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Involvement of heparan sulfate and related molecules in sequestration and growth promoting activity of fibroblast growth factor

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Abstract

Heparan sulfate proteoglycans (HSPGs) are ubiquitous macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of cells of vertebrate and invertebrate tissues [1, 2]. The basic HSPG structure consists of a protein core to which several linear heparan sulfate (HS) chains are covalently attached. The polysaccharide chains are typically composed of repeating hexuronic and D-glucosamine disaccharide units that are substituted to a varying extent with N- and O-linked sulfate moieties and N-linked acetyl groups [1, 2]. Beside serving as a scaffold for the attachment of various ECM components (e.g., collagen, laminin, fibronectin), the binding of HS to certain proteins has been suggested to induce a conformational change which may lead to the exposure of novel reactive determinants or conversely stabilize an inert protein configuration [1–4]. Of particular significance is the interaction of HS with fibroblast growth factors (FGFs), mediating their sequestration, stabilization and high affinity receptor binding and signaling [3–7]. Cellular responses to FGFs may hence be modulated by metabolic inhibitors of HS synthesis and sulfation, HS-degrading enzymes, and synthetic mimetics of heparin/HS. In the present review we focus on the involvement of HS in basic FGF (bFGF) receptor binding and mitogenic activity and its modulation by species of heparin, HS, and synthetic polyanionic 'heparin-mimicking' compounds. The results are discussed in relation to the current thoughts on the dual involvement of low and high affinity receptor sites in the growth promoting and angiogenic activities of bFGF and other heparin-binding growth factors.

Involvement of heparin and heparan sulfate in angiogenesis

Heparin, heparan sulfate (HS) and related polysaccharides have long been implicated in the angiogenic process. The earliest indication that heparin may be involved in the regulation of angiogenesis was the finding that mast cells accumulate at the site of tumor angiogenesis before capillary ingrowth [8]. Mast cells or the medium in which they were incubated significantly stimulated the locomotion of capillary endothelial cells *in vitro*. This activity was attributed to heparin since it was abolished by protamine and heparinase [8, 9]. Heparin also played a key role in the purification of the first tumor derived angiogenic factor which was found to be bFGF [10]. Subsequently, several other growth promoting factors were purified based on their affinity to heparin, constituting a growing family of heparin-binding growth factors, of which many are highly angiogenic [8, 11]. It soon became apparent that the heparin-affinity of these growth factors was more than just a useful technique for purification. Both heparin and HS were found to

i) potentiate the mitogenic effect of acidic FGF on vascular endothelial cells *in vitro* [12],
ii) stabilize and protect FGFs from inactivation [6, 13]; and
iii) function as low affinity receptors that sequester bFGF and facilitate its interaction with high affinity signaling receptors on the cell surface [7, 14].

The heparin affinity of FGFs also appears to be the basis for their storage in basement membranes (BM) and the ECM of cells and tissues, where they are bound to HS and can be released in an active form by species of heparin and heparin-like molecules and by heparin/HS degrading enzymes [4, 15, 16]. The released FGF may then stimulate cell proliferation in processes such as neovascularization, tissue repair and restenosis. Specific sequences of HS have been found in the nucleus [17]. It was suggested that fragments of HS may act as natural chaperones to shuttle bFGF or other growth factors to different cellular compartments [8]. Under certain conditions, heparin may also inhibit angiogenesis, either by itself or in conjunction with 'angiostatic' steroids [8, 18].

Heparan sulfate sequestration and release of bFGF

Our studies on the control of cell proliferation by its local environment focused on the interaction of cells with the ECM produced by cultured corneal and vascular endothelial cells (EC) [4, 15]. This ECM closely resembles the subendothelium *in vivo* in its morphology and molecular composition. Vascular EC plated in contact with the subendothelial ECM, no longer require the addition of soluble FGF in order to proliferate [15]. This observation, together with the presence of HS as a major glycosaminoglycan (GAG) in the subendothelial ECM raised the possibility that ECM contains heparin-binding growth factors that are tightly bound and stabilized by the ECM' HS. Indeed, bFGF was extracted from the subendothelial ECM produced *in vitro* [15] and *in vivo* [16], suggesting that ECM may serve as a reservoir for bFGF. Immunohistochemical staining was applied to study the localization of bFGF in various tissues. Immunoreactive bFGF was identified in BM of the cornea [16] and in BM underlying endothelial [19] and epithelial [20] cells. Because the bFGF gene lacks a consensus signal peptide, the mode of its deposition into ECM is not clear. It was suggested that intracellular FGF may be released in response to a mild cell damage and certain stress conditions associated with tissue injury, irradiation, inflammation, shear force, heat shock and tumor necrosis. The released factor may then be sequestered from its site of action by means of binding to HS [21] and possibly to bFGF receptor proteins in the ECM [22], and saved for emergencies such as wound repair and neovascularization [4, 23]. It appears that bFGF binds primarily to HS in ECM and BM since the majority of the bound growth factor was displaced by heparin, HS, or HS-degrading enzymes (i.e., heparanase), but not by unrelated GAGs or enzymes [21, 24].

The involvement of sulfate groups in bFGF sequestration by the subendothelial ECM was studied by growing the ECM producing cells in the presence of chlorate, a potent inhibitor of sulfation [25] (Figure 1). Both the bFGF content and growth promoting activity of sulfate depleted ECM were less than 10% of native ECM, indicating that sulfate moieties of HS are involved in bFGF sequestration and growth promoting activity of the ECM. Basic FGF is also sequestered by HS on cell surfaces as revealed by

i) immunohistochemistry [19],
ii) release by glycosyl phosphatidylinositol specific phospholipase C (PI-PLC) [26, 27], and
iii) displacement by heparin from the lumenal surface of blood vessels [24, 28].

Heparanase, an endoglycosidase that specifically degrades HS was found to be a most efficient specific releaser of active bFGF from ECM and cell surfaces [27, 29] (Figure 1). Our studies suggest that heparanase activity expressed by metastatic tumor cells and activated cells of the immune system may not only function in cell migration and invasion, but at the same time may elicit an indirect neovascular response by means of releasing the ECM-resident FGF [4, 23]. Apart from HS-degrading enzymes, active bFGF is released from ECM by thrombin [30] and by plasmin [31], as a noncovalent complex with HSPG (Figure 1). Kinetic and thermodynamic con-

siderations suggest, however, that an enzymatic degradation event may not be necessary for interaction of HS-bound bFGF with the high affinity signaling receptor on the cell surface.

Despite the ubiquitous presence of bFGF in normal adult tissues, EC proliferation in these tissues is usually very low, with turnover time measured in years. This raised the question of how bFGF and possibly other growth factors are prevented from acting on the vascular endothelium continuously and in response to what signals do they become available for stimulation of capillary EC proliferation. Truncated FGF receptors were identified in the BM of retinal vascular endothelial cells [22]. The presence of high affinity FGF receptors in the ECM may explain, in part, why the proliferation of FGF target cells is so low *in vivo* despite the presence of bFGF in the adjacent basement membrane [22]. It appears that the matrix stores of bFGF are sequestered by both low affinity HSPG and truncated high affinity FGF receptors. It is conceivable that restriction of FGFs in ECM and BM prevents their systemic action on the vascular endothelium, thus maintaining a very low rate of EC turnover and vessel growth. On the other hand, release of bFGF from storage in ECM may elicit a localized EC proliferation and neovascularization in processes such as wound healing, inflammation and tumor development [4, 23]. It should be also noted that under normal *in vivo* situation, the lack of EC proliferation in response to ECM-resident bFGF may be simply due to the closely apposed and contact inhibited configuration of the cells. However, once the cells are released from contact inhibition (i.e., in response to stress conditions and tissue injury) cells that remain bound to the ECM, but are no longer growth arrested, become susceptible to stimulation by the ECM-bound bFGF until they regain their characteristic contact inhibited cobblestone morphology.

Other ECM-bound growth- and differentiation-promoting factors

It is now recognized as a general concept that the ECM and in particular HSPG provides a storage depot for many of the known growth factors, enzymes and plasma proteins [3, 4]. Unlike bFGF and other members of the heparin-binding growth factors, transforming growth factor β (TGF-β), binds to HSPG on the cell surface (betaglycan) and ECM (decorin) through the core protein rather than HS moieties [3]. Primary human bone marrow cultures produce bFGF that is sequestered by HSPG of the stromal matrix and cell surface. This HSPG may serve as a reservoir for bFGF from which it is released in an active form, primarily by PI-PLC [26]. HS in the marrow stroma also binds IL-7, IFNγ, IL-3 and GM-CSF [4, 32]. These cytokines, once bound, can be presented in a biologically active form to hematopoietic cells, thereby providing a mechanistic explanation for the dependence of hematopoietic cells on intimate contact with stromal cells. Likewise, proteoglycans on EC can capture pro-adhesive cytokines (i.e., IL-8, MIP-1β) and present them to passing leukocytes that have come into contact with the endothelium after the initial tethering step of the adhesion cascade [33] and prior to their actual transmigration. It should be noted that the platelet heparanase has recently been identified as a member of the CXC family of chemokines [34]. We have demonstrated that while at slightly acidic pH values (pH 6.4–6.8) heparanase may participate in cell invasion and bFGF release, at a physiological pH the enzyme appears to act as a pro-adhesive molecule that may organize the recruitment of cells towards an extravascular loci [35].

Differential involvement of cell surface- and ECM-associated HS in bFGF receptor binding and growth promoting activity

Based on previous studies it appears that the Kd value for interaction of bFGF with the cell surface HS is lower [36] than for interaction with HS in the ECM [21], suggesting that ECM-bound bFGF may interact first with HS on the cell surface and only then presented to high affinity cell surface receptors. Because the cell surface HSPG, unlike that of the ECM is mobile in the plane of the membrane and can turn over more rapidly by shedding and internalization, it may readily replenish its bFGF

from the ECM reservoir, which appears to serve more as an efficient large capacity bFGF storage depot in the vicinity of cells [37]. In support of this functional difference between HSPG in the cell surface and ECM are our recent studies applying chlorate treated nonsulfated cells and ECM [25] and HS degradation fragments released from cells and ECM. We investigated whether HS in ECM may function as accessory receptors directly accelerating the arrival of bFGF at its high affinity signaling receptors, in a manner similar to HS on the cell surface. For this purpose, the involvement of the cell surface HS was abolished by treating endothelial cells with chlorate. Chlorate treated cells expressing nonsulfated HS side chains failed to respond to bFGF when plated on regular tissue culture plastic [14, 38] or on sulfate depleted ECM [25]. A slight mitogenic response was obtained when the chlorate treated EC were seeded in contact with native ECM and best results were obtained when heparin was added to the culture medium [25]. These results indicate that ECM-resident bFGF can not be efficiently presented to high affinity signaling receptors when the cell surface HS is nonsulfated, suggesting that the ECM'HS may not function efficiently as an accessory low affinity receptor capable of presenting the growth factor directly to the signaling receptor. In other words, it appears that HS on the cell surface play an active role in the presentation of ECM-resident bFGF to its high affinity cell surface receptors. Soluble heparin may fulfill a similar function, as demonstrated by its ability to stimulate the proliferation of chlorate treated endothelial cells in response to ECM.

A difference between cell surface- and ECM-derived species of HS in their ability to promote the mitogenic activity of bFGF was also demonstrated in our studies on the growth promoting activity of HS degradation fragments released by bacterial heparinase III from ECM vs. cell surfaces. In these experiments we applied a cytokine dependent, HS-deficient lymphoid cell line engineered to express the mouse FGF receptor-1 [39]. It was found that HS fragments released from the surface of vascular endothelial and smooth muscle cells by heparinase III were capable of serving as accessory receptors participating in a dual receptor mechanism characteristic of bFGF. In contrast, little or no such activity was associated with HS fragments released by heparinase from the subendothelial ECM. It is conceivable that species of HS derived from cell surfaces contain the appropriate saccharide sequences capable of promoting bFGF receptor binding, dimerization and activation, which are not found in HS degradation fragments released from ECM. Altogether, these studies demonstrate that properly sulfated HSPG associated with the cell surface and ECM act in concert to regulate the bioavailability of active bFGF and possibly other effector molecules to their signal transducing receptors.

The presence of two classes of receptors for a given growth promoting factor is not unique for FGF. For example, TGFβ, neurotrophin and insulin growth factor 2 (IGF-2) bind with high affinity to transmembrane signaling receptors and with low affinity to cell surface receptors that cannot transmit signals alone, but somehow modulate the ability of the growth factor or the signaling receptor to generate a biological response [40]. The low affinity nonsignaling receptors are thought to deliver and present the ligand to high affinity signaling receptors which may then trigger biological responses [40].

Role of low affinity HS sites in bFGF receptor binding and activation

Examining the role that cell- or ECM-associated HSPG play in mediating the biological activities of FGFs is complicated because all cells that express FGF receptors also display HSPG on their surface or ECM. Therefore, to better assess the involvement of HS in FGF signaling, it was necessary to remove the HS or to impair its ability to interact with FGFs. This was brought about by HS-degrading enzymes or metabolic inhibitors of HSPG synthesis [14, 41] and sulfation [25, 38] (Figure 1). Alternatively, soluble FGF-receptors and HS-deficient FGF-receptor negative cells were transfected with individual FGF receptors and the ability of these receptors to bind FGFs and mediate their biological activities was examined in the absence and presence of exogenously added heparin or HS. Us-

ing these approaches it was reported that cell surface heparin-like molecules are necessary for bFGF high affinity receptor binding [7] and growth promoting activity [38, 39]. Recently, however, Roghani et al., showed that heparin increases the affinity of bFGF for FGF-receptor-1 (FGFR1) by a factor of 2–3, but is not essential for the interaction itself [42]. This contention is in contrast with the prevailing opinion, but in agreement with previous studies showing bFGF-receptor binding in the absence of heparin [43, 44]. It is not clear whether heparin is required for bFGF mediated c-fos induction, but it is necessary for stimulation of DNA synthesis and cell proliferation [42, 45], suggesting that heparin/HS mainly stabilizes the FGF molecule and hence affect primarily long term effects of the growth factor. This was supported by the finding that the dissociation rate of the bFGF-receptor complex is decreased in the absence of HS [44]. At present, it is not clear what is the source of discrepancy between the various studies. Since these studies utilized different cell types, different receptor isoforms or different growth factors, it is rather difficult to assess which of these variables could account for some of the discrepancies. Alternatively, the inability of bFGF to bind to its high affinity receptors is due to damage occurring during purification of the growth factor or its radiolabeling procedure [42]. Impairment of biological activity during purification or iodination was recently reported for vascular endothelial growth factor (VEGF) as well. It was initially thought that heparin is required for binding of VEGF to its tyrosine kinase receptor. However, part of the effect of heparin is apparently due to restoration of the biological activity of the damaged growth factor [47]. Since heparin and HS protect FGFs from inactivation by heat and oxidation [13], this protective effect could account for some of the opposing conclusions in experiments designed to assess the importance of heparin/HS in FGF-receptor binding.

It was recently proposed that the primary function of the low affinity receptors is to reduce the dimensionality of ligand diffusion from three to two dimensions [40]. In other words, binding of ligands such as bFGF to abundant low affinity cell surface receptors that are restricted to lateral mobility in two dimensions, increases the local concentration of the bound ligands, thereby enhancing the probability of their interaction with the less abundant high affinity signaling receptors [40]. Because the binding sites on FGF for HS and for the signaling receptor are distinct, dissociation from the low affinity HS receptors may not be necessarily required for ligand binding to the high affinity receptor, resulting in the formation of a ternary complex [40]. The interplay among the three participants of the trimeric complex (i.e., bFGF, HS, FGF-receptor) is poorly understood and the possible modes of interactions are a matter of debate. While FGF may bind to both receptors at the same time, evidence for a direct specific interaction between FGFR1 and heparin/HS were obtained [14, 48]. This was supported by the identification of both activating and inhibitory heparin sequences for bFGF [38]. It was proposed that binding of HS to both bFGF and FGFR1 and formation of a trimeric complex is a prerequisite for receptor dimerization and activation [38]. Receptor dimerization leads to an increase in kinase activity, resulting in autophosphorylation and the induction of diverse biological responses [45]. Heparin binds to FGF in a multivalent manner and induces the oligomerization of bFGF [39]. Consequently, in the case of monomeric ligands such as members of the FGF family, binding to HS and formation of a multivalent FGF-HS complex is obligatory for FGF-induced receptor dimerization and subsequent activation [45]. It should be noted that a stoichiometry of 2:1 (2FGFR: 1FGF) was also reported [44]. It was suggested that bFGF binds its receptor in a manner similar to that reported for growth hormone [49] while heparin stabilizes the complex. On the basis of these considerations and regardless of the exact dual receptor mechanism of action, inhibition of bFGF interaction with low affinity HS accessory receptors is likely to suppress bFGF mediated cell proliferation and neovascularization. In fact, heparin/HS degrading enzymes (bacterial heparinase I and III) were shown to inhibit both neovascularization in the chick embryo and proliferation of capillary endothelial cells mediated by bFGF [50].

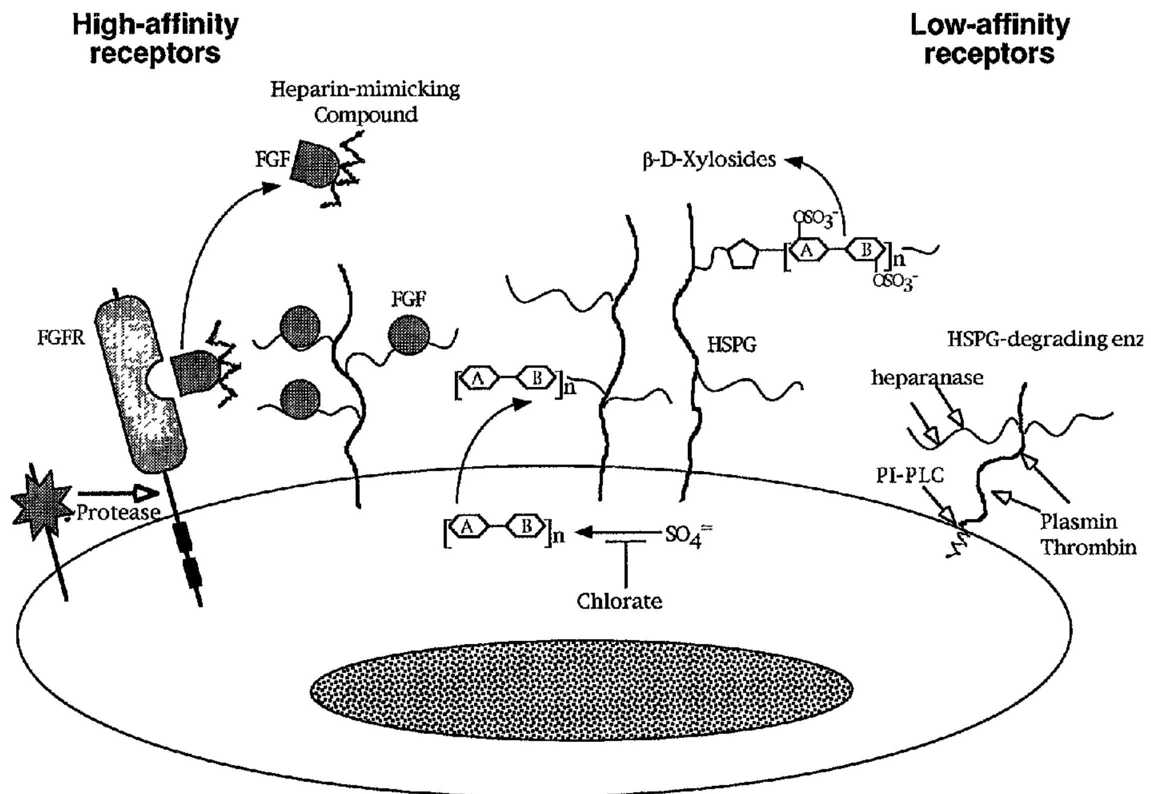

Figure 1. Modulation of bFGF receptor binding and activation by metabolic inhibitors of HS synthesis and sulfation, HS-degrading enzymes and heparin-mimicking compounds. Modulation of bFGF binding to low affinity cell surface receptor sites can be brought about by i) enzymes that degrade the HS side chains (e.g., heparanase) or core protein (e.g., plasmin, thrombin, PI-PLC) of HSPG (right), and ii) soluble primers (β-D-xylosides) of HS synthesis, and metabolic inhibitors (e.g., chlorate) of sulfation (center). Binding of bFGF to high affinity cell surface receptor sites can be modulated by i) heparin-mimicking compounds (i.e., compound RG-13577) that bind the growth factor and prevent receptor binding and/or dimerization, and ii) proteolytic enzymes (e.g. gelatinase A) that cleave the extracellular domain of the receptor and release soluble forms of the receptor (left).

Species of HS as potential inhibitors or differential modulators of FGF-receptor binding and dimerization

The effect of heparin on the biological activity of FGFs was initially examined by adding heparin to cells expressing both FGF receptors and HSPG. In such cells, heparin or HS can either stimulate or inhibit the biological activities of FGFs. Heparin, for example, potentiate the binding of bFGF to FGFR1 and FGFR4. By contrast, binding of bFGF to the keratinocyte growth factor (KGF) receptor and its ability to induce a mitogenic signal in keratinocytes was inhibited by heparin [51]. While various purified preparations of cell surface HSPG (i.e., syndecan, fibroglycan, glypican) failed to promote and potentiate receptor binding and mitogenic activity of bFGF [52], perlecan, the large basal lamina proteoglycan, was recently found to potentiate, at exceedingly low concentrations, high affinity binding of bFGF to HS-deficient cells and to soluble FGF receptors [53]. The marked difference in the capacity of various species of HSPG to potentiate receptor binding of bFGF led us to investigate the possible role of 'non-active' HSPG preparations as potential inhibitors of heparin-mediated bFGF receptor interactions. While anti-proliferative sulfate rich HS oligosaccharides, isolated from arterial tissue inhibited heparin induced bFGF-receptor binding, sulfate poor arterial derived HS oligosaccharides with no antiproliferative activity, were ineffective [52]. Such an inverse relation between the ca-

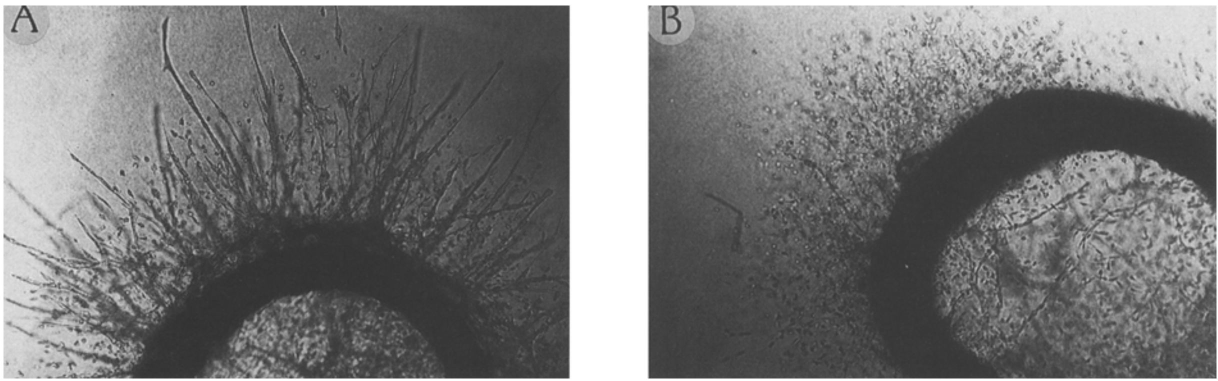

Figure 2. Effect of compound RG-13577 on microvessel tube formation. Rat aortic rings embedded in collagen gels were incubated (12 days, 37° C) in the absence (A) and presence (B) of 25 μg/ml compound RG-13577. Microvessels branching from the aortic ring and giving rise to loops and networks are seen in (A). In the presence of RG-13577, single cells migrate from the ring toward the periphery, but fail to align into microvessels (B).

pacity of heparin/HS to promote receptor binding and the ability of certain species of HS to suppress this specific interaction, strongly suggest that species of cell surface- and ECM-derived HSPG may also function as FGF growth suppressers by direct interference with the interaction between bFGF and receptor promoting species of HS and heparin [52].

The FGFs represent a multi-ligand and multi-receptor family in which one receptor can bind several ligands with high affinity. The question of how the interaction of the various ligands with the same high affinity receptor is coordinated has not been elucidated. That HSPG may be involved in this coordination was suggested by the finding that heparin potentiates the mitogenic activity of acidic FGF but inhibits that of KGF in cells that express the KGF receptor [54]. Both growth factors bind the KGF receptor with high affinity. Treatment of cells that naturally express the KGF receptor and cells that were engineered to overexpress it with either chlorate or heparinase reduced the binding of acidic FGF by 80% and at the same time enhanced the binding of KGF to high affinity cell surface receptors [54]. Thus, a cell associated HS, depending on its local concentration, may either promote or restrict the binding of a certain FGF to its high affinity receptor. This suggests a regulatory role for HS as coordinators of the interaction of FGFs, KGF and other heparin-binding growth factors with their signaling receptors and of the related cellular responses [54].

Heparin-mimicking compounds

In an attempt to identify potent mimetics that can regulate abnormal FGF signaling, we have synthesized a series of negatively charged, non-sulfated aromatic compounds that mimic many of the effects of heparin [55–57]. These non-toxic polyanionic non-sulfated compounds were found to compete with bFGF binding to HS on the cell surface and ECM (Figure 1). Compound RG-13577 (polymer of 4-hydroxyphenoxy acetic acid and formaldehyde ammonium salt, Mr~ 5,800) and related compounds were also found to inhibit the proliferation of vascular smooth muscle cells [56] and to revert the transformed phenotype of bFGF-transfected cells [55]. Direct interaction between compound RG-13577 and bFGF was suggested by the ability of the former to release bFGF that is bound to ECM or heparin-Sepharose [55]. Compound RG-13577 also inhibited bFGF receptor binding (Figure 1), as demonstrated in cross-linking experiments between vascular smooth muscle cells and ^{125}I-bFGF [57]. In subsequent experiments we investigated whether heparin mediated dimerization of bFGF is inhibited by the polyanionic heparin-mimicking compounds. Unlike heparin, compound RG-13577 alone failed to induce dimerization of bFGF. Moreover, it abrogated the dimerizing effect of heparin [57]. Next, we have utilized HS deficient CHO cell mutants and demonstrated that heparin-mediated dimerization of FGF receptors on the surface of

these cells was prevented when the cells were incubated with bFGF and heparin in the presence of compound RG-13577. These effects may be held responsible for the antiproliferative activity of compound RG-13577 and related 'heparin-mimicking' molecules. The anti-angiogenic effect of compound RG-13577 was demonstrated by its ability to inhibit the outgrowth of microvessels from aortic rings embedded in a three dimensional collagen gel. While branching microvessels forming a capillary network was developed at the periphery of untreated aortic rings (Figure 2A), in the presence of compound RG-13577 single cells were migrating from the aortic ring, but they failed to align into microvessel tubes (Figure 2B).

The development of define, non-toxic anionic compounds may therefore provide a new strategy to interfere with the mitogenic and angiogenic activities of heparin binding growth factors. We propose that synthetic heparin analogs that bind to FGF but fail to mediate its oligomerization, could effectively inhibit bFGF-heparin/HS induced activation of the FGF signaling receptor. Such analogs may therefore provide a potential avenue for pharmacological intervention with undesirable effects of heparin-binding growth factors in processes such as tumor angiogenesis, diabetic retinopathy, and restenosis of balloon injured blood vessels.

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