression and the associated complications are limited [16]. These treatments include the inhibition of angiotensin-converting enzyme, the blocking of angiotensin receptor, the optimal control of blood pressure and the control of metabolic acidosis [9, 16]. At present, there are no novel safe and efficient therapeutic targets for the prevention or reversal of renal fibrosis. Thus, new strategies to control this process are highly imperative [17], and several promising therapeutic pathways are being explored [17–19].

After initial insults, the damaged kidney activates a series of events in order to repair the damage. Initially, the accumulation of extracellular matrix maintains the three-dimensional stability and functionality of all the renal structures (glomeruli, tubules, interstitium, and vasculature). Unfortunately, in severe and/or chronic injuries, extracellular matrix accumulation becomes excessive, thus altering the renal structures and their functions. The accumulation of extracellular matrix in the tubulo-interstitial compartment is referred to as tubulo-interstitial fibrosis, whereas the accumulation of extracellular matrix in the glomeruli is referred to as glomerulosclerosis.

There are several factors occurring in the induction and accumulation of fibrosis, including various molecules, cytokines/growth factors and cellular elements [8]. At a macroscopic level, renal fibrosis is characterized by the infiltration of inflammatory cells, the activation and proliferation of mesangial cells and fibroblasts together with the loss of renal parenchyma resulting in tubular atrophy, capillary loss and podocyte depletion [8]. Altogether these events culminate in irreversible organ damage.

In this context, heparanase may significantly influence the development of renal fibrosis and the progression of CKD through its multiple roles in the biological pathway of kidney fibrogenesis [10]. In the kidney, heparanase is up-regulated in

response to several stimuli both at a tubular and glomerular level. Proteinuric animal models identified increased heparanase levels in puromycin amino nucleoside-induced nephrosis, streptozotocin-induced diabetic nephropathy and adriamycin nephropathy [19–21]. Since heparanase degrades heparan sulfate in the glomerular basement membrane, these studies [19, 21] proved that it takes part in the development of proteinuria. Proteinuria represents a stress factor for tubular cells which can have direct toxic and paracrine effects leading to fibrosis [22]. Also, these *in-vivo* studies proved that the current pharmacological therapy to control CKD with angiotensin receptor modulates heparanase expression.

Heparanase is also up-regulated in several human nephropathies: diabetic nephropathy, membranous glomerulonephritis, IgA nephropathy, dense deposit and minimal change disease [19]. Heparanase gene expression is up-regulated by transcription factors such as Sp1, GA-binding protein, EST1, EST2, EGR1 and down-regulated by p53 and DNA methylation [23]. Among these transcription factors, Sp1 and EGR1 are involved in the development of renal fibrosis [24–26]. Thus, we envision that heparanase expression could also be controlled by these two factors in the kidney. Heparanase expression is also regulated by multiple endogenous molecules such as tissue necrosis factor-α (TNF-α) [27, 28], free radicals [20], pro-inflammatory cytokines, oxidized LDL or fatty acids [29, 30], angiotensin II and aldosterone [31], all elements considered triggers and mediators of the progression of renal fibrosis [32–34].

As previously mentioned, diabetes is one of the main causes of renal fibrosis and several diabetes-associated features up-regulate heparanase expression in the kidney. High glucose increases heparanase expression in endothelial cells and podocytes [35, 36], which contribute to the degradation of heparan sulfate in the glomerular basement membrane and the consequent proteinuria. Proteinuria is then responsible for increased heparanase expression at the tubular level [37] via the megalin-mediated PI3K/AKT pathway [37, 38]. In diabetes, there is a great production of advanced glycation end-products (AGEs), which are proteins or lipids that become non-enzymatically glycated and oxidized by aldose sugars. AGEs are responsible for heparanase up-regulation in macrophages [39], endothelial cells [40] and proximal tubular epithelial cells [37]. This process is mediated by binding to the specific receptor (RAGE) [39, 40] and activation of the PI3K/AKT pathway [37, 39].

In the kidney and, in particular, in tubular epithelial cells, heparanase expression is also increased by two important pro-fibrotic growth factors: fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGF-β) [41, 42]. In the case of FGF-2, heparanase expression is regulated via the PI3K/AKT pathway [42]. Overexpression of heparanase at the tubular level is responsible for heparan-sulfate degradation and the modulation of syndecan-1 expression, the most abundant heparan sulfate proteoglycan in epithelial cells [37]. This situation can increase the availability of FGF-2, TGF-β and other pro-fibrotic factors, stored in an extracellular matrix reservoir, creating a fibrogenic vicious circle [10, 43].

An important phenomenon associated with renal fibrosis is the partial epithelial-to-mesenchymal transition of tubular cells (EMT) [44] and both FGF-2 and TGF-β represent some of the strongest activators [45]. Epithelial cells undergoing EMT are

characterized by the loss of epithelial proteins including E-cadherin, zonula occludens-1 and cytokeratin and the acquisition of mesenchymal markers such as α-SMA, fibronectin, vimentin, and fibroblast-specific protein-1 [46].

It has been demonstrated that heparanase is necessary for FGF-2 to activate the PI3K/AKT pathway leading to EMT and for the establishment of FGF-2 autocrine loop [42, 47]. In detail, heparanase-silenced tubular cells express a higher amount of syndecan-1 which, when anchored to plasma membranes, represents an inhibitor of FGF-2 receptor binding. Also, in heparanase-silenced tubular cells, FGF-2 is unable to increase the production of matrix metalloproteinase 9 (MMP9) and heparanase which mediate the conversion of syndecan-1 from inhibitor to a potent activator of FGF-2 signaling [42]. The central role of heparanase in the regulation of renal fibrosis induced by FGF-2 has also been proven by showing that heparanase inhibitors (sulodexide and fucoidan) can block FGF-2-induced EMT of tubular cells [47, 48].

Investigators also showed that the lack of heparanase delays the induction of EMT by TGF-β and reduces its autocrine loop. Moreover, heparanase is necessary for pro-fibrotic factors (i.e., FGF-2, albumin, AGE) to up-regulate the expression of TGF-β in tubular cells [41]. Also, *in-vivo* evidence confirmed that diabetic-heparanase-knockout mice did not display an increased TGF-β production and hence did not develop renal fibrosis [49].

Ischemia/reperfusion (I/R) injury, another cardinal element in the induction of renal fibrosis, is an event characterized by a decrease in tissue perfusion and subsequent restoration of blood flow as occurs in myocardial infarction, major vascular surgery, organ transplantation and acute kidney injury (AKI) [50]. In particular, the hypoxic phase caused a reduction of nutrients and this altered normal cell metabolism/energy homeostasis [51]. The extent of the damage is influenced by the magnitude and the duration of ischemic insult, resulting in activation of cell death programs including apoptosis, necrosis and autophagy-associated death [52]. Following oxygen reperfusion, endogenous ligands from necrotic and apoptotic cells enhance the activation of innate and adaptive immunity thus exacerbating inflammation [53]. In the kidney, I/R injury is responsible for both AKI and delayed graft function (DGF) in transplantation [54], and both these events correlate with the development of tubulo-interstitial fibrosis [55]. Hypoxia by itself [56] and hypoxia-generated reactive oxygen species (ROS) [57], pro-fibrotic cytokines and growth factors [58] alter tubular-cell physiology, sustaining the fibrotic process and activating the EMT process [56, 59].

It has been proven *in-vitro* and *in-vivo* that I/R up-regulates heparanase in renal tissue and tubular cells. Moreover, heparanase can regulate the activation of EMT programs in tubular cells [60], and this phenomenon can be controlled by strategies targeting heparanase [61]. Interestingly, it has been demonstrated in a mouse model that heparanase overexpression is responsible for a stronger acute injury in response to I/R (worse renal function and stronger tubular damage). Heparanase overexpression also increased the production of pro-inflammatory (i.e., IL6) and pro-fibrotic factors (i.e., TGF-β). In addition, treatment with PG545, a potent heparanase inhibitor, ameliorated the I/R-induced acute renal damage [62] (Abassi and Goligorsky, Chap. 28 in this volume).

The mechanisms that underlie I/R injury are complex, multifactorial and deeply intertwine. I/R induces inflammatory cells recruitment and production of cytokines, chemokines, and other pro-inflammatory factors. Macrophages are the predominant infiltrating cells in kidney I/R injury. They express TLR-2 and -4 that are activated after the release of danger-associated molecular patterns (DAMPs) by damaged parenchymal cells eliciting the production of a large amount of pro-inflammatory cytokines and chemokines [63, 64]. TLR-2 and -4 are constitutively also expressed by tubular epithelial cells and their expression increases after I/R injury, being involved in the production of pro-inflammatory factors [65]. Furthermore, IFN-γ, TNFα and granulocyte-macrophage-colony stimulating factor (GM-CSF) secreted by NK cells promote the full activation of M1 macrophages [66]. Macrophages also play a role during the reparative phase by switching their phenotype from M1 to M2. The uptake of apoptotic cells by macrophages and regulatory T cells increases the production of anti-inflammatory cytokines such as transforming growth factor β (TGF-β) and IL-10, promoting the polarization of macrophages into M2 phenotype [67, 68] which suppresses the inflammatory response and induces a proliferative repair phase [69].

A confirmed event induced by I/R injury is up-regulation of heparanase at the tubular and glomerular levels [62, 70]. Heparanase then modulates tubular cell apoptosis and DAMPs generation. DAMPs and molecules generated from necrotic cells can, in turn, activate TLRs both on macrophages and tubular cells. Heparanase also regulates TLRs expression in both cell types. Tubular cells in response to direct hypoxic stimuli and TLR activation produce pro-inflammatory cytokines which attract and activate macrophages thereby sustaining the inflammation. These events are prevented by silencing or inhibition of HPSE. High levels of HPSE, moreover, facilitate M1 polarization of infiltrated macrophages, a condition that sustains the additional release of cytokines and growth factors by macrophages, thus worsening parenchymal damage and sustaining partial EMT of tubular cells - a condition that over time leads to fibrosis [59].

Chronic renal damage after I/R injury is characterized by glomerulosclerosis, tubular interstitial fibrosis, and tubular atrophy [71] associated with loss of microvasculature [72]. In human patients that survived up to 5 years after transplant, a predominant finding on protocol biopsies is interstitial fibrosis/tubular atrophy [73]. The same features have also been observed in an I/R-injured chronic mouse model [74].

It is noteworthy to underline that heparanase inhibition by Roneparstat (Noseda and Barbieri; Cassinelli, Torri and Naggi, Chaps. 20 and 21 in this volume) completely abrogated I/R-induced chronic renal damage and restored normal kidney histology in mice sacrificed 8 weeks after I/R injury. In particular, HPSE inhibition fully prevented the development of fibrosis [74]. Loss of microvasculature in chronic interstitial renal damage causes chronic oxygen deprivation to the tubule-interstitial compartment, contributing to the loss of functional mass after renal I/R. This effect was reduced in response to heparanase inhibition, possibly via HIF1-α [60, 75]. In this *in-vivo* model, it has been demonstrated that heparanase inhibition significantly reduces inflammation, EMT, oxidative stress, nitric oxide, and endothelin signal alterations [74], suggesting that heparanase supervises multiple mechanisms leading to renal fibrosis Fig. 27.1.

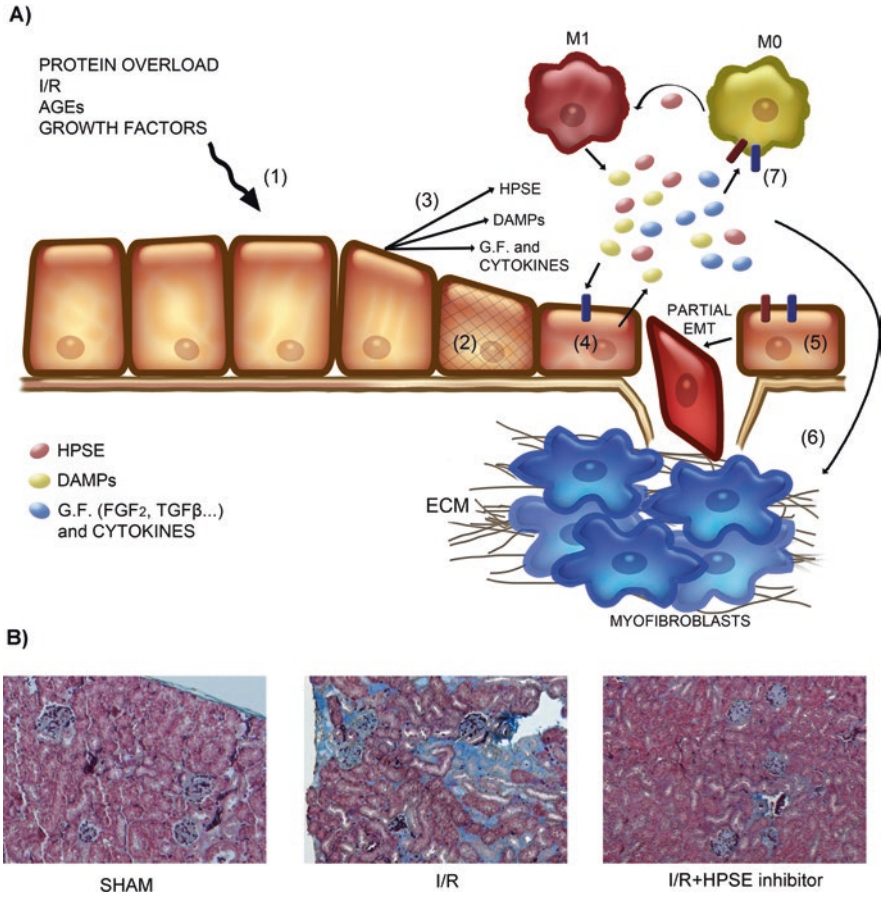

Fig. 27.1 Putative mechanisms of action of HPSE in kidney fibrosis development
(A) A series of injurious stimuli (1) (proteinuria, hypoxia/reoxigenation (H/R), advanced glycosylation products (AGEs), growth factors such as FGF-2 and TGF-β induce multiple effects in renal parenchyma cells. (2) Tubular cells may undergo apoptosis and cell cycle arrest (3) but also they start to release increased amount of HPSE, DAMPs, growth factors (FGF-2 and TGF- β) and pro-inflammatory cytokines (TNFα and IL-1 β). (4) The release of cytokines is also amplified by the direct interaction of DAMPs with TLRs expressed on tubular cells. (5) The presence of growth factors can induce partial-EMT in tubular cells, (6) increased extracellular matrix accumulation and fibroblasts proliferation generating the tubulo-interstitial fibrosis. (7) The enriched environment in pro-inflammatory cytokines increase the number of infiltrating macrophages. HPSE sustain their polarization toward an M1 phenotype releasing additional inflammatory mediators that fuel a vicious loop. **(B)** The inhibition of HPSE can control several of these events. Indeed mice subjected to renal hypoxia/reoxigenetion (I/R) injury develop fibrosis over the time. The treatment with HPSE inhibitor (Roneparstat®) prevented this phenomenon and protected parenchymal cells which shows a histological picture similar to controls (SHAM)

27.3 Peritoneal Fibrosis

Peritoneal dialysis (PD) is an established form of renal replacement therapy available for patients with end-stage renal disease and represents an important alternative to hemodialysis treatment especially in young patients with a long life expectancy and a good chance of having a kidney transplant [76]. Indeed, PD offers greater flexibility, allowing patients to continue working, preserves their residual renal function and has a lower cardiovascular impact than hemodialysis [76, 77].

In PD, the peritoneal membrane acts as a permeable barrier for exchange of solutes and water but long-term exposure to dialysis solutions, as well as episodes of peritonitis, can cause acute/chronic inflammation and injury to the peritoneal membrane, which undergoes progressive fibrosis, angiogenesis, and vasculopathy, a known cause for the loss of peritoneal ultrafiltration capacity [78, 79]. It has been shown that long exposure to hyperosmotic, hyperglycemic, and acidic dialysis fluids during PD can induce progressive sub-mesothelial thickening, reduction and distortion of the vascular lumen with hyalinization, thickening of the basal capillary membrane, deposition of ECM and of advanced glycation end products (AGE) within the arterial wall, greater synthesis of pro-inflammatory cytokines and reactive oxygen species, inhibition of cell proliferation, and DNA damage [80–82]. When exposed to bio-incompatible solutions, all the cellular components of the peritoneum (mesothelial cells, macrophages, mast-cells, dermal fibroblasts, endothelial cells, and resident macrophages) actively participate in the induction of inflammatory and fibrotic responses. A primary role in the induction of peritoneal fibrosis is played by the epithelial-to-mesenchymal transition of mesothelial cells, termed mesothelial-to-mesenchymal transition (MMT) [83].

It has been found that high glucose and PD-solutions up-regulate the expression of heparanase in mesothelial cells. High glucose and PD solutions activate, at the same time, MMT in mesothelial cells, and heparanase inhibition prevents these events. In addition, heparanase inhibition reduced VEGF production (a proangiogenic factor which reduces the ultrafiltration capacity of the peritoneum) and preserved trans-mesothelial resistance and albumin permeability [84].

27.4 Liver Fibrosis

The liver is one of the organs most affected by fibrotic processes. Liver fibrosis generally results from a process of chronic damage that can affect both hepatocytes and biliary tract cells and whose final result is cirrhosis and organ failure [85]. Among the main causes that trigger liver fibrosis are viral hepatitis B or C, parasitic infections, alcohol abuse, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis, cholestatic disorders, and metabolic dysfunction. Whatever the causative agent of the damage, a protracted lesion directed to the hepatic parenchyma induces the activation of inflammatory and pro-fibrogenic responses that develop into liver fibrosis. Apoptotic bodies arising from the death of parenchymal cells, DAMPs, ROS and

inflammatory mediators mediate the recruitment of inflammatory cells (lymphocytes and monocytes) from the blood which, in turn, induce the activation of liver macrophages (Kupffer cells) and hepatic stellate cells (HSC) [86]. Activated Kupffer cells and HSC are primarily responsible for liver fibrosis. Kupffer cells promote inflammatory and fibrogenic responses through the release of cytokines, chemokines and growth factors that exacerbate inflammation and participate in the activation of HSC, a central event during liver fibrosis. Hepatic stellate cells (HSC) are liver pericytes located in the perisinusoidal space between endothelial cells and hepatocytes (Disse space). They are usually quiescent and contain lipid droplets of vitamin A. In the injured liver, quiescent HSC undergo trans-differentiation, becoming cells similar to fibrogenic myofibroblasts and maintaining their activated state in response to paracrine and autocrine stimulation [87]. This phenotypic change transforms HSC into cells that begin to express α-SMA, increase the ability to contract, proliferate and synthesize and secrete a large amount of ECM proteins (mainly collagen and fibronectin) that progressively accumulate in the liver [88]. TGF-β is the most potent stimulus for the synthesis of collagen type I and ECM proteins while cell proliferation is mainly driven by PDGF, FGF-2, EGF, and VEGF. Activated HSC are the major but not unique producers of fibrotic ECM in the injured liver [89]. The pro-metastatic and pro-tumorigenic role of HPSE has been widely demonstrated in liver cancer. Several studies have reported upregulation of HPSE by tumor cells in liver biopsies derived from patients with hepatocellular carcinoma. Furthermore, a direct correlation between HPSE levels, tumor stage, tumor size, portal vein invasion, and tumor invasiveness has been observed [90, 91]. Concerning chronic non-cancerous liver disease, the involvement of HPSE has so far been poorly demonstrated, and controversial results have been obtained both at the histological level on human tissues and in animal models of liver fibrosis. A comparative study on the expression of HPSE in normal, cirrhotic and cancerous livers showed an insignificant difference in mRNA and protein levels between normal and cirrhotic tissues compared to its levels in tumor tissue, suggesting that HPSE could only play a role in the late stage of carcinogenesis but not in the development of pre-cancerous fibrosis/cirrhosis [90]. In a model of thioacetamide-induced hepatic fibrosis in rats, HPSE protein levels were increased in two independent studies even if the results were discordant regarding the fibrogenesis stage of up-regulation [92, 93].

In a different animal model of carbon tetrachloride-induced fibrosis (CCl4), our research group showed that HPSE expression increased in mouse livers after a few weeks of treatment but with a tendency to decline during disease progression. Immunostaining for HPSE was limited in fibrous liver tissues to necro-inflammatory areas and co-localized with macrophage markers F4/80 and TNF-α [94]. Treatment of macrophages with TNF-α in culture induced the expression and release of HPSE which consequently regulated the expression of α-SMA and fibronectin in hepatic LX-2 stellate cells. Finally, we were able to demonstrate that HPSE activity increased in the plasma of patients with liver fibrosis, but these values were inversely correlated with liver stiffness. These results suggest the involvement of HPSE in the early stages of the reaction to liver damage and indicate inflammatory macrophages as an important source of HPSE. The release of HPSE appears to play a key role in

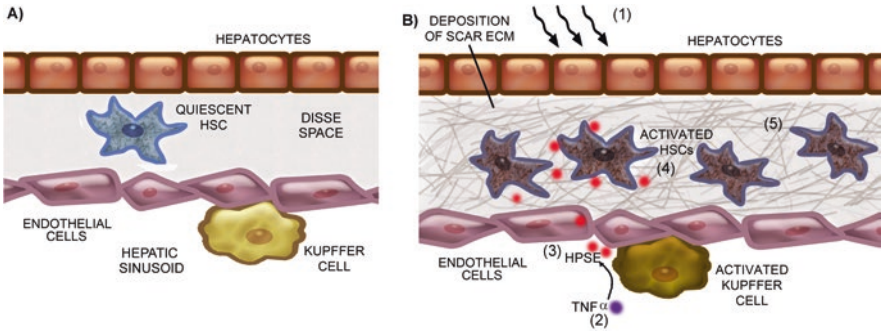

Fig. 27.2 Putative mechanisms of action of HPSE in liver fibrosis development
(A) In normal healthy liver, human stellate cells (HSC) lie between the sinusoids and the hepatocytes in the space of Disse. Normally they store Vitamin A in lipid droplets. Kupffer cells are specialized macrophages located in the liver, lining the walls of the sinusoids. They form part of the mononuclear phagocyte system. **(B)** In response to a chronic insult (1), active Kupffer cells secret inflammatory cytokines (2). Among them, TNF-α is the most critical and it induces secretion of HPSE by macrophages (3) which, in turn, regulates the activation of human stellate cells into myofibroblasts (4) to the extent of determining a dramatic alteration in extracellular matrix (ECM) composition. Hepatic stellate cells activation leads to accumulation of fibrillar extracellular matrix in the Disse space (5) and this impairs hepatocyte function, leading to deterioration of organ physiology. Figures artwork by Dr. Andrea Donadon (University of Padova)

macrophage-mediated activation/transition of hepatic stellate cells (HSC) into myofibroblasts. Taken together, these pieces of experimental evidence seem to suggest that targeting HPSE could provide a new therapeutic option in the treatment of liver fibrosis Fig. 27.2.

27.5 Lung Fibrosis

Pulmonary fibrosis is associated with several diseases, including scleroderma, sarcoidosis, infection, bronchiolitis obliterans syndrome (BOS) after lung transplantation and as a result of environmental exposures (e.g., silica dust or asbestos). The main pathogenesis of pulmonary fibrosis is that the injury to alveolar epithelial cells activates pulmonary fibroblasts, provoking their transformation into matrix-producing myofibroblasts. The replacement of normal lung parenchyma with fibrotic tissue causes an irreversible decrease in oxygen diffusion capacity [95]. It has been recently proved that HMGB1, a DAMP passively released from necrotic/damaged cells as a result of early unavoidable allograft injuries, activate fibroblasts to myofibroblasts and this event is dependent on heparanase activity. Moreover, HMGB1 through its receptor, RAGE, activates NF-κB and increases heparanase expression. The up-regulated heparanase, by cleaving heparan sulfate, releases TGF-β stored in the ECM, thereby supporting the progression of pulmonary fibrosis [96].

27.6 Conclusions

Fibrotic diseases continue to be a major worldwide health problem with a significant economic impact on overall health spending. As far as the pathogenesis of fibrotic diseases is concerned, considerable progress has been made, but they remain an important challenge considering that there are very few drugs able to counter these pathological events. Besides TGF-β, considered to be the main inducer of the fibrotic process, many other mediators such as cytokines and signaling molecules are involved, constituting a complex network of signaling pathways that must be considered when searching for new effective therapies. Heparanase is one of these players that in the last few years has increasingly been shown to play a role in fibrosis. Its involvement in the fibrotic process appears to depend on the fact that HPSE promotes the release and spread of various HS-linked molecules such as growth factors, cytokines, and enzymes. Several molecules with inhibitory activity towards HPSE are currently in clinical trials as anti-cancer drugs and, for some of them, a certain pharmacological efficacy and good tolerability for the patient have already been demonstrated. It is therefore hoped that drugs aimed at inhibiting its activity may have therapeutic efficacy not only in the oncology field but also for fibrotic organ diseases for which the involvement of HPSE has been proven.

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Chapter 28
Heparanase in Acute Kidney Injury

Zaid Abassi and M. S. Goligorsky

28.1 Introduction

Acute kidney injury (AKI), aka "acute renal failure" or "acute tubular necrosis", is a syndrome of acute renal functional impairment of varying severity which, depending on the nature of the insult –nephrotoxic, septic, or ischemic – may or may not be accompanied by the commensurate morphologic changes [1]. The causes of this syndrome could be pre-renal (i.e., cardiac failure or extracellular volume depletion), post-renal (i.e., blockage of the urine outflow), and intrinsic renal (i.e., toxic, septic or ischemic). All three categories of diseases, acutely affecting renal functions, invariably injure the kidney by a combination of hemodynamic and tubular alterations differing in their intensity. Acute pre-renal injury to the kidney affects predominantly renal circulation with the attendant tubulopathy. Acute post-renal injury to the kidney affects tubules with the attendant renal circulatory abnormalities. Acute intrinsic kidney injury affects both to a variable degree. The functional coupling of these two pathogenic mechanisms has been emphasized by H. Valtin [2], "both the renal vasculature and the tubular system run in series and are intertwined both anatomically and functionally, initial damage to the tubules will quickly involve the vessels, and vice versa." Despite such anatomic and functional coupling, comparative analysis of the transcriptome in mouse models of ischemic and volume-depleted AKI (both manifesting with the similar degree of renal failure) showed profoundly distinct gene profiles [3].

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The most common morphologic findings include dilation of the tubules and flattening of distal and proximal tubular epithelium with the desquamation of proximal epithelial cells, presence of granular and brownish-reddish casts, focal infiltration and edema of the proximal tubular epithelium and the interstitium, mitotic figures in the proximal and distal epithelial cells, and occasional tubular necrosis. These modest morphologic abnormalities are in sharp contrast to functional failure. The latter is characterized by a precipitous drop in glomerular filtration rate exceeding the reduction in renal blood flow, increased proximal tubular and interstitial pressure, thus counteracting the filtration pressure [4].

The incidence of AKI has been steadily increasing, especially among elderly hospitalized patients and critically ill subjects and may escalate in ICU to 78% [5, 6]. When developed, AKI is associated with the eight-fold increased mortality [7, 8]. The most vulnerable populations include diabetic patients, groups with other preconditions or surgical interventions and elderly subjects. Subsets of AKI are graft rejection and GVHD, acute-on-chronic, and AKI-CKD continuum.

Given the frequency and implications of this syndrome, rigorous investigations were conducted through years, which yielded, among other important findings, insights into the role of heparanase in its pathogenesis. This particular subject will be the center point of the current brief overview.

28.1.1 Heparanase Secretion in Stress

The cellular processing of heparanase involves exocytic and endocytic compartments, including early and late exocytic vesicles, endocytic vesicles, and lysosomes. Endocytic traffic of components of glycocalyx, such as heparan sulfate and proteoglycans and their self-assembly into heparan sulfate proteoglycans, as well as the transport of (pro)heparanase, have been previously reviewed [9–11]. This pathway, in addition to lysosomal degradation of endocytosed material, includes recycling of endosomal cargo. Briefly, synthesized pre-pro-heparanase is initially trafficked to the ER, shuttled to the Golgi apparatus and eventually secreted in the form of 65kD pro-heparanase [12]. This latent form of the enzyme interacts with syndecans and undergoes endocytosis. The active form of heparanase is generated by lysosomal cathepsin-induced cleavage of pro-enzyme yielding 50kD and 8kD fragments, both required for its activity. Heparanase has a low-pH optimum of 5.0–6.0 to cleave the glycosidic bond between β-D-glucuronic acid and N-(sulfated)-D-glucosamine preferentially targeting the boundary between highly- and mixed-sulfated regions of HS, thus producing highly sulfated end-domains and yielding mixed sulfated saccharide products of a size sufficient to bind proteins. Synthetic pathways of heparanase partially overlap with those of HS and MMP-9. This intricate traffic route highlights the mechanisms and sites of possible aberrations under stress conditions during endocytic, lysosomal, and ER transits.

28.2 ER Stress

Endoplasmic reticulum (ER) is responsible for the storage and release of calcium and folding and secretion of proteins, including heparanase, and biogenesis of lipids. These functions become impaired due to ER stress induced in the kidney by ischemia/reperfusion injury, NSAIDs, aminoglycosides, cisplatin, calcineurin inhibitors, cyclosporine A and tacrolimus, and some heavy metals (cadmium, mercury, lead) [13]. Accumulation of unfolded proteins triggers a default unfolded protein response (UPR). UPR initially affords cytoprotection and, if prolonged, leads to autodestruction. Cytoprotective effects of UPR are accomplished via upregulation of chaperones, glucose-regulated proteins GRP78 and GRP94, by inositol-requiring protein-1 (IRE1) kinase and protein disulfide isomerase (PDI), both assisting in protein folding, and by suppression of translation through phosphorylation of eukaryotic translation initiation factor 2α, thus reducing protein synthesis and burden of folding. When stress is prolonged these pathways become deactivated triggering pro-apoptotic pathways [14]. In ischemia/reperfusion kidney injury, eIF2α phosphorylation exerts a renoprotective effect, and overexpression of the chaperone GRP78 protects cultured epithelial cells against hydrogen peroxide, iodoacetamine and 2,3,5-tris(glutathione-S-yl)hydroquinone, all potent oxidants inducing ER stress [15]. It remains to be established how ER stress accompanying AKI affects the processing of pre-pro-heparanase.

28.3 Lysosomes

Reactive oxygen species, a common companion of cell stress, and many nephrotoxic agents with lysosomotropic effects result in lysosomal membrane permeabilization, often preceding mitochondrial membrane permeabilization and release of cytochrome c [16]. This, in turn, leads to the activation of acidic sphingomyelinase and generation of ceramide, triggering autocatalytic proteolysis by the released cathepsins.

Gentamycin-induced AKI is of special interest, as it is associated with the enlargement of the lysosomal compartment where the antibiotic is concentrated together with phospholipids and the chaperone HSP73, forming myeloid bodies typical of tubular phospholipidosis. Gentamycin binds to and interferes with chaperone activity of HSP73 [17]. Another condition typically associated with the distension of lysosomes and vacuolization and swelling of the proximal tubular epithelia is represented by osmotic nephrosis – AKI initiated by contrast media, dextrans or sucrose [18]. Immunoglobulin G therapy can lead to osmotic nephrosis due to the presence of IgG stabilizers, sucrose, and sorbitol, at 10% each. Volume expanders like low-molecular-weight dextran and mannitol, especially when combined with cyclosporine or furosemide, can result in osmotic nephrosis and AKI. Another volume expander, hydroxyethyl starch, results in about 40% chance

of developing osmotic nephrosis and AKI. Ionic and non-ionic hyperosmolar radiocontrast media, first and second generations of iodine-containing contrast agents, in combination with dehydration, are well-known causes of osmotic nephrosis. In all these cases, proximal tubular cells are swollen by accumulating vacuoles, which fuse with the lysosomes but fail to undergo complete digestion. The above-mentioned loss of pH gradient may have direct consequences for not only the maturation of the enzyme but also its ability to cleave HS. This side of the equation remains poorly investigated.

28.4 Heparanase Synthesis, Secretion, and Activity in AKI

A few available studies point out at several features of heparanase in AKI (Masola et al., Chap. 27 in this volume). Firstly, unbiased microarray analyses of renal transcriptome following AKI did not reveal any dramatic changes in the level of heparanase message in the immediate-early (up to 4 h), early (4–24 h), and intermediate-late (48 h and longer) stages post-injury [19]. A modest increase in heparanase mRNA is observed in mice with ischemic AKI [20, 21] 48 h after the insult, but much higher gene expression is observed in heparanase transgenic (Hpa-tg) mice 72 h after ischemia. In a study of septic mice, the heparanase message level was found to be modestly induced at 4 h and further increased 24 h after initiation of polymicrobial sepsis using colon ligation and puncture model [22]. In either of these conditions, the activity of heparanase was dramatically increased, thus accounting for the elevated levels of HS fragments in the circulation (see below). Importantly, in both cases application of heparanase inhibitors reduced pathologic sequelae of ischemia or sepsis. These findings are consistent with the current view of predominantly epigenetic regulation of the enzyme gene expression [23]. All these findings bring about an unresolved question. In the face of developing lysosomal dysfunction with the collapse of pH gradient, one would expect that impaired processing of pro-heparanase and reduction of heparanase activity would ensue; yet the existing evidence indicates the opposite – an increase in enzymatic activity under stress conditions. It is possible that there exist chronologic shifts in heparanase activity reflecting the state of lysosomal dysfunction and its restoration in the course of a disease.

28.5 Non-catalytic Actions of Heparanase

Besides its catalytic activity toward HS, heparanase exerts several non-catalytic actions, including endothelial cell migration/invasion, infiltration of immune cells, coagulation, fibrosis, autophagy, exosome pinching, and inflammation. These effects are thought to be mediated by membrane receptors still to be identified [24]. Binding of the pro-Hpa (65 kDa) to these receptors activates downstream signaling

which includes Akt, PI3K, ERK, p38 and Src [25–27] (Ilan et al., Chap. 9 in this volume). Notably, heparanase can also translocate into the nucleus where it is capable of regulating gene expression (Khanna and Parish, Chap. 3 in this volume).

28.6 Heparanase Actions on Glycocalyx and ECM

The targets of heparanase, HS chains, are either bound to glycocalyx proteoglycan core proteins, like syndecans, or proteoglycans located in the extracellular matrix, like perlecan. Active heparanase, therefore, not only trims HS bound to proteoglycans comprising glycocalyx but also cleaves those attached to the extracellular matrix proteins. It is remarkable how broad is the list of HS-binding proteins; it includes more than 300 interacting partners which can be liberated upon HS degradation [28]. Through the degradation of HS, heparanase is involved in an array of functions which are mediated by the released biologically active molecules, such as growth factors and cytokines bound to HS, interaction with heparinase-binding proteins and activation of kinases as Src, Akt, p38 MAPK, modulating the activity of FGF-2 and TGF-β signaling pathways, and activation of FGF-2 signaling [29]. One of the important binding partners of HS is albumin, which electrostatically interacts with the glycocalyx. The loss of electronegativity of the surface layer, therefore, is accompanied by liberation of albumin and attached to it endothelial survival factor, sphingosine-1-phosphate. When HS chains abundantly decorate proteoglycans, as it happens in Hpa-knockout mice, sepsis-induced degradation of pulmonary endothelial glycocalyx and endothelial hyperpermeability are inhibited [30]. In contrast, mice overexpressing heparanase exhibit reduced leucocytes crawling, adhesion, and infiltration of inflamed sites, presumably as a result of the defective ability of truncated HS chains in the glycocalyx to ligate chemokines, as well as due to the increase in “heparin-resembling” HS fragments endowed with anti-inflammatory properties [23]. Because HS interacts with integrins, thus participating in cell adhesion and motility, these functions could be critically affected by changes in heparanase activity. Furthermore, both HS and heparanase are important modulators of syndecan-1 and vice versa. Syndecan-1 regulates pro-heparanase internalization and endosomal-lysosomal processing leading to its activation [31]. On the other hand, HS and elevated heparanase activity regulate shedding of syndecans [32]. Yet, the mode of heparanase action in the extracellular environment with a near-neutral pH remains a vexing question.

28.7 Heparanase and Activation of TLR and Inflammation

One of the hallmark action of heparanase/ HSPGs system is to orchestrate inflammation [33]. Specifically, heparanase plays a pivotal role in the inflammatory response, mainly due its enzymatic activity on ECM and the subsequent release of

harbored in it pro-inflammatory cytokines, including IL-2, IL-8, bFGF and TGF-β, and via modulating the interaction between the leucocytes and endothelial cells surface and stimulation of leucocyte recruitment, rolling process and extravasation [34–36]. It should be emphasized that neutrophils and activated T-lymphocytes possess HS degrading activity, features essential for their extravasation and infiltration into inflamed tissues. This degrading activity was later attributed to heparanase [37]. Additional studies have demonstrated that non-enzymatic activities are behind some of the heparanase pro-inflammatory effects such as cell adhesion and signal transduction [12]. Upregulation of heparanase activity/abundance has been detected in endothelial and epithelial cells of inflamed tissues as in chronic colitis, Crohn's disease, sepsis-associated lung injury, chronic pancreatitis, rheumatoid arthritis, keratoconic cornea, neuro-inflammatory response in subarachnoid hemorrhage (SAH), and Herpes Simplex infection [38–43]. For instance, colitis is characterized by heparanase upregulation in enterocytes, where it promotes monocyte-to-macrophage activation and sustained chronic inflammation [38]. Similarly, chronic and acute kidney injuries induced by I/R sustain inflammatory characteristic as evident by Hpa-induced macrophages polarization via cathepsin L of tubular origin. The mechanisms underlying Hpa-induced macrophage activation is not fully characterized; active heparanase regulates activation of macrophage via TLR2 and TLR4 by HS degradation fragments leading to increased levels of TNF-α thereby creating chronic inflammatory conditions fostering macrophage-mediated renal injury and heparanase up-regulation. In this context, it was reported that intact HS inhibits TLR4 signaling and macrophage activation, whereas HS fragments are thought to play a key role in the activation process [44–47]. Experimental model of I/R renal injury is characterized by enhanced levels of heparanase in both tubule and glomeruli [48]. Heparanase then induces tubular cell apoptosis and Damage Associated Molecular Patterns (DAMPs) production. DAMPs, heparanase-released HS-fragments, and molecules generated from necrotic cells activate TLRs both on macrophages and tubular cells. In response to direct hypoxic stimuli and TLR activation, tubular cells produce pro-inflammatory cytokines, which attract and activate macrophages and the presence of high levels of heparanase facilitates M1 polarization of infiltrated macrophages, which worsen parenchymal damage [48]. Recently, we examined whether heparanase plays a role in acute pancreatitis (AP), one of the most common diseases in gastroenterology [49]. In this study (Khamaysi et al., Chap. 28 in this volume), we provided evidence that pancreatic heparanase expression and activity are significantly increased following cerulein-induced AP. Moreover, pancreas edema and inflammation, as well as the induction of cytokines and signaling molecules following cerulein treatment were attenuated markedly by PG545 and SST0001 (heparanase inhibitors - see below), implying that heparanase plays a significant role in AP. Notably, all the above features appear even more pronounced in transgenic mice overexpressing heparanase. In line with its pro-inflammatory role, upregulation of heparanase during AP was associated with the recruitment of neutrophils and enhanced expression of various cytokines including TNFα and Il-6, key cytokines implicated in experimental and clinical pancreatic

injury [50]. Notably, induction of TNFα increased IκB phosphorylation and nuclear translocation of p65, which were prominently decreased by PG545.

28.8 Heparanase and Coagulation

Degradation of HS by heparanase causes remodeling of extracellular matrix (ECM), and release of numerous biologically active molecules including cytokines, growth factors and other sequestered components which activate several physiological and pathophysiological processes including blood coagulation [33]. Concerning the latter, it has been shown that heparanase is involved in hemostasis, as was evident by remarkable upregulation of tissue factor (TF) in both in vitro and in vivo models of overexpression of this enzyme and enhancement of the generation of activated factor X (FXa) [51, 52] (Nadir, Chap. 33 in this volume). Support for potential linkage between heparanase and TF was derived from leukemic patients, where direct correlation between these two parameters was found [53]. Moreover, the addition of heparanase to primary cultured endothelial cells, which usually do not express TF, caused substantial enhancement of their coagulation activity [53]. Based on these studies, it was suggested that pro-heparanase hitched to cellular HS, where it induces a pro-coagulant effect. Moreover, heparanase interacts with TF pathway inhibitor (TFPI) localized to the cell surface of EC and tumor cells, resulting in dissociation of TFPI and subsequently enhanced cellular coagulation activity [54]. Likewise, by using plasma from patients treated with low molecular weight heparin (LMWH), it was demonstrated that pro-Hpa abolishes the anticoagulant effect of unfractionated heparin and the Factor Xa inhibitory activity of LMWH [51, 55]. Similarly, pro-coagulant effects of pro-heparanase were also mimicked by a peptide corresponding to its major functional heparin-binding domain (HBD-1, residues Lys158 Asp171) [52]. The anticoagulant activity of heparanase could not solely be attributed to its ability to degrade the heparinoids since this process takes hours and requires low pH. Therefore, non-enzymatic mechanisms underlying the involvement of heparanase in hemostasis are more appreciated, including (i) Heparanase can sequester heparin, (ii) The time course of Hpa-induced heparin-neutralization is faster than that of heparin degradation, and (iii) Active platelet-derived heparanase is practically inactive under physiological pH. Moreover, (iv) it reduces unfractionated heparin to about the size of LMWH, which is still highly active as a cofactor for FXa inhibition by AT, and (v) Heparanase-derived peptide (HBD-1) does not possess any enzymatic activity [51]. Most recently, Bayam et al. [56] demonstrated that increased heparanase levels might be responsible for obstructive prosthetic valve thrombosis (PVT). Furthermore, treatment with unfractionated heparin (UFH) significantly increased circulatory heparanase levels, thus attenuating the treatment efficacy as it may be associated with a high risk of thromboembolism and increased thrombus burden in PVT patients. Collectively, these findings suggest that heparanase can be used as pro-coagulant and a therapeutic antidote against heparinoid anti-coagulant activities. (Nadir, Chap. 33 in this volume).

28.9 Heparanase in AKI

In light of the inflammatory, fibrotic and ECM remodeling actions of heparanase, it is not surprising that increasing evidence supports the involvement of this enzyme in the pathogenesis of AKI of various etiologies (Masola et al., in this book). The involvement of Hpa/HSPGs in the pathogenesis of AKI has not been studied in depth so far. Nevertheless, the emerging data linking heparanase and inflammatory responses, suggest a role of this enzyme in AKI pathogenesis. Lygizos et al. [57] found that glomerular heparanase is activated during sepsis and contributes to septic AKI. Specifically, the authors induced polymicrobial sepsis in mice using cecal ligation and puncture (CLP) in the presence or absence of competitive heparanase inhibitors (heparin or non-anticoagulant N-desulfated re-N-acetylated heparin [NAH]). CLP-treated mice revealed early activation of glomerular heparanase with coincident loss of glomerular function, as indicated by increased blood urea nitrogen (BUN) and decreased GFR. Administration of the above heparanase inhibitors 2 h before CLP attenuated the deleterious consequences of sepsis, suggesting that glomerular heparanase is active during sepsis and contributes to septic renal dysfunction via uncharacterized mechanisms. In line with these findings, bilateral renal ischemia/reperfusion (I/R) in syndecan-1 deficient mice resulted in increased initial renal failure and tubular injury compared with wt mice [58]. Macrophage and myofibroblast numbers, tubular damage, and BUN were increased and tubular proliferation reduced in the kidneys of syndecan-1 deficient mice compared with wt mice 14 days following injury, suggesting that syndecan-1 promotes tubular survival and repair in murine I/R injury. Support for this notion is derived from the observation that knockdown of syndecan-1 in human tubular epithelial cells in vitro reduced cell proliferation [58]. Selective binding of growth factors suggests that syndecan-1 may promote epithelial restoration [58]. In an additional study, these authors have shown that within 24 h after renal I/R, HSPGs expressed at the abluminal side of peritubular capillaries are induced to bind L-selectin and the monocyte chemoattractant protein-1, facilitating monocyte extravasation [59]. In agreement with these findings, these authors showed that early monocyte/macrophage influx was impaired in HSPG2(Delta3/Delta3)xCol18a1(−/−) mice, providing keen evidence for the concept that not only endothelial but also microvascular basement membrane HSPGs can influence inflammatory responses. A vicious circle of Hpa-driven molecular events promoting chronic inflammation and renal injury has recently been described [60, 61]. This circle is fueled by heterotypic interactions among glomerular, tubular, and immune cell compartments. It appears that latent heparanase, over-expressed by glomerular cells and post-translationally activated by cathepsin L of tubular origin, sustains continuous activation of kidney-damaging macrophages. Briefly, active heparanase governs macrophage activation via activation of TLR2 and TLR4 by HS degradation fragments [47], leading to increased levels of TNF-α thereby creating chronic inflammatory conditions fostering macrophage-mediated renal injury and reinforcing heparanase up-regulation. Also, an interplay between heparanase and the endothelin system has been established. Most recently, Garsen et al. [62] have

demonstrated that activation of ET-1 signaling induces heparanase expression in podocytes thus resulting in damaged glycocalyx, proteinuria, and renal failure in experimental diabetes. It should be emphasized that the endothelin system is activated in several disease states characterized by vasoconstriction, proliferation, inflammation, ECM remodeling, and fibrosis, including CKD and AKI [63, 64]. Notably, all the components of the ET system are upregulated in AKI, and its inhibition attenuates the severity of the disease [65, 66]. These findings allude to the likelihood that a similar interplay between ET-1 signaling and heparanase exists in AKI. In this context, we have demonstrated that hypoxia/reoxygenation (H/R) significantly increased the expression of α-SMA, VIM, and FN, EMT-markers in wild type (WT), but not in Hpa-silenced tubular cells. Noteworthy, EMT was prevented in WT cells by SST0001 treatment. In agreement with the in vitro results, I/R induced a remarkable up-regulation of EMT markers in Hpa-tg mice after 48–72 h. In contrast, these observations were absent in WT animals [21] (Fig. 28.1). Next, we examined the involvement of heparanase in the pathogenesis

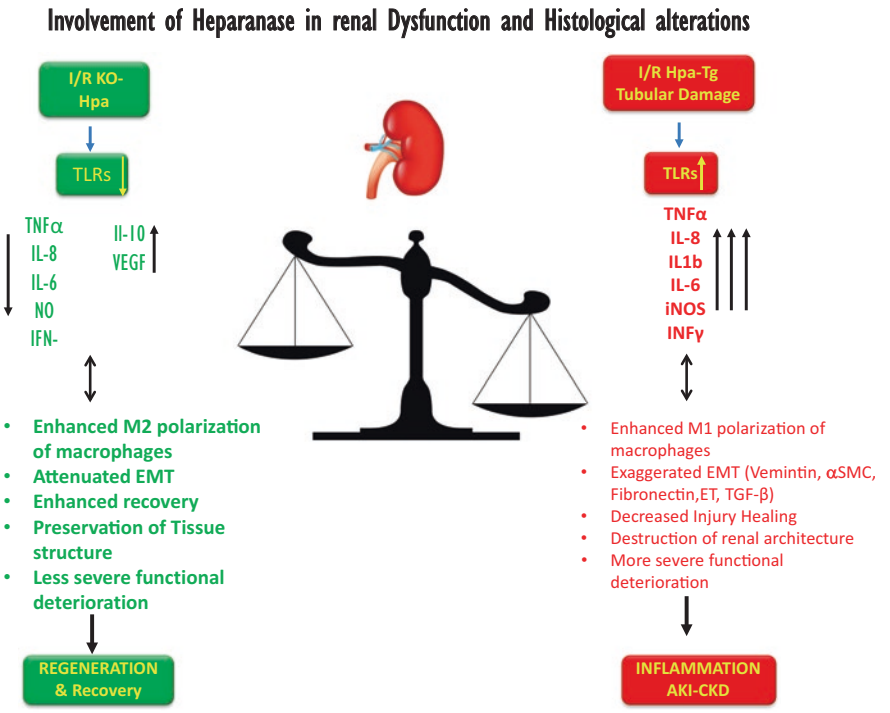

Fig. 28.1 Comparative characteristics of the course of acute kidney injury (AKI) in mice with heparanase knockout vis-à-vis transgenic mice. Briefly, compared to KO-Hpa, transgenic animals (Tg-Hpa) exhibit enhanced renal activities of pro-inflammatory and epithelia mesenchymal transforming (EMT), leading to decreased Injury healing following I/R, exaggerated destruction of renal architecture, and subsequently more severe functional and excretory deterioration (see text for more details)

of ischemia-reperfusion (I/R) AKI in a mouse model and the protective effect of PG545, a potent heparanase inhibitor. As expected, I/R induced tubular damage and elevation in SCr and BUN to a greater extent in heparanase over-expressing transgenic mice (Hpa-Tg) vs. WT mice [20]. Moreover, TGF-β, vimentin, fibronectin, and α-smooth muscle actin, biomarkers of fibrosis, and TNFα, IL6 and endothelin-1, biomarkers of inflammation, were upregulated in I/R induced AKI, primarily in Hpa-Tg mice, suggesting an adverse role of heparanase in the pathogenesis of AKI (Fig. 28.1). Remarkably, pretreatment of mice with PG545 ameliorated kidney dysfunction and the up-regulation of heparanase, pro-inflammatory (i.e., IL-6) and profibrotic (i.e., TGF-β) genes induced by I/R. In an additional study by Masola et al. [48] the cross talk between macrophages and HK-2 renal tubular cells during in vitro hypoxia/reoxygenation (H/R) was examined. Furthermore, these authors evaluated *in vivo* renal inflammation, macrophage polarization, and histologic changes in mice subjected to mono-lateral I/R and treated with SST0001 for 2 or 7 d. The in vitro studies demonstrated that heparanase sustained M1 macrophage polarization, the release of damage-associated molecular patterns in post-H/R tubular cells, the synthesis of pro-inflammatory cytokines, and the up-regulation of TLRs on both epithelial cells and macrophages. Furthermore, heparanase induced partial EMT in HK-2 renal tubular cells by M1 macrophages, which was abolished by heparanase inhibitor. In agreement with these findings, inhibition of heparanase *in vivo* reduced inflammation and M1 polarization in mice undergoing I/R injury, partially restored renal function and normal histology, and reduced apoptosis. Taken together, our results demonstrate that heparanase plays a harmful role in the development of renal injury and kidney dysfunction as was evident by EMT (Fig. 28.1) [29] and macrophage polarization, suggesting heparanase inhibition as a promising therapeutic maneuver for AKI (Masola et al., Chap. 27 in this volume). The involvement of heparanase in AKI is of special interest, as AKI may advance to CKD. In this context, heparanase activity/abundance has been also documented in diabetic and nondiabetic proteinuric kidney diseases [67, 68]. Heparanase expression was shown to be upregulated in a number of animal models of renal diseases, including passive Heymann nephritis [69], puromycin aminonucleoside nephrosis (PAN) [70], adriamycin nephropathy (ADR-N) [71, 72], anti-glomerular basement membrane (GBM) nephritis [73], and diabetic nephropathy [74]; and in glomerular epithelial and endothelial cells cultured in ambient high glucose concentration [75]. Likewise, increased heparanase activity was detected in urine samples from diabetic patients with microalbuminuria [76–78], nondiabetic nephrotic syndrome, chronic kidney diseases (CKD) and kidney transplant patients [76]. Interestingly, neutralization of heparanase activity, using either a sulfated oligosaccharide inhibitor (PI-88) or anti-Hpa antibodies, resulted in reduced proteinuria [79]. Similar findings were reported by Gil and colleagues [60] who demonstrated that Hpa-KO mice failed to develop albuminuria and renal damage in response to streptozotocin-induced diabetes mellitus. Furthermore, albuminuria was attenuated in diabetic mice treated with heparanase inhibitor [60]. These findings are in line with emerging evidence that heparanase is involved in the progression of CKD mainly via activation of profibrotic biological signals including FGF-2 and TGF-β and consequently renal EMT [80].

28.10 Heparanase in Kidney Transplantation

Since kidney transplantation is associated with renal I/R [81], it is appealing to assume that heparanase/HSPG system is involved in the pathogenesis of delayed graft function (DGF) and chronic allograft nephropathy (CAN). Indirect support for this notion is derived from the observation that heparanase plays a key role in EMT and macrophage polarization following renal I/R damage [20, 21, 82]. Furthermore, Barbas et al. have shown that HS is a novel biomarker for acute cellular rejection, where it is released from the ECM during T-cell infiltration of graft tissue via the enzymatic action of heparanase [83]. A clinical study has shown that plasma HS levels increased significantly in kidney transplant recipients with biopsy-proven acute cellular rejection compared with healthy controls, recipients with stable graft function, and recipients without acute cellular rejection [83]. Similarly, high levels of HS were found in the blood of mice that experienced rejection of cardiac allografts, along with upregulation of heparanase expression in activated T-cells [83]. The expression of Syndecan-1, a transmembrane HS proteoglycan, syndecan-1 sheddases (ADAM17, MMP9) and heparanase was significantly up-regulated after renal transplantation [58, 84]. Increased epithelial syndecan-1 in allografts correlated with low proteinuria and serum creatinine, less interstitial inflammation, less tubular atrophy, and prolonged allograft survival [58]. Celie et al. [59] confirmed the relevance of microvascular basement membrane HSPGs to EC damage in experimental and clinical renal ischemia/reperfusion also in human renal allograft biopsies. It was found that loss of endothelial expression of the extracellular endosulfatase HSulf-1 may be a likely mechanism underlying the induction of L-selectin and monocyte chemoattractant protein-1 associated with peritubular capillaries in human renal allograft rejection. Also, clinical study revealed a significant correlation between urinary heparanase and plasma levels of this enzyme in transplanted patients. The abundance of heparanase in these patients may lead to kidney damage [77]. Collectively, these findings suggest Hpa/HS as a novel biomarker of acute cellular rejection in solid organ transplantation. Moreover, the association between heparanase, proteinuria and decreased renal function could be translated into new therapeutic options aimed at attenuating chronic renal allograft nephropathy, leading to improved graft survival and patient outcome.

28.11 Use of Heparanase Related Biomarkers for AKI Detection

In general, biomarkers reporting the activity of heparanase may belong to at least three categories: (1) actual mRNA abundance; (2) protein expression, and (3) abundance of appropriately cleaved HS in body fluids. Studies by Abassi et al. [20] revealed increased abundance of heparanase and its elevated activity in animals with ischemic AKI. With the advent of mass spectroscopy-based detection of glycosaminoglycans

(GAGs) in the urine, mainly for the diagnosis of different forms of mucopolysaccharidosis, this technique is gaining popularity in monitoring HS degradation products in diagnosis and prognosis of diverse diseases. In the study by Schmidt and co-authors [85] urinary GAGs (HS, chondroitin sulfate and hyaluronic acid) were measured in patients with sepsis within 24 h of admission to the intensive care unit. These investigators showed that indices of GAG fragmentation and sulfation correlated with the development of AKI. In 100 patients after open heart surgery, high-end mass spectroscopy revealed that cathepsin L, as well as NGAL and cystatin C, are among potential biomarkers of AKI and major adverse kidney events [86]. Reduced urinary excretion of GAGs in women during the first trimester of pregnancy, was measured using electrophoresis on cellulose acetate strips, and it was demonstrated that it has predictive value as an early marker of preeclampsia [87]. Comparative analysis of AKI developing in kidney transplantation from donors after cardiac death vis-à-vis living donors showed that renal microvascular perfusion after the establishment of anastomosis was 42% lower in kidneys that were ischemic prior to the transplantation [88]. This was associated with shedding of the glycocalyx (reduced RBC exclusion zone) and increased production of syndecan-1 and HS.

28.12 Novel Heparanase Mechanisms-Based Therapies for AKI

In light of the well-established role of Hpa/HSPG in the pathogenesis of various diseases, several inhibitors of heparanase were developed and even reached clinical trials for cancer treatment [89–91]. Among the best-studied heparanase inhibitors, are PG545, SST0001, and PI-88, among other HS mimetics and heparin-derived substances and oligosaccharides [92]. (Hammond and Dredge; Noseda et al., Chhabra and Ferro, Chaps. 19, 21 and 22; in this volume). Also, glycosaminoglycans such as sulodexide inhibit heparanase [93]. The promising results that were obtained so far with these inhibitors in both experimental and clinical studies are not restricted to various malignant diseases, but also to kidney injury. In this regard, it has been demonstrated that heparanase inhibition by either SST0001 or PG545 exerts nephroprotective effects against I/R –induced experimental AKI as was evident by lowering SCr and BUN levels along with improvement in renal histological features and preventing progression to CKD [20, 21, 94]. In all these studies, the heparanase inhibitors were given as pretreatment, and therefore the efficacy of these inhibitors as post-treatment agents in restoring kidney function and reversing renal remodeling/fibrosis remains to be investigated. Yet, the ability of heparanase inhibitors to abolish FGF-2-induced EMT and TGF- β upregulation in AKI models bear promising potential also in CKD [95] including diabetic nephropathy [60, 96, 97]. Most recently, a pioneer study has demonstrated that aspirin has an inhibitory effect on heparanase [98]. Interestingly, this finding may be responsible for the anti-inflammatory effects of NSAIDs in general and aspirin in particular [99, 100].

28.13 Summary and Future Perspective

This brief overview on heparanase in AKI incorporates current knowledge on its involvement in inflammation, coagulation, matrix remodeling, and fibrogenesis as related to renal ischemia-reperfusion, nephrotoxicity, sepsis and kidney transplantation complicated by delayed graft function and chronic allograft nephropathy. An intricate pathway of heparanase synthesis, processing and activation are depicted as potentially vulnerable during the course of AKI. Importantly, application of heparanase inhibitors has gained traction in ameliorating AKI. As evident from the above description and a brief history of studies on the role played by heparanase in AKI, it should not come as a surprise that the subject remains in its infancy. Despite significant in-roads into the pathogenesis of AKI and involvement of heparanase, many questions remain open for future investigations. These include, but are not limited to the following:

1. The details on intracellular traffic of pre-pro-heparanase require clarification.
2. The ultimate fate of HS fragments of various sizes in regulating HSPG functions, TLR4 signaling, and trafficking of HSPG awaits resolution.
3. Broader incorporation of diverse nephrotoxic agents into the list of conditions associated with heparanase dysregulation is needed.
4. The optimal timing of pharmacologic intervention to regulate heparanase activity awaits elucidation and justification.

These and other questions will, undoubtedly, illuminate the course of further preclinical and future clinical investigations of heparanase in AKI and other renal diseases.

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Chapter 29
Heparanase in Acute Pancreatitis

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29.1 Epidemiology and Subsets of Acute Pancreatitis

Acute pancreatitis (AP) is one of the most common diseases in gastroenterology, where about 1–2% of all hospitalized patients are diagnosed with the disease [1]. The incidence of AP per 100,000 population ranges from 14 to 45 cases per year and is rapidly increasing worldwide [2–4]. Among the leading etiologies of AP are gallstones and alcoholism, which are responsible for about two third of all causes of AP [2, 5]. While gallstones induce obstructive AP, the mechanisms underlying alcoholism-induced AP are multifactorial, including both direct toxicity and immunological aspects [6]. Although endoscopic retrograde cholangiopancreatography (ERCP) is the ideal choice for treatment of certain types of AP, including gallstone pancreatitis and certain cases of cholangitis superimposed on gallstone pancreatitis or in patients with documented choledocholithiasis [5], this procedure may induce AP by itself, as it is responsible for about 5–10% of all cases [5]. In fact, 2–20% of patients who undergo ERCP develop AP, depending on population heterogeneity, endoscopic expertise, and procedural differences [7]. Hypertriglyceridemia (>1000 mg/dL) is the third or fourth most common cause of pancreatitis worldwide, accounting for up to 10% of all cases and up to 50% of cases in pregnancy [8].

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Drugs are responsible for 5% of all AP cases [5, 9]. Among the most common drugs that may induce AP, are azathioprine, 6-mercaptopurine, didanosine, valproic acid, angiotensin-converting enzyme inhibitors (ACE-I), losartan, and mesalamine [5, 9]. Also, hypercalcemia increases the risk for AP development [10]. Additionally, certain genetic polymorphism is associated with a higher rate of AP, yet it is difficult to determine their exact contribution to the pathogenies of this clinical entity [5].

29.2 Pathogenesis and Cellular Mechanisms of Acute Pancreatitis

Although the precise mechanisms by which different etiologies induce AP remain vague, there is a consensus concerning the involvement of cytokines (e.g., TNF-α, IL-1β, IL-6, IL-8, and IL-10) once the disease is initiated [11] (Fig. 29.1). In this context, numerous studies demonstrated activation of various inflammatory mediators during AP. For example, tumor necrosis factor alpha (TNF-α), a pro-inflamma-

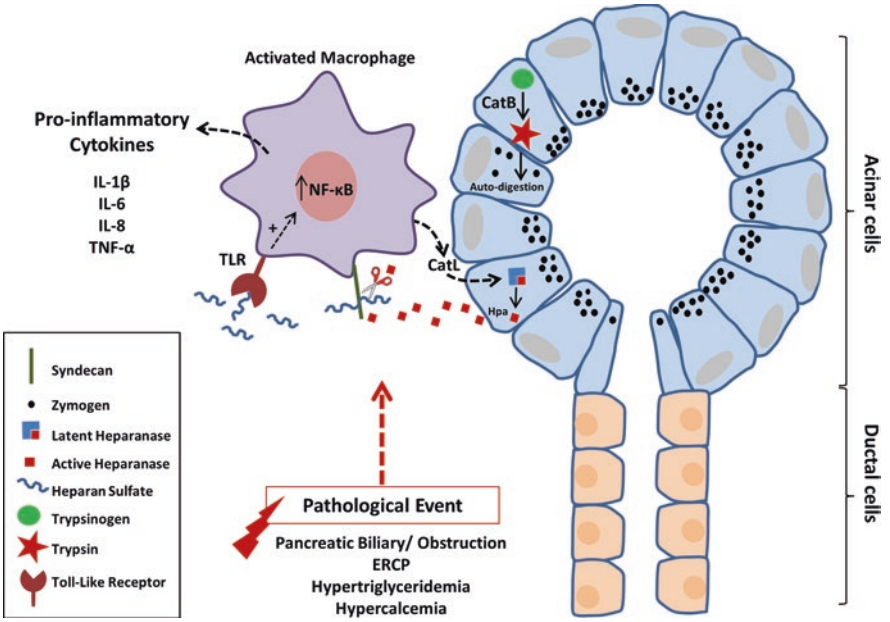

Fig. 29.1 Potential mechanisms of Hpa action in acute pancreatitis**.** Insults of various etiologies induce recruitment of macrophages and other inflammatory cells that possess Cathepsin L activity, which converts latent Hpa into active Hpa. The latter degrades HS and produces HS fragments, leading to increased levels of TNF-α, IL-1β, and IL-6, thereby creating chronic inflammatory conditions fostering macrophage-mediated pancreatic injury and up-regulation of Hpa expression in the acinar cells. This vicious cycle eventually leads to premature conversion of trypsinogen into trypsin and subsequent autodegradation

tory cytokine produced and secreted by activated macrophages and other cell types [12–14] is elevated in the pancreatic tissue and serum of mice with cerulein-induced AP. Increased TNF-α correlates with the acuteness of pancreatic injury and inflammation [15]. Another pro-inflammatory cytokine secreted primarily by macrophages is interleukin1β (IL-1β) [16], whose levels reflect the severity of pancreatic tissue damage and inflammatory response [17]. Interleukin 6 (IL-6) and interleukin 8 (IL-8) are also pro-inflammatory cytokines contributing to the severity of AP [18–20] (Fig. 29.1). Notably, high levels of IL-8 may persist for a long time during AP and are used to monitor the severity of the disease over time [21]. On the other hand, interleukin 10 (IL-10) is a potent anti-inflammatory cytokine opposing the production of pro-inflammatory cytokines and free oxygen radicals [22, 23]. In line with its anti-inflammatory effects, IL-10 treatment attenuated cellular necrosis in experimental pancreatitis [23], suggesting a protective role against AP [1].

Being an exocrine gland [24], the pancreas produces and secretes digestive enzymes as inactive zymogens [25]. Obstruction of the pancreatic duct likely results in abnormal secretion within the pancreas of zymogen granules abundant with digestive enzymes, leading to pathological digestion and inflammation of the organ [26]. Notably, vacuoles containing digestive and lysosomal enzymes generated by merging of zymogen granules with lysosomes play an important role in the resulting autodigestive injury [27]. Lysosomes contain different hydrolytic enzymes, including the cathepsin family of proteases. Cathepsin B and L participate in the accumulation of active trypsin in acinar cells, leading to a pathological cascade of autodigestive injury [28] (Fig. 29.1). This series of events results in inflammatory response, which in turns induces AP [28–30]. Evidence found in individuals with a rare genetic disorder called hereditary pancreatitis, support the notion that trypsin is an integral part of the cascade of autodigestion in AP. Furthermore, hereditary pancreatitis, caused by mutated trypsinogen gene, leads to an abnormal trypsin activity since the protease is undegraded [31]. However, the development of experimental AP following administration of cerulein occurs even in mice lacking trypsinogen isoform 7, suggesting that AP can progress independently of trypsinogen activation [32]. Moreover, it is not clear whether trypsin is the direct source of acinar cell damage in AP, or it is merely a primary stimulus for the activities of different damaging factors [33].

Several pathological cellular changes such as cytosolic calcium (Ca^{+2}) elevation, mitochondrial dysfunction, impaired lysosomal function, damaged autophagy, and endoplasmic reticulum (ER) stress have been observed during AP [28, 34, 35]. A recent study suggested that mitochondrial dysfunction in experimental AP involves two different pathways with relevance to AP induction. One of the pathways involves mitochondrial dysfunction occurring following induction of AP by cerulein, resulting in abnormal elevation in cytosolic Ca^{+2}. Another pathway exerts mitochondrial dysfunction with no Ca^{+2} overload observed in experimental AP induced by administration of L-arginine or in response to choline deficient, ethionine-supplemented diet [34].

An additional characteristic of AP is impaired autophagy, apparently due to damaged lysosomal function caused by impaired cathepsin processing [35]. Autophagy

is an essential cytoprotective process [36], which is responsible for degradation of cell organelles through the formation of autophagosomes and autolysosomes. Under normal conditions, cargo present in lysosomes is degraded by cathepsin L and cathepsin B [28, 37, 38]. However, AP damages the processing of these lysosomal proteases and causes accumulation of cathepsins immature forms, leading, among other effects, to impaired activity of cathepsin L, which degrade trypsin and trypsinogen [28, 35, 39, 40]. Pathological accumulation of trypsin in acinar cells results in the above mentioned autodigestive injury.

Furthermore, ER stress has been demonstrated in the course of AP. Pancreatic acinar cells contain massive rough ER [41] to produce and provide high amounts of proteins necessary for basal secretion of digestive enzymes [42]. Acinar cells tend to develop ER dysfunction [43]. Studies applying a murine model of AP revealed that impaired autophagy leads to several pathological cellular changes including ER stress, oxidative stress, and accumulation of damaged mitochondria. The absence of autophagy-related protein 7 (ATG7) in pancreatic epithelial cells of mice results in swollen and enlarged ER, manifestations that are in line with the observed initiation of unfolded protein response (UPR) [42]. Collectively, AP is a multifactorial clinical state which involves inflammation, biochemical derangement, and deleterious histological alterations. Although heparanase is involved in these aspects of AP, its role in the pathogenesis of this disease was only recently addressed by our group [44].

29.3 Acute Pancreatitis – Current Treatments

Despite the high incidence of AP, the therapy and management of the disease, especially severe AP, remains suboptimal as evident by high mortality rate [45, 46]. This gloomy situation is largely attributed to the vague understanding of the pathogenesis of AP and lack of efficient therapy. Concerning the latter, the current treatment of AP relies mainly on supportive measures including fluid substitution, dietary restrictions, analgesics, and antibiotics [47, 48]. In the early phase of AP, aggressive crystalloid solution (5 to 10 ml/kg) is administered to compensate for third-space loss and intravascular volume depletion [5]. Prophylactic antibiotic treatment is not recommended in mild AP and is a matter of debate even in severe AP unless there is confirmed infection or necrotizing AP [5]. Concerning nutritional changes, it was reported that early enteral nutrition improves the outcome [49].

Unfortunately, there are no pharmacological therapies for AP, and even the applied medications are largely ineffective. These include inhibitors of pancreatic secretion (somatostatin and it's analog, octreotide), L-arginine, calcium ion antagonists, and various inflammatory mediator inhibitors [50–52]. Concerning the latter, NSAIDs, especially diclofenac, have been tested in both experimental and clinical AP [53]. The rationale behind this therapeutic approach is supported by the fact that AP, especially severe AP, may cause damage to other organs because of systemic inflammation and cytokines, including TNF-α and IL-6 [54, 55]. The effects of NSAIDs in experimental animal models of AP have been contradictory and did not

affect the mortality rate [53]. Similarly, clinical studies have demonstrated that prophylactic indomethacin given prior to ERCP yielded only marginal beneficial pancreatic-protective effects [53]. The efficacy of diclofenac, another NSAID, has been tested immediately post ERCP, where the patients received either diclofenac suppositories or placebo. The incidence of AP was lower in the group receiving diclofenac compared to the placebo group [56]. Collectively, although not well established and despite the non-clear cut findings, it seems that NSAIDs exert a beneficial effect as prophylaxis against AP, where both diclofenac and indomethacin are administered as suppositories during this disease state. In contrast, neither somatostatin nor octreotide exerted overt beneficial effects in experimental and clinical AP, where both did not reduce the mortality rate [53]. There are few promising anti-inflammatory and antioxidant drugs such as IL-10, anti-TNF-α, nitric oxide (NO) donors, and N-acetyl-cysteine that were tested in animal models of AP where they exhibited anti-inflammatory effects, pending keen evidence for their efficacy at the clinical level [53].

29.4 Heparanase and Activation of Toll-like Receptors During Inflammation

The extracellular matrix (ECM) contains various cytokines, chemokines, and growth factors sequestered by means of binding to HS and affecting inflammatory responses at multiple levels: (i) Sequestration of pro- and anti-inflammatory substances in the ECM, (ii) Modulation of leukocyte interactions with the endothelium and ECM, and (iii) Initiation of innate immune responses through interactions with Toll-like receptors, primarily TLR4 [57–64].

Increasing evidence suggests that Hpa affects activities of various types of innate immunocytes, including neutrophils, macrophages, dendritic and mast cells [65–71] (Elkin, Chap. 17 in this volume). Neutrophils are important effectors in acute inflammatory responses, including AP. In a mouse model of sepsis-associated inflammatory lung disease, rapid induction of heparanase activity was demonstrated in pulmonary microvascular endothelial cells [70]. This was associated with degradation of the glycocalyx, a thin gel-like layer that coats the luminal surface of blood vessels, leading to increased availability of endothelial cell surface adhesion molecules and consequently improved neutrophil adhesion and extravasation [70]. Hpa inhibition prevented endotoxemia-associated glycocalyx loss and neutrophil adhesion and therby attenuated sepsis-induced acute lung injury and mortality in mice [70]. Likewise, reduced infiltration of neutrophils and eosinophils was noted in Hpa-knockout lungs exposed to prolonged smoke inhalation or subjected to allergic inflammatory model [72, 73]. Our group has demonstrated that administration of PG545, a potent inhibitor of Hpa, to mice prior to cerulein-induced AP markedly reduced neutrophil recruitment to the pancreatic tissue [44].

These findings are in line with the anti-inflammatory effects demonstrated for Hpa-inhibiting compounds (i.e., heparin, heparin-mimicking compounds) in animal models [74–79], further supporting the involvement of the enzyme in inflammatory reactions. Additionally, the avid infiltration of neutrophils to Hpa overexpressing (Hpa-Tg) vs. wild-type (WT) pancreas in response to cerulein further ties heparanase levels to the amplitude of inflammatory reactions. This is also reflected by augmented levels of TNF-α, IL-6, and NFκB in Hpa-Tg vs. WT pancreas, signaling determinants that are central to AP [80]. Noteworthy, heparanase enhances the IL-6/p-STAT3 axis also in inflammation (macrophages)-driven colon [68] and pancreatic cancer [81], indicating that the pro-inflammatory function of Hpa is mediated by diverse types of immunocytes, including macrophages. The latter is a vital component of the innate immune system found in various tissues, including the pancreas, and fulfilling a critical role in immune surveillance [82–84]. During inflammation, the infected tissue releases cytokines, chemokines, and growth factors [84, 85] which then activate blood monocytes and attract them to the affected site where they differentiate into macrophages and mediate tissue remodeling and repair [84–87]. Macrophages are versatile, and there is no single marker that can ascribe them to a given subclass [84–86, 88–90]. However, two main subpopulations were identified, consisting of classical M1 and alternative M2 macrophages. While M1 macrophages are involved in inflammation, pathogen clearance, and antitumor immunity, M2 macrophages exert anti-inflammatory and pro-tumorigenic properties [84, 87, 91–93]. The pro-inflammatory and antitumor immunity exerted by M1 macrophages are driven by the Th1 cytokine interferon-γ, bacterial moieties such as lipopolysaccharide (LPS), and TLR agonists [94, 95]. M1 macrophages produce pro-inflammatory cytokines (i.e., IL-6, IL-12, IL-23, TNF-α) [91, 94, 96], engulf and degrade bacteria and pathogens, and present the resulting peptides extracellularly to MHC class I and II complexes in a process called “phagocytosis.” The presented peptides are then recognized by T helper cells [83, 86, 87, 97–99]. In contrast, M2 macrophages are activated by certain cytokines such as IL-4, IL-10, or IL-13, and function in constructive processes, including wound healing and tissue repair [100, 101]. In this context, Hpa is implicated in activation of macrophages resulting in increased production of pro-inflammatory cytokines (i.e., TNF-α, MCP-1, IL-6, IL-1β) [67, 68, 81, 102], playing a role when acute inflammation is not properly resolved and turn into a chronic phase. Importantly, prominently decreased levels of cytokines were produced and secreted by macrophages isolated from Hpa-KO mice [103]. Mechanistically, Gutter-Kapon et al. have recently identified a linear cascade that starts with heparanase-mediated activation of TLRs at the cell membrane, continues with Erk/p38/NFκB/JNK activation, leading to increased c-Fos levels and induction of cytokine expression [103]. While the relevance to AP awaits further investigation, one should bear in mind that macrophages provide a major source of proteolytic enzymes such as cathepsins, MMPs, and serine proteases that play a role in cell invasion and ECM remodeling [94, 96]. Notably, our group has shown that experimental AP was associated with upregulation of Cathepsin L expression and abundance [44], whereas pretreatment with PG545 abolished the enhancement in Cathepsin L, suggesting an interplay between Hpa and Cathepsin L, most likely at

the macrophage level. It should be noted that along with pro-inflammatory cytokines (IL-6 and TNF-α), cerulein-induced AP was associated with enhanced expression of seemingly anti-inflammatory (IL-10) cytokines, suggesting that inflammation and tissue regeneration are simultaneously ongoing in this model of AP. It should be kept in mind, though, that classification into pro-inflammatory and anti-inflammatory cytokines is far too simplistic. The amount of a given cytokine, the nature of the target cell and the activating signal, the timing and sequence of cytokine action, all may dictate if a given cytokine will behave like a pro- or anti-inflammatory cytokine [104]. For example, a pro-inflammatory effect of IL-10 has been reported in some cases [105, 106]. Given its pluripotent effects, Hpa appears to exert both pro-inflammatory and anti-inflammatory effects, depending on the context. This is best exemplified in studies showing that neuroinflammation is inhibited in response to Hpa [107, 108] (Li and Zhang, Chap. 25 in this volume). The inhibitory effect is likely due to disruption of chemokine gradients and the resulting impaired extravasation of blood-borne immune cells upon degradation of HS on the surface of vascular endothelial cells [69].

29.5 Heparanase in Acute Pancreatitis

Despite the high prevalence of AP (2% of all hospitalized patients) and its enhanced morbidity and mortality rate [33, 109, 110], the mechanisms underlying the pathogenesis of this gastroenterological disease remains largely elusive [109]. In light of the involvement of Hpa in various inflammatory diseases [111], we hypothesized that Hpa might be involved in the pathogenesis of AP. This notion is supported by the fact that Hpa is present extracellularly, where it can efficiently promote inflammation, a hallmark feature of AP. As mentioned above, cleavage of HS by heparanase facilitates structural alterations in the ECM and thereby promotes cell invasion associated with inflammation, tumor metastasis, and angiogenesis [111–115]. Moreover, understanding the initial triggering events of AP may lead to the development of novel, specific, and effective remedy. For this purpose, we applied a common model of AP, namely cerulein-induced AP (3–5). A substantial portion of the present knowledge concerning AP has been gained from animal models or isolated cells of the diseased pancreas [33, 80]. Specifically, several experimental animal models have been developed over the last decades including induction of AP by cholinergic agonists such as carbamylcholine (carbachol), CCK and its analogs, or by scorpion venom [33, 80, 116]. One of the most common models of AP is AP-induced by the administration of excessive doses of pancreatic secretagogue such as CCK in rats, leading to a clinical and biochemical pattern of acute interstitial pancreatitis [117]. A prominent characteristic of this model is the development of excessive edema as early as 1 h after the onset of the disease, and induction of tissue inflammation [33, 80, 109]. Cerulein is a CCK analog derived from the Australian tree frog *Litoria caerulea* and is one of the best-characterized models of AP in mice [33, 80, 109].

Our study [44] provides keen evidence that pancreatic Hpa expression and activity were remarkably increased following cerulein administration to WT and Hpa-Tg mice. It should be emphasized that this pattern of heparanase induction was also observed in human chronic pancreatitis [118], signifying a clinical relevance of our mouse model. Moreover, pancreas edema and inflammation (i.e., recruitment of neutrophils), as well as induction of enzymes (amylase, lipase), cytokines (TNFα, IL-6), and signaling molecules (i.e., NFκB, phospho-STAT3) were enhanced in the pancreatic tissue following induction of the disease (Fig. 29.2). Strikingly, all these

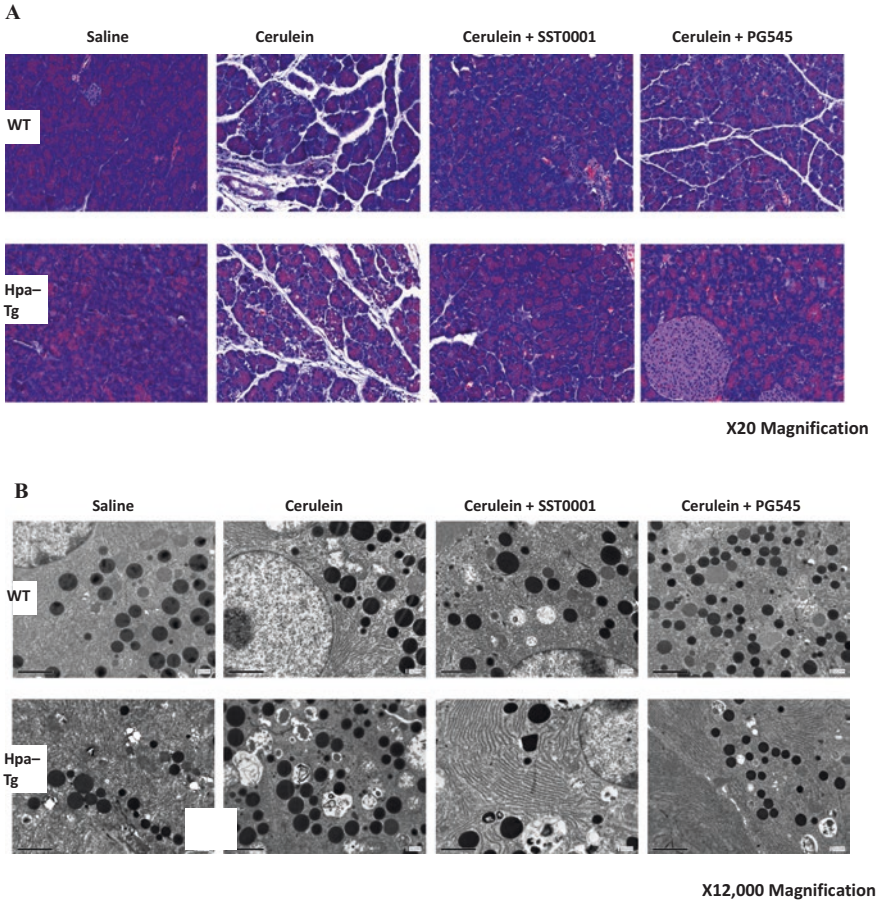

Fig. 29.2 (**A**) Histological analyses. WT and Hpa-Tg mice were injected with either saline or cerulein in the presence or absence of PG545 or SST0001. Pancreas tissues were collected 24 h after that, and 5-micron sections from formalin-fixed, paraffin-embedded samples were stained for H&E. Shown are representative photomicrographs at X20 original magnification. (**B**) Electron Microscopy**.** WT and Hpa-Tg mice were injected with either saline or cerulein in the presence or absence of PG545 or SST0001. Pancreas tissues were collected 24 h thereafter. Pancreatic tissues from the various experimental groups were fixed in 3.5% glutaraldehyde and processed for EM as described [44]. Sections were examined with a transmission electron microscope (Jeol 1011 JEM), at 80 KV. X12,000 Magnification

biochemical, histological and molecular manifestations of cerulein-induced AP were attenuated markedly by Hpa inhibitors (Roneparstat, Pixatimod) (Noseda and Barbieri; Hammond and Dredge, Chaps. 21 and 22 in this volume), suggesting a pivotal role of heparanase in the pathogenesis of AP (Fig. 29.2) [44]. We further demonstrated that this model of AP is characterized by enhanced expression of cathepsin L, a key enzyme in heparanase processing and activation (122) and that PG545 (=Pixatimod) abolished this increase. Notably, all the above features were more profound in Hpa-Tg mice, suggesting that these mice can be utilized as a most sensitive model system to further reveal the molecular mechanisms by which heparanase underlies AP. We further found that Hpa-KO mice showed decreased lipase levels in response to cerulein as compared to Hpa-Tg animals. Taken together, Hpa emerges as a potential new target in AP, and Hpa inhibitors, now in phase I/II clinical trials in cancer patients, may prove beneficial in AP. Concerning the latter, the ability of PG545 to pronouncedly attenuate the elevation lipase and amylase in cerulein-induced AP further signifies the potency of this compound. Likewise, SST0001 (= Roneparstat), another well-characterized Hpa inhibitor, exerted similar pancreatic-protective effects in this model, strongly implying that heparanase plays an important role in the course of AP and that its inhibition may pave the way for a new potential therapeutic approach for AP.

While our study implicates Hpa with AP, it should be envisioned in a broad perspective that ties Hpa with fundamental functions of the pancreas. More specifically, it has been shown that HS is essential for the survival of pancreatic beta cells; In vivo, autoimmune destruction of islets was associated with the production of catalytically active Hpa by islet-infiltrating mononuclear cells, and loss of islet HS [119, 120]. Furthermore, treatment with Hpa inhibitor (PI-88, Chhabra and Ferro, Chap. 19 in this volume) preserved intra-islet HS and protected mice from type I diabetes [119, 120]. Thus, Hpa inhibitors may turn even more important for the management of chronic pancreatitis, protecting beta cell islets from destructive heparanase produced by pancreatic acinar cells and/or inflammatory cells (Simeonovic et al., Chap. 24 in this volume).

29.6 Novel Heparanase Mechanism-Based Therapies for Acute Pancreatitis: Heparanase Inhibitors

In light of the unambiguous involvement of Hpa in the pathogenesis of AP [44], the impact of Hpa inhibitors has been investigated in an experimental model of the disease. Specifically, we have demonstrated that AP is characterized by up-regulation and activation of pancreatic Hpa, which was abrogated by Pixatimod (formerly PG545) and Roneparstat (formerly SST00010) [44], Hpa inhibitors that are being examined in cancer clinical trials (Noseda and Barbieri; Hammond and Dredge, Chaps. 21 and 22 in this volume). In line with these findings, the elevation of amylase and lipase was markedly attenuated by Pixatimod and Roneparstat in both WT and Hpa-Tg mice. Likewise, the enhancement of pancreatic edema index and tissue

inflammation, hallmarks of AP, were substantially reduced following Hpa inhibition by the above-mentioned inhibitors. Aspirin has also been demonstrated to inhibit Hpa activity both *in vitro* and *in vivo* [121]. Interestingly, this novel finding may be held responsible for the anti-inflammatory effects of NSAIDs in general and AP in particular [122]. Based on that, we hypothesized that a combination of Hpa inhibitor and Aspirin could ameliorate AP more efficiently than each drug alone. We have found that while pretreatment with either Aspirin, Pixatimod or Roneparstat alone reduced pancreatic inflammatory response, autophagy, and amylase and lipase serum levels in both WT and Hpa-Tg mice, combination of Aspirin with either PG545 or Roneparstat completely abolished AP at the biochemical, inflammatory and histological levels in both subgroups of animals (Fig. 29.2) [123]. Noteworthy, electron microscopy analyses revealed that pancreatic cells are decorated during AP with an increased number of cytoplasmic vacuoles typical of autophagosomes, and the vacuole size was increased substantially following cerulein treatment, suggesting that cerulein enhances zymophagy and autophagy (Fig. 29.2B). Indeed, the levels of LC3, the most commonly used marker of autophagy, was increased following cerulein treatment. Importantly, the number and size of autophagosomes and levels of LC3 were markedly decreased by PG545, suggesting that heparanase functions to promote autophagy in AP, as noted previously in cancer cells [124]. In fact, PG545 alone or in combination with Aspirin abolished the deleterious ultrastructural alterations induced by cerulein, yielding a nearly normal appearance of the rough endoplasmic reticulum, Golgi apparatus, and mitochondria [44]. Trehalose has been shown to alleviate experimental pancreatitis manifestations [34]. We hypothesized that combined treatment with Hpa inhibitor and aspirin or Trehalose could ameliorate AP more efficiently than each drug alone. Indeed, pretreatment with Pixatimod, Roneparstat, Aspirin or Trehalose reduced pancreatic inflammatory response, autophagy, and ultrastructure alterations as evident by mitochondrial swelling and ER stress along with a remarkable reduction in amylase and lipase serum levels in both WT and Hpa-Tg mice that underwent cerulein-induced AP. Noteworthy, a combination of Aspirin or Trehalose with either Pixatimod or Roneparstat completely abolished AP, as was evident by reversing the biochemical, inflammatory and histological perturbations in both WT and Hpa-Tg mice (Fig. 29.2) [123]. Collectively, these findings imply that Hpa plays a substantial role in AP and that Hpa inhibitors can serve as a therapeutic strategy for the treatment of AP.

29.7 Summary and Perspectives

Remodeling of HS by Hpa may affect several aspects of inflammatory reactions, such as leukocyte recruitment, extravasation, migration towards inflammation sites, release of cytokines and chemokines anchored within the ECM or cell surface, as well as activation of innate immune cells. Therefore, it is not surprising that the available data implicate the enzyme in the pathogenesis of AP. Apparently, the ability of Hpa inhibitors to attenuate cerulein-induced AP appears superior compared

with other nonspecific compounds tested in this experimental model, lending optimism that these Hpa-inhibiting compounds will prove of clinical efficacy for AP and other Hpa-driven diseases. Additional studies are needed to establish whether Hpa inhibitors will exert a similar beneficial effect also when given after the induction of AP.

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Chapter 30
Involvement of Heparanase in Endothelial Cell-Cardiomyocyte Crosstalk

Rui Shang, Nathaniel Lal, Karanjit Puri, Bahira Hussein, and Brian Rodrigues

30.1 Introduction

Globally, approximately 425 million people are affected by diabetes, with an annual mortality rate that stands at 4 million [53]. Alarmingly, the World Health Organization predicts that by 2030, diabetes will be the seventh leading cause of death [81]. In Canada, the number of individuals with pre-diabetes and diabetes (T1D and T2D) has reached 10.7 million, with over $3.6 billion of our national health care budget used to manage this chronic disease [30]. Therapeutic management of blood glucose and its monitoring are the foundational basis of diabetes treatment. However, this practice does not exquisitely match the physiological control of glucose homeostasis. As a result, people with diabetes are prone to developing long-term complications like cardiovascular disease (CVD), which accounts for 50% to 80% of diabetes-related deaths [34, 113]. Coronary artery disease (CAD) and atherosclerosis are primary reasons for the increased prevalence of CVD, with increased ROS, dyslipidemia, and endothelial cell (EC) dysfunction contributing towards this accelerated diabetes-induced macrovascular disease and subsequent heart failure [47]. However, patients with T1D and T2D have also been diagnosed with reduced or low-normal diastolic function and left ventricular hypertrophy in the absence of vascular defects, termed diabetic cardiomyopathy [9, 57]. The etiology of cardiomyopathy is complex, with early changes in cardiac metabolism being considered a major culprit [62, 105, 132]. This includes reduced glucose (GL) consumption and a switch to utilizing more fatty acid (FA) for ATP. In the diabetic heart, heparan-

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ase is an EC secreted protein that helps with this metabolic switching of substrates [126]. In this chapter, we will discuss diabetic cardiomyopathy and its initiation by aberrant fuel metabolism as well as how EC, a "first-responder" to hyperglycemia, communicates with the underlying cardiomyocyte using heparanase, and how this molecular cross-talk between these two cells is terminated following diabetes.

30.2 Diabetic Cardiomyopathy

In patients with Type 1 (T1D) or Type 2 (T2D) diabetes, heart disease is a primary reason for mortality, with vascular abnormalities playing a significant role in its development [79]. However, clinical trials support the idea that diabetes is also associated with heart failure in the absence of CAD, defined as diabetic cardiomyopathy (DCM) [105]. The early stages of DCM include structural abnormalities like ventricular hypertrophy and cardiac fibrosis, and the development of diastolic dysfunction with normal ejection fraction [57]. With the progression of diabetes, DCM advances to systolic dysfunction and heart failure with reduced ejection fraction. Similar to this clinical evidence, DCM has also been reported in animal models of T1D [52, 78] and T2D [1, 16, 20]. Given that rodents are resistant to atherosclerosis, these models provided strong supporting evidence for the occurrence of cardiomyopathy following diabetes. Cardiomyopathy is a complex disorder with multiple factors associated with its development [57]. These include an accumulation of connective tissue and insoluble collagen, autonomic neuropathy [56, 94], increased oxidative stress [56], microvascular dysfunction [108], mitochondrial dysfunction [21, 133], advanced glycation end products [56], abnormalities in Ca^{2+} handling [116], ER stress [137], RAAS activation [56] and apoptosis [65]. The premise that changes in cardiac metabolism can contribute towards the etiology of DCM has also been proposed.

30.3 Cardiomyocyte Metabolism under Physiological Conditions

The heart has a high demand for ATP and hence demonstrates substrate promiscuity, enabling it to utilize multiple sources of energy, including amino acids, lactate, ketones, GL and FA [77]. Among these, GL and FA are the major participants from which the heart derives most of its energy. Accordingly, in a basal setting, GL contributes to approximately 30% of ATP generation with FA accounting for the remaining 70% [77].

30.3.1 Glucose

For GL to yield ATP, it requires uptake, glycolysis and mitochondrial oxidation. In the cardiomyocyte, glucose uptake is dependent on glucose transporters like GLUT1 and GLUT4 [4]. The former has a predominant plasma membrane localization and is responsible for insulin-independent GL uptake whereas the latter is the dominant transporter in the adult heart and accounts for insulin-dependent transport of GL (Fig. 30.1). Thus, in response to insulin, there is vesicular recruitment of GLUT4 from its intracellular pool to the plasma membrane to mediate GL uptake [6, 46]. Once across the plasma membrane, GL is phosphorylated by hexokinase to glucose-6-phosphate (G-6-P), the first step of glycolysis which eventually generates pyruvate in the cytoplasm and two mol-

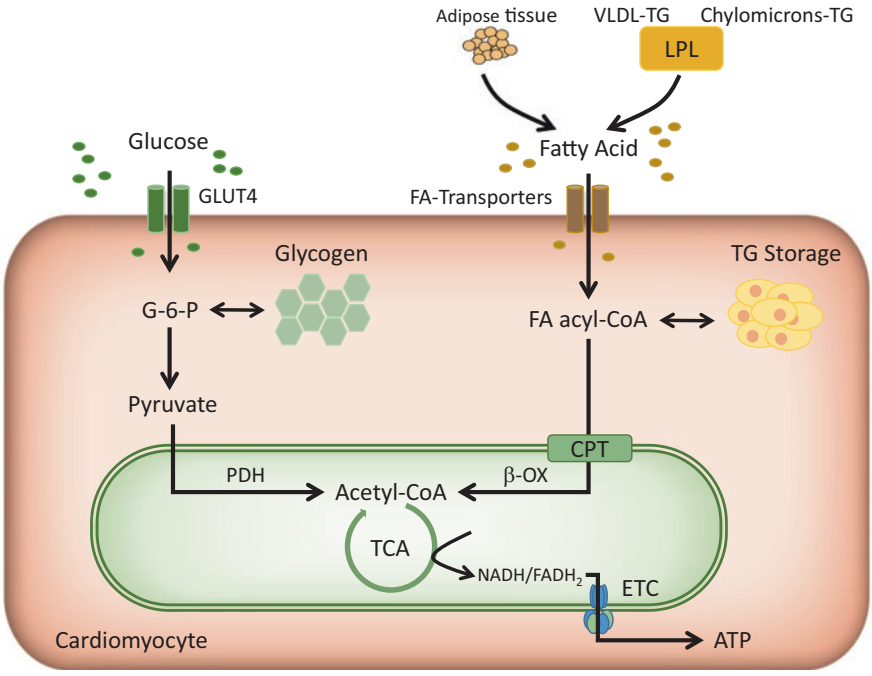

Fig. 30.1 Substrate utilization by the cardiomyocyte. In the cardiomyocyte, two of the major substrates that are used for ATP generation include glucose and fatty acids (FA). Glucose uptake into the cardiomyocyte relies on a GLUT4 transporter. Following its entry into the cell, glucose can either be stored as glycogen, or undergo glycolytic and oxidative metabolism under the control of pyruvate dehydrogenase (PDH). FA is the preferred energy substrate of the cardiomyocyte and is generated by lipoprotein lipase (LPL) hydrolysis of circulating lipoproteins or adipose tissue lipolysis. FA entry into the cardiomyocyte is through a number of FA transporters including CD36, $FAPB_{PM}$, and FATP. Within the cardiomyocyte, FA can undergo storage in the form triglycerides (TG), or enter into the mitochondria to undergo β-oxidation (β-OX). The acetyl-CoA produced from glucose or FA enters the TCA cycle and electron transport chain (ETC) to generate ATP

ecules of ATP per glucose molecule [75]. Following its import into the mitochondria, pyruvate is converted into acetyl-CoA by the multi-enzyme pyruvate dehydrogenase complex (PDH) [9]. Further processing of acetyl-CoA in the tricarboxylic acid (TCA) cycle results in the generation of NADH and $FADH_2$, release of electrons into the electron transport chain and finally, oxidative phosphorylation (OXPHOS) to generate most of the ATP (34 molecules) related to GL metabolism [75] (Fig. 30.1). It should be noted that depending on the demand for energy, glucose can also be stored as glycogen for future utilization by the cardiomyocyte [124].

30.3.2 Fatty Acids

The heart has no capacity to synthesize FA, and thus relies on obtaining it from endogenous and exogenous sources [9]. Regarding intrinsic provision of FA, breakdown of endogenous cardiac TG by lipases is one source of FA. Related to extrinsic delivery, lipolysis of stored TG within adipose tissue releases FA into the circulation [48, 84], for transport to the heart. This process is initiated through the action of adipose triglyceride lipase (ATGL), which is the first and rate-limiting step hydrolyzing TG to release diacylglycerol (DAG) and FA. Hormone-sensitive lipase (HSL) cleaves DAG into monoacylglycerol (MAG), with the release of the second FA. The final step is the hydrolysis of MAG by monoacylglycerol lipase (MGL) to generate the third FA [84]. All of these FA are delivered via the circulation to the heart for β-oxidation and ATP production. It should be noted that for the uptake of this exogenous FA into the cardiomyocyte, sarcolemmal FA transporters are required and include fatty acid translocase (FAT/CD36), fatty acid binding protein ($FABP_{PM}$), and FA transporter protein (FATP) [25, 109]. In the cardiomyocyte, FA are converted to fatty acyl-CoA, which can be transported into the mitochondria by carnitine palmitoyltransferase (CPT1/CPT2). Mitochondria serve as a powerhouse in the cardiomyocyte, where fatty acyl-CoA undergoes β-oxidation to generate acetyl-CoA, which can be used in the TCA cycle/ETC to yield ATP [76, 77]. Master regulators that oversee cardiac FA metabolism include AMP-activated protein kinase (AMPK) [8] and peroxisome proliferator-activated receptor-α (PPARα) [28, 90]. In addition to adipose tissue, another extrinsic resource of FA is that carried by TG-rich lipoproteins, such as chylomicron derived from the gut, or very low-density lipoprotein (VLDL), synthesized in the liver [88]. The release of FA from this lipoprotein bound TG requires lipolysis that is catalyzed by lipoprotein lipase (LPL) [88] (Fig. 30.1).

30.4 Lipoprotein Lipase (LPL)

Given that 90% of plasma FA are contained within lipoprotein-TG and that LPL has a robust expression in the heart, LPL-mediated lipolysis of TG-rich lipoproteins is suggested to be a principal exogenous FA source for cardiac utilization [69, 100]. Lipoprotein clearance by LPL proceeds at the apical surface of EC lining the coronary vascular lumen. Despite this critical function, EC do not synthesize LPL [12]. Instead, the majority of this enzyme is produced in cardiomyocytes and subsequently secreted onto heparan sulfate proteoglycan (HSPG) binding sites on the myocyte cell surface. Localization at the myocyte surface is a clever arrangement as it provides the cell with a rapidly accessible reservoir, thus precluding the need for *de novo* synthesis when LPL is required [33, 35]. From the myocyte surface, LPL traverses the interstitial space [7], and glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) at the basolateral side of EC transports it to the apical luminal surface [140]. Out here, GPIHBP1 also functions as a platform to enable vascular LPL to hydrolyze the TG core of lipoproteins to FA; the released FA is then transported across EC into the cardiomyocyte for ATP production [93, 124] (Fig. 30.2). More recently, an added function of GPIHBP1 has emerged whereby it reduces the unfolding of the catalytic domain of LPL by

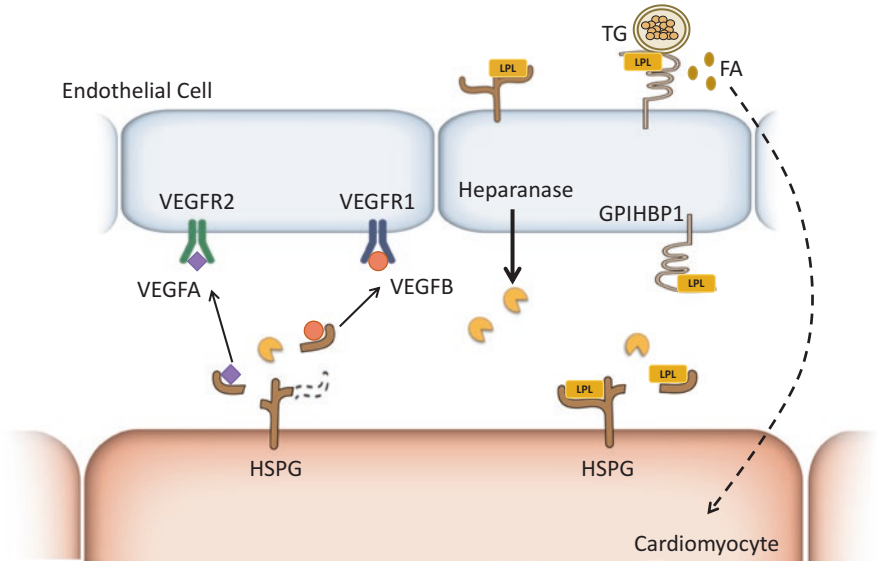

Fig. 30.2 Crosstalk between endothelial cells and cardiomyocytes. Following its secretion from the endothelial cell (EC), heparanase cleavage of cell surface HSPGs on the cardiomyocyte releases LPL. This enzyme is captured by GPIHBP1 present on the basolateral side of EC for transport to the apical luminal side of the coronary blood vessels. Out here, GPIHBP1 also acts as a platform for LPL hydrolysis of circulating TG to release FA for the cardiomyocyte. Heparanase is also capable of releasing HSPG bound growth factors (VEGFA and VEGFB), which can bind to their respective receptors on the EC, initiating their actions to support LPL function

angiopoietin-like protein 4 (ANGPTL4), consequently stabilizing LPL activity [86, 87].

LPL activity is rapidly responsive to numerous physiological conditions and does so in a tissue-specific manner. Hence, in fasting, LPL activity decreases in adipose tissue but increases in the heart [31]. As a result, FA generated from circulating TG is diverted away from storage, and towards meeting the metabolic demands of cardiomyocytes. Consequently, LPL fulfills a "gate-keeping" role by regulating the supply of FA to meet tissue requirements. However, surplus FA provision to tissues other than adipose tissue can trigger cellular demise. Not surprisingly, cardiac-specific LPL overexpression causes severe myopathy characterized by lipid oversupply and deposition, muscle fiber degeneration, excessive dilatation, as well as impaired left ventricular function in the absence of vascular defects, a situation comparable to DCM [71, 136]. Interestingly, the loss of cardiac LPL also causes cardiomyopathy [12, 89]. Hence, although the specific knockout of cardiac LPL increased glucose metabolism, neither this effect nor albumin-bound FA could replace the action of LPL. As a result, the cardiac ejection fraction decreased [12]. Altogether, these studies indicate the importance of cardiac LPL and suggest that disturbing its innate function is sufficient to induce cardiac failure. Although vascular LPL augments FA availability, this energy substrate still requires an abundant oxygen supply for its oxidation. Therefore, it is imperative that if excess FA are being supplied, there are strategies available to prevent incomplete FA oxidation, inhibition of glucose utilization and potential cell death. These could include **a)** promotion of capillary size and coronary artery density (to ensure that a limitation in O_2 delivery to the cardiac muscle is not an issue), **b)** alleviation of metabolic inflexibility (to re-establish the physiological utilization of energy substrates like glucose), and **c)** prevention of cell demise (associated with increased FA oxidation and TG accumulation). Members belonging to the VEGF family of proteins are unique in their ability to modulate O_2 delivery, regulate metabolic reprogramming and inhibit cell death.

30.5 Vascular Endothelial Growth Factors

The VEGF family consists of 6 growth factors; VEGF A-E and placental growth factor. Of these multiple growth factors, VEGFA and VEGFB are notable in that they are abundantly expressed in the heart [68, 118].

30.5.1 Vascular Endothelial Growth Factor a

VEGFA, originally described as a vascular permeability factor [60, 110], exists in various homodimeric isoforms created by alternative splicing and proteolytic cleavage, and exhibit different biological properties such as receptor binding or their

affinity to HSPG [95]. They include $VEGFA_{121}$, $VEGFA_{145}$, $VEGFA_{165}$, $VEGFA_{183}$, $VEGFA_{189}$, and $VEGFA_{206}$. All of these except $VEGFA_{121}$ (which is soluble and diffusible) have distinct heparin-binding domains, impacting their ability to bind to cell surface HSPG [13]. Additionally, the most predominantly expressed isoform is $VEGFA_{165}$ [64]. Receptors that mediate the action of VEGFA include the tyrosine kinase VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) [41]. Of these, it is VEGFR2 that is largely responsible for VEGFA function to promote angiogenesis and vascular permeability [111] (Fig. 30.2). Interestingly, VEGFR1 has a ten-fold higher binding affinity for VEGFA but weaker kinase activity [123] and is considered a negative regulator of VEGFA action [27, 39]. It should be noted that in addition to these two receptors, co-receptors like NRP1 and NRP2 are required for efficient VEGFA signaling [38, 112]. On binding of VEGFA to VEGFR2, the receptor dimerizes and autophosphorylates resulting in the activation of several downstream pathways. Examples of these include PLCγ-PKC-MAPK pathway to promote EC proliferation [111], PI3K/Akt signaling to increase GLUT1 expression [138], EC migration and endothelial nitric oxide synthase (eNOS) [22], that contributes towards blood vessel formation and vasodilation, and Src which is necessary for influencing vascular permeability [115]. Related to metabolism, VEGFA increases multiple glycolytic enzymes such as lactate dehydrogenase-A and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 [135] (Fig. 30.3).

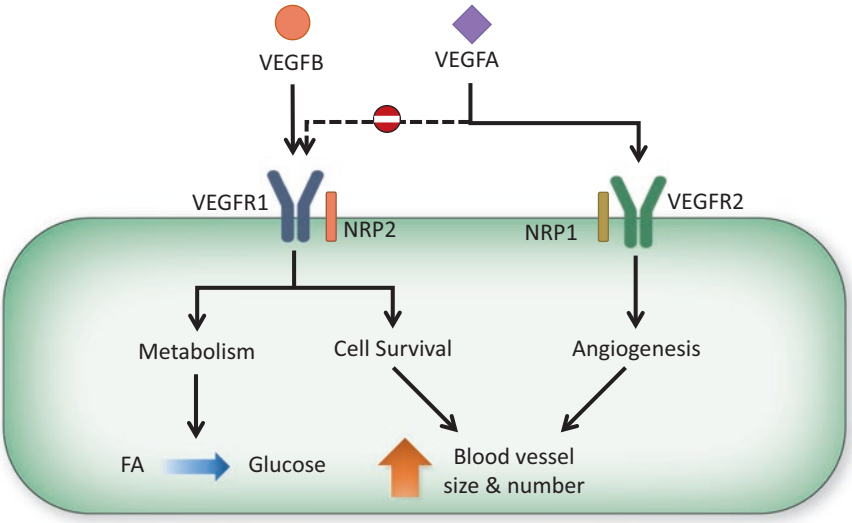

Fig. 30.3 Interplay between VEGFA and VEGFB to affect heart function. VEGFB binding to VEGFR1 (in the presence of coreceptors NRP1/2) initiates downstream signaling that activates metabolism and cell survival. The effects of VEGFA are possible through its binding of VEGFR2 promoting angiogenesis. VEGFA is also capable of binding to VEGFR1 with higher affinity, but this action is incapable of inducing any effect. With overexpression of VEGFB in the heart, VEGFA is displayed from VEGFR1 to VEFGR2, such that in these animals the increase in blood vessel size and number is likely due to the dual effects of both these growth factors

These observations indicate that VEGFA is a key regulator of vasculogenesis and angiogenesis and its knockout is embryonically lethal due to vascular abnormalities [24, 40].

30.5.2 Vascular Endothelial Growth Factor B

Intriguingly, unlike other members of the VEGF family, VEGFB uncharacteristically does not promote angiogenesis [59, 85, 144]. However, with more recent studies acknowledging a role for this growth factor in sensitizing tissues to VEGFA-induced angiogenesis [63, 103], together with its recognized impact on metabolism and cell survival [144] (a function that is especially relevant under pathological conditions [63]), VEGFB may be critical for providing protection against the threat of diabetes-induced cardiomyopathy.

Existing as two isoforms, $VEGFB_{167}$ encompasses over 80% of transcripts (at least in the mouse heart) and contains a highly basic C-terminal heparin-binding domain allowing it to be cell-surface bound [73]. The other isoform, $VEGFB_{186}$ has a hydrophobic C-terminal making it freely soluble [80, 91]. VEGFB is highly expressed in heart and skeletal muscle, with limited expression in most other tissues [73]. The action of VEGFB is coordinated through its binding and activation of a receptor called VEGFR1 [10] (Fig. 30.2). As VEGFB shares 47% of its amino acid sequence with VEGFA [92], initial experiments focused on a role for VEGFB in angiogenesis, studies that were largely futile. A more contemporary idea is that VEGFB does target angiogenesis, but indirectly by enhancing the action of VEGFA [19, 63].

Even though VEGFB shares significant homology and receptor binding with VEGFA, it uncharacteristically does not initiate angiogenesis directly. Interestingly, VEGFB, by binding to VEGFR1, displaces and increases the bioavailability of VEGFA to bind VEGFR2 and induce angiogenesis through activation of Erk/Akt/mTORC1 signaling [58]. Accordingly, VEGFR1 knockout is embryonically lethal as in its absence [42], VEGFA binds exclusively to VEGFR2 promoting uncontrolled angiogenesis and hyper-vascularization. Likewise, just two weeks post-adeno-associated virus (AAV) VEGFB transduction in mice increased vessel size and capillary density in adipose tissue [103]. The study reasoned that these reported effects of VEGFB were not a result of direct action but a consequence of the growth factor binding to VEGFR1, decreasing the availability of this receptor to bind VEGFA. As a consequence of less available VEGFR1, increased VEGFA action on VEGFR2 enhances capillary network (Fig. 30.3). Interestingly, this enriched capillary network observed with AAV VEGFB displayed a normal pattern compared to AAV administration of VEGFA which revealed abnormal vasculature, implying a functional rather than pathological impact of VEGFB on blood vessels. Finally, cardiac-specific overexpression of VEGFB significantly increased capillary size and number of arteries [63].

An interesting property of VEGFB is its reported effect on energy substrate metabolism. VEGFB gene expression has a similar expression pattern to OXPHOS genes in mitochondria, likely as a consequence of the same upstream regulator-peroxisome proliferator-activated gamma receptor co-activator 1α (PGC-1α) [49, 83]. In transgenic (Tg) rats with cardiac-specific overexpression of VEGFB, glucose uptake into the heart was increased, and so were the intermediate products related to glycolysis and glycogenolysis indicating that these hearts were using more glucose [63]. Similarly, in mice injected with adeno-associated virus-producing $VEGFB_{186}$ and fed with high-fat diet (HFD), these animals had a lower fasting plasma insulin and improved glucose and insulin tolerance suggesting better insulin sensitivity [103]. A decrease in serum glycerol further supported this observation, given the role of insulin in inhibiting adipose tissue lipolysis. Conversely, deletion of VEGFB decreased insulin sensitivity and lowered glucose clearance in adipose tissue. This effect of VEGFB could be either direct or secondary to improved vascular perfusion (through VEGFA/VEGFR2 signaling) and thus augmented insulin delivery to the target organs. Unlike its effect on glucose metabolism, cardiac-specific overexpression of VEGFB decreased the gene expression of proteins involved in FA metabolism [63]. When considered with a reduction in AMPK phosphorylation seen in these hearts, VEGFB overexpression switches the heart from FA to glucose utilization (Fig. 30.3).

In patients who had suffered an acute myocardial infarction (MI), those that had lower plasma VEGFB prior to discharge exhibited greater left ventricular dysfunction and heart failure six months post-MI [29]. Comparably, hearts from patients with ischemic heart disease or dilated cardiomyopathy undergoing heart transplant displayed reduced VEGFB mRNA compared to donor hearts that were not used for transplant [63]. Experimentally, although knockout of the VEGFB gene has limited consequences when exposed to cerebral ischemia, these mice showed a 40% greater increase in infarct size and severity of brain dysfunction compared to wildtype animals [114]. Additionally, when primary retinal EC and aortic smooth muscle cells isolated from these $VEGFB^{-/-}$ mice were cultured in serum-free medium or under H_2O_2-induced stress, they displayed increased apoptosis, and VEGFB treatment of these cells reduced this effect [114]. Unlike $VEGFB^{-/-}$ animals, cardiomyocyte-specific transgenic overexpression of VEGFB in rats produced significant cardioprotective alterations [63]. VEGFB Tg hearts exhibited physiological hypertrophy with no difference in ejection fraction, fractional shortening or maximal exercise capability. Intriguingly, exposing these Tg animals to MI revealed a less severe decrease in ejection fraction and fractional shortening at both 1 and 4 weeks post-MI. Furthermore, post-mortem analysis confirmed a substantial decrease in infarct size in Tg hearts from both male and female rats [63]. Lastly, given as recombinant protein or expressed via viral vectors, VEGFB has demonstrated significant cardioprotection against H_2O_2-induced oxidative stress [67], transverse aortic constriction [51], chronic left ventricular pacing via an external pacemaker [96], doxorubicin-induced cardiomyopathy [102], and hypoxia/reoxygenation stimulated apoptosis [142]. Collectively, evidence from clinical and animal studies

have acknowledged a favorable role for VEGFB in cell survival. It is possible that these favorable effects of VEGFB are related to potential antioxidant properties of this growth factor [11]. VEGFB is shown to increase multiple essential antioxidant genes including Gpx1, Sod1, Prdx5, Prdx6-rs1, Txnrd3, Sod2, and Gpx5. Additionally, it also reduces oxidative stress genes like Ptgs1, Nox4, and Ncf2, and thus ROS generation.

As outlined above, although the roles of VEGFB to increase blood vessel size, indirectly promote angiogenesis, regulate energy metabolism and support cell survival are emerging topics (Fig. 30.3), its function (or lack thereof) during diabetes has yet to be clearly established [67]. In the diabetic heart which exhibits increased microangiopathy (damage to the small arteries and capillaries [5, 50, 66]) and cardiomyocyte demise [18, 23, 43], hallmark conditions associated with this disease, a decline in VEGFB may well bear some responsibility for these effects. Indeed, our recent study indicated that following streptozotocin (STZ)-induced T1D in rats, a well-established model of DCM [37, 57], analysis of cardiomyocyte VEGFB mRNA and protein expression revealed a robust decrease in the production of this growth factor, together with blunted VEGFR1 signaling [67]. Loss of VEGFB and its downstream receptor signaling was an early event after hyperglycemia was sustained with disease progression, and suggested for the first time that ineffective biological functioning of VEGFB may contribute towards the progression of DCM [67].

Given this unique ability of the myocyte cell surface HSPG to capture and retain LPL, VEGFA and VEGFB, unique proteins that work together to provide but also protect the heart against excessive FA utilization, detachment of these proteins is a prerequisite for their function, and is likely mediated by enzymatic cleavage of cardiomyocyte HSPG by heparanase.

30.6 Heparanase

HSPG is comprised of a proteoglycan core protein with attached linear polysaccharide heparan sulfate (HS) side chains at the cell surface and in the extracellular matrix [55]. They function not only as structural proteins but also as anchors that act electrostatically to bind several bioactive molecules [54], like LPL and growth factors such as VEGFs [70]. As indicated earlier, localization of these functional proteins on this HSPG binding site offers the cell a readily releasable pool, in lieu of *de novo* synthesis when the proteins are required. Heparanase 1 (hitherto referred to as heparanase), with its exceptional ability to degrade HS [14], is capable of releasing and coordinating the functions of LPL, VEGFA and VEGFB, to regulate cardiac metabolism. It should be noted that heparanase 2 (Hpa-2), a novel heparanase homolog which lacks enzymatic activity, also exists and inhibits heparanase [72].

30.6.1 Heparanase 1

Heparanase is an endoglycosidase that is synthesized as an inactive, latent (L-Hep, 65 kDa) enzyme that undergoes cellular secretion [2]. Following its interaction with HSPG, there is rapid internalization of this complex, accumulation in endosomes and fusion to lysosomes [36, 45]. After undergoing proteolytic cleavage in lysosomes, a 50 kDa polypeptide is formed that is at least 100-fold more active (A-Hep) than L-Hep [3, 97]. Within the acidic compartment of lysosomes, A-Hep is stored until mobilized (Fig. 30.4). In the presence of high glucose (HG), we reported a redistribution of lysosomal heparanase from a perinuclear location towards the plasma membrane of EC, together with elevated secretion into the medium [127]. In addition, we determined that ATP release, purinergic receptor activation, cortical actin disassembly, and stress actin formation were essential for HG-induced A-Hep secretion [126]. Unlike HG, high FA provokes nuclear translocation of heparanase in EC, reducing its secretion [128] (Fig. 30.4).

In normal physiology, heparanase has a function in embryonic morphogenesis, wound healing and hair growth [141]. However, research from our lab has also suggested an additional function of heparanase in regulating cardiac FA utilization

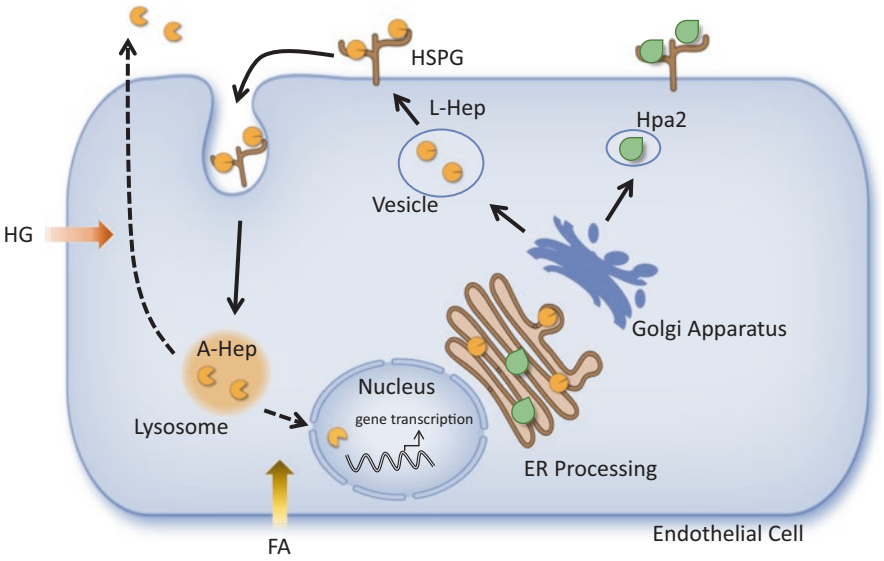

Fig. 30.4 Heparanase processing in endothelial cells. In the EC, inactive heparanase 1 (latent heparanase; L-Hep) and heparanase 2 (Hpa2) are first processed in the endoplasmic reticulum (ER), shuttled to the Golgi and subsequently secreted from vesicles to the cell surface. Once secreted, heparanase and Hpa2 rapidly interacts with cell membrane HSPGs. L-Hep undergoes endocytosis and conversion into active heparanase (A-Hep) by proteolytic processing in the lysosome, while Hpa2 remains stabilized on the cell membrane bound to HSPGs. From the lysosome, high glucose (HG) stimulates the secretion of A-Hep, whereas high FA promotes translocation of A-Hep into the nucleus to affect gene transcription

[130]. In cancer, degradation of HS chains by the increased expression of heparanase is associated with extracellular matrix and basement membrane disruption [32]. The loss of this physical barrier facilitates tumor cell invasion [120].

In the heart, heparanase-induced disruption of HSPG would allow for LPL displacement, its onward movement to the vascular lumen and provision of LPL-derived FA, a key energy source for the heart [9]. Also, we found that L-Hep generates signals in myocytes to reload LPL from an intracellular pool, to replenish that surface LPL released by A-Hep [130]. In the heart, cardiac myocytes are also a major source of VEGFA [26, 143] and VEGFB [67], which can be captured by myocyte cell surface HSPG following their secretion. Intriguingly, our study confirmed that it was L-Hep rather than A-Hep that acted as an effective stimulus for releasing VEGFs from this unique cell surface pool [143]. Overall, our studies demonstrate a unique responsibility of heparanase in cardiac metabolism by releasing myocyte i) LPL for forward movement to the vascular lumen to provide the heart with FA, ii) VEGFA to promote angiogenesis that improves delivery of O_2 and insulin, and iii) VEGFB to modulate energy substrate utilization and inhibit cell death. Our data also suggest that following its secretion from EC and uptake into cardiomyocytes, heparanase can promote cell survival which could be uniquely beneficial to the heart by providing protection against cellular stresses, like diabetes [125].

30.6.2 Heparanase 2 (Hpa-2)

McKenzie and colleagues reported the cloning of a heparanase homolog termed Hpa2 [82]. Even though Hpa2 shares ~40% homology with heparanase, it lacks enzymatic HS-degrading activity typical of heparanase, since Hpa2 does not undergo proteolytic processing in the lysosome, given that the linker region and cleavage site are not well conserved [72]. Alternative splicing of the HPSE2 gene creates three variants, including full-length Hpa2c (592 aa), Hpa2a (480 aa) and Hpa2b (534 aa). Notably, only Hpa2c is secreted and able to interact with heparin and cell surface HS, due to the lack of extra glycosylation sites in the other isoforms. Intriguingly, Hpa2c physically interacts with heparanase and displays even higher affinity towards heparin/HS, resulting in competition for HS binding and interference with heparanase enzymatic activity. Additionally, in the presence of exogenous Hpa2c, there is inhibition of heparanase uptake as evidenced by reduced L-Hep and A-Hep level in the lysate samples. Also, immunofluorescent staining indicates that exogenously added Hpa2 co-localized with cell surface HSPGs. Unlike heparanase, on binding to HSPG, rather than rapid internalization, Hpa2c remains on the cell surface (> 4 h) before degradation or shedding [72] (Fig. 30.4). Interestingly, tumor cells that overexpress Hpa2 exhibit a marked decrease in lymphatic and blood vessel densities mediated by reduced expression of ld-1, VEGFA and VEGFC [131]. Independent of its heparanase-inhibiting activity, studies also implicate Hpa2 in regulation of gene expression, promotion of tissue fibrosis, cell differentiation, DNA repair and ER stress [15, 122, 131].

Given the role of EC heparanase in communicating with the underlying cardiomyocyte, any perturbation in the molecular crosstalk between these two cells is expected to initiate metabolic dysfunction and heart diseases.

30.7 Aberrant Fuel Utilization Following Diabetes

Following diabetes, glucose utilization is impaired, so the heart is compelled to use FA almost exclusively for ATP generation [9, 77]. To restore metabolic equilibrium and prevent cardiac damage, it is important that the relationship between LPL, VEGFA and VEGFB, and their modulation by heparanase is maintained to prevent or delay heart dysfunction seen during diabetes. In diabetes, the use of GL as an energy substrate is impaired at multiple steps. This includes a reduction in the gene and protein expression of GLUT4, an impairment in the ability of insulin to promote translocation of GLUT4 from its intracellular localization to the plasma membrane [4, 9], and an inhibition of PDH activity which results in the uncoupling of glycolysis from GL oxidation [9, 101]. Under these conditions, the heart is obliged to use FA almost exclusively for ATP. One of the adaptive mechanisms for the cardiomyocyte to meet this increased FA demand is to deploy LPL to the vascular lumen, which allows LPL-derived FA to be supplied to the myocytes [104]. This idea is supported by our studies that demonstrate that following moderate diabetes with hyperglycemia when circulating albumin-bound FA or TG have yet to increase, high glucose causes the release of heparanase from EC [127]. This released heparanase can facilitate LPL detachment from the cardiomyocyte cell surface for onward movement to the vascular lumen [126, 130]. This promotes a robust expansion of heparin-releasable LPL at the coronary vascular lumen. The increase in LPL at this location was immediate and unrelated to gene expression in cardiomyocytes, given that total LPL protein remained unchanged in the diabetic heart [99, 107]. Interestingly, hyperglycemia simply encourages rapid filling of all of the unoccupied EC surface HSPG attachment sites with LPL that had moved from the cardiomyocyte cell surface to the vascular lumen [98]. To replenish the enzyme that had moved from cardiomyocyte, we showed exaggerated LPL processing in the cardiomyocyte that involved a head-to-tail dimerization which is prerequisite for ensuing enzymatic activity and secretion [44, 129]. Although this adaptation might be profitable in the short-term to compensate for limited glucose utilization, it is potentially catastrophic over a protracted duration. That is because regrettably, excessive FA utilization contributes to ROS generation whose malicious effects have been implicated in cell death [121, 134]. Additionally, given that mitochondrial metabolism of FA requires more oxygen than glucose [61], and DCM is characterized by decreased capillary density and impaired myocardial perfusion (the diabetic heart exhibits increased microangiopathy - damage to small arteries and capillaries [5, 50, 66]), there is incomplete FA oxidation, storage as TG and resultant lipotoxicity [17, 62, 119, 132].

Compelled to use FA, the diabetic heart would need mechanisms to overcome this mismatch between FA delivery and O_2 provision. VEGFA-derived blood vessel formation with the assistance of VEGFB is a valuable asset allowing for complete FA oxidation and prevention of their storage as TG. It should be noted that in addition to sensitizing the heart to VEGFA action, VEGFB is also known to turn on genes protective against cell death [74], a function that could help towards vascular and physiological cardiac hypertrophy [63].

With chronic or severe diabetes, heparanase action is severely impeded through multiple mechanisms that are largely affected by the high FA seen under this condition [128]. These include (a) decreased production in the heart [67], (b) an increase in lysosomal permeability and incomplete activation of heparanase, (c) nuclear translocation in EC with reduced secretion from this cell, and (d) impaired glucose metabolism in EC, a likely reason for the microangiopathy seen in the diabetic heart [128]. With this impaired function of heparanase, there is a failure to release VEGFA and VEGFB from the cardiomyocyte, which also has a significant impact on the vasculature followed by insufficient oxygen delivery. In the diabetic heart, when oxygen delivery cannot match the augmented FA uptake and hence utilization, and when FA supply exceeds the mitochondrial oxidative capacity of cardiomyocytes, FA is stored as TG. Under these conditions, TG synthesis pathways are overwhelmed leading to the generation of toxic metabolites, including ceramides, diacylglycerols, and acylcarnitines (lipotoxicity). It should be noted that electrons derived from excessive FA utilization, when coupled to reduced antioxidant activity, results in ROS production and decreased efficiency of ATP production [117]. Increased ROS production is also a by-product, resulting in oxidative damage to the cellular components. Following diabetes, compromised glucose utilization also leads to rerouting of this substrate into accumulation as glycogen resulting in cardiac structural and physiological impairments [106]. Intracellular enlargement of glucose and its metabolites also potentiate O-linked protein glycosylation and interfere with protein functionality (glucotoxicity) [139]. Collectively, this imbalance of substrate utilization by the diabetic heart provokes cardiomyocyte death (glucolipotoxicity), which can contribute to contractile dysfunction and a potential causative factor towards the development of DCM.

30.8 Conclusion

Following diabetes, the heart shifts to predominantly using FA for ATP. Heparanase, with a repertoire of functions, can be released from EC in response to HG. It affects myocyte metabolism and does so by interacting with its partners, LPL and VEGFs, amplifying FA delivery and utilization by the diabetic heart. When the supply of FA exceeds the flexibility of the heart to utilize this excess FA, it produces a dramatic change in cardiac gene expression largely related to TG storage, oxidative stress,

and cell death, leading to the development of a cardiomyopathy. Accordingly, treatment of this cardiac syndrome will require a rethinking of our therapeutic strategies, from a focus on controlling blood GL to re-establishing normal cardiac metabolism. Gaining more insight into the biology of heparanase during diabetes, together with an understanding of how this "band of proteins" (heparanase-LPL-VEGFs) cooperates in the diabetic heart, may assist in devising novel therapeutic strategies to restore metabolic equilibrium, curb lipotoxicity, and help prevent or delay heart dysfunction seen during diabetes.

Conflict of Interest None declared.

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Chapter 31
The Lacritin-Syndecan-1-Heparanase Axis in Dry Eye Disease

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31.1 Introduction

A translation of the 300 BC Salt 825 papyrus reads 'The god Re wept and tears from his eyes fell on the ground and turned into a bee' [1]. In another myth, Re's tears turned into a man [1]. Today, we appreciate tears for their remarkable importance in ocular surface homeostasis. Loss of homeostasis associated with acute or chronic tear deficiency and/or instability is known as 'dry eye disease,' affecting 5–7% of the world's population including 30% of the elderly [2, 3]. Little is known of its biological basis with most attention paid to downstream inflammation.

Tears constitute a thin film of at least 1800 different extracellular proteins and numerous species of lipids [4, 5]. In this chapter, we review an effort to address the biological basis of dry eye via an approach that began as an unbiased biochemical screen and led to the discovery of homeostasis-restorative 'lacritin'[6], a tear protein whose active form is selectively deficient in dry eye [5]. Lacritin targets cells via a heparanase-dependent syndecan-1- receptor complex [7]. Discovery of the lacritin-syndecan-heparanase axis brings new insight to the biology of the eye, and also potentially to the homeostasis of other organs and their diseases with lacritin peptides now detected in plasma, cerebral spinal fluid and urine [8–10].

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31.2 The Approach

31.2.1 Discovery of Lacritin

One can think of the surface of the eye as unique in multiple ways. It is arguably the most environmentally challenged wet epithelium [11], and is inclusive of the most densely innervated epithelium [12] and at the level of the cornea is the only wet epithelium that with underlying stroma is avascular [13]. Further, it is the only wet epithelium where the covering fluid is both sterile [14] and refracts most of entering light for sight [15]. As a model for epithelial and neuronal homeostasis, the surface of the eye is unmatched. Understanding precisely how homeostasis is maintained, and therefore might be restored in dry eye, is a challenge.

Our search began in 1992 using primary cultures of rat lacrimal acinar cells - taking advantage of peroxidase in secretory granules as a simple, yet sensitive enzymatic endpoint in 96 well tear secretion assays. Typically, isolated acinar cells de-differentiate and lose their capacity to respond to secretagogues, a loss we found was suppressed by plating on a 10 mM EDTA extract of Engelbreth-Holm-Swarm sarcoma matrix from mice [16]. This was indicative of a tear secretion supportive activity that appeared attributable in part to a lower molecular weight fraction against which rats were immunized for secretion blocking monoclonal antibodies. The best one was not optimal for affinity purification but gave rise to a candidate N-terminal sequence encoded by a GC-rich oligonucleotide that unfortunately failed in screens of a human lacrimal gland cDNA library. However, we were intrigued by one 'non-specific' but novel cDNA with very selective lacrimal and salivary gland expression that was cDNA cloned to full-length, and then manufactured as a bacterial recombinant protein taking care to ensure lack of contaminating bacterial lipopolysaccharide. Surprisingly, it enhanced acinar cell 'constitutive' but not 'regulated' tear protein secretion in a 1.4–14 nM dose-dependent manner [6]. It also triggered basal tearing without irritation on eyes of normal rabbits (80–4000 nM; [17]) and NOD.Aire$^{-/-}$ dry eye mice (4000 nM; [18]). Basal tearing is the form of tearing deficient in dry eye disease. Further, a semi-purified version from rhesus monkey tears provoked monkey acinar cell tear lipocalin and lactoferrin secretion without suppression by dry eye inflammatory cytokines tumor necrosis factor and interferon-γ [19]. When generated as an 'elastin-like polypeptide' fusion protein for slow release it triggered tear β-hexosaminidase secretion (10, 20 μM) by isolated rabbit lacrimal acinar cells, and tearing after injection (100 μM) into lacrimal glands of NOD dry eye mice [20]. We named this new tear protein 'lacritin' of the gene 'LACRT' on human 12q13 [6]. NCBI currently lists thirty-eight lacritin orthologs including fifteen non-primate orthologs [4] from the orders Carnivora, Chiroptera, Equidae [21], Lagomorpha, Scandentia, and Ursidae - yet none to date from Rodentia. That lacritin is effective on mice [18, 20] and rats (Hirata, Laurie, unpublished) despite lack of apparent endogenous expression implies cross-species conservation of its receptor and signaling elements, and offers potential insight into the evolution of the mammalian ocular surface. All known lacritin functions are summarized in Table 31.1.

Table 31.1 Application site

Lacritin function	Lacrimal gland	Eye	Refs
Tear secretion	Peroxidase secretion by rat lacrimal acinar cells. Lipocalin-1 and lactoferrin secretion by monkey lacrimal acinar cells; no interference by interferon-γ and tumor necrosis factor. β-Hexosaminidase secretion by isolated rabbit lacrimal acinar cells. Tearing by NOD mice after injection into lacrimal glands.	Basal tearing by normal rabbits. Tearing by NOD.Aire$^{-/-}$ dry eye mice (combined basal and reflex tearing since controls and lacritin treated also receive IP pilocarpine).	[6, 17, 18, 20]
Restoration of homeostasis	Diminished number of lacrimal gland lymphocytic foci in NOD.Aire$^{-/-}$ dry eye mice.	Restored corneal barrier function on NOD.Aire$^{-/-}$ dry eye mice eyes. On interferon-γ and tumor necrosis factor stressed human corneal epithelial cells, transiently stimulated autophagy to restore oxidative phosphorylation by mitochondrial fusion. This benefit was lost when C-terminal 25 amino acids were removed ('C-25') from lacritin, or reduced following I98S, F104S, L108S/L109S/F112S or F112S point mutation, or following preincubation in xyloside. Using the same interferon-γ and tumor necrosis factor stressed human corneal epithelial cells, normal human tears rescue, but not normal human tears depleted of lacritin. Dry eye tears do not rescue, in contrast to dry eye tears spiked with lacritin but not lacritin C-25.	[18, 23]
Cell proliferation	Acinar cells from embryonic day 14.5–17.5 mouse lacrimal gland explants (Makarenkova and Laurie, unpublished).	Corneal wound healing of NOD mice. Proliferation of subconfluent human corneal epithelial cells.	[22, 31]

31.2.2 Restoration of Homeostasis

Human lacritin is an N- and O-glycosylated [21] secreted protein of 119 amino acids with signal peptide excised [6]. Its 25 amino acid C-terminus is distinguished by an amphipathic α-helix that is necessary for activity [22, 23] and conserved among orthologs [4]. Truncation generates the negative control 'C-25' [22]. In addition to triggering basal tearing, we began to wonder whether lacritin can act directly

on cells to promote or restore health. Exploration in the context of dry eye inflammatory cytokines interferon-γ and tumor necrosis factor and human tears, initially took advantage of the propensity of the transcription factor FOXO3 to translocate between the nucleus and cytoplasm as a simple marker of cell health. When nuclear, cells are often stressed or dying, whereas the opposite is true when cytoplasmic [24]. FOXO3 was cytoplasmic in human corneal epithelial cells treated with normal human basal tears in the presence of interferon-γ and tumor necrosis factor, reflecting the importance of basal tearing in promoting homeostasis. Surprisingly, this benefit was completely lost when basal tears were immunodepleted of lacritin [23]. One potential weakness of this experiment was that lacritin immunodepletion might have removed lacritin binding proteins responsible for the benefit. We, therefore, tested human dry eye tears for which the active form of lacritin is selectively deficient or even absent [5]. FOXO3 was nuclear in cells treated with dry eye basal tears in the presence of interferon-γ and tumor necrosis factor. Spiking in 10 nM lacritin, but not C-25, was sufficient for FOXO3 cytoplasmic translocation [23], suggesting restoration of health. That respective lacritin immunodepletion and add-back were sufficient to lose or regain human basal tear pro-homeostatic activity implied that no other tear protein seemed to share this property.

How does lacritin restore health? It is well known that FOXO3 as a transcription factor can induce autophagy [25], a self-catabolic process by which damaged proteins and organelles are captured in autophagosomes for lysosomal destruction to in turn restore health [26]. In 2010, Zhao et al. [27] using HCT116 colon and H1299 non-small cell lung cancer cells reported that acetylation of family member FOXO1 with stress is a prerequisite for cytoplasmic ligation of autophagy mediator ATG7 to in turn stimulate autophagy. We wondered whether autophagy might be the mechanism by which lacritin rescued stressed cells, and attempted to replicate their observation. Interferon-γ and tumor necrosis factor were sufficient to promote stress-dependent acetylation of FOXO1, but FOXO1 surprisingly failed to bind ATG7 [23]. We then added 10 nM lacritin or C-25. Lacritin, but not C-25, promoted ligation and subsequent autophagy within minutes [23] suggesting that additional modification - possibly lacritin-dependent phosphorylation of FOXO1 - was necessary. Indeed, lacritin activates the FOXO modifying kinase AKT, and no ligation was observed when lacritin was added in the presence of the AKT inhibitor 'AKTVIII' (Wang, Laurie, unpublished). Further, AKT is constitutively active in HCT116 [28] and H1299 [29] cells. With slightly faster kinetics and different mechanism, lacritin also stimulates autophagy via FOXO3. Here, lacritin- dependent acetylation of FOXO3 was necessary for ligation of upstream autophagy mediator ATG101 [23]. We followed autophagic flux by (i) monitoring conjugation of phosphatidylethanolamine to cytoplasmic LC3-I to form LC3-II in Western blots, and (ii) by loss of the EGFP signal in interferon-γ and tumor necrosis factor stressed human corneal epithelial cells expressing a mCherry/EGFP double tagged LC3 construct. Via both approaches, it was apparent that lacritin (but not C-25) transiently stimulates autophagy within 1–10 min, and that cells return to baseline autophagy just after 24 h [23]. Further, co-expression of the mCherry/EGFP double tagged LC3 construct with toxic huntingtin mutant Htt103Q-mCFP or non-toxic Htt25Q-

mCFP confirmed that the purpose of lacritin-stimulated autophagy is to rid cells of toxic proteins [23]. By doing so, lacritin restored oxidative phosphorylation by elevating mitochondrial 'spare respiratory capacity' through enhanced mitochondrial fusion [23]. Particularly remarkable was the relative speed by which autophagy was transiently accelerated and oxidative phosphorylation restored. It was also apparent that stress was a prerequisite of lacritin-dependent autophagy.

Validation of this activity in animal models offers promise for human dry eye. NOD.Aire$^{-/-}$ dry eye mice suffer from autoimmune disease of multiple different organs, including lacrimal and salivary glands. Like human dry eye, inflammation-associated loss of epithelial junctional complexes exposes the subepithelial stroma to topical fluorescein dye or to the food dye lissamine green as a measure of 'corneal barrier function'[30]. Epithelial crevices develop leading to an irregular corneal surface. Over three weeks of treatment, lacritin (but not C-25) restored corneal barrier function in five of seven NOD.Aire$^{-/-}$ dry eye mice eyes, and diminished the number of lacrimal gland lymphocytic foci, although the total number of infiltrating CD4+ T cells did not change [18]. Similar benefit has been observed in NOD.Aire$^{-/-}$ dry eye mice eyes treated with 19- ('Lacripep™') or 25-amino acid synthetic peptides derived from lacritin's C-terminus [Chen FYT et al., unpublished]. The severity of epithelial defects has led some to approach dry eye as a problem of corneal wound repair. In NOD dry eye mice, an algerbrush- generated 2 mm corneal defect significantly worsens over 12–24 h without treatment. Yet, eyes treated with lacritin elastin-like polypeptide repaired fully by 24 h, and at 12 h matched that of the 24 h repair by a mixture of positive control EGF and bovine pituitary extract [31]. This lacritin mitogenic activity is selective for human corneal epithelial, HEK293 and human salivary ductal (HSG)/HeLa cells. Not responsive were human epidermal (A431), pure HeLa, foreskin fibroblast (H368), fibrosarcoma (HT1080), erythroleukemia (K-562), noninvasive breast carcinoma (MCF7), melanoma (SK-MEL and WM-164), Leydig (TM3), Sertoli (TM3), mouse fibroblasts (NIH3T3) and human glioma (U-1242-MG and U-251-MG) cells [22]. Thus, the tear protein lacritin is capable of multiple functions, all of which are directed to promoting and restoring health on the surface of the eye. Reactivating basal tearing helps the eye recover, but so does transient stimulation of autophagy for restored oxidative phosphorylation, and lacritin's mitogenic activity toward epithelial repair (Table 31.1).

31.3 Cell Surface Targeting: Lacritin-Syndecan-1-Heparanase Axis

Understanding how lacritin targets cells could shed insight on disease onset. Lacritin's low nanomolar health promoting and mitogenic activities gave confidence to the attempted enrichment of biotinylated surface binding protein(s) on lacritin columns, yielding a prominent 190 kDa band identified by mass spectrom-

etry as a multimer of syndecan-1 [32]. Validating lacritin-syndecan-1 pull-downs revealed an unusual affinity for the post-heparitinase/chondroitinase ABC pellet and no affinity for syndecans-2 and -4. This differed from FGF2 in which all three syndecans pulled down equally well, and were distributed in the supernatant digest [32]. The implication, therefore, was that lacritin preferred a largely deglycanated version of syndecan-1 and that the core protein was an essential element in the interaction. Indeed, lacritin purified syndecan-1 presented as a relatively discrete band in contrast to the heterogeneous smear associated with FGF2, and distinct pools of lacritin- versus FGF2-bindable syndecan-1 could be differentiated via sequential pull-downs [32]. Heparitinase was sufficient to switch the affinity of FGF2-bound syndecan-1 to lacritin, in keeping with Sepharose CL-6B gel filtration of $Na_2{}^{35}SO4$-labeled heparan sulfate chains from lacritin bound syndecan-1 predominantly of ~4–5 kDa versus ~40 kDa for FGF2 [32].

Lacritin triggers calcium signaling for mitogenesis within 20 seconds in a pertussis toxin inhibitable manner [22], and FOXO3 acetylation within 1 min [23]. Appreciating that lacritin signaling was much more rapid than, and unlike, that usually associated with syndecan-1, we wondered whether syndecan-1 was capable of mediating lacritin function. To examine this, competition and siRNA knockdown studies were coupled with cell proliferation assays. The bacterial recombinant syndecan-1 ectodomain construct HS1ED (as soluble competitive inhibitor), and siRNA knockdown of syndecan-1 (but not syndecan-2) each abrogated lacritin-dependent 'human salivary gland'/HeLa cell proliferation in a dose-dependent manner [32]. Thus, syndecan-1 is essential and likely can pair with a $G\alpha_i$ or $G\alpha_o$ coupled receptor(s), per the inhibitory capacity of pertussis toxin.

Deglycanated syndecan-1 is unstable [33] and not detectable as an immature, intracellular form [32]. Since heparanase is expressed by corneal epithelial cells [34] and detectable in tears (Romano, Laurie, unpublished), the involvement of heparanase was explored by siRNA in 'human salivary gland'/HeLa cells. siRNA knockdown of heparanase, but not heparanase 2 mRNA erased lacritin-dependent cell proliferation in a dose-dependent manner that was rescued by addition of exogenous heparitinase or heparin-purified heparanase [32]. Thus, heparanase serves as an 'on-switch' for lacritin-syndecan-1 ligation (Fig. 31.1). Although secretion of active heparanase is ATP-dependent [35] in a 25 mM glucose-dependent manner [36, 37], regulation of heparanase activity in the context of the lacritin-syndecan-1-heparanase axis has not yet been explored.

With the affinity of lacritin for syndecan-1 heparanase-regulatable, further attention was paid to the mutual ligation site. Truncation analysis narrowed lacritin binding to syndecan-1's fifty N-terminal amino acids [32], with further focus on N-terminal amino acids 20–30 inclusive of two heparan sulfate substitution sites and the hydrophobic sequence 'GAGAL' [7]. The corresponding sequences in syndecans-2 and -4 are respectively GADED and GDLDD which are less hydrophobic by the Kyte & Doolittle scale. Swapping GAGAL out for 'GADED' or 'GDLDD' in syndecan-1 largely abrogated lacritin binding, indicating that GAGAL is the core protein specifier, as per its conservation among orthologs [7]. We wondered whether α-helicity of lacritin's C-terminal amphipa-

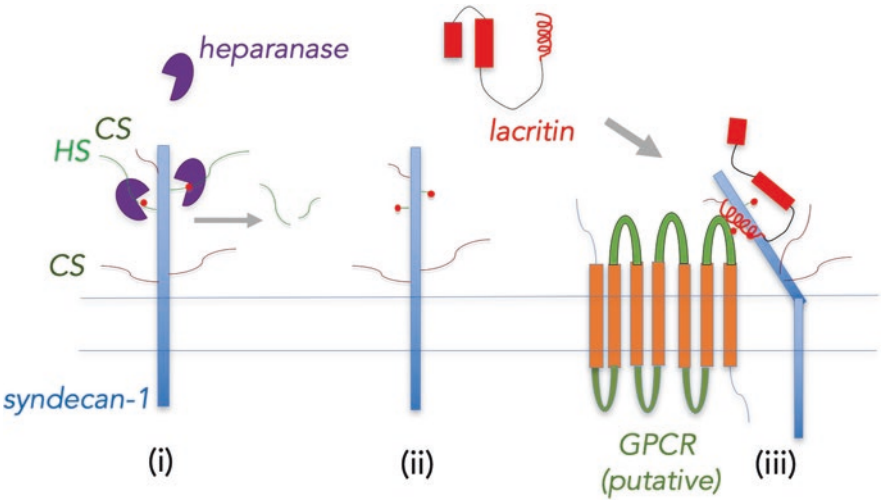

Fig. 31.1 Lacritin-syndecan-1-heparanase axis. (**i**) Heparanase deglycanation of syndecan-1 gives rise to (**ii**) syndecan-1 with heparan sulfate proteoglycan stubs of ~4–5 kDa with 3-O sulfation groups (red circle). A short chondroitin sulfate chain substitutes in place of heparan sulfate on syndecan-1's N-terminus. (**iii**) Lacritin's C-terminal amphipathic α-helix targets the exposed core protein sequence GAGAL, 3-O-sulfation group(s), and the N-terminal short chondroitin sulfate chain. Pertussis toxin inhibits lacritin dependent calcium signaling, suggesting the involvement of a G-protein coupled receptor (GPCR)

thic α-helix might be influenced by GAGAL. As monitored by circular dichroism, this was indeed the case with interaction involving lacritin hydrophobic face residues leucines-108, −109 and phenylalanine-112 whose joint affinity for syndecan-1 was absent after each had been mutated to serine [7]. Other interactions were suggested by loss of affinity of lacritin E103S/K107S and K111S for syndecan-1 [7]. These might interact with a 3-O-sulfation group on the heparanase generated heparan sulfate stub and with a short chondroitin sulfate chain substituted in place of heparan sulfate at syndecan-1's N-terminus, as per the blocking capacity of single chain anti-heparan HS4C3 and chondroitin sulfate IO3H10 antibodies [38] and point mutation of heparan and chondroitin sulfate substitution sites [7]. Thus, heparanase is the 'on-switch' for lacritin targeting of syndecan-1. It exposes two of three elements for ligation: (i) the syndecan-1 specific sequence GAGAL that interacts with the hydrophobic face of lacritin's amphipathic α-helix, and (ii) likely 3-O-sulfation of the heparanase-generated heparan sulfate stub. A third binding element is an N-terminal chondroitin sulfate that is uncommonly substituted in place of heparan sulfate [39]. This requirement would be expected to diminish the availability of syndecan-1's for ligation.

31.4 Clinical: Deficiency or Absence of Active Lacritin Monomer in Dry Eye

When tears are blotted for lacritin, several bands are noted: (i) ~9, 10 and 12 kDa C-terminal fragments, (ii) ~25 kDa monomer and (iii) dimer and trimer of 50 and 75 kDa, respectively or even larger multimers [40]. Dimer, trimer, and multimers develop as a consequence of constitutive tissue transglutaminase cross-linking, largely involving lysines 82 and 85 as donors and glutamine 106 as acceptor [41]. Since glutamine 106 resides in the syndecan-1 binding domain, dimer, trimers, and multimers are incapable of binding syndecan-1 or do so with low efficiency [41], and are inactive (Romano, Laurie, unpublished). Tissue transglutaminase expression is elevated in human dry eye [42], as is transglutaminase 1 in a mouse desiccating stress model of dry eye [43], and is the most likely reason for lacritin monomer deficiency or absence in dry eye. Proteomic analyses have documented selective lacritin deficiency in tears of individuals suffering from aqueous deficient dry eye, aqueous deficient dry eye with meibomian gland disease, blepharitis, climatic droplet keratopathy, contact lens-related dry eye, Fusarium keratitis and primary Sjögren's syndrome dry eye [4]. Its deficiency in primary Sjögren's syndrome tears can be particularly striking [44] McKown, Romano, unpublished]. Thirty-nine other tear proteins (of ~1800) are deficient in dry eye diseases, but none are known to share lacritin's properties. Since lacritin is a basal tearing secretagogue, the absence or deficiency of some tear proteins may be a consequence of the unavailability of lacritin monomer. For example, secretion of lipocalin-1 and lactoferrin is in part lacritin-dependent [19], and deficiency of both has been reported in aqueous deficient and Sjögren's Syndrome dry eye, as well as in meibomian gland disease [5]. To test the hypothesis that dry eye might be in part a lacritin deficiency disease, 'A Double-Masked, Randomized, Multi-Center Phase 2 Study to Evaluate the Efficacy and Safety of Lacripep™ in Subjects with Dry Eye Associated with Primary Sjögren's Syndrome' (NCT03226444) was initiated in the summer of 2017 with full enrollment now complete (results not available at time of writing). 'Lacripep™', a 19-amino acid synthetic peptide representing lacritin's C-terminal amphipathic α-helix appears to be equally active as lacritin, and was tested at two concentrations versus vehicle.

31.5 Concluding Remarks

In an effort to address the biological basis of dry eye, an unbiased biochemical secretion screen was initiated in 1992 that, with considerable serendipity, made possible the discovery of lacritin. As a tear protein that contributes to basal tearing and ocular surface health and yet is selectively deficient in dry eye, lacritin offers a paradigm shift in our appreciation of how homeostasis of the eye surface may be regulated, and disease initiated. Exploration of its cell surface interactions uncovered a

previously unknown heparanase 'on-switch' mechanism by which lacritin targeting of syndecan-1 is dependent, and about which there is much to learn. Although our focus is on the eye where lacritin expression predominates, expression in invasive breast cancer has been suggested [45], and mass spectrometry has detected lacritin C-terminal fragments in plasma, cerebral spinal fluid and urine [8–10]. Thus, the lacritin-heparanase-syndecan-1 axes may have wide relevance.

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Chapter 32
Heparanase, Heparan Sulfate and Viral Infection

Alex Agelidis and Deepak Shukla

32.1 Heparan Sulfate

HS is a major constituent of all mammalian tissues, existing as linear polymeric chains attached to various proteoglycans at the cell surface [1]. A complex biosynthetic process is responsible for the production and modification of HS, which takes place through coordinated action of several glycosyltransferases and sulfotransferases. HS is composed of repeating disaccharide units of glucosamine and uronic acid, with variable additions of sulfate groups and other modifications [2] (Fig. 32.1). As a result of its high sulfation, HS has an extreme negative charge density, and thereby binds with a large variety of extracellular ligands, including growth factors, cytokines, and enzymes [3]. Any disruption of these interactions releases sequestered factors to the local environment, thus engaging their respective receptors and prompting downstream effects. Despite the widespread nature of HS, little is known about how its modifications and cleavage regulate important cellular processes and signal transduction pathways.

HS has been known for many years to serve as a major attachment receptor for a large number of human viruses, including dengue virus, hepatitis C virus, human immunodeficiency virus, human papilloma virus, and essentially all herpes viruses [4–9]. In a seminal study of its kind it was found that a rare modification of HS described as 3-O-sulfation can interact with an essential HSV envelope protein to trigger viral penetration into cells [10]. This expanded the established role of HS

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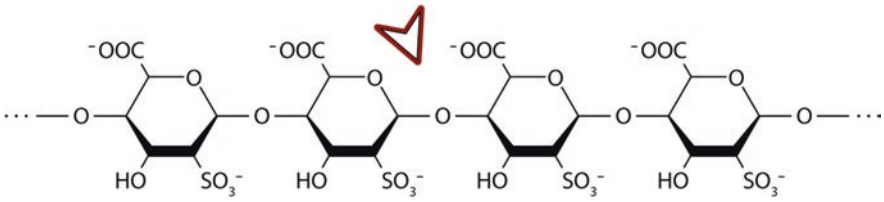

Fig. 32.1 Structure of Heparan Sulfate
Arrowhead indicates HPSE cleavage site

from a viral attachment molecule to an essential receptor required for entry. Viral attachment to the cell surface and entry are the first stages in the process of viral infection, and as such has been the subject of numerous studies aiming to block this initial interaction [11–14].

32.2 Stages of Viral Infection

Much of the early work on HS's important role in virus lifecycles was gleaned from work with herpes simplex virus type 1 (HSV-1), a prototypic double-stranded DNA virus. We understand today that HSV-1 entry into cells is a complex multi-step process involving various viral glycoproteins and host cell receptors. In HSV-1 infection, the initial association with HS is accomplished through the binding of viral glycoproteins B and C. Viral attachment is highest at cellular membrane projections such as filopodia, which express larger amounts of HS and allow for virus transport to the cell body in an actin-dependent process termed viral surfing [15]. Higher expression of the gD receptors nectin-1, herpes virus entry mediator, and 3-O-sulfated HS at the cell body brings the viral fusion complex components into closer association. Modification involving the addition of sulfate groups at the 3-OH position of HS by multiple 3-O-sulfotransferases (3-OST) imparts the ability of this molecule to bind gD, thus allowing viral entry. Pioneering experiments using HSV-1 entry-resistant Chinese hamster ovary cells showed that overexpression of 3-OST-3_B was sufficient to make these cells susceptible to viral entry [10]. It was subsequently found that multiple 3-OSTs are capable of producing this modification and render cells susceptible to infection, with tissue-specific expression and functions [16, 17].

After entry, viral genetic material makes its way into the host cell cytoplasm and finally the nucleus by way of membrane fusion or phagocytosis-like uptake [18]. Upon entry into the cell, viral tegument proteins inhibit normal cellular functions and start the viral replication process. One tegument protein, *vhs*, or virus-host shut-off protein, acts immediately to degrade host messenger RNAs, resulting in a decrease in host protein synthesis [19]. Meanwhile, the viral capsid is transported along microtubules to the nucleus, where it interacts with nuclear pores to uncoat and release its genetic material for replication [20].

Once in the nucleus, viral gene expression begins in a sequential process with transcription of α (immediate early), β (early) and γ (late) genes. The viral tegument protein VP16 is an important initial driver of immediate early gene transcription, and these α genes, namely ICP0, ICP4, ICP22, ICP27, and ICP47, in turn, serve as transcriptional activators of the β genes, which mostly serve in replication of the viral genome. Finally, the γ genes generally encode structural components that subsequently make up the viral capsid and surrounding glycoproteins [21]. Upon successful production of viral progeny and packaging of viral genetic material inside capsids, thousands of new virions undergo processes of primary envelopment and de-envelopment at the nuclear membranes, then tegumentation and secondary envelopment in the cytoplasm and trans-Golgi network, then exocytosis and cell-to-cell spread through interaction with the plasma membrane.

In a lytic infection, thousands of progeny virions are produced, and once the replicated nucleic acids are packaged in their respective protein capsids, they have been observed to leave the cell via various interactions with the cell membrane, or at the latest stages of infection through cell lysis. Given our current stepwise understanding of the viral entry process, relatively little is known about the steps and requirements of viral egress from cells. Recent studies have generated further understanding of this final stage in the viral life cycle with the discovery of HPSE and HS as major regulators of viral release.

32.3 Roles of Heparanase in Viral Infection

Heparanase (HPSE) is an endoglycosidase with the unique ability to degrade HS present at multiple cellular sites, including the extracellular matrix, plasma membrane, and nuclear envelope. Various external conditions and cellular signals have been reported to control HPSE expression and activity, such as hypoxia [22], inflammation [23, 24], oxidative stress [25], and multiple transcription factors [26, 27]. HPSE overexpression has historically been associated with various forms of invasive cancer and other inflammatory pathologies [24, 28, 29]. By breaking down epithelial and endothelial basement membranes, HPSE produces increased vascular permeability and leukocyte extravasation and liberates HS-bound cytokines and growth factors normally sequestered in the ECM, thus promoting angiogenesis and inflammation in the surrounding area [30, 31]. Still, a majority of studies on HPSE have focused on its role in driving cancer, particularly in promoting metastasis through its enzymatic degradation of the extracellular matrix.

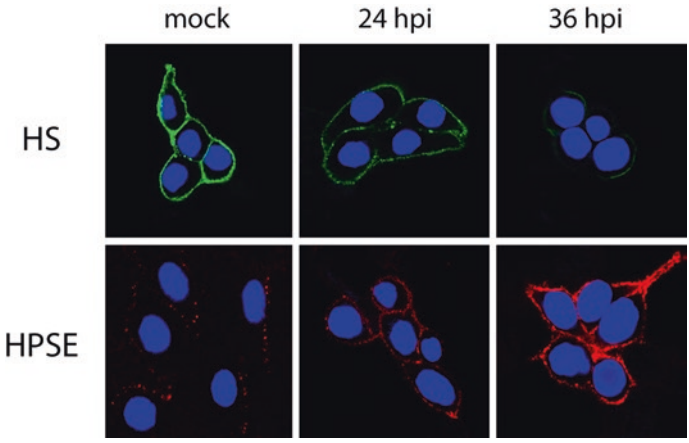

Fig. 32.2 Dynamics of HS and HPSE at the cell surface upon HSV-1 infection
HS is indicated in green, HPSE is indicated in red, DAPI stain for nucleic acid is indicated in blue

32.3.1 Heparanase Is Upregulated upon Infection and Drives Viral Release

The role of HPSE in the viral life cycle was first understood through the examination of HSV-1 viral egress. An initial observation was that HS levels at the cell surface dramatically decrease with progression of infection [32]. Using techniques of flow cytometry and immunofluorescence imaging, it was found that at late times in a productive infection HS was virtually absent from the cell surface. For further explanation of this process, the authors looked to the only known mammalian enzyme capable of this HS-degrading activity. Indeed, the loss in HS was found to occur in concordance with an increase in HPSE expression (Fig. 32.2). In a variety of mammalian cells including human corneal epithelial cells, HeLa cells, mouse embryonic fibroblasts, and Vero cells, Hadigal et al. [32] observed a significant increase in HPSE protein expression. This upregulation of HPSE was observed at the transcriptional level by quantitative PCR and HPSE-promoter luciferase assays. Given that HPSE is known to be produced from a preproprotein, the multiple forms of HPSE protein were analyzed. By western blot, it was apparent that expression of both active (50 kDa) and latent (65 kDa) forms of HPSE were significantly increased in infected cells, with the latent form showing an even more dramatic upregulation in expression. Importantly, Hadigal and colleagues' initial report of HPSE function in viral infection showed that HPSE transcription is similarly upregulated by all other herpesviruses tested, including herpes simplex virus-2, cytomegalovirus, bovine herpesvirus, and pseudorabies virus [32]. It was subsequently demonstrated in detail that HSV-2 infection of a model vaginal epithelial cell line drives transcriptional upregulation of HPSE, and leads to increased HPSE protein expression, thus validating the broader impact of these findings [33].

The observed changes in HS and HPSE expression indicated that some viral factor or cellular response is capable of driving increased expression of HPSE upon HSV infection. Hadigal et al. showed that one such factor from the host side is NF-κB, which is translocated to the nucleus upon infection, binds to the HPSE promoter, and drives transcription of HPSE [32]. Using immunofluorescence microscopy of cells infected with GFP-tagged HSV-1, NF-κB was observed in the nucleus of only the green (infected) cells. Manipulation of the NF-κB system by transfecting a constitutive inhibitor (S32A/S36A IκBα) alongside HPSE-promoter luciferase construct showed inhibition of promoter activity at baseline and with infection, thus proving direct involvement of NF-κB in the regulation of HPSE transcription. Similar results were subsequently reported with HSV-2 infection of vaginal epithelial cells, with nuclear translocation of NF-κB serving as a major mechanism of enhanced HPSE expression [33]. HPSE has been observed to be potently regulated by NF-κB in various other systems [34–36], so this confirmatory finding demonstrated the value of using virus infection as a tool to probe underlying mechanisms of cell biology.

As ocular herpetic infection is a major unresolved cause of infectious blindness, the authors explored the roles of this host protein in the transparent and accessible model system of the mouse cornea. Knockdown of HPSE using shRNA in mouse corneas and in human corneal epithelial cells resulted in significantly decreased viral egress, quantified by plaque assay of ocular washes and cell culture supernatants [32]. Likewise, overexpression of HPSE in these systems resulted in exaggerated viral release. Thus, it was proposed that HPSE behaves as a molecular switch in viral infection, which transforms the cell from a virus-permissive mode in which viral attachment and entry are favored, to a virus-deterring mode which allows for viral detachment and egress (Fig. 32.3). An analogous mechanism exists for influenza virus, which encodes its own neuraminidase enzyme, with the major function of degrading cell surface sialic acid residues to allow for viral detachment and spread to neighboring cells [37]. This viral egress system is the major target of the neuraminidase inhibitors oseltamivir, zanamivir, and peramivir, which have been FDA-approved to treat influenza A and B since 1999 [38]. In the case of herpesviruses, it does not appear that any enzyme capable of cleaving HS chains is encoded in the viral genome; rather upregulation of the host gene HPSE provides this essential function to the virus.

These initial findings constituted the first report of a host cell protein acting as a driver of viral release. Since this initial breakthrough, other investigators have replicated and built upon this work in various viral systems. Guo and colleagues recently observed that porcine reproductive and respiratory system virus (PRRSV) drives a similar increase in HPSE expression in porcine alveolar macrophages, also acting through upregulation of NF-κB [39]. Subsequently, these authors demonstrated that inhibition of PRRSV replication with pyrithione zinc was due to disruption in the levels of NF-κB and HPSE production in cells [40]. Another recent study demonstrated that HPSE also promotes tissue dissemination of vaccinia virus, a DNA virus closely related to smallpox, further demonstrating the key role of HPSE in the spread of HS-binding viruses [41]. Given that PRRSV and vaccinia virus are

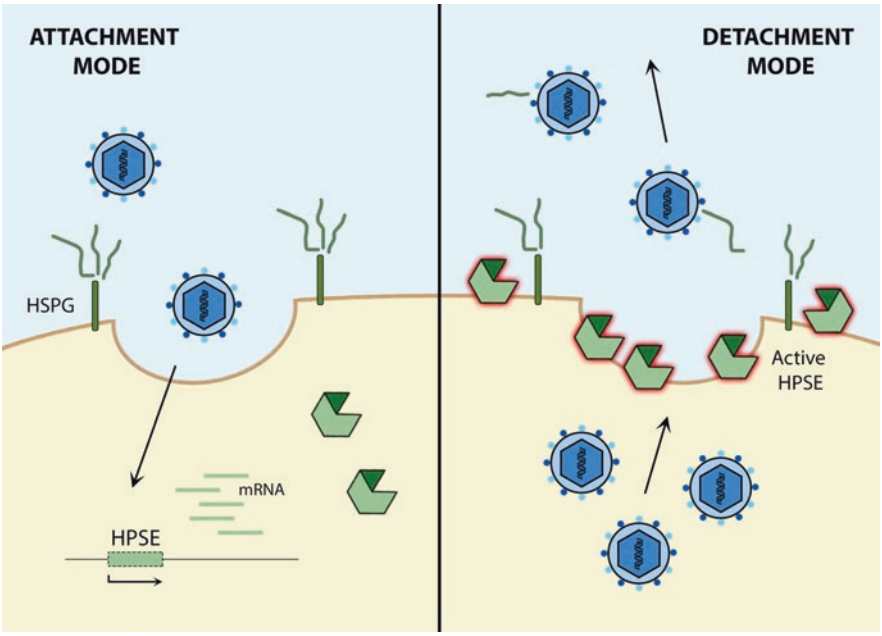

Fig. 32.3 Model of HPSE role in viral detachment and release from cells
Non-infected cells are in a resting attachment mode, where viral particles bind to heparan sulfate proteoglycans (HSPG) to gain entry. After infection, HPSE is transcriptionally upregulated. At the cell surface, HPSE cleaves HS residues to allow viral particles to be released to the extracellular environment or neighboring cells

highly unrelated to herpesviruses, these findings indicate that upregulation and activation of HPSE may be a strategy common to a broad range of viral species to increase spread and transmission.

32.3.2 Active Heparanase Drives Hallmark Features of Viral Pathogenesis

Recent work by Agelidis et al. demonstrated that through multiple mechanisms, active HPSE drives the hallmark processes of viral pathogenesis, with a particular focus on herpetic keratitis given the peculiar immuno-inflammatory nature of this ocular disease [42]. The cornea is generally avascular and lacks circulating red and white blood cells, a feature which is essential for its transparency and ability to transmit light to the retina. Any obstruction of corneal clarity, as occurs with cataract, trauma or inflammatory cellular infiltration, can lead to blindness, and preventing these threats to clear vision remains an important area of active research. Herpetic keratitis has been postulated to result from a loss of immune tolerance,

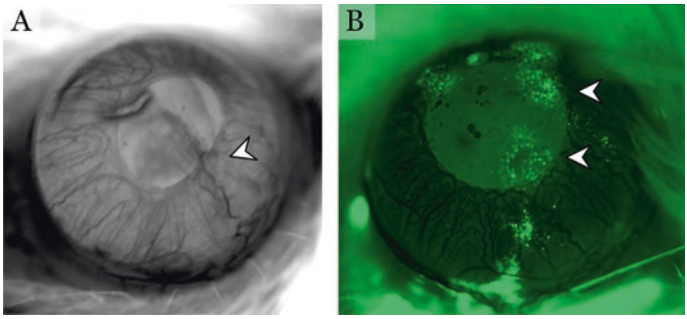

Fig. 32.4 Evaluation of ocular pathology with GS3-HPSE transfection
(**A**) Corneal ulceration and neovascularization in mouse that received corneal transfection of GS3-HPSE and HSV-1 infection. (**B**) Fluorescent virus replication in mouse that received cornea GS3-HPSE and GFP-HSV-1 infection

where some resident molecule causes a breach in the normal barrier between blood and cornea by driving excessive pro-inflammatory signaling.

Using corneal transfection of a previously described constitutively active mutant of HPSE (GS3, triple repeat of Gly-Ser replacing HPSE linker peptide) [43], the authors showed that increased active HPSE in the cornea leads to exaggerated disease upon infection [42]. Disease severity was measured based on the extent of corneal opacity and size of epithelial lesions. Ulceration of the corneal epithelium and neovascularization were dramatically increased with GS3-HPSE treatment (Fig. 32.4). Furthermore, cellular infiltration into the draining (submandibular) lymph nodes of these mice was quantified by flow cytometry at 7 days post-infection and showed increased total cellularity and increased proportions of Gr-1$^+$ neutrophils. These results provided the first indication that active HPSE is an important immunomodulatory factor that is harnessed by the virus to enhance pathogenesis in a tissue environment.

The observed intensified corneal ulceration could be accounted for by defective wound healing; with this in mind, the authors investigated the healing capacity of tissues and cells in the presence of exaggerated HPSE expression. It was observed that corneal tissues and cells exhibited significantly delayed wound healing upon overexpression of active HPSE, in the absence of any infection [42]. Measurement of fluorescein stained tissue damage indicated that non-transfected and empty vector-transfected mouse corneas healed within 24 h of circular wound application. This healing process of the cornea is known to normally occur rapidly as the corneal stem cells at the limbus proliferate to fill in any gap in tissue architecture caused by superficial epithelial abrasion [44]. Meanwhile, corneas that received GS3-HPSE transfection before wounding still showed a cellular defect after 72 h, indicating a significant delay in wound healing. Similar findings were also observed *in vitro*, using a scratch assay method in the absence and presence of HSV-1 infection. A pipette tip was dragged across the surface of a monolayer of cells, and the percentage of original scratch width was quantified over time. As HPSE is well-known for its roles of extracellular matrix turnover, it became clear that overactivity would

account for slower adhesion and migration of cells to fill a cellular void. This appears to be an important mechanism by which active HPSE in the cornea, induced by virus or otherwise, can drive the progression of disease conditions.

Further experiments indicated that the HPSE's role in driving disease likely extends beyond its known enzymatic activity at the cell surface. In the same study, Agelidis et al. documented that HPSE translocates to the nucleus upon HSV-1 infection of corneal epithelial cells and there regulates the localization of transcription factors and downstream signaling molecules. The authors observed increased transcription of pro-inflammatory mediators upon GS3-HPSE transfection, in the absence and presence of infection, which by western blot and immunofluorescence studies they indicated was due to increased translocation of NF-κB to the nucleus. The precise mechanism of these findings remains to be understood, but it is clear that enhanced inflammatory signaling is one method by which HPSE drives pathological changes observed in corneal infection. The authors concluded this study by demonstrating that the use of a documented commercially available HPSE inhibitor, OGT 2115, is effective in decreasing viral spread and pro-inflammatory factor production in response to HSV-1 infection. Transcription of IL-1β, IL-6, and TNF-α was significantly decreased after OGT 2115 treatment, and a reduction of about ten-fold in plaque numbers was achieved. Subsequent work by Hopkins et al. showed that OGT 2115 is also effective against HSV-2 infection *in vitro*. These compelling results suggest that restricting HPSE with OGT 2115 or other known inhibitors may be an effective strategy in a therapeutic or combination therapy against viral infection.

32.4 Roles of HPSE in Pathogenesis Validated across Viral Families

Since the publication of these pioneering discoveries detailing novel roles of HPSE in viral infection, multiple groups have reported similar findings in additional viral systems. Puerta-Guardo et al. demonstrated that HPSE is upregulated by dengue virus to drive increased vascular permeability and pathogenesis. As a member of the *Flaviviridae* family and relative of Zika and West Nile viruses, dengue virus is composed of a small single-stranded RNA genome that encodes 3 structural proteins and 7 non-structural proteins. The virus is transmitted by bite of the *Aedes aegypti* mosquito and is the cause of dengue fever, which is reported by the World Health Organization to have a worldwide incidence of 100 million cases per year [45]. Major symptoms include high fever, muscle and joint pain, vomiting, and diarrhea. Dengue infection can also progress to severe dengue, otherwise known as dengue hemorrhagic fever, which affects half a million individuals per year and has a current mortality rate of 2.5%. Severe infection is unique in that it is characterized by increased vascular permeability, bleeding, and hypovolemic shock. Unfortunately, existing treatments for this infection are intravenous fluids and supportive care, and there remains a critical need for better understanding of the mechanisms of pathogenesis.

With a focus on the integrity of vascular structures, Puerta-Guardo et al. [46] showed that secreted dengue virus nonstructural protein NS1 disrupts the endothelial glycocalyx layer (EGL), resulting in degradation of sialic acid and heparan sulfate. The authors demonstrated that NS1 protein upregulates various extracellular matrix-degrading enzymes, including HPSE and cathepsin L, driving increased fluid leakage characteristic of severe dengue hemorrhagic fever [46]. Additional studies showed that inhibition of the EGL-degrading enzymes sialidase, cathepsin L and heparanase, using a combination therapy of zanamavir, cathepsin L inhibitor and OGT 2115, decreased vascular leakage after NS1 exposure *in vitro* and *in vivo* [47]. It will be interesting to see if an analogous therapeutic inhibiting glycocalyx breakdown can provide similar benefit clinically.

32.5 Conclusions and Future Directions

Microbial infections are complex multifactorial processes requiring consideration of the biological features of the invading pathogen as well as those of the host. While a majority of therapeutics have historically targeted microbial products, we are learning more of the importance of host factors in determining outcomes of infection. Known as major homeostatic regulators of the extracellular environment, HS and HPSE have recently been directly implicated in the pathogenesis of several unrelated viruses. As it remains unclear how HPSE controls such a range of biological processes and pathologies, further analysis of this host protein's role in viral infection will give clues regarding its important binding partners and roles in the regulation of gene expression. Future studies will look to detect levels of HPSE in patients with a goal of understanding its potential value as a biomarker or prognostic of disease. Additionally, it has been postulated that multiple inflammatory disorders, including herpetic keratitis, have an autoimmune component, and that some autoantibody response against a dysregulated host factor may drive exaggerated inflammatory pathways. In keratitis and other disorders, HPSE could be such a target of autoimmune dysfunction. It is likely that HPSE will continue to emerge as a master host-encoded virulence factor that once activated enhances viral spread and triggers downstream inflammatory cascades.

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Chapter 33
Heparanase in the Coagulation System

Yona Nadir

33.1 The Coagulation System

The coagulation cascade is a host defense system that maintains the integrity of the high-pressure closed circulatory system. To prevent excessive blood loss, the hemostatic system, which includes the plasma coagulation system, platelets, and vascular endothelial cells, is recruited. The coagulation system is a cascade of serine proteases. Each coagulation factor activates the following one by proteolytic cleavage. Tissue factor (TF) is a transmembrane protein. It resides on the cell surface of all cells, except endothelial cells and blood cells. In the later, once an injury or activation of these cells occur (i.e., trauma), TF is immediately exposed on the cell surface and together with factor VII, activates factor X. Activated factor X (Xa) further activates prothrombin to thrombin (factor VIIa), that will cleave fibrinogen to form the protein fibrin, as presented in Fig. 33.1. Thrombin will also activate an enhancement pathway of the coagulation cascade named the intrinsic pathway that includes factor XII, XI, IX, and VIII, that can further augment fibrin formation. Additionally, thrombin is the strongest activator of platelets. Thus, once the coagulation system is activated, thrombin is formed and platelets are activated. Activated platelets tend to adhere to fibrin and form platelets aggregates that contribute to the thrombus formation.

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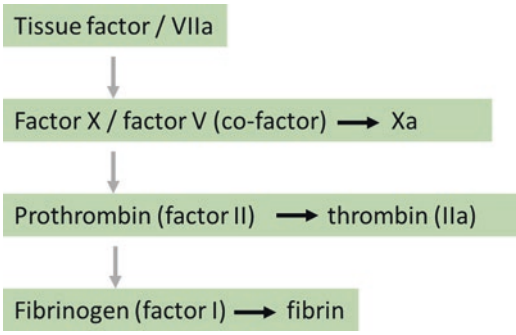

Fig. 33.1 The coagulation cascade**.** The first protein to start activation of the coagulation cascade is tissue factor. The product of the activation is fibrin

33.2 Tissue Factor, Heparanase and Tissue Factor Pathway Inhibitor

Tissue factor (TF), a 47 kD membrane-located glycoprotein, is the main cellular initiator of blood coagulation. It is a high-affinity receptor and cofactor for plasma factor VII and is expressed in most body cells. Under physiological conditions, endothelial cells and circulating blood cells are devoid of measurable TF expression, while extravascular cells at the sub-endothelial layer of the blood vessel wall show constitutive TF expression, forming a hemostatic barrier prepared to activate coagulation when vascular integrity has to be repaired [1]. TF is promptly induced in response to various stimuli such as growth factors (e.g., fibroblast growth factors, platelet-derived growth factor, and vascular endothelial cell growth factor) [2] [3] [4], cytokines (e.g., interferon gamma) [5], hypoxic conditions [6] and endotoxin [7].

Nowadays, it is well recognized that TF has extra biological activities apart from initiating the coagulation system. TF was shown to promote angiogenesis; tumor cells with TF over-expression demonstrate enhanced expression of vascular endothelial cell growth factor (VEGF) [8]. Breast, lung, and colorectal carcinomas that stain positively for TF present high microvessel density and high VEGF levels in correlation to reduced survival [9–11]. These studies point to an association, but not a direct role of TF in angiogenesis. Inactivation of the TF gene results in pathological circulation and disorganization of the vessel wall in embryos, implying that TF has an effect on blood vessel development during embryogenesis [12].

According to our previous results [13], over-expression of heparanase in tumor-derived cell lines resulted in a two-fold increase in TF expression levels, and a similar trend was observed in mice over-expressing heparanase in vivo. Similarly, exogenous addition of heparanase to endothelial or tumor-derived cells induced enhanced TF expression and activity. Remarkably, TF expression also resulted in response to enzymatically inactive heparanase, indicating that this effect was independent of heparanase enzymatic activity. The regulatory effect of heparanase on TF induction involves activation of the p38 intracellular signaling pathway. A positive correlation between heparanase activity and TF expression levels was found in

blasts collected from 22 acute leukemia patients [13]. This positive association indicates that in addition to its well-known function as an enzyme facilitating cell invasion, heparanase also contributes to the regulation of TF gene expression and its related hemostatic pathways.

TFPI, the only known endogenous modulator of the blood coagulation initiator TF, is a plasma Kunitz-type serine protease inhibitor [14]. TFPI concentration in plasma is elevated in acute myocardial infarction patients [15]. There are also studies indicating increased plasma levels of TFPI in relation to diabetes mellitus [16], disseminated intravascular coagulation [17], and other diseases, such as cancer [18, 19] and renal diseases [20]. We have demonstrated [21] that exogenous addition or overexpression of heparanase by transfected cells induces release of TFPI from the cell surface membrane and its accumulation in the cell culture medium. Notably, the *in vitro* studies were reinforced by increased levels of TFPI in the plasma of transgenic mice over-expressing heparanase. Furthermore, elevated TFPI levels have been noted in the plasma of cancer patients [18], reflecting, probably, induction of heparanase expression and elevation of its plasma levels as revealed by ELISA. In tumor cell lines and HUVEC, release of TFPI from the cell surface correlated with enhanced TF activity. This effect was evident already 30 min following heparanase addition, and prior to the induction of TF [13] or TFPI expression. Hence, heparanase augments local coagulation activity by two independent mechanisms: TFPI dissociation from the cell surface [21] and induction of TF expression [13]. Both effects comprise secretion of heparanase, but no enzymatic activity. We proposed that the mechanism involves release of TFPI due to its direct interaction with the secreted heparanase, as clearly evident by co-immunoprecipitation (co-IP) studies using purified recombinant proteins, malignant cell lines lysates, and tissues derived from heparanase overexpressing transgenic mice, reflecting a functional interaction between TFPI and heparanase.

Recently, we revealed that heparanase might serve as a cofactor of TF, indicating that heparanase is directly involved in activation of the coagulation system [22]. Our findings are supported by studies demonstrating that heparanase augments the level of factor Xa in the presence of TF/VIIa complex, an effect which was independent of heparanase enzymatic activity. The newly identified direct involvement of heparanase in the coagulation system was verified by applying three different methods, including a chromogenic assay, western blot analysis, and thromboelastography. Apart from the ability of heparanase to increase Xa levels in normal human plasma, a statistically significant positive correlation was found in patients with acute leukemia vs. healthy donors between the plasma levels of heparanase and Xa. Thus, increased Xa generation in the presence of heparanase was also shown to be relevant in the clinical setting. To clarify the heparanase mechanism of action, we have validated a direct interaction between TF and heparanase, as revealed by co-IP and far-western blot analyses. Heparin was demonstrated to completely obliterate heparanase procoagulant activity most probably through disruption of the TF-heparanase interaction, as demonstrated by co-IP experiments. This finding broadens our knowledge of heparin anticoagulant activity indicating that heparin not only enhances anti-thrombin activity but also inhibits the procoagulant effect of

heparanase. Subsequent to our publication, Baker et al. published that in the absence of vascular injury, wild type and heparanase overexpressing (Hpa-tg) mice had similar times to thrombosis in a laser-induced arterial thrombosis model. However, in the presence of vascular injury or stent insertion, the time to thrombosis was dramatically decreased, and the thrombus size was significantly augmented in Hpa-tg mice. They concluded that heparanase is a dominant mediator of thrombosis in the setting of vascular injury and stent-induced flow disturbance [23].

Tissue factor pathway inhibitor-2 (TFPI-2) is a serine protease inhibitor with inhibitory activity toward TF-VIIa complex, activated factor XI, plasmin, and certain matrix metalloproteinases. As a member of the same Kunitz-type, serine protease inhibitor family similar to TFPI, TFPI-2 contains three tandemly arranged Kunitz domains with a high degree of conservation [24]. Previously we demonstrated [22] that TFPI and TFPI-2 attenuated the effect of heparanase, but even at high doses failed to fully abolish the procoagulant effect of heparanase. Importantly, TFPI-2 was a stronger inhibitor than TFPI in the presence of heparanase. Given the high levels of both heparanase and TFPI-2 in placenta [25–27], a potential regulatory role in placental hemostasis is proposed.

Platelets are an essential part in thrombus formation. We showed that heparanase was released from platelets only upon induction of the activated coagulation protein - thrombin while other platelets activators such as epinephrine, ATP, ADP, ristocetin, collagen, serotonin, arachidonic acid, and lipopolysaccharides (LPS) exerted no such effect. Level of heparanase in a platelet was found to be 40% higher than in a granulocyte. Heparanase released from platelets or granulocytes augmented factor Xa generation by three folds. We propose that the mechanism involves thrombin receptor protease-activated receptor (PAR)-1 activation *via* ERK intracellular pathway that induces heparanase release [28]. Thus heparanase regulates activation of the coagulation system and the thrombin formed during activation further enhances heparanase release from platelets as depicted in (Fig. 33.2).

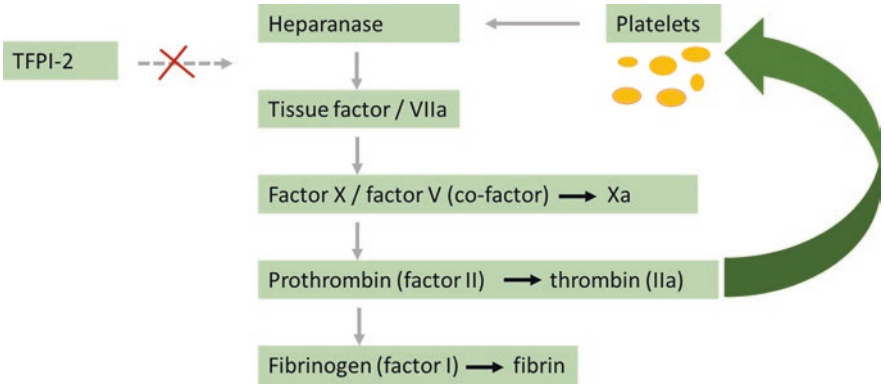

Fig. 33.2 Heparanase activates the coagulation system**.** Once the coagulation cascade is activated and thrombin is formed, platelets release heparanase in response to thrombin, further augmenting the system

Cui et al. shed additional light on the effect of platelet heparanase. They demonstrated that platelets from mice overexpressing heparanase (Hpa-tg) exhibited a much stronger adhesion activity as compared to control platelets, likely contributing to higher thrombotic activity in a carotid thrombosis model[29]. This group also found that in heparanase transgenic mice (Hpa-tg), hematological analysis of blood samples revealed a significantly higher number of platelets in comparison with wild-type mice, while no significant difference between the two groups of mice was found in the number of leukocytes and red blood cell. Concomitantly, megakaryocytes from Hpa-tg mice produced more and shorter heparan sulfate fragments that were shed into the medium. Further, thrombopoietin level was elevated in the liver and plasma of Hpa-tg mice. Together, the data indicate that heparanase expression promotes megakaryopoiesis [30].

The JAK intra-cellular signaling is a common pathway used by many cytokines. The JAK family has four members [31], but JAK-2 is the predominant JAK activated in response to type I cytokine ligands (e.g., granulocyte macrophage–colony-stimulating factor, erythropoietin, thrombopoietin, growth hormone, interleukin-3, interleukin-5, and prolactin) [32, 33]. We reported that JAK-2 activation induced heparanase up-regulation *via* the erythropoietin receptor [34]. By applying erythropoietin receptor positive cell lines, heparanase procoagulant activity and level were shown to be higher in cells transfected to overexpress JAK-2 V617F mutation compared to control, and the effect was reversed using JAK-2 inhibitors (VZ3, Ruxolitinib). Exogenous erythropoietin increased while JAK-2 inhibitors attenuated the heparanase procoagulant activity and level in parental tumor cells. These results point to JAK-2 involvement in heparanase procoagulation enhancement and up-regulation through the erythropoietin receptor.

33.3 Heparanase Inhibitory Peptides and Heparanase Procoagulant Peptides

Previously, we have generated new peptides, at a length of 14 amino acids, derived from the solvent accessible surface of TFPI-2 and found to attenuate heparanase procoagulant activity. These peptides were studied *in vitro* by measuring activated coagulation factor X levels and co-immunoprecipitation. Although the peptides inhibited heparanase procoagulant activity, they did not affect TF activity. Subcutaneous injection of newly identified peptides to mice significantly attenuated activation of the coagulation system and inhibited clot formation in an inferior vena cava thrombosis model. Thus, TFPI-2 first Kunitz domain interrupts the TF/heparanase complex. The newly identified peptides attenuate activation of the coagulation system induced by heparanase without predisposing to significant bleeding tendency [35].

Lately, we identified the domain in the heparanase protein that is involved in activation of the coagulation system. Peptides derived from this area, at the length of 14 amino acids, were demonstrated to increase the generation of factor Xa,

shorten the time to clot formation and increase thrombus strength, while in a mouse model they shortened the bleeding time [36]. The peptides interfere with TF/heparanase complex, as no factor Xa was generated in the absence of TF. Peptides derived from TFPI-2 first Kunitz domain that inhibit the procoagulant activity of heparanase [35] significantly attenuated factor Xa generation induced by peptides derived from heparanase. These procoagulant peptides are well absorbed from the subcutis and are water soluble. In a wound-healing model involving angiogenesis, the wound became more vascular, and its size was reduced to 1/5 compared to controls, upon one week of exposure to the peptides applied topically or injected subcutaneously [36]. Non-hemostatic effects induced by the interaction of TF and heparanase may explain the effect on wound healing.

33.4 Measuring Heparanase Procoagulant Activity

In an attempt to advance the research of heparanase procoagulant activity we generated a chromogenic assay [37]. The novel analysis is based on the already proven presence of TF/heparanase complex and the ability of heparins to completely abolish the complex interaction [22]. The assay provides information on three parameters: TF/heparanase complex, TF, and heparanase activities. The assay is easy to perform; results are available in a short time and are reproducible. Apart from heparanase ELISA measuring heparanase antigen [38] and heparanase enzymatic activity assay evaluating heparan sulfate degradation, no other acceptable assay to measure heparanase in plasma exists. The assay was already verified in six hypercoagulable clinical set-ups including women using oral contraceptives [39], women at delivery [37] patients following orthopedic surgery [40], patients with lung cancer [41], shift work female nurses [42] and patients with diabetic foot [43] all showing significant differences among tested groups. Remarkably, in plasma samples, differences between studied groups were more prominent using the heparanase procoagulant assay compared to the heparanase ELISA assay [38], while the opposite was revealed in cell culter studies applying cell lysates and medium. This observation may be due to more adequate concentrations in the plasma of other coagulation proteins such as TF, VIIa and factor X or due to accumulating released heparan sulfate chains in the medium, affecting the procoagulant assay.

33.5 Pregnancy and Oral Contraceptives Increase Heparanase Procoagulant Activity

Pregnancy is an acquired hypercoagulable state that increases as the pregnancy advances and peaks at the post-partum period. Women with a prior tendency to hypercoagulable state may present with clinical symptoms of placental vascular

complications. Currently, maternal thrombophilia is believed to be the main reason for placental vascular events, although 30–50% of gestational vascular pathologies cannot be explained by the presently available studies for thrombophilia [44]. Thus, the need to study the hemostasis in the placenta, especially the dominant factors that control the delicate hemostatic balance through-out pregnancy is vital. Formerly, heparanase was demonstrated to be expressed in normal and abnormal placentas, in small fetal vessels and in a variety of trophoblasts subpopulations with diverse invasive potentials [45, 46]. High levels of heparanase in the placenta and its involvement in hemostasis and angiogenesis encouraged us to explore the role of heparanase in first-trimester placentas in relation to other hemostatic and angiogenic factors, predominantly, its effect on TF, TFPI, TFPI-2, and vascular endothelial growth factor (VEGF)-A in early pregnancy losses [47]. Twenty formalin embedded placenta samples of abortions (weeks 6–10) were examined using real-time PCR and immunostaining. Ten cases were miscarriages of women with thrombophilia and recurrent fetal losses, and ten control cases were pregnancy terminations of women with normal obstetric history. Sections obtained from miscarriages showed increased (2–three-folds) levels of heparanase, VEGF-A, and TFPI-2 compared to placentas from controls in maternal as well as in fetal placenta elements. JAR (human choriocarcinoma trophoblasts) cells incubated with exogenous recombinant heparanase or overexpressing heparanase exhibited a 2–three-fold increase in TFPI and TFPI-2 in cell lysates both at the protein and mRNA levels, with no detectable effect on VEGF-A and TF levels. Accumulation of TFPI and TFPI-2 in the cell culture medium was increased 4–6-folds, exceeding the observed induction of TFPI and TFPI-2 gene transcription. These results indicate a regulatory role of heparanase on TFPI and TFPI-2 in trophoblasts, suggesting a potential involvement of heparanase in early miscarriages.

In subsequent work, we focused on the levels of heparanase, TF, TFPI, TFPI-2, and VEGF-A in full-term placentas (weeks 36–41) of three groups: cesarean section (CS), vaginal and intrauterine growth restriction (IUGR) - deliveries. Similar to the results studying early pregnancy losses [48], applying immunostaining and RT-PCR, we revealed high levels of heparanase, TFPI-2, and VEGF-A in placentas of vaginal deliveries and IUGR compared to elective CS deliveries. IUGR may be caused by placental vascular dysfunction or by fetal abnormalities. The IUGR babies included in our study had no signs of infection and were morphologically normal, so the most plausible reason for the IUGR was placental vascular insufficiency [49]. Elective CS deliveries represent unstressed placenta condition at the end of the third trimester, while vaginal and IUGR deliveries involve placental ischemia and fetal stress. We may conclude that these changes are typical of vascular stress and may contribute to the induction of labor. Prior to initiation of term labor, the myometrium must become activated. Activation enables the myometrium to be sensitive to contractile stimuli, such as oxytocin and PGs, and to generate synchronous contractions. Critically absent is knowledge about the signals and cellular mechanisms that directly lead to myometrial activation [50]. Heparanase involvement in labor induction is a possible mechanism requiring further research.

The elevated expression levels of TFPI-2 and heparanase in normal pregnancies [27, 45, 46] may imply a key role of these two proteins in placenta development and hemostasis. Increased maternal plasma TFPI-2 levels were found in pregnancies complicated by preeclampsia and intrauterine growth restriction [51]. We have previously demonstrated that heparanase up-regulates TFPI-2 expression in trophoblasts [47]. Therefore, the increased levels of TFPI-2 in the vaginal and IUGR placentas may result from a similar heparanase effect.

Heparanase procoagulant activity was also evaluated in plasma samples of 35 third-trimester pregnant women (weeks 36–41) who were in labor or came for appointed elective cesarean section and 20 control samples which were of non-pregnant healthy women. We found that heparanase procoagulant activity was significantly higher in the plasma of pregnant women compared to non-pregnant. Differences in heparanase procoagulant activity were more prominent than changes in heparanase levels (ELISA), TF activity, factor Xa, thrombin, and free TFPI levels. Hence, heparanase was found to considerably contribute to the procoagulant state in late third-trimester pregnancy and at delivery [37].

The use of oral contraceptives (OC) is a well-established risk factor for venous thrombosis. Evidence on hormonal contraceptive results almost exclusively from observational studies pointing to a 2–six fold elevated risk of venous thromboembolism (VTE) [52]. Acquired protein C resistance ensuing from reduced levels of protein C, protein S, and elevated factor VIII, is the main explanation known to describe the increased risk of venous thromboembolism among users of OC [53]. Formerly, Elkin et al. reported that heparanase expression is upregulated in response to estrogen stimuli [54]. In that study, they indicated four putative estrogen response elements in the heparanase promoter region and demonstrated that transcription of a luciferase reporter gene driven by the heparanase promoter was significantly increased in estrogen-receptor positive MCF-7 human breast carcinoma cells after estrogen treatment. *In vivo*, exposure to estrogen increased the levels of heparanase protein in MCF-7 cells embedded in Matrigel plugs and correlated with enhanced plug vascularization [54]. According to the results mentioned above, in estrogen receptor-positive cells, estrogen increased heparanase procoagulant activity while in the absence of the estrogen receptor, this effect was not observed, supporting an estrogen receptor-dependent activity [55]. These results possibly point to a novel mechanism of hypercoagulability in women using estrogen. To gain clinical applicability to this notion, we studied the plasma of 34 women using OC and compared them to 41 women without hormonal therapy. The results imply a significant elevation in TF/heparanase activity, mostly attributed to heparanase procoagulant activity, although TF activity also increased. Evaluation of heparanase level by ELISA showed no difference between these groups. Remarkably, values were very similar to those published with the group of women at the end of pregnancy [37], consolidating the hormonal effect on heparanase procoagulant activity.

33.6 Cancer and Heparanase Procoagulant Activity

Previously we have studied the cell surface expression of TF in acute leukemia cells. We found a significant positive correlation between TF surface expression in blast cells collected from 22 acute leukemia patients with their heparanase expression levels and enzymatic activity [13], indicating a mutual effect on coagulation system activation. When plasma samples of 35 patients with acute leukemia at presentation and 20 healthy donors were studied for heparanase and factor Xa levels, a significant positive correlation was shown between plasma heparanase and factor Xa levels [22], pointing to the ability of heparanase to activate the coagulation system in cancer patients.

Increased procoagulant activity of heparanase was also studied in patients with lung cancer [56]. Plasma samples of 65 patients with non-small cell lung cancer at presentation and 20 controls were analyzed. Heparanase antigen levels were elevated in the study group compared to control. TF/heparanase activity, and even more apparent, heparanase procoagulant activity were significantly higher in the study group compared to controls. Survival of patients with heparanase procoagulant activity higher than a cut-off level of 31 ng/ml predicted a mean survival of 9 ± 1.3 months while heparanase procoagulant activity of 31 ng/ml or lower predicted a mean survival of 24 ± 4 months. Heparanase procoagulant activity was higher than 31 ng/ml in the four cases of thrombotic events detected during the follow-up period [56]. We concluded that elevated heparanase procoagulant activity in patients with lung cancer discloses a novel mechanism of coagulation system activation in cancer and that heparanase procoagulant activity can serve as a predictor for survival.

Involvement of the procoagulant domain of heparanase in tumor growth was suggested by our work using TFPI-2 derived peptides to inhibit tumor growth [57]. In that study, in three tumor cell lines, peptides inhibited tumor growth and vascularization in a dose-dependent manner, reaching a 66% reduction compared to control tumors. Furthermore, a survival advantage and decreased plasma activation of the coagulation system were observed in the treatment groups. Additionally, peptides delayed tumor relapse by six days and inhibited relapsed tumor size. *In vitro*, peptides did not inhibit heparanase degradation of heparan sulfate chains, tumor cell proliferation and migration, but significantly attenuated tube formation [57]. These results indicate that the procoagulant domain in the heparanase protein may have an effect on tumor growth, suggesting a new pathway for involvement of the coagulation system in cancer.

33.7 Additional Clinical Data Supporting the Procoagulant Effect of Heparanase

Hu et al. studied 33 patients with retinal vein occlusion and 28 control subjects. Serum heparanase concentration was studied by ELISA and serum heparanase enzymatic activity was evaluated using a heparan sulfate degrading enzyme assay kit. They found that patients had significantly higher levels of heparanase protein and enzymatic activity. This study provided a correlation between systemic heparanase protein levels and activity with a thrombotic clinical manifestation of the retinal vein [58].

Bayam *et al* enrolled 79 patients with thrombotic prosthetic heart valve who received heparin and 82 controls. Plasma samples collected from patients both at baseline and after heparin treatment and from controls at baseline only were analyzed for heparanase levels by ELISA. In the prosthetic heart valve group, 18 patients had thrombus obstructing the valves and 61 patients had non-obstructive valves, receiving heparin infusions for a median duration of 15 [7–20] days. Baseline and post-heparin heparanase levels were significantly elevated in patients with a thrombus area $\geq$ 1 cm [2] and with a recent history of a thrombotic event. The authors concluded that increased heparanase levels might be associated with a high risk of thromboembolism and increased thrombus burden in patients with prosthetic valves [59].

Increased expression of the tumor suppressor protein 53 (p53) has been implicated in vascular senescence [60]. Interestingly, Bochenek et al. reported elevated endothelial Egr1 and heparanase expression after doxorubicin-induced p53 overexpression, whereas p53 inhibition using pifithrin-α reduced TF expression. Importantly, inhibition of heparanase activity using our TFPI-2 derived peptides attenuated the increased venous thrombus formation in aged mice and restored it to the thrombotic phenotype of adult mice. These findings indicate that p53 accumulation and heparanase overexpression in senescent endothelial cells are substantially involved in mediating the elevated risk of venous thrombosis with age and that heparanase neutralization may be evaluated as an approach to attenuate the prothrombotic endothelial phenotype with age [61].

33.8 Concluding Remarks

Among the multiple effects attributed to heparanase, this protein was revealed to harbor a major role in regulating activation of the coagulation system. Evidence for increased heparanase procoagulant activity in different clinical set-ups was previously demonstrated (Fig. 33.3), encouraging developing drugs to attenuate its effect on the coagulation system. Inhibition of the heparanase interaction with TF also exerts non-hemostatic effects, including attenuation of tumor growth and vascularization [36], posing TF as a potential cell surface receptor to heparanase.

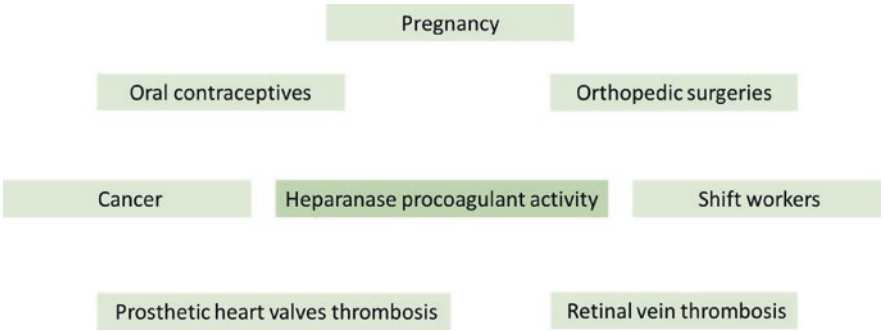

Fig. 33.3 Heparanase procoagulant activity in clinical set-ups**.** Elevated heparanase procoagulant activity was demonstrated in several hypercoagulable clinical set-ups including women using oral contraceptives, women at delivery, patients following orthopedic surgery, patients with diabetic foot, shift work female nurses, patients with lung cancer, retinal vein thrombosis and prosthetic heart valve thrombosis, showing significant differences among tested groups

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Part VII
Heparanase-2 (Hpa2)

Chapter 34
Hpa2 Gene Cloning

Edward McKenzie

34.1 History of Oxford GlycoSciences (OGS)

Oxford GlycoSystems was founded by Professor Raymond Dwek in 1988 and is credited with being the first Oxford University Glycobiology based spin-off company. The fascinating history of the company from its original concept is described in a 2008 Biochemical Society Science and Industry features article [1]. The company at that time pioneered leading technology in the area of 2D gel-based proteomics, generating glycobiology analytical tools and in drug development. In particular,

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Miglustat (OGT 918, *N*-butyl-deoxynojirimycin) was developed to treat type I Gaucher disease (GD1), gained FDA approval in 2003 and is now marketed under the trade name Zavesca [2]. In 1998 Oxford GlycoSystems underwent a name change, whilst still maintaining its OGS acronym, to Oxford GlycoSciences (OGS) to position itself more as a drug discovery focussed company. The company was acquired by Celltech in 2003, which was itself taken over a year later by UCB.

34.2 Discovery of Heparanase 2 (Hpa2/HPSE2)

OGS ran a programme from 1999–2003 to develop small molecule inhibitors of the enzyme Heparanase 1 (Hpa1/HPSE1) primarily as anti-tumor therapeutics. Early success in the development of an in-house insect cell expression system to generate large amounts of the active heterodimeric HPSE1 enzyme [3] allowed high throughput screening of internal sugar based and commercial small molecule libraries [4, 5, 6]. Initial inhibitor hits were simultaneously screened in primary *in vitro* assays against human B-glucuronidase enzyme as a non-specific glycosidase enzyme control. Moving rapidly to animal model testing studies, however, meant that it was becoming increasingly important to demonstrate either way whether homologs of Heparanase 1 existed as these would need to be counter-screened to ensure maximum HPSE1 drug target specificity.

34.2.1 Hpa2/HPSE2 Cloning

By screening an EST database (Incyte) with the HPSE1 protein sequence, three non-overlapping homologous ESTs were identified. A range of PCR oligonucleotide primers was then designed to span across these sequences using a normal mammary gland cDNA library as template. One gene product, originally termed Hpa2 at the time, but now referred to here as HPSE2a, Genbank accession AF282885) with two alternative splice forms; HPSE2b (Genbank accession AF282886) and HPSE2c (Genbank accession AF282887) were identified which encoded for proteins of length 480, 534 and 592 amino acids, respectively. The proteins contain a putative signal peptide/hydrophobic leader sequence, 2 N-linked glycosylation sites (as predicted by the NetNGlyc1.0 programme) for HPSE2a & HPSE2b (N_{237} and N_{254}) and 4 N-linked glycosylation sites for the HPSE2c isoform (N_{237}, N_{254}, $N_{275,}$ and N_{392}) [7]. As described later the more extensive glycosylation on isoform HPSE2c may account for the fact that this is the sole secreted form as the other two splice forms are retained within the cell when overexpressed in mammalian cells [8]. Interestingly a putative forth HPSE2 family member called isoform X3 of 538 amino acids has been deposited at Genbank (XP_011538332.1) but no other species homologs matching this has been described (Fig. 34.1). This fourth

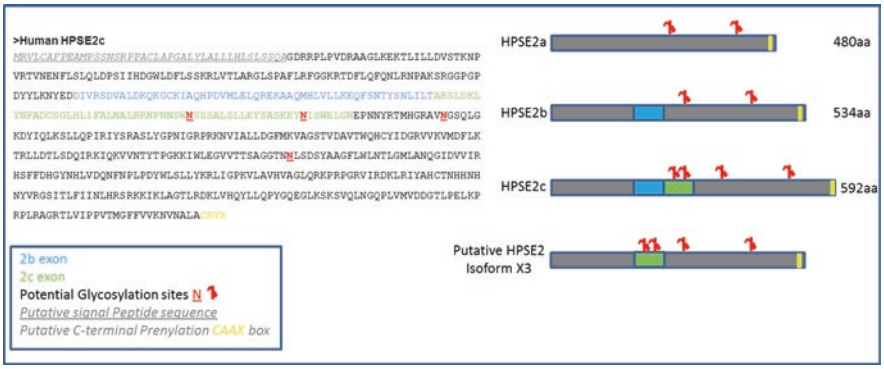

Fig. 34.1 Heparanase 2c splice form protein sequence and a schematic line up of all splice forms

form has also been reported by Vreys and David [9]. A CAAX box farnesylation site is predicted, to a high significance level, at the extreme HPSE2 C-terminus (using the PrePs Prenylation Prediction Site, P = 0.98). This suggests that the HPSE2 isoforms may well be membrane anchored but the relevance of this prediction needs formal testing. Radiation Hybrid mapping studies at OGS subsequently localized the HPSE2 gene to chromosome 10q23–24.

34.2.2 Tissue Distribution

Quantitative PCR analysis comparing HPSE1 vs HPSE2 RNAs was performed over a range of 40 different normal tissue, tumor xenograft and tumor cell line cDNAs (Fig. 34.2). A pan-specific RNA probe (covering HPSE2a nucleotide region 652–1325, downstream from the splice site) which detects the pooled expression of all three splice forms was used in these initial tissue profiling studies. Future studies examining the distribution of each splice form is needed to investigate whether specific HPSE2 forms dominate in particular tissues. HPSE1 and HPSE2 show very different patterns of mRNA distribution (Fig. 34.2). In particular, HPSE1 is restricted to hematopoietic cells and placenta where HPSE2 expression is low. The highest expression of HPSE2 was detected in the mammary gland, prostate, testis, uterus, and brain with very little or no expression in other tissues.

Northern blot analysis using a Full-length HPSE2b probe further confirmed the high expression in smooth muscle containing tissues such as the bladder, small intestine, uterus and prostate (Fig. 34.3a).

The high levels detected in normal bladder was additionally confirmed by *in-situ* hybridization (Fig. 34.3b) and this particular tissue distribution profile was later to be a pivotal linking feature in the etiology of Ochoa/UroFacial Syndrome patients [10, 11]. Immunostaining analysis revealed that HPSE2 was also expressed by normal bladder transitional epithelium [12].

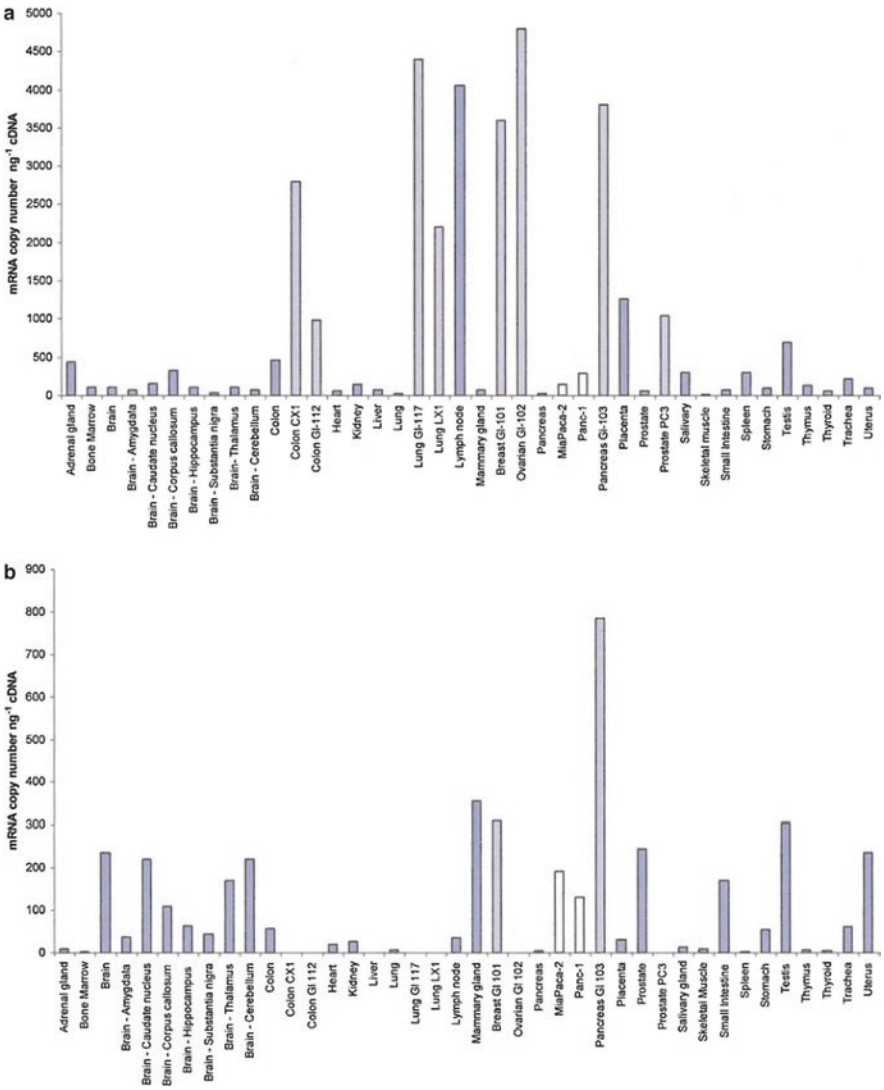

Fig. 34.2 Tissue distribution profile of HPSE1 (**a**) versus HPSE2 (**b**) RNA

A striking level of over-expression was observed in pancreatic Xenograft GI-103 compared to normal pancreas and also in pancreatic MiaPaca-2 & Panc-1 tumor cell lines (Fig. 34.2).

RNA dot-blot analysis of 20 brain sub-regions using a full-length HPSE2b probe showed highest expression in the substantia nigra (blot position A3), caudate nucleus (E2), spinal cord (E3), medulla oblongata (G2), putamen (H2), pons (H1), corpus callosum (C2) and the thalamus (C3). The highest regions of expression

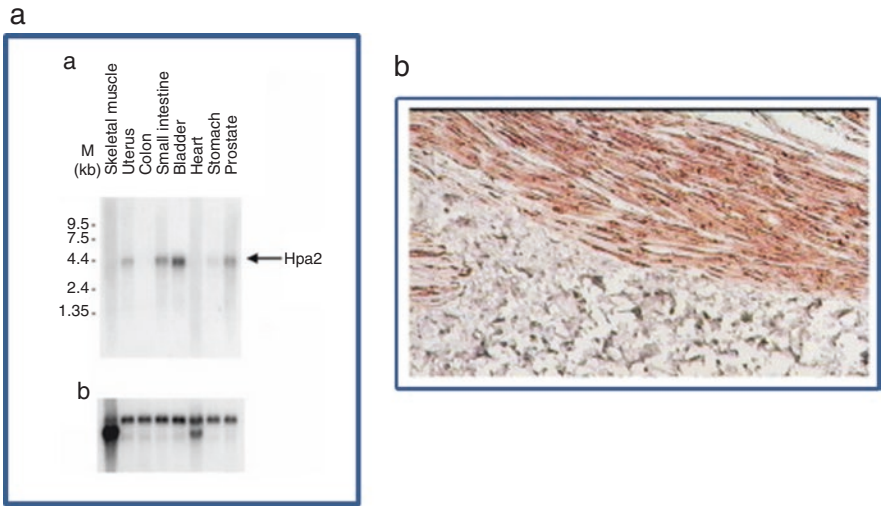

Fig. 34.3 **(a)** Northern Blot RNA Tissue profile analysis of HPSE2. **(b)** Direct *in situ* RT-PCR detection of *HPSE2* mRNA expression in human bladder tissue. Strong brown staining demonstrates expression in longitudinal smooth muscle tissue

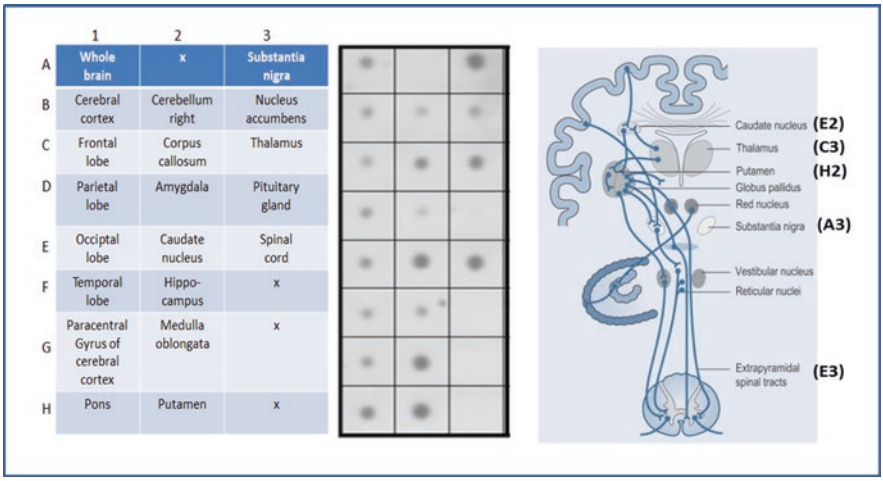

Fig. 34.4 RNA dot blot Brain region distribution of HPSE2 and its mapping to the basal nuclei and motor tracks

when mapped onto a brain region schematic show a strong linkage to the basal nuclei and motor tracks (Fig. 34.4).

Consistent with all the HPSE2 expression analyses carried out so far at the RNA level, a comparable protein profiling and tissue distribution (Fig. 34.5) were obtained using specific anti-HPSE2 antibody for tissue immunostaining. The Human Protein

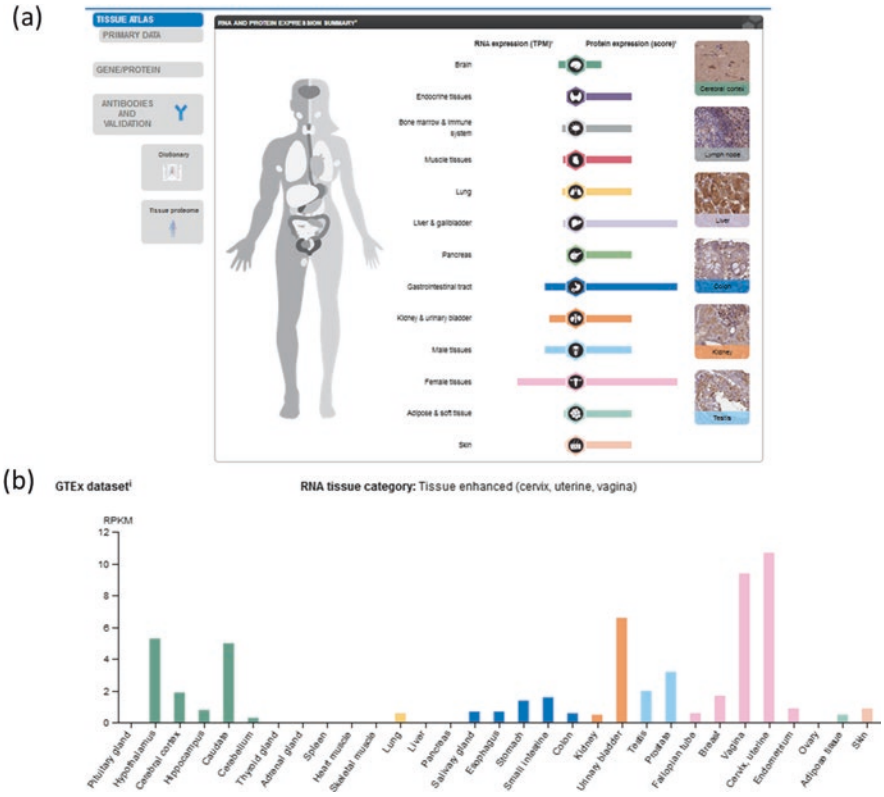

Fig. 34.5 RNA and Human Protein Atlas tissue profile (GTex dataset) for HPSE2

Atlas profile for HPSE2 (https://www.proteinatlas.org/ENSG00000172987-HPSE2/tissue) shows high levels in smooth muscle containing tissues such as the bladder and uterus and in the hypothalamus and caudate brain regions.

34.2.3 Key Amino Acid Features of a Beta-D-Endoglucuronidase Heparanase 1 Enzyme

The longest HPSE2c splice form has the greatest overall homology to HPSE1 at 42% identity. This homology spans over the 8 kDa and 50 kDa HPSE1 subunits but appears to lack a comparable intervening linker region (Ser_{110}-Gln_{157}) which is included in the latent Pro-HPSE1 [13] form. Proteolytic cleavage of the HPSE1 linker region by cathepsin L protease [14, 15] produces the active enzyme by removing the linker peptide blocking the substrate binding site. Expression of HPSE2 in mammalian cells, however so far suggests that the protein is produced as a single polypeptide chain and does not undergo internal cleavage. HPSE1 is related to

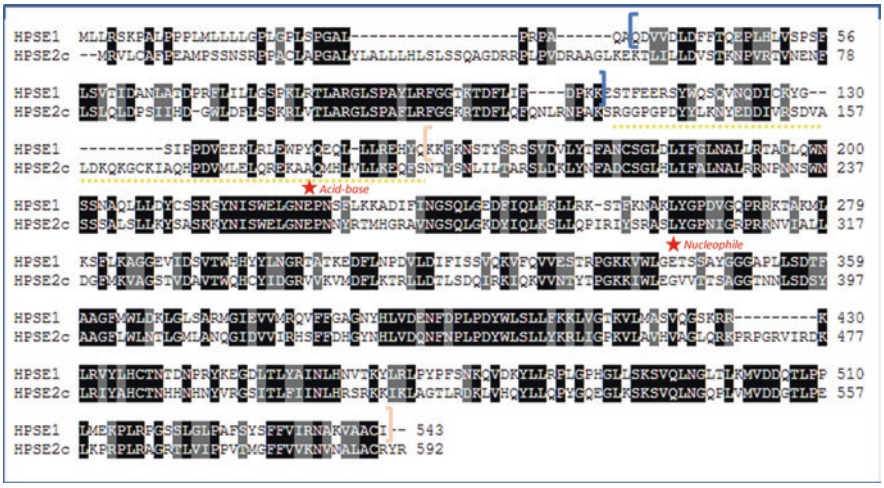

Fig. 34.6 Clustal protein alignment of HPSE1 against HPSE2C. Regions of identity or high similarity are represented by dark blocks. The HPSE1 small 8 kDa subunit region is shown between the blue brackets and the large 50 kDa subunit is shown between the orange brackets. The HPSE1 linker region is highlighted with a yellow dashed line. The key catalytic Acid-base and Nucleophile Glutamic acid residues are marked with a red star

members of the 'clan A' glycosyl hydrolases which all use general acid catalysis based mechanism to hydrolyze glycosidic bonds. Mutagenesis studies [16] identified the critical catalytic amino acid residues of HPSE1 important for cleavage of its HS substrate. These include the Acid-base proton donor E_{225} and nucleophilic E_{343} (Fig. 34.6).

34.2.4 Heparin Binding Sites

Cardin Weintraub Consensus Motif

The basic amino acid patch 1 from protein region K_{158}-D_{171} & patch 2 from region P_{271}-M_{278} within HPSE1 contain the classical XBBXBX and XBBBXXBX consensus sequences (where B = basic residue, X = hydropathic residue) and are critical for interaction with its HS substrate [17]. A third patch region (K_{411}- R_{432}) is also enriched with basic amino acids but shown not to be essential for substrate binding. These patches are not conserved in HPSE2, however; HPSE2 is also heavily enriched with basic amino acids and contains a putative basic patch region (N_{374}-N_{379}) of consensus XBBXBX (where B=Histidine).

By deletion analysis and amino acid mutagenesis (changing hydrophobic amino acids to hydrophilic ones), the hydrophobic C-terminus region of HPSE1 has been shown to be important for intracellular trafficking to the Golgi apparatus, secretion,

activation, and also for tumor cell migration [18]. Similar studies on the C-terminus of HPSE2 have not been carried out yet so it remains to be seen whether this region is also functionally conserved for trafficking and secretion of isoform HPSE2c.

CPC Clip Motif

In addition to the classical Cardin Weintraub motif, a structural signature for heparin-binding proteins has been identified [19]. The motif involves two cationic residues (Arg or Lys) and a polar residue (preferentially Asn, Gln, Thr, Tyr or Ser, more rarely Arg or Lys), with fairly conserved distances between the α carbons and the side chain center of gravity, defining a clip-like structure where heparin would be lodged. This structural motif is highly conserved and can be found in many proteins with reported heparin-binding capacity.

Scanning of the HPSE1 protein sequence reveals 5 such sites [RRK (found in Patch 1), RRK, RKT, RKS, and KRR]. The HPSE2c protein also has five sites throughout its sequence (KKY, RRN, RKN, RKK, KRT). It has already been shown [8] that HPSE2c binds HS with a very high affinity which is consistent with the presence of Cardin-Weintraub and CPC Clip motifs within the protein sequence. It is not known however whether these basic amino acid motifs allow HPSE2c to bind to other negatively charged GAGs.

34.2.5 HPSE2c Structure Homology Model

A high resolution 1.6-A^{o} crystal structure of HPSE1 has been solved [20] and comparison structures of the Apo (or Pro) and active heterodimeric forms revealed that the endo-B-glucuronidase active site cleft is exposed by proteolytic activation of the latent Pro-HPSE1 form. Oligosaccharides were complexed into the structure to help map the substrate binding sites and recognition clefts (Wu and Davies, Chap. 5 in this volume). The HPSE2c sequence was docked onto the crystal structure (Dr. Colin Levy, Manchester University) and the key contact amino acids shared between both HPSE1 and HPSE2c are highlighted in the model zoom image (Fig. 34.7). HPSE2c possesses the equivalent HPSE1 N_{224}-E_{225} acid-base group, E_{343} nucleophile (top grouping); R_{272}; G_{349}-G_{350}, $R_{303,}$ and G_{389} amino acids. It can be seen that the key amino acid sugar contact residues are also very well conserved. Certain amino acids however such as Q_{270} and K_{159} are not conserved and not found in HPSE2c.

With such a close overall primary amino conservation and especially 3D conformation similarity around the active site it might be surprising that HPSE2c does not possess even weak HS cleavage activity. Future work profiling a panel of sugar substrates is required to thoroughly investigate whether it may bind and catalyze other substrates.

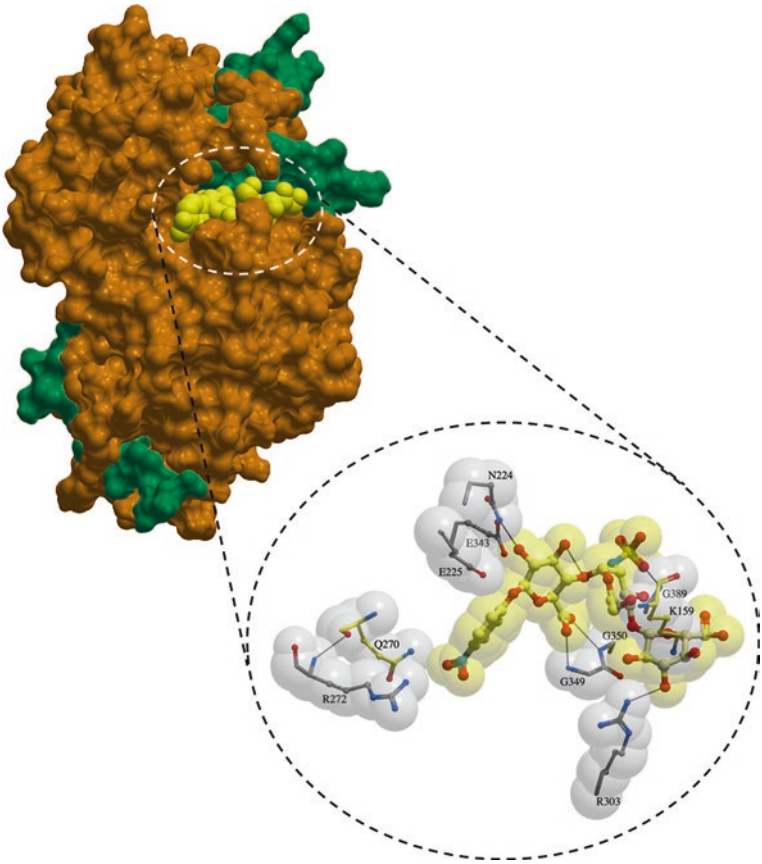

Fig. 34.7 Docking of HPSE2c onto the X-ray structure model of HPSE1**.** The active site is enlarged in the zoom to show the key contact amino acids in the active site

34.2.6 HPSE2 Expression and Functional Roles

Levy-Adam *et al* carried out a highly impressive study and still to date the most thorough expression and biochemical analysis of the HPSE2 protein. Stable HEK293 cell lines expressing c-terminal Myc epitope-tagged versions of all three HPSE2 splice forms were generated. Only the full-length HPSE2c was found to be secreted with the 2a & 2b forms both being retained within the cell. Epitope and HPSE2 specific antibodies confirmed that all proteins were produced as single poly-proteins and were not processed to heterodimeric subunits as in the case of HPSE1. Secreted HPSE2c bound tightly to the cell membrane and could only be effectively released into the media by adding exogenous heparin or HS but not a different GAG species (hyaluronan). In fact, the binding affinity of recombinant HPSE2c to heparin

sepharose beads was shown to require a far higher concentration of NaCl (1.5 M) compared to recombinant HPSE1 binding (0.5-1 M). Lysates prepared from HPSE2 overexpressed cell lines were completely devoid of HPSE1 activity when assayed at pH 5.8–7.4. Unlike the pro-form 65 kDa HPSE1, exogenously added recombinant HPSE2c was not internalized into endocytic compartments but instead was retained on the cell surface where it was found to cluster both type I transmembrane syndecan 1 & 4 HSPGs on Cal27 tongue carcinoma cells. Full-length HPSE2c protein was shown to inhibit HPSE1 enzyme activity by binding its HS interactive site more tightly and so presumably out competing. In addition, HPSE1 and HPSE2c were able to be immuno-precipitate and so there may also be a direct protein-protein inhibitory effect [8]. The roles of the 2a and 2c splice forms are still elusive and more research needs to be carried out to probe their functions within the cell. The non-enzymatic clustering of syndecans by HPSE2c had also previously been demonstrated with HPSE1 by using full-length latent protein or the KKDC peptide dimer which spans its heparin binding domain (K_{158}-D_{171}) [21].

34.3 Role of HPSE2 in Disease

34.3.1 Oncology

Head and Neck Cancer

Over-expression of HPSE2c in head and neck tumors has been shown to correlate with prolonged time to disease recurrence and inversely to tumor spread to the lymph nodes. Using FaDu pharyngeal carcinoma cells, overexpression of HPSE2c reduces tumor growth by ten fold. It also decreases vascularity by reducing Id1 transcription factor-mediated VEGF-A and VEGF-C expression by three fold. A novel blocking monoclonal antibody, mAb1c7, was discovered that prevents cellular binding of HPSE2c by blocking its interaction with HS. The ability of excess HPSE2 to reduce tumor growth was not affected by mAb1c7 and did not modify endogenous HPSE1 activity, so presumably was a result of direct interaction with an unknown protein. Interestingly, tumors that over-express HPSE2 revealed an increase in nerve bundles suggesting a role in neurogenesis which potentially may be related to the neuropathy phenotype observed in Ochoa's Syndrome [22].

Bladder Cancer

The levels of HPSE2 were found to be dramatically reduced in a bladder cancer tissue array umors with a marked increase in lysyl oxidase (LOX) staining. Interestingly, exogenous addition of recombinant HPSE2 protein to bladder carcinoma 5637 cells subjected to scratch assay was shown to inhibit cell migration and wound closure. HPSE2 appears to function as a tumor suppressor in this tumor type by maintaining

cellular differentiation and decreasing cell motility. These effects were shown to be independent of HPSE1 activity since heparanase enzymatic activity was nearly identical compared to control cells. In contrast to the role of HPSE2 in normal and bladder cancer, HPSE1 has undetectable levels in the normal bladder but is highly expressed in bladder carcinomas, correlating with disease progression [12].

Pancreatic Cancer

Over-expression of HPSE2 in an orthotopic model of pancreatic cancer has been reported to produce tumors that were eightfold smaller than controls. A number of phosphorylated signaling proteins were found to be subsequently decreased with an increase in immunostaining of ER stress markers such as BIP, CHOP and cleaved caspase-8. ER stress was also shown to upregulate HPSE2 levels thus setting up a potential apoptotic feedback loop further suggesting that HPSE2 may act as a tumor suppressor [23].

34.3.2 HPSE2 as a Biomarker

Remodeling of the ECM by upregulation of matrix metalloproteinases in conjunction with Heparanase 1 enzyme has been shown to be a key event promoting cancer cell invasion and metastasis. Matrix metalloproteinases (MMPs) that have been implicated include such as MMP-2, −9, −11, and − 14. In a study aimed at discovering novel tumor biomarker gene sets for Breast cancer, Gene expression array, and RT-qPCR analysis was carried out using 150 clinical breast cancer tissues. Out of a total of 28 candidate genes with >ten fold change in expression levels, a strong correlation of MMP11 upregulation with a corresponding downregulation of HPSE2 was found [24].

34.3.3 Alzheimer

HSPGs have been shown to co-localize with beta-amyloid in senile plaques and evidence suggests that they are important for seeding amyloid deposition and aggregation. Processing of the HS chains of HSPGs by HPSE1 enzyme reduces the amyloid burden as observed in transgenic mice studies [25]. Both HPSE1 and HPSE2 are expressed in the brain but in different sub-regions. RNA and protein levels of both heparanases were analyzed at different stages of Alzheimer disease by RT-PCR and immunohistochemistry. Both were found to be upregulated. Extracellular HPSE1 was observed only in neuritic plaques with a fragmented core, while HPSE2 appeared also in those with compact cores [26].

34.3.4 Ochoa's Syndrome

Human HPSE2 Gene Mutations

A major development for HPSE2 clinical research came in 2010 with the publication of two papers linking the disease Ochoa's Syndrome/Urofacial Syndrome to mutations in the HPSE2 gene [10, 11] (Roberts and Woolf, Chap. 25 in this volume). The majority of mutations produce frameshifts resulting in the formation of premature stop codons. In frame exon deletions were also found in exon 3 (losing 54 amino acids) and a combined double deletion of exons 8 & 9 in some patients. Three sisters born off the first cousin Pakistani parents were found to have nucleotide transduction of 1628 A-T which resulted in an amino change of asparagine to isoleucine at amino acid 543. The N_{543} occurs in an 8 amino acid patch (S_{437}KSVQL<u>N</u>G$_{454}$) that is completely conserved in HPSE1. A single point mutated version of HPSE2 that on its own is capable of producing the Ochoa's Syndrome phenotype points clearly at the critical importance of this part of the protein. The C-terminus of HPSE1 has been shown to be important for correct intracellular trafficking and it remains to be seen whether the N_{543}I mutation affects trafficking in a similar fashion or whether it destabilizes the protein by altering protein folding. A database of additional point mutants has been created by diagnostic laboratories and include changes R_{352}H and L_{457}S [27]. It is unclear, however, whether these variants have any clinical significance to the development of Ochoa's Syndrome.

Xenopus HPSE2 Morpholino Knockdown Studies

Neil Roberts et al [28] produced a knockdown of HPSE2 gene expression in Xenopus embryos using a series of antisense morpholinos which resulted in abnormal development of the gut and tail. Significantly, they also resulted in a meandering of motor nerve paths and of axon bundles. At the molecular level, FGF2 signaling and phosphorylation of ERK were upregulated.

Mouse HPSE2 Gene Knockouts

Guo et al. [29] produced HPSE2$^{-/-}$ mice and these died within 1 month of birth. They had small underdeveloped kidneys, enlarged fibrotic bladders, and abnormally high resting and voiding intravesical pressures. These phenotypes were all consistent with the Ochoa's Syndrome range of abnormalities.

LRIG2 Mutations

Soon after the HPSE2 gene link to Ochoa Syndrome, a second gene namely the Leucine-Rich Repeats and Immunoglobulin-Like Domains 2 (LRIG2) was shown by Helen Stuart *et al* to be linked to the same syndrome [30, 31]. LRIG2 is a large

132 kDa transmembrane protein (but also found in other cellular locations), implicated in neural cell signaling and tumor development. It has been speculated that LRIG2 and HPSE2 must be connected on a common biological pathway, potentially intracellular signaling, so that functional mutations in either gene is sufficient to cause the defective bladder and innervation symptoms seen in the Ochoa syndrome patients. A co-immunoprecipitation study using both native and c-terminally Myc-tagged constructs transiently transfected into HEK293 cells failed to detect a direct physical protein interaction of LRIG2 and HPSE2 splice forms (Stuart and McKenzie, unpublished). Future studies using RNAi knockdown of both proteins and a range of intracellular reporter assays are needed to fully understand how they are connected and critically determine their common pathway of action.

34.3.5 Is HPSE2 a Pseudoenzyme of HPSE1?

It may be possible that HPSE2 originally evolved from a gene duplication of the ancestral HPSE1 enzyme. This would have created an opportunity for these extra gene copies to diverge without the selective pressure of maintaining the active site protein geometry of key amino acid catalytic motifs and HS substrate binding sites required for the canonical functioning of the enzyme. The classical pseudoenzyme evolution divergence is more commonly found in kinase and phosphatase enzymes in relation to cell signaling [32]. HPSE2 may well have been in effect re-purposed overtime away from the original enzymatic function of HPSE1 whilst still retaining high glycosaminoglycan binding affinity and other non-enzymatic functions. In addition, differential splicing has been added to its newly evolved roles.

34.3.6 Does HPSE2 Have an Elusive Substrate?

It may well be that HPSE2 has an altered substrate specificity compared to HPSE1 which is related to its very different tissue distribution and glycosaminoglycan profile found in particular areas. HPSE2 has a high overall primary and structural homology compared to HPSE1 and this stretches over comparable 8 kDa and 50 kDa domains which contain a number of key conserved catalytic sites. The higher affinity for HS has been proposed to allow HPSE2 to out-compete for and displace HPSE1 substrate binding. Since both homologs have such disparate tissue distributions, how then this would only come to play when both are co-expressed within the same cell type. As described earlier, for example, normal bladder cells only express HPSE2 with low or undetectable levels of HPSE1. If HPSE2 does possess a glycosaminoglycan cleavage activity then presumably the absence of a functioning protein in Ochoa patients may provide clues based on the glycosaminoglycan chain length and distribution in, for example, bladder basement membranes or excreted urinary GAGs. By analogy, HS extracted from *Hpse1*-KO mice showed a higher molecular weight and the isolated free HS chains appeared less heterogenous

by gel filtration compared to those from wild type mice [33]. Excreted urinary GAGs, in particular, HS, chondroitin sulfate (CS) and dermatan sulfate (DS) have been quantitatively measured [34] and could be screened. Specific phage display anti- HS, CS, and DS antibodies are also available for immunostaining and ELISA assays.

Mouse HPSE2 KO mice have been generated and shown to display a Urofacial Syndrome-like phenotype with distended bladder and abnormal voiding [29]. It would be interesting to compare the GAG staining of mutant vs WT bladders from these mice. The outer umbrella cell layer of the bladder is heavily decorated specifically with CS. These cells undergo a significant shape change in cell morphology from a roughly inverted umbrella shape in the empty bladder state to one that is flat and more squamous in the filled bladder state [35].

34.3.7 Cellular Localization

HPSE1 has been found in many cellular locations from peri-nuclear (lysosomes and endosomes), nuclear, cytosolic, membrane-associated and secreted [36, 37]. Its location has also been shown to vary or be regulated depending on the cancer state of the cell with more metastatic cells secreting higher levels of protein [38]. HPSE2, similar to HPSE1, undergoes endoplasmic reticulum and Golgi trafficking leading to secretion (HPSE2c form). HPSE2 is also found in the nucleus and may well play an HPSE1-like role in DNA binding and gene transcription [39]. HPSE2c is the only family splice form that can be secreted, whilst forms 2a and 2b are retained within the cell. Secretion of HPSE2c inhibits extracellular HPSE1 cleavage of HS and also HPSE1 internalization. HPSE2c, therefore, acts as an HPSE1 brake or negative regulator. Unlike HPSE1, the HPSE2c splice form cannot be internalized once released and so is presumably degraded on the cell surface by protease action (summarised in Fig. 34.8).

HPSE2 has a predicted Nuclear Localisation Sequence (L_{504}-A_{514}; LHRSRKKIKLA) revealed using the cNLS mapper prediction software programme (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_y.cgi) with a high significance score of 12.5. Since HPSE2c demonstrates a high affinity for HS, it is anticipated that it will also have a high affinity for DNA but this remains to be formally tested by, for example, atomic force microscopy using recombinant protein.

34.3.8 Conclusion and Future Perspectives

The yin & Yang of the Heparanase Family Proteins

In Chinese philosophy, the concept of Yin & Yang describes how forces that initially appear opposing may actually be interconnected and complementary. The emerging complex roles of Heparanase 1 and 2 proteins show distinct functional differences

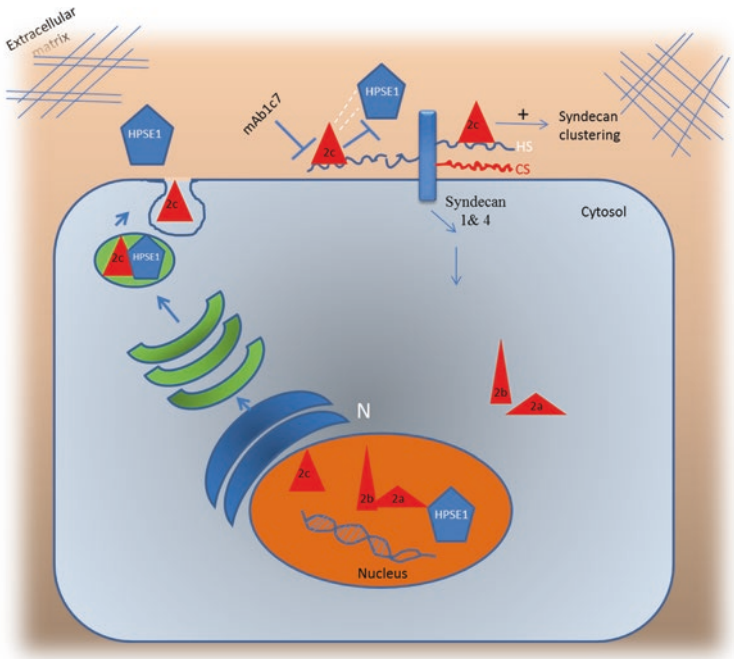

Fig. 34.8 Cellular localization and interaction map of the HPSE2 splice forms

but there is also fundamentally a common core of properties that link them together in a potential Yin & Yang type fashion. Both proteins for example bind HS with high affinity (HPSE2 > HPSE1) and have been shown to affect Syndecan clustering. HPSE1 and HPSE2 have both been detected in different cellular locations, intracellularly and also extracellular via secretion (only the HPSE2c splice form). Whilst their overall tissue distribution profiles are quite distinct there are overlap tissues however where both proteins have been detected. As well as being able to bind to HS substrate, both proteins have been shown to interact with each other directly by immunoprecipitation analysis. HPSE1 protein can be characterized as having two key functional duties; enzymatic via HS degradation and non-enzymatic by affecting cell adhesion and intracellular signaling. HPSE2, on the other hand, has been shown so far to only possess the non-enzymatic function but can act as a negative regulator by blocking HPSE1 enzymatic activity (by outcompeting HPSE1 binding to its HS substrate and/or possibly via protein: protein interactions). In terms of cancer progression and survival outcome, there is a wealth of data showing that high levels of HPSE1 correlate negatively whereas, in stark contrast, HPSE2 has been implicated as a tumor suppressor in a range of tumor types.

Linking of HPSE2 gene mutations to Ochoa Syndrome may well serve as a diagnostic route to probe further functions of this protein and its various splice forms. In particular, the single point mutation of amino acid N_{543} could well affect glycosylation and so protein stability and/or secretion. It will, therefore, be important to

create a library of HPSE2 mutated proteins and express these in eukaryotic cells to examine localization and effects on signaling and HPSE1 mediated activities.

HPSE2 is thought to function, among other options, as HPSE1 inhibitor protein, devoid of any enzymatic activity despite its extremely high binding affinity to HS. Other glycosaminoglycans have not been screened systematically to investigate whether HPSE2 could bind and potentially cleave these. Tissue distribution correlations of HPSE2 with CS suggests that it will be worthwhile to probe their potential interaction. GAG Chip array using purified recombinant HPSE2 proteins will provide full systematic profiling. Other parameters such as Ph and salt concentrations can also simultaneously be studied. Studies of this nature are needed to properly test whether HPSE2 is, in fact, a non-catalytic pseudo-enzyme of HPSE1 or whether it has evolved an alternative substrate specificity and potentially endoglycosidase function.

The current normal tissue profiling studies that exist for HPSE2 do not distinguish between the various splice forms. It will be interesting to repeat and compare the original RNA profiling studies using exon-specific probes. HPSE2 can be effectively divided into an intracellular pairing (HPSE2a & 2b) and a secreted form (HPSE2c) and profiling their specific abundance may throw up valuable functional clues. In addition, it is not clear whether a specific HPSE2 form predominates in its tumor suppressor role or whether all forms can offer this protective role. A corresponding proteomic study of normal and tumor tissues would provide another key to try and unravel where each splice forms predominate.

HPSE2 has come a very long way since its initial discovery at OGS drug discovery laboratory in the Abingdon Science Park, Oxford in 1999 and remained largely in the research shadows for the next 10 years. 2010 was a landmark year for HPSE2 with the publication of the papers by Levy-Adam on Heparanase 2 function and the Daly and Pang papers linking HPSE2 gene mutations to Ochoa Syndrome. The growing significance of HPSE2 as a tumor suppressor protein will hopefully ensure that future research into uncovering its biological mechanisms of action continues.

Acknowledgments I am very grateful to Professor John Gallagher (Manchester University and Iduron https://iduron.co.uk/) who was a major source of inspiration and Glycobiology advice throughout my time at OGS and for critical reading of this chapter.I would also like to thank Professor Paul Brenchley (Manchester University) for his invaluable advice on many aspects of the OGS heparanase [1 & 2] programme and for feedback on my chapter contribution.I would like to further thank Dr. Colin Levy (Manager of the Manchester Institute of Biotechnology X-ray Facility) for kindly modeling the HPSE2c protein structure and for many useful discussions.

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Chapter 35
Heparanase 2 and Urofacial Syndrome, a Genetic Neuropathy

Neil A. Roberts and Adrian S. Woolf

35.1 Introduction

Identification of *HPSE*, the gene that encodes the endoglucuronidase enzyme called heparanase (= heparanase-1), was reported by two research groups in 1999 [13, 38]. Both reports emphasized that the expressed gene correlated with the metastatic potential of mouse and rat tumor cells [13, 38]. One year later, McKenzie et al. [20] reported the existence of *HPSE2* (also called *HPA2*), a gene encoding the homologous protein heparanase 2. Other chapters in this book detail the cloning of *HPSE2* and its potential roles in modulating tumor biology (Ilan et al.; McKenzie, Chaps. 9 and 34 in this volume). Here, we begin by sketching out the biochemistry of heparanase 2 and then consider in more detail its role, when mutated, in causing the human congenital disease urofacial syndrome (UFS), which is thought to have a neurogenic basis. We end by considering broader roles for both the classical heparanase and also heparanase 2 in neurobiology.

HPSE2 has a 2353 base pair open reading frame generating three mRNA isoforms predicted to encode heparanase proteins of 480, 534, and 592 amino acids [20]. These are respectively called 'a', 'b' and 'c' variants, with 'a' lacking exons three and four, and 'b' lacking exon four [16]. Unlike heparanase, heparanase 2 contains a hydrophobic N-terminal putative transmembrane domain, rather than a signal peptide recognition sequence, initially suggesting it is not secreted [20].

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Thus, the three heparanase 2 isoforms were predicted to be intracellular, membrane-bound proteins [20]. The longest isoform of heparanase 2 has 40% amino acid identity with heparanase and a 59% resemblance [16]. The predicted structure of full-length heparanase 2 indicated that it could bind heparan sulfate. McKenzie [20] noted a five amino acid motif, consistent with the heparin-binding consensus sequence (X-B-B-X-B-X), spanning Asn374 to Asn379, where B indicates basic and X indicates small neutral amino acids. Moreover, the high proportion of basic amino acids (16%), particularly in the C-terminus, and a low proportion of acidic amino acids (7%) are structural motifs in heparanase 2 consistent with its binding to negatively charged glycosaminoglycans. Residues critical for heparanase enzymatic activity (Glu225 and Glu343) are also conserved in full-length heparanase 2 at Glu260, itself lost when exon 4 is spliced out in the two smaller isoforms, and Glu381 [16]. EMBL Ebi-Pfam analysis (http://pfam.xfam.org) predicts an approximately 200 amino acid glycoside hydrolase (family 79) motif spanning exons three to nine. On the other hand, the linker region, which undergoes proteolytic removal to activate the heparanase enzyme, and its proposed cleavage sites, are not well conserved in heparanase 2. Indeed, the lowest region of sequence homology is in the heparanase linker region, and Tyr156, which is essential for removal of the heparanase linker by cathepsin L is not conserved in heparanase 2 [16, 20]. Subsequent biochemical analyses of heparanase 2 confirmed that it was neither proteolytically processed, nor did it exhibit heparanase enzymatic activity at pH 5.8 or 7.4, the respective optimal pHs for heparanase and heparitinase [16]. Moreover, heparanase 2c inhibited heparanase enzymatic activity [16]. Two hypotheses were proposed to explain this effect: firstly, the greater affinity of heparanase 2 than heparanase for heparan sulfate moieties would lead to competitive inhibition for binding sites; second, the physical interaction between heparanase 2 and heparanase, as demonstrated by co-immunoprecipitation studies, could interfere with the latter protein's enzymatic activity [16]. Cell biology experiments in HEK293 cells went on to show that, despite the apparent lack of a signal peptide, heparanase 2c is secreted while the shorter isoforms are not. These latter molecules are predicted to have lesser degrees of glycosylation than full-length heparanase 2 [16], and there is evidence that glycosylation is needed for the secretion of heparanase [31]. Both overexpressed, and endogenous, heparanase 2 protein was immunodetected on the plasma membrane of HEK293 cells. This localization was diminished by the addition of heparin, suggesting that heparanase 2c is bound to cell-surface proteoglycan-bound heparan sulfate residues. When heparanase 2 was applied to Cal27 carcinoma cells, syndecan clustering was observed on the cell surface, but unlike heparanase, heparanase 2 is not internalized or detected in endocytotic vesicles [16]. It is postulated that cell surface heparanase 2 may be degraded by proteolysis, or it may be shed with bound heparan sulfate proteoglycans.

Heparanase 2 has been proposed to function as a tumor suppressor. In human head and neck carcinomas, heparanase 2 expression positively correlates with time to disease recurrence and inversely correlates with the dissemination of primary tumor cells to regional lymph nodes [16]. Similarly, high expression of heparanase 2 was associated with favorable survival of gastric cancer patients [42]. Experimental

overexpression of heparanase 2 in human cancer cells implanted into immunocompromised mice reduced growth of the resulting tumors [10]. This effect was associated with reduced vascularity and a reduction of inhibitors of DNA binding/inhibitors of differentiation 1 (Id1) expression, a transcription factor implicated in vascular endothelial growth factor expression [6]. Increased collagen deposition was observed in heparanase 2 expressing xenografts, correlating with increased expression of lysyl oxidase, an enzyme that catalyzes cross-linking of collagen and/or elastin, and which is implicated in extracellular matrix (ECM) remodeling and fibrosis [2, 27]. Surprisingly, heparanase enzymatic activity was not inhibited in cells overexpressing heparanase 2, suggesting that reduced tumor growth was not due to heparanase 2 blockade of heparanase function [10]. Further, the growth of tumor xenografts overexpressing heparanase 2 was not affected by a monoclonal antibody targeting a heparin binding domain of heparanase 2. Collectively, this suggests that heparanase 2 can function through a yet-to-be-defined mechanism independent of both heparanase enzyme activity and heparan sulfate binding. It was also noted that tumors produced by heparanase 2 expressing cells had more nerve bundles than tumors arising from parent cells. While the relevance of this observation to tumorigenesis is unclear, it is consistent with a functional link between heparanase 2 activity and neural biology. Apart from head and neck carcinomas, heparanase 2 is detected in other types of human tumors, such as breast cancer [7], colorectal adenomas [39] and thyroid carcinomas [19]. In all these scenarios, the possible functional roles of heparanase 2 remain to be explored.

35.2 Heparanase 2 and the Urofacial Syndrome

Two research groups reported mutations *HPSE2* in a human congenital disease called urofacial syndrome (UFS) [4, 23]. This inherited syndrome was first clinically described by Ochoa, a surgeon based in Colombia, who recorded families with individuals affected by both renal tract disease and facial dysmorphology [22] (Fig. 35.1). The affected individuals, generally children, were unable to fully void urine from their bladders and instead produced frequent low volume voids. At first, it was thought that there might exist a physical obstruction inside the urethra, but closer investigation revealed that this was not the case. Instead, the bladder was ‘dyssynergic’, with its detrusor muscle wall contracting against an incompletely dilated urethra (Fig. 35.2). This contrasts with healthy voiding in which the bladder empties completely through a dilated urethra [14]. The pathology results in bladder contents being retained under an abnormally high hydrostatic pressure and this increases the risk of bacterial urosepsis, with the retrograde passage of high-pressure urine from the bladder to the kidney. This can result in infection of the kidney parenchyma with loss of renal function and the risk of renal excretory failure [25]. The ‘facial’ aspect of UFS described by Ochoa consists of a normal looking face at rest but a grimace while the patient is smiling “as if in pain or sadness when they tried to smile or laugh” [22]. Ochoa intelligently speculated that the disease would

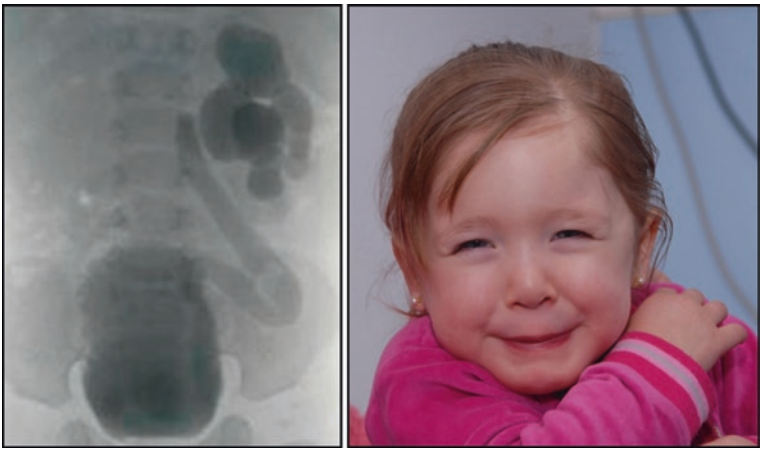

Fig. 35.1 Clinical features of urofacial syndrome**.** Left panel. Radio-opaque dye (black) introduced into the urinary bladder shows an enlarged organ as well as the retrograde passage of urine into one ureter. These are both secondary to the bladder outflow obstruction. Right panel. The child's face while smiling, demonstrating the characteristic 'grimace'. This particular individual carried biallelic *LRIG2* variants, but the phenotype is the same as for urofacial syndrome caused by *HPSE2* variants. Frames reproduced from Stuart et al. [32] under the Creative Commons License (open access)

have a genetic basis and that the mutated gene would cause a lesion in the midbrain affecting both the facial nerve motor nucleus and the 'micturition centre' [22]. However, although both are located in this part of the brainstem, they are quite separate structures, so a lesion would need to be extensive to affect both [8]. UFS can be clinically devastating but appears rare, with around 150 cases reported in the literature [21]. Its prevalence may, however, be underestimated because the facial features can be subtle and have sometimes not been recognised until late in the course of the renal tract disease.

Around one third to half of UFS patients tested carry likely pathogenic variants of *HSPE2* on chromosome 10q.24.2 (Fig. 35.3). As expected for an autosomal recessive disease, the *HPSE2* mutations in UFS are always biallelic [1, 4, 18, 23, 33, 36]. Most of the variants reported to date generate stop codons or frame shifts and so are predicted to be 'functionally null' mutations. Other mutations may shed light on the function of heparanase 2, although the biochemistry of these variants has yet to be studied. For example, one variant is an in-frame deletion of asparagine 254 [33], an amino acid predicted to be N-linked glycosylated and thus possibly enhance the endoplasmic reticulum processing [29] of heparanase 2. Another *HPSE2* variant is a missense change of asparagine 543 to isoleucine [33]; the wild type amino acid is not predicted to be glycosylated but presumably has a key function in the heparanase 2 protein. The phenotype of these individuals is similar to UFS in individuals who carry stop and frameshift mutations, so one can postulate that the in-frame deletion of asparagine 254, and the asparagine 543 to isoleucine change, are functionally null proteins if indeed they are produced.

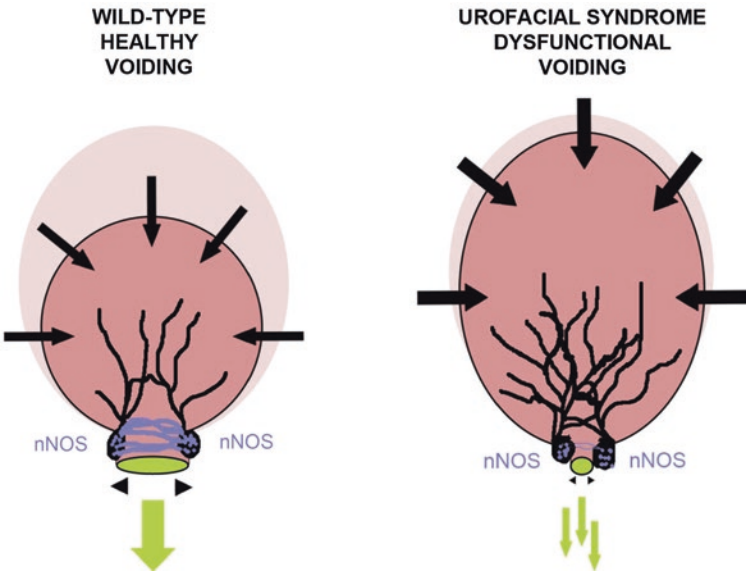

Fig. 35.2 Dysfunctional urinary voiding in urofacial syndrome**.** In the healthy bladder (left) a robust urinary stream and complete emptying are facilitated by full dilatation of the outflow tract. This is mediated by neuronal nitric oxide synthase (nNOS) nerves. In the urofacial syndrome bladder (right) the stream is poor due to failure of the outflow to fully dilate, which is associated with downregulated nNOS. The detrusor muscle in the body of the urofacial syndrome bladder is overactive, which is associated with an abundance of nerves in the bladder body. Contractile forces in the bladder body are shown by black arrows facing inward, and outflet dilatation is shown by black arrowheads facing outward. Nitrergic (nNOS) autonomic nerves around the outflow are depicted in purple, and those in the body (presumed cholinergic) are depicted in black. Urine is depicted in light green. The body of the uncontracted bladder is represented in lilac, and the voiding bladder body is shown in pink. Frames are reproduced from Roberts et al. [26] under the Creative Common Licence (open access)

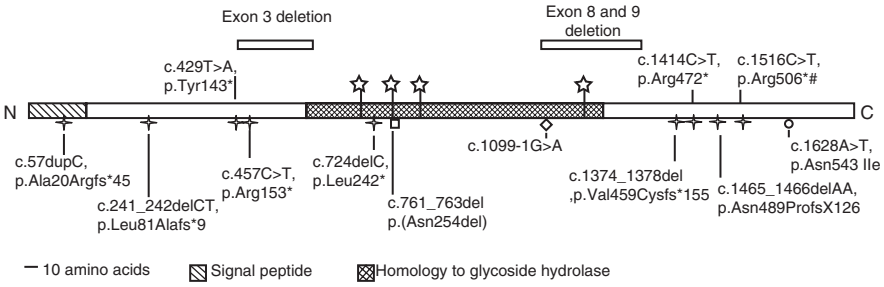

Fig. 35.3 *HPSE2* mutations in urofacial syndrome**.** Schematic heparanase 2 showing locations of mutations. Stars, nonsense or frameshift mutations; circle, missense mutation; diamond, splice-site mutations; stars above the gene show predicted N-glycosylation sites; #, founder mutation in Ochoa's Colombian cohort. Domains were predicted by Pfam and SignalP. N and C, the protein's amino and carboxy terminals, respectively. Figure modified from Stuart et al. [33] under the Creative Common Licence (open access)

Urine is produced by human kidneys from the last third of the first trimester, and from this stage bladders cyclically store and void urine. Fetal ultrasonographic anomaly screening has identified grossly dilated bladder, or megacystis, in individuals who are later diagnosed with UFS. This indicates that the bladder defect occurs before functional differentiation of the bladder is complete, and may be a developmental disorder. A first biological clue that heparanase 2 may have a neural role in renal tract biology was its immunodetection in normal human first-trimester urinary bladders; here, nerve trunks, presumed autonomic nerves, between smooth muscle bundles, contained heparanase 2 [32]. Complementary experiments in wild type mice showed that heparanase 2 was present in nerve bundles within the maturing urinary bladder and also in the pelvic ganglia that contain postganglionic autonomic nerve cell bodies which send axons into the bladder [26, 33]. The same ganglia immunostained for heparanase, although it was not technically possible to determine whether these were in the same cells in which heparanase 2 was detected. Also, in explanted wildtype embryonic pelvic ganglia, heparanase 2 was detected in regenerating neurites (Fig. 35.4).

In health, the autonomic nervous system mediates bladder filling and voiding [14]. Parasympathetic innervation of detrusor muscle in the wall of the bladder releases acetyl choline that causes muscarinic receptor-mediated contraction to drive voiding. In addition, nitrergic axons cause neuronal nitric oxide to mediate dilatation of the outflow tract during voiding (Fig. 35.2). Sympathetic innervation of the bladder outflow tract causes noradrenergic mediated contraction during the bladder filling phase to maintain continence. Two lines of *Hpse2* gene trap mice have been created and studied [11, 26, 33]. Each line has a bladder phenotype similar to the

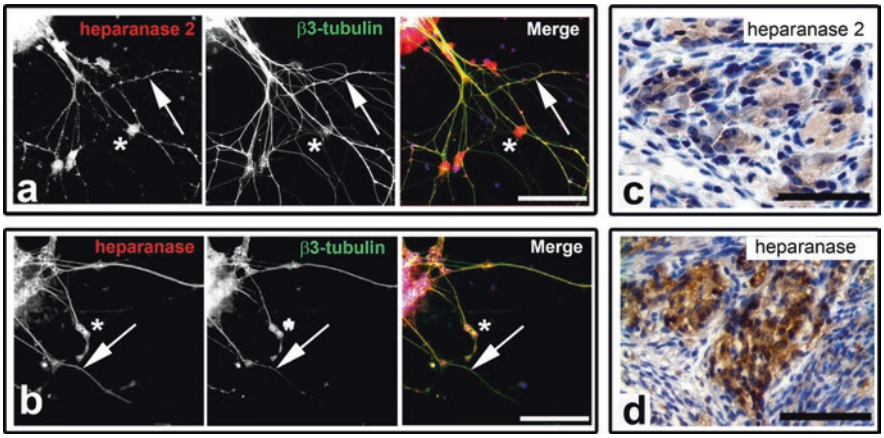

Fig. 35.4 Neural localization of heparanase 2 and heparanase. (**a,b**) Immunocytochemistry of pelvic ganglia explants. The first 2 frames in each row show signals (white) for individual proteins named in each frame. The final (merge) frame in each row shows double immunostaining, with nuclei stained with 4′6-diamidino-2-phenylindole (DAPI, blue). Bars = 100 μm. (**c,d**) Bright field immunohistochemistry of neonatal pelvic ganglion immunostained (brown) for the named protein. Bars = 50 μm. Frames are reproduced from Roberts et al. [26] under the Creative Common Licence (open access)

human UFS disease. In particular, the mice show an increased frequency of voiding, and the volume per void is less than normal (Fig. 35.5a). Also, the bladder fails to empty completely, as it should normally do, and this is accompanied by retained urine under high hydrostatic pressure [11]. Thus the *Hpse2* mutant mouse bladder is 'dyssynergic', as occurs in people with UFS. Further study revealed that the anatomical patterns of bladder nerves are abnormal in homozygous *Hpse2* mutant mice, with a decreased density of nerves, including nitrergic axons, around the top of the bladder outflow, and an increased density of nerves inside the body of the organ [26] (Fig. 35.5b). Further work is needed to unravel whether these abnormal patterns are a primary and pathogenic defect in the disease, or whether they represent changes secondary to, for example, increased hydrostatic pressure inside the bladder. Indeed, outlet obstruction caused by experimental urethral ligation causes striking secondary changes in the cell- and molecular- biology of developing bladders [34]. A different study reported that *Hpse2* mutant bladders were fibrotic, with biochemical evidence of increased transforming growth factor β signaling activity [11]. Again, whether this

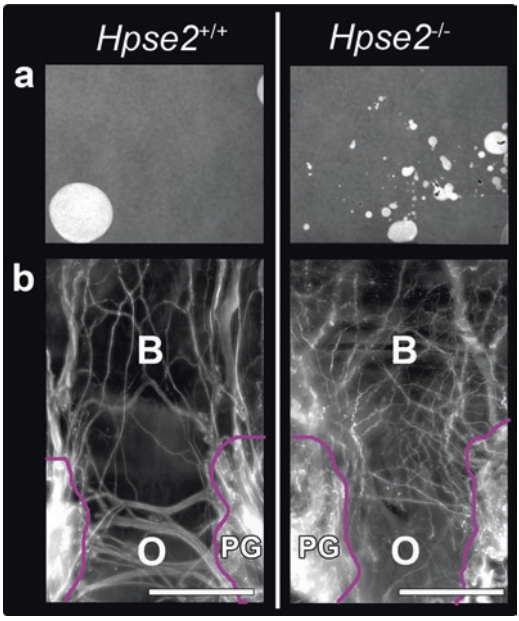

Fig. 35.5 Urination defects and aberrant nerves in the bladder of *Hpse2* mutant mouse. (**a**) voided stain on paper urination patterns produced by Hpse2$^{-/-}$ mouse (right frame) and a wild-type littermate (left frame). (**b**) Whole-mount immunostaining of one-week postnatal bladders with antibodies to peripherin, a pan-neuronal marker. Purple lines indicate borders of pelvic ganglia (PG), the bladder body is denoted by B, and the outflow by O. Neurons spanning the two pelvic ganglia and projecting into the bladder body were detected in wild-type bladders (left column). Note increased peripherin + (white) structures in the homozygous *Hpse2* mutant bladder body (right frame) versus the wild-type littermate, and less prominent peripherin + nerves in the outflow. Bars = 200 μm. Frames are reproduced from Roberts et al. [26] under the Creative Common Licence (open access)

is a primary or secondary effect remains to be explored. Molecular analysis of *Hpse2* gene trap mouse bladders showed similar levels of urothelial (*uroplakin 3A*) and smooth muscle (*alpha-smooth muscle actin* and *myosin heavy chain 11*) transcripts in homozygous and wildtype littermates during the first two weeks after birth [33].

35.3 Emerging Roles for Heparanses in Neurobiology

Given that the Xenopus *hpse2* gene is highly homologous to human *HPSE2*, and to further explore roles of heparanase 2, a knock-down model in the frog *Xenopus tropicalis* was developed [24]. Heparanase 2 was immunodetected in skeletal muscle of Xenopus embryos and the ventrolateral spinal cord, in putative cell bodies of motor neurons that supply the forming skeletal muscle. The protein was depleted by injecting into fertilized eggs a morpholino designed to perturb RNA translation or splicing. This was associated with skeletal paralysis, evidenced by the loss of hatching and escape reflexes. Immunostaining for peripheral nerves revealed abnormal patterns of motor nerves that had emanated from the embryonic spinal cord (Fig. 35.6a and b). Compared with control embryos, these axons appeared to have more circuitous paths and be less compactly bundled. Intriguingly, in heparanase 2 depleted embryos, increased *hpse* transcript was recorded. This was accompanied by an increase in growth factor signaling molecules, including *fibroblast growth factor 2* (*fgf2*) transcript and activated (phosphorylated) extracellular signal-regulated kinase (ERK). In the embryonic spinal cord of healthy embryos, immunostaining detected phosphorylated ERK in a subset of cells in the ventrolateral neural tube, in proximity to heparanase 2 immunostaining (Fig. 35.6c). This was the first study to demonstrate an *in vivo* developmental role for heparanase 2, and it supports the hypothesis that UFS has neurogenic pathogenesis. The frog data are also consistent with heparanase 2 modifying growth factor signaling, perhaps acting as a counterbalance to heparanase itself that has the ability to upregulate FGF signaling through increasing the availability of growth factors sequestered to heparan sulfate proteoglycans [37].

Other literature point to roles for heparanase in neurobiology. For example, in cell culture of the rat adrenal PC12 pheochromocytoma line, it enhances nerve growth factor-induced neurite outgrowth, modulating p38 mitogen-activated protein kinase phosphorylation [3]. Notably, application of a mutant latent heparanase protein has similar effects, suggesting that heparanase enzymatic activity was not playing a key role [3]. Heparanase has also been shown to protect against axonal degeneration following experimental sciatic nerve injury in rodents [40]. In this context, *HPSE* transcript was upregulated after acute damage, and heparanase enzymatic inhibition by OGT2115 led to accelerated axonal degeneration, associated with upregulation of genes implicated in peripheral neuropathy and Schwann cell de-differentiation. Furthermore, in the adult human central nervous system, *HPSE2* and *HPSE* transcripts were detected in the adult human brain in the temporal cortex, hippocampus, basal ganglia and cerebellum [9]. In brain tissue from individuals

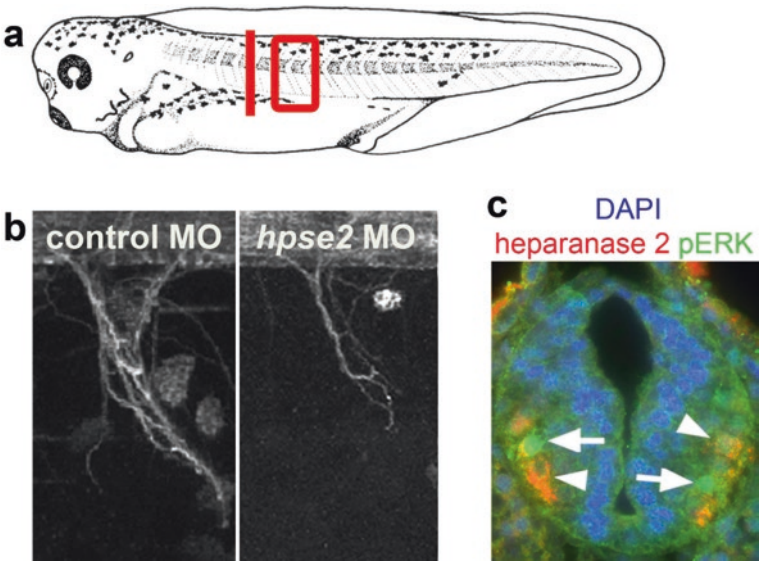

Fig. 35.6 Peripheral nerve morphology in xenopus model of urofacial syndrome**.** (**a**) stage 38 xenopus larvae, with a red box indicating the parasagittal imaging plane in b, and red horizontal line indicating the transverse section shown in c. (**b**) Visualisation of a single motor neuron in whole-embryos immunostained with anti-acetylated α-tubulin, a pan-neuronal marker. Across the top of each frame, a longitudinal section of the neural tube is evident, with anterior on the left, and a peripheral nerve emanating ventrally. Single-cell fertilized embryos were injected with either a control morpholino (left panel) or an *hpse2* specific morpholino (right panel). The resulting morphants were paralyzed, and following immunostaining displayed nerves lacking compact bundling and coherent directional extension. (**c**) pERK (green) and heparanase 2 (red) were detected in the neural tube by of wild-type stage 32 embryos following immunofluorescence staining of transverse sections. The dorsal surface is uppermost. pERK+ nuclei (two are indicated by arrows) and heparanase 2+ cells (two are indicated by arrowheads) are detected in the lateral zones of the neural tube, where motor neuron cell bodies are found. Note that cells with strong pERK immunostaining and strong heparanase 2 immunostaining tend to be mutually exclusive. Frames are reproduced from Roberts et al. [24] under the Creative Common Licence (open access)

with a history of Alzheimer disease heparanase 2 was immunodetected in atrophic neurons affected by neurofibrillary degeneration, and extracellularly in plaques [9].

35.4 LRIG2 Mutations in Urofacial Syndrome

HPSE2 mutations have to date been found in only around half of the genetically tested individuals with UFS. Some of the others [5, 32] have been found to have biallelic mutations in *LRIG2*, a gene encoding leucine-rich-repeats and immunoglobulin-like-domains 2. Putative pathogenic missense mutations have also been reported in rare individuals with a UFS-like bladder disease but who lack the

facial features of the full-blown syndrome [26]. Although mutations in either *HPSE2* or *LRIG2* can cause an apparently similar syndrome, the structure of the encoded proteins is quite different. LRIG2 belongs to a family of three single-pass transmembrane proteins, of which LRIG1 has been most comprehensively studied [30]. LRIG1 is a tumor suppressor which downregulates growth factor signaling by ubiquitination-mediated receptor tyrosine kinase degradation and inhibition of receptor tyrosine kinase recruitment to lipid rafts. LRIG1 blocks glial cell line-derived neurotrophic factor-induced neuritogenesis *in vitro* [15]. Less is known about LRIG2, but its expression is associated with poor survival in a range of cancers [17]. Studies in *Lrig2* homozygous gene-targeted mice revealed it to be permissive for glial tumor growth *in vivo* [28]. Moreover, in a glioma cell culture model, LRIG2 interacted with the epidermal growth factor receptor and positively modulated intracellular signaling [41]. The latter two studies point to possible biochemical mechanisms for LRIG2's positive association with more aggressive forms of cancer.

As for heparanase 2 and heparanase itself, LRIG2 is immunodetected in pelvic ganglia of healthy mice [26, 33], and in nerves within human fetal bladders [32]. Importantly, *Lrig2* mutant mice with homozygous excision of exon 12 were reported to have abnormal urination patterns similar to *Hpse2* mutant mice [26] and also had similar aberrant patterns of nerves in the body of the bladder and around its outflow tract [26]. Molecular analyses of *Lrig2* homozygous mutant urinary tracts by RNA sequencing revealed changes in transcripts associated with neural function [26]. In developing mouse brains, siRNA knockdown of *Lrig2* was reported to increase cortical migration of neurons [35]. Furthermore, knockdown of *Lrig2* in an optic nerve crush model induced overexuberant regeneration of axons [35]. Biochemical evidence pointed to LRIG2 modulating axonal growth by preventing the proteolytic degradation of neogenin, a receptor for repulsive guidance molecules [35]. Interestingly, LRIG2 is also known to be expressed in the facial nerve nucleus [12].

Further work is needed to define the precise roles of heparanase 2 and LRIG2 in normal and abnormal neural differentiation.

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Competing Interests The authors declare no competing interests.

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Chapter 36
The Good and Bad Sides of Heparanase-1 and Heparanase-2

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36.1 Extracellular Matrix: At the Crossroads of Cell-Cell and Cell-Microenvironment Relationships

The extracellular matrix (ECM) can be defined as the structure shared by all multicellular organisms, and it is composed of proteins and glycoconjugates that are synthesized and exported/secreted by the cells to the extracellular environment. The ECM is organized in a heterogeneous macromolecular network that does not only provide structural support, organization and tissue orientation (tissue biomechanical), but among other functions acts as substrate for cell growth, migration, proliferation, adhesion and differentiation and moreover, plays vital role in the various sensory crossroads of cell-cell and cell-environment interactions [1–5].

The ECM is composed of a wide variety of molecules, including glycosaminoglycan chains, which are generally covalently linked to a core protein giving rise to proteoglycans, fibrous structural proteins such as collagen and elastin; and fibrous adhesive proteins such as laminin and fibronectin. These components are organized into macromolecular networks that act in diverse cellular dynamics mentioned above. Such macromolecular glycoconjugates are the most abundant class of structurally diverse and heterogeneous molecules present in the ECM and cell surface, forming the so-called glycocalyx. Their diverse and heterogeneous structures are the result of the action of several glycosyltransferases, which are capable of polymerizing carbohydrate chains as well as other classes of enzymes such as sulfotransferases and epimerases, which alter their substitution pattern and stereochemistry at specific sites along the polymer. As a result, at the end of their biosynthesis, there will be structurally distinct functional chemical species.

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The ECM is at constant remodeling in response to various extracellular and intracellular stimuli, and the way such signals are transmitted, captured and interpreted, dictate and distinguish the fate of normal and pathological remodeling [3]. Hence, understanding and modulating the ECM information flux can deeply influence the development of new and improved therapeutic approaches of significance for life quality.

ECM, as previously mentioned, has a highly complex supramolecular structure, influencing the assembly, viability, and functions of cells and tissues. ECM components can influence multiple cell properties and functions directly or through its degradation products, being able to modify the cellular microenvironment and tissue function [6]. The epithelial-mesenchymal and epithelial-stromal interactions are critical in physiological and pathological processes, e.g., embryonic morphogenesis [7], tissue repair [8] and tumorigenesis [9], accompanied by dynamic changes and generating new cell-matrix interactions [10, 11]. The binding of cell surface receptors to ECM activates signal transduction pathways that regulate cell functions, including adhesion and migration [12, 13]. Several of these activities depend upon integrins, that are transmembrane glycoproteins composed of non-covalently linked heterodimers that may act at ECM receptors. Integrins require an activation process for interaction with ligands that may mediate reactions induced by their binding to ECM proteins [14–17].

Consequently, besides its structural role, ECM represents a microenvironment that can sequester growth factors and cytokines, which can facilitate rapid and localized changes in the activities of mediators in the absence of newly synthesized proteins. Moreover, the ECM plays an important role in cell-cell communication. Fibrillar and non-fibrillar components may limit or facilitate the transport of molecules across the extracellular space while regulating the interstitial hydrostatic pressure. Furthermore, extracellular matrix molecules are involved in cell signaling pathways, which are mediated by cell surface receptors. Once activated, this system can lead to ECM remodeling either by the production and activation of enzymes such as proteases and glucosidases or by de novo synthesis and structural modification of ECM components [1–3].

36.1.1 Glycosaminoglycans and Proteoglycans

Glycosaminoglycans (GAGs) are important constituents of both the ECM and cell surface. Apart from hyaluronic acid, all GAGs are present in tissues as proteoglycans, where the polysaccharide chains are covalently bound to a protein backbone. Heparan sulfate and heparin are composed of alternating units of D-glucosamine and uronic acid (β-D-glucuronic acid and α-L-iduronic acid), linked by α [1–4]-type glycosidic linkages. The glucosamine can be N-acetylated or N-sulfated and/or O-sulfated mainly at the C-6 position, and less at the C-3 position. Furthermore, the uronic acid moiety can be sulfated to various degrees at the C-2 position [18–21]. They participate in a variety of biological processes including cell-ECM interactions, cell growth, cell differentiation and malignant transformation due to their ability to bind and modulate key cell growth-related molecules, such as TGF-β (transforming

growth factor β), FGF (fibroblast growth factors), VEGF (vascular endothelial growth factor) and others [22–25].

Heparan sulfate proteoglycans (HSPGs) play an important role in cell-ECM interaction. Nearly all ECM molecules have heparan sulfate binding sites, suggesting that the balance between adhesion and cell motility rely on integrating PGs and integrin-mediated adhesion signals. HSPGs are composed of a protein backbone and one or more glycosaminoglycan chains of heparan sulfate (HS). There are subfamilies of HSPGs: transmembrane PGs (e.g., syndecans, betaglycan and CD44), PGs connected to the GPI anchor (e.g., glypicans), and PGs secreted into the ECM (e.g., agrin, several collagens, and perlecan) [26]. Syndecans are present at the cell surface and can also act as co-receptors along integrins by modulating interactions between the cytoskeleton and the ECM [25–27]. Syndecans can interact with a variety of ligands via HS chains. It is thought that these PGs are involved in vital cell functions, including cell proliferation, signaling, and recognition, as well as cell-matrix and cell-cell adhesion [28–30]. Numerous molecular interactions between heparan sulfate chains, growth factors, cytokines, and ECM molecules are known, associated in part with cell adhesion and migration mediated by integrins.

Modeling and remodeling of the matrix are driven by the local cellular milieu, including secreted and cell-associated components in a framework of dynamic reciprocity. The current collection of expertly-written chapters aims to relay state-of-the-art information concerning the mechanisms of matrix modeling and remodeling in normal physiology and disease. Even though there are many results showing that glycosaminoglycans from the extracellular matrix and cell surface play a fundamental role in controlling the proteolytic activity of several metalloproteases [31–34], as well as cysteine proteases, such as cathepsins [35–38], in this review we will focus on the role played by heparanase in the cell environment.

The interactions between cells and ECM are crucial for cell events such as growth, death, differentiation, and motility, which have importance in various biological processes such as morphogenesis, inflammation, immune response, parasitic and virus invasion, cell transformation and metastasis. The ECM composition is not static and changes in response to internal and external cell stimuli occur in order to maintain the metabolic equilibrium. Changes in ECM structure and organization are implicated in many pathobiological states, including cancer, neurodegenerative diseases, and fibrosis, among others [1–4, 39].

36.2 Heparanase: A Key Modulator of ECM Architecture at the Crossroads of Homeostasis and Diseases

36.2.1 General Aspects

Heparan sulfate chains are degraded by hydrolase, known as heparanase, which is an endo-β-D-glucuronidase that cleaves β-D-glucuronyl(1 → 4)D-N-acetylated glucosamine. Heparanase (HPSE, Hpa or Hpa1) is capable of cleaving heparan sulfate side chains of heparan sulfate proteoglycans on cell surfaces and the extracellular

matrix. As previously mentioned, heparan sulfate proteoglycans are crucial elements for normal cell physiology due to their strategic localization and interaction with ECM components, integrins, and the cytoskeleton. Heparanase, by altering heparan sulfate structure at the cell surface and ECM leads to a cascade of cellular events that affect a diversity of physiological processes, such as cell growth, adhesion, migration, and death. The repertoire of physiological and pathological activities of heparanase is growing steadily, being implicated in inflammation, neovascularization, and tumor development [40–42].

Heparanase-1 resides in the endosomal/lysosomal compartment for a relatively long time and is likely to play a role in the normal turnover of heparan sulfate [43]. Furthermore, heparanase secretion kinetics resembles that of cathepsin D, a known lysosomal enzyme, validating its lysosomal origin. Extracellular signals activate protein kinases signaling pathways leading, among other effects, to heparanase secretion [44]. Also, the uptake of heparanase is dependent on the presence of heparan sulfate proteoglycans (syndecans) at the cell surface [45].

At present, there are more than 1500 papers focusing on heparanase. Therefore, the purpose of this chapter is to give a general view of the putative biological roles of heparanase and its implications in normal homeostasis and disease situations. Heparanase-1, as an enzyme, plays a role in remodeling the ECM and basement membrane by degrading heparan sulfates and thus liberating heparan sulfate-bound proteins, such as cytokines and growth factors. On the other hand, heparanase also exhibits non-enzymatic activities due to its capacity to interact with membrane proteins such as tissue factor and tissue factor pathway inhibitor, thus playing a role in the coagulation cascade [46]. The identification of heparanase-1 in tumor cells and platelets, as well as characterization of its substrate specificity, type of uronic acid recognized by the enzyme, and inhibition by non-anticoagulant heparin molecules goes back to the ‘80s [47–56]. Other papers reported its activity and secretion by degranulating mast cells [57, 58], T and B lymphocytes, granulocytes, and macrophages [59, 60], suggesting a role for heparanase in vessel wall injuries, atherosclerosis, neovascularization, and immune responses.

36.2.2 Heparanase Favors Blood Coagulation

Heparanase-1 has been implicated in coagulation in a non-enzymatic manner. Heparanase overexpression in human leukemia, glioma, lung cancer, and breast carcinoma cells leads to increased levels of tissue factor (TF) and tissue factor pathway inhibitor (TFPI). Heparanase-1 was demonstrated to enhance tissue factor activity. Hence heparanase procoagulant activity in the plasma of patients with lung cancer reveals a new mechanism by which the coagulation system is activated in malignancy [46]. Additionally, the interaction of heparanase-1 with TFPI at the cell surface of endothelial and tumor cells, increases the coagulability due to dissociation of TFPI from the cell membrane, thus resulting in increased coagulation activity,

supporting a prothrombotic function of heparanase [61]. Peptides generated from tissue factor pathway inhibitor, which inhibit heparanase procoagulant activity, attenuate inflammation in a sepsis mouse model. Likewise, peptides inhibiting heparanase procoagulant activity significantly reduced tumor growth, vascularisation, and relapse. The procoagulant domain in heparanase-1 protein may thus play a role in tumor progression, suggesting a new mechanism for the involvement of the coagulation system in cancer [62].

Also, the ability of von Willebrand factor (VWF) to trap platelets contributes to inflammation, infection, and tumor progression. Overexpression of syndecan-1 (SDC-1) significantly supports the binding of VWF to endothelial cells. However, heparanase degradation of heparan sulfate chains or impaired synthesis of heparan sulfate, a major component of the endothelial glycocalyx, reduce platelets recruitment by VWF [63]. Therefore, the first step of hemostasis, platelet aggregation, appears to be dependent on heparanase modulation.

36.2.3 Heparanase and the Tumor Microenvironment

Increased expression of heparanase-1 seems to be a marker for various tumors [51, 64–67]. Furthermore, high levels of heparanase expression correlate with poor survival rates, as in gliomas, breast cancer, gastrointestinal tumors, and esophageal carcinomas [68–70]. The crosstalk between heparanase-1 and macrophages propitiates chronic inflammatory conditions creating a pro-tumorigenic microenvironment, as is the case for chronic inflammatory bowel disease and colon cancer, among others [42, 71]. Also, the protagonist of heparanase-1 in inflammation, neuronal disorders, and viral infection is becoming more evident [70, 72–79].

36.2.4 Exosomes

Exosomes are extracellular vesicles produced in the endosomal compartment of most eukaryotic cells and play a role in intercellular communication and signal transduction [80, 81]. Recently, the pathophysiological effects of exosomes on diseases, especially cancer, have emerged. Heparanase-1 enhances exosome secretion, alters its composition, and thereby promotes tumor progression [81–84].

Exosomes participate in multiple mechanisms that support tumorigenesis, such as ECM remodeling, angiogenesis, thrombosis, and tumor cell proliferation, being implicated in the promotion and establishment of a pro-tumorigenic metastatic niche due to their cargo, including oncoproteins and heparanase [82–84]. Using CAG cells (plasma cell myeloma), it has been shown that several exosome cargoes such as syndecan-1, VEGF and hepatocyte growth factor, are regulated by high levels of activated heparanase-1, reflecting in the spreading of tumor cells and invasion

of endothelial cells in vitro [84]. The biogenesis of a class of these vesicles depends on syntenin and syndecans [85]. Heparanase-1 acts as a regulator of the syndecan-syntenin-exosome biogenesis pathway, and the upregulation of both syntenin and syndecan has been demonstrated in cancer [86–88]. Syndecan heparan sulfate proteoglycans were found to control exosome biogenesis and endosomal-sorting complex through syntenin-1 and ALIX [87, 88]. The fact that both syntenin and heparanase are upregulated in tumors favors the proposed role of exosomes in carcinogenesis. Recent data show that anti-myeloma drugs used in the treatment of myeloma upregulate heparanase through the nuclear factor-kappa B (NF-κB) signaling pathway [89]. Additional studies demonstrated increased exosome secretion when myeloma cells were exposed to the same drugs. The chemotherapy-induced exosomes display a proteomic profile distinct from cells not exposed to the drug. Furthermore, besides an increase in the levels of heparanase, it shows a distinct localization, being present at the exosome surface and hence act on the surrounding ECM. Exosomes secreted by tumor cells, together with high levels of heparanase-1, not only alter the behavior of tumor cells but also promote alterations to nonneoplastic host cells [82]. Thus, macrophages exposed to these exosomes increase the secretion of myeloma growth-promoting factors [90] and exosomes secreted by tumor cells containing heparan sulfate, modulate the expression of heparanase-1 in circulating T-lymphocytes [92]. These and other results bring new insights into the understanding of chemoresistance [91].

36.2.5 Heparanase Inhibitors

Since heparanase-1 is known to be involved in tumor progression, inhibitors of this enzyme have been produced as novel cancer therapeutics [93]. An improved understanding of the molecular contexts favoring the action of these agents against cancer would allow a full application of their potential. Current approaches for heparanase-1 inhibition include the development of chemically modified heparins, small molecule inhibitors, natural products, synthetic oligonucleotides, and neutralizing antibodies [94–101].

Development of heparanase-1 inhibitors focused on carbohydrate-based compounds of which few are being evaluated in clinical trials for various types of cancer, including myeloma, pancreatic carcinoma, and hepatocellular carcinoma [103]. Low-sulfate oligosaccharides were less effective heparanase inhibitors than medium- and high-sulfated fractions of the same-size saccharide. While O-desulfation abolished the heparanase-inhibiting effect of heparin, O-sulfated, N-substituted (e.g., N-acetyl or N-hexanoyl) species of heparin retained high inhibitory activity [102]. Therapeutic potential of a supersulfated low molecular weight heparin (ssLMWH) showed potent anti-heparanase activity in preclinical models [104]. Synthetic glycopolymers that mimic heparin structure with reduced anticoagulant activity is another strategy to generate heparanase-1 inhibitors.

Among these glycopolymers, a sulfated poly-2-aminoethyl methacrylate grafted heparin disaccharide has shown potent efficacy in inhibition of heparanase-1 activity and microvascular endothelial cell proliferation, protecting against tumor metastasis [105]. Several heparan sulfate glycomimetics demonstrated heparanase-1 inhibition comparable to the compounds in clinical development and also inhibit metastasis and growth of human myeloma cells in mouse xenografts [106].

Roneparstat (=SST0001), a chemically-modified heparin saccharide with 100% N-acetylation and 25% glycol split with non-anticoagulant activity is known to decrease the uptake and the effects of soluble heparanase-1 [89].

PI-88 is a mixture of highly sulfated, monophosphorylated mannose oligosaccharide a heparanase inhibitor showed efficacy as an adjunct therapy for hepatocellular carcinoma [107].

PG545, a synthetic mixture of tetrasaccharide derived from heparin is also an inhibitor of the heparan sulfate-degrading enzyme heparanase-1 (Hammond & Dredge, Chap. 22 in this volume). Using a murine model of lymphoma, it was observed that the antitumor effect of PG545 is dependent on natural killer cells [108, 109]. Moreover, PG545 exhibits a strong anti-lymphoma activity, eliciting lymphoma cell apoptosis, and involving ER stress response [110].

Protein tyrosine phosphorylation plays a pivotal role in various growth factors signaling to induce cell proliferation, differentiation, and survival. Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are the two counteracting proteins, which regulate tyrosine phosphorylation. PTP1B is a ubiquitously expressed non-transmembrane phosphatase that belongs to the protein tyrosine phosphatases superfamily, and the implication of PTP1B in dephosphorylation of Src (Y530) is well documented in the progression of oncogenesis in various cancers. Therefore, PTP1B has been emerged as a promising next-generation therapeutic target to design novel, effective, and bioavailable drugs to fight against cancer [111]. A synthetic strategy that could generate libraries of biologically active condensed-bicyclic triazolo-thiadiazoles identified inhibitors of PTP1B. Among such compounds, 1,2,4-triazolo-1,3,4-thiadiazoles presents human heparanase-1 inhibitory activity [112].

Compound 1-(2-hydroxyethyl)-2-imidazolidinone was synthesized as an inhibitor of both heparanase-1 and metalloproteinase 9 (MMP-9). The inhibition of basement membrane degrading enzymes such as heparanase-1 and MMP-9 may improve the epidermal barrier function of facial skin, which is exposed to the sun on a daily basis. Therefore, 1-(2-hydroxyethyl)-2-imidazolidinone is an effective way to care for regularly sun-exposed facial skin [113].

Heparanase-1 neutralizing monoclonal antibodies profoundly attenuated myeloma and lymphoma tumor growth and dissemination in preclinical models by targeting heparanase in the tumor microenvironment [97, 103]. As previously mentioned, peptides derived from TFPI-2 inhibitory site were shown to inhibit tissue factor/heparanase-1 complex and to attenuate sepsis severity and tumor growth in a mouse model [46]. Interestingly, aspirin binds to Glu225 at the active site of heparanase-1 and inhibits its enzymatic activity, preventing tumor metastasis and angiogenesis [114].

36.3 Heparanase-2 the Ugly Duckling or the Beautiful Swan

36.3.1 Heparanase-2 Cloning

Mackenzie and coworkers cloned a new heparanase, nominated human heparanase-2 or Hpa2 which differs from Hpa1 since it does not present catalytic activity. The gene encoding heparanase-2 is located on chromosome 10q23–24. There are three isoforms of heparanase-2 originated by alternative splicing containing amino acids 592, 534 and 480, termed Hpa2c, Hpa2b and Hpa2a, respectively. These isoforms are all membrane-associated proteins containing the C-terminal portion facing the cytoplasm. Heparanase-2c is the only variant capable of being secreted, possibly because it contains specific glycosylation sites, which are absent in Hpa2a and Hpa2b. It was also observed that unlike Hpa1, heparan sulfate proteoglycans on the cell surface (i.e., syndecans) are not able to promote internalization and proteolytic processing of heparanase-2 [115].

The coding region alignment of heparanase-1 and heparanase-2 shows 40% identity, including amino acid residues critical to the catalytic activity (Glu225 and Glu343). Moreover, heparanase-2 has a high affinity for heparan sulfate. The segment comprising the HS-binding regions and catalytic site of heparanase-1 are not conserved in heparanase-2, preventing heparanase-2 from being processed by proteolysis [115, 116] (McKenzie, Chap. 34 in this volume). The HPSE2 gene presents 12 exons, comprises approximately 630 kb and is located in a region (10q23–24) that is predisposed to loss of heterozygosity, characteristic of genomic instability in cancer. Molecular defects in the occurrence of loss of heterozygosity are derived from tumor suppressor genes, which protect DNA integrity or involve chromosome segregating genes that mediate correct separation of sister chromatids into daughter cells during mitotic cell division. Segregating genes may include genes involved in the determination of centromere structure, sister chromatid cohesion and genes involved in formation of the anaphase promoter complex [117]. Therefore, loss of heterozygosity, as well as segregating genes, are potentially involved in carcinogenesis [118].

The determination of genomic segments susceptible to loss of heterozygosity in solid tumors allowed the delineation of specific regions of the genome as tumor suppressor genes favoring a molecular profile of accumulation of genetic changes in a multi-step process during cancer progression [119, 120].

36.3.2 Heparanase-2 and Urofacial Syndrome

The urofacial syndrome, or hydronephrosis, comprises an autosomal recessive congenital disorder characterized by inverted facial expressions, an unusual facial expression, in association with obstructive urinary tract disease. The clinical symptoms of the urofacial syndrome are largely confined to the urinary tract, and patients appear to grimace when they smile. The main urologic features include urinary incontinence, bladder enlargement, renal complications, and many patients also

experience repeated episodes of urinary tract infections [121, 122]. The genetic characterization of urogenital syndrome identified HPSE2 gene as the primary candidate for such pathology [123, 124, 125] (Roberts and Woolf, Chap. 35 in this volume). It was identified as a great variety of mutations (deletions and missense) in all 12 exons of the HPSE2 gene in the human genome. However, it should be noted that many of the mutations in the HPSE2 gene are not related to the clinical features of the urogenital syndrome. Thus, the urogenital syndrome is probably underestimated, particularly when urinary tract characteristics are mild. Such phenotypic variability present in the urogenital syndrome may also be the result of environmental influences and other genetic modifications [123].

The second gene that characterizes the urogenital syndrome is LRIG2, leucine-rich repeats and immunoglobulin-2-like domains, which encodes a transmembrane family of proteins that modulate a variety of signaling pathways [126]. Surprisingly, LRIG2 gene mutant mice present a normal survival rate without detectable phenotype or exhibit slowed growth and a slight increase in spontaneous mortality [127, 128]. Both HPSE2 and LRIG2 represent proteins that co-localize with a neuronal marker, β3-tubulin, present in the human bladder, which justifies their potential involvement in neuronal modulation [125] (Roberts and Woolf, Chap. 35 in this volume). However, the fact that heparanase-2 knockout mice have been able to develop urofacial syndrome strongly suggests that HPSE2 is independently related to the development of such pathology. Furthermore, the presence of HPSE2 gene in a chromosome region susceptible to loss of heterozygosity may reinforce its involvement in carcinogenesis [129].

36.3.3 What Can we Learn from Heparanase-2 Knockout/ Knockdown Studies?

The HPSE2 analog gene was identified in frogs, showing that the protein is localized in the embryos neural tube region, where the motor neurons develop. Heparanase-2 knockdown in frogs was performed by morpholino; the *Xenopus tropicalis* embryos developed skeletal muscles paralysis, and motor neurons showed significant morphological alteration. Biochemically, HPSE2 knockdown in frogs caused an increase in FGF-2 expression, enhancement in cell signaling mediated by kinases, and transcription alterations of genes associated with neurons and muscles. It was hypothesized that the primary role of heparanase-2 in this model was modulation of FGF-2 and signal transduction during neural development, corroborating the phenotype found in urofacial syndrome [130]. Deletion of both HPSE2 alleles in mice caused a phenotype similar to urofacial syndrome, with bladder distended, abnormal voiding behavior, slow growth rate, renal dysfunction, malnutrition, and the animals die one month after birth. The mice also presented severe urological alterations, and the bladder is characterized by excessive presence of fibrotic tissue correlated with an increased level of transforming growth factor-beta (TGF-β), indicating that tissue remodeling involving such mutation is also related to the signaling of TGF-β [129].

Urogenital carcinoma is highly frequent in California sea lions (*Zalophus californianus*), and the etiology of such carcinoma was extensively studied and is

clearly multifactorial [131]. Interestingly, a genetic analysis of many generations of sea lions showed a single locus associated with the occurrence of bladder carcinoma, and in a case-control study, it was shown that bladder carcinoma in sea lions was significantly associated with homozygosity at the locus of the Pv11 microsatellite. Pv11 was mapped as a microsatellite of intron 9 of the HPSE2 gene, evidencing the relationship between this microsatellite and the HPSE2 gene and suggesting that HPSE2 gene alteration could be related to bladder carcinogenesis in sea lions [132].

Microsatellites are DNA repeated sequences and the most common microsatellite in humans is a dinucleotide repeat of nucleotides C and A, which occurs tens of thousands of times throughout the genome. Microsatellites are also known as single-sequence repeats. Although the length of these microsatellites is highly variable in different persons, the number of repetitions of such sequences contributes to the fingerprint of individual DNA. Therefore, each individual has microsatellites of definite length which occur at thousands of sites within a genome. Microsatellites are high spots for mutations compared to other areas of DNA, leading to high genetic diversity. Microsatellites are widely used for DNA profiling in cancer diagnosis, fingerprint analysis, paternity test, and forensic identification, to locate a gene or mutation responsible for a particular disease. Microsatellite instability is the condition of genetic hypermutability (predisposition to mutation) that results from repair of DNA incompatibility. The presence of microsatellite instability represents phenotypic evidence that repair mechanisms are not functioning normally. The evidence of HPSE2 gene mapped as a microsatellite and correlates with bladder carcinoma in California sea lions suggest that heparanase-2 might be involved with cancer.

36.3.4 Colorectal Cancer

The first article evidencing increased expression of heparanase-2 in human cancer revealed an enhancement of heparanase-2 in colorectal carcinoma compared to the non-neoplastic tissue and inverse correlation between heparanase-2 overexpression and downregulation of syndecan-1 [133]. Considering this article Giordano questioned whether heparanase-2 would be the ugly duckling representing a tumor marker or whether heparanase-2 could be the beautiful swan acting as a mechanism to compensate for the loss of syndecan-1 from the tumor tissue [134]. By this time, other authors have reported a decrease in syndecan-1 as a worse prognosis for colorectal carcinoma with a higher incidence of liver and lymph node metastases, as well as decreased patient survival and poor histological differentiation of tumors [135, 136]. Additionally, it was known that heparanase-1 could modulate adhesion and invasion of neoplastic cells by activation of Rho, independently of its enzymatic activity, corroborating the notion that heparanase-2, due to its similarity with heparanase-1, might be associated with carcinogenesis even without having a catalytic activity [137].

The presence of heparanase-1, heparanase-2, and syndecan-1 in colorectal adenomas, suggested a possible role of these molecules in progression of benign tumors. There was an inverse correlation between heparanase-2 and syndecan-1, as well as heparanase-1 and heparanase-2, while heparanase-1 and syndecan-1 showed a direct correlation [138]. The inverse correlation between heparanase-2 and heparanase-1 in

benign colorectal tumor suggests that heparanase-2 may be related to good prognosis since heparanase-1 is directly involved in tumor metastasis.

Zhang and coworkers reported that heparanase-2 represents a favorable prognosis in colorectal cancer, observing intense cytoplasmic labeling of heparanase-2 in gastric cancer compared to non-neoplastic tissue; however overexpression of heparanase-2 indicated higher survival of patients affected by such neoplasm [139]. Otherwise, among tissues collected from patients with colon carcinoma heparanase-1 was overexpressed specifically in cases of tumor metastasis, indicating that the most poorly differentiated carcinoma tissues presented the highest expression of heparanase-1 [140]. Thus, while heparanase-1 appears to be directly related to metastatic tumors, heparanase-2 appears to be overexpressed in benign tumors and does not increase in more advanced stages. The expression of both heparanase-1 and heparanase-2 isoforms (heparanase-2a, 2b, and 2c), was determined in plasma samples from 21 patients with gastrointestinal cancer and 43 healthy individuals. The results showed a significant increase in all heparanase species in the plasma of cancer patients compared to the control group. Additionally, the enzymatic activity of heparanase-1 was increased in all individuals affected by cancer compared to the control group [141], suggesting a potential noninvasive new diagnostic assay to detect both heparanase-1 and heparanase-2.

36.3.5 Breast Cancer

The interactions between tumor cells and ECM components are essential during invasion and metastasis. Tumor cells must destruct the basement membrane in order to be able to migrate into the connective tissue. Degradation of the ECM and basement membrane releases HS-bound active cytokines, growth factors, and angiogenic factors. In fact, degradation of HS chains by heparanase-1 generates oligosaccharides that intensify the action of such growth factors, cytokines, and angiogenic factors, thus inducing cellular proliferation, inflammation, and formation of new blood vessels, contributing to the carcinogenic process. While heparanase-2 lacks HS-degrading activity, it has a high affinity towards heparan sulfate, which can alter ECM dynamics leading to deregulated cancer cell proliferation and invasion. In fact, decreased heparanase-2 expression appears to represent an excellent diagnostic marker for the molecular subtypes of luminal A, luminal B, and triple negative breast cancer. Therefore, heparanase-2 by interacting intensively with HS chains prevents the deleterious action of heparanase-1. It was reported that heparanase-2 is downregulated in tumor tissues from patients with luminal A, luminal B, and triple negative breast cancer compared to non-neoplastic tissue, while metalloprotease-11 was overexpressed in all subtypes of breast cancer [142].

The evaluation of infiltrating ductal adenocarcinomas (metastatic and non-metastatic adenocarcinomas) evidenced a significant decrease of heparanase-2 [143]. Heparanase-2, as well as heparanase-1, are overexpressed in the mononuclear fraction of peripheral blood cells in patients with breast cancer. Circulating lymphocytes obtained from healthy individuals when incubated with plasma from patients with breast cancer express a significantly higher amount of both heparanases [144].

The mechanism of induction of heparanase-1 and heparanase-2 expression in circulating lymphocytes appears to be mediated by heparan sulfate secreted by tumor cells, proving a cross-talk between the tumor, tumor microenvironment and circulating lymphocytes [92].

36.3.6 Cervical and Endometrial Cancer

Immunohistochemical analyses showed a progressive increase of heparanase-2 according to the severity of cervical lesions comparing low-grade squamous intraepithelial lesions and invasive carcinoma, while the group of non-affected individuals presented lower expression of heparanase-2. This study revealed that heparanase-2 can be used as an auxiliary biomarker and contribute to improving the histopathological diagnosis of benign cervical lesions [145]. Labeling of heparanases in endometrial tissue has demonstrated that while heparanase-1 strongly targets advanced cancer in glandular tissue, heparanase-2 revealed strong staining at the endometrial stroma that is not affected by neoplasia [146]. Again, these results corroborate the notion that heparanase-2 is involved in early stages of tumor development and is present in benign tumors.

36.3.7 Ovarian Cancer

There was no significant difference in heparanase-2 expression between benign and malignant ovarian tumors, indicating that heparanase-2 is not exclusive for malignant tumors [147]. Interesting data were obtained using fertilization methods that involve superovulation. Fertilization induces the expression of several genes which participate in endometrial remodeling and affect trophoblast migration, embryo implantation, and endovascular invasion. A study that evaluated genes related to superovulation showed a significant reduction in heparanase-2 expression [148].

36.3.8 Bladder Cancer

Immunohistochemical analysis revealed that Heparanase-2 is expressed by bladder normal transitional epithelium and its expression level decreases substantially in bladder cancer. Notably, tumors that retain high levels of heparanase-2 have been diagnosed as low grade and low stage, suggesting that Hpa2 is possibly essential to preserve cell differentiation and disrupt cellular motility. In vitro, addition of recombinant heparanase-2 inhibited bladder carcinoma cell migration. Moreover, tumors produced by bladder carcinoma cells that overexpress heparanase-2 were smaller and of lower grade than tumors produced by mock-transfected cells [149]. Interestingly, the expression of Hpa2 in bladder stromal cells correlates with collagen deposition and a marked increase in lysyl oxidase (LOX) staining. The association between heparanase-2 and LOX expression was clinically confirmed by staining of bladder

cancer biopsy samples [149]. In summary, heparanase-2 seems to function in bladder tissue to maintain cell differentiation and decrease cell motility in a manner that appears to be independent of heparanase enzymatic activity.

36.3.9 Thyroid and Head and Neck Cancer

One of the major challenges for the diagnosis of thyroid cancer is to identify ideal markers that can distinguish between differentiated thyroid carcinoma and benign lesions. Ultrasound-guided aspiration is the most appropriate method to evaluate thyroid nodules. However, a significant percentage of the cytological examination has an indeterminate classification with malignancy proportions ranging from 10 to 30%. The anatomopathological evaluation of tissues obtained by surgical resection allows good diagnosis, but in this case, the patient has already undergone thyroidectomy, and often the thyroid ablation was unnecessary because the tumor is benign. Heparanase-2 seems to be an excellent marker to differentiate benign tumors from malignant thyroid tumors. Intense labeling of heparanase-2 in the colloid secreted by follicular thyroid cells along with negative stroma staining characterizes benign tumors. Conversely, negative colloid staining and intense labeling of the extracellular matrix by heparanase-2 indicates differentiated thyroid carcinoma [150].

Levy-Adam and coworkers proposed an elegant model of heparanase-2 action and demonstrated that the interaction of heparanase-2 with HS induces inhibition of heparanase-1 activity [116]. Cells obtained from head and neck tumors that overexpress heparanase-2 are abundantly decorated with stromal cells and collagen deposition, correlating with a marked increase in lysyl oxidase expression. In this study, it was observed that the enzymatic activity of heparanase-1 was not affected in cells that over-express heparanase-2, suggesting that reduced tumor growth is not due to the regulation of heparanase-1 by heparanase-2. Furthermore, the growth of xenografts that overexpress heparanase-2 was unaffected by administration of anti-Hpa2 monoclonal antibodies that inhibit the interaction of Hpa2 with HS, together indicating that the function of heparanase-2 does not depend on heparanase-1 activity or HS binding [151]. Hpa2 overexpression in head and neck cancer cells markedly reduces tumor growth due to inhibition of vascularization. Restrained tumor growth was associated with a prominent decrease in tumor vascularity (blood and lymph vessels), likely due to reduced Id1 expression, a transcription factor highly implicated in VEGF-A and VEGF-C gene regulation [151].

36.3.10 Heparanase-2 and Alzheimer's Disease

Alzheimer's disease is a neurodegenerative disease with a high incidence that causes progressive loss of memory and cognitive dysfunction and causes death due to chronic complications. Alzheimer's disease is caused by abnormal accumulation of cytotoxic peptides called amyloid-β (Aβ) that form senile plaques and intracellular accumulation of hyperphosphorylated forms of the microtubule-associated tau protein. HS proteoglycans favor Aβ or tau fibrillization and promote resistance to proteolytic

degradation of such protein aggregates [152]. Both heparanases (heparanase-1 and heparanase-2) are overexpressed and co-localized with Aβ aggregates in degenerate neurons and are also present in the extracellular matrix at different stages of Alzheimer's disease. While heparanase-1 is present in fragmented nuclei of senile plaques composed of β-amyloid deposition, heparanase-2 is found around senile compact plates [153].

Studies have shown that the enzymatic activity of heparanase-1 appears to decrease β-amyloid deposition or block the intracellular formation of tau fibrils by promoting degradation of HS chains, whereas heparanase-2 seems to act as a heparanase-1 inhibitor, competing for HS binding. Thus, it appears that both heparanases are involved in Alzheimer's disease [154, 74] (Li and Zhang, Chap. 25 in this volume).

36.3.11 Heparanase-2 as a Tumor Suppressor

Cancer is caused by sequential pathological variations or mutations, the transformation of proto-oncogenes into oncogenes and loss of function of tumor suppressor genes. However, it is also important to consider epigenetic changes that may alter the expression pattern of certain genes. Such epigenetic changes include DNA methylation as well as histone modifications. The complex between histones and DNA comprises the structural unit of chromatin. The organization of chromatin is regulated in part by post-translational modifications of histones. The complex of proteins called Polycomb act as transcriptional repressors that promotes the silencing of specific genes by chromatin modifications. Specifically, EZH2 protein belonging to the Polycomb complex is capable of promoting methylation of Lys27 residues of histone H3, leading to repression of target genes [155]. Together, the Polycomb complex plays central roles in epigenetic silencing of stem cell target genes, tumor metastases, and cancer [156–158]. Target genes of the Polycomb complex have been extensively investigated. In a study that evaluated the signature repression of the Polycomb complex in metastatic prostate cancer, 87 genes were described as down-regulated genes that were associated with worse clinical prognosis. Strikingly, HPSE-2 gene expression is repressed by the Polycomb complex, strongly supporting the tumor suppressor activity of heparanase-2 [156].

36.4 Conclusions

HPSE gene is located on chromosome 4q21.2 and the enzyme heparanase (Hpa-1) degrades HS chains and plays a role in the normal turnover of HS proteoglycans. The oligosaccharides generated by heparanase at the cell surface and ECM lead to a cascade of cellular events that affect a diversity of physiological processes, such as cell growth, adhesion, migration, and death. Hence, heparanase is being implicated in diverse cellular systems, including inflammation, neovascularization, tissue remodeling, carcinogenesis, tumor cell metastasis, and blood coagulation. Additionally, heparanase acts as a regulator of the syndecan-syntenin-exosome biogenesis pathway and enhances exosome secretion. Inhibitors of heparanase include

chemically modified heparins, small molecules, natural products, synthetic oligonucleotides, and neutralizing antibodies, and some of these compounds are currently in clinical trials and have been produced as novel cancer therapeutics.

Heparanase-2 is located on chromosome 10q23–24, and coding region alignment with heparanase-1 showed 40% identity. Additionally, heparanase-2 has no enzymatic activity but has a higher affinity for HS compared to Hpa-1. Heparanase-2 appears to be overexpressed in benign tumors and less aggressive tumors. Apart from attenuation of heparanase-1 enzymatic activity, heparanase-2 inhibits neovascularization mediated by VEGF, independent of heparanase-1 modulation and HS binding. The fact that the HPSE2 gene is located in a chromosomal region susceptible to loss of heterozygosity, and is under the control of the Polycomb complex, strongly suggests that HPSE2 may function as a tumor suppressor. It also plays important roles in embryogenic development and survival. Altogether, heparanase-2 may be looked upon as a beautiful swan, while heparanase-1 is the ugly duckling (Figs. 36.1 and 36.2).

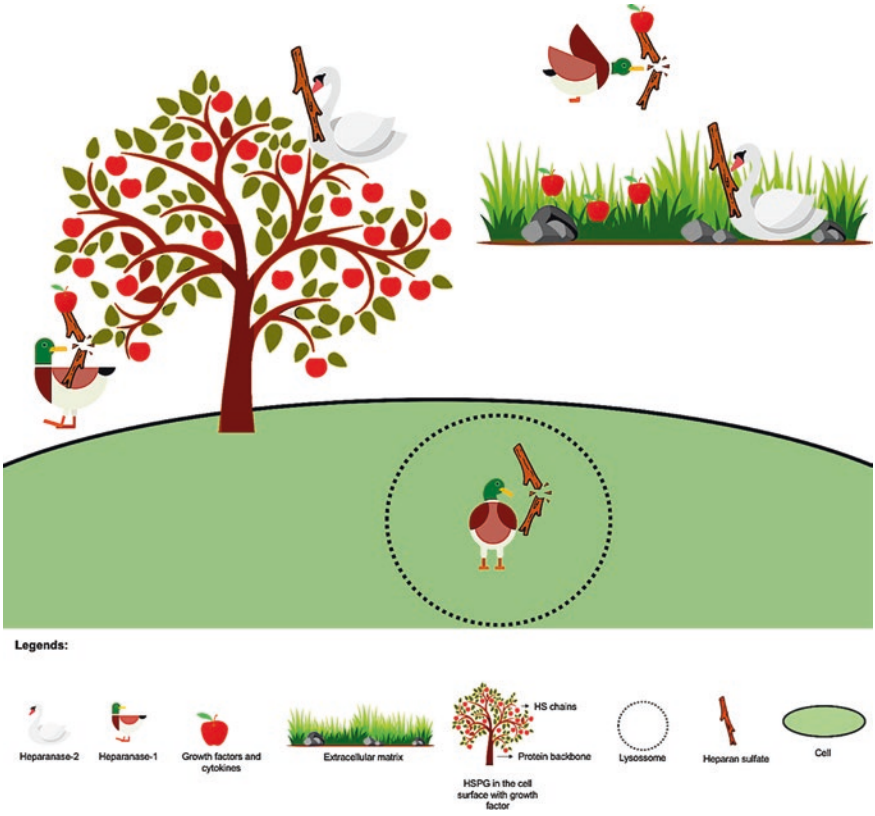

Fig. 36.1 Interaction of heparan sulfate with heparanase-1 and heparanase-2. Both heparanases interact with heparan sulfate side chains of proteoglycans (HSPG) at the cell surface and extracellular matrix. Heparanase-1 binds and degrades HS chains generating oligosaccharides with high affinity to growth factors, cytokines, and angiogenic factors, affecting cell proliferation, cell migration, angiogenesis, and inflammation. Heparanase-2 binds with high affinity to HS chains but does not cleave them. Heparanase is also involved in the lysosomal turnover of HSPG. Heparanase-2c is the only variant capable of being secreted, possibly because it contains specific glycosylation sites, that are absent in Hpa2a and Hpa2b. heparanase-2 is does not promote the internalization of HSPG.

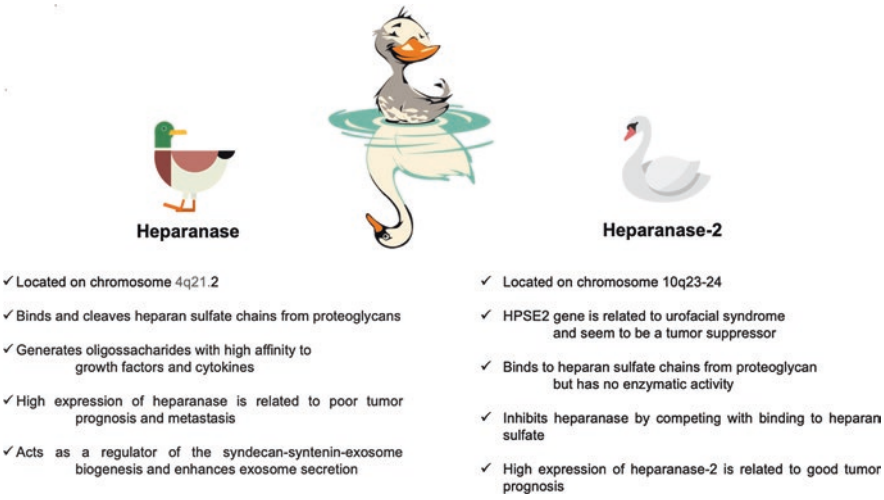

Fig. 36.2 Effects of heparanase and heparanase-2. The scheme summarizes the main characteristics and principal roles of both heparanases. While heparanase-1 is strongly associated with tumorigenesis and tumor metastasis, heparanase-2 plays a role in tissue development and appears to function as a tumor suppressor

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Chapter 37
Opposing Effects of Heparanase and Heparanase-2 in Head & Neck Cancer

Ilana Doweck and Nir Feibish

37.1 Head and Neck Cancer

Squamous cell carcinoma of the head and neck (SCCHN) is the most common cancer in the head and neck and is the sixth most common neoplasm in the world. More than 550,000 new patients are diagnosed annually with SCCHN and about 380,000 deaths are related to this malignancy [14]. In the USA, head and neck tumors account for 3% of all malignancies [43], whereas in Europe it is 4% [15]. The incidence is higher in males compared to females with a ratio of 1:2 up to 1:4, which vary geographically according to exposure to certain risk factors [5, 28]. The major risk factors for SCCHN are smoking and alcohol consumption. Furthermore, Tabaco and alcohol have a synergistic effect on the incidence of SCCHN [4, 31, 45], and the incidence of SCCHN increases with the increased consumption of both [4, 31, 45, 53]. Another important risk factor is human papillomavirus (HPV) that emerges as a significant risk factor in SCC of the oropharynx, specifically the tonsil and base of the tongue subsites. Malignant transformation is caused by HPV 16, however HPV 18, 31, and 33 are also connected to oropharyngeal carcinoma [51]. Another viral etiology that is generally accepted as the primary risk factor for non keratinizing nasopharyngeal carcinoma (NPC) is the Epstein-Barr virus (EBV). EBV-related differentiated NPC incidence has consistently increased over the past four decades in the US [1]. There is an improvement in the outcome of patients with SCCHN that is EBV or HPV related (nasopharyngeal and oropharyngeal carcinoma, respectively)

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[17, 29]. Still, the prognosis of patients with typical SCCHN that is associated with tobacco and alcohol use has not been changed significantly over the past decades [19]. SCCHN has a high propensity to lymph node metastases, especially when the primary tumor is located at the pharynx. It has been well documented that the prognosis of patients with head and neck malignancy is gravely influenced by the status of the involved metastatic cervical lymph nodes. The survival rates might drop down to half if the patient is presented with a metastatic node [41]. Tumor metastases are common among patients with head and neck cancer with uncontrolled local or regional disease, and autopsy studies revealed 40–47% overall incidence of distant metastases [25, 54]. The incidence of distant metastases among patients who remain free of disease at the local and regional sites is lower (18–20%), although still significant. With the improvement of the locoregional control of advanced head and neck cancer resulting from new treatment regimens, distant failure has emerged as the most common reason for disease recurrence [10, 38].

37.2 Heparanase

The enzymatic activity of the endo-β-D-glucuronidase heparanase is recognized for over three decades [37, 49]. Heparanase activity is considered a prerequisite for cellular invasion associated with tumor metastasis, inflammation, and angiogenesis, a consequence of heparan sulfate (HS) cleavage and remodeling of the sub-endothelial and sub-epithelial basement membrane and extracellular matrix (ECM) [23, 50]. The clinical significance of heparanase activity critically emerges from numerous publications describing induced heparanase expression in human hematological and solid tumors, and its inverse correlation with post-operative patients' survival, encouraging the development of heparanase inhibitors [12, 13, 33, 35, 39, 47, 42] (see Ferro and Chhabra, Cassinelli et al., Noseda and Barbieri, Hammond and Dredge, and Giannini et al., Chaps. 15, 19, 21, 22 and 23 in this volume). Cloning of a single human heparanase cDNA sequence was independently reported by several groups [21, 27, 46, 48], implying that one active heparanase enzyme exists in mammals. Further analysis of human genomic DNA led researchers to conclude that the heparanase gene is unique and that the existence of related proteins is unlikely [34].

37.3 Involvement of Heparanase in Head and Neck Cancer

A role of heparanase in head and neck tumors was first established in oral cavity cancer cell lines and tissues. Ikuta et al. investigated heparanase activities and levels of heparanase mRNA in both metastatic and non-metastatic human oral squamous cell carcinomas (SCC) cell lines and tissues [22]. Kurokawa et al. demonstrated a

positive correlation between heparanase enzyme activities and the invasiveness of xenografted human oral SCC cell lines [26]. Heparanase overexpression and increased activity were also reported in other types of head and neck cancers [44]. The clinical relevance of heparanase as a prognostic marker was first examined in a study that analyzed various head and neck cancer tissues derived from 25 patients. The authors concluded that the lack of heparanase in tumor cells was correlated with prolonged disease-free and overall survival [2]. Moreover, in salivary gland carcinoma, 70% of the patients with negative staining for heparanase were still alive 300 months (25 years) following diagnosis, whereas none of the patients stained strongly for heparanase survived at 300 months [3]. Nagler et al. analyzed the expression of heparanase in mobile tongue cancer tissues from 60 patients and found a three-fold increase in heparanase levels in the saliva of these patients compared to healthy controls. The authors suggested the use of salivary heparanase levels as a diagnostic and prognostic tool [36]. Recently, heparanase activity was shown to be elevated in malignant thyroid neoplasms compared to benign tumors and was proposed as an accurate diagnostic test for distinguishing between malignant and benign thyroid lesions [32]. Doweck et al. reported that the expression levels of heparanase in head and neck tumors were correlated inversely with patient outcome. The study included 74 patients with SCCHN and revealed the implication of the cellular localization of heparanase on tumor progression and patient survival. Favorable prognosis and prolonged survival were found in patients that exhibit no or weak heparanase staining (Fig. 37.1B). Notably, nuclear localization of heparanase (Fig. 37.1A, lower panel) predicted a favorable outcome compared to cytoplasmic localization of the enzyme [9] (Fig. 37.1C). The mechanism(s) responsible for heparanase induction in human head & neck tumors is not entirely clear but may involve viral infection. The first evidence of HPV protein E6 involvement in heparanase induction in SCCHN cell lines was provided by Hirshoren et al., reporting that the mechanism is p53-dependent [20].

Following the understanding that heparanase induction correlates with increased vascularity in numerous types of malignancies [11, 16, 24, 40, 42, 52], Cohen-Kaplan et al. utilized tumor specimens obtained from 65 SCCHN patients to illustrate the role of heparanase in lymph angiogenesis. In this study, heparanase staining intensity was positively correlated with lymphatic vessel density and lymph node metastasis via the elevation of vascular endothelial growth factor C (VEGF-C) [8] (Fig. 37.2). In another study by the same group, heparanase ability to enhance phosphorylation of epidermal growth factor receptor (EGFR), which in turn leads to head and neck carcinoma progression, was suggested as a second critical molecular system in which heparanase facilitates tumor growth [6]. A third signal transduction pathway, signal transducer and activator of transcription (STAT) proteins, was described as an important factor in both pre-clinical and clinical SCCHN settings. Specifically, STAT3 phosphorylation was associated with head and neck cancer progression, EGFR phosphorylation, and heparanase expression and cellular localization [7].

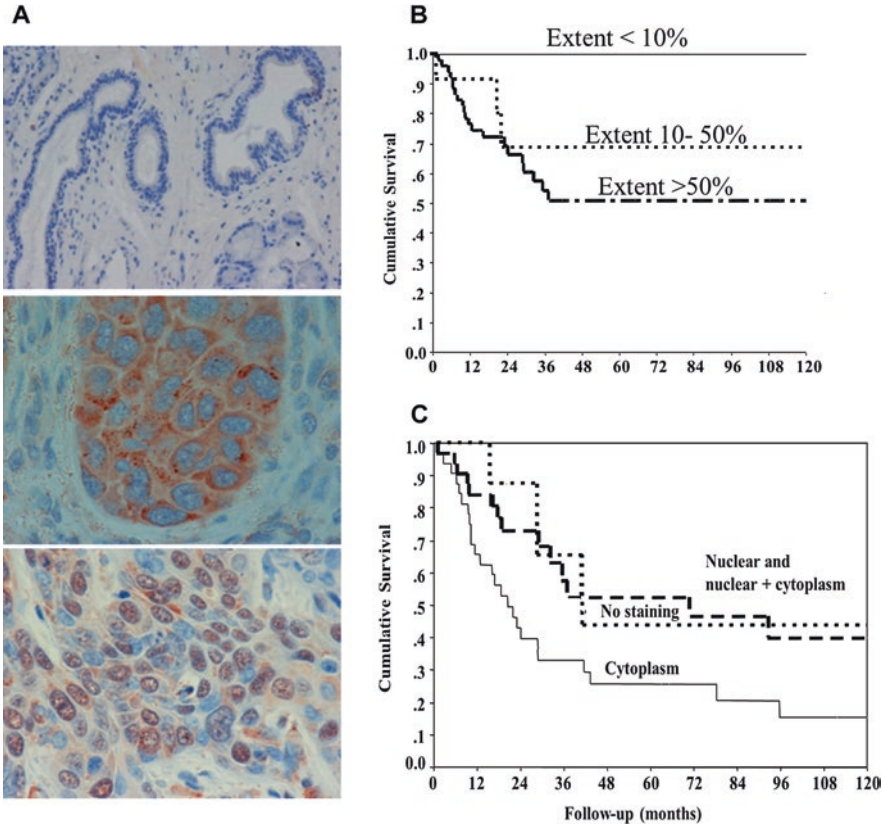

Fig. 37.1 (**A**) Immunohistochemical staining of heparanase in SCCHN tumor specimens. Formalin-fixed, paraffin-embedded 5-micron sections of head and neck tissue (**A**, upper panel) and tumors (**A**, middle and lower panels) were subjected to immunostaining of heparanase, applying anti-heparanase pAb #733. Shown are representative photomicrographs of heparanase negative normal head and neck epithelium (**A**, upper panel), and heparanase positive specimens exhibiting cytoplasmic (A, middle panel) and nuclear (A, lower panel) localization (9). (**B**). Cause-specific survival (Kaplan-Meier survival plot) of patients with SCCHN stratified by the extent of heparanase staining. Note that patients with low levels of heparanase staining (<10%) had 100% five-year cause-specific survival (9). (**C**). Overall survival of patients with SCCHN stratified by nuclear vs. cytoplasmic staining of heparanase (Kaplan-Meier survival plot). Log-Rank test, p = 0.03. (9)

37.4 Heparanase 2 in Head and Neck Cancer

Heparanase-2 (HPA2) is a close homolog of heparanase that lacks intrinsic HS-degrading activity but retains the capacity to bind HS with high affinity. It was cloned and described by McKenzie and colleagues, based on amino acid sequence homology [34] (McKenzie, Chap. 34 in this volume). HPA2 shares an overall identity of ~40% with heparanase and is not subjected to proteolytic processing as opposed to heparanase. Notably, HPA2 inhibits heparanase enzymatic activity,

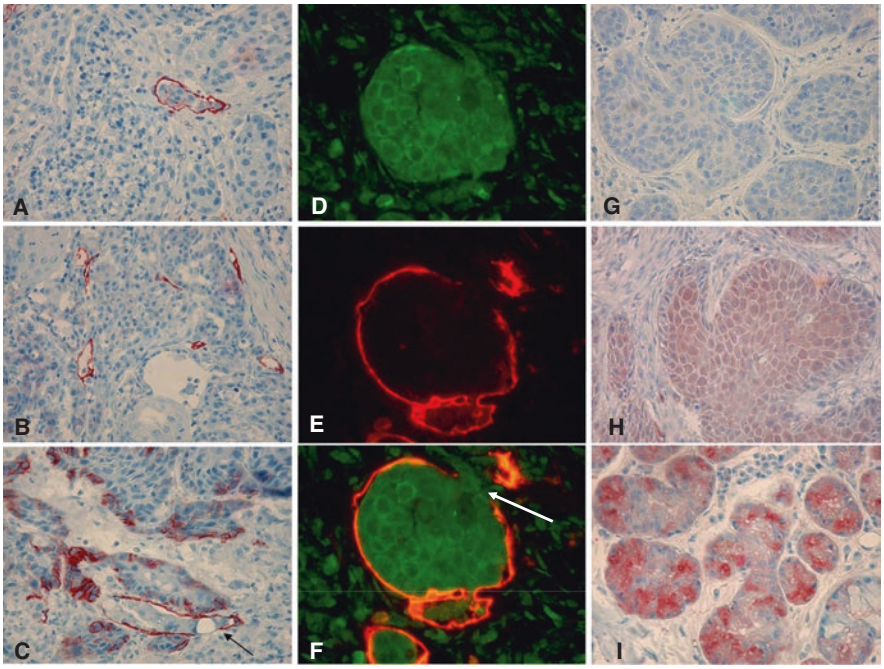

Fig. 37.2 Immunohistochemical staining of lymph vessels and VEGF-C in human head and neck tumor specimens. Formalin-fixed, paraffin-embedded 5-micron sections of 65 head and neck tumors were subjected to immunostaining, applying D2–40 monoclonal (A-C; anti-lymphatic endothelial marker) and anti-VEGF-C (G-I) polyclonal antibodies. Shown are representative photomicrographs of specimens depicting low (A; +1) and high (B; +2) number of lymphatic vessels. Lymphatic vessel (arrow) adjacent to D2–40-positive tumor cells is shown in (C). Shown are also photomicrographs of VEGF-C negative (G), and positively stained specimens scored as weak (H; +1) and strong (I; +2) intensity. D-F. Double immunofluorescent staining. Head and neck tumor specimen was stained with anti heparanase polyclonal (green, D) and D2–40 monoclonal (red, E) antibodies, illustrating heparanase-positive tumor cells inside a lymphatic vessel lumen (merge, F), penetrating the lymphatic endothelium (arrow) (8) (Permission by Int. J Cancer, John Wiley and Sons Inc.)

likely due to its high affinity to heparin and HS and its ability to associate physically with heparanase [30]. HPA2 expression was markedly elevated in head and neck carcinoma patients (Fig. 37.3), correlating with good prognosis and inversely correlating with patients' N-stage ($p = 0.02$) [30]. According to the authors, 57% of patients that stained negative (0) for HPA2 were diagnosed as N2–3 (advanced nodal metastases, which indicate advanced stage IV cancer), compared with only 13% of patients whose tumors were scored as high HPA2 ($p = 0.03$). No correlation was found between HPA2 staining and either tumor grade or T-stage. Likewise, an association was found between HPA2 staining and patients' follow-up to disease recurrence ($p = 0.006$) (Fig. 37.3). Thus, patients stained negative for HPA2 had a mean follow-up of 33.4 months, whereas the follow-up time was prolonged to 77.7 months for patients exhibiting high HPA2 staining [30]. Furthermore, the

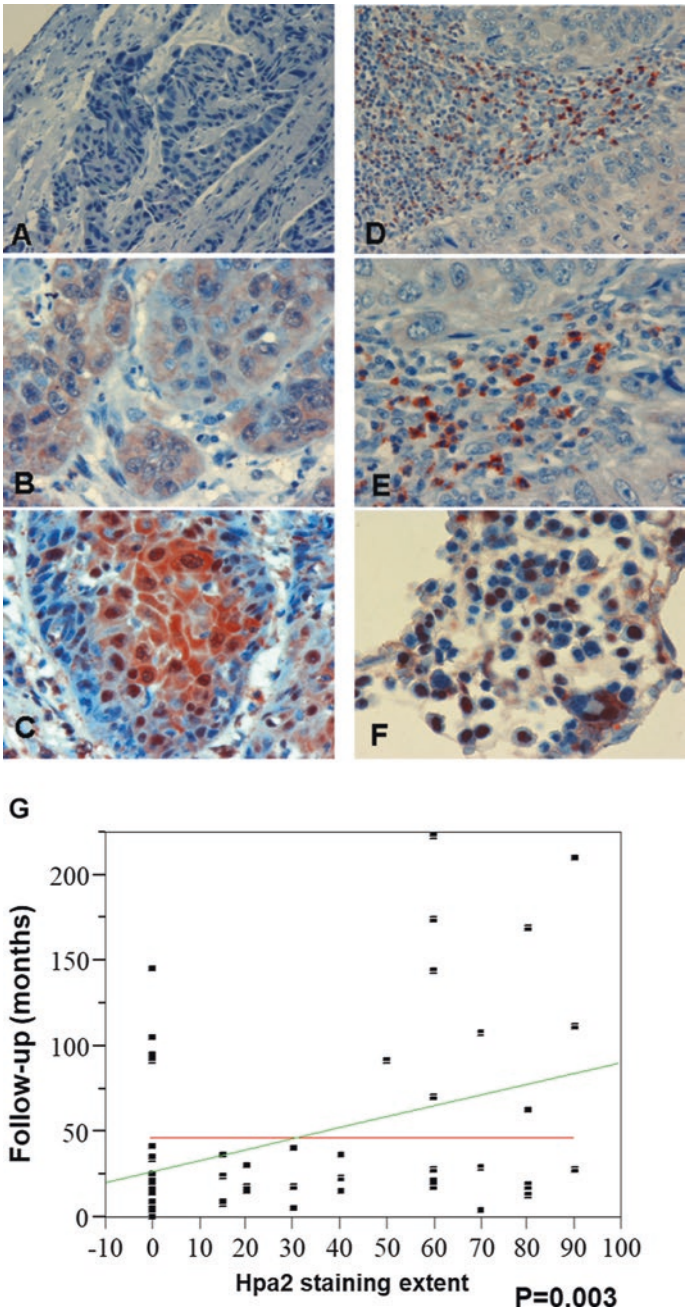

Fig. 37.3 Immunohistochemical staining of Hpa2 in human head and neck tumor specimens. Formalin-fixed, paraffin-embedded 5-micron sections of 58 head and neck tumors were subjected to immunostaining, applying anti-Hpa2 polyclonal antibody (Ab58). Shown are representative

authors reported a linear association between HPA2 staining extent and patients' follow-up time, clearly pointing to HPA2 expression levels as a favorable determinant in head and neck carcinoma [30]. HPA2 appears to inhibit tumor dissemination, suggesting that HPA2 functions as a tumor suppressor. Gross-Cohen et al. [18] provided evidence that HPA2 overexpression in head and neck cancer cells markedly reduces tumor growth. Restrained tumor growth was associated with a prominent decrease in tumor vascularity (blood and lymph vessels), likely due to reduced Id1 expression, a transcription factor highly implicated in VEGF-A and VEGF-C gene regulation. Interestingly, the authors reported that heparanase enzymatic activity was not impaired in cells overexpressing HPA2, suggesting that reduced tumor growth is not due to heparanase regulation. Furthermore, the growth of tumor xenografts by HPA2-overexpressing cells was unaffected by the administration of a monoclonal antibody that targets the heparin-binding domain of HPA2 [18], implying that HPA2 function does not rely on heparanase or HS. This implies that HPA2 mode of action is more complex than anticipated, and may involve interaction with additional cellular proteins that mediate its anti-cancer properties.

37.5 Concluding Remarks

Heparanase's pro-oncogenic features are known for over three decades and its roll in head and neck cancer has been well established in pre-clinical and clinical studies; similar conclusions were drawn from numerous studies in other types of solid and hematologic neoplasms (Vlodavsky et al., Ilan et al., Chaps. 1 and 9 in this volume). The enzyme's characteristics made it an attractive candidate both as a diagnostic tool and as a therapeutic target. Only recently, a new member of the heparanase family, HPA2, revealed the opposite set of virtues, acting as a potent inhibitor of its heparanase homolog, imposing a favorable prognosis for head and neck cancer patients. As the research concerning heparanase-HPA2 interactions is at infancy, it is yet to be affirmed whether HPA2 is indeed a tumor suppressor for SCCHN, and more work is required to translate this information into clinical practice.

Fig. 37.3 (continued) photomicrographs of Hpa2 negative (A), and positively stained specimens scored as weak (B; +1)and strong (C; +2) intensity. Positively-stained inflammatory cells adjacent to the carcinoma lesion are shown in low (D) and high (E) magnifications. In some specimens, bone marrow cells were present, exhibiting strong staining of Hpa2 (F). G. A linear, statistically-significant association ($p = 0.003$) between HPA2 staining extent and prolonged patients' follow-up. [follow up (months) = 27.4 + 0.6 x HPA2 extent (%)] (30)

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