Sajal Chakraborti · Naranjan S. Dhalla
Editors

Pathophysiological Aspects of Proteases

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Springer

Editors
Sajal Chakraborti
Department of Biochemistry
and Biophysics
University of Kalyani
Kalyani, West Bengal
India

Naranjan S. Dhalla
Institute of Cardiovascular Sciences
University of Manitoba
Manitoba
Canada

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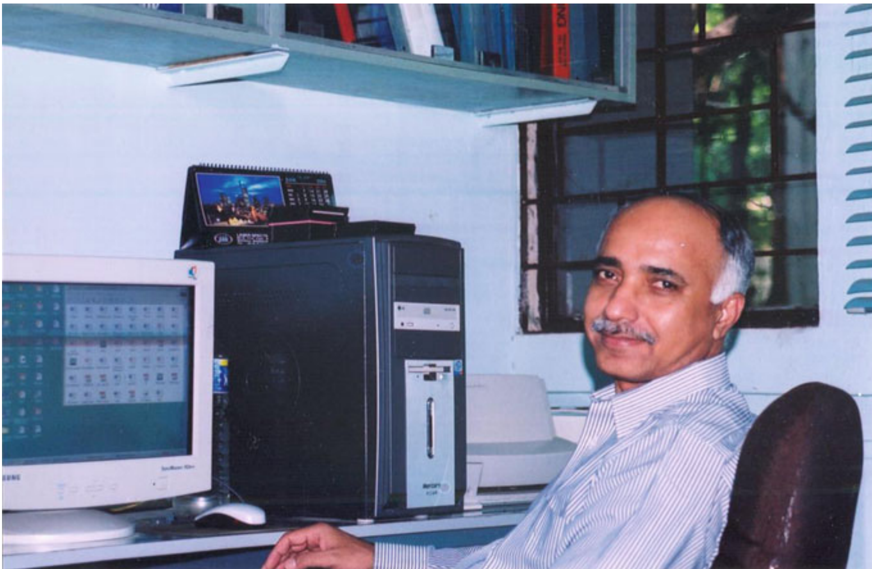

This book on Pathophysiological Aspects of Proteases is dedicated to Prof. K. Muniyappa, who was born on 8 September 1952 at Chickaballapur in the state of Karnataka, India. His individual and group achievements in the field of biochemistry and molecular biology have been spectacular and have secured him unique position in several areas of frontier research.

Prof. K. Muniyappa is a Former Chairman of the Department of Biochemistry at the Indian Institute of Science, Bangalore, and the Founding Coordinator for the National Research Associateship Program (and a similar program for the Northeast region) of the Department of Biotechnology, Govt. of India. He obtained his B.Sc. and M.Sc. degrees from the University of Mysore and doctoral degree from the Indian Institute of Science, Bangalore. He did his postdoctoral research at Yale University School of Medicine, USA. He has served as the American Cancer Society Visiting Professor at the University of Washington, Seattle and Royal Society Scholar at MRC, London. His research interests involve various aspects of chromosome biology in both prokaryotes and eukaryotes. Beyond research laboratory, his interest includes capacity building in higher education and research.

Prof. Muniyappa has received a plethora of national and international prizes and awards. To mention a few: S.S. Bhatnagar Prize (highest national award in Science & Technology), IISc Alumni Award for Excellence in Research, J.C. Bose National Fellowship, Sir M. Visvesvaraya Award by Govt. of Karnataka (highest state award), Kempe Gowda award for persons of eminence by GoK, Golden Jubilee award of the University of Mysore, Prof. Vishwanath Memorial award, American Cancer Society Eleanor Roosevelt Award, Yamagiwa International Cancer award (Switzerland) and the Rockefeller Foundation Career Development Award (USA). He is an elected Fellow of all the major Science Academies in

India, a Member of The Third World Academy of Sciences (TWAS), the Founding Member of the Karnataka State Academy for Science and Technology and Member of the Karnataka Vision Group in Science and Technology. He has been the Chairman/Member of several Task Force, PAC and Award committees of DBT, CSIR, ICMR, DST, UGC, UPSC and TWAS, and faculty/scientist selection committees of several universities and National Research Institutes.

Prof. Muniyappa undoubtedly is a legendary figure in Indian Science. Prof. Muniyappa has excellent ability to inspire and motivate young researchers. We feel honoured to dedicate this book to Prof. Muniyappa and wish him good health and success in his long fruitful activities.

Preface

Thou hast made me endless, such as thy pleasure.
This frail vessel thou emptiest again and again, and fillest it ever with fresher life. This little flute of a reed thou hast carried over hills and dales and hast breathed through it melodies eternally new. At the immortal touch of thy hands my little heart loses its limits in a great joy and gives birth to utterance ineffable. Thy infinite gifts come to me only on these very small hands of mine. Ages pass and still thou pourest and still there is room to fill.

Rabindranath Tagore (Gitanjali: Song of offerings)

This book is a product of a long-standing interest of the editors to handover compilations of chapters on a number of proteases and their pathophysiological consequences to the scientific fraternity.

The existence of proteases has been known for centuries. Their use was noted very early in our everyday life. In early days, the properties of proteases were exploited by man for food processing. Proteases from *Aspergillus oryzae* were used to modify wheat gluten, a component of bread indigestible to many people, which also affects yield of loaf processing. Gastric juice was demonstrated to be full of proteases and known to be responsible for food digestion.

Proteases play important physiological functions including cell division, regulation of functional and structural protein turnover and activation of zymogens, formation and lysis of blood clot, entry of sperm into ovum and fertilization, processing and transport of secretary proteins across membrane, regulation of gene expression and also in infection of pathogens. Based on the catalytic site on substrate, proteases are mainly classified into endoproteases and exoproteases. However, considering the mechanism of catalysis, proteases are classified into six distinct classes: aspartic, glutamic, metallo, cysteine, serine and threonine proteases.

In the recent past, inhibitors of angiotensin converting enzyme (ACE) and HIV proteases have shown substantial therapeutic success in developing drugs. However, due to difficulties of clear understanding of the selectivity of the active site of target protease, discovery of new drugs appears to be challenging. Therefore,

more basic research is needed on proteases with reference to their physiological and pathological consequences. In this book, eminent scholars illuminated us with new information, which, we believe, will surely benefit researchers to unravel, at least, some of the yet unknown complexities of proteases and their pathological implications.

This book contains twenty-nine chapters, which are contributed by established investigators from across the globe. We are grateful to all the authors for enthusiasm, energy and precious time that they spent on their respective chapters to materialize this project. We would like to thank Praveenkumar Vijayakumar, Dr. Madhurima Kahali and Vinoth Selvamani (Springer) for their persuasiveness to achieve our goal. We are also thankful to Prof. Sankar Kumar Ghosh, Vice Chancellor, University of Kalyani for his encouragement.

Kalyani, India Sajal Chakraborti
Manitoba, Canada Naranjan S. Dhalla

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About the Editors

Dr. Sajal Chakrabori is a Professor of Biochemistry in the Department of Biochemistry and Biophysics, University of Kalyani, Kalyani, West Bengal, India. He received his Ph.D. in Biochemistry from the University of Calcutta in 1982, and D.Sc. in Biochemistry from the University of Kalyani in 2004. His main areas of interest are regulation of phospholipase A2 and -D, Na+/K+ -ATPase, Ca2+ATPase and NADPH oxidase in pulmonary vascular cells; biochemistry of proteases and anti-proteases; and oxidant and calcium signalling by different stimulants such as angiotensin-1, thromboxan A2, endothelin-1 and Leukotriene D4 in regulating pulmonary vascular tone.

He received a Fulbright visiting fellowship to pursue research at the Department of Medicine, The Johns Hopkins University, USA, from 1987 to 1989, and at the Pulmonary Division, University of Utah Medical School and New York Medical College, New York, USA, from 1989 to mid-1990. He joined the University of Kalyani as a Reader in Biochemistry in 1998. Dr. Chakraborti is actively involved in research and has more than 100 publications in prominent journals to his credit. He has also contributed chapters to books published by Springer and Elsevier. He has edited four books with Springer namely: Proteases in Health and Disease; Role of Proteases in Cellular Dysfunction; Regulation of Membrane Na/K-ATPase; and Regulation of Ca2+ ATPases, V-ATPases and F-ATPases.

Dr. Naranjan S. Dhalla is Distinguished Professor at the University of Manitoba, Winnipeg, Canada. His expertise includes the subcellular and molecular basis of heart function in health and disease. He has been engaged in multidisciplinary research and education for promoting the scientific basis of cardiology, as well as training of professional manpower for combating heart disease for over 45 years.

Part I
Role of Proteases in the Development of Cancer

Role of Proteases in Breast Cancer

Sandra Radenkovic, Kristina Gopcevic, Gordana Konjevic and Vladimir Jurisic

Abstract

Proteolytic enzymes comprise five classes depending on their catalytic mechanisms: serine, cysteine, aspartic, threonine, and matrix metalloproteinases (MMPs). Proteases play an important role in all stages of cancer progression: cancerogenesis, invasiveness, and metastasis. Level and activation of proteases is associated with progression of breast cancer. Cathepsins are proteases involved in tumor formation, and activity of Cathepsin D (CatD) is considered as an independent tumor marker for breast cancer patients. Processes of apoptosis, cell proliferation, growth, angiogenesis, and metastasis are enhanced by CatD. MMPs represent a family of proteases that are involved in processes of invasion and metastasis through cleavage of basement membrane and remodeling of extracellular matrix. Considering that breast cancer had highly aggressive biology, it has been investigated and found that high activity of MMPs, particularly MMP-2 and MMP-9, is engaged in all stages of tumor progression. As proteases are involved in physiological processes such as immune response and in pathological conditions, it seems that examinations of molecular pathways of breast cancer could define a therapeutic target based on proteases.

S. Radenkovic (✉) · G. Konjevic
Department of Radiation Oncology and Diagnostics, Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia
e-mail: stankovics@ncrc.ac.rs; radenkovics6@gmail.com

K. Gopcevic · G. Konjevic
Faculty of Medicine, University of Belgrade, Belgrade, Serbia

V. Jurisic
Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

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1 Introduction

Activity, structure, and role of proteases and their inhibitors were extensively investigated over past 20 years. Enzymes called proteases are subdivided into four subclasses: serine ($\sim$180), cysteine ($\sim$160), aspartic ($\sim$25), threonine ($\sim$30), and matrix metalloproteinases ($\sim$200). Proteases in established classes cleave peptide bonds through several different mechanisms. Serine proteases, cysteine proteases, and amino-terminal nucleophile hydrolases (threonine proteases) had an enzyme side chain in the active cleft that involves in catalysis (covalent catalysis) [1]. On the other side, aspartic acid proteases and metalloproteinases have peptide-bond hydrolysis without covalent involvement, by creating a highly reactive water molecule [2].

Proteases and their function is essential for physiological processes, as well as pathological processes such as cell cycle progression, cell migration, tissue remodeling, neuronal outgrowth, hemostasis, wound healing [3]. Considering that proteolysis is essential for life, all organisms have proteases [4–6].

Considering that increased expression of proteases is involved in processes of tumor progression and autoimmmune diseases, blocking of their expression is usually therapeutic approach [7, 8]. Proteases are usually used as markers for prognosis and follow-up, and they are involved in process of cell death, such as caspases. Matrix metalloproteinases and cathepsins have an important role in cancer progression, especially in breast cancer [8, 9], but exact function must be investigated furthermore [10].

Proteases are involved in all steps of tumor formation and dissemination: tumor initiation, metastasis, and invasion. Each class of human proteases has specific endogenous inhibitor: Cystatins are inhibitors of cysteine proteases; serpins are inhibitors of serine proteases; and specific tissue inhibitors of metalloproteinases (TIMPs) are inhibitors of metalloproteinases [10, 11]. Thus, progression of malignancy is tightly connected to the activity of both proteases involved in cancer and specific inhibitors of proteases [8].

In complex process of tumor formation are included many specific proteases which make prognosis very difficult. The published investigations have shown that cancer progression is connected with activity of different proteases. Cancer cells express proteases, but they also induce its expression in non-neoplastic neighboring cells favoring tumor expansion [11]. The cells of adjacent tissue have been transformed and enhance all steps of cancer progression [8]. The cancer degradome represents a group of all enzymes include in processes of tumor formation and metastasis [12].

2 Proteases in Breast Cancer

Today, breast cancer represents an important health problem in world cause [13]. However, screening which includes mammography and ultrasound has helped detection of non-palpable lesions [14]. Also, investigation regarding tumor progression and new therapeutics enhance survival of breast cancer patients [8, 14].

Breast cancer is one of the most common cancers in women, and, even after chemotherapy, 50% of the patients die from distant relapse or metastasis [15]. Predictive factors can be used to predict response or lack of response to a particular therapy, and prognostic factors can be useful in making decisions concerning patients who should receive adjuvant therapy [16, 17].

Considering that new targets for therapy, prognosis, and follow-up are crucial for breast cancer management, further investigation regarding pathophysiology of breast cancer is necessary. Novel studies showed that insufficient expression of adhesion molecules, such as cadherins, and shorter overall survival of breast cancer patients [18]. In addition to the cadherins family, other adhesion molecules have been studied aiming at understanding their role in mammary carcinogenesis in order to associate such discoveries with the development of new therapeutic targets. MMPs as well as claudins are involved in invasive pseudopodes of tumor edge and are associated with increased metastatic potential [8].

In this review, we evaluate the role of proteases in breast cancer progression and management.

3 Cathepsins in Breast Cancer

The maintenance of the steady state in the cell requires the precise regulation of protein synthesis as well as the protein degradation. For intracellular protein degradation two systems are responsible: the lysosomal as the main system, and the ubiquitin-proteasomal system. Among the approximately 50 known lysosomal hydrolases, the aspartic, serine, and cysteine proteases are the best studied [10, 12]. In an organism, there is a dynamic equilibrium between cells necessary for growth and development and cell which are not necessary. These unwanted cells are eliminated by apoptosis, programmed cell death, with involvement of caspases. Lysosomal acid hydrolases comprise a group of 14 members of three catalytic mechanisms: cysteine (B, C, F, H, L, K, O, S, V, W, and X), aspartate (D and E), and serine (G) cathepsins [11].

Cathepsins are produced as inactive proenzymes, and the process of autolysis converts them in active forms. Cathepsins usually are exposed at the cell surface. They are able to degrade proteins of ECM in the extracellular space [19].

Cysteine protease Cathepsin B (EC 3.4.22.1) is widely distributed and functions in intracellular protein catabolism, in processing of antigens in the immune response, hormone activation, and bone turnover. The role of propeptide is to drive cathepsin precursor in the endoplasmic reticulum, to enable its hydrolysis. The

proregion inhibits cathepsin proteolytic activity that preserves its three-dimensional structure at neutral pH and maintains the precursor in an inactive state until cleaved [20]. Cathepsins are processed to the active forms either autocatalytically or by other proteases and transferred to lysosomes either through endosome maturation or through endosome lysosome fusion in late endosomes. However, it has been shown that breast cancer carcinomas as well as colon, colorectal, cervix, head and neck, liver, lung, melanoma, ovarian, pancreatic, prostate, and thyroid tumors have increased expression of CatB [10, 19, 20].

Cathepsin D (CatD), (EC 3.4.23.5) a soluble aspartyl protease, has a role in cancer development and considered as an independent tumor marker for breast cancer patients. CatD is synthesized in rough endoplasmic reticulum as preprocathepsin D (pCatD), 52 kDa inactive precursor, and after its subsequent conversion by proteolytic processing to its active form, CatD (44 kDa) becomes two-chain molecule of 31 and 14 kDa. After activation CatD is transported to the acidic endosomes, lysosomes and phagosomes [11, 21].

In breast cancer cells, unlike in normal ones, are found high levels of pro-cathepsin D due to both overexpression of the gene and altered processing of the protein. [22]. pCatD (the propeptide of CatD) is a glycoprotein secreted by human breast cancer cell lines which are hormone dependent. Two biological activities of pCatD, mitogenic on estrogen-depleted MCF-7 cells, suggesting that it is an estrogen-regulated autocrine mitogen, and an acidic proteolytic activity on different substrates and on proteoglycans and basement membranes, have been demonstrated in vitro [10]. Both activities—growth promoting and extracellular proteolytic—suggest that pCatD may have prognostic importance in breast cancer [21]. It has been reported that both pCatD and CatD induce proliferation and migration of cancer cells, fibroblasts, and endothelial cells [11, 21]. It has been evidenced pCatD may induce mitogenic response by both proteolytic-dependent and proteolytic-independent mechanisms by the induction of fibroblast and cancer cell proliferation [11]. This study confirmed upregulation of pCatD expression in estrogen receptor-positive breast cancer cell lines treated with estrogen. In an excellent study of Ohri and colleagues has been demonstrated that, as a mitogen, pCatD asts as a protein ligand rather than as an enzyme, and that expression and secretion of pCatD achieved by transfection of pCatD construct in HBL-100 cells increased the metastatic potential of this cell line [21].

CatD is distributed in lysosomes, endosomes, and phagosomes. This enzyme is primarily involved in degrading missfolded or non-functional proteins intracellularly. Up to now, many important roles of CatD in normal physiological and pathological conditions were studied [10]. Physiological functions of CatD are based on its ability to degrade intracellular proteins, and activate and degrade polypeptide hormones and growth factors. Furthermore, CatD takes part in several steps of mitosis, regeneration, activation of leukocytes, and regulation of vassal wall permeability. The proteolytic activity of the enzyme is regulated by various intralysosomal factors including pH, products of metabolism, hormones, growth factors, and specific inhibitors. Hormones, e.g., estrogens and progesterone, can

regulate the synthesis of cathepsin D and its receptor mannose 6-phosphate [10, 11, 21].

Both extracellular and intracellular activities of CatD are involved in multiple tumor progression steps [22]. Due to the lysosomal membrane permeabilization, CatD is transferred to the cytosol and through several steps leads to the release of cytochrome c from mitochondria into the cytosol, where it is inhibited by pepstatin A, which partially delay IFN-gamma and Fas/APO-induced apoptosis [8, 11]. Thus, proteolytic activity of CatD is one of the key steps in apoptotic cascade, besides Bcl-2 protein family, TNF, and p53 [23, 24]. CatD accumulation in an estrogen-positive breast cancer cells could be a consequence of higher secretion of its proenzyme [11].

Overexpression of CatD at the mRNA and protein level, demonstrated by various methodological approaches: in situ hybridization, cytosolic immunoassay, Northern and Western Blot, immunohistochemistry has been confirmed. Concentration of CatD was higher 2–50 folds, compared to its concentrations in other cells of normal mammary glands. CatD overexpression is correlated with increased risk of clinical metastasis and short survival in breast cancer patients. In the plasma of patients with metastatic breast carcinoma, the increased serum pCatD levels were detected [21]. These studies suggest that CatD stimulates cancer cell proliferation, fibroblast outgrowth, angiogenesis, and metastasis and is a marker of poor prognosis in breast cancer.

4 Cadherins

Among the mechanisms responsible for initiation and progression of tumor, the control of cellular adhesion and motility is one of the crucial mechanisms. Normal cells are connected with each other and the surrounding stroma by different adhesive molecules.

The beginning phase of metastatic cascade includes the local invasion of the tissue by tumor cells which should separate from each other, by lacking restrictions in homotypic cell adhesion and cell–cell contact inhibition. Endothelial cadherins (E-cadherins), a family of transmembrane glycoproteins, represent the best-studied molecular markers expressed in epithelial and breast cancer cells [8, 25, 26]. They are responsible for the structure of epithelial tissues by providing the connection between endothelial cells and the surrounding stroma. E-cadherin is one of the most important molecules in cell–cell adhesion in epithelial tissues. It is localized on the surfaces of epithelial cells in regions of cell–cell contact known as adherens junctions.

The extracellular domain of E-cadherin mediates calcium-dependent homotypic interaction with E-cadherin molecules on adjacent endothelial cells, while the intracellular domain binds cytosolic catenins and provides a link to the actin cytoskeleton [8, 25].

Therefore, in many carcinomas, the lack of expression of E-cadherin correlates with an invasive and dedifferentiated phenotype. Furthermore, the normal function of E-cadherins depends on their association with catenins. The loss of adhesive E-cadherin molecules and the E-cadherin–catenin complexes leads, morphologically, into impaired structure of the cytoskeleton and allows amoeboid movement and motility, but also deregulates the cell cycle and leads to cell proliferation. The observed decrease in adhesive force facilitates dispersion of carcinoma cells from the primary tumor mass. In addition to promoting passive dissemination of carcinoma cells, loss of E-cadherin function can also promote cell invasiveness [8, 25, 26].

Aside from these, there is yet another mechanism to change E-cadherin function, its proteolytic modification. E-cadherin can be altered by degradation of E-cadherin's extracellular domains by stromelysin-1 (MMP-13), a member of MMP-family that has been closely connected with tumor progression [27, 28]. Constitutive expression of active stromelysin in mammary epithelial cells results in cleavage of E-cadherin and progressive phenotypic changes in vitro, including loss of catenins from cell–cell contacts, downregulation of cytokeratins, and upregulation of vimentin and MMP-9 [8]. These changes result in unfavorable epithelial-to-mesenchymal transition of cellular phenotype. In vivo stromelysin expression promotes breast carcinogenesis that involves certain genomic changes [8, 25, 27]. Thus, a decrease in cell–cell adhesion is associated with malignant conversion, tumor cell invasion, and the beginning of metastasis.

The process of epithelial-to-mesenchymal transition (EMT) is characteristic of most carcinomas and represents the progression toward malignancy, associated with loss of epithelial differentiation and, consequently, a shift toward a mesenchymal phenotype. This process is considered as a crucial event in late stage tumorigenesis and a prerequisite for tumor infiltration and metastasis [8, 29]. EMT provides mesenchymal characteristics for epithelial cells to overcome the physical constraints imposed on them by intercellular junctions and adopt a motile phenotype. The transition from a well-differentiated epithelial phenotype to an unfavorable invasive mesenchymal phenotype may include diverse molecular mechanisms that may independently enhance motility and invasiveness. EMT is characterized by proteolytic loss of E-cadherin or endothelial to dysfunctional neural-cadherin (N-cadherin) "switching" and by increasing motility plays a crucial role during invasion and metastasis of carcinomas [27]. Without tight junctions, provided by E-cadherin, mesenchymal lacks an apical–basolateral polarity and transform to an elongated morphology. This morphology is characterized by front–back asymmetry that facilitates motility and locomotion [8, 27, 28]. At the invasive edge of the mesenchymal cells are situated filopodial extensions, enriched with integrin family receptors that interact with the ECM to enhance motility. Filopodial extensions contain MMPs, particularly MMP-2 and MMP-9, that promote metastasis by digesting components of the basement membranes and ECM [8].

Transforming growth factor β (TGF-β) is crucial protein in inducing EMT of various epithelial cell types. During EMT, many proteins are upregulated: Src kinase, integrin-linked kinase, integrin h-5, and MMP-11, MMP-12, and MMP-14

that induce cytoskeletal remodeling and promote cell motility and invasiveness [8, 27]. The plasticity of malignant carcinoma cells due to diverse molecular mechanisms that enhance EMT also allows incomplete EMT, reversion to an epithelial phenotype, and collective migration. As these mechanisms can manifest in a series of independent and reversible steps, EMT represents just one mechanism in the global metastatic carcinoma development process [8, 25]. Moreover, loss of tumor suppressor gene, p53, allows an upregulation of MMP-2 and downregulation of E-cadherin modulation of Slug and regulation of cancer cell metastasis [29, 30].

The additional loss of heterotypic adhesion of endothelial cells, maintained by β1 and β2 integrins (e.g., VLA-1), as receptors for different components of ECM, BM, a highly specialized layer of the extracellular matrix composed of dense, intertwined fibrils of collagen type IV and glycoproteins, such as laminin and heparan sulfate proteoglycan, as well as similar components in the extracellular matrix (ECM), also allows detachment and invasion [8, 28]. A fundamental difference of neoplastic cells is the loss of anchorage requirement for cell survival and growth. The anchorage-independent growth of tumor cells may result from an uncoupling of cell survival signals transduced from the ECM by attachment, coupled with activation of cell cycle progression, and is associated with neoplastic transformation [8].

5 Proteolytic Pathways

In the process of degradation of surrounding tissue tumor cells use two proteolytic systems. It has been proposed that a cascade of degradative enzymes clears a path at the advancing edges of invasive tumors, through which the cancer cells move [27]. The inactive, pro-enzyme forms of the degradative enzymes (zymogens) are locally secreted and become active by the action of their regulatory enzymes which are produced either by the tumor cells themselves or the surrounding stromal cells (fibroblasts, mast cells). Such zymogens include pro-cathepsins, plasminogens and procollagenases, heparanases and matrix metalloproteinases (MMPs). In the process of invasion, the most important pathways are MMPs and plasminogen activation system [8, 31]. Plasminogen activation is an extracellular proteolytic system, which converts the inactive plasminogen into a trypsin-like serine protease plasmin. Plasmin has very broad substrate specificity and can degrade matrix proteins and can convert other zymogens (i.e., MMPs) to their active forms by proteolysis. Two plasminogen activators have been defined urokinase plasminogen activator (uPA) and tissue-type PA (tPA) [8, 32]. It has been shown that uPA is crucial in cell migration and invasion [8, 33].

Plasminogen activation by uPA occurs at the cell surface where the inactive proenzyme of uPA is bound by its cellular receptor uPAR. Pro-uPA is activated by an unknown mechanism but remains bound to its receptor (uPAR) where it is capable of activating plasminogen. uPA is often secreted, aside from tumor cells, by stromal fibroblasts. In this way, highly focalized centers exist where controlled

areas of proteolysis can occur at the leading edge of the invading cell [8, 31]. The inhibition of receptor-bound uPA by plasminogen inhibitors, PAI-1 and PAI-2, regulates the invasive process and influences the “proteolytic activity map.” The potential importance of u-PAR for the development of minimal residual disease in solid cancer and its biological relevance for tumor cell dormancy has been described due to its influence on the angiogenic switch [8, 33].

The uPA-plasmin system has a role in the control of gelatinase (MMP-2, MMP-9) activity. MMP-2 and MMP-9 are secreted in the form of inactive zymogens that are activated extracellularly, a fundamental process for the control of their activity. Both gelatinases are associated with the cell surface, binding of uPA and plasminogen to the cell surface results in gelatinase activation without the action of other proteinases, and the inhibition of uPA or plasminogen binding to the cell surface can blocks gelatinase activation [3, 8]. Plasmin in soluble phase degrades both proteinases and proteinase activation [8, 33].

The term “cascade” warrants some revision for some other postulated proteolytic cascades. For example, in proteolytic matrix remodeling, the existence of a cascade, or even of a proteolytic pathway, is questioned by considerable biochemical and genetic evidence. Perhaps the concept of a redundant network of interactions is more appropriate in this case. It is now considered that many of the metalloproteinases once thought to degrade the matrix might in fact be limited processing enzymes, the substrates of which are cytokines and growth factors [3, 34]. It is also unclear whether the plasminogen activator–plasmin–metalloproteinase pathway exists anywhere but in vitro, and the 30-year-old concept that the matrix must be broken down to allow cell migration (in normal or in metastatic instances) is under close scrutiny and revision [34]. Indeed, the possibility that the proteolytic degradation of the extracellular matrix might not be required for migration at all has been investigated [35].

5.1 Matrix Metalloproteinases (MMPs)

MMPs are a family of structurally related zinc-dependent endopeptidases that are engaged, aside from various physiological processes such as embryogenesis, reproduction, uterine involution, angiogenesis, in pathological conditions, such as tumor invasion, by mediating degradation of basement membrane and remodeling of extracellular matrix. As invasiveness and metastasis are important biological characteristics of malignant tumors, these processes are extensively investigated and it has been shown that increased expression and activity of MMPs are among the earliest and most sustained events in tumor progression [8, 16].

In tumor progression, increased expression and activity of matrix metalloproteinases plays a role in angiogenesis, invasion, and metastasis. They are a family of zinc metalloproteinases, secreted as latent proenzymes, which are activated by proteolytic cleavage and are inhibited by the tissue inhibitors of metalloproteinases (TIMPs) [8, 36]. There are 27 structurally related known members, which differ according to their individual substrate specificities, including stromelysin 1 and 2,

gelatinase A and B, matrilysin, neutrophil collagenase, and interstitial collagenase. Gene-encoding stromelysin 3 has been identified and is specifically overexpressed in breast carcinomas. The gene was expressed from stromal cells in all of the invasive breast carcinoma tested and only in the direct vicinity of the neoplastic cells of the tumor. The transformed cells secrete a factor that induces expression of stromelysin 3 from the stromal fibroblasts and allows progression of the expanding tumor cell mass through the ECM into surrounding tissue [37].

MMPs have been subdivided into four subclasses based on their substrate specificity and cell localization:

(1) Collagenases (MMP-1 and MMP-8) degrade fibrillar collagen;
(2) Gelatinases (MMP-2 and MMP-9) have a preference for collagen type IV or denaturated collagen, i.e., gelatin;
(3) Stromelysins (MMP-3, MMP-7, and MMP-10) degrade a wide variety of ECM substrates such as proteoglycans, laminin, and fibronectin;
(4) Collagenase-3 (MMP-13) has the widest spectrum of substrates;
(5) Membrane-type MMPs (MT-1 to MT-6-MMP) integral plasma membrane proteins capable of activating MMPs (20).

MMP are secreted as soluble inactive enzymes which are activated by intrinsic Zn^{+} ions, located in the center of the molecule and by extrinsic Ca^{+}. Enzyme activity of MMPs is tightly regulated, and deregulation of MMPs activity can lead to uncontrolled degradation of ECM, a process crucial in tumor metastasis. The most commonly associated MMPs with the processes of metastasis are MMP-2 (gelatinase A) and MMP-9 (gelatinase B) due to their ability to degrade collagen type IV, a major component of vascular basement membrane [8, 31].

Also, MMPs, specifically MMP-2 and MMP-9, are essential for the switch to the angiogenic phenotype during tumor progression. It has been shown that MMP-2 and MMP-9 is expressed in breast carcinomas. Increased MMP expression required for the angiogenic switch and may predict when a non-angiogenic, microscopic, dormant tumor becomes angiogenic and invasive [8, 38].

5.2 Tissue Inhibitors of MMPs (TIMPs)

Tissue inhibitors of MMPs (TIMPs) are tight-binding inhibitors of the active forms of MMPs. There are four of homologous proteins, referred to as the tissue inhibitors of MMPs (TIMP-1 to TIMP-4) [39]. In addition to inhibiting matrix metalloproteinase (MMPs) activity, TIMP-2 has been shown to suppress mitogenesis of both host (endothelial, fibroblast) and tumor cells via receptor tyrosine kinase (RTK) inactivation. Potent anti-tumor activity of TIMP-2 is composed of three separate activities. These are direct inhibition of MMP activity, MMP-independent anti-angiogenic effects, and direct downregulation of tumor cell EGFR signaling [8, 39, 40]. It has been shown that TIMP1, although can inhibit MMP-9, stimulates tumor growth and increases proliferation of normal and tumor cells [41]. TIMP1

stimulates proliferation via MMP-independent and MMP-dependent pathways [8, 16]. Breast cancer cells have on their surface receptors for TIMP1 (CD63). Process of bounding TIMP1 for CD63-integrin β1 complex leads to intracellular signaling and activation of mitogen-activated protein kinases (MAPK) which result in cell proliferation. Growth factors activate MAPK signaling pathway and lead to cell proliferation. Studies have been shown that TIMP1 differently acts with cells and these depend on different expression of receptors for TIMP1 on cell surface. Increased expression of CD63 is connected with degree of malignance. TIMP1 also can activate Ras which also can induce tyrosine-specific phosphorylation and activate MAPK pathway [41]. In addition to the above MMP-independent mode of inducing cell proliferation, TIMP1 also via MMP can induce cell proliferation [42]. It has been shown that TIMP1 by blocking constitutive active MMPs prevents MMP-mediated degradation of newly produced growth factors and growth factors so that they cannot stimulate cell proliferation [36, 37].

In addition to the cell proliferation effects, it has been shown that TIMP1 has anti-apoptotic effect. It has been confirmed that high levels of TIMP1 expression induce constitutive activation of FAK (focal adhesion kinase). FAK is activator of PI (3) kinases which regulate Bcl2, protein important for cell survival and avoiding of apoptosis. Bcl2 and BclXL are mitochondrial membrane proteins which blocks Bax, protein which released cytochrome C from mitochondria [23]. Considering that cytochrome C activates caspases, major mediators of apoptosis, by retaining cytochrome C in mitochondria, block apoptosis. Also, it has been shown that TIMP1 increases expression of Bcl2 and BclXL. Due to this mechanism, there is a decreased sensitivity of tumor cells with high expression of TIMP1 to chemotherapeutics which act by inducing apoptosis [42].

In the beginning, it was considered that TIMP1 has anti-angiogenic effects, but in the time it has been shown that TIMP1 enhances angiogenesis. It has been confirmed that production of endostatins and angiostatins, inhibitors of angiogenesis, is mediated by MMPs. MMPs by degradation of collagens release endostatins and angiostatins from collagens [36]. Due to this mode, TIMP1 by inhibition of MMPs and block production of endostatins and angiostatins enhances tumor angiogenesis. Moreover, it has been shown that breast cancers in rats with high expression of TIMP1 are with higher volume and had better vascularization compared to controls [16, 41]. In addition, it has been shown that TIMP1 directly stimulates vascular endothelial growth factor (VEGF), which helps blood vessels growth. Mechanism by which TIMP1 stimulation of VEGF helps angiogenesis is not clarified.

Several studies have been shown that TIMP1 expression is increased in breast cancer tissue compared to benign or normal breast tissue. In addition, it has been confirmed that RNA and protein expression of TIMP1 are significantly increased in malignant breast tissue compared to benign breast tissue. It has been shown that RNA TIMP1 expression increases with invasive potential of transformed cells. Normal tissue has low expression of TIMP1, cancer in situ has higher expression of TIMP1, and invasive breast cancer has highest expression of TIMP1 [43, 44].

Invasion of tumor cells into ECM is an active process that involves four closely related processes:

1. Separation of tumor cells from each other;
2. Encroachment into local tissues by an active process involving lysis of host cells and degradation of the ECM by proteolysis;
3. Following separation from each other and degradation of the surrounding stroma, tumor cells must then be able to enter the region cleared by the action of the proteolytic enzymes by an active process of cell motility;
4. Tumor cells must also promote their own vascularization by inducing capillary growth into and around the tumor.

Cyclical repetition of these four steps leads to progressive invasion of tumors. Therefore, local invasion by tumor cells allows them to reach a blood or lymph vessel and offers them an undesired opportunity to spread to distant body sites [1, 23, 45, 46].

6 Metastatic Cascade

Although metastasis is the major cause of death in cancer patients [1, 47], it is well established that metastasis formation is an inefficient process. Studies have demonstrated that late events in metastasis formation are largely responsible for the inefficiency of this process. The number of circulating tumor cells and tumor emboli correlates with the size and age of the primary tumor. However, the number of circulating tumor cells does not correlate with the clinical outcome of metastases [1, 8, 10]. The inefficiency of tumor cells in completing the metastatic cascade is the result of the fact that successful formation of metastatic foci consists of several highly complex and interdependent steps. Each step is rate limiting in that failure to complete any of these events completely disrupts metastasis formation. The steps involved in metastasis formation are thought to be similar in all tumors and are characterized as follows:

1. Tumorigenesis. After the initial neoplastic transformation, the tumor cells undergo progressive proliferation that is accompanied by further genetic changes and development of a heterogeneous tumor cell population with varying degrees of metastatic potential. The initial growth of the primary tumor is supported by the surrounding tissue microenvironment, which eventually becomes rate limiting for further growth [1, 8, 10].
2. Angiogenic switch. As the tumor grows and central tumor cells become hypoxic, the tumor initiates recruitment of its own blood supply by a process called the “angiogenic switch” which involves the secretion of various angiogenic factors and the removal or suppression of angiogenesis inhibitors [3, 8, 10].

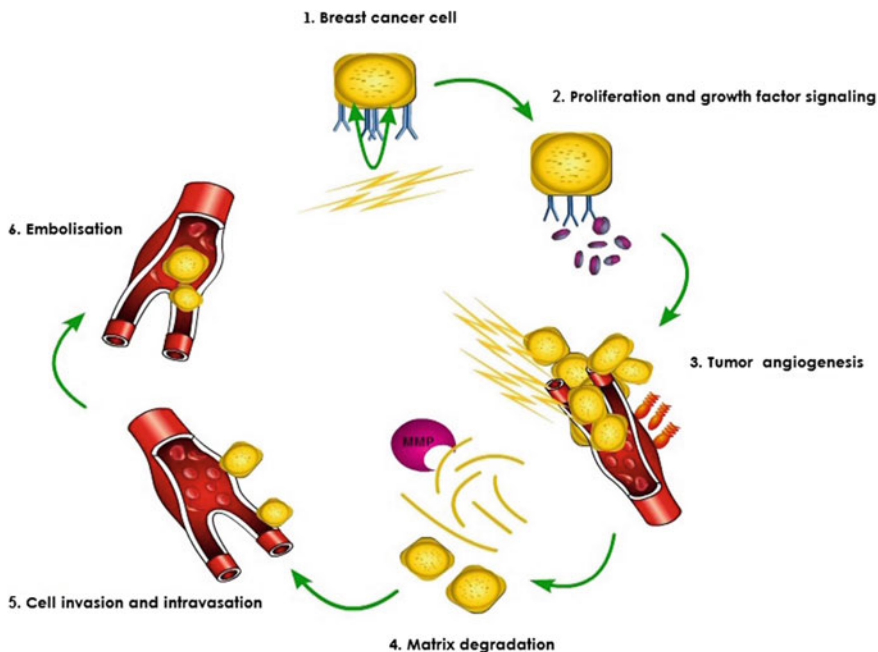

Fig. 1 Role of MMPs in metastatic cascade

3. Clonal dominance of invasive phenotype. Continued genetic alterations in the tumor cell population lead to selection of tumor cell clones with distinct growth advantage and acquisition of an invasive phenotype. Invasive tumor cells downregulate cell–cell adhesion, alter their attachment to the ECM by changing E-cadherin, integrin expression profiles, and by proteolytically altering the matrix accomplish stromal invasion. Tumor cells lose "contact inhibition" that prevents normal cells from continuing to divide when they contact their nearest neighbors. MMPs degrade the components of ECM and basement membrane and allow migration of tumor cells (Fig. 1) [8].
4. Circulating of cancer cells in blood vessels [8, 10].
5. Dormancy of cancer cells at lymph nodes and organs [8, 10].
6. Extravasation and growth at the secondary site. Arrested tumor cells proliferate in response to paracrine growth factors or become dormant. The poor growth of tumor cells after extravasation from the circulation is a major factor contributing to the inefficiency of the metastatic process.
7. Angiogenesis in metastatic foci. Finally, continued growth of the metastatic foci is also dependent on angiogenesis. The development of this neovascular network at the metastatic site enhances the metastatic potential of these cells just as it does for the primary tumor [8, 10].

8. Evasion of immune response. Tumor cell evasion of immune response in metastatic foci includes antigenic modulation and immunosuppression and prevents their eradication (Fig. 1) [1, 8, 10].

Therefore, detachment of single cells or clumps of cells from the tumor mass are associated with lack of cell adhesiveness in tumor populations, regulated by a variety of cell surface molecules such as the cadherins and integrins.

Circulating tumor cells arrests at distant sites and by a repeated process of invasion may colonize a secondary site for growth. In some instances, this organ preference of metastasis can be explained simply in terms of the anatomical relationship of the organ with the site of primary tumor growth (Ewing theory of metastasis) [3, 8]. Some cancers express organ preference that is not associated with a non-specific entrapment process, but rather with specific determinants, which actively promote the growth of the metastatic cells, thereby providing favorable soil (Padgett's theory of metastasis). Fully metastatic behavior is attributable to the expression of only one, or a few, metastasis promoting genes (e.g., CD44) or to the loss of an equally small number of metastasis-suppressing genes (e.g., nm23) [8, 10].

After arriving in a secondary site, metastatic cells begin proliferating, undergo apoptosis, or remain as solitary dormant cells. The process of metastasis, although dangerous, is extremely inefficient with the majority of the cells undergoing apoptosis and thus becoming clinically irrelevant. The cells that begin proliferating and dormant cells are responsible for cancer recurrence [8, 38]. It has been shown that tumor dormancy, a complex and still poorly understood phenomenon observed both in experimental models and in patients, has been connected with insufficient angiogenesis. A defined event, termed "angiogenic switch" characterized by an imbalance between pro- and anti-angiogenic factors, by the influence of two proteolytic systems, uPAR and MMPs, often marks interruption of the dormant state, thus triggering invasive tumor growth [8, 48].

7 Clinical Correlation and Prognosis

There is an urgent need for more efficient prognostic markers that can accurately and reliably identify breast carcinomas with potential to cause recurrence and/or death [17]. There are data that show that markers for survival prognosis could be found among the proteins that participate or regulate the processes associated with cancer progression. Possible candidates include MMPs and their tissue inhibitors, TIMPs [8, 15].

8 MMP-2 and MMP-9 in Tumor Tissue of Breast Cancer Patients

Although there is a need for diagnostic as well as prognostic markers in breast cancer, the studies did not show a new marker who would have an important impact on disease progression [9, 49, 50]. However, molecular examinations have shown that breast cancer is divided into at 4 types of tumors: luminal (ER+, PR+, and HER2−), HER2 overexpressing (ER−, PR−, and HER2+), basal-like (ER−, PR−, HER2−, and CK5/6+/EGFR+), and breast-like (ER−, PR−, and HER2−), with diverse biology and prognosis [9, 36]. It has been shown that patients with basal-like carcinomas had shorter relapse-free survival and worse survival, and the aggressive biology of basal-like carcinomas requires a new therapeutics for treatment [9, 13]. Investigation of activity of gelatinases in tumor tissue and sera of breast cancer patients could help in designing newer more adequate therapeutics.

It has been shown that gelatinase activity in the tissue of breast carcinomas patients can be a diagnostic and staging markers, but also as potential target for new therapies [37]. Radenkovic et al. have been shown that tumor tissue of breast cancer patients had higher latent and active forms of MMP-2 and MMP-9 compared to peritumor tissue (Fig. 2) [9, 50]. Also, it has been confirmed that activity of latent and active forms of MMP-2 and MMP-9 in tissue of breast carcinomas show stage-dependent increase. These results indicate that activity of gelatinases could be used as biomarker of progression of breast cancer patients [9].

Radenkovic et al. show that tumor size is positively associated with latent and active MMP-2 activity [9], while other studies show correlation between MMP-9 and tumor size. Other study investigated detection of MMP-2 and MMP-9 by immunohistochemistry and confirmed positive association of MMP-2 and tumor size [51]. Moreover, it has been shown a positive correlation between gelatinase activity and lymph node positivity of breast cancer patients [9, 52]. The shown association of gelatinase activity and tumors size and lymph node involvement

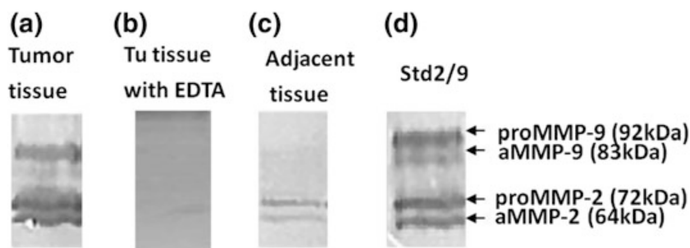

Fig. 2 Electrophoretic traces of SDS-PAGE zymography. **a** The activity of pro and active form of MMP-2 and pMMP-9 in tumor tissue of breast cancer patients. **b** Inhibited MMP-2 and MMP-9 activities in tumor tissue of breast cancer patients with EDTA. **c** The activity of pro and active form of MMP-2 and pMMP-9 in adjacent tissue of breast cancer patient. **d** Standard proteins marker

suggests that MMP-2 and MMP-9 are involves in cancerogenesis as well as in metastasis.

It has been confirmed that estrogen and progesteron receptor tumor positivity is associated with better relapse-free survival and overall survival of breast cancer patients [17, 53]. Radenkovic et al. have shown that hormone receptor tumor negativity is associated with tumor activity of active forms of gelatinases [9]. Since estrogen and progesteron receptor negative tumors are associated with worse outcome, the above-mentioned studies suggest that aggressive nature of hormone-negative tumors is associated with MMP-2 and MMP-9 activity.

Almost one-fifth of patients with breast cancer have tumors with increased expression of the HER2 [13]. The studies have been found that HER2 induces MMP-2 and MMP-9 activity and gene expression, therefore stimulating an invasiveness and migratory phenotype of breast cancer cells [8, 9]. The published studies have shown no difference of gelatinase level between patients with HER2-positive tumors in tumor tissue compared those with HER2 negative tumors [9].

It has been shown that basal-like breast cancers had poor differentiation and worse outcome and usually had haematogenic pathway of dissemination, while luminal A breast cancers are likely to spread into axillary nodes and bones and mostly had better survival [9, 13, 29]. Pro-MMP-2 is involved in the process of angiogenesis. Particularly, proMMP-2 degrades of collagen type IV and, therefore, allows the $\alpha v\beta 3$ integrin bounding of newly formed blood vessels [37]. In these sense, it was found a higher activity of proMMP-2 in tumor tissue of patients with basal-like cancers, indicating that haematogenic spread of basal-like tumors is at least in part regulated by MMP-2.

It has been found that surrounding tissue had MMP-2 and MMP-9 activities produced by stromal cells [36, 37]. It is proposed that tumor cells induce stromal cells via interleukins, growth factors, and interferons in order to produce gelatinases [29, 37]. Also, it has been shown association of higher MMP-2 activity in adjacent tissue and basal-like cancers. [9]. It seems that examinations of adjacent tissue are essential for enlightening of tumor biology.

The above results confirmed that MMP-2 and MMP-9 could be used as prognostic biomarker as well as therapeutic target in breast cancer patients [9, 10].

9 MMP-2 and MMP-9 in Sera of Breast Cancer Patients

It is suggested that MMP-2 and MMP-9 produced in tumor tissue entrance into vessels, and therefore, these enzymes could serve as potential diagnostics and staging biomarkers for breast cancer patients [15, 17, 37].

The published studies have been shown that activities of gelatinases in sera are associated with tumor size and lymph node involvement in breast cancer patients [54]. Stankovic S et al. have shown significant stage-dependent increase of MMP-2 and MMP-9 sera activity (Fig. 3) in breast cancer patients [15], and this indicates

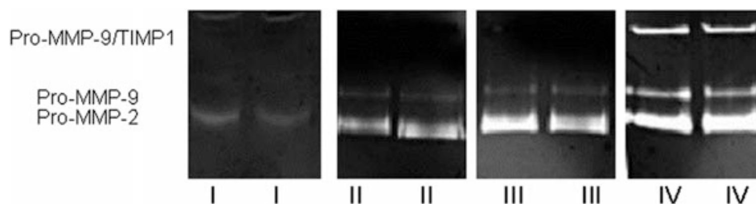

Fig. 3 Representative SDS-PAGE zymography gel showing relative abundance of MMP-2 and MMP-9 in the sera with different clinical stages of breast cancer patients (clinical stage of breast cancer *II*, *III*, and *IV*)

that these enzymes could be used as possible staging and follow-up markers in breast cancer patients. Moreover, it has been found that MMP-9/TIMP-1 complex activity is detected only in metastatic stage of the disease. This result suggests that MMP-9/TIMP-1 complex plays an important role in disease progression [8, 15]. In these sense, MMP-9 and MMP-2 were investigated as prognostic markers for breast cancer patients in many studies. It has been confirmed that plasma MMP-9 levels are inversely associated with RFS and with survival [15, 55]. All these studies suggest importance of MMP-2 and MMP-9 sera activity evaluation in breast cancer patients.

It has been shown that ER induces MMP-9 gene expression and activity and therefore enhances growth of tumor cells and progression [13, 17, 53]. However, some studies have shown a positive association of MMP-9 expression and ER positivity in breast cancer patients, while others studies did not shown any association [15, 54, 56].

HER2 represents an important prognostic marker for breast cancer patients [13, 15]. The published studies found that invasiveness of HER2-positive tumors is enhanced by MMP expression [15, 36, 37]. Moreover, HER2 expressions via stimulation of proteolytic network of enzymes help cancerogenesis, as well tumor progression in breast cancers [15, 55].

Although soluble MMPs have been shown to be produced as zymogen (proMMP-2) in cells, the fate of MMPs and TIMPs after their secretion from producing cells or spatial regulation of their molecular activations is not fully understood [15, 57]. It has been shown that cancer cells as well as tumor infiltrating lymphocytes secrete MMPs, MT!-MMP, and TIMPs and these secretions induce anti-tumor immune response in metastases [15, 57, 58]. It is proposed that MMPs produced by TIL can spread closely to cancer cell in order to activate there [57].

Activity of MMP-2 and MMP-9 in sera of breast cancer patients could be implemented as staging biomarkers. The complex network between lymphocyte and MMPs requires further investigation. The association of the increase in MMP-2 and MMP-9 activity in serum and prognostic and therapeutic parameters, tumor size and axillary lymph node status, suggests a role of MMP-2 and MMP-9 in prognostic stratification of breast cancer patients. This may be helpful not only in the

new approaches of “stromal-directed” therapy, but also in avoiding overtreatment with chemotherapy of breast cancer patients [8, 15].

10 Conclusion

Considering that breast cancer had highly aggressive biology, it has been investigated and found that high activity of MMPs, particularly MMP-2 and MMP-9, is engaged in all stages of tumor progression. As proteases are involved in physiological processes such as immune response and in pathological conditions, it seems that examinations of molecular pathways of breast cancer could define a therapeutic target based on proteases.

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The Multifunctional Post-proline Dipeptidyl Peptidase, DPP9, in Mice, Cell Biology and Immunity

Margaret G. Gall and Mark D. Gorrell

Abstract

Dipeptidyl peptidase 9 (DPP9) is a ubiquitous intracellular post-proline protease of the DPP4 (S9b) family of atypical serine proteases. Emerging data support roles for DPP9 in intracellular signalling, particularly in the epidermal growth factor receptor pathway, in immune cells, particularly in macrophages and antigen processing, and in energy metabolism. The focus of this review is the roles of DPP9 in regulating physiological and cellular processes. Such data is derived from a genetically modified mouse strain and from manipulations of cell lines. The mouse strain that lacks DPP9 enzyme activity is homozygous lethal. DPP9 alters behaviours, such as cell adhesion, of cancer cell lines. This review points to the functional importance of DPP9 in immunity, metabolism and cancer.

Keywords

Dipeptidyl peptidase · Mouse models · Neonate development
Fibroblast activation protein

Abbreviations

AMC	Amino methylcoumarin
AMPK	AMP-activated protein kinase
B-CLL	B-cell chronic lymphocytic leukaemia
CAF	Cancer-associated fibroblasts
CXCL	CXC chemokine ligand

M.G. Gall · M.D. Gorrell (✉)
Centenary Institute and Sydney Medical School, University of Sydney, Sydney, NSW 2042, Australia
e-mail: m.gorrell@centenary.usyd.edu.au

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DEN	Diethylnitrosamine
DPP	Dipeptidyl peptidase
DSS	Dextran sulphate sodium
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
ESFT	Ewing sarcoma family of tumours
FAP	Fibroblast activation protein
FGF	Fibroblast growth factor
HCC	Hepatocellular carcinoma
HFD	High fat diet
HSC	Hepatic stellate cells
IRS	Insulin receptor substrate
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
MMP	Matrix metalloproteinase
NEM	N-ethylmaleimide
NPY	Neuropeptide Y
PARP	Poly(ADP-ribose) polymerase
PGC-1α	PPAR coactivator-1α
PBMC	Peripheral blood mononuclear cells
pNA	P-nitroaniline
POP	Prolyl oligopeptidase
PPAR	Peroxisome proliferator-activated receptor
SUMO	Small ubiquitin-like modifier
TAA	Thioacetamide
TAM	Tumour-associated macrophages
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor α
VEGF-A	Vascular endothelial growth factor-A
WT	Wild-type

1 Introduction

1.1 The Dipeptidyl Peptidase 4 (DPP4) Enzyme Family

The DPP4 family of proteases has important roles in regulating physiological and cellular processes. The ubiquitous DPP4 family is the S9b protease subfamily of the prolyl oligopeptidases [1] of atypical serine proteases that contain a conserved catalytic triad of serine, aspartate and histidine [2]. Prolyl endopeptidase (PEP) and the DPP4 family enzymes possess their ability to hydrolyse a post-proline peptide bond. The DPP4 family enzymes can cut two residues from the N terminus of a protein substrate [3–5]. The presence of a proline near the N terminus of a peptide (Fig. 1) generally confers resistance against protease degradation and occurs in many different biologically active peptides, including chemokines, incretins and neuropeptides. Thus, the specialised ability of dipeptidyl peptidases to cleave the post-proline bond is useful to degrade such peptides [6].

The DPP4 protein family consists of six members: four enzymatic members and two non-enzymatic members. DPP4, DPP8, DPP9 and FAP have DPP activity, while FAP is also a post-proline endopeptidase [3–5, 7]. The two non-enzymatic members are DPP6 (DPP-X) and DPP10, also known as dipeptidyl peptidase like (DPL) protein-1 and DPL-2 [8–10] (Fig. 2).

1.1.1 DPP4

DPP4 is the prototypical and the most well-characterised member of the family. It displays enzymatic activity in both the dimeric cell surface membrane-bound form and the soluble circulating form [12, 13]. DPP4 is expressed by epithelial cells in the liver, gut, uterus and kidney, by immune organs, by capillary endothelium [14],

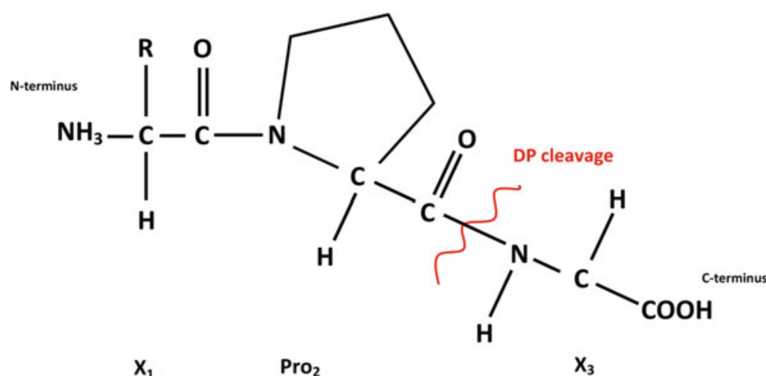

Fig. 1 Cleavage of a post-proline bond. A tri-peptide containing a proline at position two creates a bend in the peptide chain. Proline-containing peptides pose a specific problem for proteases due to structural constraints imposed by the pyrrolidine ring that prevents hydrolysis by most peptidases. Dipeptidyl peptidase cleavage by DPP4, DPP8 and DPP9 occurs C-terminal to the proline residue

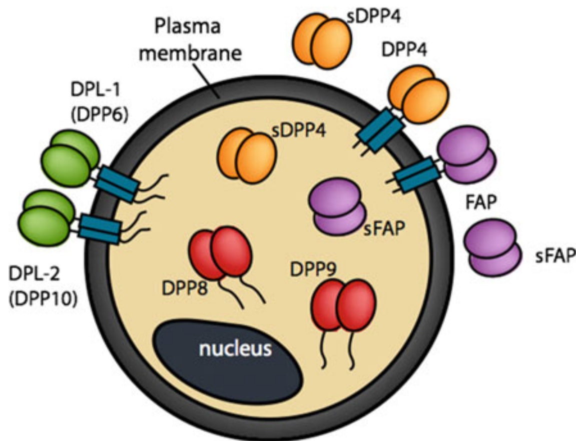

Fig. 2 Cellular localisation of the six members of the DPP4 protein family. DPP4 and FAP are type-II proteins that are membrane-bound cell surface proteases and also have soluble forms. DPP8 and DPP9 are intracellular proteases. DPP6 and DPP10 are membrane-bound, non-enzymatic proteins. Adapted from [11]

and by acinar cells of mucous and salivary glands and pancreas [15]. The soluble form is present in serum, seminal fluid, saliva, kidney, liver and bile [12, 15–18].

Functionally, DPP4 is involved in numerous processes throughout the body, including immunological, haematological, endocrine and metabolic. These include the ability to participate in chemokine inactivation and also to have a role in apoptosis, lymphocyte activation and cell migration, along with the capacity to cleave a wide range of small bioactive proteins [16, 19–22]. Most noted of these is the ability of DPP4 to rapidly inactivate the incretins glucagon-like peptide-1 (GLP-1) and glucose insulinotropic peptide (GIP) [23] leading to the inhibition of DPP4 as a successful type 2 diabetes therapy. DPP4 is also known as CD26 and, as such, has roles in the immune system in T cell activation and proliferation and T helper 1 responses to foreign antigens. Cell surface expression on T cells greatly increases following stimulation with antigen or mitogens [15, 17, 24].

1.1.2 FAP

FAP, which is also known as seprase, has 52% amino acid sequence identity with DPP4. However, FAP possesses both dipeptidyl peptidase and endopeptidase activities, which enables it to cleave a post-proline bond two or more amino acids from the N terminus of a protein (Fig. 3) [7].

While DPP4 is abundant in most tissues, FAP is at very low levels [18] with high-level expression being limited to sites of tissue remodelling and areas of stromal activation, such as in solid tumours, wound healing, tissue damage and inflammation [25, 26], during mouse embryogenesis [27] and in activated hepatic stellate cells in cirrhotic liver [28–30]. FAP has been isolated in a truncated soluble form from human plasma and serum [31, 32]. FAP activity levels in normal and

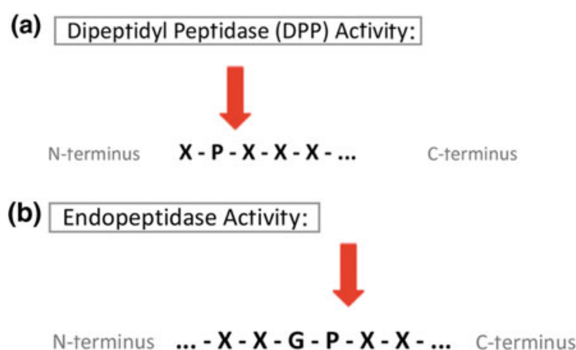

Fig. 3 Enzyme activity of FAP. **a** Dipeptidyl peptidase activity involves the cleavage of two amino acids of the N terminus of a protein following a proline residue. **b** Endopeptidase activity involves the cleavage of a post-proline bond that is more than two amino acids from the N terminus of a protein

diseased tissue from humans, mice and baboons have been measured using the novel FAP-specific substrate, 3144-AMC. In mice, uterus, pancreas, submaxillary gland and skin showed the greatest levels of FAP activity [18].

While FAP has been shown to cleave some DPP4 substrates in vitro [33], until recently there were no known physiological substrates of FAP's dipeptidyl peptidase activity. The first study to characterise the physiological DPP substrate repertoire of endogenous FAP in mammalian plasma showed a potential function of FAP in neuropeptide signalling in liver and liver cancer [34]. In addition to its dipeptidyl peptidase activity, FAP also has endopeptidase activity cleaving α2-antiplasmin [31] and denatured type I collagen [35]. The gelatinase activity of FAP most likely contributes to extracellular matrix degradation.

Because of its pattern of expression by activated stromal fibroblasts in many cancers, FAP has been studied as a potential therapeutic target in tumours. FAP roles in cancer biology have been reviewed previously [36–38]. Strategies for targeting FAP for cancer therapy and the role of FAP seem to be very contextual, so the expression pattern and substrates of FAP require greater definition to better predict the effects of targeting this protease.

1.1.3 DPP6 and DPP10

DPP6 and DPP10 have 53% amino acid similarity with each other and are highly homologous to DPP4 with sequence identities of 33 and 32%, respectively [5, 10]. They both form dimers but lack the catalytic serine and a nearby tryptophan that are essential for enzymatic activity. DPP6 has a wide tissue distribution, but DPP10, in contrast, has expression limited to human brain, adrenal gland and pancreas [5, 10, 39]. The functional effects of these proteins appear to be through binding interactions, and there is evidence of an association with neuronal diseases and asthma [10, 40, 41].

1.1.4 DPP8 and DPP9

DPP8 and its closest relative DPP9 are the most recently discovered members of the DPP4 gene family [4, 5, 42, 43]. As DPP4, DPP8 and DPP9 all have DPP4-like enzymatic activity and ubiquitous expression, the discovery of DPP8 and DPP9 has called for the reinterpretation of previous DPP4 data [4, 42]. This was necessary to determine which functions had been attributed to DPP4 that may instead be accounted for by DPP8 and DPP9.

Unlike DPP4, DPP8 and DPP9 are intracellular proteins and are therefore likely to have different biological roles. As there are currently no substrates or inhibitors that distinguish DPP8 from DPP9, their characteristics are usually outlined together but this review will focus upon DPP9. DPP9 is currently under study in many fields including cell biology, immune-biology and tumour biology [44].

2 Structure of DPP8 and DPP9

DPP8 and *DPP9* are located on human chromosomes 15q22 and 19p13.3 [42, 43] and mouse chromosomes 9 and 17, respectively. In *DPP4* and *FAP*, the sequence adjacent to the active-site serine is encoded by two exons while the homologous region in *DPP8* and *DPP9* is encoded by a single exon, which suggests that the *DPP8* and *DPP9* genes arose at an earlier evolutionary stage and may have arisen by gene duplication [43]. The amino acid homology between human and mouse DPP8 and DPP9 is 95 and 92% identity, respectively [4, 43]. No crystal structures of DPP8 or DPP9 exist, but homology models have been built using the known structures of DPP4, DPP6 and FAP [45–47]. In these models, DPP9 is depicted with an α/β-hydrolase domain containing a Ser-Asp-His catalytic triad and an 8-blade β-propeller domain containing two glutamic acids essential to functionality of the catalytic pocket (Fig. 4).

While structurally similar to DPP4 and FAP, DPP8 and DPP9 are longer at the N terminus but lack a transmembrane domain and are intracellular proteins [4, 42]. Another structural difference between DPP4 and DPP9 is that selective point mutations in the C-terminal loop can inactivate DPP9 while maintaining the dimeric structure [48]. This confirms the importance of the C-terminal loop for DPP8 and DPP9 enzymatic function. Finally, since DPP8 has been shown to have a larger substrate pocket than DPP4 [47], DPP8 and DPP9 might have a larger β-propeller domain and/or may contain a separate element of tertiary structure at the N terminus of the protein.

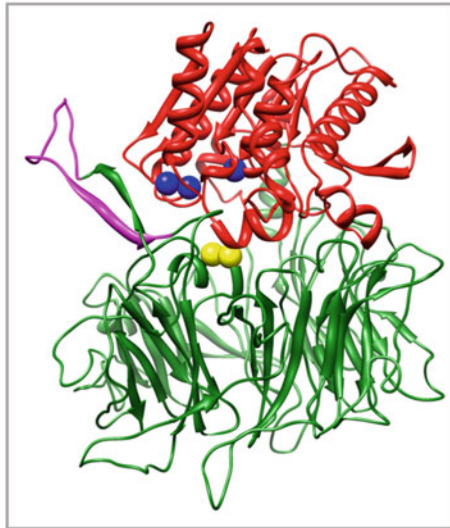

Fig. 4 A model of DPP9 protein structure. DPP9 residues 51–863 are depicted in ribbon representation of the monomer. The α/β-hydrolase domain is in *red* and the 8-blade β-propeller domain in *green*. The catalytic triad (Ser-Asp-His) is shown as *blue* spheres in the hydrolase domain and 2 glutamates essential for catalysis as *yellow* spheres in the propeller domain. An extended arm (^{285}VEVIHVPSPALEERKTDSYR^{304}) in the propeller surface of DPP9 that is critical in SUMO1–DPP9 interaction is coloured *pink*. Modified from [44, 45]

3 Expression Profiles and Tissue Distribution of DPP8 and DPP9

Both DPP8 and DPP9 have isoforms. Human DPP8 has several mRNA transcripts identified: one is abundant in testis, prostate and muscle [5, 42], while another longer transcript variant has intense signals in adult testis [49]. Bioinformatics analysis of NCBI database sequences has identified at least two other isoforms [50].

Two forms of DPP9 cDNA have been cloned, with the short form (DPP9-S) encoding 863 amino acids [4, 43] and the long form (DPP9-L) encoding 971 amino acids [4]. DPP9-S is ubiquitously expressed including high levels in liver, heart and skeletal muscle, while DPP9-L is much less abundant and is predominantly expressed in skeletal muscle. The N-terminal extension of DPP9-L contains a nuclear localisation signal (NLS) allowing it to enter the nucleus [51]. Endogenous nuclear DPP9 has been visualised in mouse fibroblasts [52].

In canine and porcine small intestine, lung, kidney and pancreas, differential relative abundance of DPP8 and DPP9 has been measured by RT-PCR analysis with DPP9 having the greater expression [53]. In adult mice, greater numbers of DPP9 transcripts are in brain, skin, colon and thymus compared with DPP4 [53, 54].

DPP8 mRNA is upregulated in human adult testis compared to foetal testis [49]. DPP8 and DPP9 is in spermatozoids embedded in the epithelium of the seminiferous tubules [55], while the mRNA is in spermatogonia and spermatids [56]. DPP9-S has been purified from bovine testes [57]. Taken together, these studies suggest a role for DPP8 and DPP9 in spermatogenesis.

Using in situ hybridisation, immunohistochemistry and enzyme assays on baboon, mouse and human tissues, the ubiquitous expression and distribution of DPP8 and DPP9 have been visualised in lymphocytes and epithelial cells in the gastrointestinal tract, liver, lung, spleen, lymph node and skin, as well as in pancreatic acinar cells, muscle, adrenal gland, testis and Purkinje cells and the granular layer of the cerebellum [56]. These comprehensive findings concord with related studies [55, 58, 59].

Analyses of mRNA and protein levels in Sprague Dawley rat and cynomolgus monkey showed similar ubiquitous expression and tissue distribution correlated with DPP8/9 enzymatic activity and also detected the DPP8 and DPP9 proteins in some capillary endothelia [14]. DPP8 and DPP9 have been shown to be in rat primary endothelial cells of aortic, endocardial and cardiac microvascular origin [60] with a greater abundance of DPP8 protein over DPP9. However, in human carotid artery endothelial cells, DPP9 has been the only DPP4-like enzyme detected using immunohistochemistry, suggesting a regulated expression of DPPs in endothelial cells.

Both DPP8 and DPP9 occur in tumours [4, 56, 61], hepatocytes [56, 62, 63], macrophages [64] and lymphocytes [56, 63, 65] but are absent from activated stellate cells [56].

Recent reviews provide more detailed information regarding the expression and distribution of DPP8 and DPP9 [19, 22, 44, 66].

4 Enzymatic Activity and Substrate Specificity of DPP8 and DPP9

Although originally thought to be monomers, both DPP8 and DPP9 are, like DPP4, catalytically active as dimers [48, 67, 68]. Both DPP8 and DPP9 have optimum activity at neutral pH of 7.0–8.5 [5, 42, 48], which is consistent with intracellular localisation. DPP8 and DPP9 have reversible changes in enzymatic activity intensity, dependent on the redox state of their cysteine moieties, with activity decreased by oxidation and increased by reduction [45, 69]. DPP9 is allosterically activated by SUMO1 [70]; however, the small peptide region of SUMO1 that interacts with DPP9 is also a non-competitive inhibitor of DPP9 activity with inhibition dependent on the DPP9 arm motif (Fig. 4) [71].

Both enzymes have similar substrate specificity, preferring a proline in the penultimate P1 position and small or basic amino acid residues at the P2 position of synthetic substrates [48, 68, 69]. Similarly, preferred natural substrates have a P1 proline preceded by an alanine in the P2 position [72], but alanine can be in P1

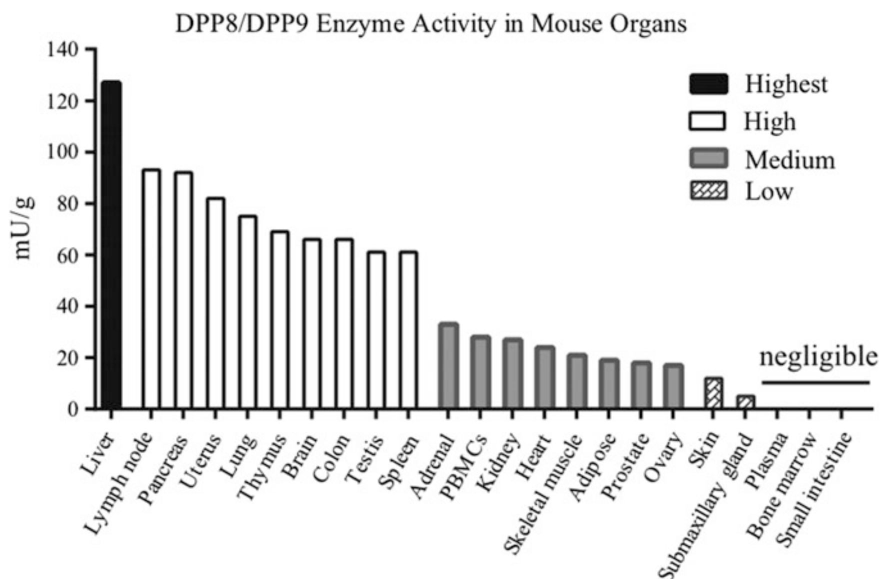

Fig. 5 DPP8/DPP9 enzyme activity in mouse organs. The approximate intensity of DPP8/9 enzyme activity was measured by Yu and colleagues [56] from mouse organs in DPP4-gene knockout mice with/without the addition of the DPP8/9 inhibitor NEM (N-ethylmaleimide). NEM-inhibited activity was subtracted from total DPP activity to estimate the activity derived from DPP8 and DPP9. Modified from [44]

[67, 73]. Ala-Pro- and Gly-Pro-containing fluorogenic and chromogenic substrates have been routinely used to detect DPP8/9 enzymatic activity (Fig. 5), but H-Arg-Pro-pNA is also hydrolysed [4, 56]. We recently showed that DPP9 is much more efficient than DPP8 at cutting after alanine in P1 [73].

DPP8 and DPP9 are able to cleave a number of naturally occurring extracellular peptides and chemokines in vitro but with reduced rates of hydrolysis compared to DPP4 [67, 73, 74]. Whether extracellular substrates of these intracellular DPPs have physiological relevance is unclear. It has previously been shown that neuropeptide Y (NPY) can be cleaved by DPP4, DPP8, DPP9 and FAP [33, 34, 67, 75, 76]. NPY extracted from rat brain can be cleaved in the presence of a DPP4 inhibitor, suggesting that DPP8 and DPP9 can cleave NPY in vivo [76]. Moreover, NPY is a substrate for DPP8 and DPP9 in intact cells, not just cell extracts, and in doing so regulates Ewing sarcoma family tumour (ESFT) cells by reducing NPY-induced cell death [77].

A role of DPP9 in antigen presentation and peptide turnover was first shown by the identification of the antigenic peptide $RU1_{34–42}$ as its first natural substrate. Silencing of DPP8 and DPP9 using siRNA resulted in increased RU1 presentation only in DPP9-silenced cells, which indicated that the $RU1_{34–42}$ antigen is an in vivo substrate of DPP9 [69].

DPP9 substrates have been sought using a degradomic approach and resulted in the identification and validation in vitro of numerous potential substrates, with adenylate kinase 2 and calreticulin confirmed as DPP8 and DPP9 substrates. Those substrates suggested a role for DPP8 and DPP9 in cellular metabolism and homoeostasis [72].

Most recently, two-dimensional differential in-gel electrophoresis (DIGE) was applied to cytoplasmic and nuclear extracts of mouse cells lacking endogenous DPP9 activity (the DPP9-GKI mouse, see below) to identify novel DPP9 substrates [73], with nine being confirmed as substrates by MALDI-TOF or immunoblotting. This study also identified a DPP9-specific consensus site for cleavage that was not recognised by DPP8, suggesting that these two proteases have different in vivo roles. The substrates included two key immune regulators, CXCL10 and IL1RA, and the putative DPP9 substrates S100-A10, SET and NUCB1, which are mediators of immunity and inflammation [73].

5 Non-Enzymatic Functions of DPP8 and DPP9

Early investigations using overexpression in the HEK-293T epithelial cell line showed potential roles for DPP8 and DPP9 in apoptosis, wound healing and cell migration. In vitro overexpression of DPP8 and DPP9 resulted in impaired cell adhesion, migration and monolayer wound healing for DPP9 and impaired migration and wound healing for DPP8, suggesting that those outcomes may result from direct protein–protein interactions or via altered expression of other proteins involved in cell adhesion and migration [78]. Both DPP8 and DPP9 also enhance apoptosis. Using enzyme-negative mutants of each protein, the role of DPP8 and DPP9 in apoptosis appeared to be independent of enzymatic activity in HEK-293T cells.

Recent work showed that apoptosis and cell death were unaffected by DPP9 knock-down or enzyme inhibition, but found that enzyme inhibition or gene silencing of DPP9 in Huh7 cells resulted in less cell mobility and adhesion compared to control cells [79], indicating that DPP9 enzymatic function is important for these processes. Differences between that recent study [79] and the early study [78] may reflect that overexpression data can be cell-type specific [30] or can be misleading.

A non-enzymatic activity that needs further exploration is the association of H-Ras with DPP9 [62]. Whether there is direct binding between these two proteins and the functional significance of this association are unknown.

6 Inhibition of DPP8 and DPP9

Many early DPP4 inhibitors were later found to also inhibit DPP8 and DPP9, raising the question of which functions attributed to DPP4 activity should be attributed to DPP8 and/or DPP9. This has led to the development of many inhibitors that are selective for DPP8 and DPP9 over DPP4 and FAP [80–85], but are not selective for DPP8 or DPP9 alone. While some isoindoline inhibitors were originally thought to inhibit DPP8 alone [82, 86], these were later shown to inhibit both DPP8 and DPP9 [81].

The safety of targeting DPP8/9 activity for therapeutic use has been controversial, with toxic effects reported in rats with the use of the DPP8/9 selective inhibitor, UAMC00132 [80], while a rebuttal study on rats and mice using vildagliptin at high doses that inhibit DPP4, DPP8 and DPP9 showed no toxicity or mortality in animals [87]. Moreover, the compound 1G244 does not cause pathological symptoms when inhibiting DPP8 and DPP9 [81]. With the recent design of two analogues of 1G244 that are 10-fold selective for DPP8 over DPP9 [84], there is good progress towards selectively differentiating between these two enzymes by inhibition of either DPP8 or DPP9, rather than both.

Alternative approaches to chemical inhibitors have been examined for targeting DPP4 and FAP. As the DPPs have non-enzymatic functions, antibodies might block cell surface or extracellular protein-binding interactions, or might remove DPP-expressing cells. Immunotargetting DPP4 is an example [88–90]. A separate approach involved a non-substrate non-apeptide, the HIV-1 Tat protein, the first natural inhibitor of DPP4, as an immunosuppressor [91]. Whether non-apeptides or antibodies can enter cells to act on cytoplasmic or nuclear DPP8 and DPP9 is unknown.

More recently, it was found that, while the small ubiquitin-like protein modifier SUMO1 interacts with DPP9 leading to allosteric activation of the peptide [70], the E67-interacting loop peptide acts as a non-competitive inhibitor [71]. This highlights the potential modulation of enzyme activity by peptides that mimic interaction surfaces. Targeting the non-enzymatic activities of DPP8 and DPP9 as a therapeutic approach would require further identification and analyses of protein-binding partners, such as those required for the localisation of DPP9 to the leading edge of moving cells [79].

7 Biological Functions of DPP8 and DPP9

Knowledge of the physiological functions of DPP8 and DPP9 is emerging with investigations in normal homoeostasis and pathophysiological conditions. Data on enzymatic activity, substrate specificity and cell and tissue distribution of these proteases provide some guidance regarding their biological functions in cell survival, cell biology, disease, inflammation and cell-mediated and humoral immunity.

7.1 DPP8 and DPP9 in Immunity

7.1.1 DPP8 and DPP9 in Innate Immunity

The non-specific defence mechanisms against pathogens include physical barriers, such as skin integrity, blood-borne chemicals and certain immune cells. DPP8 and DPP9 have been shown to have extensive in vivo expression in epithelial cells of the gut and skin and normal immunological tissues and also are expressed by all major lymphocyte populations [42, 56, 63]. DPP8 and DPP9 have also been shown to be upregulated upon B or T cell activation [63]. Several T cell leukaemia cell lines highly express DPP8 and DPP9 mRNA transcripts [4].

DPP8/9 enzyme activity has been found in human lymphocytes, monocytes, Jurkat and U937 cells [65] and also in human and mouse primary leucocytes and B and T cell lines [42, 80, 92]. DPP9 is at low levels in both M1 and M2 macrophages derived from U937 cells but abundant in the macrophage-rich regions of plaques [64, 93]. Using the mouse macrophage cell line J774 and bone marrow-derived monocytes and macrophages, an inhibitor of DPP8 and DPP9 has been shown to lessen mouse M1 macrophage activation [64].

Natural killer (NK) cells play a major role in host rejection of both tumours and virally infected cells in the innate immune system. An early study showed that inhibition of DPP4 enzyme activity results in the suppression of stimulatory cytokines causing a reduction of DNA synthesis and cell cycle progression [94]. However, the inhibitors used in that study were later shown to also inhibit DPP8/9 activity [80]; therefore, those effects were probably DPP8/9 mediated. Further support for that conclusion derives from the finding that the presence or absence of DPP4 on the cell surface of NK cells does not influence the natural cytotoxicity of these cells [95]. In a lung metastasis model, NK cytolytic function against tumour cells was less in DPP4-deficient than in WT rats [96].

7.1.2 DPP8 and DPP9 in Adaptive Immunity

The antigen-specific adaptive immune response is complex and includes formation of peripheral and tissue-resident memory leucocytes that improve the efficiency of future responses, and depends upon antigen presentation and recognition. DPP9 is the dominant protease that degrades the antigenic peptide $RU1_{34–42}$ such that downregulation of DPP9 increases the presentation of this antigen [69].

While mouse splenic B lymphocytes express little DPP4 mRNA, DPP8 and DPP9 mRNA are expressed at greater levels and stimulation with pokeweed mitogen and lipopolysaccharide of mouse splenocytes and Jurkat T- and Raji B-cell lines upregulates both proteins [63]. DPP8 and DPP9 mRNA are downregulated by dithiothreitol treatment and upregulated by mitomycin c treatment of Raji cells. Contrary to this, human Jurkat cells or peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin do not change DPP8 or DPP9 expression levels [48].

DPP8 and DPP9 have each been detected by in situ hybridisation in lymphocytes in the mantle and paracortical zones of human lymph node and interfollicular cells of baboon spleen, and DPP8/9 activity has been detected in baboon spleen and

Jurkat cells [56]. Activation and proliferation of immune cells appears to be influenced by DPP8 and DPP9 enzymatic activity, and selective DPP8/9 inhibition can reduce cytokine production due to the induction of TGF-β secretion, as well as DNA synthesis and T cell proliferation [80, 97]. This is suggestive of DPP8/9 enzyme activity having important roles in the regulation of immune function.

7.2 DPP8 and DPP9 in Cell Biology and Cell Survival

Both DPP8 and DPP9 are involved in cell-extracellular matrix interactions, but DPP8 does not influence cell adhesion [78]. While DPP8 and DPP9 are very similar intracellular enzymes and both ubiquitously expressed, DPP9 appears to have a more pronounced role in cell biology.

DPP9 mRNA is elevated in cartilage from osteoarthritis patients [98]. A study of normal and keloid-derived skin fibroblasts in vitro showed that inhibitors of DPP8 and DPP9 could suppress fibroblast proliferation and decrease secretion of both the fibrogenic cytokine TGF-β_1 and procollagen type 1, which is important in the treatment of fibrotic skin disorders and keloid scar [99].

In sarcoma cell lines, NPY-driven tumour cell death mediated by the nuclear protein modifying enzyme poly(ADP-ribose) polymerase (PARP-1) and apoptosis-inducing factor can be abolished by the overexpression of DPP8 and DPP9 and enhanced by downregulating these proteases [77]. In contrast, overexpression of DPP9 in epithelial tumour cell lines is anti-proliferative and enhances intrinsic apoptosis [62, 78]. DPP9 overexpression can also cause less epidermal growth factor (EGF)-mediated Akt pathway activation in HepG2 cells but has not been observed in cells stimulated with other growth factors, suggesting that this activation was growth factor dependent [62]. Since experiments in which DPP9 is overexpressed on the one hand and orgene silenced or inhibited on the other has produced differing data, the pro- or anti-apoptotic activity of this enzyme may depend on its in vitro culture environment and/or the cell type in which the experiment is undertaken.

The DPP inhibitor vildagliptin synergistically enhances parthenolide's action in leukaemia, lymphoma and primary human acute myeloid leukaemia cell lines [100]. This synergy was due to the inhibition of DPP8 and DPP9 rather than DPP4, suggesting that DPP8 or DPP9 inhibition might be a chemosensitising agent for leukaemia cells, as has been found for FAP in fibrosarcoma [101].

The use of a wide variety of cell lines and tissues can provide insights into the physiological and pathological roles of DPP8 and DPP9 in cell behaviour and survival.

7.3 DPP8 and DPP9 in Disease and Inflammation

There is increasing evidence for an association of DPP8 and DPP9 with disease pathogenesis (Fig. 6). In a gene expression profile study of human hepatocellular

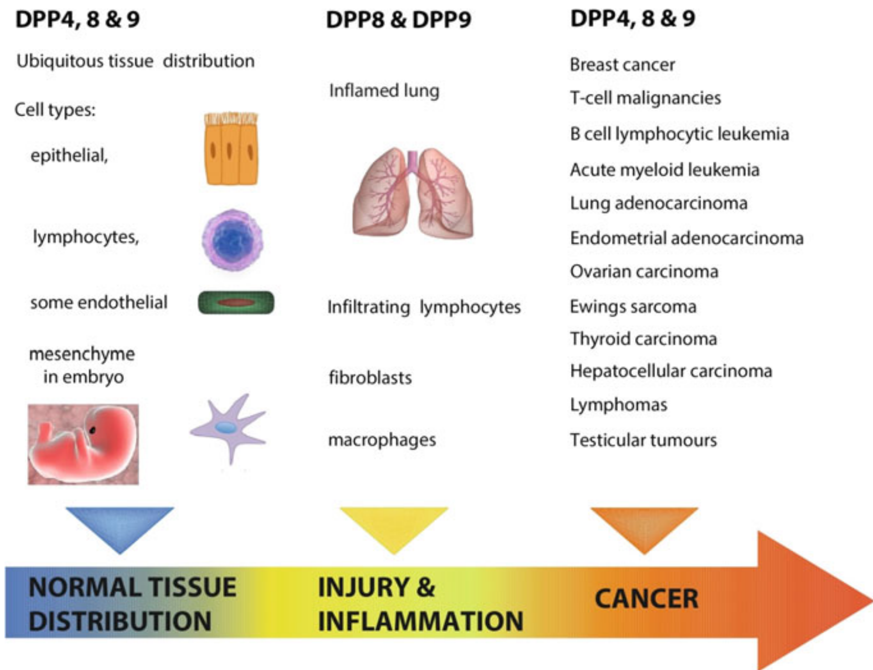

Fig. 6 Summary of studies that have detected mRNA, protein or enzyme activity of DPP4, DPP8 and DPP8 in tissues or cells without disease and increased levels in disease

carcinoma (HCC) tissue, mRNA of DPP9 showed differential expression and upregulation between non-tumour HCC-bearing liver and normal liver [102].

Expression profiling of breast and ovarian carcinoma cell lines, along with 293T and HeLa cell lines, showed ubiquitous but differential expression of DPP8 and DPP9 mRNA and protein across these cell lines. There was poor correlation between mRNA transcript and protein levels for both DPP8 and DPP9, which suggests that DPP8 and DPP9 may be regulated post-transcriptionally in breast and ovarian cancer cell lines [66]. This contrasted with normal mouse tissues, where mRNA and enzyme levels of DPP8 and DPP9 were associated [56].

DPP8 mRNA expression is greater than other DPPs in breast and ovarian cancer cell lines [66], and DPP9 mRNA is abundant in chronic myelogenous leukaemia (K-562) and immortalised cervical cancer (HeLa) cells [43], suggesting that DPP8 and DPP9 may have roles in tumour pathogenesis.

DPP8 and DPP9 mRNA is in tumour infiltrating lymphocytes and DPP9 mRNA is upregulated in human testicular tumours [56]. DPP activity inhibition partially attenuates dextran sulphate sodium (DSS)-induced colitis in mice, and DPP8 mRNA is differentially expressed during colitis development, which is suggestive of role of DPP8 in inflammatory bowel disease [103]. DPP8 mRNA and protein

expression are significantly upregulated in B-cell chronic lymphocytic leukaemia (B-CLL) cells compared to normal tonsil B lymphocytes, which suggests that DPP expression may have biological relevance in B-CLL disease states [36].

Meningiomas are inter-cranial tumours derived from the arachnoid cap cells and include a wide variety of subtypes. DPP8/9 enzymatic activity has been detected in all benign meningiomas, with elevated levels in atypical meningiomas that are associated with greater cell proliferation [61]. An aggressive group of human paediatric malignancies, ESFT, are driven by an aberrant transcription factor that upregulates specific target genes including NPY. ESFT cell death is stimulated by exogenous and endogenous NPY; however, this effect is prevented by cleavage of NPY by cell surface DPP4 and intracellular DPP8 and DPP9. Thus, DPPs are survival factors for EFST and may become potential therapeutic targets for these tumours [77].

A pathophysiologically significant role for DPP8 and DPP9 in asthma is suggested by data from a rat asthma model in which DPP8 and DPP9 and DPP8/9 enzyme activity were primarily detected in the bronchial epithelium of the airways, particularly with allergy-like inflammation [59]. DPP8 and DPP9, along with other DPPs, are elevated in human articular cartilage in osteoarthritis patients, which implicates all of these enzymes in the cascades leading to cartilage breakdown and/or collagenolysis that occurs in arthritis [104].

The expression, localisation and activity patterns of DPP8 and DPP9 have been examined in a model of transient and unilateral cerebral ischaemia in rats [105]. That study found that mRNA expression of DPP9 was diminished in the ischaemic region of the brain at 6 h and 3 days after induced ischaemia, whereas DPP8 levels remained the same at all time points. Also, DPP8 was present in activated microglia and macrophages at day 3 post-ischaemia and in astroglial cells at day 7 post-ischaemia [105]. Those data suggest that DPP8 and DPP9 have potential roles in cerebral inflammation.

As a role for DPP4 in atherosclerosis emerged [106], it was important to investigate the expression and role of DPP8 and DPP9. While DPP4 is only present in endothelial cells of plaque, DPP8 and DPP9 are also in macrophages with a significant upregulation of DPP9 in both pro-inflammatory M1 and anti-inflammatory M2 macrophages during monocyte-to-macrophage differentiation [93]. Primary endothelial cells from aortic, endocardial and cardiac microvascular regions of the rat heart contain DPP8/9 enzymatic activity, with DPP8 protein more abundant than DPP9 [60]. The localisation and expression of DPP8 and DPP9 proteins in endothelia have spatial heterogeneity, with DPP8 more highly expressed in the cardiac microvascular endothelium and DPP9 predominant in the human carotid artery endothelial cells. DPP8 and DPP9 are abundant in the macrophage-rich regions of human and mouse atherosclerotic plaques [64, 93]. M1 macrophages have a role in atherogenesis, so those data suggest potential therapeutic prospects in atherosclerosis and/or plaque rupture by inhibition of DPP9.

A genome-wide association study that focused on human pulmonary fibrosis identified *DPP9* as one of several novel susceptibility loci. Although the evidence for greater expression of *DPP9* in lung tissue of pulmonary fibrosis cases compared

to controls was slight, this group speculated that DPP9 may be involved in the integrity of lung epithelia via cell-to-cell adhesion [107]. This finding needs further investigation of whether DPP9 has a role in the development of pulmonary fibrosis.

8 DPP4 Family Mouse Phenotypes

8.1 Mouse Models

As the mouse genome is amenable to genetic manipulation, many studies involve the use of gene knockout (GKO) mice as models for investigation of the biological properties of proteins in a physiological and/or pathophysiological context. In GKO mice, the protein of interest is absent from the resultant mouse. In the DPP4 gene family, GKO mice have been created for both DPP4 and FAP. The DPP4-GKO mouse was produced by a targeted inactivation in the *DPP4* gene and resulted in healthy mice [108]. Similarly, the FAP-GKO mouse was constructed through exon deletion and also created fertile mice with no overt developmental or haematological defects or increased cancer susceptibility [109]. It has been speculated that, in the absence of these proteins, other proteins are upregulated and have compensatory roles. To date, no DPP8-GKO mice have been reported.

8.1.1 DPP9 Gene Knock-in (GKI) Mouse

Unlike GKO mice where the entire protein is absent from the mouse, in a GKI mouse the protein of interest is present but inactivated; thus, it is still able to fulfil all functions apart from the one that has been removed. By targeting the DPP9 protein catalytic site with a serine to alanine point mutation, we were able to create a DPP9 gene knock-in mouse (DPP9-GKI) that lacks DPP9 enzymatic activity while maintaining normal DPP9 protein structure and thus the non-enzymatic functions of DPP9 [52]. This DPP9-GKI mouse exhibits neonate lethality, suggesting that DPP9 enzymatic activity is essential for early neonatal survival in mice [52]. However, no morphological defect or cause of death has been identified. Further studies using neonatal liver and gut from DPP9-GKI mice compared to WT have shown that DPP9 enzymatic activity influences the expression of many genes [110].

Taqman PCR arrays and qPCR analyses of neonatal liver revealed differential expression of genes involved in cell growth (epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), vascular endothelial growth factor-A (VEGF-A), innate immunity (tumour necrosis factor (TNF)-α, interleukin-1 β, IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha)) and metabolism signalling pathways, such as insulin receptor substrate-2 (INS-2). Neonatal liver and gut showed differential expression of the master transcription coactivator peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) and genes involved in long-chain-fatty-acid uptake and esterification, long-chain fatty acyl-CoA binding, trafficking and transport into

mitochondria, lipoprotein metabolism, adipokine transport and gluconeogenesis in the DPP9-GKIhomozygote neonatal mice compared to WT. In addition, immunoblots showed that DPP9 enzyme activity can modulate AMP-activated protein kinase phosphorylation in the Huh7 human hepatoma cell line [110].

The differential expression of genes involved in cell growth, innate immunity, lipid metabolism and gluconeogenesis suggests roles for DPP9 enzymatic activity in the regulation of immune and metabolic pathways in the neonate [110].

9 Conclusion

DPP9 is emerging as an interesting and unique atypical serine protease that has potential roles in tumours, leukaemia, atherosclerosis, inflammation and energy metabolism and in T, B and NK cells and endothelial cells. Numerous studies have been reported now that detail DPP9 expression and its upregulation in brain, joint, liver, lung and gut inflammation. However, a dominant homoeostatic or disease role for DPP9 has not been identified. Despite strong expression in muscle, no function in muscle or heart has arisen. The enzyme activity of DPP9 has been implicated in neonate survival, metabolism and immune system, and in macrophage function, EGF receptor signalling and cell migration.

The impediments to a greater understanding of DPP9 are the lack of any selective enzyme inhibitor, specific enzyme assay or adult GKO or GKI mice.

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The γ-Secretase Protease Complexes in Neurodegeneration, Cancer and Immunity

Caroline Coleman-Vaughan, Arijit Mal, Abhijit De and Justin V. McCarthy

Abstract

The intramembrane-cleaving proteases (I-CliPs) are necessary for the proteolytic cleavage of several transmembrane proteins and initiation of divergent signalling events. The tetrameric γ-secretase protease complexes, comprised of presenilin and three other subunits, represent a major subclass of the I-CliPs. The γ-secretase protease complexes are involved in regulated intramembrane proteolysis, an evolutionary conserved and important signal transduction process encompassing the sequential proteolysis of transmembrane substrates that are central to many physiological processes, including embryonic development, haematopoiesis, and normal functioning of the nervous and immune systems. Deregulated intramembrane proteolysis of certain substrates is proposed to be associated with neurodegeneration, cancer and impaired immune function. In this chapter, we summarise the major biochemical and functional properties (structure, catalytic mechanisms, substrate specificities, and regulation) of the γ-secretase protease complexes. We also present evidence for a role of γ-secretase protease complexes in neurodegeneration, cancer and inflammatory disease and consider the use of γ-secretase inhibitors as prospective therapeutics in several diseases.

C. Coleman-Vaughan · J.V. McCarthy (✉)
Signal Transduction Laboratory, School of Biochemistry & Cell Biology, University College Cork, ABCRF, Western Gateway Building, Cork, Ireland
e-mail: jv.mccarthy@ucc.ie

A. Mal · A. De
Molecular Functional Imaging Laboratory, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Kharghar, Navi Mumbai, India

J.V. McCarthy
School of Biochemistry & Cell Biology, University College Cork, ABCRF, 3.41 Western Gateway Building, Western Road, Cork, Ireland

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1 Introduction

With the identification and characterization of the γ-secretase protease complexes and parallel acceptance that specific enzymes can catalyse the proteolysis of proteins within the cell membrane, the concept of "regulated intramembrane proteolysis" was defined [1, 2]. Regulated intramembrane proteolysis (RIP) is now considered an evolutionary conserved and fundamental signal transduction process central to a diversity of cellular processes, such as cell differentiation, cell adhesion, cellular stress responses, protein degradation, growth factor secretion, axon guidance, neurite outgrowth, mitophagy, lipid metabolism and transcriptional regulation [3–8]. RIP is vital for several biological events, including embryonic development, haematopoiesis, and normal functioning of the nervous system and the immune system. Highlighting its biological importance, RIP is under austere regulation [6, 9], and deregulation is associated with the pathogenesis of diseases, such as Alzheimer's disease (AD), cancer and autoimmune disorders.

RIP is a two-step proteolytic process initiated following the proteolytic cleavage of type I or type II transmembrane proteins (Fig. 1). Referred to as ectodomain shedding, this cleavage occurs either constitutively or in response to ligand binding, close to the transmembrane domain (TMD) within the substrate ectodomain. Proteases collectively termed "sheddases" [10] including members of the a disintegrin and metalloproteases (ADAMs) [11], matrix metalloproteases (MMP) [12], neutrophil derived proteases (SDPS) and the aspartyl proteases β-site APP-cleaving enzymes 1 and 2 (BACE1 and BACE2) [13], perform the ectodomain shedding of most reported substrates. This initial cleavage results in extracellular release of a soluble ectodomain and generation of a membrane-anchored stub, which is subsequently cleaved within its TMD, called intramembrane proteolysis. Members of the family of intramembrane cleaving proteases (I-CliPs) are responsible for the hydrolysis of peptide bonds in the hydrophobic lipid bilayer of many transmembrane proteins and the release of extracellular peptides and an intracellular domain (ICD) into the cytosol (Fig. 1) [14].

An ever-increasing number of transmembrane protein substrates that undergo RIP have been reported, including growth factors, cytokines, cell adhesion proteins, growth factor and cytokine receptors, viral proteins and signal peptides [5, 8, 15]. Amongst the best studied and characterised RIP substrates are functionally important proteins such as amyloid-β precursor protein (APP) [16], Notch [17, 18], E-Cadherin [19], tumour necrosis factor-α (TNFα) [20], interleukin-1 (IL-1)

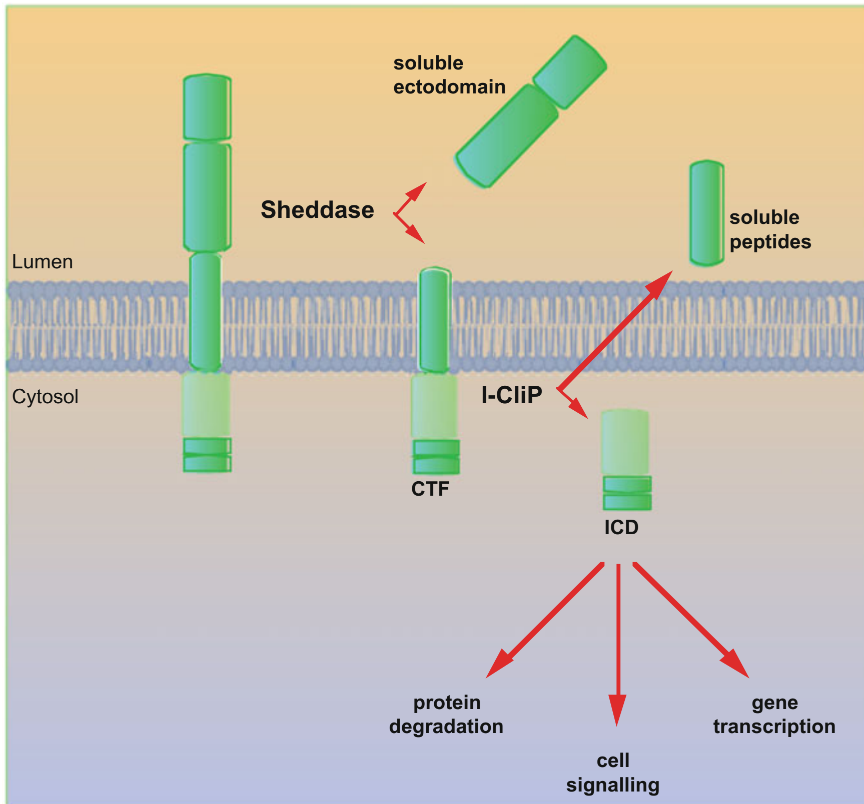

Fig. 1 Schematic depicting regulated intramembrane proteolysis. The sequential proteolysis of single-span transmembrane protein with type-I or type-II orientation, involving intramembrane-cleaving proteases (I-CliPs) is referred to as regulated intramembrane proteolysis. First, cleavage within the substrate extracellular domain, close to the transmembrane domain, by proteases collectively termed "sheddases" release the soluble ectodomain and generate a membrane-bound fragment, which subsequently undergoes a second cleavage within its transmembrane domain, called intramembrane proteolysis. Members of the family of I-CliPs are responsible for the hydrolysis of peptide bonds in the hydrophobic lipid bilayer of many transmembrane proteins and the release of extracellular soluble peptides and an intracellular domain (ICD) in the cytosol. The ICD can possess substrate-specific biological activities, including roles as a cytosolic effector in cell signalling and nuclear gene transcription

receptor, type I and II (IL-1RI and IL-1RII) [21–23], Insulin-like growth factor 1 (IGF-1) receptor [24], epidermal growth factor (EGF) [25], ErbB4 [26], p75 neurotrophin receptor (p75^{NTR}) [27–29], CD44 [30] TREM2 [31] and epithelial cell adhesion molecule (EpCAM) [32] amongst others, (reviewed in [5, 8, 15]). Arising from RIP, the soluble protein fragments generated; ectodomains and ICDs can possess biological activities with signalling characteristics distinct from the full-length substrate or are rapidly degraded (Fig. 1). For example, the shedding of growth factors provides for the generation of a soluble signalling molecule that can

activate target cells [20, 33, 34]. For other substrates, such as p75^{NTR}, TNFR1, IL-1RI and IL-1RII, it is a means of regulating receptor-mediated signalling, as shedding of the soluble receptor ectodomain reduces cell surface availability of receptors and desensitises cells to further stimulation [21, 22, 35]. Furthermore, because the soluble receptor ectodomain can still bind to circulating ligands, ectodomain shedding also provides a mechanism for regulating the amount of soluble ligand available to bind and signal through membrane-bound receptors. For other RIP substrates, such as Notch and EpCAM, it is the newly generated ICD that possesses biological activity [17, 32]. In both cases, the EpCAM ICD (EpICD) and Notch ICD (NICD) translocate to the nucleus and facilitate the transcriptional regulation of target genes. For other RIP substrates, such as TNFα which is cleaved by SPP, both the soluble ectodomain and ICD are biologically active and initiate both extracellular and intracellular signalling events in different cells [20, 33, 34]. In many cases, the newly generated ICD is very unstable and short-lived, suggesting that certain ICDs do not possess a signalling function and are rapidly degraded. Thus, from the characterisation of numerous substrates, RIP is proposed to have two principal opposing functions, namely the initiation of signalling events that are distinct from functions of the full-length substrate and degradation of membrane proteins. Given the diversity of substrates and biological signalling pathways involving RIP, deregulation of this proteolytic pathway is also important in the pathogenesis and progression of several diseases.

2 Proteolytic Processing of Amyloid β-Precursor Protein (APP)

Much of our understanding of γ-secretase and RIP come from studies focused on the proteolytic processing of APP and generation of amyloid-β (Aβ) peptides, a defining characteristic in the molecular aetiology of AD. APP, a type I transmembrane glycoprotein, has three major isoforms, of 695, 751 or 770 amino acids, which are derived from alternative splicing of a single gene product (reviewed in [36]). In neurons APP695 is the predominantly expressed form, while APP751 and APP770 are mainly expressed in glia cells and other non-neuronal cells. APP undergoes consecutive cleavage events in its extracellular domain by α-secretase (ADAMs) or β-secretase (BACE1) and intramembrane proteolysis by γ-secretase to generate AICD. Aβ peptides are generated from APP during the late secretory pathway, in which active BACE1 is highly concentrated (reviewed in [37]). Therefore, like many other RIP substrates, the proteolysis of APP is closely associated to APP trafficking within the cell and the spatial distribution of specific proteases.

APP can undergo either proteolysis in its extracellular domain by α-secretase (ADAMs) or β-secretase (BACE1), which are referred to as the non-amyloidogenic and amyloidogenic pathways, respectively (Fig. 2). In neurons, the amyloidogenic pathway is important in the pathogenesis of AD, while the

non-amyloidogenic pathway is more prevalent in most cell types. In this pathway, APP is first cleaved by α-secretase, resulting in the release of a soluble N-terminal fragment (sAPPα) and formation of a membrane-bound 83 amino acid C-terminal fragment (αCTF or C83). It has been shown that γ-secretase completes sequential endopeptidase-like and carboxypeptidase-like cleavage events (reviewed in [38]), which are exemplified in the proteolysis of APP. First the APP αCTF undergoes endoproteolysis by γ-secretase at the juxtamembrane region, which is referred to as the ε-cleavage site, generating the APP intracellular domain (AICD) and the p3 peptide. In the amyloidogenic pathway, following an initial cleavage by β-secretase (BACE1), which releases sAPPβ and generates a membrane-bound 89/99 amino acid βCTF, which is then proteolytically cleaved by the same γ-secretase at the ε-cleavage site to produce AICD and two major longer Aβ products (Aβ49 and Aβ48). Next γ-secretase effects carboxypeptidase-like processive cleavage of Aβ49 and Aβ48 resulting in the generation of Aβ peptides of different sizes arises due to a series of progressive γ-secretase cleavage steps, every 3–4 residues. Starting with ε-cleavage and generation of Aβ49, which is processed primarily at the ζ-cleavage site to generate Aβ46 and γ-cleavage sites to generate Aβ43 and Aβ40 peptides. In a similar manner Aβ48 is cleaved to produce Aβ45, Aβ42 and Aβ38 respectively, [39–41] (Fig. 2). This proposed model clearly explains that successive proteolysis of substrates by γ-secretase and highlights the importance of the ε-cleavage site in determining the size of Aβ peptides that will be produced following subsequent carboxypeptidase-like processive cleavage by γ-secretase, which ultimately contribute to the formation of amyloid plaques in AD. Like APP, many other substrates undergo γ-secretase cleavage at ε- and γ-cleavage sites. Therefore, like APP, it has been demonstrated for the Notch receptor that extracellular small peptides, referred to as Notch Aβ-like peptides (Nβ), are produced and secreted during RIP of Notch [42]. This also leads to the suggestion that the release of Aβ- or Aβ-like peptides is a common feature of γ-secretase activities during RIP.

3 Intramembrane Cleaving Proteases

Four major classes of I-CLiPs have been discovered each with distinct membrane topologies and embedded active sites within their TMDs (reviewed in [43, 44]). These include the rhomboid serine proteases, the metalloprotease site 2 protease (S2P) family and the intramembrane aspartyl proteases, including the signal peptide peptidase (SPP)-like (SPPL) family and the γ-secretase proteases. All the I-CLiPs are presumed to be enzymatically active as monomeric or dimeric proteins, with the exception of γ-secretases, which is a tetrameric protein complex, including the catalytic subunit presenilin [45]. Signature catalytic motifs are preserved across family members in the TMD of the I-CliP proteases. The S2P catalytic activity is dependent on its conserved HExxH motif, which contributes two histidines and one glutamate, and a distally located conserved aspartate residue [45]. Rhomboids contain a (Ser, His, Asn) catalytic triad, however others suggest that the Asn residue

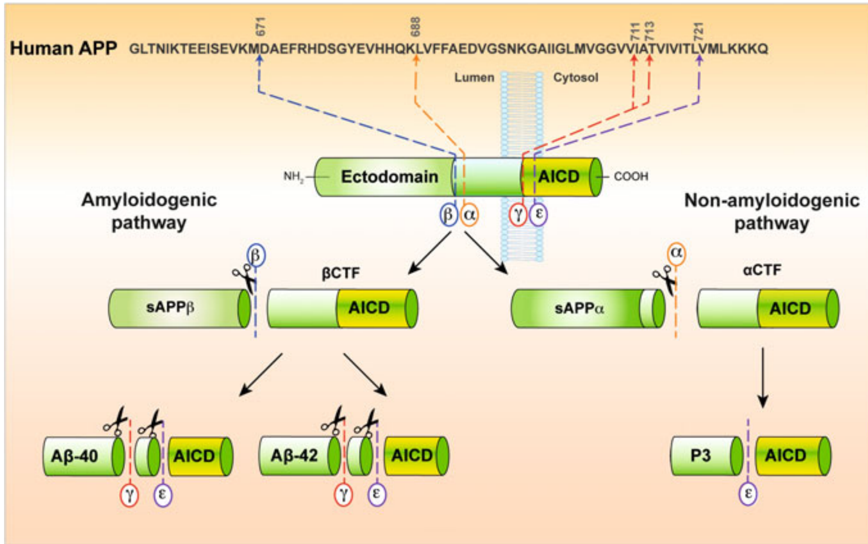

Fig. 2 **Regulated Intramembrane Proteolysis of the Amyloid β-Precursor Protein**. The amino-acid sequences adjacent to and within the transmembrane domain of APP are shown together with the principal sites of cleavage that occurs during regulated intramembrane proteolysis. In the non-amyloidogenic pathway, α-secretase cleavages APP 16 residues from the cell surface to release the soluble α-cleaved N-terminal fragment (sAPPα) and generate the 83 amino acid membrane-bound α-cleaved C-terminal fragment αCTF (C83). In the amyloidogenic pathway, β-secretase cleaves APP to release the shorter soluble β-cleaved N-terminal fragment (sAPPβ) and the longer 99 amino acid membrane anchored β-cleaved C-terminal fragment, βCTF (C99). The αCTF and βCTF remnant are further processed by the γ-secretase protease. APP αCTF is cleaved by γ-secretase at the ε-cleavage site, near residue 721 of APP, generating AICD and the p3 peptide. In the amyloidogenic pathway, βCTF is cleaved at the ε-cleavage site to produce AICD and two major longer Aβ products (Aβ49 and Aβ48) which undergo γ-secretase mediated processive cleavage to generate Aβ peptides of different sizes The γ-secretase cleavage of APP near residues 711 and 713 in APP, when combined with cleavage at the β-secretase site, generates amyloid-β peptides $Aβ_{40}$ and $Aβ_{42}$, respectively

is not necessary for enzymatic activity, and favour a catalytic dyad model of proteolysis [4]. SPP and the γ-secretase proteases are polytopic membrane proteins and members of the GxGD-type aspartyl protease family with two conserved aspartate residues that define the catalytic site [46]. With the exception of the rhomboid proteases, in general all I-CliPs require a preceding ectodomain cleavage step to generate a truncated version of the initial substrate [47]. However, recently substrates of γ-secretase that require direct cleavage without ectodomain shedding have also been reported [48]. Although structures of representative members of the I-CliP family have been solved, the full mechanism of substrate recognition and cleavage are still poorly understood [47].

Given that both γ-secretase and SPP are members of the same I-CliP subfamily, it's not surprising that they share many similarities, however many differences have also been identified (Fig. 3) (reviewed in [43, 44, 49]). Briefly, SPP functions

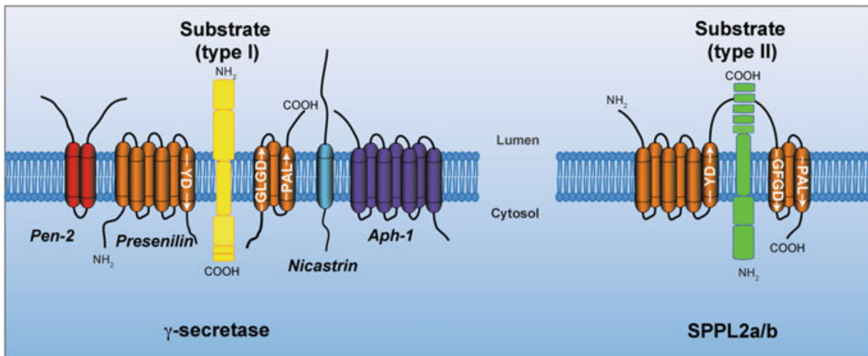

Fig. 3 Schematic comparing the membrane topologies of γ-secretase and the signal peptide peptidase (SPP) intramembrane cleaving aspartyl proteases. Topologies of γ-secretase tetrameric protease complex (*left*) and of SPPL2a/b, a member of the signal peptide peptidase (SPP/SPPL) family of aspartyl proteases. The γ-secretase protease is composed of four proteins including, presenilin (PS1 or PS2), nicastrin, Pen-2 and Aph-1 (Aph-1a/Aph-1b). Presenilin requires endoproteolysis to produce catalytically active presenilin NTF/CTF heterodimers. Note the opposite membrane orientation of type I (γ-secretase) and type II (SPPL2a/b) substrates. *Arrows* indicate the inverted orientations of their respective GxGD and YD active sites and PAL motif

without the requirement for additional cofactors for their activity, but may exist as an active homodimer [50–52]. However, the presenilins require endoproteolysis and at least three other additional proteins to form an active γ-secretase protease. The two proteases display distinctive substrate recognition profiles, where γ-secretase cleaves type I transmembrane substrates, SPP cleaves type II transmembrane proteins. Both share the highly conserved catalytic motifs (GxGD and YD), containing the two critical catalytic aspartate residues located on adjacent TMDs [20], and the C-terminus PAL motif, which is necessary for catalytic activity [53, 54]. SPP and γ-secretase do however display different membrane topologies, which account for the inverted orientation of the conserved catalytic motifs and PAL motif, and which may account for the difference in substrate specificity (Fig. 3). Certain specific γ-secretase inhibitors such as L-685,458 also significantly reduced SPP activity, and L-685,458 and helical peptides mimicking the substrate-docking site block SPP activity supporting the conclusions that these proteases have similar biochemical and structural similarities (reviewed in [49]).

4 γ-Secretase Protease Composition and Structure

In the early 1990s Haass and Selkoe first described "γ-secretase" a proteolytic enzyme, which in combination with BACE1 cleaved APP within the TMD resulting in the generation of Aβ peptides associated with AD pathology [55]. Subsequent

studies performed in families of early onset AD patients led to the discovery of mutations in two homologous Presenilin genes *Psen1* and *Psen2* which encode the proteins Presenilin 1 (PS1) and Presenilin 2 (PS2), respectively [56–58]. In addition to the presenilins, the mature γ-secretase complexes have since been characterized to include three additional proteins that are essential for stability and activity of the γ-secretase protease: nicastrin, anterior pharynx-defective-1 or 2 (Aph-1/Aph-2) and presenilin enhancer-2 (Pen-2) (Fig. 2) [59–63]. The observation that reconstitution of these four polypeptides in *Saccharomyces cerevisiae*, a cell type devoid of intrinsic γ-secretase activity, produced enzymatic activity has led to the conclusion that these four proteins are minimally required for γ-secretase protease activity [64].

The presenilins (PS1 and PS2) are highly homologous evolutionary conserved multi-transmembrane aspartyl proteases with a proposed nine TMD topology with the amino-terminus in the cytosol and the carboxyl-terminus within the luminal/extracellular space [62, 63]. The presenilins are synthesised as catalytically inactive full-length proteins with a short half-life. Catalytic activity and incorporation into γ-secretase protease complexes requires endoproteolytic cleavage between TM6 and TM7 to produce a 27–28 kDa amino-terminal fragment (NTF) and 16–17 kDa carboxyl-terminal fragment (CTF) [65, 66], which together form stable and active presenilin NTF/CTF heterodimers. Human presenilins contain two highly conserved aspartate residues on adjacent TMDs, TM6 and TM7, which are located at D257 and D385 in human PS1 and D263 and D366 in human PS2, respectively [67], which are indispensable for γ-secretase protease activity. The C-terminus PAL motif in TM9 of presenilins also contributes to the catalytic activity and the formation of the γ-secretase complex [53, 54]. Despite sharing 67% amino acid identity, PS1 and PS2 are not redundant. Deletion of PS1 is lethal in mice while PS2 deficiency is associated with a very mild phenotype linked to pulmonary fibrosis [68, 69].

Nicastrin encodes a 78 kDa type I membrane protein with a large extracellular domain, transmembrane helix and smaller cytoplasmic domain. Nicastrin is highly glycosylated, resulting in an apparent molecular weight of ~130 kDa. It is proposed that glycosylation of nicastrin is required for proper trafficking of the mature complex [70, 71]. It is often considered the 'gatekeeper' of the γ-secretase protease complex [72, 73]. The ectodomain is proposed to bind to the free amino-terminal of ectodomain-shed proteins, essentially acting as a scaffold protein important in substrate recognition. The amino-terminus of nicastrin interacts with the carboxyl-terminus of presenilin, facilitating assembly of the complex [69, 74]. The final two subunits, Pen-2 and Aph-1, are less well studied. Pen-2 is a ~12 kDa protein that spans the membrane twice, with N- and C-terminus domains facing the lumen of the endoplasmic reticulum (ER). The incorporation of Pen-2 into the complex is critical for the final maturation of γ-secretase as it triggers endoproteolysis of the presenilin holoprotein into its stable heterodimer [69]. Binding of Pen-2 is mediated through the fourth TMD of presenilin proteins and this event subsequently prompts the final steps of the γ-secretase maturation process with the concomitant final glycosylation of nicastrin and the release of the γ-secretase enzyme complex from the ER to the *trans-Golgi* [75]. Aph-1 is a ~25 kDa protein

with seven TMDs. Aph-1 is proposed to function in γ-secretase complex stability, activity and selectivity [66, 76]. Two human *Aph-1* homologous genes have been identified on human chromosomes 1 (*Aph-1a*) and 15 (*Aph-1b*) [60, 61], and *Aph-1a* is also expressed in two isoforms (*Aph-1aL* and *Aph-1aS*) via alternative splicing [77]. One additional homologue *Aph-1c* was identified in mice [78].

This quartet of proteins exists as a 1:1:1:1 heterodimer and several gain and loss of function studies, reconstitution experiments in yeast and mammalian cells have been performed, which collectively suggest that all these proteins stabilize each other in the assembly of catalytically active γ-secretase protease complexes [77]. Assembly of γ-secretase into functional complexes occurs in a stepwise manner in the ER, where nicastrin maintains stability of the assembling complex and regulates its intracellular trafficking. Aph-1, is required for initiating assembly of the complex and proteolytic activity [77]. Deficiency of either *Nicastrin* (*Nct*) or *Aph-1* decreases the levels of Pen-2 and PS1 NTF/CTF heterodimers, and disrupts their intracellular trafficking [77]. Pen-2 is destabilized in conditions of *Psen* deficiency, and in conditions of *Pen-2* deficiency full length PS1 accumulates. Subcellular localization motifs present in the TMDs of PS1, Pen-2 and nicastrin responsible for ER retrieval/retention prevent trafficking of unassembled subunits beyond the ER [79, 80]. These signals are concealed in properly assembled γ-secretase complexes thereby allowing trafficking to the *Golgi* apparatus and final subcellular localizations. These observations demonstrate that the four proteins cross-regulate each other and can influence the assembly, stabilization, and trafficking of the protease complex. Until recently details on the structure have been hampered due to difficulties in expressing and purifying intact γ-secretase [81]. Recently a 3D structure of an intact human γ-secretase complex has been shown at 4.5 angstrom resolution [82], and an atomic-resolution (3.4 angstrom) cryo-EM structure was also reported [83].

Like the presenilins, *Aph-1* homologues share overlapping but non-redundant roles. While deficiency of *Psen1* or *Aph-1A* is associated with embryonic lethality [68, 69, 84], *Psen2*- or *Aph-1b*-knockout mice mainly develop as normal. Thus, given the diversity of subunit homologues and distinct phenotypes from knockout mice, it is proposed that at least six distinct γ-secretase complexes can exist in humans [85, 86]. Consistent with this, the levels of expression of the presenilins and Aph-1a/Aph-1b can differ between cell types and tissues, and different γ-secretase complexes can also co-exist within the cell [78, 87]. Recent studies have demonstrated that PS1-containing γ-secretase complexes are mainly localized to the plasma membrane and cell surface [88, 89], while PS2-containing γ-secretase complexes are restricted and compartmentalised in the late endosome/lysosome [88, 89]. Additionally, the subcellular distribution of PS2-containing γ-secretase complexes differs according to the Aph-1 subunit composition, where PS2/Aph-1aS- and PS2-Aph-1b-containing γ-secretase complexes were observed in late endosomes and lysosomes, while PS2/Aph-1aL containing complexes were never detected in this subcellular space [88]. Biochemical evidence also shows that the different γ-secretase complexes differentially cleave substrates, including APP [89, 90] and Notch [89]. More specifically, PS1-containing γ-secretase complexes were

shown to favour cell surface substrates, such as N-Cadherin, while PS2-containing complexes preferentially cleaved substrates that localize to the late endosome/lysosome, such as tyrosinase-related-protein (TRP1) and premelanosome protein (PEML) [89]. Similarly, while both Aph-1a and Aph-1b containing complexes are equally capable of cleaving synthetic APP and Notch, from in vivo studies it is clear that the Aph-1a-containing complexes are essential for Notch proteolysis during embryogenesis, while Aph-1b-containing complexes apparently do not have a substantial role in Notch signalling in peripheral tissue. Therefore, since the more restricted compartmentalization of PS2-containing complexes contribute to the generation of the AD-related intracellular pool of Aβ peptides [91–93], and given that *Aph-1b* is predominantly expressed in the brain with limited effects on Notch signalling, this potentially highlights PS2-containing complexes and/or Aph-1b-containing complexes as attractive drug target for AD.

5 γ-Secretase Enzyme Regulation

The cleavage of a substrate is a strictly controlled activity that involves either the direct regulation of the protease activity or substrate modifications (localization or post-translational modifications) that inhibit or enable proteolysis. The γ-secretase proteases cleave over 100 type-I transmembrane proteins (reviewed in [7, 15]). Despite its apparent promiscuous nature, γ-secretase enzymatic activity is strictly controlled by a number of mechanisms as intricate and diverse as its functions. As discussed previously, γ-secretase activity is dependent on the accurate assembly of its four indispensable subunits. The removal of any of the four subunits results in a reduction of active complex formation and loss of enzymatic activity. Furthermore, many proteins that interact with the complexes can influence enzymatic activity [94], including CD147, phospholipase D1, TMP21, GPR3, γ-secretase activating protein (GSAP), syntaxin-1, Arc, voltage-dependent anion channel 1 (VDAC1), contactin-associated protein 1 (CNTNAP1), TPPP, NDUFS7, Erlin-2, β-arrestin-1, β-arrestin-2, Hif-1α and Nexin 27 [94–96].

Post-translational modifications (PTM) of γ-secretase complex components or their substrates can enable or inhibit substrate recognition and cleavage by the γ-secretase proteases (reviewed in [7, 97]). Post-translational modifications can also regulate trafficking of substrates and their γ-secretase-generated cleavage products between distinct subcellular membrane compartments. The presenilin proteins undergo proteolysis (endoproteolysis and caspase cleavage), phosphorylation and ubiquitination [7, 98, 99]. These modifications have been shown to not only determine subcellular localization [100], and selectivity of binding-partners [101], but also regulate γ-secretase complex assembly and enzyme activity [67]. For example, PS1 is ubiquitinated by SEL-10 and tumour necrosis factor receptor associated factor 6 (TRAF6), which regulate γ-secretase activity and substrate cleavage [27, 98, 102]. Additionally, glycosylation of nicastrin is also essential for

the trafficking and correct subcellular localization of γ-secretase complex components [68].

As summarized earlier, subsequent to APP ectodomain shedding, the remaining membrane-anchored APP-CTFs can be processed by intramembrane proteolysis or proteasomal degradation [103]. It has been reported that the heterogeneity of APP-CTFs is in part attributed to post-translational modification, specifically APP-CTF phosphorylation (reviewed in [104, 105]). APP has eight potential phosphorylation sites [106], seven of which are phosphorylated in AD brains, i.e., Y653, S655, T668, S675, Y682, T686, and Y687 (APP695 isoform numbering) [106, 107]. The phosphorylation of APP at T668 is mediated by neuronal cyclin-dependent protein kinase 5 (cdk5) [107], p34cdc2 protein kinase (cdc2) [108], glycogen synthase kinase 3β (GSK-3β) [109], or c-jun N-terminal kinases (JNK) [110–112], while phosphorylation of APP-CTFs by JNK has been shown to enable association with PS1, enhance their proteolysis by γ-secretase [112], and regulate the nuclear translocation of AICD [113]. Similar to APP, post-translational modification of other γ-secretase substrates has been shown to affect intramembrane proteolysis. For example, palmitoylation of $p75^{NTR}$ and CD44-CTFs, ubiquitination of IL-1R1 [23], $P75^{NTR}$ [27] and Notch, and phosphorylation of CD44-CTFs haves been shown to regulate their stability to enhance processing by γ-secretase [114].

The specificity of γ-secretase complexes is also determined by the compartmentalization and spatial co-expression of complexes and substrates. γ-secretase activity is observed at the *trans-Golgi* network, endocytic organelles and plasma membrane (reviewed in [115]). It was recently shown that the N-terminus of PS2 contains a highly conserved unique acidic-dileucine sorting sequence ($E_{16}RTSLM_{21}$) [89], which fits the acidic-dileucine motif [D/E]xxxL[L/I/M] present in other proteins that are transported to late endosomes/lysosomes [116]. This motif enables PS2 to bind to the γ1-σ1 (AP1G1-AP1S1) hemi-complex of the AP-1 adaptor complex, which enable sorting and localization of proteins at different stages on the endomembrane system. It was shown that phosphorylation of the $E_{16}RTSLM_{21}$ motif regulated PS2 interaction with AP-1 and restricted the spatial distribution of PS2 to late endosomal/lysosomal compartments [89]. In contrast, PS-1 containing γ-secretase complexes that lack the AP-1 interaction motif are trafficked along a different route, frequenting Rab-11-positive recycling endosomes, and are more prevalent at the plasma membrane. These observations also support the long-standing proposal that intracellular trafficking and localization of γ-secretase proteases also regulates the enzyme activities of γ-secretase proteases (reviewed in [9]). PS1-containing γ-secretase complexes are enriched in lipid rafts and their activity is modulated by lipid composition [117–121], further implying that the subcellular localization of γ-secretase is critical to its proteolytic activity.

A growing body of evidence also supports the proposal that the subcellular localization of γ-secretase substrates is important for intramembrane proteolysis and subsequent signalling functions. It has been shown that following cell surface shedding of Notch ectodomain, internalization of Notch is required for intramembrane proteolysis and generation of NICD [122, 123]. Similarly, subsequent to NGF ligand-induced ectodomain shedding, $p75^{NTR}$ C-terminal fragments are localized in

early endosomes where they are proposed to undergo γ-secretase-dependent intramembrane proteolysis [124]. Other examples from our studies include the proinflammatory cytokine receptors TNFR1 and IL-1R1, which were recently identified as γ-secretase substrates [21, 125]. In our studies we have demonstrated that inhibition of dynamin-dependent endocytosis increases cell surface shedding of TNFR1 [125], and antagonises γ-secretase-dependent intramembrane proteolysis of TNFR1 [125], and IL-1R1 (unpublished). In the case of APP, inhibition of internalization was shown to antagonize the generation of sAPPβ (reduce BACE cleavage) and enhance cell surface generation of sAPPα. Of pathological relevance, endocytic disturbance has been described as an early characteristic of AD pathology (reviewed in [126]), and proposed to significantly induce endosomal accumulation of APP and BACE1, which may lead to the exacerbation of Aβ pathology [127]. Collectively, these examples highlight the importance of proper spatial distribution and the compartmentalization requirement of both γ-secretase complexes and substrates for appropriate intramembrane proteolysis, and suggest the existence of similar tissue, cell or subcellular spatial requirements for RIP of other substrates.

6 Alzheimer's Disease and Neurodegeneration

AD is a multifactorial disorder, associated with progressive and debilitating loss of cognition, memory and language, and is now considered the main cause of dementia and one of the greatest health-care challenges of the Twenty-first Century. Most AD cases are sporadic and late onset (LOAD), although genetic mutations found in patients with the dominantly inherited form of early onset familial AD (FAD), account for approximately >5% of AD cases, and are caused by autosomal dominant mutations in *App* or either of the presenilin genes, *Psen1* and *Psen2*. At least 266 AD-associated mutations have been mapped to the presenilin genes, with 277 identified in *Psen1* and 39 in *Psen2* (http://www.alzforum.org/mutations). Pathologically, AD is defined by the occurrence of profuse proteinaceous deposits; extraneuronal amyloid plaques and intraneuronal neurofibrillary tangles [128]. Amyloid plaques are a fibrous aggregate principally composed of Aβ peptides, whereas neurofibrillary tangles are composed of aggregated, hyperphosphorylated tau, a cytosolic protein normally associated with microtubules. Over the past 30 years of AD research extensive evidence has been presented to support the hypothesis that disproportionate accumulation of abnormally folded Aβ and tau are casually related to the neurodegenerative processes associated with AD. Genetic mutations found in patients with FAD also cause increased production and/or aggregation of Aβ, suggesting that Aβ is involved in the pathogenesis of AD. In particular, Aβ42 is predominantly deposited in the brains of AD patients. For this reason, the generation of Aβ peptides has been the primary target towards developing potential AD therapeutics, and inhibiting the enzymes that produce Aβ has been a priority and favoured strategy for AD drug discovery (reviewed in [52, 129–131]). However, the complexity of γ-secretase proteolysis and its

functionally divergent substrates has recently discouraged some efforts to directly inhibit their proteolytic activity, which can have deleterious and toxic side effects.

Prior to the identification of β-secretase and γ-secretase [16, 132], the proteases responsible for the generation of Aβ peptides, several pharmaceutical companies identified and developed distinct classes of γ-secretase inhibitors (GSIs) with favourable pharmacokinetic profiles [133–135]. These included the active site-directed small molecule transition state analogue inhibitors, which do not discriminate between different γ-secretase complexes or substrates, and none have progressed to AD clinical trials [130, 136]. The non-transition state analogue inhibitors include the well-studied N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and LY450,139 (semagacestat). However, in addition to inhibiting γ-secretase cleavage of APP and reducing Aβ peptide formation, these GSIs also inhibited the cleavage of Notch, in some cases to a similar or greater extent that APP (reviewed in [130, 131, 134, 137]). Recently, Eli Lilly were forced to prematurely terminate a phase III trial of LY450,139 (semagacestat) in a large cohort of patients with probable AD, due to severe side effects including, weight loss, worsening cognitive function scores, infections and increased incidences of skin cancer [138], some likely due to Notch-related toxicities [139]. Clinical trials with second-generation GSIs designed to have reduced antagonistic effects on Notch (referred to as Notch-sparing compounds or γ-secretase modulators (GSMs)), have been reported for avagacestat [140] and begacestat [141], however both were discontinued. Similarly, clinical trials with, ELND006 and ELND007 were prematurely stopped due to non-mechanism-based liver toxicity [142]. However, clinical trials with other GSM compounds, NIC51- and CHF-5075, which exhibited acceptable safety profiles in phase I trials, are now progressing through phase 2 clinical trials (http://clinicaltrials.gov). Because GSMs have less effect on Notch or other substrates, they should have fewer undesirable side effects and discovering more potent and safe GSMs is still a key strategy towards the development of a γ-secretase-based therapy for AD.

Another class of compounds, the nonsteroidal anti-inflammatory drugs (NSAIDs) were fortuitously identified to lower Aβ42 levels in vitro [143]. One such NSAID, ibuprofen was also shown to reduce plaque burden and brain inflammation in a mouse model of AD [144]. Subsequently a decade of research into the clinical suitability of NSAIDs and derivatives thereof in the treatment of AD has lead to several small clinical trials in AD with inconsistent or limited clinical benefits (reviewed in [129, 130]). Others have developed peptidomimetic compounds that interact with a proposed substrate-binding site on γ-secretase, however these have not progressed to clinical trials [145]. These failures have forced researchers and pharmaceutical companies to reanalyse the validity of γ-secretase as a viable therapeutic target and to search for more γ-secretase complex-specific inhibitors, substrate-specific inhibitors, or inhibitors that target the carboxypeptidase-like activity but spare the endopeptidase activity (reviewed in [130]). These efforts should deliver improved and safer therapeutics for AD and other disorders with which unfavourable γ-secretase activity have been recognised (reviewed in [5]).

7 Cancer

The γ-secretase protease complexes have an important role in onset and progression of many cancers, as many of its substrates are highly implicated in disease pathogenesis and malignancy. Nearly a century after its discovery [146], the evolutionarily conservation and functional significance of the Notch signalling cascade was well established in multiple cellular processes, such as embryonic development, neurogenesis, cell fate determination, proliferation, differentiation, and self-renewal in adult tissue [18, 147]. In mammals there are four Notch receptors (Notch 1–4), which are synthesised in the ER as inactive single peptide precursors. Three sequential proteolytic cleavage steps are required for canonical Notch receptor signalling [18]. First, the Notch peptide precursor is cleaved in the *trans-Golgi* by furin convertase (S1 cleavage), which produces non-covalently bound heterodimers composed of two fragments; an N-terminal fragment which is accessible to Notch ligands, Notch extracellular domain (NECD) and a C-terminal transmembrane fragment (NEXT), which is comprised of an extracellular stub, the transmembrane domain and NICD. There are five Notch ligands in mammals, Delta-like-ligand (DLL) 1, 3, 4 and Jagged (JAG) 1 and 2 that are transmembrane proteins expressed on the surface of neighbouring cells. Upon ligand binding to the NECD, a second proteolytic event (S2 cleavage) by ADAM10 or ADAM17 releases most of the NECD from the cell. Subsequently γ-secretase cleaves the NEXT fragment (S3 cleavage), generating the transcriptionally active NICD. As NICD enters nucleus, it displaces the co-repressor from the transcription factor CSL (CBF 1/Sn(H)/Lag 1). Upon binding with CSL, NICD forms a ternary complex with MML (Mastermind like protein 1) and further recruits transcriptional co-activators like CBP/P300 to increase the expression of target genes like *Hes1*, *C-myc* and *Igf1r* [148]. It is this S3 cleavage that can be targeted by γ-secretase inhibitor to block Notch signalling by inhibiting the release of NICD in the cytoplasm [149].

The first indication that Notch signalling had a role in human malignancy was unravelled in T-cell acute lymphoblastic leukaemia (T-ALL) cells. In the early 1990s a rare chromosomal translocation was identified involving the human Notch gene in T-ALL [150, 151]. Later, gain of function mutations in Notch1 receptors was identified in over 50% of T-ALL tumours [148, 152, 153]. Mutations were detected in the extracellular homodimerization (HD) domain and the C-terminal PEST domain in the Notch 1 receptor. Mutations in the HD domain allowed ligand-independent activation of Notch signalling, while mutations in the PEST domain stabilises the NICD and abruptly prolong Notch activation [154]. Notch signalling also plays a crucial role in tumorigenesis of several solid tumours like breast [155], colon [156, 157], pancreas [158], prostrate [159] and glioblastoma [160]. Aberrant activation of Notch signalling is mainly due to overexpression of ligand or loss of negative regulators of the pathway. Oncogenic *Ras* up-regulates Notch 1 ligand *Dll1,* and *Psen1* resulting in increased Notch 1 proteolysis and signalling [161]. Aberrant activation of Notch signalling was associated with loss of

a negative regulator of Notch pathway, called Numb [162]. Notch also promotes angiogenesis and epithelial-mesenchymal transition in tumours. It also confers radio- and chemo-resistance to cancer cells [163]. Interestingly, Notch may act as a tumour suppressor as reported in hepatocellular carcinoma, small cell lung cancer, and cutaneous squamous cell carcinoma [164]. These findings validate the targeting of Notch signalling as a possible anti-cancer therapeutic [165].

EpCAM is a 37 kDa type I transmembrane glycoprotein [166, 167], localised to the basolateral membrane in the majority of normal epithelial tissues and is over-expressed in the majority of human epithelial cancers, making it an attractive diagnostic and therapeutic target in oncology [168, 169]. EpCAM has also been identified as a marker for cancer stem cells in various tumours including breast cancer, raising further interest in its use for tumour targeting and therapy [170]. EpCAM is overexpressed in 35–42% of patients with invasive breast cancer and is a predictor of poor prognosis and survival [171]. Given this, EpCAM is a widely used immunohistochemical marker for epithelial human malignancies, and has also been used to enrich circulating tumour cells before microscopic evaluation in the only FDA-approved assay for detection of circulating tumour cells in breast cancer. Similar to Notch, EpCAM oncogenic signalling requires intramembrane proteolysis of EpCAM by γ-secretase and generation of a biologically active EpICD that triggers more aggressive oncogenesis [32, 172]. More recently it has been shown that activation of EpCAM signalling relies on the homophilic aggregation of EpCAM via cell-cell contact, resulting in regulated intramembrane proteolysis of EpCAM [173], which has recently been shown to act as the mitogenic signal transducer of EpCAM in vitro and in vivo [32]. Sequential proteolysis by the TACE/ADAM17 and PS2-containing γ-secretase complexes facilitates the generation of soluble EpEx and the biologically active cytosolic EpICD [32, 172]. EpCAM interacts with Claudin 7, which is necessary for EpCAM recruitment to tetraspanin-enriched domain (TEM) [174], where EpCAM associates with CD9, CO-029 and CD44v6 [175]. EpCAM recruitment to TEM is necessary for its intramembrane proteolysis and signalling. In human carcinoma cells, upon cleavage of EpCAM cytosolic EpICD couples with FHL2 (four-and-a-half LIM domain protein) and Wnt pathway components; β-catenin and Lef-1 forming a nuclear complex that binds DNA at Lef-1 consensus sites and induces gene transcription of target genes, including *c-Myc* and *cyclin D1*, leading to increased cell proliferation and oncogenesis in immunodeficient mice [32].

Clinically profound evidence suggests that EpCAM expression is conserved with cancer progression and distant metastasis [176, 177]. Overexpression of EpCAM in breast cancer cells results in increased proliferation and altering EpCAM expression affects in vivo tumorigenesis [178]. Knockdown of *EpCAM* by siRNA or aptamer siRNA chimeras suppress tumour initiation and tumorigenesis in mice [178, 179], and the invasive potential of breast cancer cells [180]. EpCAM is also known to confer breast cancer cells chemo-resistance [181]. In thyroid carcinoma, nuclear and cytoplasmic accumulation of EpICD is used as an index for aggressiveness and poor prognosis [182, 183], and as a marker to distinguish between metastatic and non-metastatic papillary thyroid carcinoma [184]. With

distinct differential expression of EpCAM with various cancer conditions, EpCAM is also an attractive tumour-associated surface antigen used for diagnostic imaging or for targeted drug delivery [185–191]. Thus, in view of the tremendous heterogeneity in solid tumours and the novel role of EpCAM as an oncogenic signal transducer [32], therapeutic targeting of γ-secretase mediated cleavage of EpCAM in human cancers is under consideration.

Structurally, $p75^{NTR}$ is a member of to the TNF receptor superfamily and binds all four mammalian neurotrophins. As a heterodimer with the Trk receptors, $p75^{NTR}$ activates MAPK (mitogen activated protein kinase) and PI-3K (phosphatidylinositol 3-kinase) signalling pathways to regulate cell cycle progression, survival, neurite outgrowth and differentiation reviewed in, [192, 193]. However, $p75^{NTR}$ homodimers can also activate the NF-κB (nuclear factor kappa-light chain-enhancer of activated B cells) and Akt pathways to promote cell survival and cell cycle progression [194, 195]. $p75^{NTR}$ is subjected to ectodomain shedding by ADAM17 [196, 197] and γ-secretase-mediated proteolysis [198], resulting in the generation of the $p75^{NTR}$ ICD and transcriptional control of gene expression [199], as well as induction of apoptosis [200, 201], and inhibition of neurite outgrowth [202].

Deregulation of $p75^{NTR}$ expression and signalling is associated with the pathogenesis and progression of several cancers. For example, in prostate cancer loss of $p75^{NTR}$ has been coupled to the progression of tumour growth [203, 204], $p75^{NTR}$ overexpression promotes tumour migration in melanoma and medulloblastoma [205, 206]. Indeed, $p75^{NTR}$ is used as a biomarker to recognize and isolate melanoma tumour stem cells [207]. Overexpression of $p75^{NTR}$ has also been implicated in the progression of melanoma [208], glioma [209] and medulloblastoma [210]. Expression of $p75^{NTR}$ in human gliomas can transform non-invasive cells into highly invasive gliomas [211]. Activation of $p75^{NTR}$ by NGF also induces breast cancer cell survival [212, 213]. $p75^{NTR}$ undergoes RIP in higher-grade glioma and in invasive glioblastoma cell lines [211, 214], and inhibition of γ-secretase antagonizes migration and metastasis in medulloblastoma and glioblastoma cell lines [28, 210, 214]. Similarly, inhibition of γ-secretase activity prevented spinal metastasis of medulloblastoma [210]. Collectively these studies support the development of γ-secretase inhibitors as anti-cancer therapeutics [211, 214].

As outlined above, many γ-secretase substrates have a critical function in the control of normal cell proliferation, differentiation and apoptosis; however, they are also overexpressed in many cancers. Therefore, in order to selectively target γ-secretase substrates, very specific and selective therapeutics need to be developed. Following positive proof-of-concept experiments using in vitro human cancer cell lines [215–219], and in vivo xenograft models [220], many GSIs initially developed as AD therapeutics have advanced as anti-cancer therapeutics. For example, γ-secretase inhibitors have been shown to increase survival in mouse models of leukaemia [221], to reduce osteosarcomas in vivo in mice [222], and to enhance the effect of chemotherapy in human colon adenocarcinoma cell lines [223]. Many GSIs have now progressed to phase I or II clinical trials (https://clinicaltrial.gov), alone or in combination with other standard chemotherapeutic strategies in adult

patients with advanced haematological and solid tumours. A complete list of GSIs in clinical trials for cancer was recently reviewed in, [224, 225].

A list of some GSIs used in different in vitro, in vivo or clinical studies, are provided in Table 1, for a detailed listing please refer to [225]. Among these GSIs, extensive phase I/II clinical trials of RO4929097 (Roche) [226, 227], PF-03084014 (Pfizer) [228, 229], and MK0752 (Merck) [230–232] for advanced solid tumours (breast, pancreatic, nervous system and colorectal) and haematological malignancies are being conducted where maximum tolerated dose, efficacy, toxicity and effect on overall survival rates in different cancers are being determined [224, 225, 233–237]. Phase I trials of MK-0752, an oral GSI, show that it is also well-tolerated and exhibited Notch inhibition in children with refractory and recurrent central nervous system malignancies [230, 233]. A phase I study of the non-competitive, reversible GSI PF-03084014, in patients with T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL) demonstrated a favourable pharmacokinetic profile and target inhibition, supporting further evaluation of PF-03084014 in patients with T-ALL or T-LBL.

GSIs can also be combined with other cancer therapeutic strategies such as radiotherapy and chemotherapy to achieve a better curative effect. For example, RO4929097 has also been administered in combination with other anti-cancer agents including capecitabine (ovarian cancer), gemcitabinein (pancreas, tracheal and breast cancers), cediranib (advanced solid tumours) and temsirolimus (advanced solid tumours) (reviewed in [224]). In combination with gemcitabine, PF-03084014 treatment caused tumour regression in pancreatic cancer xenograft models and target inhibition in patients with advanced solid tumours [229], encouraging further development of PF-03084014 for the treatment of patients with advanced solid tumours. GSIs are known to strongly synergize with the anti-cancer effects of several chemotherapeutic agents. In vivo studies have shown chemotherapeutic drugs in combination with GSIs have a better potency in killing cancer cells [238]. GSIs augment anti-cancer drug mediated apoptosis following mitotic arrest [239, 240], and increase the efficacy of chemotherapeutic drugs in eliminating the cancer stem cells [241, 242]. Radiotherapy is one of the important therapeutic strategies in treatment of cancer cells. Patients often receive it as a part of therapeutic or palliative treatment in combination with surgery and chemotherapy. While most of the patients respond well to radiation, sometimes reoccurrence of cancer arises. The relapse of the tumour may be result of tumour heterogeneity or acquired and intrinsic radiation resistance. Many signalling pathways requiring γ-secretase substrates are accountable for radiation treatment resistance [243–245]. Thus targeting intramembrane proteolysis of these substrates by inhibiting γ-secretase activity can increase the efficacy of radiation [246, 247]. In vitro studies have shown that cancer cells pre-treated with GSIs enhances susceptibility to apoptosis on exposure to ionising radiation [248]. GSIs increase radiation-induced cell death not only by affecting the DNA damage repair mechanism but also can act as radio-sensitizers for cancer stem cells (CSCs) [249–251]. Pre-treatments with the GSI RO4929097 also sensitized breast cancer stem cells to ionizing radiation.

Table 1 Common γ-secretase inhibitors and use in clinical trials for cancer therapy

Name	Chemical name Formula	Class	Type	Use in cancer clinical trial [165]
Z-Leu-Leu-Nle-CHO	Benzyloxycarbonyl-L-Leucyl-L-Leucine-L-Norleucinal $\mathbf{C_{26}H_{41}N_3O_5}$	Peptide isostere	TSA	N.A.
L-685458	(5*S*)-(*tert*-Butoxycarbonylamino)-6-phenyl-(4*R*)-hydroxy-(2*R*)-benzylhexanoyl)-L-leucyl-L-phenylalaninamide $\mathbf{C_{39}H_{52}O_6N_4}$	Azepine	TSA	N.A.
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester $\mathbf{C_{23}H_{26}F_2N_2O_4}$	Azepine	Non-TSA	N.A.
Compound E	*N*-[(1*S*)-2-[[(3*S*)-2,3-Dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]amino]-1-methyl-2-oxoethyl]-3,5-difluorobenzeneacetamide $\mathbf{C_{27}H_{24}F_2N_4O_3}$	Azepine	Non-TSA	N.A.
LY-411575	N2-[(2S)-2-(3,5-Difluorophenyl)-2-hydroxyethanoyl]-N1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-L-alaninamide $\mathbf{C_{26}H_{23}F_2N_3O_4}$	Azepine	TSA	N.A.
Semagacestat (LY450139)	(2*S*)-2-hydroxy-3-methyl-*N*-((1*S*)-1-methyl-2-{[(1*S*)-3-methyl-2-oxo-2,3,4,5-tetrahydro-1*H*-3-benzazepin-1-yl]amino}-2-oxoethyl)butanamide $\mathbf{C_{19}H_{27}N_3O_4}$	Azepine	TSA	N.A.
RO-4929097	2,2-dimethyl-N-[(7S)-6-oxo-5,7-dihydrobenzo[d] [1] benzazepin-7-yl]-N′-(2,2,3,3,3-pentafluoropropyl)propanediamide $\mathbf{C_{22}H_{20}F_5N_3O_3}$	Azepine	TSA	– Advanced solid tumours – Refractory solid tumours – Epithelial ovarian cancer – Melanoma – Pancreatic adenocarcinoma – Colorectal cancer –Renal cell carcinoma – Progressive and malignant glioblastoma – Metastatic sarcoma

(continued)

Table 1 (continued)

Name	Chemical name Formula	Class	Type	Use in cancer clinical trial [165]
MRK-003	(3′R,6R,9R)-5′-(2,2,2-trifluoroethyl)-2-((E)-3-(4-(trifluoromethyl)piperidin-1-yl)prop-1-en-1-yl)-5,6,7,8,9,10-hexahydrospiro[6,9-methanobenzo [8] annulene-11,3′-[1, 2, 5] thiadiazolidine] 1′,1′-dioxide $\mathbf{C_{25}H_{31}F_6N_3O_2S}$	Sulfonamide	Non-TSA	N.A.
MK-0752	3-[4-(4-chlorophenyl)sulfonyl-4-(2,5-difluorophenyl)cyclohexyl]propanoic acid $\mathbf{C_{21}H_{21}ClF_2O_4S}$	Sulfonamide	Non-TSA	– Advanced solid tumour – Breast cancer – Pancreatic cancer – Paediatric brain tumour (CNS) – Ovarian cancer – Recurrent and metastatic colorectal cancer
PF-03084014	(2S)-2-[[(2S)-6,8-difluoro-1,2,3,4-tetrahydronaphthalen-2-yl]amino]-N-[1-[1-(2,2-dimethylpropylamino)-2-methylpropan-2-yl]imidazol-4-yl]pentanamide $\mathbf{C_{27}H_{41}F_2N_5O}$	Selective	Non-TSA	– T-ALL – T cell lymphoblastic lymphoma – Advanced solid tumour

Arising from the high recurrence rates and availability of limited therapeutic options, the prognosis for glioblastoma patients is very poor [252, 253]. Thus γ-secretase-dependent Notch and the $p75^{NTR}$ signalling have been implicated in the invasive phenotype of glioma, and in vivo studies demonstrate that γ-secretase inhibition may have direct clinical application for the treatment of malignant glioma. Indeed, in a glioblastoma multiform mouse model, combination of the clinically approved GSI RO4929097 with standard of care (radiotherapy and chemotherapy with temozolomide) reduced tumour growth and prolongs survival compared to dual combinations [244, 254]. Similarly, by combining treatment of the GSI MRK003, with the late stage autophagy inhibitor chloroquine, a significant induction in apoptosis and reduction in growth was noted in glioma neurospheres [255]. Most encouraging are results from a phase I trials of MK-0752, in children with refractory and recurrent central nervous system malignancies [230, 233], which show inhibition of Notch target and good drug tolerance. These findings indicate that GSIs alone or combined with standard of care treatment have an anti-glioma effect and encourages further translational and clinical studies.

Common side effects of treatment with these drugs include nausea, fatigue, diarrhoea, or anaemia. One major side effect of systemic administration of GSI in mice is diarrhoea [256]. Notch inhibition on GSI treatment in mice resulted in rapid increase of secretary goblet cell at the expense of adsorptive cells and consequently diarrhoea. However, diarrhoea can be prevented by co-administration of glucocorticoid [257]. GSI treatment also affects the immune function by altering B-cell versus T-cell decision within the earliest uncommitted lymphocyte stem cell population in the thymus. This defect in the immune system as a side effect of GSIs treatment is primarily due to inhibition of Notch signalling, however inhibition of E-cadherin and CD44 are also implicated. Inhibition of Notch signalling for cancer treatment can paradoxically increase the risk of skin cancer [258]. Given that only phase I or II trials are currently being conducted, progression of GSIs to phase III trials in large multicentre cohorts of cancer patients and the results of these trials are required before the full potential clinical benefits of GSIs can be determined.

8 γ-Secretase-Mediated Intramembrane Proteolysis in the Immune System

There is increasing evidence that suggests that the presenilins and γ-secretase-mediated proteolysis play an important role in regulating the immune system with the γ-secretase complex components preferentially expressed within the cells and tissues of the immune system including dendritic, myeloid cells and monocytes, bone marrow, tonsils, and spleen, respectively [5, 8, 259, 260]. A number of studies using in vivo murine models of presenilin-deletion or -mutations have shown a role for presenilin in neuroinflammation and the peripheral immune system, and clearly indicate the existence of a critical minimum presenilin threshold, below which a reduction in *Psen* gene dosage and function result in

serious deficits in innate and adaptive immunity. The partial-deficient mice (*Psen1*$^{+/-}$*Psen2*$^{-/-}$), are normal until approximately 6-months of age, where after the majority of mice develop age-associated myeloproliferative and autoimmune diseases [68]. These mice develop pathologies such as dermatitis, splenomegaly, glomerulonephritis, keratitis and vasculitis, with increased $CD4^{+}$/$CD8^{+}$ T-cell ratio and B-cell infiltrates in several tissues similar to symptoms observed in human systemic lupus erythematous [261]. Similarly, reducing levels of γ-secretase by at least 30% in nicastrin-deficient mice (*Nct*$^{+/-}$ or *Nct*$^{+/-}$*Psen1*$^{+/-}$) is associated with age dependent (>15 months) hyper-proliferation of granulocytes and reduced T-cell populations, both indicators of splenomegaly [262]. In contrast, *Psen1*$^{+/-}$*Psen2*$^{+/-}$ mice remained relatively healthy.

Furthermore, conditional-deficiency in *Psen1 and Psen2* in T- and B-lymphocytes, cells of the adaptive immune system, antagonized the selection and development of T-cells by disrupting T-cell receptor (TCR) signalling [263], while B-cells presented with a deficit in both lipopolysaccharide (LPS) and B-cell antigen receptor-induced proliferation and signal transduction events [264]. To study the role of *Psen1 and Psen2* in adulthood, tissue-specific *Psen* knockout models were needed to circumvent the developmental abnormalities carried by *Psen1*-deletion in the whole animal. Several groups created postnatal *Psen* conditional double knockout mice (cDKO) where they had ubiquitous loss of *Psen2* in all the tissues but only lacked *Psen1* in the forebrain. These mice have impaired hippocampal memory formation and reduced synaptic plasticity with neurodegeneration apparent by 6-months of age. Microarray analysis of gene expression profile of cDKO mouse cerebral cortex revealed differential expression of several inflammatory genes including, complement components, MHC histocompatibility proteins, cathepsins, chemokines, CD antigens and GFAP, implying that neurodegeneration in cDKO mice may not be due to Aβ accumulation, but might be regulated by neuroinflammation [265, 266]. Notably, presenilin deficiency in the brain is associated with inflammation in the brain and periphery, which are associated with elevated leukocytes and pro-inflammatory mediators in the serum [267]. These cDKO mice studies have also identified a role for *Psen2* in negatively regulating the microglia-mediated inflammatory response, with interferon (IFN)-selectively up-regulating *Psen2* expression and *Psen2* deficiency resulting in enhanced cytokine expression [268]. Most of the hematopoietic defects in γ-secretase defective murine models have been attributed to the Notch signalling pathway, being consisted with the requirement for Notch in proper haematopoiesis.

Immune function depends on rapid signalling events that enable the activation and amplification of signalling responses to antigen. Proteolytic processing by ADAMs and MMPs are established regulatory processes associated with both innate and adaptive immunity. Both ADAMs and MMPs are involved in aspects of membrane protein ectodomain shedding, immune-cell migration, cytokine and chemokine maturation, lymphocyte maturation, clonal expansion, migration and effector function (reviewed in [269–271]). As outlined earlier, the proteolytic activity of sheddases is often followed by γ-secretase intramembrane proteolysis, and many innate immune receptors and ligands have now been identified as

γ-secretase substrates (Table 2), although the direct function of many of their cleavage products remains to be determined. Nonetheless, this is proposed to further enable cells of the immune system to proteolytically regulate cell-type specific immune activities (reviewed in [272]), and emphasise an important role for γ-secretase proteolysis in the pathophysiology of infectious diseases (reviewed in [273]).

Work in our group has demonstrated that several pro-inflammatory cytokine receptors undergo γ-secretase proteolysis. We have shown that following TACE-mediated ectodomain shedding of TNFR1, the membrane-bound

Table 2 List of γ-secretase substrates relevant to innate and adaptive immune system

Substrate	Known immune functions	References
Signalling receptors		
IL-IRI	Pro-inflammatory cytokine, interleukin-1 (IL-1) receptor and signalling	[21]
IL-1RII	Interleukin-1 (IL-1) receptor (antagonist)	[22]
IL-6R	Interleukin-6 (IL-6) receptor. Regulation of the immune response, acute-phase reactions and haematopoiesis	[275]
CSF-1	Colony stimulating factor-1, also known as macrophage colony-stimulating factor (M-CSF). A secreted cytokine that influences hematopoietic stem cells to differentiate into macrophages or other related cell types	[276]
IFNαR2	Subunit of Type 1 interferon-α (IFN-α) receptor	[301]
TNFR1	Pro-inflammatory cytokine, tumour necrosis factor (TNFα) receptor. Pro-inflammatory and cell death signalling pathways	[125]
TREM2	Triggering receptor expressed on myeloid cells 2 (TREM-2). Role in chronic inflammations and may stimulate production of constitutive rather than inflammatory chemokines and cytokines. Homozygous mutations in TREM2 cause rare forms of dementia. Missense mutation (R47H substitution) confers a significant risk of Alzheimer's disease	[31]
Cytokines		
CXC16	Transmembrane chemokine Scavenger receptor on macrophages induces chemotactic response	[282]
CX3CL1 (fractalkine)	Transmembrane chemokine	[282]
Immune antigens		
CD43 (Leukosialin)	Cell adhesion molecule. Major glycoproteins of thymocytes and T lymphocytes	[283]
CD44	Cell adhesion molecule. Cell surface glycoprotein, hyaluronan receptor	[284] [286]
HLA	Human leucocyte antigen (HLA). MHC Class I protein	[302]

(continued)

Table 2 (continued)

Substrate	Known immune functions	References
HLA-A2	MHC Class I protein Immune response, T-cell development, synaptic plasticity and refinement	[303]
NOTCH 1-4	Regulation of cell differentiation and embryonic development	[304–306]
Pathogen receptors		
SDC1	Syndecan 1 (SDC1), Herpes simplex receptor. Receptor for HIV-1, Hepatitis E and human papilloma virus (HPV)	[302]
SDC2	Syndecan 2 (SDC2), Herpes simplex receptor	[302]
SDC3	Syndecan 3 (SDC3), Herpes simplex receptor. Receptor for HIV-1 and human papilloma virus (HPV)	[302, 289, 288]
NOTCH 1-4	Signalling receptors, Cell proliferation and differentiation Activated by Epstein Barr Virus	[304] [305, 306]
CD44	Cell adhesion molecule Entry receptor *for C. neoformans,* lymphocyte activation, recirculation and homing	[286]
CD46	Receptor for adenoviruses, measles virus, human Herpes Virus 6 (HHV-6), Streptococci, and Neisseria. Also cleaved by a protease secreted by *P. gingivalis*. Complement and T cell regulatory functions	[291, 290]
Desmoglein-2	Adenovirus receptor	[302, 307]
LRP1	Lipoprotein receptor 1 (LPR1). Receptor for rhinovirus, *Pseudomonas aeruginosa* and exotoxin A	[308]
LDLR	Low density lipoprotein receptor (LDLR). Receptor for rhinovirus, Hepatitis C and vesicular stomatitis virus	[302]
VLDLR	Very low density lipoprotein receptor (VLDLR). Receptor for rhinovirus	[309]
ERBB4	Epidermal growth factor receptor tyrosine kinase. Receptor for vaccinia and other poxviruses	[310]
NGFR ($P75^{NTR}$)	Neurotrophin receptor. Receptor for rabies virus	[29, 311]
Nectin-1α	Immunoglobulin superfamily member that mediates cell-cell adhesion. Receptor for herpes-simplex virus (HSV) and pseudorabies virus	[312]
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10). Cytotoxin *Staphylococcus aureus* α-hemolysis-mediated cell injury	[313, 314]
Dystroglycan	Member of multiproteindystrophin-glycoprotein complex Receptor for the lymphocytic choriomeningitis, Lassa fever viruses and *Mycobacterium leprae* (the Leprosy pathogen)	[302]

TNFR1 CTF is subsequently cleaved by γ-secretase, and internalization of TNFR1 CTF is a requirement for proteolysis. Furthermore, using in vitro and in vivo model systems we have shown that loss of presenilin expression and γ-secretase activity, inhibits TNFα-induced c-Jun N-terminal kinase (JNK) activation, and reduces TNF-induced apoptosis [125]. We have also demonstrated that the

cytokine receptor IL-1R1, which is required for IL-1α/β pro-inflammatory signalling, undergoes constitutive or metalloprotease-mediated ectodomain shedding and is a substrate for γ-secretase cleavage [21]. We reported that inhibition of γ-secretase activity antagonises IL-1β-induced cytokine secretion, suggesting that γ-secretase regulates cytokine responsiveness. Previously IL-1R1 has been localised to the nucleus and within the IL-1R1 ICD nuclear localisation sequences have been reported. Based on these findings we hypothesise as with other substrates that the IL-IR1 ICD could act as a transcription factor that affects IL-1β-induced gene expression in the nucleus in conjunction with the usual signal transduction pathway [21]. IL-1RII, which is a decoy receptor that binds excess soluble IL-1β, and antagonises IL-1R1 signalling, is also cleaved by α- and β-secretase and is a substrate for γ-secretase proteolysis [22]. Elevated soluble IL-1RII levels are observed in the cerebrospinal fluid of patients with AD, which may be explained by the similar proteolytic processing as APP by γ-secretase [274]. Another member of the interleukin receptor family, the IL-6 receptor (IL-6R) is sequentially cleaved by ADAM17/TACE and γ-secretase at the plasma membrane. IL-6 mediated signals are important for the coordination of the immune system and IL-6 deregulation can lead to chronic inflammation [275].

The colony-stimulating factor 1 (CSF-1) receptor has an essential role in the haematopoiesis of cells of the immune system especially mononuclear phagocytes, such as macrophages and monocytes. CSF-1 is cleaved by γ-secretase and is regulated by Toll-like receptor (TLR) activation [276]. A similar system could be in operation in microglia and the activation of TLRs in response to Aβ peptides could lead to microglial activation via promotion of CSF-1 receptor proteolysis by TACE and γ-secretase. In our lab we have shown in bone marrow derived macrophages that down regulation of *Psen2* transcription is paralleled by reduced *tlr4* mRNA levels and loss of LPS-induced *tlr4* mRNA transcription regulation with reduced inflammatory cytokine expression [277]. Conversely, in studies with brain-specific knockouts of the presenilins, it was found that *Psen2* was critical in inhibiting microglia-mediated inflammation and with *Psen2* deficiency resulting in enhanced cytokine expression [268]. From the data, a model is emerging whereby the functions of TLRs and γ-secretase in inflammation and microglial activation combine to regulate the inflammatory response. Dysfunction of this system in the brain could be fundamental to the pathophysiology of AD.

Of particular relevance to the pathogenesis of AD, recently two independent groups identified variants of the *Trem2* gene, which encodes triggering receptor expressed on myeloid cells-2 (TREM2), which increase susceptibility to late onset AD [31]. TREM2 undergoes regulated intramembrane proteolysis [31]. A model has been proposed by which *Trem2* variants contribute to the pathogenesis of AD by deregulation of Aβ phagocytic ability and altering the pro-inflammatory response of microglia [278]. It has also been shown that haplodeficiency of *Trem2* or *Trem2* variants reduced the ability of microglia to surround and compact early Aβ deposits, thereby developing a neuro-protective barrier between early Aβ deposits and vulnerable neuronal cells [279, 280]. Importantly, a recent study reported that inhibition of γ-secretase activity interferes with TREM2-dependent

signalling and phagocytic activity [281], further highlighting an important functional connection between γ-secretase, TREM2 and the pathogenesis of AD.

Recruitment of leukocytes to a site of infection is an early immune response following acute or chronic inflammation. The proteolytic activity of ADAMs and MMPs is a consistent occurrence in the generation of chemokine and cytokine gradients for immune cell recruitment (as reviewed in [270]). The ADAMs (ADAM10 and ADAM17/TACE), are involved in the shedding of biologically active chemokines, such as CX3CL1 (fractalkine) and CXCL16. Importantly both CX3CL1 (fractalkine) and CXCL16 have also been identified as γ-secretase substrates [282]. Ectodomain shedding of CX3CL1 and CXCL16 serves to enhance neutrophil recruitment to sites of infection. The generation of a chemokine gradient is often associated with the cell-adhesion properties of immune and endothelial cells. CD43 is a major glycoprotein of thymocytes and lymphocytes that is cleaved by γ-secretase during neutrophil activation. Unlike other substrates CD43 is first cleaved by cathepsin G, not a metalloprotease [283]. The extracellular domain of CD43 can regulate inflammation by preventing neutrophil adhesion on endothelial cells. CD43 cleavage by γ-secretase was found to be required for neutrophil adhesion, thereby regulating CD43-mediated signalling [283]. CD44 is a widely distributed cell surface adhesion antigen that is also a γ-secretase substrate [284]. It has been shown that the CD44 ICD translocates to the nucleus of macrophages and promotes the activation of NF-κB and macrophage fusion.

As the γ-secretase complex is localized to dendritic cells that are primarily responsible for the processing of antigens and presentation to B and T cells, it has been shown that many γ-secretase substrates are important in the lifecycle of many pathogens, with some γ-secretase substrates acting as an entry receptors or as scavenger receptors by binding to pathogen membrane components, such as binding of phosphatidylserine to CXCL16 [285]. The earlier mentioned CD44 glycoprotein is also an entry receptor for the opportunistic yeast *Cryptococcus neoformans* the causative agent of the infection cryptococcosis that can manifest as a secondary infection for AIDS patients [286]. The γ-secretase substrates nectin-1α, syndecan 1 and 2 are herpes simplex viral receptors [287], and the HIV-1 and human papilloma viruses also attach to dendritic cells via the syndecans, SDC1 and SDC3 [288], both also reported to be substrates for intramembrane proteolysis [273, 289]. CD46 another γ-secretase substrate of the immune system, which is responsible for protecting the cell against complement-mediated injury, also has a role in differentiation of $CD4^+$ into T-regulatory 1 cells that secrete IL-10, supressing immune response and therefore preventing autoimmunity. Similar to CD44, CD46 is also a target of many pathogens including adenoviruses, measles virus, human herpes virus 6 (HHV-6) and Streptococci [290]. Elevated γ-secretase cleavage of CD46 stimulates CD46-dependent T cell responses in response to *Neisseria gonorrhoeae* and *Neisseria meningitidis* infection [291].

There are several recent experimental studies that have demonstrated that GSIs may be potential therapies for inflammatory disease. For example, in vitro and in vivo studies have shown that GSIs can antagonise lipopolysaccharide-induced Notch signalling and attenuated the LPS-induced inflammatory response [292].

GSIs were shown to alleviate acute airway inflammation of allergic asthma in mice by down-regulating Notch-dependent Th17 cell differentiation [293]. Collectively these findings suggest a strong link between γ-secretase activity and the immune system and may in the future provide therapeutic potential in the treatment of immune-related diseases.

9 Conclusions

It was generally accepted that signalling events-mediated by cell surface receptors, including Notch, EpCAM, G-protein-coupled receptors, TLRs, $p75^{NTR}$ and death receptors (TNFR1, FasR and TRAIL-R1/2) are initiated and terminated at the plasma membrane. This dogma has now been challenged [294–300], and a new model proposes that following cell surface receptor activation and propagation of a signalling event, through the combined enzymatic activity of "sheddase" and γ-secretase protease complexes, receptors can also propagate distinct signalling events facilitated by the generation of soluble receptor ectodomains and intracellular ICD. The combined enzymatic activity of "sheddase" and γ-secretase protease complexes have defined regulated intramembrane proteolysis, a fundamental signal transduction process.

Since the discovery of the presenilin genes and their association with FAD, great progress has been made in understanding the structure, assembly, catalytic mechanisms, substrate specificities, and regulation of the γ-secretase protease complexes. A greater appreciation for the high degree of regulation surrounds assembly, localization, expression and activity of the γ-secretase protease complexes, and how γ-secretase protease complexes and their substrates affects cellular processes have emerged and reinvigorated efforts to develop therapeutic modulators and inhibitors of γ-secretase protease activity. Challenges for the future include the advancement of our understanding of the diversity of γ-secretase protease complexes in humans and translation of this knowledge into practical therapeutics. These efforts should not only provide better and safer therapeutics for AD, but also other diseases such as breast and haematological cancers, immune disorders, acne inversa, cardiomyopathy and kidney disorders, with which adverse γ-secretase activity and signalling have been attributed.

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The Role of Urinary Proteases in Bladder Cancer

Paulo Bastos, Sandra Magalhães, Lúcio Lara Santos, Rita Ferreira and Rui Vitorino

Abstract

Bladder cancer (BCa) is one of the most prevalent malignancies worldwide. Risk factors for BCa are well established and include smoking and infections, which can lead to immune system activation, altered gene expression patterns, proteolytic activity, tissue damage, and, ultimately, cancer development. Urine has become one of the most attractive diagnosis samples, and, notably, urine profiling by mass spectrometry allows the simultaneously analysis of multiple enzymes and their interactors, substrates, inhibitors, and regulators, providing an integrative view of enzymatic dynamics. Most BCa-associated enzymatic alterations take place at the level of proteases, being MMP-9, MMP-2, urokinase-type plasminogen activator, cathepsin D, and cathepsin G already related to BCa development and progression. Herein, we overview the role of

Paulo Bastos and Sandra Magalhães are equally contributed.

P. Bastos · S. Magalhães · R. Vitorino (✉)
iBiMED—Institute for Research in Biomedicine, Department of Medical Sciences, University of Aveiro, Aveiro, Portugal
e-mail: rvitorino@ua.pt

S. Magalhães · R. Ferreira
QOPNA, Mass Spectrometry Center, Department of Chemistry, University of Aveiro, Aveiro, Portugal

L.L. Santos
Experimental Pathology and Therapeutics Group—Research Center, Portuguese Oncology Institute-Porto (IPO-Porto), Porto, Portugal

R. Vitorino
Department of Physiology and Cardiothoracic Surgery, Faculty of Medicine, University of Porto, Porto, Portugal

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proteases and the classes more studied in BCa pathogenesis, as well as the methodologies used for assessing protease amount and activity in urine samples, highlighting its advantages and limitations, and the value of urinary proteases as disease biomarkers.

1 Introduction

Bladder cancer (BCa) is the ninth most common cancer worldwide with an estimated incidence above 400,000 new cases *per* year. BCa incidence rises with aging and is more frequent in men, which account to be the seventh most common cancer in this gender [1]. In women, BCa is the seventeenth most common type of malignant disease [1]. Whereas men are three to four times more likely to develop BCa, women have worse prognosis [2, 3]. This sexual dimorphism may be related to differences in the exposure to carcinogens such as tobacco or to genetic, hormonal, and anatomical factors [2]. There are several risk factors involved in the pathogenesis of this disease [4]. According to World Health Organization (WHO), cigarette smoking and exposure to aromatic amines are the most important risk factors [5, 6]. In fact, the risk of developing BCa in smokers is about 2–6 times higher compared to nonsmokers [5]. In turn, occupational exposure to carcinogens like aromatic amines is responsible for 25% of all BCa cases [5–7].

BCa is more frequent in developed countries where, in 2012, approximately 10 among every 100,000 individuals were diagnosed with BCa. Among developed regions, higher incidence rates have been reported in both Europe and North America [1]. As a consequence of its high incidence, prevalence, and aggressiveness, BCa is one of the most expensive diseases to treat and to manage [8–11]. Most of this economic burden results from the high number of invasive procedures performed (e.g., cystoscopies) and from the several hospitalizations required for its management [8–11]. Taken together, incidence, mortality, prevalence, and costs of BCa make this disease a public health problem, even though it is not officially regarded as such.

Currently, BCa is classified as a multifactorial disease that can be divided into three histological subtypes: urothelial cell carcinomas, adenocarcinomas, and squamous cell carcinomas [5, 12]. In developed countries, namely USA, France, and Italy, urothelial cell carcinomas constitute about 90% of BCa cases, while the incidence of the remaining types of BCa is much lower, ranging from 1.1 to 2.8% for squamous cell carcinomas and from 1.5 to 1.9% in the case of adenocarcinomas [5]. Urothelial cell carcinomas can be classified into low-grade BCa and high-grade BCa, a classification that is based on the histological analysis of the bladder. Low-grade BCa is less aggressive and rarely invades bladder muscle tissue or metastasizes to other parts of the body, and patients rarely die from it. On the other hand, high-grade BCa commonly invades muscle wall and metastasizes, therefore

requiring more aggressive treatments. Consequently, high-grade BCa is responsible for almost all BCa-related deaths [7]. Another way to classify BCa is based on the invasion of the bladder muscular layer. Nonmuscle-invasive BCa is often treated by the surgical removing the tumors, sometimes combined with localized chemotherapy [7, 13]. Patients with nonmuscle-invasive BCa have a 5-year survival rate ranging from 82 to 100% for early-stage BCa. On the other hand, muscle-invasive BCa is much more aggressive, and treatment involves surgical removal of the bladder as well as aggressive radiotherapy and chemotherapy treatments [7, 13]. Even with the complications associated with surgery, surgical intervention for tumor removal remains the most effective treatment, highlighting the lack of specific therapeutic targets and effective pharmacological agents. However, even complete resections are followed by recurrence rates of 70% for superficial bladder tumors, and intravesical chemotherapy can attenuate this extremely high recurrence rate by only 15% at most. Nevertheless, this type of BCa has a poorer prognosis, so that only 5–10% of patients with metastasis live more than 2 years after diagnosis [14].

The proportion of squamous cell and transitional cell bladder carcinomas has been changing, at least in some populations, most likely due to increased exposure to distinct etiologic factors, especially smoking [15]. In areas where schistosomiasis is endemic (e.g., Sudan, Egypt), squamous cell carcinoma accounts for 75% of all malignant bladder tumors. However, the increased smoking prevalence has been responsible for a significant shift toward urothelial/transitional cell carcinoma (six-fold increase over squamous cell carcinoma) [15, 16]. This observation has important repercussions as urothelial/transitional cell carcinoma patients are poorer candidates for surgical intervention and present increased risk of recurrence [16]. In addition, other risk factors for developing BCa include family history of the disease, genetic mutations in *HRAS*, *RB1*, *PTEN/MMAC1*, *NAT2*, and *GSTM1* genes, exposure to arsenic, and certain drugs used in chemotherapy like cyclophosphamide [5, 7].

There are several pathways involved in bladder tumorigenesis. At a molecular level, it is known that mutations in *FGFR3*, *TP53*, *RB1*, *ERBB2*, *and PTEN* genes are involved in bladder carcinogenesis [17, 18]. Also, some oncogenic miRNAs are upregulated, and tumor suppressor miRNAs are downregulated in BCa patients [17]. At the metabolome level, like in other types of cancer, the Warburg effect contributes to BCa development, progression, and aggressiveness [19]. Neoplastic cells have a strong dependence on glycolysis in conditions of normoxia, and BCa cells display an upregulation of genes involved in glucose uptake, like GLUT1 and GLUT3 transporters and Akt, which promotes the PI3 K/Akt/mTOR pathway [19]. There is also an overexpression of pyruvate-related and pentose phosphate pathway-related genes, and glycogen metabolism is enhanced during BCa development. Simultaneously, there are alterations in lipid metabolism, such as increased fatty acid synthesis and oxidation, changes in the tricarboxylic acid cycle and ketogenesis activity [19], as well as the hyper activation of proteases, which may lead to tissue damage and invasion, immune system and apoptosis evasion, tumorigenesis and cancer metastasis [20, 21].

Early detection of BCa is not always easy, since BCa is commonly asymptomatic at the early stages. Also, the first sign of BCa is usually painless hematuria, which is shared by other diseases [5, 12, 18]. Moreover, other symptoms may include abdominal pain, fatigue, weight loss, and urinary frequency and urgency. Thus, diagnosis of BCa can be performed by ultrasounds, computed tomography scans, and noninvasive urine cytology [5, 12]. Urinary cytology has the advantage of being a noninvasive approach, but despite its high specificity, it lacks sensitivity to detect low-grade tumors, so that cystoscopy is always done to corroborate the diagnosis [5, 12].

One of the biggest and most promising challenges in BCa research is the development of a diagnostic tool based on biofluids' profiling. Not surprisingly, urine is the most promising biological fluid, once it can be collected in high amounts by a noninvasive and simple procedure [12]. Also, in the case of BCa, urine better reflects alterations taking place in the bladder because it is in direct contact with the tissue [12]. Several studies have explored the diagnosis value of biomarkers for urine assay in BCa [22–25]. Among the identified putative biomarkers are a few proteases, reflecting the role of proteolytic systems in carcinogenesis and metastization [22, 23]. However, the huge interplay between multiple proteases and its inhibitors makes the assay of proteases in urine complex and unpredictable. In this chapter, we will explore the role of proteases in BCa pathophysiology and how these enzymes might be assessed in biofluids for the diagnosis and management of this disease.

2 An Update of the Existing Biomarkers for the Clinical Management of BCa

Currently, there are three FDA-approved urinary tests for the screening and surveillance of BCa. NMP22 Bladder Check Test® is an enzyme immunoassay for the detection of NMP22, a nuclear matrix protein recognized as an urothelial-specific cancer biomarker [26, 27]. This test has the advantage of being inexpensive, rapid, and operator-independent. However, it leads to a high number of false positives [23, 27]. The FDP test is another FDA-approved one that detects increased urinary concentration of fibrin/fibrinogen degradation products (FDPs), which are associated with malignant tumors [23, 27]. However, the NMP22 test lacks sensitivity (68%) and displays a high rate of false positives, particularly in patients with hematuria. So, it was recently removed from the market [23]. Lastly, BTA stat and BTA-TRAK are two tests that detect the bladder tumor antigen (BTA) in urine. BTA is the human complement factor H-related protein, and it is produced by human BCa cells but not by normal epithelial cells [26]. These tests were approved for surveillance only, since both display a high rate of false positives. Despite its high sensitivity for low-grade lesions, the sensitivity of BTA tests for high-grade lesions is worse than cystoscopy [23]. Even so, it is possible to

improve the sensitivity of these tests by combining them, but the rate of false positives remains high [26].

Therefore, efforts continue to be made for the identification of biomarkers that help to improve the clinical management of BCa. Still, there is a general awareness that it will be difficult, if not impossible, to identify a single biomarker with high specificity and sensitivity (a golden bullet). Consequently, emphasis has been given to the combination of multiple protein markers that when analyzed together in a multimarker panel may allow the diagnosis of BCa and improve the clinical management of this type of cancer. Considering the role of the proteolytic systems in bladder carcinogenesis and metastization, proteases might be seen as markers to be included in such diagnosis panels. Because proteolytic activity is regulated at multiple levels, including gene expression, enzyme compartmentalization, regulation by modulators and degradation [28], the biological significance of urinary proteases, and quantitative alterations are difficult to interpret and not readily predictable. The urinary levels of primarily intracellularly acting proteases may not accurately reflect local mRNA expression since these proteases may be enriched in the tissues and poorly secreted in urine [29–40]. In turn, urinary levels of highly secreted proteases are subjected to the influence of multiple factors including the rate of secretion, tumor burden, and transcription regulation [41–43]. As such, the exploitation of quantitative changes in the urinary protease levels has revealed a far more challenging avenue than initially envisioned. Still, proteomics appears to be the most promising approach to characterize urinary proteases and promises to allow BCa screening, diagnosis, monitoring, therapy management, and the identification of novel pharmacological targets.

3 Urine Proteases Profiling for Biomarker Screening

When studying proteins and peptides in urine samples, different enrichment procedures are usually applied, given different but complementary results. The identification and characterization of putative urinary biomarkers may be performed with gel-based or gel-free approaches (Table 1). Gel-based approaches usually involve protein separation by 1D/2D Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), whereas gel-free approaches include capillary electrophoresis (CE) or liquid chromatography (LC). Nevertheless, gel-free and gel-based approaches might be combined with each other into a strategy known as GeLC. Starting from a complex sample such as urine, 1D/2D SDS-PAGE allows accurate separation of proteins according to their molecular weight and/or isoelectric point, resolving hundreds of proteins, then subjected to MS-based analysis, immunoblot detection, and/or zymography-based activity assays (Table 1) [29–31, 34, 36–38]. One of the main shortcomings of gel-based approaches results from the high-salt concentrations in urine (mainly urea), as well as the presence of lipids and glycosaminoglycans, which interfere with proteomic analysis (particularly during isoelectric focusing in 2D SDS-PAGE). Despite the versatility of gel-based

approaches, these may become daunting when dealing with complex samples, particularly for high-throughput analyses. Consequently, LC has become the platform of choice for protein separation from urine samples of patients with BCa, providing better resolution and allowing easier sample manipulation (Table 1) [30, 32, 33, 35–37]. Furthermore, distinct chromatography-based techniques such as dual-lectin affinity, strong-cation exchange, and reverse-phase chromatography exploit unique physicochemical properties, which allow the enrichment of particular urinary subproteomes and the identification of novel enzymes as well as enzyme inhibitors [24, 32]. Similar to gel-based strategies, gel-free approaches can also be followed by MS analysis (with or without prior digestion), immunoblotting, and/or zymography (Table 1).

Table 1 Procedures employed in bladder cancer urine proteomics

Procedure	Distinguishing features	References
1D/2D sodium dodecyl sulfate-Polyacrylamide gel electrophoresis	High-throughput technique (hundreds to thousands of proteins) Useful for highly abundant and soluble proteins, allowing the separation of complex mixtures Very acid or basic proteins can be accurately resolved Allows bands/spots excised for MS analysis Lacks dynamic range, reproducibility, and sensitivity Bad for poorly abundant (enzyme inhibitors) or poorly soluble (membrane) proteins Laboriously coupled to MS analyzers	[29–31, 34, 36–38]
Capillary electrophoresis	Allows the study of naturally occurring peptides Small amounts of sample required (nL) Very high speed (high voltage allowed) and resolution without band broadening due to high surface area and heat dissipation Easily coupled to MS analyzers	[55–58]
Zymography	Detects enzymatic activity Allows for multiple enzymes/substrates monitoring simultaneously Allows prior molecular weight- and isoelectric point-based protein resolution, tissue sample in situ zymography, and analyses using protease-activated fluorogenic probes that allow the monitoring of multiple MPPs/fluorophores and the mapping of MMPs' activity	[48–53]

(continued)

Table 1 (continued)

Procedure	Distinguishing features	References
Chromatographic separations	High-resolution techniques Separation based on several characteristics (mass, volume, isoelectric point, hydrophobicity) Susceptible to ion suppression by the presence of salts High retention (e.g., column-to-column) time variability Easily coupled to MS analyzers	[30, 32, 33, 35–37]
Western blot and other immunological assays	Allows relative and absolute quantification of multiple proteins High resolution and sensitivity Targeted approach High costs Targeted (misses much information) Does not separate isoforms Requires previous knowledge of its targets	[30, 36–38]

3.1 Immunoaffinity Assays

Immunoaffinity or antibody-based approaches can be used either to isolate intended targets or to deplete unwanted ones, and these techniques have been exploited during all steps of urinary biomarker discovery, from targeted sample depletion and protein isolation (e.g., immunoprecipitation) to biomarker validation (e.g., Western blot, Dot blot) [30, 33, 44]. In addition to be highly sensitive and specific, immunoaffinity assays are, above all, the methods of choice required for biomarker validation. Particularly, as will be discussed in the subsequent sections, immunoaffinity assays such as Western blot have been successfully used for biomarker validation using urine samples from BCa patients, including the proteases cathepsin D, cathepsin G, cathepsin L, matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), a disintegrin and metalloproteinase with thrombospondin motifs 7 (ADAMTS7), metalloproteinase inhibitors (e.g., TIMP1), among others.

In recent years, classical blotting techniques have started to be replaced by surface plasmon resonance imaging, which employ antibody arrays for the multiplexed detection of protein biomarkers [45, 46]. This technology has been applied to the detection of BCa in its early stages, through the assessment of cathepsin D/protein ratio in both serum and urine [47].

3.2 Zymography

Zymography has assumed a leading role when studying urine proteases because it allows protein resolution based on molecular weight and isoelectric point,

enzymatic activity analysis, and the screening of substrates and enzyme inhibitors. Despite this versatility, zymography-based studies of proteolytic alterations in BCa patients have relied almost exclusively on PAGE-based resolution of urinary proteins, incubation with a substrate more or less specific for a group of proteases (e.g., gelatin zymography to access the activity of MMP-2 and MMP-9), and estimation of enzymatic activity by substrate hydrolysis quantification [48–50]. Also, zymography allows multiple analyses to be performed, either simultaneously or sequentially, thus largely expanding the repertoire of possibilities for analysis [51, 52].

By analyzing enzymatic activity levels, when applied to specimens collected from BCa patients, zymography has provided biomarker-based models with specificities of up to 100%, identifying molecular players not amenable to be monitored by other techniques [48–50, 53]. The concentration of urinary proteases may not accurately reflect proteolytic activity [48, 49]. Notably, zymography allows to study and to quantify enzymatic activities in addition to enzymatic concentrations, thus providing information regarding quantitative changes as well as qualitative alterations. As such, zymography becomes an invaluable tool for the analysis of functional aspects, as seen for BCa where enzymatic trafficking is impaired and protease/inhibitor complexes or protease dimers may be frequent, with repercussions to the proteolytic function [48–50, 53].

3.3 Peptidomics

The screen of native peptides (peptidomics) in urine, particularly polar, charged, or chargeable small ones, has mostly relied on CE analysis. CE-based urine peptidomics aims to screen the peptides differentially expressed across distinct populations, providing an indirect window to assess the proteases modulated by BCa. Such indirect urine profiling approach has been proven capable of accurately discriminating cancer patients from patients with other organ-related nonmalignant conditions [54]. Together with bioinformatics tools, CE may serve for the discovery and validation of multimarker panels [55–57] and for the comparison of excreted urinary peptides to endogenous "housekeeping" peptides [55, 56]. CE-based native urinary peptide profiling has been applied for the detection of primary (with up to 91% sensitivity and 68% specificity) and recurrent (with up to 87% sensitivity and 51% specificity) urothelial BCa [57].

Peptides shown to most accurately allow the detection of BCa were collagen fragments ($\sim$60%), distantly followed by hemoglobin, apolipoprotein A-I peptides, membrane-specific heparin, sulfate proteoglycan core protein, a disintegrin and metalloproteinase domain-containing protein 22 (ADAM22), and a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1). Together, these peptides reflect increased proteolytic activity, extracellular matrix remodeling, collagen degradation, and hematuria [57].

However, there are some sampling issues that should be considered in the analysis of proteome data retrieved from proteome profiling of urine samples

collected from patients with BCa. Urine proteomics applied to the search of BCa biomarkers have focused most extensively on older patients (over the age of 60) [32, 34, 36], which reflect the clinical phenotype of the majority of patients but reduce the likelihood of finding markers for early diagnosis purposes. Gender also influences the urine proteome profile. Females tend to present higher albumin and transferrin urinary concentration and a more complex urinary subproteome (composed by the less abundant proteins) [58]. Therefore, the pathophysiological role and the prognosis and diagnosis value of a given BCa-related urinary biomarker may not be suitable across all age-groups or across genders.

4 Urinary Proteases Associated with Bladder Cancer

Urine has become one of the most attractive diagnosis samples, involving noninvasive collection of unrestricted quantities [59]. Generally, less than 150 mg of proteins is excreted in urine, mainly due to glomerular filtration, and partially by tubular reabsorption, secretion, and degradation [60, 61]. Around 70% of urinary proteins are of kidney origin [62], a percentage modulated by diseases. In addition to kidney diseases, recent findings show that each and several diseases can be accurately discriminated by a unique protein fingerprint. From more than 2,500 proteins identified by MS-based urinalysis as differentially expressed across human diseases, approximately 15% (approximately 360 proteins) result from malignant diseases, particular BCa (58% or 209 proteins of these approximately 360 malignancy-associated differentially expressed urinary proteins) [63, 64]. Indeed, by pooling data retrieved from ten MS-based studies concerning differentially expressed urinary proteins from patients with BCa [29–38], 209 proteins were highlighted. Among these, 44 proteins were classified as enzymes, 21 of which are hydrolases and from these 13 are proteases (Tables 2 and 3). These proteases/peptidases are listed in Table 3 together with its function and expression variation (up/down) in BCa. Likewise, the expression patterns of several genes have been determined by microarrays in close to 1,500 BCa patients and revealed several proteases as putative diagnostic and prognostic BCa markers (Table 3) [65–75]. However, as also depicted in Table 3, alterations in proteases mRNA do not always reflect those concerning the concentrations of proteases neither their activity. For instance, while both levels and the activity of MMP-2 and cathepsin G are upregulated in urine samples from BCa patients, their mRNA expression levels are downregulated (Table 3), which may reflect increased stimulation-dependent activation (by, for instance, inflammatory and stress–response signaling pathways), stimulation-independent activation or enhanced intrinsic hydrolytic activity or alterations in enzymatic/lysosomal trafficking (see the following section and Table 3).

The role of proteases goes behind protein digestion with recognized important functions in several signaling pathways (Tables 2 and 3; Figs. 1 and 2), namely those involved in DNA replication and translation processes, cellular proliferation,

Table 2 Classes of enzymes identified in the urine of bladder cancer human patients detected by mass spectrometry and corresponding enriched biological processes

Type of enzyme	Number	Enriched biological processes	Enzymes
Oxidoreductase	6	Prostaglandin metabolic process, hypochlorous acid biosynthetic process, daunorubicin metabolic process, doxorubicin metabolic process, polyketide metabolic process, peroxisome fission	ACOX1, AKR1C2, CP, DHRS2, NOS2, MPO
Transferase	11	Peptidyl-pyroglutamic acid biosynthetic process, using glutaminyl-peptide cyclotransferase, activation of phospholipase A2 activity by calcium-mediated signaling, UDP-glycosylation, glutamate catabolic process to aspartate or 2-oxoglutarate, L-kynurenine metabolic process, double-strand break repair via alternative nonhomologous end joining, ectopic germ cell programmed cell death, positive regulation of catenin import into nucleus	GOT2, CD38, EGFR, F13A1, GSTT1, ART3, PLK2, PRKDC, QPCT, TGM4, UGGT1
Isomerase	2	DNA topological change, cyclooxygenase pathway	PTGDS, TOP1
Lyase	4	Positive regulation of neurotrophin production, double-strand break repair via classical nonhomologous end joining, regulation of organelle transport along microtubule, positive regulation of inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity, protein heterotetramerization, DNA demethylation, DNA ligation	XRCC6, CA1, APEX1, ADCY2
Hydrolase	21	Regulation of humoral immune response, positive regulation of epidermal growth factor receptor signaling pathway, positive regulation of ERBB signaling pathway, regulation of complement activation, regulation of protein activation cascade, antibiotic metabolic process, positive regulation of vascular smooth muscle cell proliferation, negative regulation of tumor necrosis factor (ligand) superfamily member 11 production, positive regulation of B cell proliferation, endodermal cell differentiation, positive regulation of neurotrophin production, biofilm formation, response to lactam/staurosporine/fluoxetine, regulation of smooth muscle cell-matrix adhesion, negative regulation of cell cycle checkpoint, negative regulation of DNA damage checkpoint, negative regulation of cysteine-type endopeptidase activity involved in apoptotic signaling pathway	ADAMTS7, CTSD, CPN1, CTSG, CD38, CFB, C2, DDX39B, MASP2, MMP-2, MMP-9, PGA5, PON1, ALPP, PTPRC, LTF, DPEP1, VNN2, APEX1, PLAU, XRCC6

Table 3 Proteases detected in distinct protein and mRNA levels in the urine of bladder cancer human patients

Type of protease	Protein name	Gene name	Uniprot accession number	Protein variation	mRNA variation	Bladder cancer-associated biological processes/pathways/responses
Membrane dipeptidase	Dipeptidase 1	DPEP1	P16444	↓	↓	Dipeptides hydrolysis, eicosanoid synthesis
Lysine carboxypeptidase	Carboxypeptidase N catalytic chain	CPN1	P15169	↑	↓	Steroid biosynthesis
Serine endopeptidase	Cathepsin G	CTSG	P08311	↑	↓	ACE inhibitor pathway/renin–angiotensin system, lysosomal trafficking
	Complement C2	C2	P06681	↑	↑	Complement and coagulation cascades, pertussis, *Staphylococcus aureus* infection
	Complement factor B	CFB	P00751	↑	–	Complement and coagulation cascades, *Staphylococcus aureus* infection
	Urokinase-type plasminogen activator	PLAU	P00749	↓	↑	NF-kappa B signaling pathway, complement and coagulation cascades, Wnt signaling pathway, osteopontin signaling, transcriptional misregulation in cancer, proteoglycans in cancer
	Mannan-binding lectin serine protease 2	MASP2	O00187	↓	↓/↑	Complement and coagulation cascades, *Staphylococcus aureus* infection
Aspartic endopeptidase	Cathepsin D	CTSD	P07339	↑	↓/↑	Lysosomal trafficking
	Pepsin A-5	PGA5	P0DJD9	↑	–	Protein digestion and absorption
Metalloendopeptidase	A disintegrin and metalloproteinase with thrombospondin motifs 7	ADAMTS7	Q9UKP4	↑	–	Cartilage oligomeric matrix protein degradation
	Matrix metalloproteinase-9	MMP-9	P14780	↑	↑	TWEAK signaling pathway, osteopontin signaling, angiogenesis, AGE/RAGE pathway, matrix metalloproteinases signaling, leukocyte transendothelial migration, estrogen signaling pathway, transcriptional misregulation in cancer
	Matrix metalloproteinase-2	MMP-2	P08253	↑	↓	AGE/RAGE pathway, matrix metalloproteinases signaling, leukocyte transendothelial migration, GnRH signaling pathway, estrogen signaling pathway

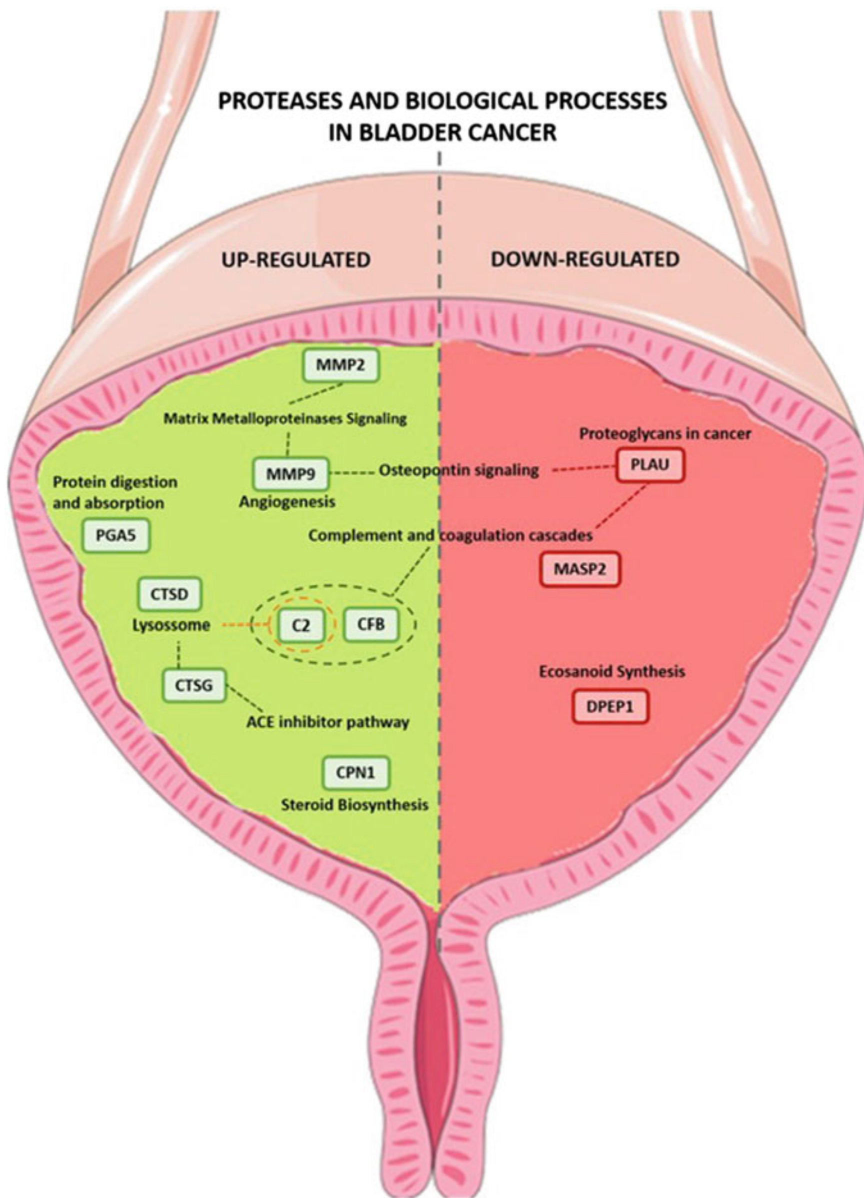

Fig. 1 Proteases modulated by bladder cancer and associated biological processes and putative biomarkers with diagnosis and prognosis value. Only a few representative examples are depicted. For a more comprehensive overview, see Table 3. Figure was designed using *Servier Medical Art*

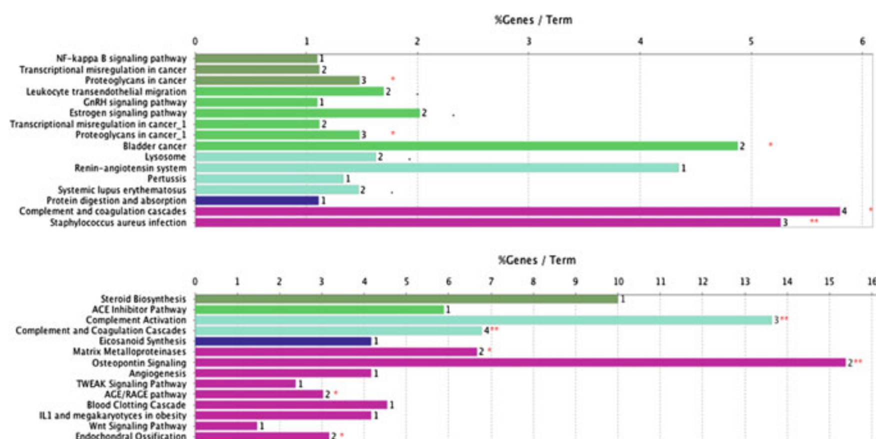

Fig. 2 Main pathways involving differentially expressed urinary proteases/peptidases modulated in bladder cancer human patients. Analysis performed using ClueGo plugin for Cytoscape. *Upper Graph* KEGG pathways. *Lower Graph* Wiki pathways. The number after bars corresponds to the number of genes contributing for those processes' enrichment. Bars marked with an asterisk (*) correspond to processes considered significantly changed (p-value < 0.05) by the built-in algorithm

tissue remodeling, angiogenesis, fertilization, hemostasis, blood coagulation, apoptosis, necrosis, immunity, organelle recycling, and cellular senescence [76–78]. Nevertheless, the human "degradome," which results from protein digestion, has been suggested to play a critical role in the pathogenesis of some diseases. Even thought, only 2% of protein coding genes contribute to such "degradome" [76–84]. These proteases are divided into cysteine proteases, serine proteases, aspartic proteases, threonine proteases, or metalloproteases [85]. Since the scope of this chapter is the biological role and clinical implications of urinary proteases in BCa, a brief description of proteases' families follows.

4.1 Cathepsins

Cathepsin proteases comprise a diverse family of hydrolytic enzymes, which are expressed at the cell surface and/or secreted into the extracellular milieu. These proteases are classically known for its role in lysosomal protein turnover and extracellular matrix degradation [86]. Despite this classical view, cathepsins encompass many and much more diverse functions. For instance, cathepsin S plays an important role in MHC-II-mediated antigen presentation, so that animal models lacking this enzyme cannot activate the MHC-II, displaying markedly impaired antigenic peptide binding, antibody class switching, and splenocytes/dendritic cells function [87]. Moreover, while trafficking through the endocytic pathway to allow invariant chain degradation and antigenic peptide loading onto MHC-II may be mediated by either one of several cathepsins in mice, this redundancy in humans is

not observed, and therefore, alterations in the levels of one particular cathepsin may have more severe consequences than in animal models [88]. The involvement of cathepsins on progressive tissue remodeling [89] is also required for malignant disease development, progression, and metastasis [86, 90], which might explain the upregulation of these enzymes in BCa (Table 3).

Most members of this family of proteases (B, C, F, H, L, K, O, S, V, W, and X) display proteolytic activity at cysteine residues, while cathepsins A and G are serine carboxypeptidases and cathepsins D and E are aspartic proteases [91]. In turn, the activity of these proteases is regulated by its corresponding inhibitors cystatins and serpins [91].

Upregulated in the urine from BCa patients (Fig. 1), cathepsin G is a serine peptidase highly expressed in immune cells, most notably in neutrophils and mast cells, but also in monocytes and dendritic cells [92, 93]. Therefore, the high levels of cathepsin G, detected by MS profiling of urine from BCa patients (Table 3), are most likely of immune cell origin (rather than tumor cell origin) and may account for some of the biological alterations observed in BCa patients, such as NF-kappa B signaling pathway, eicosanoid synthesis, leukocyte transendothelial migration, and transcriptional deregulation (Fig. 2; Table 3).

Cathepsin G is known to cause extensive damage to host tissues due to its poor specificity [94]. Animal models of ischemia lacking cathepsin G can survive, while the condition is lethal to most wild-type animals. For instance, while neutrophil infiltration and the levels of CXCL1 and CXCL2 chemokines and myeloperoxidase are equal in both groups immediately after induction of ischemia, mice lacking cathepsin G do not suffer from the severe tubular necrosis, tubular cell apoptosis, and fibrosis characteristic of normal mice, showing that cathepsin G sustains tissue pathology and fibrosis after stress, inflammation, and injury induction [94].

Cathepsin G derived from neutrophils is coreleased into phagolysosomes, thereby helping to fight of infections [95]. In fact, studies with animal models suggest that cathepsin G activation is required for survival upon infection by *Aspergillus fumigatus* and *Staphylococcus aureus* [95, 96]. Likewise, both membrane-bound and secreted forms of cathepsin G colocalize and are coreleased with other peptidases [97, 98], possessing antimicrobial activity and being the main neutrophil-derived antibacterial mediators [95]. Accordingly, its antimicrobial activity seems to be partially enhanced by and dependent on other peptidases like elastase [95], and its surface expression may increase up to 30-fold upon stimulation [97]. Therefore, infections may trigger the upregulation of cathepsin G as a host protective mechanism, but may also cause tissue damage and promote tumorigenesis (Fig. 2) or metastization once cancer has been established.

Compared to other human proteases, cathepsin G is less selective by combining both chymotryptic and tryptic hydrolytic activities [99], and while mutations/evolutionary alterations responsible for this duality confer it a broader activity spectrum, these may also contribute for its lower potency [100]. Irrespectively, its broad spectrum of activity most likely contributes for extracellular matrix degradation, complement activation, proteoglycan degradation, and lysosomal signaling alterations in BCa (Fig. 2; Table 3). It should be noted that most of what

is known about cathepsin G activity results from in vitro assays or animal studies, which may hamper the interpretation of its hypothetical implications in BCa. For instance, in contrast to human cathepsin G, murine cathepsin G displays a chymotryptic bias, lacks tryptic activity to a large extent, and is more specific, but significantly more potent [100].

The major activator of cathepsin G and related peptidases is the lysosomal cathepsin C (CTSC) or dipeptidyl peptidase I (DPEP1) [101], which was also found in increased levels in the urine from BCa patients (Fig. 1; Table 3). Increased levels of DPEP1 and the corresponding activated peptidases are associated with exaggerated immune responses and tissue damage. In contrast, animal models lacking DPEP1 are protected from these effects upon the induction of inflammatory response and display decreased expression of TNF-α and IL-1β [101], also involved in MMP-mediated BCa progression as discussed in the following section. Its tryptic activity, in turn, activates proteinase-activated receptors, calcium mobilization, and neutrophil–platelet interactions at sites of injury or inflammation [102], as well as complement proteins [103] and prourokinase plasminogen activator (PLAU) [104], which are in high levels in the urine of BCa patients (Table 3). Owing to its peptidase activity, human cathepsin G hydrolyzes angiotensin-(1-10) (angiotensin I) to yield angiotensin-(1-8) (angiotensin II) [105, 106] and activates metallopeptidases [107], thereby accounting for the alterations observed in the renin–angiotensin and lysosomal signaling (Fig. 2; Table 3). Such metallopeptidases include MMP-2, which is also in high content in the urine from BCa patients (as described in the next section), and the regulation of both substrates may therefore be intimately interconnected [107]. Taken together, cathepsin G, as well as its promoter and its substrates, is increased in urine samples from BCa patients and seems to be involved in the promotion of extracellular matrix component degradation (e.g., collagen, laminins) and local tissue damage, allowing malignant cells to infiltrate the bladder tissues and to disseminate into the blood circulation.

In contrast to cathepsin G (a serine protease), cathepsin D is an aspartyl protease involved in cellular components' recycling and apoptosis control. Procathepsin D is found in the Golgi complex and is proteolytically inactive but the intermediate and mature forms of cathepsin D are enzymatically active [108]. These forms are found in endosomes and lysosomes, respectively, and are responsible for the autophagy and apoptosis pathways [109], thus accounting for the enriched processes illustrated in Table 3 and Fig. 2. For instance, cathepsin D is known to function as an anti-apoptotic mediator in human malignant glioblastoma cells, inducing autophagy under stress conditions and conferring cancer cells resistance against genotoxicity mediated by oxidative agents such as hydrogen peroxide [110]. However, the proteolytic activity of cathepsin D also allows the release of growth factors that act by promoting tumor cell growth [109]. Cathepsin D mediates both metastasis and recurrence in breast cancer [111, 112] and metastasis/dissemination in laryngeal and pancreatic cancers [113, 114]. It was proposed as a marker of vascular and microvascular density in breast and ovarian tumors [115, 116]. However, in contrast to cathepsin G, which appears to be mostly of immune cells' origin, cathepsin D has been described as secreted primarily by cancer cells [117, 118]. In a small

cohort of patients with BCa (15 patients), it was shown that the activity of cathepsin D was significantly increased in serum samples [119]. The urinary concentration of cathepsin D is approximately equal to that of serum in both BCa patients and healthy subjects [47]. In another small cohort of patients (21 subjects), tissue cathepsin D analyzed by immunohistochemistry displayed a significant but inverse correlation with tumor grade and stage. These data reinforce the involvement of this protease in the early stages of the disease and local tissue invasion, but its expression decreases once high grade and high invasiveness are attained [120]. A disengagement between the regulation of cathepsin D and its inhibitor cystatin C was also reported [120]. Subsequently, in a more comprehensive study comprising 68 BCa patients, both serum and urinary concentrations of cathepsin D were found significantly higher, using the surface plasmon resonance imaging (SPRI) biosensor [47]. The serum cathepsin D/creatinine ratio was reported to be significantly higher in superficial tumors (Ta+T1) compared to invasive tumors (T2+T3). This observation makes cathepsin D of particular value for detecting BCa in its early stages. Of uttermost importance, urinary cathepsin D/protein ratio was significantly higher in primary versus recurrent (2.24-fold) and low-grade versus high-grade (1.67-fold) tumors, reinforcing its diagnosis value for BCa in the initial stages [47]. Overall, BCa displayed significantly higher serum (eightfold) and urine (sevenfold) cathepsin D concentrations when compared with healthy controls, and differences remained significant even when normalized to total protein and creatinine levels [47].

Similar to cathepsins D and G, urinary cathepsin L is present in significantly higher urinary levels in patients with urothelial carcinoma compared to normal controls, even in patients with negative cytology [40]. Urinary cathepsin L levels were higher in patients with higher grade, being significantly associated with tumor invasiveness. Also, the associations with BCa presence and aggressiveness were maintained after adjusting cathepsin L levels for urinary creatinine content [40]. Perhaps of uttermost value, voided urinary cathepsin L was proven to be an independent predictor of BCa recurrence and invasiveness at advanced stages, outperforming both cytology and NMP22 (an FDA-approved marker for BCa at initial stages) [40]. These results were in accordance with increased cathepsin L mRNA expression in tissue samples from BCa patients [39]. Cathepsin L expression in tumor cells is under distinct control mechanisms compared to normal cells, as the transcription factors responsible for basal cathepsin L expression (NF-Y, Sp1, Sp2, and Sp3) are not responsible for its overexpression in tumor cells [121]. The 5′ region of the cathepsin L gene encompasses CpG islands, which regulate promoter activity, and demethylation of these CpG islands seems to be positively associated with cathepsin L-dependent types of malignant diseases, but not independent ones [121]. Therefore, one possible mechanism (albeit not tested to date) contributing for the upregulation of cathepsin L in BCa may encompass epigenetic alterations, particular DNA demethylation, explaining the transcriptional misregulation depicted in Table 3 and Fig. 2.

Overall, the upregulation of cathepsins and their inhibitors is known to mediate multiple stages of tumorigenesis, carcinogenesis, and angiogenesis [90, 122]. In addition to directly carrying out extracellular matrix and basement membrane

degradation, cathepsins also disrupt intercellular adhesion proteins (e.g., E-cadherin, at adheren junctions) and activate proteolytic cascades in which the activity of other proteases such as MMPs and urokinase plasminogen activator is enhanced [86, 122]. Therefore, the levels of cathepsins and, more specifically, the cathepsin/CIP (cathepsin inhibitor) ratio may be used as a suitable marker of malignancy [39]. Nevertheless, among the cathepsin protein family, cathepsins G and D seem to be the best potential urinary biomarkers for BCa (Fig. 1; Table 3).

4.2 Matrix Metalloproteinases

MMPs are zinc-dependent endopeptidases crucial for many cellular processes, from physiological and developmental to pathological ones [123, 124]. Accordingly, these enzymes are particular important in regulating the local cellular and tissue microenvironment, not only by carrying out extracellular matrix remodeling but also by mediating several physiological processes through signaling regulation [125]. There are 23 human MMPs catalogued to date, most of which display the conserved propeptide, catalytic, and hemopexin-like C-terminal domains. Accordingly, MMPs are expressed as inactive propeptides with their zinc ions in the catalytic sites attached to the propeptide domain, being activated by the cleavage of this domain. Even though the cleavage of the prodomain requires the action of another proteolytic enzyme (intracellular furin or extracellular MMPs/serine Proteases), modifications taking place at the cysteine residue of the propeptide domain may also compromise such interactions and thus activate MMPs [126, 127].

In the setting of BCa, uncontrolled proliferation and compromised apoptosis are attributed in a large extent to the upregulation of zinc-dependent endopeptidases MMP-2 and MMP-9 (Fig. 1; Table 3) [128]. The proteolytic activity of MMPs is responsible for the cleavage of ligands, and corresponding receptors are responsible for conveying proapoptotic signals, thus allowing tumor cells to evade apoptosis [129]. These changes mediate both tumor cell migration and the development of new tumor-related blood vessels [53, 130]. So, MMP-2 as well as MMP-9 has been regarded as promising biomarkers for BCa [21, 49, 131, 132]. MMP-9 acts synergistically with ADAM metallopeptidases and epidermal growth factor receptor (EGFR) in order to degrade E-cadherin and liberate β-catenin, allowing its translocation into the nucleus and thus enhancing cellular proliferation [133, 134]. Also, tumor cells may use transforming growth factor-β (TGF-β) signaling via MMP-mediated proteolytic conversion, allowing tumors to evade immune surveillance and thus leading to local invasion and cancer metastasis [135, 136]. Similarly, ADAM-mediated proteolytic cleavage of tumor-associated major histocompatibility complex class I-related proteins MICA and MICB, which suppresses NK cell-mediated cytotoxicity, seems to require the activity of MMPs [137, 138]. Inflammatory cell-derived MMP-9 increases the bioavailability of vascular endothelial growth factor (VEGF), which is the most potent inducer of tumor angiogenesis [139].

High molecular weight forms of MMPs are also found in increased levels in the urine of BCa patients, most notably MMP-9 dimers and MMP-9 conjugated with its inhibitor TIMP1 [53], and MMP-2 conjugated with the MMP inhibitor TIMP2 [132]. The overexpression of MMP-9 promotes the upregulation of its inhibitor TIMP1 as a countermeasure. However, when MMP-9 is in excess over its inhibitor TIMP1, the formation of MMP-9 disulfide-bonded dimers takes place, forming a more stable and slowly activating MMP-9, which is less susceptible to inactivation [140]. MMP-9/NGAL conjugates seem to protect MMP-9 from autodegradation and are associated with the formation of cancer metastasis [53, 141, 142]. MMP-2, MMP-9, MMP-9/NGAL complexes, and MMP-9 dimer are overexpressed in high-grade BCa patients compared to low-grade ones, and MMP-9/TIMP1 complex is overexpressed in larger tumors compared to smaller ones [48]. Also, MMP-2 monomer, MMP-2 conjugated with its inhibitor, and MMP-2 fragments are significantly associated with high-grade/*lamina propria* invasion [132], while the presence of MMP-9/TIMP2 discriminates malignant tumors from benign ones [50]. The misbalance given by the ratio MMP/MMP inhibitor, rather than upregulation of any MMP independently, seems to be characteristic of BCa [30]. Moreover, in addition to enzyme monomers and enzyme complexes, immunoblotting approaches have revealed the presence of multiple MMP-2 fragments in the urine of BCa patients, which are also associated with the disease, particularly with transitional cell carcinoma cases [132].

More than measuring the levels of MMPs, MMPs' proteolytic activity assessed by zymography has assumed a paramount role in discerning the involvement of MMPs in BCa. In these studies of enzymatic activity, close to 65% of BCa patients present increased urinary proteolytic activity compared to healthy controls [48, 49]. The assessment of the proteolytic activity of multiple MMPs has provided specificities of up to 100% for BCa detection [48, 50]. Most of the proteolytic activity characteristic of urine samples from BCa patients seems to be carried out by MMP-9, followed by MMP-9/NGAL conjugates and MMP-2 and MMP-9 dimers. When MMP-9 dominates, the maximum sensitivity resulting from urine MMPs profiling for BCa detection seems to be attained with MMP-9 (62.1%), followed by MMP-9/NGAL conjugates (60.9%) and MMP-2 (54.5%) and MMP-9 dimers (53%) [79]. Irrespectively, MMPs are positively correlated with each other. So, the increased levels of a particular MMP upregulate the remaining one. In this regard, MMP-9 seems to suffer the highest positive influence, being more sensitivity and more susceptible to a larger number of factors [79, 141]. In fact, the expression of MMP-9 is enhanced by inflammatory cytokines such as TNF-α and interleukins, being its increase more susceptible to alterations triggered by inflammatory conditions (e.g., cystitis) and less specific to BCa itself [143], as illustrated by patients with cystitis who excrete significantly more MMP-9 but not more MMP-2 than controls [141]. In contrast, MMP-2 allows a better discrimination between BCa patients and healthy subjects [139, 143], being more specific and less influenced by cancer-unrelated factors [79]. However, MMP-2 and MMP-9 have failed to accurately identify BCa in the early stages/superficial bladder carcinoma (stages CIS, Ta, and T1). Urinary MMP-2 seems to be a suitable biomarker for high BCa tumor

grade (G3), while both MMP-2 and MMP-9 are suitable for advanced BCa stage (T2 or greater) [21]. Moreover, data normalization to total protein instead to creatinine increases the sensitivity and specificity of these makers because total protein is less influenced by hematuria, which is commonly found in BCa patients [144]. Also, combining urine cytology with urine protease profiling increases sensitivity from approximately 75% (given by conventional screening approaches) to more than 95%, and if the MMP-9/TIMP-2 or MMP-2/TIMP-2 ratios are used, a sensitivity close 100% might be attained [50].

4.3 Serine Proteases

Serine proteases represent more than one-third of all identified proteases and include 40 families of well-known proteases, such as trypsin and thrombin [145, 146]. This family of enzymes, which have a serine in the catalytic site, is involved in several biological processes from food digestion to inflammation response [146]. Thrombin-like proteases are a class of serine proteases involved in blood coagulation and fibrinolysis and include thrombin, plasminogen activators, and plasmin [146]. Urokinase-type plasminogen activator (PLAU or uPA) is a serine protease that in addition to regulate fibrinolysis also modulates innate and adaptive immune responses (Table 3; Fig. 2), acting as an endogenous antibiotic (e.g., bacteriostatic against *Staphylococcus aureus*) [147] and leading to severe compromised T cell activation and proliferation if absent [148]. Its antimicrobial activity is mediated by the serine protease domain [149]. Moreover, PLAU is not only a marker of severe infection, but also a good predictor of mortality (76% sensitivity and 69% specificity for fatal sepsis) [150].

Despite the weak proteolytic activity, conversion of plasminogen to plasmin by uPA makes it a molecule of high interest for the study of tumors, since plasmin is related with various malignant properties of cancers [151]. In fact, the urokinase plasminogen activator system, which comprises PLAU, is involved in several steps of cancer development and metastization (Table 3; Figs. 1 and 2), including ECM degradation, cell proliferation, migration and adhesion, angiogenesis and intravasation [152–154]. PLAU can activate the precursor forms of MMP-9 and other matrix metalloproteases (such as MMP-3 and MMP-12), leading to ECM remodeling. ECM remodeling is essential to angiogenesis and consequent tumor growth [152]. However, a study in human prostate cancer cells shows that PLAU may have an antiangiogenic role, due to antiangiogenic activity of angiostatin, produced by PLAU/plasmin system [155]. While there are two types of plasminogen activator (PLAU and tissue-type plasminogen activator), only PLAU promotes cell migration and proliferation via its growth factor domain [156]. In contrast, large reductions in the levels of its receptor (approximately 70%) have been shown to induce a dormancy state in human epidermoid carcinoma (HEp3) cell lines [143]. PLAU also induces cell migration, via its kringle domain, by acting as a chemotactic agent [157]. Therefore, a continuum comprising infection-induced PLAU upregulation and PLAU-induced cellular proliferation/chemotaxis can be discerned. In BCa,

PLAU modulates many signaling pathways, including NK-kappa B, TWEAK signaling pathway, and Wnt signaling pathway, as highlighted in Table 3 and Fig. 2. Furthermore, PLAU levels were suggested as a parameter for BCa prognosis. In fact, evidences show that patients with BCa displaying higher PLAU levels have lower survival rates than patients with low levels of PLAU [158].

5 The Added Value of Urine Proteases for Bladder Cancer Diagnosis

In order to better integrate the role of proteases in the pathogenesis of BCa (Fig. 1) together with its clinical value, Table 3 overviews the differential involvement of urinary proteases in BCa development and progression. As can be depicted from this figure, MMP-2 and -9, PLAU, and cathepsins D and G seem to be involved in all stages of BCa, including tumorigenesis and angiogenesis, cancer progression, invasion, and metastasis. Therefore, the detection of these 4 proteases would most likely be indicative of BCa, independently of its stage. Moreover, these proteases might be seen as therapeutic targets in the set of BCa. However, the majority of clinical trials with MMP inhibitors has failed, most likely because these were introduced only in advanced stages of the disease [127]. In the search of more potent and specific agents, near-infrared polymer-based proteolytic beacons were tested in animal models and were able of detecting tumors as small as 0.01 cm^2. Techniques employing visible or near-infrared fluorescence resonance energy transfer fluorophores to detect and measure MMPs' proteolytic activity allowed the detection of tumors at early stages with increased sensitivity [159]. Packard and colleagues have demonstrated that using a high-resolution fluorescent biosensor based on MMP (2, 9, or 14)-mediated peptide cleavage and fluorescence emission, the protease activity can be localized at the polarized leading edge of migrating tumor cells (rather than further back on the cell body) [160]. Similarly, biocompatible fluorogenic MMP substrates allow assessing the efficacy of MMP inhibitors in tumors without the requirement for tissue biopsies [161]. Using this approach, MMP inhibition has been shown to take place within hours after treatment initiation using a potent MMP inhibitor as prinomastat [161]. Furthermore, radiolabeled imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) may be employed for accessing MMPs' activity in order to screen patients and diagnose cancer [162, 163]. Nevertheless, as biomarkers assessed in biological fluids, MMPs and corresponding inhibitors are rather unspecific for Bca. So, envisioning to improve the clinical management of BCa, the most sensitive, specific, and robust panels of urinary biomarkers must combine: urinary MMP-2 and MMP-9, their inhibitors and ratios thereof, cathepsins G, D, L, and B, and PLAU (Table 3). However, one should keep in mind that these markers will definitively become more robust if combined with conventional markers of tumorigenesis and malignancy [164, 165].

6 Urinary Proteases as Therapeutic Targets in the Management of Bladder Cancer

Cathepsin, MMPs, or PLAU is not primary therapeutic targets in the clinical management of BCa, despite the available broad-spectrum inhibitors particularly for MMPs (e.g., batimastat, marismastat, salimatat, prinomastat, and tanomastat). These MMP inhibitors (MMPIs) have been tested for the treatment of cancers such as leukemias, lymphomas, testicular, lung, gastrointestinal, oropharangial, once inhibit malignant growth by enhancing fibrosis around malignant lesions. By doing so, MMPIs prevent tumor invasion, apoptosis, and angiogenesis. However, these therapies induce significant side defects, which limit its clinical applicability [166].

Despite not directly targeting protease activity, most of BCa therapies have impact on the regulation of proteases' expression and activity. For instance, tyrosine kinase inhibitors were reported to modulate proteases in the set of bladder cancer. For example, the treatment of bladder cancer cell lines with sunitinib impaired cathepsin B activation and stimulated a lysosomal-dependent necrosis, whereas pazopanib induced cathepsin B activation and autophagic cell death [167]. Glucocorticoids also impact the activity of proteases in BCa. Corticosterone, prednisone, and dexamethasone were shown to inhibit the expression of MMP-9 in bladder cancer cell lines with impact on cell invasion but marginal effect on cell growth [168].

Intravesical treatment with Bacillus Calmette-Guerin (BCG) is a clinically established and effective therapy for superficial bladder cancer and CIS though the mechanism of BCG immunotherapy is largely unknown. BCG was suggested to bind to fibronectin, near the carboxyl terminal region and adjacent to the heparin-binding domain, and protect this region of the molecule from tumor proteases [169]. More recently, the antitumor effect of BCG on bladder cancer was associated with BCG-induced cytokines TNF-α and INF-γ. Curiously, these cytokines were shown to induce MMP-9 expression and increase its enzyme activity in J-82 bladder cancer cells, potentially enhancing the invasiveness of bladder cancer in certain conditions [170]. Cathepsin B was also shown to mediate the BCG effect. The BCG-induced increase of this protease content and activity was related to the apoptosis of T24 and MB49 cell lines [171].

Immunotherapies with antibodies targeting PD-1 receptor expressed on T cells and its ligands, PD-L1 and PD-L2, have been recently proposed for the clinical management of BCa. Specifically, atezolizumab attaches to PD-L1 on the surface of tumor cells and prevents it from interacting with PD-1 receptors on immune cells and thus unleashes the immune system (ASCO 2016). These therapies also have impact on protease activity once targeting MMP-dependent cleavage of PD-1 ligands on fibroblasts, inhibiting inflammation in tissues [172]. Nevertheless, few experimental evidences exist on this topic once these immunotherapies are starting to be implemented for the treatment of BCa.

In vivo evidences of the impact of these therapies on the levels and activity of these proteases in urine will help to disclose the biomarker value of urinary proteases for monitoring patients' response to therapeutics.

7 Conclusions

Protease hyperactivation is a hallmark of BCa, leading to tissue damage and invasion, immune system and apoptosis evasion, tumorigenesis, and cancer metastasis. So, changes in the levels of proteases in biofluids as urine might help in the management of this malignancy. However, one should be aware that alterations in the gene expression patterns of proteases do not accurately reflect enzyme levels and activity. MS is particularly suitable for the detection of quantitative and qualitative alterations of proteases in urine samples during exploratory research, as it provides an integrative view of the multiple molecular mediators, promoters, and inhibitors that play a key role in BCa. In addition, zymography- and immunoaffinity-based approaches are required for activity assays and validation of proteases as biomarkers, respectively. The application of these methodological approaches has shown the involvement of several proteases in tumor development, progression, and dissemination. Nevertheless, these proteases have not been therapeutically targeted with success, but novel sensors are being developed and will hopefully allow the successful monitoring of urine proteases and therapy efficacy.

In conclusion, a fingerprint of proteolytic activity can be discerned in the urine of BCa patients, one comprising alterations in MMP-2 and MMP-9 activities, their inhibitors and ratios thereof, cathepsins G, D, L, and B, DPEP, and PLAU. Despite promising, the exploration of these proteases as biomarkers for the clinical management of BCa is still in its infancy. Future studies associating urine protease profile with BCa stage, patients' age and gender, and response to therapeutics are needed to support their diagnosis and prognosis value.

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Endogenous Proteases in Tumoral Progression

Dalton Dittz Júnior, Fernanda de Oliveira Lemos and Ana Candida Araujo e Silva

1 Introduction

Tumor progression is a well-controlled process which comprises the steps from the origin of a tumor cell to its ability to invade and metastasize. During invasion, tumor cells need to detach from primary tumor and, as a result of a local proteolysis, to cross the basement membrane and migrate to vascular system [1]. The local proteolysis is facilitated by releasing proteases bound to cell surface or secreted from cells (tumoral and non-tumoral) present in tumor microenvironment. Thus, endogenous proteases have been associated with tumor progression because of their contribution during invasion and metastasis [2]. When tumor cells reach the circulatory system (intravasation), they disseminate through both vascular and lymphatic vessels, move out from the vasculature (extravasation), and colonize the surrounding tissue (metastasis) [3]. The balance between proteases and antiproteases, in vivo, seems to be the key for many diseases like cancer. During tumor initiation, growth, invasion, and metastasis, all five classes of endogenous proteases (serine, cysteine, aspartate, threonine, and metalloproteases) are involved [4]. In this review, we will discuss these protease classes separately, considering its participation in tumor progression.

D. Dittz Júnior (✉)
Pharmacology Department, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
e-mail: daltondittz@gmail.com

F. de Oliveira Lemos
Pharmacology Department, School of Medicine, Yale University, New Haven, CT, USA

A.C. Araujo e Silva
Mucuri Medicine School, Federal University of Jequitinhonha and Mucuri Valleys, Teófilo Otoni, Minas Gerais, Brazil

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2 Serine Proteases

Over one-third of all known proteolytic enzymes are serine proteases. The name is due the presence of a nucleophilic serine (Ser) in the active site of these enzymes. According to MEROPS classification that divides proteases into clans based on catalytic mechanism and families on the basis of common ancestry, serine proteases are grouped into 13 clans and 40 families [5]. The family S1, clan PA, is the largest one and perhaps the best characterized group of proteases. These enzymes share structural similarities with trypsin and chymotrypsin, also members of this family. In the active site, there is a triad formed by Ser 195, His 57, and Asp 102 that is essential for catalytic activity [6, 7]. Trypsin and chymotrypsin cleave polypeptide chains at positively charged (Arg/Lys) or large hydrophobic (Phe/Trp/Tyr) residues, respectively. Most clan PA proteases have trypsin-like substrate specificity and prefer Arg or Lys side chains at the P1 position of substrate [8].

The family S1 is partitioned into two subfamilies, S1A and S1B, that share a common architecture but are philogenetically distinct and play different functions. The S1B proteases control the intracellular protein turnover and have a limited distribution in plants, prokaryotes, and the archaea. In contrast, the S1A proteases participate in a variety of extracellular processes and controlling many physiological processes as digestion, coagulation, immunity [9]. Examples of S1A members include trypsin, chymotrypsin, elastase, thrombin, urokinase-type plasminogen activator (uPA) system, tryptase, chymase, cathepsin G, matriptase, granzymes, and kallikrein [4, 7].

Activation of the most trypsin-like proteases requires proteolytic cleavage of an inactive zymogen precursor, involving the removal of one or more peptide ligations in N-terminal portion. In all known members of the family, the cleavage occurs in the same position (between residues 15- and 16-chymotrypsinogen numbering) [7]. The proteolysis is controlled by about 20 natural structurally diverse inhibitor families. Inhibitors are grouped according their mechanism of action in three different types: canonical (standard mechanism) and non-canonical inhibitors, and serpins. Serpins, similar to the canonical inhibitors, interact with their target proteases in a substrate-like manner; however, cleavage leads to an irreversible covalent acyl–enzyme complex, huge conformational changes in inhibitor, and disruption of protease active site [10]. A strict regulation of serine protease activities is essential to maintain physiological process. The abnormal activity of proteolytic enzymes promotes excessive tissue destruction and/or the aberrant processing of other proteins and peptides resulting in pathological disorders, as chronic inflammation, coagulation disturbs, and cancer [4, 11]. Following, we discuss about the role of the main serine proteases in cancer.

2.1 Trypsin

For a long time, trypsin was known only as a digestive enzyme. However, the detection of trypsin in patients that undergone pancreatectomy suggested that this serine protease is produced in other sites [12]. Later, trypsin expression was reported in epithelial cells in stomach, small intestine, colon, lung, liver, skin, kidney, neuronal cells, spleen, and inflammatory cells [13]. Trypsin is secreted as zymogen (trypsinogen) and in four isoforms with great structural homology: Trypsinogen-1, -2, and -3 are common in epithelial tissues and trypsinogen-4 in the brain [13, 14]. Trypsin activation is dependent on trypsinogen cleavage by enteropeptidases present on duodenal lumen and enterocytes and can be prevented by a antiprotease mediator pancreatic secretory trypsin inhibitor (PSTI), also named Kazal inhibitor [15]. Loss of balance in trypsin/PSTI activation leads to pathological conditions, as pancreatitis, and indicates increased risk for developing pancreatic adenocarcinoma [13]. The same peptide is secreted by tumor cells and is often referred to as "tumor-associated trypsin inhibitor" (TATI). TATI is a low molecular weight (6 kDa) coexpressed with trypsin in many cancer forms, and an elevated serum level is associated with poor prognosis. In colorectal cancer, TATI was indicated as a prognostic marker and its presence indicates poor tumor differentiation [16].

Trypsin is also overexpressed in many tumor cell type of ovary, prostate, lung, stomach, colon where it involved with proliferation and metastasis [4, 17]. These effects are mediated through activation of protease-activated receptor-2 (PAR-2). Proteinase-activated receptors (PARs) are G-protein-coupled receptors activated by many proteases. They comprise of four receptors that are activated by thrombin (PAR-1, -3, and -4) or by multiple trypsin-like enzymes, such as trypsin itself and mast cell tryptase (PAR-2). The activation is an irreversible phenomenon involving cleavage of the amino-terminal exodomain of the receptor by the protease, producing a new amino-terminal sequence that binds the core receptor and activates transmembrane signaling through G-proteins [17].

Cellular proliferation and differentiation are stimulated through PAR-2-driven signals and could contribute to the uncontrolled cellular growth in gastric and colorectal mucosa and invasion of pancreatic cancer by expressions of MMP-2, MMP-7, and MMP-9. These proteases promote degradation of extracellular matrix (ECM), facilitating the invasion, angiogenesis, and metastasis. The participation of PAR-2 in cancer invasion is suggested by the increased activity of MMP-2 and occurs via MMP/EGFR/MAPK/ERK1/2 pathway [17]. PAR-2 may also promote prostaglandin E_2 release through Ca^{2+} channel activation resulting in EGFR-stimulated cell proliferation [13, 18]. PAR-2 is also overexpressed by breast, gallbladder, lung, kidney, uterine, and cervical cancers and glioblastoma tumors, but the exact contribution of this receptor in the pathogenesis of these cancers remains unclear. In contrast, PAR-2 exerts a tumor-protective role in skin carcinogenesis [19]. An in vitro study with pancreatic carcinoma cells showed trypsin-induced proliferation and migration. These events were promoted via PAR-2 activation and the consequent increase of MMP-2 expression [18]. Further,

recent findings showed that trypsin is overexpressed in head and neck cancer cells and can promote pain via PAR-2 activation [20].

2.2 Thrombin and Urokinase-Type Plasminogen Activator (UPA) System

Thrombin and uPA systems are known for their role in regulating hemostatic and fibrinolytic pathways. In addition, these enzymes also control many other processes including wound healing, ECM remodeling, angiogenesis, tumor invasion, and metastasis [21–23]. In addition to cleaving fibrinogen, thrombin activates cells through a proteolytic mechanism involving PAR-1, PAR-3, or PAR-4 activations [17]. PAR-1, -3, and -4 are expressed in epithelium, neurons, astrocytes, and immune cells [24, 25].

The expression of PARs (mainly PAR-1 and PAR-2) has been implicated in the occurrence of several types of human cancers and correlated with degree of invasiveness and prognostic [17, 19, 26]. PAR-1 expression has been detected in human pancreatic and breast cancers, colon adenocarcinoma, laryngeal carcinoma cell line, and melanoma [17, 26, 27]. In breast cancer, PAR-1 expression levels were directly correlated with the degree of invasiveness in primary breast tissue, especially in infiltrating ductal carcinoma. PAR-1 activates MMP-1 that stimulates cell migration and invasion of breast and gastric cancer cells, facilitating metastasis processes. Previous studies demonstrated that thrombin acts as a mitogen for vascular smooth muscle cells by activation of the nuclear factor kappa B (NF-κB) or driven by the release of bFGF by these cells. In osteosarcoma cell, thrombin induced in vitro expression and association of beta 1-integrin with MMP-9 in the cell surface localization of the protease by the integrin promotes tumor cell invasion [28].

The uPA system has been related to the tumor cell in tumor cell invasion and metastasis in cancer, specially breast, lung, and ovarian types [22, 29, 30]. This system consists of the serine protease urokinase (uPA), its cell surface receptor (uPAR), the serpin plasminogen activator inhibitors (PAI-1 and PAI-2), and the serine protease precursor plasminogen. Urokinase is produced as zymogen, denoted prourokinase, that is converted into its active form by plasmic cleavage. Active uPA cleaves plasminogen, thereby generating proteolytically active plasmin that in turn degrades components of the ECM as fibrin, fibronectin, laminin, and proteoglycans or activates certain matrix-degrading enzymes, as MMPs [30].

Based on this ability to inhibit uPA activity, it was expected that PAI-1 would suppress cancer progression. However, consistent data from clinical studies suggest that PAI-1 is also involved in mediating cancer progression. A recent investigation reported that a specific inhibitor of PAI-1 blocked angiogenesis and tumor progression in mice, evidencing a possible protumor effect of PAI-1 [31]. This is corroborated by a previous study that demonstrated defective angiogenesis occurred in PAI-1 deficient mice [32]. In addition to angiogenic promotion, PAI-1 can display antiapoptosis activity. In vitro, recombinant PAI-1 inhibited cytotoxic

drug-induced apoptosis in prostate and leukemia cell line [33] and protects endothelial and other types of cells from apoptosis [34].

Finally, the overexpression of uPA, PAI-1, or uPAR is associated with advanced breast, lung, and ovarian cancer and can be used as an independent prognostic factor in several studies. They have been correlated with a short progression free and overall survival time for patients [35, 36]. Indeed, uPA and PAI-1 are among the best validated prognostic biomarkers available for breast cancer, and their dosage can drive the treatment. Low levels of both uPA and PAI-1 in lymph node-negative patients indicate a low risk of disease recurrence, and thus, patient may be spared from the toxic side effects and costs of adjuvant chemotherapy. In the opposite, lymph node-negative patients with high levels of these markers should be treated with adjuvant chemotherapy [30].

2.3 Tryptase, Chymase, and Cathepsin G

Tryptase, chymase, and cathepsin G (cath-G) are neutral serine protease trypsin-like present in secretory granules of mast cell (MC) and are involved in inflammatory process, angiogenesis, tissue repair, host defense, linking adaptive and innate immunity. Tryptase is stored in four different fully active forms in MC granules: α-(released from MCs in the bloodstream); β-(concentrated in the secretory granules of MCs and released only after degranulation—major content); γ- and δ-tryptase [37].

Human chymase is synthesized as an inactive pre-proenzyme, which is then converted to the proenzyme. The activation occurs into the secretory granules and a thiol proteinase; dipeptidyl peptidase I is necessary in this process. Chymase is enzymatically inactive at pH 5.5, and its optimum enzymatic activity is achieved at a pH of 7–9 following release into the interstitial tissues. Connective tissue and the submucosa are the major sites of MC-containing chymases [38].

Tryptase and chymase are involved in angiogenesis by activating MMPs as MMP-9, plasminogen activators or releasing growth factors by degrading ECM components or release matrix-associated growth factors. All these actions contribute to angiogenesis as well as invasion and metastasis of tumor cells. Tryptase also is a mitogen for human dermal microvascular endothelial cells with a significant increase of capillary growth, which is suppressed by specific tryptase inhibitors, and stimulates releasing of proangiogenic factors such as IL-1, IL-6, IL-8, stem cell factor, and TNF-α [37].

An evidence of the participation of tryptase in tumor progression is that tryptase-positive MCs increase in number and vascularization increases in a linear fashion in solid tumors, such as human malignant melanoma, endometrial carcinoma, breast cancer, uterine leiomyomas, gastric cancer, colorectal cancer, pancreatic ductal adenocarcinoma. In B cell non-Hodgkin's lynphomas and benign lymphadenopathies, tryptase-positive mast cell counts correlate with the development of angiogenesis and both with malignancy grades [37, 39–43].

Cath-G is a 26-kDa neutral serine protease found in the azurophil granules of neutrophils and a subset of monocytes. It is the major serine protease released by

activated neutrophils, acting through hydrolysis of a list of proteins (such as chemoattractants), ECM, and hormonal factors, playing an important role in inflammation [44, 45]. Cath-G is found in lung and breast tumors and has been implicated in tumor progression and metastasis by facilitating angiogenesis and tumor cell dissemination. A mechanism purposed is the stimulus of VEGF and MCP-1 secretion via TNF-beta signaling activation [46]. Another mechanism is the facilitation in forming cell aggregates as verified in vitro with MCF-7 mammary adenocarcinoma cells is exposed to a supernatant from culture of neutrophil activated, or in the presence of these cells, there is spontaneous formation of aggregates. It is postulated that these aggregates disseminate in the bloodstream and form tumor emboli at distant sites, potentially leading to the establishment of metastatic foci. In the presence of activated neutrophil supernatant, with inhibition of cath-G, the aggregation is abolished, suggesting the participation of cath-G on this process and the dependence on proteolytical activity [47, 48].

2.4 Kallikreins

Kallikreins form a group of 15 members (KLK 1-15) of serine protease S1 family, sharing similarities with trypsin and chymotrypsin. They are present in diverse tissues and biological fluids, playing important roles in inflammation, reproductive function, blood clotting, fibrinolysis, and cancer. These enzymes are categorized in tissue kallikrein and plasma kallikrein, differing significantly in molecular weight, substrate specificity, immunological characteristics, gene structure, and type of kinin released from kininogen cleavage [49, 50].

Currently, the potential roles of kallikreins in cancer progression have been investigated. These enzymes are thought to promote invasion of tumor cells by degrading ECM components or cleaving cell–cell adhesion proteins (e.g., E-cadherin). Indeed, KLK1, 2, 4, 5, 6, and 14 can activate protease-activated receptors (PARs) and stimulate cellular migration, contributing to tumor spreading. In vitro studies showed that KLK1, 3, 10, and 13 increase the invasiveness of tumor cells [51] and KLK6 also is involved in human melanoma progression. Although KLK6 was not detectable in tumor cells, KLK6 protein expression was detected in adjacent cells to primary melanomas, cutaneous metastatic lesions, and benign nevi lesions, indicating a possible paracrine function of extracellular KLK6 during cancer development. This was evidenced by the observation of a recombinant KLK6 protein-promoted melanoma cell migration and invasion, with an intense intracellular Ca^{2+}-flux, through PAR1 activation [52]. Despite activities in angiogenesis and proliferation, some kallikreins can play dual role: as promoter or suppressor tumor. It is possible that KLK3 protein regulates oxygen balance in tumors, inhibiting cell migration, and tube formation of endothelial cells in vitro and presents effects on inflammation. T cell proliferation and dendritic cell maturation may be inhibited by KLK3, indicating immunosuppressive effects of this protein. By the way KLK3 seems to stimulate IFN-γ secretion by natural killer

cells, displaying a proinflammatory action. Thus, classifying kallikreins as tumor promoters or suppressors is still difficult, needing other functional studies [51].

Because of the number and roles of kallikreins, there is an increasing interest for use as diagnostic markers and for therapeutic targets. Currently, for prostate cancer diagnoses, prostate-specific antigen (PSA) is a useful biomarker and KLK2 and KLK11 have been emerging for this purpose. In ovarian cancer, KLK5, 6, 7, 10, 11, and 14 have been considered as biomarkers [53]. Despite their known importance, many of the functions of kallikreins need to be enlightened [52].

2.5 Matriptase

Matriptase is a type II transmembrane serine protease expressed in endothelial, neural, and white blood cells and in a wide range of epithelial tissues, such as gastrointestinal and respiratory tracts, and in the epidermis. Structurally, matriptase contains a serine protease domain, four low-density lipoprotein–receptor domains, a transmembrane domain, and two CUB domains [54–56]. It plays a critical role in skin formation, epidermal differentiation, and skin function. Matriptase-ablated mice die shortly after birth, due to a severe dehydration that results from an impaired epidermal barrier function [57, 58]. Matriptase is nearly ubiquitously coexpressed with the Kunitz-type transmembrane serine protease inhibitors, HAI-1 and HAI-2, in adult and embryonic tissues [9], and both HAI-1 and HAI-2 display potent matriptase inhibitory activity in purified systems [59, 60].

Overexpression of matriptase mRNA or protein was detected in a wide variety of benign and malignant tumors of epithelial origin, as gastric, pancreatic, breast, cervical, ovarian, endometrial, and prostate carcinomas [61–67]. Overexpression of matriptase is a significant predictor for poor prognosis in endometrial breast cancer [66] and correlated with tumor aggressiveness in ovarian cancer [68]. An increased matriptase/HAI-1 ratio was indicative of the poor clinical outcome of advanced-stage tumors, suggesting that loss of protease inhibition may play a role in the late stages of the disease [61]. It has been suggested that the ratio of these two gene products may serve as a promising biomarker for prostate cancer progression and a potential marker for establishing the efficacy of therapeutic and chemopreventive interventions [69]. The molecular mechanisms of matriptase-induced epithelial carcinogenesis remain to be determined. In cell-free or cell-based assays, matriptase can activate several proteins that have been previously associated with malignant progression, including prohepatocyte growth factor (pro-HGF), pro-uPA, PAR-2, suggesting that matriptase can be involved in angiogenesis and degradation of ECM [7, 56, 70].

3 Cysteine Protease

3.1 Cathepsin

Cathepsins (cath) B, C, F, H, K, L, O, S, V, W, and X form a class of the cysteine proteases which contain a highly conserved cysteine residue in their active sites. Cathepsins are optimally active and stable at slightly acidic pH and inactive at neutral pH, with the exception of cath-S. Cathepsins are synthesized as preproenzymes, and the signal peptide is removed during the translocation to the endoplasmic reticulum, where cathepsins are also *N*-glycosylated. Procathepsins are transported to the endosomal/lysosomal compartments and proteolytically processed to the active forms, either autocatalytically or with the help of other proteases [71–73]. It has already been described that cysteine cathepsins have an important role in the cancer progression of solid tumors and metastasis development. In agreement with this, the levels of cystatins, natural cysteine protease inhibitors, can also vary quite widely in different cancers [74].

Cathepsin expression is frequently increased in patient sera and in tumors in comparison with normal tissue and is significantly associated with poor prognosis in breast, lung, head and neck, and colorectal cancers, melanoma, and many other cancers [75]. Cathepsins most commonly associated with cancer are cath-B, C, H, L, S, and X [72].

Numerous studies have been reported that cath-B and -L are capable of degrading the proteins of the basement membrane and extracellular matrix (ECM), such as laminin, fibronectin, and collagen IV, thus facilitating tumor cell invasion and metastasis. In addition, these cathepsins can activate other enzymes, such as metalloproteases and urokinase-type plasminogen activator, which act downstream in the proteolytic cascade and cause even more extensive degradation of the ECM [71, 75–81]. Cysteine cathepsins also degrade cell adhesion molecules, such as E-cadherin. E-cadherin can be specifically cleaved and removed from the cell surface by the cath-B, -L and -S, and the decrease of this cell adhesion protein correlates with a more invasive tumor phenotype [71].

Cathepsins can also regulate angiogenesis. As their participation in ECM degradation facilitates capillary formation, cysteine cathepsins are involved in the generation of pro- and antiangiogenic factors and exposure of cryptic protein sequences that play a functional role in angiogenesis. Tumor neovascular formation was directly related to cath-B, S, L and X overexpression [71, 75, 77, 81].

Besides participation in invasion, metastasis, and angiogenesis, cysteine proteases have a pivotal role in the regulation of innate and adaptive antitumor immune response. Their high concentration in tumor-infiltrating innate immune cells, such as macrophages, mast cells, and granulocytes, aids tumor cells in tissue remodeling, angiogenesis, and consequently, metastasis [76].

In contrast to tumorigenic effect of cathepsins, these enzymes have a proapoptotic activity in several tumor cell lines. Cathepsins have been shown to participate in apoptosis event promoted by tumor necrosis factor-a (TNF-a) or TNF-related

apoptosis-inducing ligand (TRAIL) in human cervical carcinoma, murine fibrosarcoma, ovarian cancer, prostatic cancer, and hepatoma cells. It is important to highlight that apoptosis induced by TRAIL shows to specifically target cancer cells without damaging normal cells and tissues, interesting observation for cancer therapy [81].

3.2 Calpain

Calpain is a well-conserved family of Ca^{2+}-dependent cysteine proteases that the enzymatic activity is regulated by cytosolic calcium [82–84]. Differently from other proteases that totally degrade the substrate, calpains conduct a limited process, after which the modified target protein may acquire an additional basic function or a novel function. Fifteen different calpain isoform genes have already been identified in human [82, 85]. The representative members of the calpain family, μ–calpain and m–calpain, are heterodimers consisting of a catalytic (80 kDa) subunit and a regulatory subunit (28 kDa). Some isoforms of calpain are ubiquitously expressed, such as μ–calpain and m–calpain, and others are expressed in a tissue-specific manner, such as calpain 9, which is found in the digestive tract [82–84].

Aberrant expression of calpain has been implicated in tumorigenesis. Overexpression of calpain was observed in different cancer type, such as schwannomas and meningiomas, renal cell carcinoma and colorectal adenocarcinomas, uterine cervical neoplasia, sarcomas, and carcinosarcomas. However, the downregulation of calpain is also related to cancer, e.g., in melanoma and gastric cancer [86]. In addition, aberrant level of calpastatin, and ubiquitously expressed endogenous inhibitor of μ–calpain and m–calpain, has been also observed in tumor tissue, e.g., in endometrial cancer [83]. The relative level of expression of various calpain system members within a number of different tumors is implicated in disease prognosis and progression in a number of cancers, including breast, ovarian, pancreatic, and gastro-esophageal tumors [84].

The calpain family has an established role in mediating cellular migration through their ability to regulate focal adhesion dynamics in an enzymatic-dependent manner. Calpain associates with focal adhesion structures and directly cleaves focal adhesion proteins, e.g., FAK (Focal Adhesion Kinase), talin, paxillin, fodrin, ezrin, vinculin, the actin-regulating protein cortactin, the intermediate filament protein vimentin, the protein tyrosine phosphatase PTPN23, and the cell–cell adhesion molecule PTPμ [82–84]. Calpain-2, up-regulated in localized and in metastatic prostate cancer, can also disrupt the E-cadherin adhesion complex and facilitate tumor cells invasion [82]. Inhibition of calpain has been shown to stabilize peripheral focal adhesions and subsequently decrease the rate of retraction at the rear of the cell, inhibiting migration. Calpain can also regulate the membrane protrusion at the leading edge of migrating cells by modifying actin cytoskeleton, such as cortactin [83]. Several studies also suggest that calpains are involved in matrix metalloprotease (MMP) expression and secretion, in particular MMP-2 and

MMP-9, which are important for the degradation of the extracellular matrix [82, 84].

Calpain has also been shown to play a role in the regulation of the cell cycle at a number of important steps. In breast cancer, calpain has been shown to cleave cyclin E, a protein required for the transition from G1 to S phase, to a hyperactive low molecular weight form. In addition, cyclin E overexpression has been shown to activate m-calpain [83–85]. Calpain activity also promotes hyperphosphorylation of retinoblastoma (RB) and alters the levels of cyclins A and D, as well as cyclin-dependent kinase 2 (CDK2). Calpain can cleave the CDK inhibitor p27 in a MAPK-dependent process; the cleavage of p27 is involved in G1/S transition. Furthermore, calpain can interfere with the interaction between protein phosphatase 2A (PP2A) and AKT to prevent forkhead box O (FOXO)-mediated cell death. Interestingly, PP2A can also negatively regulate calpain during cell migration [83].

Calpain has been implicated in both cell death and survival. As a prosurvival effect, calpain is able to cleave wild-type p53, regulating protein stability to prevent p53-dependent apoptosis. Growth arrest-specific protein 2 (GAS2), a protein which is cleaved during apoptosis to allow rearrangement of the actin cytoskeleton, can physically associate with calpain to prevent p53 cleavage resulting in enhanced p53 stability. In addition, calpain can promote survival through activation of NF–κB by cleavage of its inhibitor IκBα [82–85]. Calpain is responsible for the proteolysis of various substrates that can sensitize cells to apoptosis, such as the transcription factor Myc. Cleavage of Myc by calpain converts the active protein to Myc-nick, which is a transcriptionally inactive [83, 84].

In addition to survival, calpain has a clear role in some cellular apoptotic pathways. Calpain-mediated proteolysis of a number of caspases (caspase-3, -7, -9, -10, and -12) has been demonstrated; however, this can result in activation (caspase-3, -7, -10, and -12) or inactivation (caspase-9) of these cysteine proteases. Furthermore, calpain is implicated in proapoptotic pathways by the cleavage of a number of other apoptosis-associated proteins such as BAX, BCL-2, JNK, JUN, CDK5, APAF1, and FOS [82–85]. Besides, mitochondrial-located calpain is implicated in caspase-independent apoptosis—calpain cleavage of apoptosis-inducing factor (AIF) allows the mitochondrial release of AIF. Autophagy-related gene 5 (Atg5) protein is an ubiquitin ligase required for the formation of autophagosomes. Calpain can cleave Atg5 to switch autophagy to apoptosis via Atg5 translocation to the mitochondria, which results in cytochrome c release [83, 84].

3.3 Caspases

Caspases are a family of cysteine-dependent aspartate-specific proteases, which contain cysteine residue at their active site and cleave their substrate at position next to aspartate residue. Protein cleavage by nucleophilic cysteine residues is a well-conserved mechanism among proteases, but the specific affinity for aspartate is unique to caspases [87, 88]. Caspases are largely known for their role in controlling cell death (caspase-2, -3, -6, -7, -8, -9, and -10) and inflammation (caspase-1, -4, -5,

-11, and -12) [89, 90]. The apoptotic caspases are subdivided into the initiators (caspase-2, -8, -9, and -10) and the effectors (caspase-3, -6, and -7) in which the initiators are activated by dimerization, while the executioner caspases require proteolytic cleavage of prodomains by activated upstream caspases. Activated caspases result in intracellular proteolysis of cytoskeletal components and proteins vital for organelle integrity toward cell execution (apoptosis). The two main pathways signaling the execution of apoptosis are the extrinsic (death receptor-mediated) and intrinsic (mitochondrial) [87, 90, 91]. Considering the importance of caspase to the apoptosis, it is intuitive that loss of these proteases promotes tumor development. However, caspases can also interfere in other tumorigenic events, such as proliferation, invasion, and migration.

Caspase-1 and -2 are tumor suppressor enzymes frequently downregulated in human cancers. Caspase-1 is especially downregulated in prostate cancer. It was reported that androgen-independent prostate cancer cell lines overexpressing caspase-1 are more prone to death induced by irradiation. About caspase-2, the low expression of this caspase is correlated with poor prognosis in some cancers, such as gastric and colorectal cancers, whereas the high caspase-2 levels are associated with remission and survival in adults with acute lymphoblastic leukemia and acute myeloid leukemia [89].

About caspase-3, it was observed that these gene variant alleles are associated with squamous cell carcinomas of the head and neck, endometrial cancer, non-Hodgkin's lymphoma, and multiple myeloma. Upregulation of caspase-3 was observed in malignant breast cancer compared to non-malignant breast cancer tissue. Despite the increase of tumor cell apoptosis, the increase of caspase-3 exacerbates the secretion of PGE2, causing the repopulation of tissue with tumor cells and resistance to radiotherapy [89, 90, 92].

Despite mutations in caspase-6 and caspase-7 are uncommon in most human cancers, they are especially important to colon and gastric cancers. Somatic mutations in caspase-6 and -7 genes associated with reduced apoptosis can promote susceptibility to lung cancer and endometrial cancer [89, 92]. Differently from caspase-6 to -7, mutations in caspase-8 gene are not rare, being found in 5% of invasive colorectal carcinomas, but not adenomas, and in hepatocellular carcinomas, advanced gastric cancers, malignant neuroendocrine tumors, and small cell lung carcinoma. Intriguingly, a specific polymorphism in the caspase-8 gene, D302H, is associated with significantly reduced overall risk breast cancer [89, 90].

About caspase-9, it was found a positive correlation between polymorphisms in caspase-9 genes and the risk of lung cancer in smokers, evidencing a case of gene–environment interaction [92]. Regarding caspase-10, it is known that inactivating caspase-10 genes has been detected in non-Hodgkin's lymphoma, acute lymphoblastic leukemia, and multiple myeloma, as well as colon, breast, lung, hepatocellular carcinoma, and gastric cancers [89].

4 Aspartate Protease

4.1 Cathepsin D

Cathepsin D (cath-D) is a soluble lysosomal aspartic endoproteinase that requires an acidic pH to be proteolytically active (pH 4.5–5.0) [93–95]. Human cath-D is synthesized as a 52-kDa pre-procathepsin-D that is converted to a procathepsin-D (pcath-D) 48-kDa single-chain intermediate within endosomes and, then, to the fully active mature protease in lysosomes. In some physiological and pathological conditions, pcath-D/cath-D escapes normal targeting mechanism and is secreted from the cells [94]. In contrast to other tissue proteases (e.g., serine proteases and metalloproteinases), no endogenous cath-D tissue inhibitor is known in mammal [95].

High levels of cath-D and pcath-D were reported in several human tumors such as prostate, breast, endometrial, and ovarian tumors; colorectal, lung, bladder, and gastric carcinomas and melanoma. Clinical reports have even made positive associations between level of cath-D or pcath-D and tumor size, tumor grade, tumor aggressiveness, incidence of metastasis, prognosis, and a degree of chemoresistance [94].

Several reports have indicated that cath-D and pcath-D stimulate cancer cell proliferation. The mitogenic effect of cath-D can be explained by its enzymatic activity. Several growth factors, growth factor receptors, and extracellular matrix (ECM) components have been found among cath-D substrates. Moreover, cath-D digests various chemokines and may therefore attenuate antitumoral immune response. Regarding pcath-D mitogenic effect, the secreted pcath-D could be converted to the active enzyme in an extracellular milieu because of the fact that the extracellular pH of tumors is moderately acidic. The cell proliferation effect of cath-D can be also explained independently of enzymatic activity. Some studies demonstrated that enzymatically inactive pcath-D mutants stimulate growth of cancer cells in vitro and in vivo in the same manner as wild-type pcath-D, and the growth-promoting effect of pcath-D on cancer cells is not inhibited by pepstatin (an aspartic protease inhibitor). Then, it is hypothesized that pcath-D secreted from cancer cells serves as an autocrine growth factor for cancer cells since pcath-D can bind to cell surface [93–96]. The mitogenic activity of cath-D and pcath-D can also explain the vessel formation observed in different solid tumors. Cath-D can release proangiogenic growth factors from ECM, e.g., bFGF. In addition, a direct stimulation of endothelial cell growth is speculated, via a yet unidentified cell surface receptor, that could be present on both cancer cells or endothelial cells [94, 95]. In contrast to be observed in several tumor tissues, pcath-D secreted by prostate cancer cells was shown to have a possible role in generating angiostatin via proteolysis—a specific inhibitor of angiogenesis in vitro as well as in vivo [96].

In addition to the mitogenic effects of cath-D on cancer cells and endothelial cells, numerous studies demonstrated the involvement of cath-D in cancer invasion and metastasis. Different in vitro and in vivo tumor models showed that metastasis

stimulation by cath-D seems to be more dependent on cell proliferation, favoring the growth of micrometastases, rather than on the invasive potential [95].

About the involvement of cath-D in apoptosis, it has already described that cath-D can either promote or prevent apoptosis. The enzymatic activity is implicated in the mechanism of proapoptosis effect of cath-D. Cath-D mediates apoptosis induced by many apoptotic agents since it is released from the lysosome to cytosol, leading in turn to the mitochondrial release of cytochrome c into the cytosol, activation of procaspase-9 and -3, cleavage of Bid at pH 6, or Bax activation independently of Bid cleavage. Numerous studies have shown that pepstatin A, an aspartic protease inhibitor, could partially delay apoptosis induced by different proapoptotic agents [93, 95]. Regarding the antiapoptotic effect of cath-D, the mechanism has not been elucidated yet. Some studies have shown that xenografts of cancer cells overexpressing cath-D displayed less tumor apoptosis than mock-transfected cancer cells and that cath-D protected human neuroblastoma cells from doxorubicin-induced cell death [93].

4.2 Cathepsin E

Cathepsin E (cath-E) is an intracellular aspartic protease that is highly homologous to the cath-D. However, differently from cath-D, cath-E is a non-lysosomal protease predominantly expressed in cells of the immune system such as macrophages, lymphocytes, microglia, and dendritic cells. Cath-E is localized in plasma membranes, endosomal structures, endoplasmic reticulum, and Golgi apparatus [97–99].

Several adenocarcinomas and squamous cell carcinoma show high levels of cath-E expression and secretion, including lung, gastric, pancreatic ductal and bladder adenocarcinomas, and lung squamous cell carcinoma [97, 99–101]. However, the clinical significance of the increased cath-E expression in tumorous tissue has been controversial. The high expression of cath-E is related to a poor prognosis in some premalignant tumors, such as lung carcinoma, pancreatic ductal adenocarcinoma, and colorectal adenomas, whereas cath-E is associated with higher survival rates in hepatocellular carcinoma, breast and bladder cancers [99, 102].

The positive outcome in cancers that overexpress cath-E can be explained by the fact that cath-E prevents tumor growth and metastasis by inhibiting angiogenesis and augmenting the immune response [98, 100]. In carcinoma cell lines, cath-E induces tumor growth arrest and apoptosis by catalyzing the release of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) from the cell surface and by increasing the expression of interleukin-12 and endostatin, antiangiogenic proteins [97].

5 Threonine Proteases (Proteasome)

The ubiquitin proteasome system (UPS) is responsible for 80–90% of the degradation of all eukaryotic cellular proteins [103, 104]. This system is composed by ubiquitin, ubiquitination enzymes, deubiquitination enzymes (DUBs), and proteasome. First, ubiquitin monomers are conjugated to other proteins by a ubiquitin-activating enzyme (E1), resulting in their activation. The complex formed is transferred to a ubiquitin-conjugating enzyme (E2 or UBC) and then to the target protein. An ubiquitin ligase (E3) is required in the last step, which is responsible by selecting a protein for ubiquitination [105].

Human 26S proteasome consists of a proteolytic core particle (20S proteasome) and 19S regulatory particles (19S regulatory complex) that cap 20S at both ends. The proteasome is a threonine protease, where the N-terminal threonine of the β-subunit bonds in target proteins [105]. A polyubiquitinated protein is recognized by the 19S proteasome that unfolds and liberates the protein from the polyubiquitin chain. Following, 19S promotes the translocation of the protein into a chamber inside 20S proteasome for degradation. Through DUB action, ubiquitin molecules are recycled and the peptides generated are used for antigen presentation or degradated into amino acids that are used for new protein synthesis [103, 105].

The UPS is involved in every cellular function, including cell proliferation, apoptosis, migration, and invasion, and its deregulation may lead to cancer development. Overexpression of UPS has been identified in many types of cancer, including colorectal and gastric cancers [104]; novel antitumor agents based on inhibition of proteasome inhibitors have been studied, and some were approved to treat multiple myelomas (e.g., carfilzomib, marizomib, ixazomib, oprozomib) [103]. Programmed cell death is promoted when proteasome is inhibited, due to accumulation of misfolded and ubiquitinated intracellular debris, and the minor degradation of proapoptotic factors. NF-kB is a cytosolic protein that is inactivated by IkB family inhibitors. When IkB is phosphorylated, it becomes a target for degradation by the 26S proteasome. Thus, NF-kB is able to translocate to the nucleus which displays an antiapoptotic effect. If proteasome is inhibited, IkB availability is bigger, resulting in consistent inhibition of NF-kB leading to an increase in apoptosis [103, 105].

6 Matrix Metalloproteinases

Matrix metalloproteinase (MMP) is a family of enzymes with conserved methionine residue in the active site and depends on the zinc ion for enzymatic reactions. Members of MMP family include the “classical” matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs), and ADAMs with thrombospondin motifs (ADAMTS) [106]. According to their substrate specificity, MMPs are classified as collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11) and a heterogeneous group containing

matrilysin (MMP-7), metalloelastase (MMP-12), enamelysin (MMP-20), endometase (MMP-26), and epilysin (MMP-28). In addition, a set of MMPs are anchored to the cell surface (referred as MT-MMP-14, -15, -16, -17, -24, and -25) with their catalytic site exposed to the extracellular space [107]. The MMP activity is also controlled by inhibitors in the ECM and in the circulation such as the four tissue inhibitors of MMPs (TIMP-1–4) and α2-macroglobulin [108].

6.1 Metalloproteases in Cell Proliferation

One of the main tumor cell characteristics is the constant progression through cell cycle, which is sustained by the release of growth-stimulating signals from itself and neighboring cells. Although the primary function of MMPs is the remodeling of ECM, they also act on non-matrix substrates as cell surface. As a result of its proteolytic activity, MMPs and also ADAMs participate in the releasing of cell surface growth factors as TGF-β, TGF-α, TNF-α, EGF, and FGF which will stimulate cell proliferation. The membrane-bound enzymes capable of cleaving extracellular portions of transmembrane proteins, releasing the soluble ectodomains (as growth factor) from cell surface, are known as "sheddase." The balance between MMPs and TIMPs governs the cleavage and the release of many important growth factors and cell surface receptors [109, 110].

The MMP-7, matrilysin, has been associated with the release of soluble HB-EGF (heparin-binding epidermal growth factor) and the cleavage of all six members of the insulin-like growth factor-binding protein (IGF-BP) family releasing these free grow factors which promote cell growth and survival [111, 112]. MMP-8 is associated with human ovarian tumor proliferation as a result of degradation of type I collagen, and its overexpression seems to be an indicator for poor prognosis [113, 114]. On the other hand, MMP-8 prevents metastasis in melanoma and lung carcinoma in mice through the modulation of cell adhesion and invasion [115, 116]. Among MMPs that act as "sheddases," ADAM is the main family with this characteristic. In epidermal keratinocytes, ADAM-10 is involved in the releasing of the chemoattractant CXC-chemokine ligand 16 (CXCL16) from cell membrane. In addition to the sheddase activity of ADAM-10, epithelial cells with this metalloproteinase overexpressed have the proliferation enhanced as a result of an increase in the activity of β-catenin [117]. ADAM-12 regulates cell proliferation and migration by shedding heparin-binding epidermal growth factor-like growth factor (HB-EGFR) allowing it to bind to EGFR [118, 119]. Although ADAM-13 is known by its sheddase property, it also degrades the ECM, and its protease activity is determinant for neural crest cell migration [120].

The primary activity of TIMPs is the regulation of MMP activity, but its participation in cell signaling events also proliferation and apoptosis (as inhibitor) has been described [121, 122]. TIMP1 and TIMP2 inhibit FGF-2- and VEGF-induced proliferation in endothelial cells by increasing the activity of phosphatases on FGFR1 and VEGFR [123, 124]. The mitogenic activity of TIMP3 and TIMP4 is

not as extensive as for other TIMPs, although these inhibitors are expressed in tumor cells [125, 126].

6.2 Metalloproteases in Invasion

During tumor invasion cells, even those from stroma exchange enzymes and cytokines that modulate the local ECM and stimulate cell migration. One of the ECM modulations is the breakdown of connective tissue barriers made by collagens, laminins, fibronectin, vitronectin, and heparan sulfate proteoglycans, which requires the MMP activity [109]. Boire et al. [127] reported that MMP-1 cleaves PAR-1 receptor in breast cancer cells promoting its invasion and tumorigenesis and contributing to their metastatic potential. MMP-7 and MMP-3 may further promote tumor invasion by cleavage of E-cadherin, a cell adhesion molecule, leading to decreased cellular adhesion and increased cell mobility [107]. On the contrary, in experimental squamous cell carcinoma mouse models, MMP-8 was found to have antitumor and antimetastatic through the modulation of tumor cell adhesion and invasion [128, 129], also observed in melanoma cells by enhancing their adhesion to the ECM [115]. In breast cancer and melanoma, MMP-9 leads to the activation of TGF-β and breakdown of type IV collagen contributing to cell invasion and proliferation [130, 131]. Although MMP-11 is not capable of degrading the major ECM, it promotes tumor cell invasion, in a proteolytic-dependent manner [132]. After a vascular injury, MMP-14 is capable of degrading fibrin interrupting the mechanism of repair and allowing endothelial cell invasion [133]. Importantly, MMP-14 can serve as prognostic factor, and its expression is strongly associated with cancer progression and metastasis and poor prognosis of patients with melanoma [134]. Interestingly, expression of MMP-16 is associated with rapid fibrin and poor collagen invasion suggesting that MMP-16 might be important for infiltration of melanoma cells in perivascular space which is frequently abundant with fibrin [135].

ADAMs and ADAMTS, detected in numerous tumors, also contribute to their invasion and metastasis by degrading ECM [136]. Through an G-protein-coupled receptor–EGFR transactivation, ADAM10, ADAM15, and ADAM17 have been shown to promote tumorigenesis and cell invasion [137]. In contrast, Ungerer et al. [138] showed that overexpressed ADAM-15 in melanoma cells reduced invasion and growth in vitro, suggesting a tumor suppressor role for ADAM-15 in melanoma. In pancreatic cancer, the overexpression of ADAMTS-1 seems to be involved in tumor progression characterized by a local invasion and lymph node metastasis [139]. Moreover, an upregulation of ADAMTS-1 levels is achieved by breast tissue-associated fibroblasts contributing to cancer cell invasion [140]. Protumor and metastatic activity of ADAMTS-1 also may be associated with proteolytic action of MMP-1 and the release of the membrane-bound epithelial growth factor (EGF-like) factors and heparin-binding epidermal growth factor (HB-EGF) [141, 142]. Protumor activities have also been reported for ADAMTS-12 which

potentiate trophoblast invasion by disrupting $\alpha_v\beta_3$ integrin-mediated cell adhesion to ECM [143].

The balance between MMP and TIMP plays a crucial role in tumor progression and is associated with an inhibitory effect of TIMPs on tumor cell invasion and metastasis [144]. In astrocytoma cells, TIMP-1 reduced the growth rate and inhibited cell invasion [145], as well as prevented metastasis of gastric cancer cells [146] and invasion and growth of mammary carcinoma cells [147]. Recombinant TIMP-2 also inhibited the invasion but had no effect on growth of fibrosarcoma cells [148, 149]. The overexpression of TIMP-4 in breast carcinoma has been associated with a reduction in lymph node and lung metastasis by suppressing tumor growth and invasion [150]. Thus, the expression pattern of individual TIMPs as well as the cell type may define the role of these inhibitors in tumor progression in vivo.

6.3 Metalloproteases in Angiogenesis

The process of angiogenesis involves a complex and dynamic interaction between endothelial cells and extracellular environment and is modulated by soluble angiogenic factors, cytokines, and insoluble ECM components [151].

MMPs play a wide and complex role in angiogenesis since endothelial cells produce many MMPs during the formation of new blood vessels in physiological and pathological conditions. In this context, the interaction between MMP-2 and $\alpha_v\beta_3$ integrin is essential to restrict the enzyme activity to the surface of newly forming vessels [152]. Through cleavage of laminin-5, MMP-2 increases endothelial cell migration [153] and the release of VEGF that stimulates angiogenesis, in physiological conditions and also during tumor development. The release of VEGF which switches cells from vascular quiescence to angiogenesis has also been shown for MMP-9 and MMP-14 in tumors [154, 155]. Complementarily, MMP-9 promotes proteolytic degradation of vascular basement membrane proteins during the formation of new blood vessels enhancing cell survival and angiogenesis [156]. The expression in the peritumoral area of MMP-3, MMP-7, MMP-9, and MMP-12 has been shown to generate angiostatin from plasminogen, acting as a limit for tumor-induced angiogenesis [157–159].

TIMPs have been proposed to play a regulatory role during angiogenesis as inhibitors of MMPs. In Burkitt's lymphoma, Ehrlich cells and Kaposi's sarcoma-derived KS-IMM cells which overexpress TIMP1 in tumor cells show decreased tumor angiogenesis and growth [160–162]. In cutaneous and squamous cell cancers, the stromal and adjacent to the tumor expression of TIMP-1, TIMP-2, and TIMP-3, represents a limit to tumor invasion and tumor-induced angiogenesis [163–165]. Reed et al. [166] showed that TIMP1 can also inhibit angiogenesis without changes in collagenase or gelatinase activity. TIMP-2 (in melanoma cells) and TIMP-3 (in human breast carcinoma cells) have been associated with the reduction of FGF-2- and VEGF-induced proliferation through blockage of its receptors contributing to decrease of invasion and angiogenesis in these cell lines [167, 168].

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Tripeptidyl Peptidase I and Its Role in Neurodegenerative and Tumor Diseases

Mashenka B. Dimitrova, Dimitrinka Y. Atanasova
and Nikolai E. Lazarov

Abstract

Tripeptidyl peptidase I (TPPI) is a lysosomal enzyme widely distributed in mammals and humans. Its genetically determined deficiency causes the classical late-infantile form of neuronal ceroid lipofuscinosis, a fatal hereditary neurodegenerative disease associated with severe symptoms and early death, usually in the second decade of life. Many studies also show that TPPI is differentially regulated under various pathological conditions such as malignancy, neurodegeneration, ischemia, and inflammation, pointing at possible enzyme involvement in the pathogeneses of these entities. This chapter focuses on the TPPI participation in neurodegenerative and neoplastic diseases.

Keywords

Tripeptidyl peptidase I · Genetically determined diseases · Neurodegeneration
Tumor diseases

M.B. Dimitrova
Institute of Experimental Morphology, Pathology and Anthropology with Museum,
Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

D.Y. Atanasova · N.E. Lazarov
Institute of Neurobiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

N.E. Lazarov (✉)
Department of Anatomy and Histology, Faculty of Medicine, Medical University of Sofia,
2, Zdrave Street, 1431 Sofia, Bulgaria
e-mail: nlazarov@medfac.acad.bg

D.Y. Atanasova
Department of Anatomy, Faculty of Medicine, Trakia University, 6003 Stara Zagora,
Bulgaria

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1 Introduction

Tripeptidyl peptidase I (TPPI; EC 3.4.14.9) is a serine-type lysosomal protease sequentially removing tripeptides from the unmodified *N*-terminal of polypeptides and small proteins at pH around 4.5 [1, 2]. The enzyme can also act as an endopeptidase depending on the acidity of the medium [3, 4]. It is the first mammalian member of the S53 family of pepstatin-insensitive sedolisin serine-carboxyl peptidases (reviewed in [5, 6]). In humans, TPPI is encoded by the cln2 gene mapped to 11p15 [7], which mutations are associated with the fatal autosomal recessive disease classical late-infantile neuronal ceroid lipofuscinosis [8]. Therefore, TPPI is also called CLN2 protein. Different approaches have been applied to study the enzyme structure, catalytic mechanism and maturation, including studies on recombinant human TPPI [9–11], site-directed mutagenesis [12–14], and crystallographic structures of TPPI pro-enzyme [15, 16]. According to those studies, the TPPI molecule possesses a subtilisin-like fold and an octahedrally coordinated Ca^{2+}-binding site [14]. Its catalytic domain contains the active nucleophile Ser475 in a catalytic triad Ser475-Glu272-Asp360, distinct from the one of the classical serine proteases (Ser-His-Asp) but typical of sedolisin proteases. Furthermore, Asp360 and Asp276 participate in the formation of the oxyanion hole and are responsible for the acidic pH optimum of TPPI [15, 16], whereas Trp542 is critical for maintaining the proper tertiary structure of both the precursor and mature TPPI [14]. Studies on the *N*-glycosylation of the enzyme show that TPPI is a highly glycosylated protein with the oligosaccharide at Asn286 being crucial for folding and lysosomal targeting [12, 17]. Like most lysosomal proteases, TPPI is synthesized as an inactive precursor and is targeted to lysosomes through a mannose-6-phosphate-dependent pathway, where its maturation takes part involving autoprocessing or proteolytic activation [10, 11]. The prosegment of TPPI that is removed in the lysosomal compartment acts as a slow binding potent inhibitor of the parent enzyme [18]. Among the synthetic inhibitors, the most powerful and enzyme-specific inhibition is found for Ala-Ala-Phe-chloromethyl ketone [2, 19]. The substrate specificity of TPPI is thoroughly studied to show that the enzyme prefers bulky hydrophobic amino acid residues at P1 position and positively charged amino acids at P3 [20] and that its activity is much higher to substrates containing amino acid at P1’ compared with the commonly used synthetic substrates like methylcoumaryl amides [4]. Comprehensive studies on the enzyme specificity, including ones from our laboratory, provide guidance for the synthesis of specific enzyme substrates [21–23]. Natural substrates of TPPI are not fully identified, but it is known that the enzyme participates in the hydrolysis of collagen type I [1, 2], cholecystokinin-5 and cholecystokinin-8 [24, 25], angiotensin II and glucagon [2], angiotensin III and neuromedin B [26, 27], substance P [19], and the subunit c of the mitochondrial ATP synthase [19, 28]. Analyses on the presence of orthologs of human TPPI in the genomic sequences of a number of eukaryotic species have been performed to show that the enzyme is highly conservative and widely distributed in higher organisms [29].

Localization and activity of TPPI in different organs and tissues of laboratory rats and mice have been studied using biochemical assays, immunohistochemistry, and enzyme histochemistry. Our results, and data from other laboratories, show high enzyme activity in the neurons and glial cells in the central nervous system (CNS) [30–32] as well as in a number of peripheral organs such as the spleen, kidney, liver, pancreas, lungs [30, 33], male and female reproductive organs [34], and the carotid body [35]. Similar studies in human autopsy materials [36, 37] reveal an equally wide tissue distribution of the enzyme in humans. Furthermore, it has been shown that the enzyme is developmentally regulated in rats [32, 38] and mice [39] as well as in humans [36, 37]. According to Kopan et al. [27], TPPI participates in the degradation of neuropeptides entering cells through receptor-mediated endocytosis. Other authors assume a possible involvement of the enzyme in the breakdown of unnecessary mitochondria by intracellular autophagy in the stomach and in intracytoplasmic digestion of the protein content in secretory granules in excess through crinophagy in the pancreas [30]. Recently, it has also been shown that TPPI participates in receptor-mediated apoptosis [40, 41]. In addition, other studies have demonstrated that TPPI is differentially regulated in pathological processes (reviewed in [42]). The aim of the present chapter, therefore, is to summarize the current state of understanding on the role of TPPI in the pathogenesis of neurodegenerative and oncological diseases.

2 Genetically Determined Deficiency of TPPI

Genetically determined deficiency of TPPI causes the hereditary neurodegenerative disorder classical late-infantile neuronal ceroid lipofuscinosis (LINCL). It is inherited in a recessive autosomal manner and represents one of the forms of neuronal ceroid lipofuscinoses (NCLs). NCLs are a group of at least 14 genetic diseases caused by mutations in the genes encoding the so-called CLN proteins (for a recent review see [43]). NCLs are classified according to the age of onset, clinical course, and the mutated gene involved [44, 45]. They share some common features like accumulation of ceroid lipopigment in membrane-bound lysosomal residual bodies, a loss of neurons in the CNS, mental retardation, motor deterioration, seizures, and shortened life expectancy [46]. Despite the similarities, there are also substantial clinical variations in NCLs depending on the affected genes and specific types of mutations. Comprehensive reviews on NCLs have been published [47–54]. Latest achievements in the studies of NCLs are recently collected in a special issue of Biochimica et Biophysica ACTA (BBA)—Molecular Basis of Disease (BBA Volume 1852, Issue 10, Part B, Pages 2235–2338, 2015)—entitled "Current Research on the Neuronal Ceroid Lipofuscinoses (Batten Disease)" [55].

The symptoms of LINCL appear at the age of 2–4 years (late infancy) when the enzyme reaches its adult activity levels in the CNS and most probably becomes crucial for the neuronal functions [36, 37]. The symptoms develop gradually and include epileptic seizures, mental decline, impaired locomotor functions, visual

loss, and usually death in the second decade of life [48, 56]. Some mutations have been documented to cause a later onset and considerably milder phenotype [57, 58] due to the presence of residual TPPI activity [58, 59]. Earlier diagnoses of LINCL as well as other NCLs have been largely based on histopathological technics including ultrastructural analyses of the lipopigment deposits. Most of the storage material represents the subunit c of the mitochondrial ATP synthase [19, 28] although other proteins, lipids, and various components are also included [60]. In LINCL, storage bodies form curvilinear profiles sometimes accompanied by fingerprint-like deposits [49]. Those curvilinear bodies can be observed both in the CNS and extraneural tissues. Recent methods of diagnosis are much more complex and include electroencephalography, electrophysiological studies, electroretinography, enzyme assays in cultured skin fibroblasts as well as genetic analysis (reviewed in [50]). Although LINCL is still incurable, therapies are available, mostly directed at reducing the symptoms. Animal models of the disease could serve an important and useful tool for developing novel therapies. The available animal models for all types of NCLs including LINCL have been recently reviewed by Bond et al. [61]. Mice bred through biotechnological modifications targeting cln2 gene [62] have been used to test an enzyme replacement therapy [63, 64] and adeno-associated virus gene therapy [65, 66]. The results show attenuated neuropathology and a longer lifespan. Experimental therapies are promising and need careful clinical trials to achieve progress in the treatment of LINCL patents [67].

3 TPPI in Other Neurodegenerative Diseases

The similarities in NCLs have led to the search of some functional relationships or direct interactions between CLN proteins, which are deficient in individual diseases (reviewed in [53]). The first attempt to identify possible protein–protein interactions was made using a yeast two-hybrid system, and they revealed no evidence for molecular connections between CLN proteins [68]. Later on, co-immunoprecipitation and in vitro binding assays of CLN proteins from transfected COS-1 cells over-expressing them, provided evidence for a direct interaction of the TPPI and CLN5 protein. The latter is responsible for the Finnish variant of LINCL [69, 70]. Recently, however, based on uncertainties in the experimental procedures, these results were questioned [53]. As a matter of fact, it is still unclear whether possible interactions between CLN proteins might be of any biological significance [53].

Immunohistochemical studies on autopsy materials have shown that TPPI is over-expressed in both glial cells and neurons of individuals suffering from the infantile (CLN1) and juvenile (CLN3) forms of NCLs when compared to age-matched controls [37]. According to the authors, this increase may be due to either a brain reaction to the lack of important lysosomal proteins or phagocytosis of cellular components of damaged tissue or both. Using a biochemical method, an increased TPPI activity has also been detected in brains of patients suffering from

infantile (CLN1) and adult (CLN4) NCLs [71]. Similarly, TPPI activity in CLN5-deficient fibroblasts (Finnish variant of LINCL) has been found to increase by about 35% as compared to normal fibroblasts [69]. All these results indicate a possible involvement of TPPI in compensatory mechanisms of other forms of NCLs.

Mutations in the cln2 gene encoding TPPI have recently been identified to cause autosomal recessive spinocerebellar ataxia 7 (SCAR7), a rare, slow progressive hereditary disease [72, 73]. Only six cases have been described so far, five of which are in members of the same family. The clinical symptoms of SCAR7 resemble to some extent those of LINCL but are much milder and slowly progressing, resulting in an almost normal life expectancy. The symptoms include dysarthria, limb ataxia, and cerebellar atrophy but no eye anomalies or epilepsy. Ultrastructurally, curvilinear profiles have not been detected either. It has been supposed that mutations in cln2 causing a reduction of the TPPI activity, and not a dramatic loss of the enzyme functions, are responsible for SCAR7 [73].

TPPI has been reported to degrade the synthetic amyloid-β (Aβ) peptides 1–42 and 1–28 in vitro [19]. Since senile plaques in Alzheimer disease (AD) contain Aβ42, and studies have been performed on the TPPI localization and activity in AD. Earlier studies have shown a presence of amyloid precursor protein (APP) in the storage material in different forms of NCLs [74]. The authors suppose that CLN proteins are involved in the degradation pathway of APP and their deficiency leads to an abnormal APP processing. However, CLN-enzymes are lysosomal and therefore are inactive in neutral pH media outside the lysosomes. Using biochemical studies with a TPPI selective substrate, the enzyme activity has been found to increase about two-fold in AD brains when compared with normal or age-matched controls [71]. On the other hand, using immunohistochemistry it has been shown that TPPI possesses decreased expression levels in brains of patients with AD as well as in aged controls, particularly in areas of senile plaques and in neurons with neurofibrillary tangles [37]. In this study, high TPPI expression has been detected only in glial cells associated with certain senile plaques. These results are not necessarily controversial since the biochemical increase in the enzyme activity might for instance be due to gliosis. According to Kida et al. [37], TPPI may be involved in the intracellular degradation of Aβ. Recent research has shown that TPPI-deficient fibroblasts from LINCL patients have a decreased degradation by macroautophagy and impaired formation of autophagosomes resulting from an up-regulation of Akt-mTOR signaling pathway [75]. Thus, TPPI appears to be an important participant in this lysosomal pathway for the turnover of organelles and long-lived proteins. Furthermore, it is known that macroautophagy is abnormally activated in AD neurons and is one of the earliest events observed even before the formation of senile plaques. According to similar studies, it might also represent an additional pathway for generation of Aβ [76]. Whether TPPI is implicated in such pathogenic processes in AD is yet to be explored in further studies.

Hypoxic stress has been recognized to be one of the main causes of neurodegeneration to various extents. It may result from different disorders like cardiac arrest, brain injury, stroke, or even ingestion of harmful chemicals. Recent studies

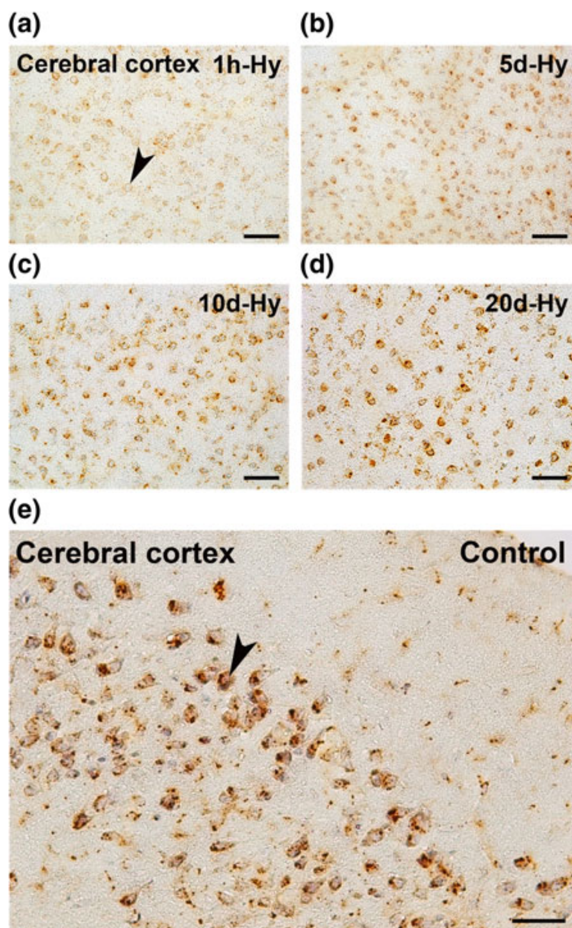

Fig. 1 Histochemical imaging of TPPI activity in cryostat sections of the rat cerebral cortex at different time periods after sodium nitrite-induced hypoxic shock using the substrate glycyl-L-prolyl-L-metionyl-5-chloro-1-anthraquinonylhydrazide. The enzyme activity in cortical neurons (*arrowheads*) is very low or missing one hour post-treatment (**a**). Later on, it increases slowly at the fifth (**b**), tenth (**c**), and twentieth (**d**) day after animals treatment to almost reach its control levels (**e**). Scale bars = 50 μm

have shown that TPPI-deficiency is accompanied by a decrease in the catalase activity and intensive accumulation of reactive oxygen species [75] as well as a substantial mitochondrial fragmentation in response to oxidative stress [41]. Furthermore, it has been revealed that TPPI activity increases in the embryonic cells of goldfish after 24 h of austere hypoxia, and the enzyme has been recognized as one of the possible target genes of hypoxia-inducible factor-1 in fish [77]. Based on these studies, recently we followed up the consecutive alterations of TPPI activity in the brain of rats, which have undergone an acute hypoxic stress throughout a

single injection of sodium nitrite, a simple and efficient model to study the impact of hypoxic insult on the brain [78, 79]. We found reduced activity levels of the enzyme in the CNS neurons almost immediately after the hypoxic event which slowly returned to their normal values 2–3 weeks after the treatment (Fig. 1). These results may be representative for a possible participation of TPPI in the brain response to hypoxia [80]. In this respect, studies on TPPI expression pattern in autopsied human brains after stroke have revealed a very weak enzyme expression in the neurons adjacent to the necrotic area but substantial enzyme levels in the microglia and astroglia [37]. Stroke results from a severe hypoxic shock leading to necrotic foci in the affected areas, and in line with this, neurons in less affected adjacent areas show a similar low TPPI expression which coincides with our results. The biological significance of TPPI activity reduction in brain hypoxia should be a subject of more detailed studies.

4 TPPI in Tumor Diseases

Most of the studies on TPPI deal with LINCL or other neurodegenerative diseases, whereas the enzyme involvement in tumors is relatively less explored. In fact, one of the earliest studies on TPPI was performed on the enzyme isolated and purified from human osteoclastomas [1]. It has been shown that TPPI participates in the degradation of collagen and probably other matrix proteins. Moreover, the TPPI specific inhibitor Ala-Ala-Phe-chloromethyl ketone reduces the ability of isolated osteoclasts to form resorption lacunae in bone slices in vitro. This might give an opportunity for the use of specific enzyme inhibitors as therapeutics for inhibiting excessive bone resorption in osteoclastoma.

Further, the enzyme activity levels have been measured in a large number of primary breast carcinomas isolated at surgery and compared to those in normal breast tissue specimen extracted at reductive mammoplasty [81]. The results reveal up to 17-fold higher enzyme activity in the tumor tissues which correlates positively with the established biomarkers in breast cancer such as cathepsin D, estrogen, and progesterone receptors. Specific methods for measuring the enzyme activity in tissue homogenates and in blood samples have also been proposed [82] in order to use them for diagnostic or prognostic purposes in breast cancer.

Additionally, in an experiment designed to establish the individual enzyme profiles and possible biochemical differences between adenocarcinomas of the gastroesophageal junction and squamous cell carcinomas of the lower third of the esophagus, the activities of a number of lysosomal enzymes including TPPI have been measured [83]. The findings have demonstrated higher TPPI activity levels in tumor tissues in the lower esophagus as compared to those in the bordering intact mucosa. According to the authors, lysosomal enzymes act synergistically to facilitate the tumor invasion into adjacent tissues.

Moreover, TPPI expression has been evaluated in colorectal carcinomas at various clinical stages using immunoblot analyses, immunohistochemistry, and quantitative RT-PCR [84]. Immunocytochemical studies have shown that the enzyme is localized mainly in the invasive front of the tumor and has a significantly elevated expression in both liver and lymph node metastatic foci. The authors suggest that TPPI is expressed in cancer metastases as a matrix protease. Since the expression levels of TPPI are associated with advanced clinical stages and occurrence of distant metastases, a diagnostic and prognostic value of TPPI has been proposed for colorectal carcinomas.

Increased TPPI expression has also been reported in other tumors, such as thyroid adenocarcinoma, liver cancer, meningioma, mesothelioma [37]. Using enzyme histochemical method in a rare malignant epithelial neoplasm, i.e., pancreatic acinar cell carcinoma, we revealed a higher TPPI activity in tumor acinar cells with varying grades of differentiation and intensity of the stromal cell reaction (Fig. 2).

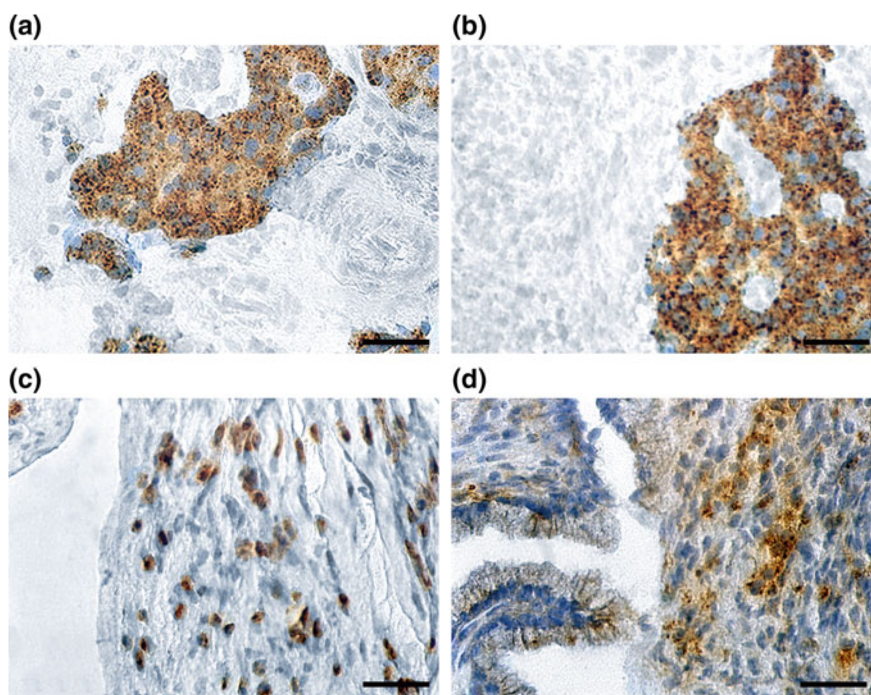

Fig. 2 TPPI activity in acinar cell carcinoma of the pancreas. The tumor acinar cells in well-differentiated (**a**), moderately differentiated (**b**) and undifferentiated (anaplastic) cancer (**c**) exhibit enzyme activity. Note the diffuse mononuclear cell infiltration around the neoplastic focus (**b**) and the marked periductal stromal cell reaction (**d**). Scale bars = 50 μm

All the studies on solid tumors described above show substantially elevated TPPI expression in the areas of tumor invasion into the adjacent tissues and/or in metastases. In this respect, it seems tempting to conclude that the enzyme is involved in the degradation of the extracellular matrix components, thus opening free spaces for the tumor growth and metastasizing, and probably for the formation of tumor vessels. However, the lysosomal localization and acidic pH optimum of TPPI are not in favor of such an assumption. On the other hand, secretion of TPPI from cells over-expressing it has been documented although the precise mechanisms involved are not fully understood [10]. Similarly, secretion of other acid hydrolases has also been reported and different mechanisms have been proposed [85, 86]. Therefore, TPPI in the extracellular matrix obviously appears in an inactive form as a pro-enzyme unable of autoactivation in neutral media [10]. More recent studies of Golabek et al. [87] have shown that the TPPI pro-enzyme binds to polyanionic glycosaminoglycans such as heparin, dextran sulfate, heparan sulfate, and chondroitin sulfate B, and these substances exert a protective effect on the enzyme molecule. They not only protect TPPI from heat- or alkaline pH-induced degradation, but also facilitate the enzyme activation at pH up to 6.0. Since sulfated polyanionic glycosaminoglycans may be present in the extracellular matrix, these findings may explain the possible TPPI role as an extracellular (matrix) protease in tumor diseases.

5 Conclusion

In summary, TPPI activity is critical for neuronal functions. Its genetically determined deficiency leads to classical late-infantile neuronal ceroid lipofuscinosis, a lysosomal storage disorder which is associated with a progressive loss of neurons and photoreceptor cells, severe symptoms, and short lifespan. Some mutations in the TPPI encoding gene lead to autosomal recessive spinocerebellar ataxia 7, a milder hereditary neurodegenerative disease. A number of studies also show that the enzyme is involved in the pathogenic mechanisms of other neurodegenerative diseases, e.g., Alzheimer disease, as well as in neurodegeneration induced by brain hypoxia. TPPI involvement in tumors is less explored, but available studies reveal its possible diagnostic or prognostic value in different types of solid tumors. The role of the enzyme in pathological processes such as tumor invasion and metastasizing are to be elucidated in further studies.

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Calpain Activity and Expression in Human Colonic Tumors

Rajendra K. Sharma, Umashankar Das, Sreejit Parameswaran, Sujeet Kumar and Jonathan R. Dimmock

Abstract

Families of cysteine proteases which are activated by Ca^{2+} are known as calpains. These enzymes can contribute to the metastatic behavior of certain tumors. In particular, we have found from Western Blotting and immunohistochemistry that the activity and expression of *m*-calpain is higher in colorectal tumors than non-malignant colonic mucosa. Furthermore, the expression of calpastin and high molecular weight calmodulin-binding protein, which are inhibitors of calpains, is reduced in colon cancers. These findings are of great importance in the design and development of anticancer drugs as well as providing diagnostic value as an indicator of the development of colorectal cancer. This chapter is devoted principally to the discoveries in our laboratories of calpain and its inhibitors in relation to human colonic neoplasms.

Keywords

Calpains · Calpastatin · High molecular weight calmodulin-binding protein
Colonic tumors

R.K. Sharma (✉) · S. Parameswaran · S. Kumar
Department of Pathology and Laboratory Medicine, College of Medicine,
University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada
e-mail: rajendra.sharma@usask.ca

U. Das · J.R. Dimmock
College of Pharmacy and Nutrition, University of Saskatchewan,
Saskatoon, SK, S7N 5C9, Canada

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1 Introduction

Cancer is one of the insidious diseases and leading causes of death worldwide. Cancer is characterized by uncontrolled growth and spread of abnormal cells that can result in death if the spread is not controlled. In the Western world, major cancer deaths occur due to colon cancer [1]. The sequencing of the human genome had made it possible to understand the genetic basis of cancer. The underlying mutations and the lack of certain gene functions are some of the leading causes of cancer. The introduction or manipulation of genes into cancer cells where the gene function has been impaired can restore gene function and inhibit tumor progression.

2 Calpains

Recently, the calpains are recognized for their important role in various diseases including cancer [2, 3]. Calpains are intracellular Ca^{2+}-activated proteases which act as important mediators of the action Ca^{2+} [4, 5]. The calpain family (EC 3.4.22.17; Clan CA, family CO_2) consists of at least 16 proteins, all of which are coded for by an independent gene [6, 7]. These genes lead to the formation of various calpain homologs which have such functional domains as Zn-finger and Ca^{2+}-binding domains [4].

Cysteine proteases, *μ*-calpain and *m*-calpain (also known as calpains I and II), require micromolar and millimolar concentrations of Ca^{2+}, respectively [4]. Both of these enzymes have a subunit of 28 kDa which has a regulatory function as well as a catalytic subunit of 80 kDa. There are different ways of regulating *m*-calpain, and its activity is inhibited by calpastatin [8–10]. The high molecular weight calmodulin-binding protein (HMWCaMBP) was discovered in our laboratory [11] and shown to be homologous to calpastatin based on calpain inhibition, amino acid analysis, sequence homology, and antibody reactivity [12].

Calpain has four catalytic domains and two regulatory domains [4–10]. Activation by Ca^{2+} leads to autolysis of the N-terminus of domain 1, and thus, the requirement of Ca^{2+} is less. This process leads to the subunits dissociating. Hence, the regulation of calpain specificity and activity includes autolysis [4]. When Ca^{2+} is absent, there are two subdomains IIa and IIb in the protease domain which are folded into one Ca^{2+} binding site [4–10]. In the calpain family of enzymes, this binding site is the most conserved which suggests that it has an essential role in bioactivity. Both *μ*- and *m*-calpains have protease domains which display Ca^{2+}-dependent protease activity and do not require other domains. Structural studies revealed Ca^{2+} binding to domains IIa and IIb. In fact, all domains, i.e., IIa, IIb, III, IV, and VI, bind at least one Ca^{2+} although with different affinities [4–10].

The calpains have been implicated in the phenomenon of metastasis [2, 3], as well as cell cycle control, mitosis, ischemia reperfusion, muscle contraction, myoblast fusion in addition to cell migration, spreading, and adhesion [13–17]. An important way that post-translational modification occurs is by proteolysis

mediated by calpain which affects cell proliferation and migration, apoptosis, and other physiological processes [10]. Substrate activity may be changed by the proteolysis of substrates by calpains. The intramolecular signals which lead to fast proteolytic degradation by *m*-calpain are the PEST sequences which are segments along the polypeptide chain that are rich in proline (P), glutamate (E), serine (S) and threonine (T). Proteolysis of calcineurin (CaN), which is mediated by *m*-calpain, resulted in increased activity and strong immunostaining of CaN in ischemic-perfused rat hearts [18, 19]. Both μ- and *m*-calpain react strongly with CaN in epileptic chickens but not normal birds [20, 21], while *m*-calpain interacts with N-myristoyltransferase (NMT) in epileptic chickens [21, 22]. It is of interest to note that of the two isoforms of NMT, it is NMT2 which reacts more strongly with *m*-calpain than NMT1 [21, 22].

3 Calpains in Pathological Conditions

The calpains cause a number of proteins in cells to undergo proteolysis [4] such as tyrosine phosphatase 1B, pp60c-Src, protein kinase, and various signaling enzymes [23–25]. Most of these proteins are believed to be involved in the development of human tumors. Thus, the calpains appear to have an important regulatory role to exercise in cancer formation. For example, greater amounts of μ-calpain were found in human renal cell carcinomas which had metastasized to peripheral lymph nodes than in tumors which had not undergone metastasis [26]. The invasiveness of some human prostate cancers has been activated epigenetically by *m*-calpain, and targeting this protein reduces tumor progression [27]. Caspase-9 which is specific for gastric cells is down-regulated in some cancers although its effect on differential status or the development of cancers has yet to be clarified [28, 29]. In chronic lymphocytic leukemia cells, the activity and expression of μ-calpain but not *m*-calpain nor calpastatin were elevated compared to non-malignant cells [30].

Various proteins are substrates of calpain including the non-receptor kinase focal adhesion kinase (FAK) and protein kinase C both of which are signaling molecules [31–33]. In addition, calpain is a substrate for several cytoskeletal proteins, the protooncogenes c-fos and c-jun as well as the tumor suppressor protein p53 [31–33]. V-Src-induced morphological changes are caused by a focal adhesion disassembly and cleavage of FAK which is mediated by calpain [33]. This v-Src activation leads to alterations in cellular proliferation, survival, motility, adhesion, and morphology [34, 35]. In chicken embryo fibroblasts, this activation leads to increases in the concentrations of *m*-calpain and decreased amounts of calpastatin [36]. The same study revealed that the activation of the v-Src oncogene led to a feedback loop mechanism of calpain activation [36]. In various human colorectal and breast solid tumors, activation of Src has taken place [37, 38]. Various protein–protein interactions have been observed in our laboratories. Thus, *m*-calpain interacts with NMT1 and caspase-3 couples with NMT2 in human colorectal tumors [39, 40]. Furthermore, NMT1 is inactivated by *m*-calpain by proteolysis [41].

While apoptosis occurs in healthy individuals, its reduced occurrence is found in a number of pathological conditions including cancer [4–10]. To some extent, apoptosis and cell proliferation may be controlled by calpains which do so by determining the level and duration of the transduction signals [8]. Nevertheless, the role of calpains in apoptosis is debatable. A number of factors such as stress conditions, expression of various oncogenes, and cell adhesion regulate the level of p53 which is a tumor suppressor gene and influences the susceptibility of a cell to apoptosis [42, 43]. In this regard, calpains have cleaved p53 in vitro by proteolysis [32, 44]. Furthermore in the case of colorectal cancer, apoptosis was reduced which may have been due to the increased level of *m*-calpain acting on p53. Similarly, the cleavage of Bax by calpain enhances the pro-apoptotic effect of Bax [45]. In addition, the transformation of procaspase-3 and procaspase-7 into caspase-3 and caspase-7 is mediated by calpain [46, 47], and the reduced levels of caspase-3, caspase-7 and caspase-9 in human colon cancers have been reported [48]. On the other hand, when the calpain inhibitor II was added to neoplastic lymphoid cells, the caspase system was activated, thereby enhancing apoptosis [49]. In colon cancers, apoptosis appears to be controlled by the extent of cross-talking between calpain and caspases.

This chapter summarizes the overexpression and elevated activity of calpain in human colonic tumors that is carried in our laboratory.

4 Calpain and Its Inhibitor Calpastatin in Colon Cancer

We have found that in human colorectal adenocarcinoma, there is an increased activity and expression of *m*-calpain as well as a reduction in the expression of calpastatin [50]. The activity of calpain and protein expression in human colorectal adenocarcinoma has been examined with a view to finding the role of *m*-calpain in this tumor. Fifty patients were evaluated although only six cases were reported. Calpain activity was greater in this tumor than in non-malignant mucosa in 83% of the cases ($p < 0.05$, Fig. 1a). In particular, the activity of *m*-calpain was greater in polyps than non-malignant tissues but less so than in cancers (Fig. 1a). Western Blot analysis revealed that *m*-calpain was weakly expressed in non-malignant mucosa, but a higher expression was found in the tumors (Fig. 1b). The expression of *m*-calpain was 2- to 3-fold greater in colorectal neoplasms than in the respective non-malignant mucosa ($p < 0.05$) as determined by a quantitative analysis of the 80 kDa band (Fig. 1b). On the other hand, there was no change in the expression of *m*-calpain at the 28 kDa small subunit [50]. The expression of *m*-calpain was higher in polyps than in non-malignant tissues, but no significant changes were noted in the other normal tissues (Fig. 1b, c).

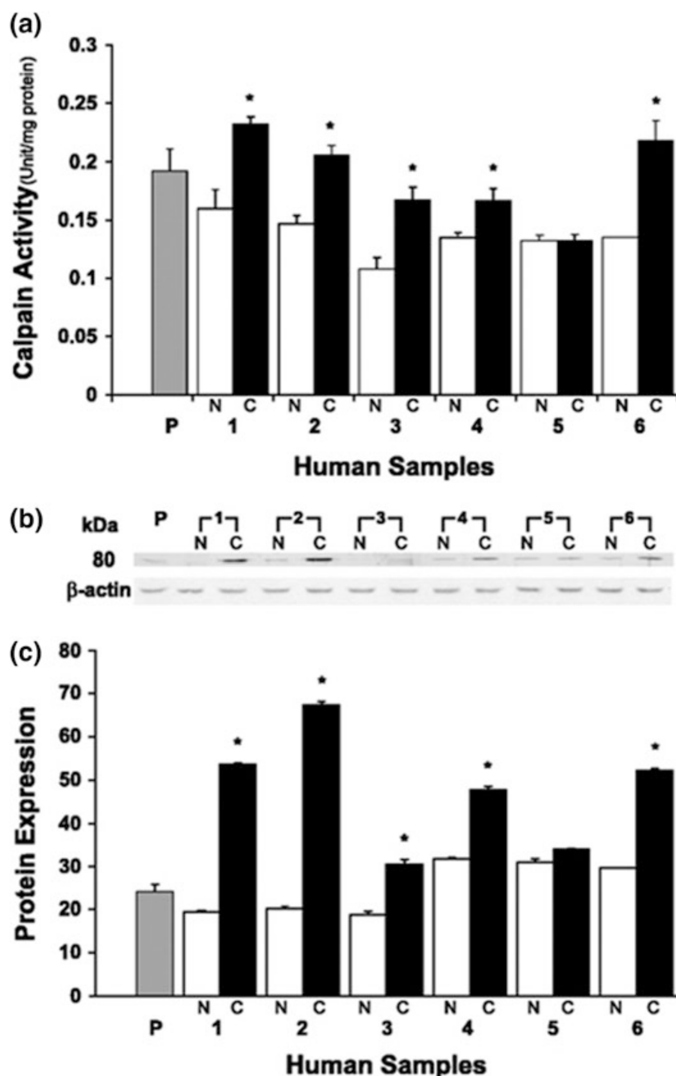

Fig. 1 Calpain activity and protein expression in human colorectal patients. **a** Calpain activity in normal (N), polyp (P), and cancer (C) tissue. **b** Western Blot analysis of *m*-calpain in human colorectal patients in normal (N), polyp (P) and cancer (C) tissue, **c** quantitative analysis of **b**. For details see Lakshmikuttyamma et al. [50]

The strong staining for *m*-calpain in colorectal adenocarcinoma was demonstrated by immunohistochemistry (Fig. 2c). When sections of the mucosa which were remote from the tumors were examined, small reactivity (<10% of the expression of the proteins) was observed (Fig. 2a). In contrast to tumors, both immunoreactivity

and staining were lower in polyps (Fig. 2b). These investigations clearly reveal that *m*-calpain activity and protein expression are higher in human colonic tumors.

Calpastatin is an endogenous polypeptide which controls the activity of calpain [9, 10, 12] by inhibiting both of the major forms of calpain [51]. Therefore, we examined the expression of calpastatin to determine whether it regulates calpain activity in colorectal adenocarcinomas [50]. In non-malignant mucosa, Western Blot analysis indicated strong expression of calpastatin whereas in colorectal neoplasms there was a weak expression of this endogenous polypeptide (Fig. 3a).

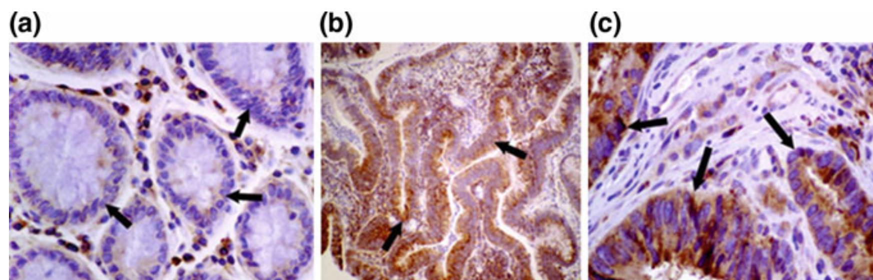

Fig. 2 Immunohistochemical analysis of *m*-calpain using anti-*m*-calpain. **a** Normal mucosa showing a mild degree of focal staining. **b** Polyps showing moderate staining. **c** Colorectal adenocarcinoma showing a positive staining. Original magnification: ×120. For details see Lakshmikuttyamma et al. [50]

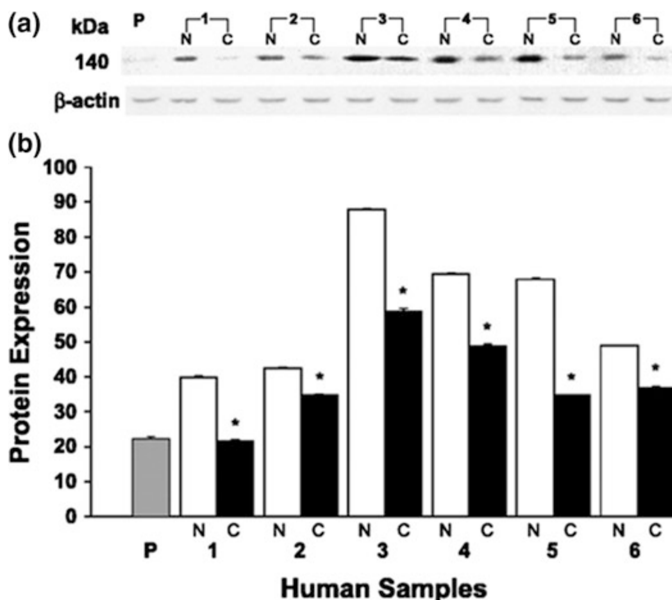

Fig. 3 Expression of calpastatin in human colorectal patients. **a** Western Blot analysis of calpastatin in human colorectal patients in normal (N), polyp (P), and cancer (C) tissue. **b** Quantitative analysis of **a**. For details see Lakshmikuttyamma et al. [50]

In fact, quantitative analysis demonstrated that there is a twofold increase in the expression of calpastatin in non-malignant cells compared to neoplastic tissues (Fig. 3b). Immunochemistry revealed that calpastatin was expressed weakly in colon tumors (Fig. 4b) while non-malignant mucosa displayed moderate to strong staining (Fig. 4a). A weak cytoplasmic positivity was noted for calpastatin in invasive carcinoma with lowered intensity in the invasive component (Fig. 4b).

As discussed earlier, our laboratory was the first to discover HMWCaMBP [11]. We have established that HMWCaMBP is homologous with calpastatin based on its ability to inhibit calpain as well as antibody reactivity, amino acid analysis and sequence homology [12]. However, it is interesting to observe that HMWCaMBP not calpasatin contains the calmodulin-binding domain [11]. Therefore, we compared the immunohistochemical studies of HMWCaMBP with calpastatin in human colonic tumors. In addition, there is a mild reactivity of HMWCaMBP in colorectal adenocarcinoma as demonstrated by histochemistry (Fig. 5b). On the other hand, there was strong staining of the mucosa which was remote from the tumor (Fig. 5a).

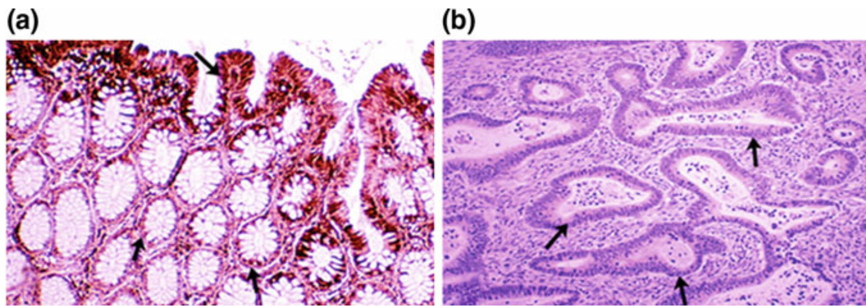

Fig. 4 Immunohistochemical staining for calpastatin using anti-calpastatin. **a** Normal mucosa showing a marked degree of focal staining. **b** Colorectal adenocarcinoma showing a mild degree of reactivity. Original magnification: ×120. For details see Lakshmikuttyamma et al. [50]

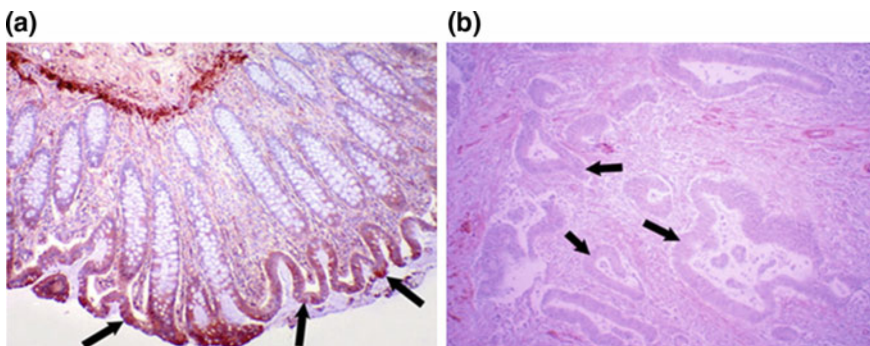

Fig. 5 Immunohistochemical staining for HMWCaMBP using anti-HMWCaMBP. **a** Normal mucosa showing a marked degree of focal staining. **b** Colorectal adenocarcinoma showing a mild degree of reactivity. Original magnification: ×120. For details see Lakshmikuttyamma et al. [50]

A correlation was noted between the lowered expression of HMWCaMBP and calpastatin in human colorectal adenocarcinomas on the one hand and the increased activity and expression of *m*-calpain on the other hand.

5 Conclusion

Our findings suggest that the development of colorectal adenocarcinoma may be influenced by the increased expression of *m*-calpain. There are many issues to be addressed regarding the proliferation of cells in colonic neoplasms including the ways in which calpain expression is increased and the effect of calpain on cell signaling. The increased activity and moderate staining of *m*-calpain in polyps reveal its use as an immunological marker for the early detection of colorectal adenocarcinoma. It is conceivable that based on their ability to inhibit calpain, the overexpression of HMWCaMBP and/or calpastatin may be a useful procedure in treating colon cancer.

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Role of Cathepsins, in Particular Cathepsins B and D in Breast Cancer: Mechanisms and Clinical Implications

Anuradha Ratna and Salil K. Das

Abstract

Consistent with the biological relevance of cathepsins B and D, their overexpression, translational or post-translational modifications, and mitogenic effects are hallmarks of breast cancer. Several studies have established a link between increased expression of cathepsins B and D and tumor growth, invasion, and metastasis. This review outlines the potential role of these two cathepsins, specifically in breast cancer, and different mechanisms involved during tumor progression and metastasis. The development of various therapeutic strategies, including specific inhibitors, gene targeted therapy, and antibodies against cathepsins in an attempt to curb breast tumor progression holds a promising future. However, from a translational perspective, further extensive studies are needed in order to unravel the therapeutic abilities of cathepsins B and D in controlling breast cancer.

Keywords

Cathepsin B · Cathepsin D · Breast cancer · Overexpression · Metastasis Biomarker · Cancer therapy

A. Ratna · S.K. Das (✉)
Department of Biochemistry and Cancer Biology, Meharry Medical College, Nashville, TN, USA
e-mail: sdas@mmc.edu

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1 Introduction

The term "cathepsin" originated from the Greek word for "digesting" [1]. Originally it was isolated from the stomach mucous membrane as an intracellular acidic protein. Cathepsin(s) were purified from spleen in 1940, and their lysosomal association was established in 1955 [2, 3]. These intracellular peptide hydrolases are a class of globular proteases, although several cathepsins also have extracellular functions [4]. Active forms of cathepsins are localized in endosomal or lysosomal vesicles, cell membranes, and/or secreted and localized in pericellular environments as soluble enzymes involved in cleaving the extracellular matrix (ECM) proteins laminin and type IV collagen, and cell-adhesion proteins such as E-cadherin and matricellular proteins [5–8]. According to their active site amino acid, cathepsins can be divided into three sub-groups: cysteine (B, C, H, F, K, L, O, S, V, W and X/Z), aspartate (D and E) and serine (G) cathepsins [9]. The lysosome relies on these protein hydrolases and other enzymes to carry out intracellular degradation before recycling cellular constituents. The functional implication of cathepsins was made possible by various genomic, proteomic, and imaging tools as well as by the generation and in-depth analysis of knockout and transgenic mice [10]. According to these studies, cathepsins act as not only redundant, homeostatic enzymes involved in the turnover of proteins delivered to the lysosome by endocytosis or autophagocytosis, but also play a critical role in the proteolytic processing of specific substrates. Thus, cathepsins contribute to distinct physiologic processes, such as antigen presentation in the immune system [11], collagen turnover in bone and cartilage [12, 13], and neuropeptide and hormone processing [14]. In addition, ectopic or excessive expression and activity of cathepsins promotes the development of several common diseases in humans, including cancer. Human cysteine cathepsins are highly up-regulated in a wide variety of cancers. Several studies have shown a correlation between cathepsin proteolytic activity and neoplastic transformation, tumor invasion, and metastasis through the destruction of ECM components and basement membranes [15]. Here we emphasize specifically, cathepsins B and D as these are the most thoroughly studied enzymes. However, neither the functions of these two cathepsins in cancer, nor their roles in tumor cells and the tumor-associated cells that contribute to neoplastic progression have been clearly defined. This chapter focusses on the role played by cathepsins B and D and their therapeutic potential, particularly in breast cancer.

2 Cellular Location of Cathepsin B and Its Function

Cathepsin B (E.C. 3.4.22.1) is a lysosomal cysteine protease belonging to the papain family of enzymes which is constitutively expressed in normal cells [16]. Cathepsin B is localized on cell membranes, and secreted and localized in endosomal or lysosomal vesicles, suggesting that its enzymatic substrates and functions might change according to its localization. Cathepsin B possesses both endopeptidase,

and exopeptidase (carboxydipeptidase) activity [17, 18]. The presence of an occluding loop which interferes with the binding of substrates to the active site contributes to the dual activity of cathepsin B [16]. At acidic pH, the occluding loop, partially blocks the active site of the molecule and prevents the entry of large substrates, yet allows the access of carboxy terminus of proteins. Thus, cathepsin B displays its carboxydipeptidase activity at acidic pH. Among the extracellular roles, cathepsin B is involved in the cleavage of ECM proteins such as laminin and type IV collagen [19], tenascin C [20], and cell-adhesion proteins such as E-cadherin [21]. At neutral pH, cathepsin B functions as an endopeptidase [22]. The loop is displaced leaving the active site to be available for the entry of large substrates. Cathepsin B is overexpressed in many human malignancies by tumor cells and tumor-associated cells at the mRNA and protein levels [23–25]. Using transgenic murine models of pancreatic and mammary carcinomas, a causal relationship has been established between cathepsin B and tumor initiation, proliferation, angiogenesis, invasion, and metastasis [26, 27]. Cathepsin B was found to promote growth of both tumor cells and tumor-associated macrophages in transgenic models. Unravelling the detailed mechanisms underlying increased expression of cathepsin B in tumors is a requisite for therapeutic targeting of cathepsin B.

3 Cellular Location of Cathepsin D and Its Function

Cathepsin D (E.C. 3.4.23.5) is a member of the aspartic proteases family that function in intracellular catabolism at lysosomal compartments. Initially, the inactive pre-pro-cathepsin D (43 kDa) is synthesized in rough endoplasmic reticulum, which is in turn cleaved and glycosylated to form pro-cathepsin D (52 kDa) containing N-linked oligosaccharides with mannose-6-phosphate (M6P) residues. Pro-cathepsin D is then directed to intracellular vesicular structures such as lysosomes, endosomes and phagosomes where it undergoes a sequence of proteolytic cleavage and autocatalysis to form mature cathepsin D containing a heavy chain (34 kDa) and a light chain (14 kDa) [28]. Further, lysosomal trafficking of cathepsin D may be either dependent on the M6P receptors or independent of M6P receptors where it undergoes endocytosis (Fig. 1).

In the past two decades, beyond its original "housekeeping" role, the participation of cathepsin D in a multitude of biological processes has been documented. It aids in the proteolytic events that are critical in controlling diverse biological processes, such as apoptosis, cell cycle, cellular differentiation, morphogenesis and tissue remodeling, immunological processes, fertilization, neuronal outgrowth, and angiogenesis [29]. Various pathological conditions, including atherosclerosis, cancer, neurological and skin disorders may occur due to the deficiency, altered regulation, or post-translational modification of cathepsin D [29]. In particular, deregulated synthesis, post-translational modifications, and over-secretion of cathepsin D, along with its mitogenic effects, are well established characteristics of cancer [29]. Overexpression and hyper-secretion of Cathepsin D is evident in

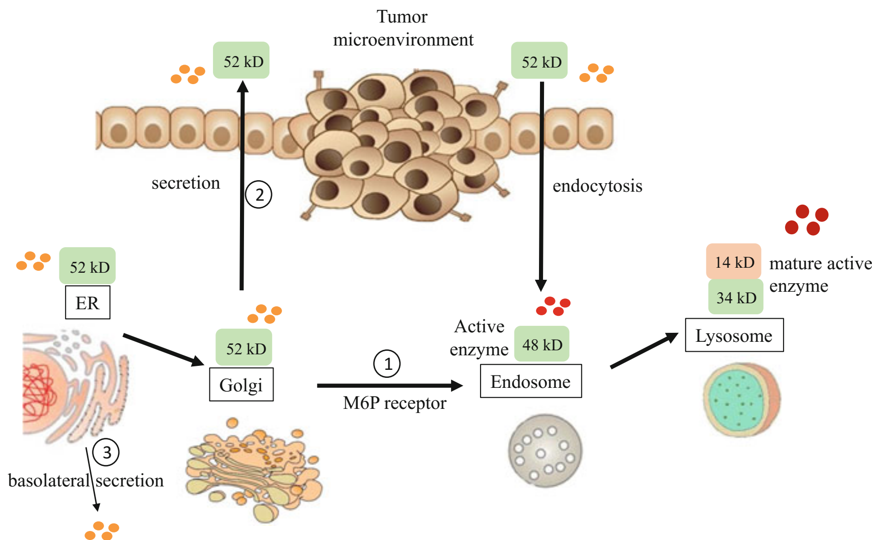

Fig. 1 Model depicting localization of cathepsin D in cancer cells. The majority of these pathways is found to be altered in breast cancer. (*1*) In cancer cells, the reduced acidification of endosomal/lysosomal compartment results in the improper processing of cathepsin D, leading to increased secretion of pro-cathepsin D. Pathways (*2*) and (*3*) are highly elevated causing excessive degradation of ECM [29, 30]. *ECM* extracellular matrix; *ER* endoplasmic reticulum; *M6P* mannose 6-phosphate; *kD* kiloDalton

different types of cancer (breast cancer, ovarian cancer, endometrial cancer, head and neck cancer, bladder cancer, malignant glioma, melanoma). Overexpression of Cathepsin D by cancer cells leads to its accumulation in the cells where it may modify the tumor micro-environment by affecting stromal cell behavior and/or degrading ECM components, and the pro-cathepsin D is hyper-secreted in the tumor micro-environment [30]. Therefore, cathepsin D activity can be blocked by targeting extracellular cathepsin D located in the cytosol, and/or intracellular cathepsin D located in intracellular vesicles or nucleus (Fig. 1). Cathepsin D is being explored extensively due to its overexpression in advanced stages of solid tumors causing aggressive metastasis and deteriorating the pathological condition.

4 Role of Cathepsins B and D in Breast Cancer

In cancer cells, lysosomes are redistributed from the perinuclear area to the cellular periphery, where they can release cathepsins or be secreted into the extracellular space [24, 31]. The secreted cathepsins facilitate tumor cells to disintegrate the stroma and intravasate into lymphatic or blood vessels, thus promoting metastasis. Cathepsins promote invasion and migration of cancer cell through ECM

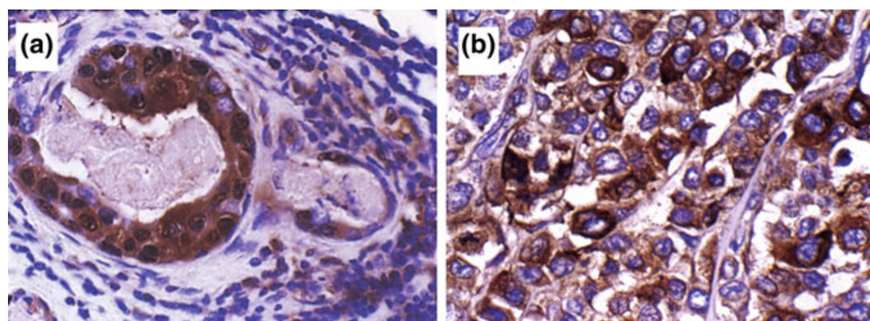

Fig. 2 Immunohistochemical staining of infiltrating ductal breast carcinoma. **a** Section stained with anti-cathepsin B antibody (original modification ×400). **b** Section stained with anti-cathepsin D antibody (original modification ×400) [38]

components in different types of cancer, including breast cancer [32]. It has also been reported that cathepsins initiate proteolytic cascades of serine-proteases and matrix metalloproteinase (MMP) [33, 34]. Besides, cathepsins may enable tumoral invasion, through proteolysis of E-cadherin, and resulting in a loss of its function in different tumors [35, 36]. Consequently, it leads to ECM remodeling, angiogenesis, invasion cancer metastasis. Expression, redistribution and/or secretion of cathepsins B and D have been reported to parallel the malignant progression [37]. The immunohistochemical study done by Castiglioni et al. reported an over expression of cathepsins B and D in most of the tumor samples as compared with normal breast epithelium from the same patients (Fig. 2) [38]. The mechanisms underlying cathepsin upregulation ranges from gene amplification to post-transcriptional modification [24]. However, the exact molecular mechanisms by which cathepsins affect cancer progression still remains elusive. The potential roles of cathepsins B and D are depicted in Fig. 3.

Aberrant overexpression and redistribution of cathepsin B has been reported in invasive and metastatic cancers, including breast cancer, melanoma and colorectal cancer [23–25]. There are reports that link cathepsin B to apoptosis, tumor-associated inflammation, angiogenesis and metastasis by contributing to the altered intracellular protein metabolism of cancer cells and to proteolytic cascades in the tumor microenvironment [39]. Cathepsin B is a prognostic marker in several types of cancer and its increased expression by tumor cells is correlated with poor outcomes in breast cancer [23, 40–42]. Cathepsin B interacts with cystatins [43, 44] and annexin II tetramer (p11) [45] and thus, responsible for the proteolytic activation and subsequent degradation of ECM.

Different mechanisms have been attributed to the upregulation of cathepsin B in tumors. Chromosomal loci for various cysteine cathepsins are prone to mutations; however, a novel amplicon has been reported only for cathepsin B. The overexpression of cathepsin B in adenocarcinomas of the esophagus has been demonstrated where tumor cells express transcript variants of cathepsins B due to the use of alternative promoters and alternative splicing [46–48]. A comprehensive study on

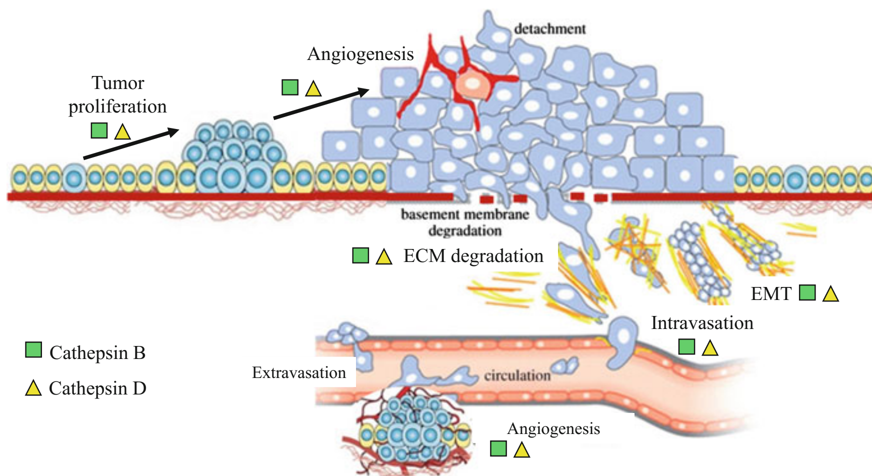

Fig. 3 Potential role of cathepsins B and D in breast cancer progression. *ECM* extracellular matrix; *EMT* epithelial-mesenchymal transition

the transcriptional regulation and the presence of transcript variants of cathepsin B in human cancers, is necessitated. One of the study showed that cathepsin B promoter contains many Sp1 sites [49], and these Sp1 sites increase in tumor cells [50]. Some other independent studies concluded that the varying expression of cathepsin B depends mainly on the different levels of Sp1 [51]. Cathepsin B also plays a role in autophagy and tumor cannibalism that could be possibly involved in specific immune resistance [52], enabling tumor cell proliferation and infiltration. This could be a possible reason for the necrotic cores of highly infiltrative tumors. Cathepsin B is responsible for initiating the proteolytic cascade involving uPA (urokinase plasminogen activator), plasminogen and plasmin [53]. Cathepsin B suppresses the inhibitors of active proteases, thus it appears to support a promalignant phenotype. For example, in human articular chondrocytes, cathepsin B destroys the inhibitors of MMPs [e.g., TIMP1 (tissue inhibitor of metalloproteinases 1) and TIMP2] and maintains its high level, leading to ECM degradation and angiogenesis [54]. It was also reported that inducers of monocytic and granulocytic differentiation [e.g., calcitriol (D3), sodium butyrate (NaB) and all-trans retinoic acid (RA)] increased cathepsin B mRNA levels in a dose-dependent manner [55]. From all these findings, we can conclude cathepsin B as a multifaceted molecule with diverse function and is regulated at various cellular levels which are still not clearly understood.

Numerous studies have documented that cathepsin D promotes breast cancer cell proliferation [56, 57], and metastasis [58, 59]. Cathepsin D expression is increased by 2- to 50-fold in breast cancer cells, compared to other cells, such as fibroblasts or normal mammary glands [60]. To investigate the causes of elevated secretion of cathepsin D in tumor cells, multiple studies have focused on and compared the protein structure, glycosylation, and proteolytic activity of cathepsin D in normal and cancer cells. It has been anticipated that cathepsin D can activate pro-cathepsin B

and/or degrade cystatins which triggers the proteolytic cascade leading to ECM degradation [61]. Nevertheless, many studies have highlighted the role of cathepsin D in promoting distant metastasis via an indirect mitogenic activity rather than stimulation of local invasion and extravasation [32]. The in vitro mitogenic potential of cathepsin D in MCF7 cells when cultured using purified pro-cathepsin D [56] is debated [62] but confirmed in vivo following cDNA transfection [32, 63]. Cathepsin D has been suggested to be an independent prognostic factor in women with stage I breast cancer [64].

Different molecular mechanisms have been put forward to explain the mitogenic activity of cathepsin D. Studies performed in human breast cancer cells have explained the mitogenicity of pro-cathepsin D. Cathepsin D acts as a competitor and interferes with the interaction between IGF2 (insulin-like growth factor) and M6P moieties of the M6P/IGF-2 receptor. As a result, IGF2 is displaced from the IGF1 receptor, leading to the activation of mitogenic IGF1 receptor pathway [57, 65]. Alternatively, it has also been proposed that the catalytic activity of secreted cathepsin D may be implicated in releasing growth factors, such as FGF2 (fibroblast growth factor), from the ECM [66]. In the case of ER (estrogen receptor) positive breast cancer, an increased number of large acid vesicles containing cathepsin D cause tumor invasion. Elevated level of cathepsin D in primary tumors is indicative of local recurrence or distant metastasis [67]. In ER-positive breast cancer, both estrogen and growth factors stimulate cathepsin D protein and mRNA accumulation [68–70]. Like other steroid-responsive genes, estrogen induced accumulation of cathepsin D mRNA is mainly due to increased initiation of transcription [70]. Since estrogen and growth factors stimulate the growth of ER-positive tumors, the induction of cathepsin D appears to be associated as well. Contrary to other estrogen-induced genes such as pS2, the progesterone receptor and wild type BrCa1, cathepsin D is also overexpressed in ER-negative breast cancer [68]. In triple-negative breast cancer cathepsin D is reported to be a key biological marker along with a high Ki-67 index [71]. It has been shown that cathepsin D is constitutively overexpressed in ER-negative breast cancer cell lines. The molecular mechanism behind overexpression of cathepsin D in ER-negative breast cancer cells may engage local reorganization of the chromatin structure of the cathepsin D promoter [72].

The prognostic significance of cathepsin D and optimal methodologies to measure cathepsin D in breast cancers is controversial. There exists ample evidence associating an elevated level of cathepsin D in tumor extracts with poor overall and relapse-free survival [73, 74]. Henry et al. reported that immunohistochemically assessed cathepsin D in tumor cells was associated with a favorable prognosis in node-positive patients [75]. In a separate study based on immunoassays and enzymatic assays, cathepsin D was also reported as a prognostic indicator for node-negative breast cancer patients [76]. In contrast, Isola et al. in a study involving 262 node-negative patients showed that tumor cell-associated cathepsin D expression was associated with a poor prognosis [77]. Another group suggested that cathepsin D determinations were not of prognostic value using immunoblotting and immunohistochemical methods [78]. After using the double antibody

immunoradiometric (IRMA) assay for measuring cathepsin levels, the same group concluded that cathepsin D is of doubtful value in predicting risk of early relapse or death for patients with newly diagnosed invasive breast cancer. Thus, the literature suggests that either cathepsin D is not a potent prognostic readout or the techniques for its optimal measurement has not been unambiguously defined.

5 Therapeutic Targeting of Cathepsins B and D in Breast Cancer: Panoply of Possibilities

The role of cathepsins in the progression and metastasis of cancer has been well documented. There is growing evidence that cathepsins may have a therapeutic potential for reducing the malignant progression of tumor cells and for treating some kinds of metastatic cancer because ablation or inhibition of cathepsins in tumor models decreased or delayed metastasis [27, 79, 80]. A range of therapeutic strategies has been employed to inhibit the proteolytic activity of cathepsins with the aim to restrain the metastatic infiltration mediated by them (Fig. 4). Among the strategies developed, chemical inhibitors, gene therapy, and use of cathepsin antibodies against cancer have shown promising developments.

5.1 Cathepsin Inhibitors

Each cathepsin has a different cleavage-bond specificity for substrate proteins, thus allowing for the development of cathepsin—specific inhibitors for targeting different family members [81]. These inhibitors are capable of selectively suppressing the cathepsin activity and inhibiting tumor growth. For example, some studies have reported the therapeutic efficacy of anti-cathepsin B and D in cancer [81, 82]. Zhang and colleagues showed for the first time that cystatin M significantly delayed breast tumor growth in mammary fat pads and lowered the metastatic burden at secondary

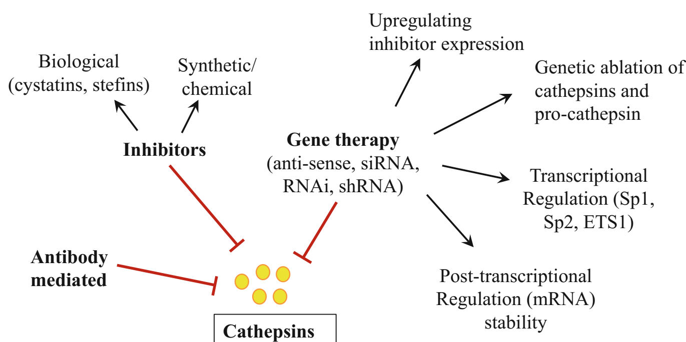

Fig. 4 Schematic representation of strategies employed to target cathepsins in breast cancer. *siRNA* small interfering RNA; *RNAi* RNA interference; *shRNA* short hairpin RNA; *Sp1* specificity protein 1; *ETS1* E26 transformation-specific 1

sites in SCID mice orthotopically implanted with breast cancer cells expressing cystatin M.

Small molecule inhibitors that target cathepsins B and D have been tested in vitro. Multiple in vitro experiments has reported that the inhibition of specific cysteine cathepsins results in a reduced invasion of tumor cells through matrigel or ECM in different types of cancers, including melanoma, glioblastoma, colon, prostate, and lung cancers [83, 84]. Studies have shown that both intracellular and extracellular inhibitions of cathepsins can inhibit invasion, suggesting that both the secretion of active enzyme and intracellular degradation pathways are important. Due to the extensive study of cathepsin B and D functions, and progress in the atomic understanding of cathepsin structure, a variety of inhibitors have been developed to combat these enzymes in breast cancer. However, even with different inhibitors being identified and developed, none of them has been proven as a successful therapeutic candidate.

One study conducted in nude mice reported that intraperitoneal administration of a highly selective cathepsin B inhibitor, such as CA-074, reduced the metastatic potential of breast cancer cells [80]. In another study, a novel approach termed activity-based protein profiling (ABPP) was developed to screen compound libraries for activity, inhibiting multiple cathepsins in defined tissues simultaneously [85]. This approach has highlighted a particular compound, ASM7, which has good activity against cathepsin B (IC50: 40–70 nM), and could be useful to reevaluate the available inhibitors for required specificity. Parker, et al. showed that expression of cystatin A may lead to the inhibition of cathepsin B, subsequently causing a reduction in distant metastasis in breast tumor cells. [86]. Another research group showed that depletion of cathepsin B was able to completely reverse the invasive phenotype of MCF7 cells and HER2-expressing SKBR-3 and MDA-MB-453 cells [40]. In addition, combining cathepsin inhibitors with other approaches may lead to better clinical strategies to treat breast cancers and metastasis. Shree, et al. demonstrated a tumor protective role of cathepsin B in breast tumors, and they showed that the use of compound JPM (research grade cathepsin B inhibitor) and the chemotherapeutic drug Taxol, significantly impaired tumor growth [87].

Nowadays, interest in the development of potent inhibitors of aspartic peptidases such as cathepsin D is increasing. Unlike other cathepsins (e.g. cysteine or serine), no mammalian endogenous lysosomal or cytoplasmic cathepsin D inhibitor has been identified, whereas the majority of exogenous cathepsin D inhibitors is synthetic compounds produced by microorganisms, plants and lower animals [88, 89]. Numerous studies are being conducted to discover novel cathepsin D inhibitors for the treatment of breast cancer. Earlier, in an attempt to develop cathepsin D inhibitors with desirable biological activity, McConnell et al. constructed a library of cathepsin D inhibitors with varying physical properties that improved their potency and half-life in vivo [90]. Anantaraju et al. performed an energy-based pharmacophore virtual screening and molecular docking studies to discover novel cathepsin D inhibitors [91]. These molecules were efficient to inhibit cell growth in the triple-positive and TNBC (triple-negative breast cancer) cell

lines. Recently, one group used the structure-activity relationship of a vectorized cathepsin D inhibitor, JMV4463, a pepstatin bioconjugate containing a vector composed of four AMPA (AMPA, ortho-aminomethylphenylacetyl) units, to inhibit the proteolysis of critical cathepsin D substrates involved in tumor cell proliferation [92]. Although many of the cathepsin D inhibitors will be limited in their therapeutic usefulness, with a large enough pool of active compounds it is possible that a few may someday prove to be drug candidates for the treatment of cancer. To date, cathepsin D inhibitors have not reached clinical trials, and there remains more to explore about their role and involvement in breast cancer chemotherapy.

5.2 Gene Therapy

Breast cancer is a very heterogeneous and multigenic disease clearly requiring new therapeutic strategies, targeting specific genes and proteins actively engaged in pathophysiology of breast cancer rather than conventional cytotoxic chemotherapy, which is often hampered by cytotoxicity or resistance. In order to combat this treatment barrier, attempts at specialized, targeted therapies are emerging. There are several prospective strategies using gene therapy, including: (a) expression of a gene to induce apoptosis or enhance tumor sensitivity to conventional drug/radiation therapy; (b) introduction of a tumor suppressor gene to compensate for its loss/deregulation; (c) blockade of an oncogene expression by using siRNA/shRNA approach; and (d) enhancement of tumor immunogenicity to stimulate immune cell recognition [93].

Besides, the use of chemical and biological inhibitors of cathepsin, a more advanced tool such as gene therapy is being used. The reduction in tumor growth, invasion, metastasis, and angiogenes due to either increased expression of the endogenous inhibitors of cysteine cathepsins or downregulated expression of cathepsin B lays the foundation for targeting cysteine cathepsins genes in cancer. Here we mention some of these genetic approaches carried out to downregulate or silence the expression of cathepsin B in various carcinomas, including breast cancer. Gene targeting strategies is being used towards cathepsin B expression either directly or indirectly through endogenous inhibitors (the cystatins and stefins) or associated transcription factors in human colon and prostate carcinomas, gliomas, and melanomas [94, 95]. In the literature, there are reports of genetic downmodulation of cathepsin B carried out in transgenic mice, however, studies employing gene-targeted approaches in human breast cancer are scarce. The antisense, siRNA, and shRNA technologies have been carried out in different transgenic murine models of carcinomas in order to downregulate the expression of cathepsin B. Studies have reported a reduction in motility and invasion of osteosarcoma cells due to antisense downregulation of cathepsin B, [96] and reduction in bone metastasis in a murine mammary carcinoma model due to shRNA downregulation of cathepsin B [80].

Disruption of cathepsin B gene production in the mouse model of mammary carcinoma (MMTV-PyMT) was found to delay the onset, reduce the growth of

primary mammary tumors and decrease the volume of the lung metastases [26]. Studies have aimed at the downregulation of cathepsin B function by increasing the expression of the endogenous inhibitor [24]. Alternatively, studies in which cystatin C and stefin/cystatin A were overexpressed in tumor cell lines demonstrated increased invasion and metastasis of tumor cells of epithelial and mesenchymal origins [97, 98]. In line with these reports, silencing cystatin M expression in a metastatic oral cancer cell line resulted in the inhibition of cysteine cathepsin activity, further causing an increase in the migration and invasion of cancerous cells [99]. Genetic ablation of cystatin C in the RIP1–Tag2 mouse model of pancreatic cancer has shown to increase the number and size of pre-malignant lesions, and tumor vascularity [100].

Different classes of proteases (cathepsins, MMPs, uPAR) interact with each other and take part in proteolytic pathway. Strategies targeting more than one class of proteases during tumor progression might prove to be more efficient than targeting a single cathepsin. Rao and colleagues carried out simultaneous downregulation of cathepsin B and MMP9 (34) and of cathepsin B and uPAR140 in SNB19 glioblastoma cells [101]. They reported that intratumoral injections of plasmid DNA expressing hairpin RNAs for these enzymes resulted in regression of pre-established tumors. Using a similar approach, Gondi and Rao showed that RNAi-mediated downregulation of cathepsin B and uPAR inhibited cell proliferation and initiated a partial extrinsic apoptotic cascade [102]. These studies demonstrate the effectiveness of RNAi-mediated downregulation of cathepsin B and other proteases in retarding tumor burden, invasion, and angiogenesis. It is evident that the downregulation of cathepsin B and other tumor-associated molecule simultaneously exhibit an additive effect when compared to the downregulation of cathepsin B alone.

Targeting the transcriptional regulation of cathepsins could be another promising approach. Transcription factors Sp1, Sp3, and those of the ETS family have been associated with the transcriptional regulation of cysteine cathepsins [24]. ETS1, known to be expressed in invasive tumors, regulates cathepsin B transcription. Therefore, exploiting transcriptional regulation of cathepsins might considerably decrease the expression of cathepsins in tumors and tumor-associated cells. However, clinically relevant and more robust studies need to be done to develop cathepsin B in the human breast cancer treatment.

Similarly, different approaches have been made to inhibit cathepsin D production or its action in breast cancer. Cathepsin D is associated with breast cancer progression, as well as it acts as a rate-limiting factor in stimulating in vitro and in vivo tumor growth. One study reported that reduced expression of cathepsin D in MDA-MB-231 breast cancer cells, by transfection of antisense cathepsin D cDNA, significantly decreased anchorage-independent growth, tumorigenicity, and lung colonization in nude mice as compared to mock-transfected cells [103]. An additional targeting strategy is based on inhibition of proteolytically inactive pro-forms of cathepsins. Overexpression of pro-cathepsin D, has been associated with highly invasive malignancies, including breast cancer. Various in vitro and animal studies have shown that the transfection of pro-cathepsin D affects the tumor cell

development, and that the rate of tumor growth was directly linked to the level of pro-cathepsin D expression [32, 104]. Vashishta et al. [58] tested the efficacy of the anti-pro-cathepsin D hammerhead ribozyme as a potential gene therapy agent in human breast cancer. Anti-pro-cathepsin D ribozyme used specifically to inhibit pro-cathepsin D in breast cancer cells not only inhibited invasion and growth but also induced apoptosis of MDA-MB-231 cells [58]. These findings reveal that cathepsin D is a potential candidate for new breast cancer therapy. The development of such novel therapies in addition to other existing therapies targeting genetic alteration in cancers might prove to be more efficacious in clinical setting and patient selection for this therapy would rely on the current assay for cathepsin D in primary breast tumors. Despite preclinical advancement with respect to both enhanced targeting and expression in a tumor-selective manner, multiple hurdles, including non-specific expression, low-efficiency delivery, and biosafety prevent the success of cathepsin and their inactive pro-forms in the clinic.

5.3 Antibody-Targeted Therapy

In the last decade, the use of antibody therapy in the field of oncology has shown very promising results [105]. One of the most promising and exciting fields in modern anti-cancer therapy involves the use of monoclonal antibodies which, once administered to the patient, will selectively and efficiently target a particular protein involved with the proliferation of tumor cells. Due to their high specificity, antibodies represent a promising method for interfering with a single target molecule, such as cathepsins, with high selectivity. Although research into neutralizing antibodies against protease targets is still budding, some examples of this drug class in preclinical and early clinical development are available. These include neutralizing antibodies against cathepsin B (Mab 2A2) [106] and uPA [107] for cancer treatment. Mab 2A2 was capable of impairing the intracellular and extracellular activities of cathepsin B and thus reducing the invasion of MCF-10A neo T cells (ras-transformed human breast epithelial cells). Significant inhibition of cell invasion by cathepsin B neutralizing Mab 2A2 suggests its application in antitumor therapy.

The involvement of pro-cathepsin D and cathepsin D in cancer progression is well documented in the literature, and their effects can be inhibited both in vitro and in vivo by antibodies targeting proenzyme or active enzymes. Breast cancer cells secrete enzymatically inactive pro-cathepsin D and the region of pro-cathepsin D responsible for its mitogenic activity was localized to its activation peptide corresponding to amino acids 27-44 [108]. The proteolytic cleavage of the activation peptide generates enzymatically active cathepsin D but it retains a portion of activation peptide [109]. There is data showing regression in tumor growth when the activation peptide is blocked by specific antibodies. One study demonstrated that using both anti-activation peptide and anti-27-44 peptide monoclonal antibodies inhibited its mitogenic potential and also inhibited the growth of human breast tumors in athymic nude mice [108]. Additionally, specific inhibition of

pro-cathepsin D by addition of anti-pro-cathepsin D antibodies specifically inhibited its mitogenic activities [110]. Blocking the activities of either the activation peptide of pro-enzymes or the active form of cathepsins by specific antibodies or analogs functioning as antagonists might be a valuable tool in breast cancer inhibition. With the emergence of new technologies, and as the detailed physiological function of cathepsins in cancer are revealed and updated, it should become easier to develop specific antibodies against them.

6 Conclusions

The broad influence of cathepsins B and D on various aspects of breast tumor development and progression proves them as an attractive target for breast cancer therapy. It is evident that targeting these cathepsins has significant therapeutic potential. From a translational perspective, the integration of cathepsin-targeting strategies into established chemotherapeutic regimens seems to hold substantial promise. However, more detailed studies are necessitated to determine if focussing these two cathepsins, may be useful in attenuating the progression of breast cancer. For cathepsins to emerge as a successful druggable target, a detailed knowledge is required about the tumor type, individual patient, and the course of tumor progression when anti-cathepsin therapies might be most effective. It still remains elusive whether the functions of cathepsins B and D change during the course of tumor progression; whether these enzymes come from tumor cells, or tumor-associated cells, or both; the mechanisms by which cathepsins interact with the tumor, and tumor microenvironment. In the next decade, we hope to achieve an integrated understanding of cathepsin activity in the tumor microenvironment and determine specific instances for which the reliance of cancers upon cathepsins represents a fatal, exploitable weakness. If cathepsins are to be targeted therapeutically, extensive studies are still needed to define the multiple and temporal roles of cathepsins B and D in breast cancer, including its cellular localization and interactions with other pathways contributing to malignancy.

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PAR1-Mediated Apoptosis and Tumor Regression of Breast Cancer Cells by *Vibrio cholerae* Hemagglutinin Protease

Tanusree Ray and Amit Pal

Abstract

Recent therapies of cancer have different side effects, so present scenario demands new therapeutic strategies. Bacterial toxins have been reported to show promising results in cancer treatment. *Vibrio cholerae* hemagglutinin protease (HAP) regresses tumor growth in Swiss albino mice by programmed cell death of mouse breast cancer cells. Treatment with HAP (one µg/week for three successive weeks) reduced solid tumor and enhanced survival of Ehrlich ascites carcinoma (EAC)-induced Swiss albino mice. A good therapeutic agent should specifically kill malignant cells without any effect on the survival of healthy normal cells. HAP induced PAR1 activation in mouse breast cancer cells (EAC). Overexpression of PAR1 has been reported in different malignant cells when compared to normal cells. HAP-induced PAR1 activated the downstream signaling pathways by nuclear translocation of p50-p65 and the phosphorylation of p38 which caused the activation of NFκB and MAP kinase pathways. The NFκB and MAP kinase activation enhanced the cellular ROS levels. The basal ROS level is reported to be higher in malignant cells as compared to normal healthy cells. Malignant cells cross the threshold level of ROS faster than the normal cells and switch on the cascades of cellular apoptosis. HAP-induced PAR1-mediated apoptosis of malignant cells without altering normal healthy cells makes it a good therapeutic agent for cancer treatment.

Keywords

HAP · Apoptosis · Tumor regression · PAR1 · NFκB · MAP kinase

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T. Ray · A. Pal (✉)
Division of Pathophysiology, National Institute of Cholera and Enteric Diseases,
P-33, CIT Road, Scheme-XM, Beliaghata, Kolkata 700010, India
e-mail: apal7364@rediffmail.com

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1 Introduction

Present-day cancer therapies affect both malignant and healthy cells. These therapies are also responsible for different side effects and may also develop drug resistance. Therapies that can kill specifically malignant cells, without damaging the healthy cells, can overcome these problems. Different targeted strategies are used in cancer therapy such as gene therapy, hormone therapy, inhibitors of signal transduction, and apoptosis inducers [1]. Malignant cells have strategies to bypass apoptosis [1, 2]. Apoptosis inducers destroy these strategies and kill malignant cells. Pediatric leukemia and certain solid tumors are diminished by induction of apoptosis by different drugs' treatments [3]. In a recent study, we have shown that *Vibrio cholerae* hemagglutinin protease (HAP) induced apoptosis in mouse breast cancer cells Ehrlich ascites carcinoma (EAC) by targeting specific PAR1 receptor.

Bacterial toxins have been used for cancer therapies. Bacterial toxins can control cell proliferation, differentiation, and apoptosis. These properties are associated with malignancies and inhibit normal cell controls. Bacterial toxins per se are reported to show its efficacy for cancer therapy [1]. *Clostridium perfringens* type A strain secretes Clostridium perfringens enterotoxin (CPE) [2, 3], which can kill pancreatic cancer cells and inhibit tumor growth in vivo [4–6]. Adenylate cyclase (AC) toxin from *Bordetella pertussis* induced apoptosis [7] in different malignant cells. Proteases play important roles in cellular processes and are widely related to different pathological conditions including cancer. Proteases are associated with cancer prognosis, cellular invasion, and metastasis [8]. A protease from *Serratia marcescens* kums 3958 showed antitumor property [9]. In our recent studies, we have shown that *V. cholerae* hemagglutinin protease (HAP) enhanced cellular ROS level and induced the intrinsic pathway of apoptosis and also regresses tumor growth in mice model [4]. HAP is a major protease in *V. cholerae*, and purified HAP showed cytotoxic effects on HeLa cells and also showed hemorrhagic fluid accumulation in rabbit ileal loop [10].

Protease signaling is mostly regulated by protease activated receptors (PARs). Protease signaling is initiated by the activation of PARs through the protease-mediated cleavage of PARs at its N-terminal domain [11]. Proteases have been reported to enhance tumor progression by activating the invasive property of malignant cell leading to metastasis [12]. However, recent studies also suggested its role in tumor regression [9]. PARs are the class of G-protein-coupled receptors (GPCRs) [13, 14]. There are four different subtypes of PARs, PAR1 – PAR4. The expression of PARs is generally higher in cancerous cells than in normal cells [15]. PARs are involved in both progression of cancer and apoptosis of cancerous cells depending on different stimuli [13, 14, 16].

PAR1-induced apoptosis has been reported in different neuronal, fibroblasts, endothelial and epithelial tumor cells. PAR1 expression is higher in breast cancer cells when compared to normal cells [17]. The role of PAR1-mediated cancer

progression and its therapeutic property in cancer prevention are still not clear. The functions of microbial proteases are diverse, and they are involved as antimicrobial peptides [18], destruction of the protective mucosal barrier [19], and in apoptosis of target cells [20].

Vibrio cholerae hemagglutinin protease (HAP) caused PAR1 activation and triggered its downstream NFκB and MAP kinase signaling pathways. These pathways enhanced cellular ROS level and induced apoptosis. These strategies of HAP make it a good therapeutic precursor for cancer therapy [21].

2 HAP Induces Intrinsic Apoptotic Pathway in Malignant Cells and Causes Tumor Regression in Murine Model

2.1 Purified HAP Induces Intrinsic Pathway of Apoptosis in Malignant Cells Without Effecting the Normal Healthy Cells

In presence of HAP, different malignant cells like EAC and RAW 264.7 cells show apoptotic response (Fig. 1a–d). 1 μg/ml of purified HAP induced apoptotic response in EAC and RAW 264.7 cells, but interestingly, this dose of HAP is unable to induce apoptotic response in normal mice peritoneum macrophages (Fig. 1e, f). HAP induced overexpression of p53. Moreover, it increased the ratio of proapoptotic Bax to antiapoptotic Bcl-2 in EAC cells. HAP treatment results in activation of caspase 3 and 9 with significant increase in cytochrome c level in mitochondria-free cytosolic fraction of HAP-treated cells. HAP induces intrinsic apoptotic pathway in mouse breast cancer cells (Fig. 1g, h).

2.2 HAP Treatment Regresses Solid Tumor in Mice

Solid tumor was developed subcutaneously at the right thigh muscle by injecting 2×10^6 number of EAC cells. Once the solid tumor reaches 15 mm in diameter (1.76 cc), 1 μg of HAP was inoculated into the tumor site. After three successive weeks of treatment with 1 μg of HAP, the tumor size diminished significantly, whereas in positive control group, the tumor size increased from 15 to 35 mm in 60 days (Fig. 2a). HAP treatment significantly decreased the tumor volume. In positive control group, the tumor volume increased significantly from 1.76 cc (at 0 day) to 14.75 cc (at 60 days). In the HAP-treated group, the tumor volume decreased from 1.76 to 0 cc at 30 days (Fig. 2b). When HAP was inhibited with EDTA, it failed to diminish tumor volume. Similar effect was observed when only buffer was used instead of HAP (Fig. 2a, b).

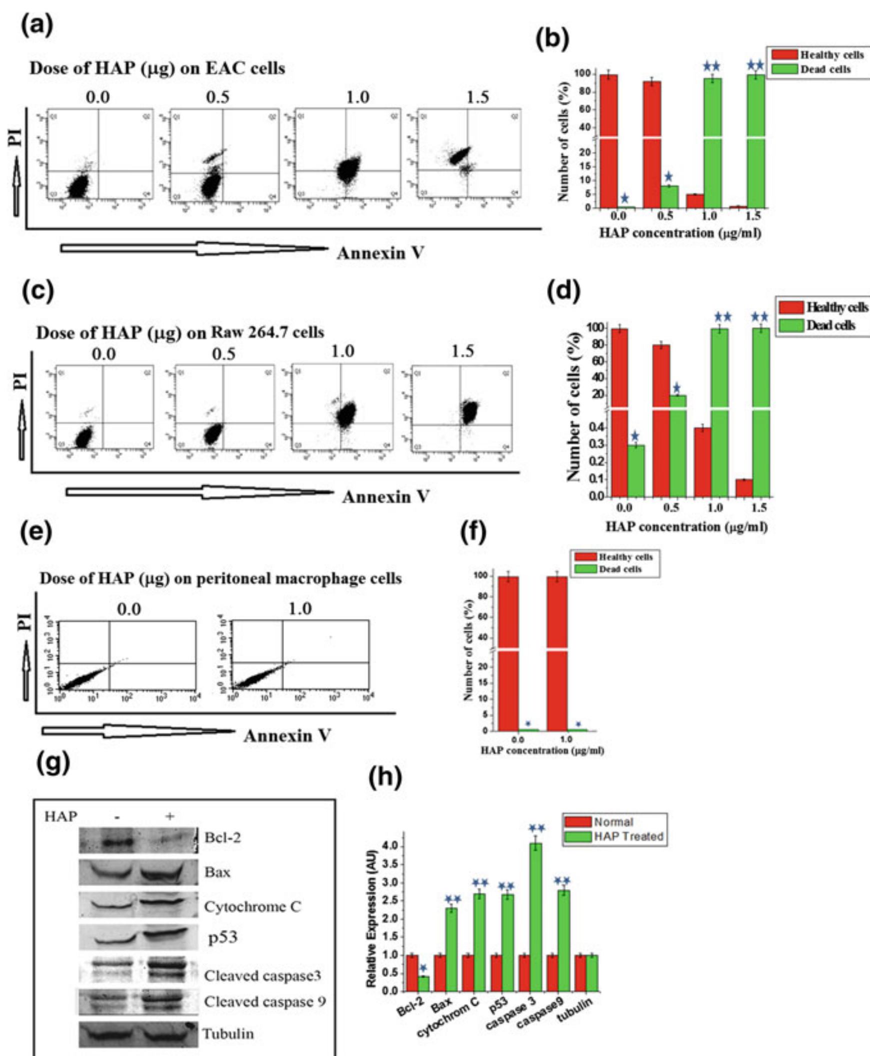

Fig. 1 HAP induced intrinsic pathway of apoptosis in mice macrophages and breast cancer cells. **a**, **c**, **e** Dose-dependent response of HAP on EAC, RAW 264.7, and mouse peritoneal macrophage cells analyzed by FACS to study cellular apoptosis. **b**, **d**, **f** The above results are graphically represented in the bar diagram. In all panels, $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$. **g** Western blot and **h** densitometric analysis on expression levels of different proteins of apoptotic pathway in HAP-induced and HAP-uninduced cells

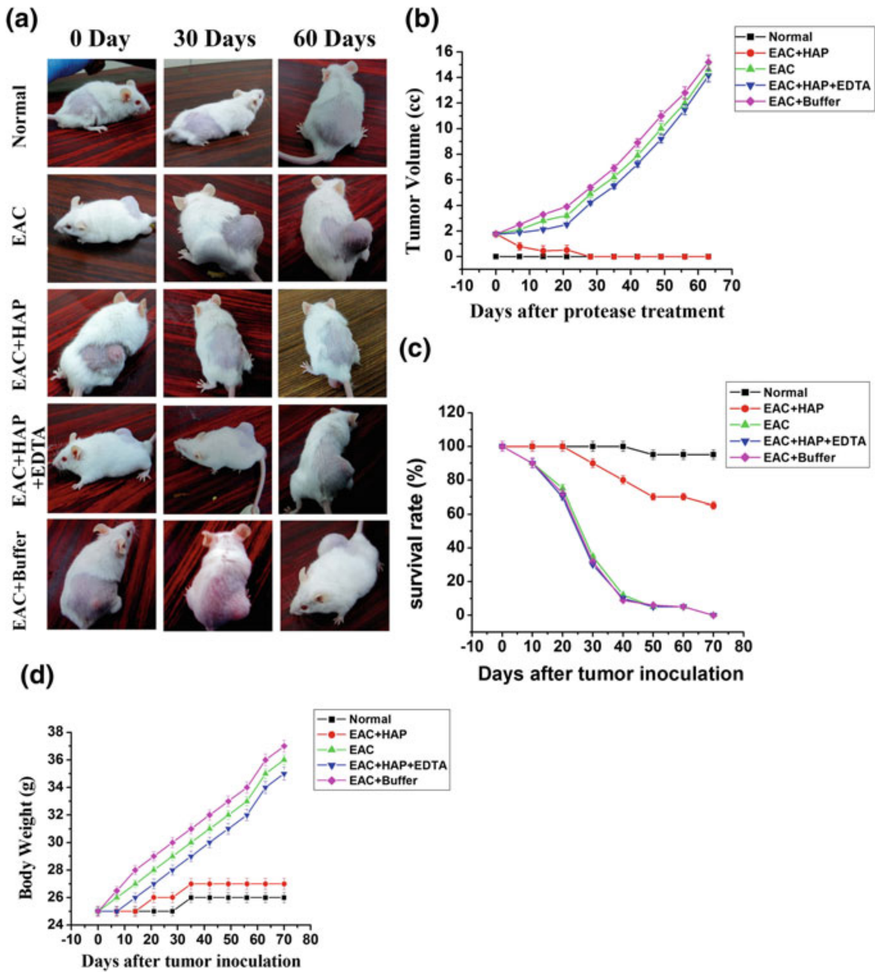

Fig. 2 Effect of HAP on EAC-induced solid tumor and intraperitoneum mice model. **a** Antitumor effect of HAP on EAC-induced solid tumor. The 0 day indicates tumor size of 15 mm in diameter which is diminished after HAP treatment. Tumor control group, buffer control group, and EDTA mediated HAP inhibited group showed significant increase in tumor size. **b** The above results are graphically represented showing the reduction in tumor volume with respect to time. HAP in presence of EDTA and only buffer showed no effect on tumor regression. **c** Effect of HAP in EAC-induced intraperitoneum mice model showed significant increase in survival rate. HAP in presence of EDTA and only buffer showed similar survival rate as tumor control group. **d** Effect of HAP in EAC-induced intraperitoneum mice model showed no significant increase in body weight. Tumor control group and EDTA treated in presence of HAP showed increase in body weight. In each panel, error bars were calculated based on results obtained from minimum of three independent experiments

2.3 HAP Increased the Survival of EAC-Induced Mice

The survival kinetics and the changes in body weight were observed in intraperitoneal EAC-induced mice. Tumor was developed with 10^6 numbers of EAC cells. In the tumor control group, the survival rate of mice was decreased to 30% and then to 5% after 30 and 60 days, respectively. HAP treatment (1 μg HAP/weekly for successive 3 weeks) maintained the survival rate of mice to 90 and 70% after 30 and 60 days, respectively (Fig. 2c). The body weight of tumor control group increased with time (from 25 g at 0 day to 45 g at 60 days), and HAP-treated EAC-induced mice did not show significant changes in body weight (Fig. 2d). Inactivated HAP (by EDTA) treatment and the buffer (25 mM Tris, pH 7.4) control group failed to increase the survival rate of mice (Fig. 2c) and also showed increase in body weight (Fig. 2d). These results proved that the protease activity of HAP is responsible for its antitumor effect.

3 Effect of HAP on Liver, Kidney, and Thigh Muscle of Intraperitoneally EAC-Induced Mice

Histological examination (using hematoxylin-eosin staining) revealed healthy cellular structure of liver, kidney, and thigh muscle fibers. However, in tumor control group, damaged and destroyed cell morphology was found in liver, kidney, and thigh muscles. HAP treatment recovered this damage and helped to regain healthy tissue structure in liver, kidney, and thigh muscle (Fig. 3a).

Presence of apoptosis or necrotic cells was identified by ethidium bromide (EB) and acridine orange (AO) staining of thigh, liver, and kidney tissues of normal, tumor-bearing, and HAP-treated animals. The presence of EB-positive apoptotic or necrotic cells was highly present in tumor-bearing animals as compared to normal and HAP-treated animals (Fig. 3b).

Ki-67 is a nuclear protein that is present in the nucleus of all stages of proliferating cells except G_0 stage and dead cells. The absence of Ki-67 in EAC control group indicates the measure of thigh, liver, and kidney damage. The presence of Ki-67 in the nucleus of normal and HAP-treated cells proved the recovery of damaged cells after HAP treatment (Fig. 3c).

The liver function was tested by the measuring of ALT and AST. Kidney functionality was measured by urea and creatinine levels in the serum sample of animals. The level of ALT and AST was increased significantly, and urea and creatinine levels were significantly decreased in the tumor control group compared to the normal group. There was no significant change in urea, creatinine, ALT and AST levels in HAP-treated group as compared to the normal group (Fig. 3d).

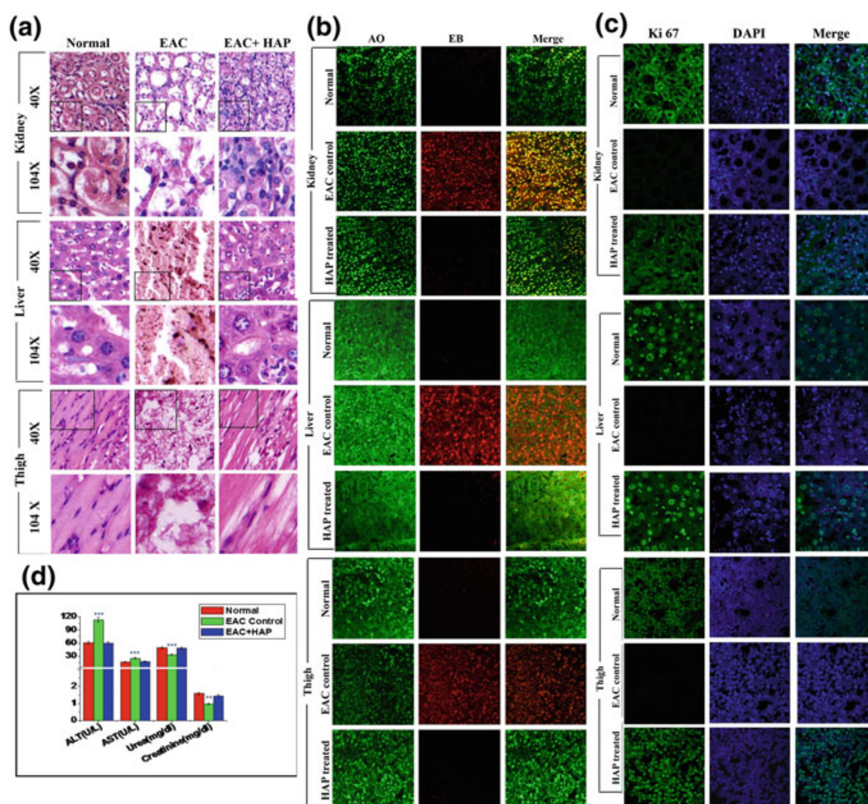

Fig. 3 Effect of HAP on histological and biochemical parameters of tumor induced mice. **a** Histological studies by H&E staining showed that treatment with purified HAP improved the damaged architecture of liver, kidney, and thigh muscle caused by tumor induction. **b** AO and EB staining showed that treatment with purified HAP improved the cell viability in liver, kidney, and thigh muscle caused by tumor induction. **c** Immunohistochemistry of Ki-67 indicated that HAP treatment caused cellular proliferation of damaged liver, kidney, and thigh muscle. **d** Biochemical tests of liver and kidney functionality (ALT, AST, urea and creatinine) indicated the recovery of liver and kidney cells from EAC-mediated damage after HAP treatment

4 Molecular Mechanism of HAP Induced Apoptosis of Malignant Cells

4.1 HAP Causes PAR1 Activation and Induces Downstream Signaling of NFκB and MAP Kinase Pathway

Real-time PCR revealed 30-fold overexpression of PAR1 RNA level as compared to the other PARs (Fig. 4a, c) in RAW 264.7 (Fig. 4a) and in EAC cells (Fig. 4c) after 1 h treatment with 1 μg/ml of HAP. HAP treatment did not cause any

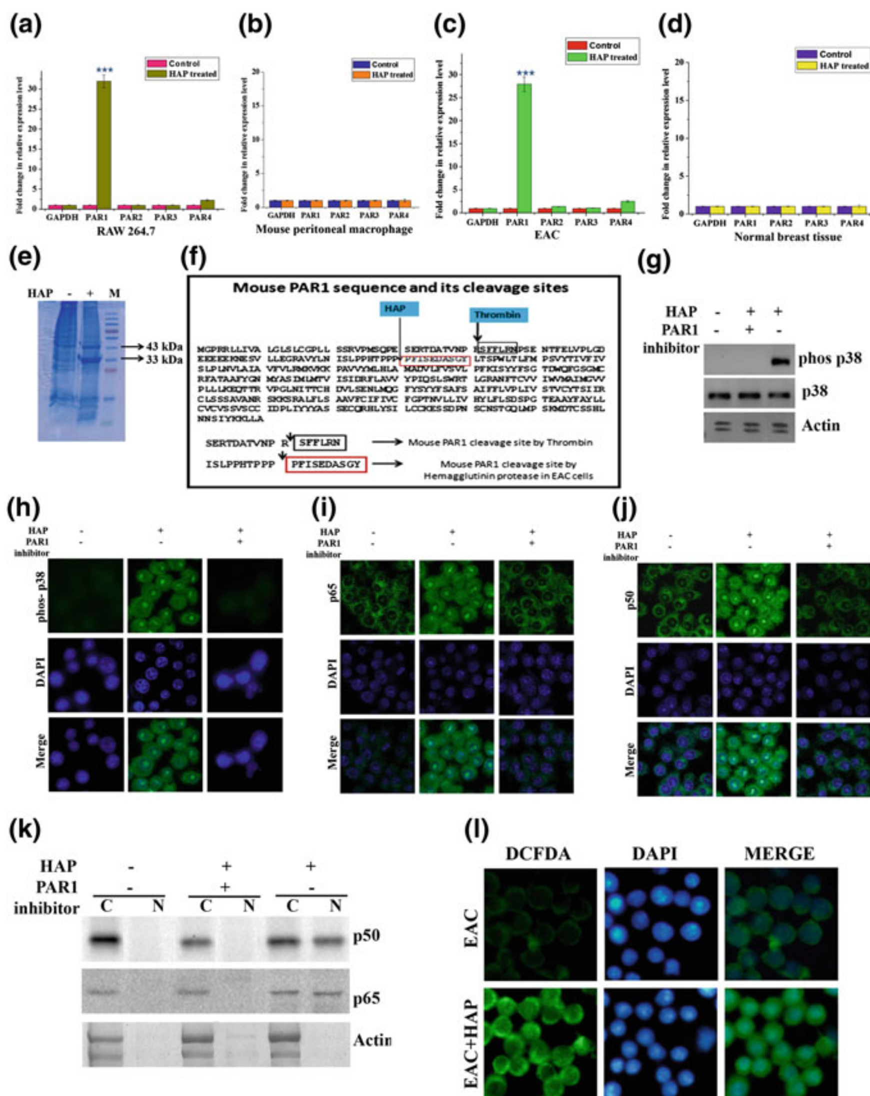

Fig. 4 HAP-induced PAR1 activation causes downstream signaling of NFκB and MAP kinase pathway. **a**, **c** Real-time PCR showed almost 30-fold overexpression of PAR1 RNA compared to the other PARs in RAW 264.7 and EAC cells upon HAP treatment. **b**, **d** There was no significant change in PARs expression level in mouse peritoneal macrophage cells and in normal breast tissues on HAP treatment. **e** HAP treatment causes overexpression of 43 and 33 kDa protein bands in EAC cells compared to untreated cells. **f** N-terminal sequencing of both the bands revealed homology with PAR1. The N-terminal sequence of the cleaved 33 kDa band is "PFISEDASGY". **h**–**j** Immunofluorescence assay revealed that HAP-mediated p38 phosphorylation and the nuclear translocation of p50 and p65 were inhibited by PAR1 inhibitor. **g**, **k** Western blot analysis confirmed the inhibition of HAP-mediated phosphorylation of p38 and the nuclear translocation of p50 and p65 by PAR1 inhibitor. **l** DCFDA staining observed under fluorescence showed that HAP increased the cellular ROS level in EAC cells

alteration of PARs' expression level in mouse peritoneal macrophage cells (Fig. 4b) and in normal mouse breast tissue (Fig. 4d). PAR1 activation has been well studied with thrombin, and it has been shown to induce tumorigenesis and metastasis. In our study, we have found HAP causes PAR1 activation. HAP treatment caused overexpression of 43 and 33 kDa protein bands when compared to normal EAC cells (Fig. 4e). N-terminal sequencing of 43 and 33 kDa bands showed homology with PAR1, whereas the 33 kDa band showed a new N-terminal sequence "PFI-SEDASGY" (Fig. 4f).

HAP causes NFκB activation by the nuclear translocation of p65 and p50. HAP also induces MAP kinase pathways by phosphorylation of p38. Use of PAR 1 inhibitor blocked both the pathways, which indicates HAP-mediated PAR1 activation induces NFkB (Fig. 4i–k) and MAP kinase pathway (Fig. 4g, h). Several evidences showed that p38 and NFκB enhance cellular ROS level and induced intrinsic apoptotic pathway [22].

4.2 HAP Treatment Increased Cellular ROS Level that Reduced the Viability of Malignant Cells

Cellular ROS level is higher in malignant cells when compared to normal cells [23, 24]. HAP treatment enhanced cellular ROS level, and as a result after HAP treatment, malignant cells crossed their threshold ROS level faster than the normal cells. Increased ROS level induced apoptosis [25, 26]. DCFDA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative DCFH and is thereby trapped within the cells. In the presence of ROS, DCFH is oxidized to highly fluorescent DCF. DCFDA staining showed that HAP treatment enhanced ROS level in EAC cells compared to untreated cells and caused apoptosis (Fig. 4l).

5 Discussion

Breast cancer is one of the most common malignancies in females [27]. Present-day therapeutic strategies have several flaws for complete remission of the disease [28, 29]. The use of targeted inhibitor of oncogenic proteins and cytotoxic agents has increased the rate of success of cancer treatment [30, 31].

Malignant cells divide faster than normal cells, and chemotherapeutic drugs target these cells. In this process, they also kill proliferating healthy cells. Natural products isolated from plants, marine organism and microorganism, are reported to show anticancer activity [32, 33]. *V. cholerae* hemagglutinin protease showed anticancer property by inducing intrinsic apoptotic pathway in malignant cells. It diminished the solid tumor in EAC-induced mice model and enhanced the life span of the cancerous mice. Protease from *S. marcescens* kums 3958 also showed antitumor activity [9], and its degenerating effect of tumor tissue was due to its

proteolytic activity [34]. HAP is required in a very low dose to show its antitumor activity. 1 μg/ml of HAP induced apoptosis in malignant cells. HAP treatment caused overexpression of proapoptotic Bax and downregulation of antiapoptotic Bcl-2. The release of cytochrome c into the cytosol from the mitochondria and the activation of caspase 9 along with caspase 3 suggest that HAP triggers activation of intrinsic pathway of apoptosis [35, 36].

EAC cells are widely used for tumor development and to study the anticancer activity of compounds in vivo [37, 38]. HAP treatment significantly diminished the tumor size in mice and increased the survival rate (70%) of the mice when compared to the tumor control group. HAP treatment affected the viability of malignant cells. The histological studies revealed that HAP treatment improved the cellular morphology and architecture of tissues in HAP-treated group compared to tumor-bearing group, suggesting regression of tumor in mice models.

Cellular ROS level of cancerous cells is higher than normal cells [23, 24]. HAP treatment increased cellular ROS level in EAC-induced mice; as a result, malignant EAC cells crossed the threshold level of cellular ROS earlier than normal cells. Increased ROS initiates the process of apoptosis [25, 26]. Due to generation of high ROS, there is a change in mitochondrial membrane potential [39] resulting in the release of cytochrome c into the cytosol [40, 41]. The ratio of proapoptotic Bax to antiapoptotic Bcl-2 is increased leading to the activation of caspase 9 and caspase 3. HAP treatment activates the intrinsic pathway of apoptosis of EAC cells.

Protease signaling is regulated via the activation of protease activated receptors (PARs). PARs function in cell proliferation, metastasis, and apoptosis depending on different stimuli. PARs are G-coupled receptor. PAR1 is reported to play a role in metastatic process of different cancers [42–45]. Thrombin is a well-known activator of PAR1 [46, 47]. PAR1 becomes activated when thrombin cleaves a specific site on the N-terminal domain (R41-S42). Synthetic peptides that are derived from the first few amino acids of thrombin-mediated cleaved N-terminus (SFLLRN) can act as intramolecular ligand of PAR1.

HAP induced PAR1-mediated apoptosis of EAC cells. HAP cleaved the N-terminal sequence of PAR1 at a novel site. The sequence of this novel N-terminal cleaved site is “PFISEDASGY”. The role of this peptide to induce the downstream signaling of PAR1 is still unknown.

Thrombin-mediated PAR1 activation has been well studied [48]. PAR1 activation-induced different downstream signaling pathways such as PI3K [49], protein kinase C [50], c-Jun N-terminal kinase (JNK) [51], p38 MAPK [52], RhoGTPase [53], and nuclear factor NFκB [54] have been reported. HAP enhanced ROS level that activates the intrinsic apoptotic pathway [55] in EAC cells.

This is for the first time we have shown that *V. cholerae* hemagglutinin protease (HAP) induced apoptosis by PAR1 activation in cancer cells. HAP causes PAR1 activation that induces NFκB and MAP kinase signaling. These pathways enhance cellular ROS level that activates the intrinsic apoptotic pathways (Fig. 5). PAR1 expression in normal cell is lower than that in the malignant cells [48]. Thus, HAP is considered as a good therapeutic agent to specifically kill malignant cells, whereas the normal healthy cells remain unaltered in the same environment.

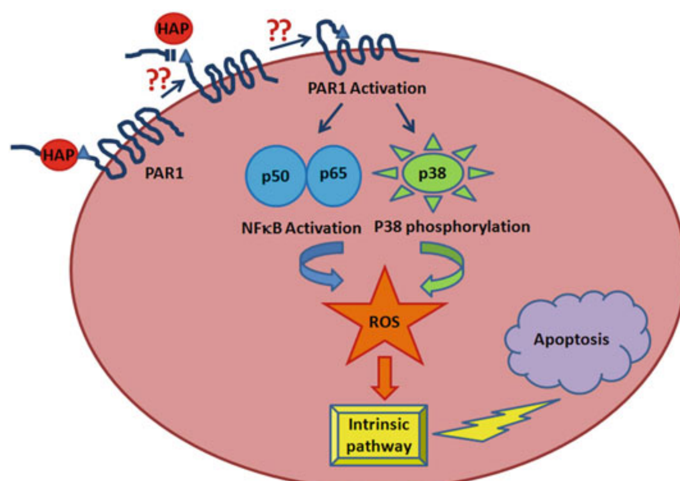

Fig. 5 HAP induced PAR1-mediated apoptosis of breast cancer cells by activation of NFκB and MAP kinase pathways. HAP induced PAR1 cleavage and activation, though the exact "tethered ligand" that binds intramolecularly to trigger transmembrane signaling is unknown but this receptor activation induced phosphorylation of p38 and nuclear translocation of p50 and p65 in breast cancer cells. The activation of MAP kinase and NFκB signaling enhance the cellular ROS level and triggered the intrinsic pathways of apoptosis

6 Conclusion

Conventional tumor therapies are relatively non-specific and show dose-limiting side effects and also develop drug resistance. Several proteases and peptides have been reported to have antitumor activity [56–58]. For being a good therapeutic agent, it should kill malignant cells specifically without altering the survival of normal healthy cells. This is possible by targeted strategies that specifically disrupt oncogenically active cell surface receptors and endogenous signaling molecules [42]. Protease signaling is mostly regulated via the activation of protease-activated receptors (PARs). PARs showed the property for cell proliferation and apoptosis of malignant cells depending on stimuli [15, 45]. In our earlier study, *V. cholerae* hemagglutinin protease (HAP) showed PAR1 induced apoptosis via MAP kinase and NFκB pathways [21]. We have found HAP induced a new PAR1 cleavage site "PFISEDASGY" but the exact "tethered ligand" that interacts with the receptor itself and induces downstream signaling is still unknown. Since HAP-mediated PAR1 activation caused apoptosis in different mouse malignant cells, so we hypothesized that the "tethered ligand" sequence will provide a novel proapoptotic peptide that may be able to induce PAR1-mediated apoptosis. Due to the advancement in the large-scale synthesis of peptides, it will be possible to make peptide-based anticancer drugs more affordable to patients, though this technique needs more practice and research. We are hopeful that this peptide will kill

malignant cells without altering the survival of normal healthy cells via targeting PAR1 that is known to be overexpressed in malignant cells. This proapoptotic peptide can be used for an alternate strategy for cancer therapy.

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Matrix Metalloproteinases (MMPs) in Cancer Initiation and Progression

Sanjeev Kumar Maurya, Nitesh Poddar, Pallavi Tandon and Ajit Kumar Yadav

Abstract

Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing peptidehydrolase, which are actively involved in degradation of the extracellular matrix (ECM), organ development, and tissue remodeling to maintain homeostasis of tissue. Through degradation of ECM in tumor, MMPs provide fundamental base for further tumor cell metastasis. The complex constitution of tumor microenvironment permits various types of regulatory mechanism and expression of cascades of MMPs. Through which various functions of MMPs can be determined. The physiological role of MMP enzymes can be determined by their location and time frame of its activity during tumor progression. According to the recent studies which have revealed the diverse functions of MMPs other than ECM degradation, MMPs are known to play a major role in regulation of many signaling pathways. Their participation in such pathways helps in altering cell physiology as well as in combating disease. MMPs regulate initiation of apoptosis in tumor cells through cleavage of ligands or receptors. There are evidences which support MMPs role in angiogenic and lymph-angiogenic processes. Most of the studies suggest the major involvement of MMP-2, MMP-9, and MMP-14 in tumor angiogenesis, and to a smaller extent, MMP-1 and MMP-7 are also known to be involved. MMPs also play a prominent role in generation of growth signals, apoptosis regulation, tumor vasculature, initiation of neoplastic progression, invasion and metastasis, metastatic niche formation, and MMPs orchestrate inflammation in cancer. Some other non-proteolytic

S.K. Maurya (✉) · N. Poddar · P. Tandon
Department of Biotechnology, Invertis University, Bareilly, Uttar Pradesh, India
e-mail: sanjeev.m@invertis.org

A.K. Yadav
Department of Pharmacy, Invertis University, Bareilly, Uttar Pradesh, India

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functions of MMPs are also important to be considered in cancer. Wide range of MMPs role in cancer initiation and progression also provides wide range of therapeutic opportunity for cancer treatment.

Keywords
Matrix metalloproteinase · Oncogenesis · Vasculature · Angiogenesis
Tumor growth · Metastasis · Invasion

1 Introduction

Wide range of physiological, biochemical pathways and genes is involved in maintenance of normal physiology of the cell. Undesirable alterations in vital genes, which control important metabolic/physiologic pathways, in cell lead to understand growth of tissue cells [1]. The cell microenvironment is analogous to the inflammatory response, which reassures angiogenesis, alterations of the extracellular matrix (ECM), and tumor cell mobilizations [2]. Knowing the complex interplay among malignant cancer cells and the adjoining stroma, the molecular mechanisms represent some of the major challenges in cancer research. The recent studies highlight the role of extracellular proteinases, such as matrix metalloproteinases (MMPs) that facilitate changes in the microenvironment of tumor cells during cancer progression. These enzymes control arrange of physiological processes and signaling pathways, and thus they act as great mediators in molecular communication between tumor and stroma. Concerning the failure of MMP inhibitors as targets for anticancer therapy in different clinical trials, development of the new understandings into the roles of these extracellular proteinases in cancer, which depends on the circumstances, may either conqueror endorse tumorigenesis or even act freely of their proteolytic action. In Fig. 1, the interest in MMPs research continually increases with better understanding and knowledge about its potential for diagnosis and therapy in different type of tumors.

In this book chapter, we collected literatures from current developments and studies regarding MMPs and its role in tumor initiation and progression. This chapter will help to answer various questions that are very relevant to understand the role of MMPs in special reference to cancer. What are characteristics of MMPs family? What are the mechanisms of MMPs activity? How they play a role in cancer initiation, progression, growth signaling, apoptosis regulation, tumor vasculature, neoplastic progression, tissue invasion and metastasis, metastatic niche formation, inflammatory response, and other non-proteolytic function? These questions when answered with all available experimental and literature data will increase our understanding about the role of MMPs and its utilization for treatment of cancer.

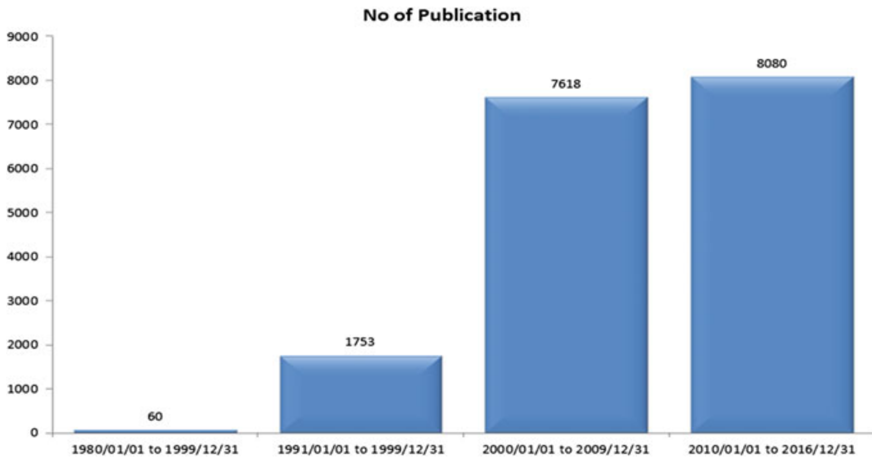

Fig. 1 No. of publication: initially, the scientific interest in MMPs was low but as potential of MMPs discovered MMPs research shoot-up

2 Introduction About MMPs and Its Family

MMPs are a member of zinc-dependent endopeptidases family, firstly defined nearly half a century ago [3] in amphibian during metamorphosis. In the face of intensive study in vitro, in cell culture and in animal models, the standard physiological roles of these extracellular proteases have been identified. They play a crucial role in numerous physiological courses including tissue remodeling and organ development [4], in the regulation of inflammatory processes [5], act on pro-inflammatory cytokines and chemokines, in degradation of the extracellular matrix and in diseases such as cancer [6] (detailed classification described in Fig. 2).

Till now 26 human MMPs are known. On the basis of their structure and specificity, these MMPs are classified into five classes: collagenases, gelatinases, stromelysins, matrilysins, and membrane-type (MT-MMPs) (described in Table 1).

The broad structural outline of MMPs illustrates three domains which are common to nearly all MMPs, the N-terminal pro-domain, the catalytic domain, and the hemopexin-like domain at C-terminal which is connected via a flexible hinge region to the catalytic domain. The membrane-type MMPs (MT-MMPs) combine with a supplementary transmembrane domain that anchors the main cell surface plasma membrane. Pro-MMPs are primarily inactive state because of structural protection by cysteine amino acid present on pro-domain with the zinc ion of the catalytic site. After digestion of this interface through cysteines which are typically arbitrated by proteolytic elimination of the pro-domain cysteine residue. After this modification, the enzyme gets converted into proteolytically active enzyme. Sequence-specific cleavage by convertases occurs in the pro-domain that contains a

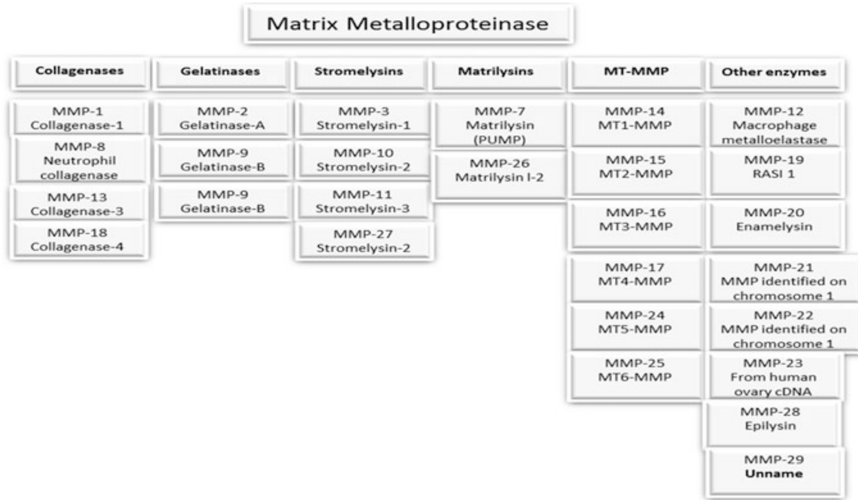

Fig. 2 Classification of MMPs: MMPs are classified in 6 groups depending on their mechanism of action, preferred substrate, and structure

Table 1 The matrix metalloproteinase family: different class of MMPs and their natural substrate in or out of the cells in a tissue

Enzymes	MMPs	Position	Primary substrate
Interstitial collagenase	MMP1	Secreted	Substrates include Col I, II, III, VII, VIII, X, gelatin
Gelatinase-A, 72 kDa gelatinase	MMP2	Secreted	Substrates include gelatin, Col I, II, III, IV, VII, X
Stromelysin 1	MMP3	Secreted	Substrates include Col II, IV, IX, X, XI, gelatin
Matrilysin, PUMP 1	MMP7	Secreted	Membrane associated through binding to cholesterol sulfate in cell membranes, substrates include: fibronectin, laminin, Col IV, gelatin
Neutrophil collagenase	MMP8	Secreted	Substrates include Col I, II, III, VII, VIII, X, aggrecan, gelatin
Gelatinase-B, 92 kDa gelatinase	MMP9	Secreted	Substrates include Gelatin, Col IV, V
Stromelysin 2	MMP10	Secreted	Substrates include Col IV, laminin, fibronectin, elastin
Stromelysin 3	MMP11	Secreted	MMP-11 shows more similarity to the MT-MMPs, is convertase-activatable and is secreted therefore usually associated to convertase-activatable MMPs. Substrates include Col IV, fibronectin, laminin, aggrecan

(continued)

Table 1 (continued)

Enzymes	MMPs	Position	Primary substrate
Macrophage metalloelastase	MMP12	Secreted	Substrates include elastin, fibronectin, Col IV
Collagenase 3	MMP13	Secreted	Substrates include Col I, II, III, IV, IX, X, XIV, gelatin
MT1-MMP	MMP14	Membrane-bound	Type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin
MT2-MMP	MMP15	Membrane-bound	Type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin
MT3-MMP	MMP16	Membrane-bound	Type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin
MT4-MMP	MMP17	Membrane-bound	Glycosyl phosphatidylinositol-attached; substrates include fibrinogen, fibrin
Collagenase 4, xcol4, xenopuscollagenase	MMP18	Not known	Not known
RASI-1, occasionally referred to as stromelysin-4	MMP19	Not known	Not known
Enamelysin	MMP20	Secreted	Not known
X-MMP	MMP21	Secreted	Not known
CA-MMP	MMP23A and B	Membrane-bound	Type-II transmembrane cysteine array
MT5-MMP	MMP24	Membrane-bound	Type-I transmembrane MMP
MT6-MMP	MMP25	Membrane-bound	Glycosyl phosphatidylinositol-attached
Matrilysin-2, endometase	MMP26	Secreted	
MMP-22, C-MMP	MMP27	Secreted	
Epilysin	MMP28	secreted	Discovered in 2001 and given its name due to have been discovered in human keratinocytes. Unlike other MMPs this enzyme is constitutively expressed in testis and at lower levels in lung, heart, brain, colon, intestine, placenta, salivary glands, uterus, skin.

consensus sequence, in which is degraded intracellularly by furin or extracellularly by different MMPs or serine proteinases, for example, plasmin [7, 8]. Some proteins functionally correlated with MMPs are termed as a disintegrin and metalloproteinase (ADAM) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families of metzincin proteinases. ADAMs play an important role in fertilization, development, and tumor [9, 10]. Most ADAMs are membrane-bound and perform their functions in the pericellular space. Even though all of the ADAMs has a metalloproteinase domain, but only half of them display

proteolytic activity, signifying that ADAMs perform their role by shedding connection accomplices or by interceding the organic capacity in a non-proteolytic manner. The ADAMTS enzymes consist of a protease domain, disintegrin domain, and one or more than one thrombospondin domains [11].

ADAMTS enzymes are generally secreted and are soluble in nature. These enzymes perform central roles in ECM arrangement with other members, ovulation, and tumor [12]. The physiological function of MMPs is based on the nearby adjust among different MMPs and their physiological inhibitors. Considerable amount of energy reserves of the human body are assigned for the inhibition of unregulated extracellular proteolysis by MMPs and different proteinases. For instance, high centralizations of the proteinase inhibitors α2-macroglobulin (α2-MG), α1-proteinase-inhibitor (α1-PI), and α1-chymotrypsin (α1-CT) are combined in the liver and sent into the blood, where these predicaments to the dynamic site of various proteinases [13–15]. The resultant proteinase-inhibitor complexes are targeted to a scavenger receptor and are finally engulfed by macrophages. Most significant physiological inhibitors of MMP are tissue inhibitors of metalloproteinases (TIMPs), which are moreover differentially expressed in tumor cells, and stromal cells [16–18]. TIMP-1, TIMP-2, TIMP-3, and TIMP-4 form 1:1 stochiometric buildings with dynamic MMPs prompting hindrance of proteolytic action. Like MMPs, the proteolytic ADAM and ADAMTS relatives are additionally restrained by particular TIMPs [19].

The outflow of metalloproteinases and their inhibitors in the tumor smaller scale condition are stopped adversely (Fig. 3). Although different cancer cells/tissues express various types of MMPs, ADAMs, and TIMPs family, the major sources of these proteinases are the stromal cells infiltrating the cancer [20]. The various types

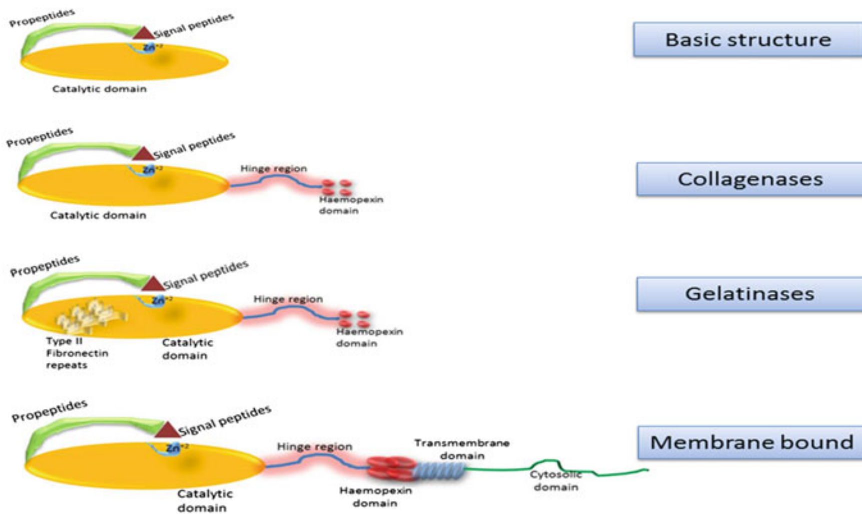

Fig. 3 General structure of MMPs

of stromal cells secrete a particular pair of proteinases and proteinases inhibitors, which are finally discharged into the extracellular space, and help to change the microenvironment around the tumor cells. The MMPs function and activity potential may be governed by their cellular sources, for example, infiltrating neutrophil derived MMP-9 is more readily active in contrast to other cell types [21].

3 Leading MMP Activity

The complex constitution of cancer microenvironment permits various types of regulatory mechanism as well as expression of cascades of MMPs which help us to decide their numerous roles. MMPs activity, proteolysis can be regulated at various levels like compartmentalization, transformation from proactive zymogen to fully active functional catalyst, and at last the existence of precise inhibitors. The most crucial step of MMPs activity is the conversion of inactive enzymes, known as pro-enzymes such as zymogen into a regulatory proteolytic enzyme. Though the understanding of MMPs physiological activity is increasing at a good pace, but still we require a lot of experimental data supported by factual data to get clear-cut knowledge of MMPs activation as well as its regulatory mechanism. There are numerous proteinases that facilitate MMP activation such as plasmin, furin, and other active MMPs [22–24]. Inflammatory cells releasing hyperactive proteinases causing significant injuries in surrounding tissues [5]. For instance, MMPs also degrade plasmin-suppressing serpin proteinases inhibitors. This initiates the changeover of pro-MMPs into active MMPs. Such as, α2-AP is inactivated by MMP-3, whereas serpins, for example, α1-PI and α1-CT are inactivated by several MMPs, this delays the proteolytic action of extracellular proteinases that are restrained by these particles [25]. In another illustration, solidified working of MMP-9, α1-PI, and neutrophil elastase happens in skin rankle arrangement, in which MMP-9 capably destroys α1-PI, an utilitarian serpin inhibitor of neutrophil elastase and further serine proteinases [26, 27]. This supports elastase intervened grid debasement that begins in dermal–epidermal division and rankling [27]. On the other hand, their active oxygen species (ROS) influence working of MMPs. By which huge amounts of ROS occur at the tumor site due to the inflammatory response. These ROS are secreted by regulatory neutrophils and macrophage [28]. This leads to the active function of MMPs via route of oxidation of the pro-domain cysteine [29] while the inactivation occurs by hypochlorous acid (HOCl) through modification of amino acids of catalytic domain. A hemeprotein myeloperoxidase, secreted by neutrophils, monocytes, and macrophages, uses hydrogen peroxide to generate hypochlorous acid [30]. The compartmentalization of MMPs at normal physiological circumstances frequently commands their natural capacities. A few MMPs connect with outside receptors, for example, integrin or think to specific zones of ECM which upgrades MMP's action by expanding their nearby focus and furthermore may limit their accessibility to endogenous inhibitors [31]. The trial confirmations proposed that connection of MMP-2 to integrin αvβ3 by means of its

hemopexin space is basic structure's cell intrusive activity [32]. For instance, high convergences of active MMP-14 on the cell surface of obtrusive malignancy cells demonstrate critical characters in cell relocation [33–35]. Likewise, there may be different instruments to aggregate extracellular proteinases at clear places around tumor small-scale condition. Neutrophilic granulocytes, amid cell initiation, are framed and known as neutrophil extracellular traps (NETs). These are web-like organizations with high proteinases convergence of MMP-9 and leukocyte elastase limited on the extracellular chromatin framework [36, 37]. These NETs demonstrate chiefly to battle bacterial contaminations and pathogenesis of immune system infections [22, 38]. Whether the wellsprings of NETs are neutrophils, in case of tumor smaller scale condition, is not clear yet. Likewise, their contribution in malignancy is yet to be determined by compartmentalization and confining proteinase action to certain tumor locales [39]. Mechanical powers (hydrostatic pressure, shear stress, compression, and tension) add to tumor movement [40–42] perhaps by hardening proteolysis of ECM components. Above powers may loosen up and open the basic compliance of substrate proteins, in this way allowing acknowledgment and cleavage by MMPs.

The well-known major controller of primary hemostasis and blood clotting is the Von Willebrand factor (VWF) which is produced in the form of ultra-long chain consisting of hundreds of VWF monomers. The bulky size of this primarily secreted multimeric complex makes it more prone to attacking by high shear forces in the blood stream at spots of rapture. These shear forces cause conformational variation in multimeric complex which leads to exposure of broke site, recognized by ADAMTS-13. The resultant smaller fragments of VWF begin blood clotting process [43]. Tumor advancement is frequently described by expanded tissues stiffness, raised interstitial liquid weight, and changing blood stream conditions [40]. Thus, it is likely that comparable to instruments subject to mechanical strengths are turned out to be the overseeing components for MMP work in the tumor-miniaturized scale condition.

4 MMP Function in Cancer

The physiological role of MMP enzymes is determined by their location and time frame of their activity during cancer progression. In many tumors, the facts still remain hidden that from where and when these enzymes display their proteolytic activity in the cancer microenvironment. In this way, there is a developing need to build up some non-intrusive innovative techniques to manage these inquiries. These techniques would utilize imaging tests in light of MMP-particular exercises. It will be simpler to beat specialized obstacles of clinical criticalness, for example, the finding of early-stage tumors with enhanced affectability by controlling the proteolytic movement of MMPs, the recognizable proof of tumors that open new plausibility to utilize metalloproteinase inhibitors (MPIs) as anticancer medications.

Due to technical advancements in recent time, imaging MMP movement is generally in light of fluorescent optical imaging modalities including fluorescent resonance energy transfer (FRET), radio marked imaging, for example, positron discharge tomography (PET), single photon emanation figured tomography (SPECT), and attractive reverberation imaging (MRI) [44]. Fluorogenic MMP molecules in vivo display that cancers have elevated MMP activity compared to non-tumor-bearing animals [45]. Thus, the evaluation of the effectiveness of MPIs on MMPs activity can take place in whole tumors in vivo [46]. Quantifiable imaging techniques like PET and SPECT are routinely used for detection of cancer, classification, and staging in the treatment as well as in the detection of on spot MMP activity in vivo. Such as, an 18 F-labeled PETMMP-2 inhibitor that specifically binds with cells in tumor models of breast cancer in mice using a specific MMP activity in cancer detection has been used [47]. Radiolabeled ^{123}I-MMP inhibitors specific for the MMPs can recognize MMP-2 and MMP-9 motion using SPECT [48]. Tc-anti-MMP-14 a radiolabeled 99 m monoclonal antibody used as a probe is detected by radiolabeled SPECT in cells bearing malignant mammary tumors in rodents [49]. Gadolinium-based paramagnetic complexity specialists that convey affectability to the MMP proteolytic cleavage turn out to be less hydrophilic on proteolytic cleavage bringing about a discernible change interestingly with MMP-2 [50]. MRI facilities are normally accessible in clinical focuses and are used to detect tumors. This is the most commonly used method as a diagnostic tool for cancer patients. Now, due to these technologies it is easy to constrict the site of MMPs activity in tumor microenvironment by the help of higher resolution probes. Scientists use cell-penetrating peptides linked with fluorescent cargo that are activated by proteolysis and accumulated in area of high MMPs activity [51]. This experiment has been successfully used to visualize MMP-2 and MMP-9 activity in mouse xenograft model and cell culture systems [51, 52]. Known as proteolytic beacons, fluorogenic substrates built on self-quenched and near-infrared FRET pairs, were developed to reduce absorption and scattering and increase tissue penetration. It showed critical increasing MMPs activity in experimental models of colon and pancreatic cancers [53, 54]. For instance, MMP-7 activity imaging in vivo by a specific wavelength of infrared polymer-based proteolytic beacon can sense tumors of 0.01 cm^2 in rodent model [44, 53]. It is hypothesized that such probes can be developed to image MMPs activity at subcellular level in extracellular matrices in vitro. This new type of high-resolution investigation mechanism provides site-specific reporting about the insights of protease activity and cells migration through the extracellular matrices. Imaging of MMPs activity is a promising strategy particularly in the development of MPIs as anticancer drugs. Although many technical challenges with further in vivo imaging of MMP activity are in the way, yet according to the survey, most of the clinical trials with MPIs became unsuccessful, probably due to handling of advanced-stage tumors. Imaging with profound MMP probes will greatly help to govern the time span of MMP activity in which MPI administration is required to be operative. As the understanding and technological advances will grow, this technique may gain a new level.

5 Role of MMPs in Cancer

From last 40 years, MMPs are known to act as a major factor in cancer progression as MMP-mediated ECM degradation leads to cancer cell invasion and metastasis [55]. The idea of MMPs inhibition leading to the suppression of tumor invasive potential was a major turn in MMPs research and its implementation into clinical trials. After so many years of research, the actual tumor suppression potential is yet to be unraveled, but prominence of MMPs in tumor progression cannot be ignored [2]. The recent studies suggest wide range of roles that MMPs perform other than ECM proteolysis. It regulates and participates in many signaling pathways which alter cell physiology and disease. MMPs arbitrate an extensive variety of biological properties on their neighboring tissue (Fig. 4). In the subsequent segments, we will be dealing with the consolidated knowledge about the physiological or pathological processes moderated by MMPs, which are known to have a great influence on the tumor microenvironment.

5.1 MMPs Affect Growth Signals

General feature of tumor cells is uncontrolled proliferation. Principally, there are two basic ways to accomplish this condition: by obtaining independence of external growth promoting signals (growth activator) or by becoming insensitive to anti-growth signals (growth suppressor). The exact balance of two processes decides fate of cells. Any imbalance leads to the occurrence of diseases. In the ECM microenvironment, MMPs may play important role in disturbing the

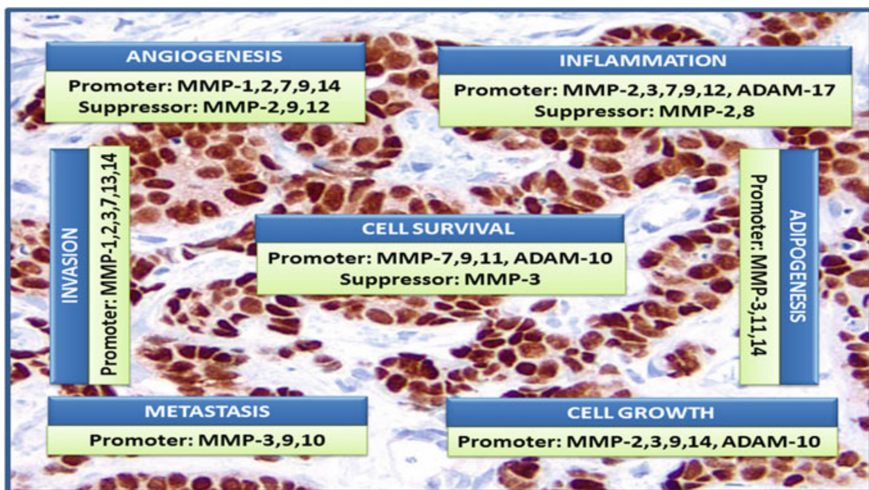

Fig. 4 Summary of different MMPs functions in cancer initiation and progression

balance between growth and anti-growth signals. For instance, TGF-β usually exerts tumor-suppressive properties by applying cytostasis and differentiation in one central signaling pathway that plays a very crucial role in tissue homeostasis.

During the course of malignant tumor progression, mutations accumulated in genome lead to unresponsiveness of TGF-β receptor system to TGF-β factor which results in increased invasion and metastasis [56, 57]. Proactive TGF-β converts into active TGF-β form due to proteolytic conversion by furin or other proteinases, such as MMP-9, which generally comes into play during cellular inflammation [58]. Similarly, TGF-β1 is proteolytically activated by MMP-14 and MMP-2 [59]. Then again, MMP-2, MMP-9, and MMP-14 indirectly modify TGF-β bioactivity by chopping the ECM component latent TGF-β-binding protein1 (LTBP-1), thus making ECM-bound TGF-β free [60–62]. Provided the tumor cells frequently obtain non-responsiveness to TGF-β, this proposes that proteolytic activation of TGF-β by MMPs has tumor-promoting effect leading to increased development of stroma-mediated invasion and metastasis of the tumor. In another example, epidermal growth factor receptor (EGFR) ligands are effective driver so f cell proliferation and important watch dog of tissue homeostasis. Genetic mutations leading to malfunctioning of the molecules involved in this system are commonly witnessed in breast cancer and other malignant diseases [63, 64]. ADAM proteinases are known for their ability to regulate EGFR pathway. For instance, ADAM-10 starts discharge of soluble EGF while ADAM-17 acts as a converter of preforms of TGF-α and epiregulin into their respective active forms. Activation of EGFR upregulates MMP-9 which further degrades E-cadherin. It is a strong regulator of numerous cellular functions like cell-to-cell adhesion and differentiation. Relationship inbetween EGFR, MMP-9, and E-cadherin plays a significant role in ovarian tumor and metastasis due to the co-localization specificity of EGFR and MMP-9 in an area of reduced E-cadherin [65].

The proteolytic cleavage of E-cadherin by MMPs or ADAM has a strong influence on cancer cell proliferation. As a matter of fact, ADAM-10 helps in the detachment of E-cadherin which consequently leads to changeover of β-catenin to the nucleus, causing increased cell proliferation [66]. In addition to the role of ADAM-10, overexpression of MMP-3 in mammary epithelium initiates a cascade of subsequent events including the cleavage of E-cadherin which leads to epithelial–mesenchymal transition [67, 68]. Here, the MMPs inhibitors may slowdown hyperactive MMPs and may prevent tumor growth and metastasis. Consolidated action mechanism of inhibitors of these metalloproteinases with inhibitors of EGFR and HER-2/neu kinases synergistically prevents the growth of human breast cancer xenografts [69]. Above studies help to unravel the hidden facts that correspond with proteolytic speeding up of cellular growth and put forth that exact inhibition of these metalloproteinases can prove to be beneficial in inhibiting unregulated cell growth and uncontrolled cell proliferation.

5.2 MMPs and Apoptosis

Non-occurrence of programmed cell death or apoptosis leads to increased cell number and tumor size. Fas receptors, one of the extracellular receptors, initiate proteolytic cascades of intracellular caspases after binding with Fas ligand which therefore prompts the selective corruption of subcellular part and nuclear DNA, i.e., the conduction of normal apoptosis. MMPs obstruct initiation of apoptosis in tumor cells through cleavage of ligands or receptors. In doxorubicin-treated cancer cells, MMP-7 degrades Fas ligand from the surface [70, 71]. This leads to the lowered effect of chemotherapy on tumor due to hindered apoptotic process. Non-small cell lung cancer patients show resistance to chemotherapy that can be predicted with the help of predictive markers such as MMP-7 expression [72]. Likewise, ADAM-10 hinders/halts apoptosis initiation by cytotoxic lymphocytes via the deprivation of Fas ligand, thus Fas receptor-triggered cell death of target cells is interfered [73]. In pancreatic ductal adenocarcinoma, MMP-7 and Fas ligand interaction may play important role. According to the experimental studies, mice with deficiency of MMP-7 or having non-functional Fas ligand showed reduced metaplasia [74, 75].

MICA and MICB, tumor-associated major histocompatibility complex class-I protein, can be proteolytically degraded by ADAM-17 resulting into the suppression of NK-cell mediated cytotoxicity toward the cancer cells, and hence, ADAM-17 can potentially interfere with anti-tumor directed immune response [76]. It remains unknown whether MMPs show same interfering mechanism like ADAM-17, but the studies have suggested that use of MMPs inhibitors in combination with interleukin-15 in small cell lung cancer cells can act in overcoming tumor resistance issue [77]. Above experimental demonstration shows tumor-promoting role of MMPs by blocking receptor transmitted or lymphocyte-mediated apoptosis.

5.3 MMPs and Angiogenesis in Tumor

Solid tumor vasculature is fundamentally different from that of regular vasculature, and special features of tumor vessels serve as decent targets for cancer therapies. Circulating vasculogenic progenitor cells emerging from local blood vessels form tumor vasculature.

Tumor cells can penetrate these newly formed blood vessels because they are often irregular and leaky in nature due to lack of pericyte cover. In addition to blood capillaries, lymphatic endothelial cells have less-developed junctions, large inter-endothelial gaps, and impaired basement membranes. Angiogenic factor and invasive margin are critical for stimulation of angiogenesis and lymph-angiogenesis in tumor [78]. In following paragraphs, we have cited the evidences that support of MMPs role in angiogenic and lymph-angiogenic processes [79]. According to most of the studies, MMPs involved in tumor angiogenesis are MMP-2, MMP-9, and MMP-14, and to a smaller extent MMP-1 and MMP-7. There are a number of MMPs known for their expression in all tumors. Each MMPs contribute to distinct

vascular events in same tumor [45]. The bioavailability of vascular endothelial growth factor (VEGF), one of the potent factors of tumor angiogenesis and therapeutic target, is regulated by MMP-9 [80, 81] for its receptor VEGFR2. This process is known as angiogenic switching. Angiogenic switching by MMP-9 comprises a complex interaction related factors. In a mouse experimental model of glioblastoma, CD45-positive bone marrow-derived cells are recruited by the hypoxia inducible factor-1α (HIF-1α) to initiate angiogenesis. MMP-9 action provided by CD45-positive myeloid cells is vital and adequate for the angiogenic switching by enhancing VEGF bioavailability [82].

It is found that VEGF obstructs tumor cell movement along blood vessels, but it encourages perivascular tumor cell infiltration into the brain parenchyma [83]. MMP-3, MMP-7, MMP-9, or MMP-16 cleave matrix bound VEGF which leads to the formation of modified VEGF molecules with modulated bioavailability, this changes vascular patterning of tumors in vivo. In MMP-9 deficient mice, tumor transplanted to irradiated tissue did not show angiogenesis in vivo, while the tumor growth was restored by transplanting CD11b-positive myeloid cells from the bone marrow of MMP-9 sufficient mice. This experiment proved that MMP-9 is undoubtedly very much required for tumor vasculogenesis [84]. Therefore, during chemotherapy and radiotherapy of tumor, MMP-9 could become an important target for adjunct therapy to increase the response of tumors to radiotherapy.

Neutrophilic granulocytes deliver MMP-9 for performing a specific role. Neutrophil derived pro-MMP-9 does not form complex with another protein TIMP-1 and thus, it is easily activated to initiate tumor angiogenesis [85]. The angiogenic function of MMP-9, derived from neutrophil, needs its active and hemopexin domain, and finally activates basic fibroblast growth factor (FGF)-2 [86]. It is interesting to note that MMPs produced by different cells types can function differently. The release of neutrophil derived TIMP-1 free MMP-9 may be an important factor in the pro-angiogenic effect. So it can be put forward like this, the number of neutrophils is directly proportional to the increase in the intensity of occurrence of angiogenesis. Same as, a high number of neutrophils in fibro sarcoma correlates with high microvessel density in tumor, and [87] while reducing neutrophils in pancreatic cancer significantly decreases angiogenic switching in dysplasia [88]. Above results provide an insight of vital role of infiltrating neutrophils in the initiation of tumor angiogenesis. The degradation of ECM components and other extracellular molecules may generate different fragments with different bioactivities, which may inhibit angiogenesis [89].

For instance, naturally active endostatinis produced via proteolysis of type XVIII collagen by MMP-3, MMP-7, MMP-9, MMP-13, and MMP-20 [90, 91]. Further, the proteolysis of collagen Ivα3 through MMP-9 generates the monomeric NC1 domain called tumstatin, a powerful suppressor of neovascularization. This is established in tumor angiogenesis and amplified tumor growth in MMP-9-lacking mice [92]. Significant amounts of angiostatin can be produced by the degradation of plasminogen by MMP-2, MMP-9, and MMP-12, a product with anti-angiogenic function [93]. Angiostatin production by MMP-12 shows suppressive effects on outgrowth of lung metastases [94].

Collectively, MMPs can produce both anti-angiogenic as well as promoting signals. The effects of MMPs on angiogenesis might be diverse depending on the time frame of MMP expression and the availability of substrates. MMPs regulate vascular stability and permeability. Predominantly, MMP-14 seems to facilitate the vascular reaction to tissue injury and tumor development by activation of TGF-β [95]. Lymph-angiogenesis is known to play a very important role in tumor biology, provided it is directly related to the development of lymphatic metastases. MMPs definitely have overall influence on lymph-angiogenesis as supported by the application of broad-spectrum MMP inhibitors [96]. For lymph metastases, active VEGF bioavailability is an important factor which is provided by MMPs, especially by MMP-9. The three-dimensional culture system using mouse thoracic duct fragments embedded in a collagen gel provided experimental proof of the involvement of MMPs in lymph-angiogenesis.

Amplified MMP-1, MMP-2 [98], and MMP-3 [99] expression are correlated with lymphatic invasion and lymph-node metastases. Attenuation of angiogenesis and lymph-angiogenesis is achieved by inhibition of MMP-2, MMP-9, and MMP-14 [96]. MMP-2 deletion may affect lymphatic vasculature in a negative way, but may not affect aortic vasculature [97]. The literature and experimental evidences are insufficient to prove the link between lymph-angiogenesis and MMPs. Further detailed studies are required to reveal the facts.

5.4 MMPs and Adipocyte in Cancer Progression

Adipocytes are well-known constituents of tumor stroma and play a vital role in cancer progression. Adipokines, the adipose secretory products, are involved in negative consequences of adipose tissue expansion in diabetes, cardiovascular disease, and cancer [100, 101]. Further, adipokines such as leptin control the secretion and activation of MMPs, for instance, leptin encourages MMP-13 expression in glioma cells causing enhanced migration and tumor invasion [102]. Cancer cell and human adipose tissue stem cell are co-cultured to produce CCL5, which consequently increases breast cancer cell invasion associated with MMP-9 activity [103]. MMP-3 is known to be greatly involved in adipocyte differentiation in mammary gland during post-lactational involution. MMPs or TIMPs are expressed by fibroblastic adipogenic progenitor cells. Specific pre-adipocytes induce transcription of a number of MMP's and TIMP's mRNA (MMP-2, MMP-3, MMP-13, and MMP-14 and TIMP-1, TIMP-2, and TIMP-3) while fully developed adipocytes are involved in the expression of MMP-2 [104].

It has been observed that mice carrying a mutated MMP-3 or mammary glands of transgenic mice that overexpress MMPs inhibitors showed increased differentiation and hypertrophy of adipocytes [105]. Development of lipodystrophic null mice occurs due to aborted white adipose tissue development because of the absence of MMP-14.

MMP-14 also contributes to the coordination of adipocyte differentiation [106]. Recently secretome, a member of adipocyte, is found to improve MMP-2 activity [107].

Remodeling of extracellular milieu of adipogenesis and tumor microenvironments, where adipocytes are the key stromal components, is governed by Wdnm-1-like protein (a distant member of the whey acidic protein/four disulfide core [WAP/4-DSC] [108]. So far, only MMP-11 induced by adipose tissue is known to directly affect cancer progression. MMP-11 controls adipogenesis by decreasing pre-adipocyte differentiation and reverting adipocyte differentiation on tumor infiltrates the adjacent environment. Adipocyte dedifferentiation favors cancer cell survival and tumor progression due to collection of no-malignant peritumoral fibroblast-like cells at specific sites. It was speculated that this MMP-11-mediated crosstalk between infiltrating cancer cells and nearby adipocytes/pre-adipocytes emphasizes on its special role during tumor desmoplasia and signifies a molecular correlation between obesity and cancer [109].

5.5 MMPS and Neoplastic Progression

Loss of cell–cell adhesion and enhanced cellular mobility leads to cancer progression due to the combined effects of tumor invasion and epithelial–mesenchymal transition (EMT) programmers. Over expression of several MMPs, including MMP-3, MMP-7 and MMP-14 leads to carcinoma formation [6]. MMP-3 overexpression enhances EMT and induces genomic instability in cultured mammary epithelial cells leading to all stages of malignant transformation, neoplastic progression, and mammary carcinomas in transgenic mice [67, 110]. These things are related with the occurrence of an alternatives splice product of Rac1 that later on encourages the secretion of reactive oxygen species by mitochondria [68].

5.6 Tissue Invasion and Metastasis

The occurrence of metastasis is the resultant outcome of majority of cancers due to the infection of cancer cells from the primary tumor outgrowth to distant sites, i.e., secondary tumors (Fig. 5). The beginning of metastasis includes the invasion of the tumor into the outer tissue leading to intravasation of cancer cells into blood or lymphatic vessels from where they distribute into secondary organs. For a tumor cell to metastasize, four events are known to occur. They are:

(i) Dispassion from the primary tumor and later invasion into tissue stroma.
(ii) Entry to the blood vessel, such as the endothelial basement membrane or lymphatic system (intravasation) to cross other sites in the body.
(iii) Exudation from the circulation (extravasation).
(iv) Development to metastatic colonies. For distant setting it needs receptive sites called pre-metastatic niche.

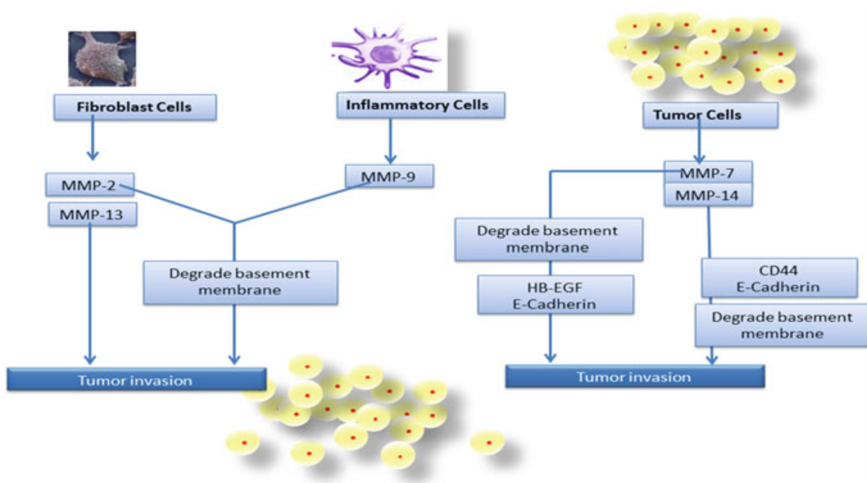

Fig. 5 Representative path and involvement of MMPs in cancer invasion and metastasis

This process has a complex relationship with inflammatory cells and hematopoietic progenitor cells [111]. In the presence of MMP-1, a special mechanism of MMP-mediated signal transduction is initiated which facilitates the detachment of primary tumor cells. In breast cancer model, MMP-1, extracted from tumor infiltrating fibroblasts, cleave PAR-1 which initiates cancer cell migration and invasion of tumor cells [112].

This demonstrates a prominent role of stroma-derived proteinases in progression of tumorigenesis, supported by particular signal transduction on cancer cells. One of the most common locations for metastasis is bone, often leading to mortality. Osteolysis and dissemination into bone tissue depend on MMPs expressed at the interface between tumor and stromal cells. In a rodent model of prostate cancer, osteoclasts expressed by MMP-7 at the tumor–bone interface twitches osteolysis and subsequently bone metastasis [113]. Close interaction between activated osteoclasts and osteoblasts permits binding of RANKL to its receptor RANK on osteoclast progenitors leading to osteoclast differentiation. Highly metastatic cell line, MDA-MB-231, of human breast cancer, was prepared by knocking down MMP-1 and ADAMTS-1 which showed similar effects like MMP-7 action [114]. Osteolysis and metastasis in bone are the results of activation of RANKL pathway, and this pathway is activated by ADAMTS-1 and MMP-1 which proteolytically engage EGF-like ligands. These studies support the idea of MMP-1 gene as being a piece of the multigenic program that mediates occurrence of bone metastasis in breast cancer cells [115].

Altogether, these proteinases can become potential therapeutic targets to prevent metastasis to the bone in breast or prostate cancer. Though MMPs play diverse roles in biological aspects yet these are known to play central role in degradation and remodeling of ECM creating a way through the marginal tissue for invasion and

metastasis. Current studies by high-resolution multimodal microscopy have validated the significance of ECM remodeling by MMP-14-driven pericellular proteolysis, which competently prepares the tissue to facilitate single-cell and subsequently collective-cell migration and invasion [34].

A number of proteolytic ECM enzymes, like MMP-1, MMP-2, MMP-13, and MMP-14 and cathepsins B, K, and L have been involved in this process; but MMP-14 may be solely very important and rate-limiting in collagen turn over [34, 116]. It is known that metastatic cancer cells become protease-independent from protease-dependent invasion by using an ameboid migration mode [34]. However, it is not clear that whether the ameboid migration is only applicable under the in vitro conditions [116]. This advocates that MMPs produced by tumor-associated macrophages facilitate intravasation of cancer cells into the blood stream. Also MMPs derived from myeloid cells help in intra and extravasation of metastatic cancer cells.

5.7 MMPS and Metastasis Preparation

Metastatic cancer cells most likely have their preference for their distant locations, i.e., secondary sites. L most-preferred secondary sites for metastasis are lungs, liver, or bone. These migrating cells require some open condition, known as a metastatic specialty that is particularly suited for tumor cells at the far-off organ. During the development of this microenvironment, MMPs and other proteinases become active in the formation of a metastatic niche. Fibroblasts produce soluble factors which trigger the formation of a metastatic niche for secondary tumors [111]. These secretions, before disseminated tumor cells, become noticeable at distant organs; therefore the name of this process is given “pre-metastatic niche.” Infiltration of VEGFR1-positive cells that confirm metastasis-supporting microenvironment occurs at these secondary metastatic sites due to the secretion of increased levels of fibronectin. According to recent studies, the factors, for example, fibronectin and so forth are discharged by the essential tumors that start the metastatic specialty development. Primary tumors secrete VEGFA, TGF-β, and tumor necrosis factor-α (TNF-α) that trigger the expression of S100 chemokines by lung endothelium that helps in myeloid cells movement to various sites [117]. The mechanisms and proper reasons for why above factor triggers chemokines expressions in specific tissues are not known. Another factor playing a significant role is serum amyloid A3(SAA3) which works as an upstream regulator of chemokine S100 throughout this process.

Infiltrating myeloid cell’s mediate inflammatory response that initiates formation of pre-metastatic niche.

It is speculated that SAA3 prompts toll-like receptor4 (TLR4) signaling in penetrating myeloid cells leading to beginning of the nuclear factor-κ-B (NF-κB) pathway [118]. NF-κB pathway activates production of MMPs by specific cells like, stromal cells [119], which helps various cellular microenvironment alterations.

Undoubtedly, MMP-9 plays an unprecedented role in development of the metastatic niche [111], which is closely and most probably related with its stability to release VEGF and thus promotes angiogenesis [80]. MMP-9 discharges solvent Kit-ligand to enlist stem and progenitor cells from the bone marrow [120] which may have a specific significance in this unique situation; provided the niche-forming progenitor cells express c-Kit [111]. Hypoxic breast cancer cells secrete Lysyloxidase (LOX) which can play role in metastatic site creation due to crosslinking of collagen IV proteins in basement membrane structures. This leads to the employment of tumor cells to these locations [121], perhaps owing to changed tissues stiffness [122]. This infiltrating tumor cell exudes MMPs that destroy collagen fiber and discharge peptides that can pave the bone marrow driven cells and metastasizing tumor cells to these destinations. Simultaneous production of MMP-3 and MMP-10 is upregulated altogether with angiogenic modulator angiopoietin-2 in premastatstic tissue even before myeloid cells are utilized to these destinations [123]. Silencing by in vivo, RNA impedance of MMP-3, MMP-10 and angiopoietin-2 diminish vascular penetrability and penetration of myeloid cells in lungs. It creates the impression that these three variables synergize in the destabilization of the pneumonic vasculature and in this way advancing metastasis. These outcomes demonstrate that essential part of extracellular proteolysis in pre-metastatic specialty arrangement. Though thorough studies are required to explain these extremely important pathways modulated by these proteinases yet it appears that the action of MMPs in this example critically involves the inflection of inflammatory responses.

5.8 MMPs and Inflammation

Recent findings suggest that MMPs are the chief controllers of innate and acquired immunity. As speculated, inflammatory responses are one of the contributing factors to cancer. Numerous chronic inflammatory diseases (like pancreatitis and Crohn's disease) are known to increase chances of occurrence of cancer [124]. In the consequent portion, we will explain the part of MMPs and their capacities and how MMPs adjust the capacity of cytokines and chemokines and what impacts this insusceptible administrative capacity may have on tumor microenvironment. Knockout mice experimental model gives strong evidence that MMPs play a crucial role in acute and chronic inflammation [5]. TNF-α is the most studied genius provocative cytokine communicated as layer bound precursor(pro-TNF-α) on macrophages and T cells. Translation of pro-TNF-α to TNF-α requires proteolytic cleavage by ADAM-17/TACE (TNF converting enzyme), or by MMPs including MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, MMP-14, MMP-15, and MMP-17 [125]. It is found that in some special circumstances, MMP-7 can act as TNF-α-generating convertase instead of ADAM-17 which is known to play a major role in generation of above convertase [126]. In many tumors TNF-α is upregulated many folds and it advances growth cell survival in NF-κB-subordinate way [127].

These suggest that generation of functional TNF-α by MMPs and ADAM-17 is an essential venture to begin tumor-advancing course. A significant number of individuals from the CCL/monocyte chemoattractant protein (MCP) group of chemokines are severed by MMPs, which especially abandon them into non-activating receptor antagonists with inflammation-dampening effects [128]. For example, CCL8/MCP-2 is processed by MMP-1 and MMP-3. Undoubtedly, the proteolytic cleavage of CCL8 can hinder the anti-tumor ability of this chemokine in a melanoma model [129]. MMP-8, MMP-9, and MMP-12 alter the bioactivity of CXCL11/I-TAC, a strong Th1 lymphocyte-pulling in chemokine [130]. This literature reveals that proteolytic cleavage of a chemokine can probably have a strong impact in a clinically significant location of tumor development. It is found out after vigorous studies that MMP-mediated C-terminal cleavage abolishes the antagonist function and removes heparin-binding capacity of CXCL11 while N-terminal truncation of CXCL11 leads to inactivation of the chemokine. This creates a potent receptor antagonist, thereby solubilizing the chemokine from the ECM. These studies reveal the importance of myeloid cell resulting MMPs in the direction of T cell reactions, due to which they may have significant consequences on the adaptive anti-tumor immune response. Expression of CXCR7, biological receptor of CXCL11, on several tumor cells can generate signals for growth as well as survival [131]. Proteolytic fragments of MMPs cleaved ECM components exert chemotactic properties; similarly, macrophage elastase MMP-12 produces neutrophil-attracting peptides by degrading elastin [132]. A fragment N-acetyl Pro-Gly-Pro (PGP) is generated from ECM which stops working during lungs inflammation. This tripeptide shows demonstrate chemotactic action through enactment of CXC chemokine receptors on neutrophilic granulocytes [133]. Another factor known as chemotactic PGP, generated by MMP-8, manages neutrophil enrollment at inflammation site [134, 135]. Also MMP-8 secreted by neutrophils plays a vital and phenomenal tumor stifling part in a mouse model of cancer-causing agent initiated skin disease [136]. MMP-8 deficient mice have a tendency to develop chronic inflammation and therefore, the loss of MMP-8 contributes mechanistically to expanded weakness of skin adenocarcinoma and melanoma arrangement in people [137]. It is found out that MMP-8 expression in tumor cells tightens their linkage to the ECM, and therefore it may lead to the repression of metastatic activities of tumor cells [138]. Neutrophils penetration in tumor-miniaturized scale condition as a rule connects with poor anticipation [139]. Like other inflammatory cells, neutrophils have one of a kind capacity of detecting the focus slope of chemokines of CXCL1/KC, a homolog of CXCL8 that is known to shape buildings with the heparan sulfate proteoglycan syndecan-1 on interstitial cell surfaces. MMP-7 continuously changes the CXCL1 bioactivity by cutting syndecan-1 from the cell exterior, consequently discharging chemotactic complexes of syndecan-1 and CXCL1 [140]. CXXL1 syndecan-1 forms focus inclination of dissolvable chemotactic complex in ECM. Consequently, MMPs coordinate the employment of leukocytes as a vital segment of tumor-related irritations.

6 Non-proteolytic Function of MMPs

Nowadays, the most evolving zone of attention is the non-synergist capacity of MMPs. It is not surprising to realize that about portion of all ADAMs indicates proteolytic capacity, whereas other half capacities in non-proteolytic way [141]. The structural domain of MMPs, hemopexin, is very imperative for their non-proteolytic usefulness. TIMP-1 and TIMP-2 bind on hemopexin domain of MMPs and perform hemopexin-mediated function. MMP-2 activation needs [142] TIMP-2 which is attached with single molecule of MMP-14 through its synergist area and furthermore is associated with pro-MMP-2 through its hemopexin sphere. At last, a moment atom of MMP-14 then chemically actuates MMP-2 [143]. During immune or cancer cell migration, numerous member of MMPs family take part to mediate this migration. Recent literatures suggest that they intervene chemotaxis even without utilizing their proteolytic area. Inactive precursors of MMP-2 and MMP-9 increase cell movement in a trans-well chamber assay. MMP-9 hemopexin domain is required for MMP-9 interceded epithelial cell movement in this measure [144] MMP-9. Upregulation of MAP kinase and PI3 kinase pathways is speculated to be included in this non-proteolytic capacity of MMP-9. MMP-14's cytoplasmic tail performs a relocation advancing capacity on macrophages. When this cytoplasmic tail is removed, it impairs the movement of macrophages amid in vitro relocation through Matrigel [145]. Recent analysis done by using the technique of hereditary adjustment of one of the two MMP qualities of Drosophila melanogaster revealed that in spite of the fact that the synergist area is required for all MMP capacities, the hemopexin space is extraordinarily required in tissue intrusion amid transformation; however, not for tracheal redesigning [146]. Disfunctioning of MMP-12 in mice prompt flawed finish bacterial clearance and upgraded mortality when contaminated with gram-negative and gram-positive microorganisms. One of a kind four amino corrosive grouping inside the hemopexin-like C-terminal space of MMP-12 shows antimicrobial properties. A few MMPs may interface with other extracellular parts without instigating proteolytic cleavage. MMP-14 associates with C1q segment of supplement framework in a ligand-receptor way without making C1qr and C1qs proteinase action. It is proposed that this connection counteract initiation of supplement proteinase course [147]. The exact purpose of binding of MMP-14 and C1q is still not clear and also how it helps in tumorigenesis is yet to be elucidated. In ECM, MMPs bind with integrin family proteins on cell surface receptors. Pro-MMP-1 interacts with $\alpha 2\beta 1$ integrin in a non-proteolytic manner on neurons [147]. Reduced AKT dephosphorylation after MMP-1 incubation, recommends that integrin binding, rather than proteinase activity, is significant for MMP-1-mediated cytotoxicity. MMP-9 is known to promote B cell survival, in chronic lymphocytic leukemia, in a non-proteolytic fashion via its hemopexin domain by getting attached to the surface receptors $\alpha 4\beta 1$ and CD44v which actuate intracellular flagging including Lyn enactment and STAT3 phosphorylation that forestalls B cell apoptosis [148]. Consolidating the facts, these experimental data are just providing a suitable start to understand the

non-proteolytic functions of MMPs. More elucidations and revelations of facts are required to clarify this principle and to assess its role under in vivo conditions. However, it is tempting to find out that non-proteolytic functions of MMPs, may be, were the main reasons behind the failure of early clinical trials using inhibitors of the MMP catalytic domains as anticancer therapeutics.

7 Conclusion and Future Prospective

Organization of MMPs with numerous cancers makes them as potent therapeutic target during the treatment of cancer. The idea of obstructing of MMP-interceded angiogenesis and metastasis laid the reason for advancement of first medication, and additionally, various little atom metalloproteinase inhibitor (MPI) sedates in stage III clinical trials. MPIs in these trials did not prove to be potential drugs as they failed to treat cancer patients. Many reasons were found out, and it was speculated that MPIs are, may be, just restricted to be beneficial in early stages of cancer. MPIs were used as anticancer drugs because of their ability to truncate interstitial relocation of metastatic cancer cells. Later on, it was found that these cancers can change to an ameboid-like protease-independent movement mode by shaping actin-rich bulges [34]. Thus, MPIs lost their ability to inhibit the transitory conduct of metastatic tumor cells. But the fact remains hidden regarding the migration mode whether that is actually relevant for tumor cell relocation under in vivo situation in the nearness of a normally crosslinked collagen lattice.

According to a study supported by strong evidences, MMP-14 plays a major role in the migration and invasion of metastatic tumor cells; hence, MMP-14 is a promise target for tumor cells [116]. These all findings proved that researchers can use MPIs as effective anti-invasive drug that can inhibit MMP-14. Additionally, MMPs are likewise known to meddle with apoptosis acceptance, particularly after chemotherapy, as they break Fas ligand from the surface of disease cells as appeared for MMP-7 [70]. In experimental trials, MPIs were given to patients who were at the last stage of cancer. This was done to check whether even at the last stage these MPIs can actually help in survival of patient. Due to such studies drugs like Bevacizumab (Avastin, anti-VEGF monoclonal antibody) were developed. These were known to interfere with the tumor vasculature, thus helped to inhibit tumor growth. These drugs were approved by FDA to use in combination with chemotherapy for the treatment of various metastatic cancers. The recent studies on MMP-9 suggest that it plays a dominant role in angiogenesis switch. MMP-9 also helps in the regulation of bioavailability of VEGF tumors, thus unravels the beneficial effect of MPIs on tumor. A few reviews likewise propose that MMP-9 additionally fills in as a powerful ECM fragment generator like tumstatin which goes about as a suppressor of tumor vasculature arrangement. This prompts expanded tumor development in MMP-9-insufficient mice [92]. This leads to the discovery of the fact that one MMP can have contradicting effects in different tumor types. This highlights the necessity for the use of specific type of MPIs with proper

consideration and detailed examined assessment of various types of cancers. The preliminary study suggested that the major sources of MMPs are non-malignant stromal cells. These studies were based on the usage of cancer cell lines that could overexpress certain members of the MMPs family. But these studies were not applicable under in vivo conditions. In fact, the cellular source of each MMP is given due importance as the activity of the released enzyme varies substantially between cell types. When assessing the expression patterns of MMPs in cancer types, these cellular sources of each MMP are taken into consideration to select which type of MPI should be used for the treatment. In this manner, the part of MMPs has stretched out from being powerful proteinases that debase ECM to particular modulators of angiogenesis as well as fine-tuners of cell flagging pathways and the incendiary reaction. Discovery of several regulatory effects of MMPs in the microenvironment of stromal cells has been proved to be the major breakthroughs in MMPs research. MMPs influence adipocyte work, which is likely to be involved in fat-rich tumor destinations, for example, breast. The Inflammatory response is directed in different ways subsequently the enrollment of incendiary cells is encouraged prompting the modification of the capacity of chemokines and the bioavailability of imperative pro-inflammatory cytokines. Talking about a settled connection among irritation and malignancy [124], it was hypothesized that intercession with MMP-interceded immunoregulatory capacities could wind up plainly worthwhile for disease patients. For example, the movement of a few sorts of tumor is contributed by TNF-alpha factor [148]. Inhibition of TNF-α activation using MPIs may help in reducing the intensity of inflammatory response in the tumor microenvironment. The generation of the pre-metastatic niche helps us to know the effects of MMPs. MMP-2, MMP-3, and MMP-9 are well-known because of their capacity to add to the foundation of metastasis-inclined destinations at tumor far-off organs [121]. These analyses help us to understand that MPIs can be used in many cancers at preliminary stages, i.e., prior to the full initiation of tumor-associated inflammation and before the infection reaches to the distant organs. The failure of various broad-spectrum MPIs as anticancer drugs is attributed to the various tumor-suppressing functions of these MMPs [149] and for the link between MMP-8, loss-of-function mutations and melanoma in humans. Some MMPs are known to carry out biological functions other than proteolytic cleavage as they specifically bind to certain target molecules, for instance via their hemopexin domain. Small molecule MMP inhibitors are not as effective as the bigger molecules of MMP as they are certainly ineffective to interfere with a non-proteolytic role of MMPs.

The ultimate goal of many future researchers is that they can really prove to be a breakthrough in MMPs studies, which is the advance in active site-directed inhibitors or antibodies those were precise for single MMPs without any interaction with other MMPs. The relocation and incursion of endothelial cells in fibrin and collagen gels can be inhibited by monoclonal antibodies which are raised against the catalytic domain of MMP-14. These antibodies are speculated to specifically site those functional non-catalytic domains of MMPs. Cytotoxic agents such as anthrax toxin can be activated with the help of MMP to target the tumor

vasculature. The validation of these agents is done by testing them in MMP-deficient animals. With the help of specific probe designed for MMPs and their activity can view by these imaging probes. Due to the usage of probes along with minimal invasive imaging techniques will help us to visualize MMP function during progression of tumors.

It should be noted that these imaging techniques are very useful as they help us to understand the tumor progression at all instants of time. The cell signaling pathways which are responsible for the control of cell growth and cell fat gets deregulated due to the accumulation of multiple independent mutations which lead to the development of oncogenic state. Defining the cancer subtypes, recurrence of disease and response to specific therapies has become possible now due to DNA microarray-based gene expression techniques. Various gene expression profiles for the analysis of oncogenic pathways are also used nowadays. Here, expression level of genes reflects the activation status of several oncogenic pathways. When a large number of human cancers are studied, these gene expression signatures can help in identifying patterns of pathway deregulation occurring in tumors and also relevant associations with disease outcomes can also be found. When these individual gene expression signatures are combined across several pathways then the coordinated patterns of pathway deregulation that distinguish between specific cancers and tumor subtypes can be obtained. Combining tumors based on pathway signatures further define prognosis in respective patient subsets that indicate the patterns of oncogenic pathway deregulation underlying the development of the oncogenic phenotype which further reflect the biology and outcome of specific cancers. This associated knowledge of pathway deregulation and therapeutic action of therapeutic agents helps in providing an opportunity to make use of these oncogenic pathway signatures to guide the use of targeted therapeutics.

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Proteolytic Networks at the Crossroads of Cancer Cell Life and Death: Cancer Stem Cell Deciding Cell Fate

Poulami Khan, Shruti Banerjee, Apoorva Bhattacharya, Dipanwita Dutta Chowdhury, Apratim Dutta and Tanya Das

Abstract

Over the years, proteases have been implicated in the development of tumors. The proteolytic network, which critically modulates the functioning of a normal cell, is often dysregulated in cancers. In the recent past, the identification of a subpopulation of cancer cells, termed as cancer stem cells (CSCs), has helped gain a better understanding of the complex mechanisms involved in cancer development, progression, as well as recurrence. In this context, it is of considerable importance to comprehend the pivotal role of proteases in regulating the fate of cancer cells via the CSCs. In fact, the proteolytic network influences cancer cell's fate via CSC and its associated niche, which coordinates the functions of CSCs. In this chapter, we have emphasized on the dynamic role displayed by the proteases in regulating numerous steps of tumorigenesis commencing from tumor initiation, angiogenesis, invasion and metastasis. Apart from this, CSCs also execute a survival mechanism with the help of proteases, upon induction of apoptosis. We have also revisited the mechanisms underlying the contribution of proteases in tumor drug resistance, which ultimately leads to cancer relapse, and the role of CSCs in the same. Similarly, proteases are also intricately involved in inflammation and immune surveillance of CSCs. Given the important role of proteases in carcinogenesis, further development of antiprotease therapeutics may enable better treatment procedures and minimize the risk of recurrence. This chapter has, therefore, epitomized the complex crosstalk involving proteases, CSCs and its niche.

The authors Shruti Banerjee and Apoorva Bhattacharya have contributed equally towards this chapter.

P. Khan · S. Banerjee · A. Bhattacharya · D.D. Chowdhury · A. Dutta · T. Das (✉)
Division of Molecular Medicine, Bose Institute, P-1/12 CIT Scheme VII M, Kolkata 700054, India
e-mail: tanya@jcbose.ac.in

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Angiogenesis · Cancer stem cells · Drug resistance · Immune surveillance Metastasis · Microenvironment · Proteases

1 Introduction

In the recent past, a stark rise has been observed globally in cancer incidence and mortality [1]. Despite considerable progress in cancer detection and treatment procedures, elimination of this deadly disease is becoming increasingly difficult. Cancer recurrence is an emerging problem which further complicates disease-free survival of patients. The recent concepts of cancer stem cells (CSCs) provide a better comprehension of various mechanisms exhibited by the tumor populations. CSCs are a subpopulation of cancer cells which exhibit stem-like characteristics and are solely responsible for imparting various traits which result in tumor aggression, resistance, and relapse [2–4]. It is interesting to note that the features exhibited by the CSCs are mainly driven by the tumor microenvironment [5] and the interaction between CSCs and tumor microenvironment facilitates tumor growth, development, and resistance. In fact, different cell types present in the CSC niche execute critical functions which enhance the survival and proliferation of tumor. The CSC niche accommodates fibroblasts, pericytes, endothelial cells, immune cells, etc., which interact in a convoluted manner and succor the CSCs for inducing malignancy [6–8]. Therefore, the microenvironment of the tumor plays an essential role in tumor initiation, angiogenesis, invasion and metastasis. Keeping in mind the critical contribution of CSC niche in maintaining tumor growth, it is important to discuss the means by which the supportive tumor environment executes its functions. It is well accepted that the stromal cells which are part of the tumor microenvironment regulate the tumor growth by secreting various cytokines, chemokines, growth factors, ECM proteins, proteases, etc. [9, 10]. Among which, the proteases exhibit a paramount role in malignant progression [11–13]. At present, we know that tumor progression is associated with enhanced expression, enhanced activity, and altered localization of proteases in cells. Consequently, here we would like to discuss the role played by the proteolytic networks in regulating the cancer cell fate via CSC niche.

2 Proteases

Proteolysis, which is one of the utmost required biological reactions, is accredited to a category of enzymes called proteases. In normal cells, proteases regulate different biological processes. In fact, the equilibrium between proteases and antiproteases plays important role in maintaining normal cellular function; disruption of which results in diseases like cancer both at primary and metastatic sites [14]. Deregulation of proteolytic activity has been elucidated in numerous cancer cases and often corresponds to poor patient prognosis [15]. Recent findings claim a positive

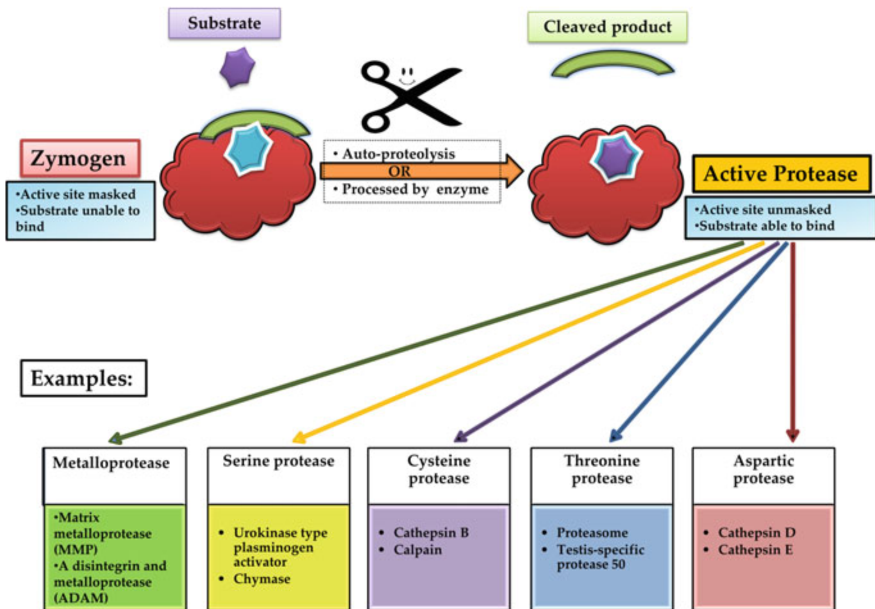

Fig. 1 Activation of a protease from its zymogen state by autoproteolysis or processing by other enzymes. The five major groups of proteases involved in cancer progression are depicted with examples

interconnection between the amount of secreted proteases and aggressiveness of tumor. It is often observed that the cancer cells have the ability to foster the expression of proteolytic enzymes in adjacent normal cells, thereby directing them in favor of tumor growth and maintenance [16]. The function of proteases in cancer progression has been defined as "cancer degradome" after recent identification of the entire gamut of proteases due to the accessibility of the genome sequence of different organisms [17]. There are at least 569 proteases in human degradome which have been categorized into five catalytic classes (Fig. 1): 194 metalloproteases—that use a metal, usually zinc (e.g., meprin, gelatinases A and B), 176 serine proteases—that use serine alcohol (e.g., urokinase-type plasminogen activator, chymase, plasmin), 150 cysteine proteases—that use a cysteine thiol (e.g., cathepsins B, L, S, K, Q; bleomycin hydrolase; caspases), 28 threonine proteases—that use a threonine secondary alcohol (e.g., proteasome), and 21 aspartic proteases—that use an aspartate carboxylic acid (e.g., cathepsins D and E) [18]. Proteases are normally synthesized as inactive precursor called a zymogen or proenzyme, which gets converted to mature, active enzymes by limited but regulated proteolysis of the proenzyme either by a different enzyme or by autoproteolysis (Fig. 1).

These enzymes target diverse substrates, thereby regulating numerous cellular functions that are indispensable for cell survival and death in organisms [13]. However, not all of them are linked with cancer [19, 20]. Actually, the complex process of tumor formation involves diverse changes in the normal cell, beginning from tumor initiation, angiogenesis, growth, metastasis, and invasion into secondary site as well as induction of multidrug resistance. Available reports mainly

demonstrate the role of proteases in tumor invasion and metastasis. However, studies are now emanating which imply that proteases are involved in all these above-mentioned steps, thereby aiding tumorigenesis and its progression [18]. This discussion, therefore, raises the possibility of using protease inhibitors as anticancer drugs. Choi et al. [21] and Turk [22] have separately reviewed the pros and cons of targeting proteases in order to develop novel therapeutics. In contrast, López-Otín and Matrisian [23] have reviewed the potential role of proteases as tumor suppressors.

Further, it is interesting to note that the proteasome complexes influence induction, maintenance, and exit of self-renewal as well as pluripotency in both mouse and human [24]. The proteasome complex as well as the proteasomal degradation pathway represents a major system controlling many cellular processes. Even though most proteasomal substrates are required to be ubiquitinated prior to being degraded, there exist some deviations in this normal phenomenon, mainly when the proteasome engages in a normal role in the post-translational processing of the protein [25]. In addition, Yasutaka et al. [26] demonstrated ubiquitin proteasomal system as a key regulator of stem cell pluripotency. In connection with this, lung tumor spheres are reported to be enriched in cells with low proteasome activity [27].

Above discussion signifies that the proteases mediate a complex role in carcinogenesis and its management. Studies related to the proteasomal network in influencing cancer cell fate by regulating CSCs within tumor niche, therefore, highlight the emergence of a new and important field of research. Therefore, in this chapter, we would like to portray the role of the proteolytic networks which regulate tumor initiation, angiogenesis, invasion and metastasis, etc., via the CSC niche.

3 Cancer Stem Cells

Recently, there has been a paradigm shift in the concept of cancer biology, where accumulating evidences show that a minority subpopulation of the cancer cells residing inside a tumor are the root cause of several facets of tumorigenesis including tumor initiation, angiogenesis, metastasis drug resistance, and tumor relapse (Fig. 2) [28–31]. These cells have the capacity to self-renew and to produce the heterogeneous lineages of cancer cells forming the bulk of tumor. Owing to their properties that they share with normal stem cells, e.g., self-renewal and differentiation, or their ability to initiate and promulgate tumors in vivo, this subpopulation of cells has been given the nomenclature as cancer stem cells (CSCs). The other synonymous terms that are used to describe CSCs are “tumor-initiating cell” and “tumorigenic cell” [32, 33]. CSCs having tumorigenic potential and drug-resistant phenotypes have been identified in many human malignancies. Extensive investigation conducted over the last few years on the existence of CSC has gained immense attention due to its profound impact on cancer progression.

In fact, in a tumor mass, CSCs retain the potential to create all the differentiated cell populations that form the original tumor. These tumor-initiating cells that represents less than 5% of the total tumor mass, have been observed to play vital

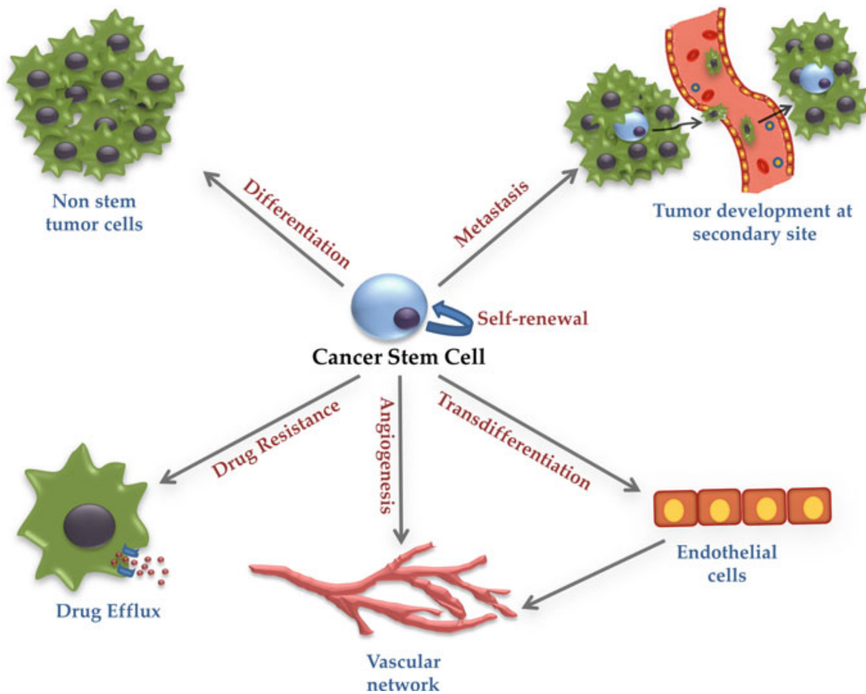

Fig. 2 Functional network of cancer stem cells: CSCs have the ability to self-renew as well as differentiate into the tumor cells to drive tumor initiation. CSCs are also the major culprit behind metastasis, drug resistance, and tumor angiogenesis

role in tumor-induced angiogenesis by shedding the major pro-angiogenic factor VEGF that invites endothelial cell proliferation, migration, and network formation [34]. Very recent reports signify that CSCs even contribute in VEGF-independent neo-angiogenesis by transdifferentiating into endothelial-like cells having potency for network formation [35, 36]. Melanoma-specific CD133^{+} CSCs have been observed to transdifferentiate into an endothelial-like state when cultured under endothelial differentiation-promoting conditions [37]. Accumulating evidence suggests a considerable role of these self-renewing CSCs in inherent resistance to traditionally used chemotherapy [38]. In addition, clinically, the residual risk of recurrence has been found to be higher in cancer patients with higher number of CSCs that acquire chemoresistance [39]. Moreover, intrinsic CSCs are endowed with higher migration potential than non-stem cancer cells due to the suppression of the tumor suppressor, E-cadherin [40]. According to Mukherjee et al. [41], intrinsic non-migratory CSCs convert non-stem cancer cells to migratory CXCR4^{+} CSCs with aggravated migration property, thereby shaping them for metastasis and recurrence in cancer patients. Besides, two major aspects of CSC physiopathology are in vivo quiescence and plasticity [42]. There exist different theories regarding the generation of cancer stem cells in a tissue by (i) the mutation in stem cells,

(ii) the mutation in non-stem cancer cells, and (iii) the dedifferentiation of non-stem cancer cells to CSCs. Recent findings also demonstrated that these undifferentiated, self-renewing drug-resistant cells themselves induce dedifferentiation of non-stem cancer cells to CSCs via paracrine signaling by shedding different growth factors, cytokines depending on the microenvironmental conditions [39, 41] and thereby increasing CSC pool in a tumor mass. Recent report demonstrates microRNA (miRNA) regulation of self-renewal and differentiation of CSCs of various types of cancers, e.g., lung cancer, head and neck cancer, breast cancer [43]. Development of miRNA-based therapeutics may, therefore, help control CSC population in diverse forms of cancers in the future. Furthermore, the CSCs are strongly regulated by the circumambient microenvironment, which also influences tumor responses. Above discussion signifies that CSCs play critical role in tumor development and progression. Therefore, it is necessary to understand how these clinically important CSCs are controlling the cell fate decision.

4 Cancer Stem Cell Niche

The highly specialized microenvironment where CSCs reside within the tumor and have their fates decided is referred to as the cancer stem cell niche. Within the tumor microenvironment, they have been identified as anatomically distinct population of cells. The CSC niche includes the non-stem cancer cells (NSCC) of the tumor, the adjacent stroma, blood vessels, fibroblasts, immune cells, extracellular matrix proteins, and secretory factors that decide cell proliferation, self-renewal, and fate of CSCs, either directly or indirectly (Fig. 3) [44]. Reportedly, any stem cell has limited function outside its predefined niche because of its dependence on the local microenvironment for signals determining cell growth, proliferation, and differentiation [45]. The niche of CSCs in the primary tumor potentially decides the progression of tumors to a more malignant state [46]. The CSC niche may be acquired by CSCs by either utilizing the tissue-specific somatic stem cell niche that is already present, or expressing the soluble factors which signal between the specific microenvironment and CSCs. The cells of the surrounding stroma provide the basic support framework essential for maintaining the tumor microenvironment. The paracrine and autocrine functions of the cytokines released by mesenchymal stem cells (MSCs), which are multipotent stromal cells, enhance cancer stemness. Impeded access to the blood vascular system results in lack of oxygen (hypoxia) for the tumor which enhances stemness through dedifferentiation and activation of stemness-promoting genes [47]. Moreover, the reactive oxygen species (ROS) produced due to hypoxia enhance epithelial to mesenchymal transition (EMT) and CSC survival [48]. Cancer-associated fibroblasts (CAFs) present in the CSC niche can cause increased proliferation, ECM production, and stemness via various signaling pathways like WNT and Notch [49]. Although Notch signaling impedes cells from responding to differentiation factors, it aids in stem cell maintenance and cell fate determination [50]. On the other hand, tumor-associated

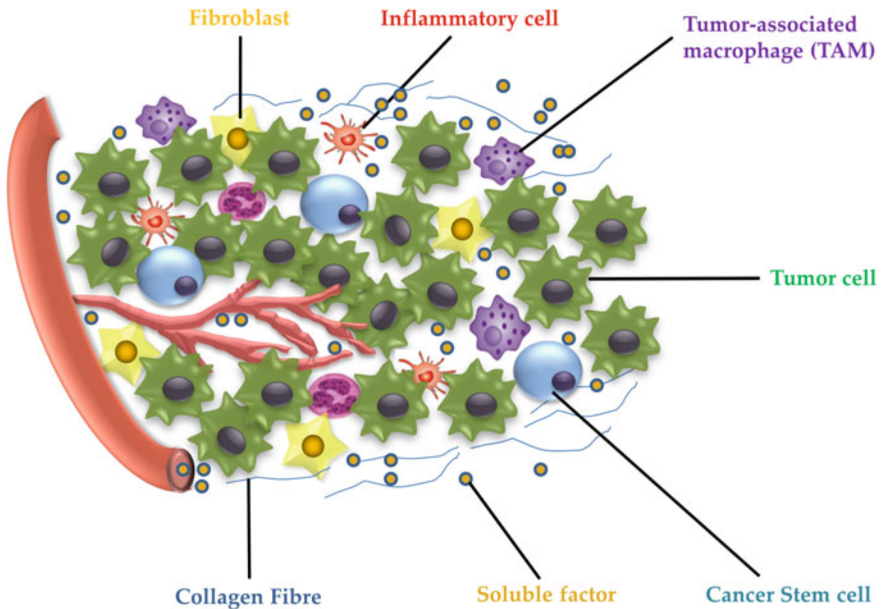

Fig. 3 Tumor microenvironment and the CSC niche depicting various cellular subtypes and extracellular matrix involved in CSC functional network

macrophages (TAMs) present in the tumor milieu secrete transforming growth factor beta (TGF-β) that can cause immunosuppression by recruiting regulatory T cells (Tregs) [51] TAMs show plasticity in their phenotypes and may act as alternatively activated M2 macrophages by supporting tumor growth. They also induce ECM breakdown, tumor invasion and metastasis [52, 53]. Matrix metallo-proteinases (MMPs) degrade the ECM components in tumors and up-regulate invasion, angiogenesis, and metastasis in tumor cells by releasing cytokines from the ECM [54, 55].

Hence, all these numerous factors contribute to the final structure and function of the microenvironment. The CSCs are, therefore, armed with an arsenal of techniques through which it can manipulate its niche and the tumor microenvironment to assist with the initiation, invasion and metastasis of cancer. While on the other hand, the components of the CSC niche can regulate the growth, proliferation, self-renewal, and differentiation of CSCs via its vast array of signaling networks. An elevated interdependency is thus observed between these two essential facets of cancer: the CSC and its niche.

5 Tumor Initiation

Initiation of cancer is due to abnormal proliferation of neoplastic cells upon genomic alterations. Cancer-causing mutations alter the normal cell functioning, ultimately resulting in tumor development. The process of tumor initiation and subsequent progression is a multistep process involving different hallmarks [2]. Previous concepts mainly highlighted the role of genetic aberrations in inducing tumors. However, with the progress in research, the significant contribution of epigenetic changes has gained importance in context to cancer initiation [56]. In an interesting finding, the molecules involved in axon guidance were observed to regulate cancer initiation and progression. Eph receptors and ephrins significantly contribute in modulating the important steps of tumor initiation and progression. Upon subsequent knockdown of these molecules, tumor initiation was found to be perturbed [57]. Also, the immune system plays a crucial role in tumor initiation. In fact, immune system has been found to display a dual role in cancer initiation and progression, i.e., in certain cases, it exhibits tumoricidal response while in others furnishing tumor-enhancing effect [58]. The inflammatory response by the immune cells also significantly contributes to the process of tumor initiation and hence has been discussed in later sections. Further research in the domain of cancer biology has successfully identified a heterogeneous population of cells inside the tumor mass. Even though the cell of origin is unknown to a large extent, it is speculated that different subtypes represent discrete cells of origin during the time of tumor initiation [4]. The heterogeneity existing in the disease is largely attributed to the CSCs. These CSCs are responsible for the generation of diverse cell populations within the tumor [3]. Experimentally, CSCs have been identified as the cell population that has the ability to generate a tumor in xenograft models of human cancer [32]. Even though the detailed molecular mechanisms of cancer initiation are poorly understood, Boumahdi et al. [59] unveiled the essential role of SOX2 in controlling tumor initiation and CSC properties. Therefore, the CSCs contribute significantly in initiating the tumor. However, it is extremely important to discuss the role of the microenvironment which supports the initiation process. As already discussed, the CSC niche is a complex environment which houses various cell types that aid the process of carcinogenesis. It is acknowledged that the factors secreted by the CSC niche help in the self-renewal and maintenance of CSCs [60]. Moreover, the proteases secreted from the tumor microenvironment play a pivotal role in the initiation process. In this regard, reports have illustrated the role of Taspase1 in initiating cancer (Fig. 4). This protease has been reported to regulate the proliferation of cancer cells in which it is often up-regulated [61]. In contrast, down-regulation of Taspase1 disrupts cell cycle and enhances the expression of CDK inhibitors. This protease is mainly involved in the cleavage of MLL (mixed lineage leukemia) [62]. Therefore, Taspase1 has been identified as a novel therapeutic target. Further

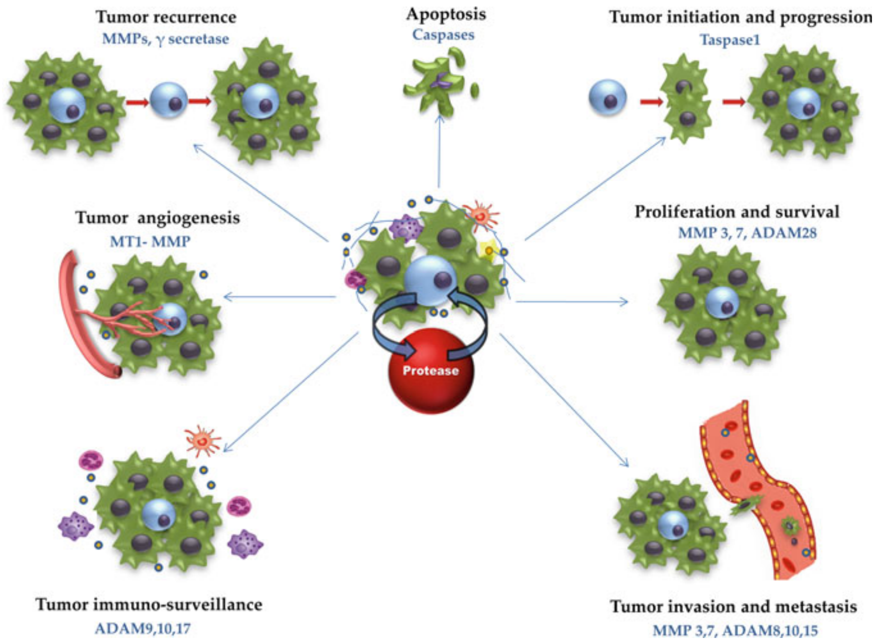

Fig. 4 Involvement of different proteases in CSCs that drives the hallmarks of cancer: tumor initiation and progression, proliferation and survival, invasion and metastasis, tumor angiogenesis, tumor recurrence, tumor immune surveillance, and regulation of apoptosis

research will help determine whether inhibitors of Taspase1 can be effective against CSCs [63]. Interestingly, Kumar et al. [64] have demonstrated using a biophysical model how extracellular matrix (ECM) proteolysis is responsible for changing the spatial distribution and number of CSCs. In this manner, ECM proteolysis plays a crucial role in regulating the growth of tumor as well as its relapse. In fact, ECM proteolysis has been observed to increase tumor growth. It is interesting to note that the CSCs in association with the tumor microenvironment play a critical role in ECM proteolysis. Pericellular proteolysis is known to be a leading process in generating a cancer-promoting extracellular milieu. The degradation of ECM components is achieved by the pericellular proteases [65]. Therefore, the regulation of these pericellular proteases is largely mediated by the tumor microenvironment. As mentioned above that ECM proteolysis regulates the distribution of CSCs, it will not be out of context to associate CSC and its niche with regulation of ECM proteolysis by pericellular proteases. Thus, the above discussion mainly highlights the critical role of CSCs and its associated niche in initiating carcinogenesis via proteases.

6 Tumor Angiogenesis

The development of tumor essentially depends on angiogenesis. Survival, proliferation, and metastatic spread of cancer require ample supply of nutrients, oxygen, and removal of waste material, thus making angiogenesis one of the most crucial steps in the development of cancer. In fact, angiogenesis is a prerequisite for metastasis since tumor cells must penetrate blood vessels and travel along the blood stream in order to reach secondary sites and colonize [66]. The means by which new blood vessels form is known as neo-angiogenesis, and it is mandatory for a continuous supply of oxygen, nutrients, and immune cells and also for the removal of waste products [66]. Since tumor growth and metastasis depend on angiogenesis, angiogenic factors are receiving a lot of attention. During the period of rapid growth, tumor cells release chemical signals that trigger angiogenesis [66]. In a study, done by Muthukkaruppan et al. [67], it was shown that when cancer cells were positioned in an area where angiogenesis was feasible, they grew in size (more than 2mm3), while they did not grow in an area devoid of angiogenesis. In the absence of adequate blood supply, tumor cells may become necrotic or even apoptotic [68, 69]. Hence, angiogenesis is essential for the progression of cancer. Activators and inhibitors are both involved in the regulation of angiogenesis. For example, tumor neo-angiogenesis is triggered not only by up-regulation of angiogenic factors but also by down-regulation of antiangiogenic molecules [70]. The CSC niche is also vital in inducing angiogenesis since endothelial cells, that control vascular function, are an essential part of the CSC niche [71, 72]. Many reports have depicted that CSCs via the CSC niche regulate the angiogenesis. Two most potent angiogenic factors are VEGF and bFGF [73]. Among the VEGF family members, VEGF-A has major role in angiogenesis. VEGF-A binds to VEGFR1 and VEGFR2 to trigger angiogenesis [74]. The role of VEGFR2 in angiogenesis is well defined while that of VEGFR1 is not that much understood. Recently, the role of CSCs in tumor angiogenesis has caught a lot of attention. This is due to the fact that CSCs survive various antiangiogenic therapies like Avastin (monoclonal antibody targeting VEGF-A) and Sunitinib (VEGFR inhibitor) due to the overexpression of drug efflux pumps, thereby causing tumor relapse. Bao et al. [75] demonstrated that glioma CSCs stimulate angiogenesis via VEGF. Recent studies by Tang et al. [35] have clearly depicted the role of CSCs in promoting angiogenesis. Ovarian CSCs have also been found to even transdifferentiate into endothelial cells and promote angiogenesis via CCL5 signaling. Given the important role of CSCs and its niche in angiogenesis, we would like to highlight the role of proteases in modulating angiogenesis.

Hypoxia has a major role in CSC-induced angiogenesis since experiments have shown that hypoxia enhances the CSC population in a tumor. Recently, the role of hypoxia in regulating the self-renewal property of CSCs by enhancing the activity of stemness proteins like Oct-4, Sox-2, and Nanog has been reported [76]. It is believed that hypoxia induces VEGF secretion from CSCs triggering angiogenesis though the exact mechanism is not clear. Hence now, it is widely believed that by

targeting hypoxia, we can actually inhibit CSC-induced angiogenesis. In addition, the equilibrium between the proteases and their inhibitors plays the role of a critical determinant in angiogenesis [77]. Proteases are involved in the degradation of the ECM, thus allowing endothelial cells to invade the tissues and penetrate the tumor stroma. In this regard, pericellular proteases have been reported to play an essential role in angiogenesis (Fig. 4). They include serine proteases, membrane-type matrix metalloproteinases (MT-MMPs), membrane-bound aminopeptidases, and cysteine cathepsins [78]. Reports demonstrate major roles of MT1-MMP, MMP-2, and MMP-9 in initiating angiogenesis [79]. Among these metalloproteases, MT-MMPs are predominantly membrane-bound while MMP-2 has the ability to localize to the membrane by binding to αvβ3-integrin and MMP-9 normally localizes to the membrane utilizing CD44 [80]. The overexpression of MT1-MMP has been found to be associated with increased tumor growth and vascularization in vivo [81]. The role of MT1-MMP in tumor angiogenesis is caused by various mechanisms like the activation of αvβ3 integrin, which leads to protection of the endothelial cells from apoptosis by fibrinolytic activity [82]. It is well known that VEGF is a crucial regulator of angiogenesis [83]. A subset of MMPs like MMP9 is proposed to cleave the ECM components and the c terminal region of VEGF, thereby releasing the biologically active VEGF from the ECM. MMP9-mediated VEGF release leads to its higher interaction with VEGFR at the quiescent blood vessels, thereby turning on the angiogenic switch [65]. Proteases are also shown to play differential role in regulation of angiogenesis by cleaving plasminogen and collagen XVIII to release angiostatin and endostatin, respectively, which have inhibitory effect on angiogenesis [84, 85].

Proteases trigger angiogenesis by activation of angiogenic growth factors, followed by degradation of the endothelial cell matrix and ECM [86]. They are also involved in the modification of the properties of cytokines and angiogenic growth factors. Angiogenesis requires migration of endothelial cells, which in turn is dependent on the protease–protein complexes which congregate at the migrating cell front [65]. Multiprotein complexes are formed in the lamellipodia and focal adhesions of migrating cells by utilizing MT1-MMP and urokinase (u-PA), thus favoring proteolysis, which provides adequate support for the survival and migration of endothelial cell [87]. However, uncontrolled proteolysis results in the loss of endothelial cell–matrix interaction, thereby hampering neovascularization [88]. MMP-9 and cathepsin L recruit accessory cells, which are derived from blood or bone marrow, for augmenting neovascularization [89]. During proteolysis, fragments of ECM and homeostatic factors are generated and these possess antiangiogenic properties [90]. Understanding the role of proteases in neovascularization is essential for recognizing new targets for inhibiting angiogenesis. The proteases are also thought to play a critical role in the vasculogenic mimicry, which is recently reported as a tumor vascular paradigm, where the CSCs form vessel-like networks that provide adequate blood supply to the growing tumor, independent of angiogenesis [91]. Since increasing evidences suggest that the neovascularization

and vasculogenic mimicry are associated with the CSCs, it can be well predicted that regulation of their niche by different proteases may be responsible for their angiogenic fate.

7 Tumor Invasion and Metastasis

Tumor invasion and metastasis are two major hurdles in the battle against cancer, which ultimately drive cancer relapse, poor prognosis, and high mortality. Metastasis is one of the most common types of complexities associated with cancer, treatment failure, and high mortality. During tumor progression, tumor cells undergo epithelial to mesenchymal transition (EMT) that involves reduced expression of epithelial markers and enhanced expression of the mesenchymal ones. The cascade of metastasis is a multistep process which includes local invasion, EMT—which induces stemness in cancer cells, intravasation, extravasation and colonization and rehabilitation at a distant location [92]. The ECM, being an important part of tumor niche and microenvironment, has captured the interest of researchers from a long time, since proteolysis and remodeling of ECM seem to work as key factors in cancer metastasis and invasion.

Interestingly, recent reports signify that although the tumor tissue comprises of heterogeneous population of cells, only CSCs exhibit selective growth advantage to form tumors at distant metastatic sites [93]. Thus, the crosstalk between CSC niche, epithelial mesenchymal transition (EMT) process, and CSC is coordinated for initiating the metastasis at secondary tissues is of great therapeutic interest. In this regard, the role of matrix metalloproteinases (MMPs) is being extensively studied because of their potential to degrade and remodel extracellular matrix (ECM) and hence leading to an increase in tumor invasion and metastasis (Fig. 4). Moreover, enhanced expression of pericellular proteases is seen in many cancers and has long been associated with poor prognosis and patient survival. A flock of pericellular proteases, which can degrade all ECM constituents including proteoglycans, collagens, and non-collagenous glycoproteins, have been well identified and characterized [55]. In addition to primary tumor progression and invasion, these pericellular proteases also play significant roles in tumor cell incorporation into the circulation. It also modulates invasion and tumor formation at the distant secondary sites. Disruption of vascular barriers by various pericellular proteases has been reported. For example, MMP17 facilitates the detachment of pericytes from blood vessels, thereby aiding intravasation of breast cancer cell leading to an increase in vascular leakage [94]. Besides degradation of ECM, disruption of intercellular junction proteins and cell adhesion molecules like E-cadherin is also brought about by proteases, thereby diminishing physical barriers. E-cadherin is shown to be cleaved by MMP 3, MMP 7, ADAM 10, ADAM 15, and also by Cysteine cathepsins B, L, S [95–98, 65]. Cleavage of the cell attachment molecules abolishes cell–cell adhesion, thus promoting tumor cell invasion and metastasis. MMP1 is known to cleave and activate protease-activated receptor-1 (PAR-1) present on

endothelial cells, leading to an increase in endothelial permeability and ultimately transendothelial migration of cancer cells [99]. Degradation of Claudin and Occludin (important cell junction proteins) by MMP 2 and 9 breaks the blood–brain barrier and thereby promotes CNS invasion of cancer [100]. All these accumulated evidences are indicating toward a huge role of proteases, through a plethora of molecular mechanisms, in tumor invasion and metastasis.

Perceptually, $CD44^{+}CD24^{low/-}$ breast cancer stem cells showed increased expression of metastasis-related genes and induced lung metastasis in vivo [101]. Induction of EMT program in immortalized human mammary epithelial cells (HMLEs) results in an increased potential to form tumor spheres, and in the expression of stem cell-like markers [102]. The microenvironment of CSCs dictates the behavior and fate of the tumor [55]. CSCs interact with adjacent cells and ECM that regulate the rate of self-renewal, proliferation, and differentiation of CSCs and thus the destiny of tumor mass. MMPs have the ability to modulate adult stem cell niches by cleaving, degrading, and rearranging ECM molecules. For example, MMP14 (MT1-MMP) modulates HIF-mediated gene transcription of cytokines and chemokines inside the hematopoietic stem cell niche, thereby regulating its behavior [103]. In addition, MMP3 is shown to be involved in the sustenance of adult epithelial stem cells in the mammary gland by binding and inactivating non-canonical Wnt ligands [55]. The remarkable loss of tumor progression by knocking down of CSC marker CD44v6 (a variant of CD44 known for metastasis promotion) has been documented to depend on its capacity to collaborate with associating integrins and proteases [104, 105]. This demonstrated a novel role for the serine protease Granzyme B in up-modulating the epithelial-to-mesenchymal transition, which is an indicator of stemness gain, in colorectal cancer cells. In another study, the $CD133^{+}$ lung CSCs, that are responsible for the tumor metastasis and patients' survival, have been shown to promote the tumor invasion and metastasis via the up-regulation of MMP-9 expression [106].

Thus, the protease cascades, having intensive role in modulation of stem cell niches and remodeling of ECM, and "pre-metastatic niche formation," points toward its huge possibility in tumor invasion and metastasis by manipulating the cancer stem cells [23]. However, this area demands further attention of the researchers.

8 Proliferation and Survival

Cellular survival and proliferation are the major aspects of any developmental process including tumors. The course of tumor development involves maintenance of tumor cells and sustaining their proliferation. In this regard, CSCs are considered major contributors to cancer growth and progression although the precise roles of CSCs in governing tumor development, progression, and survival are under intense scrutiny. CSCs undergo symmetric and asymmetric divisions depending on the

requirement of the daughter cells to maintain their self-renewal capacities. In asymmetric division, old mitochondria upon segregation get distributed to the daughter cells which undergo differentiation. Contrary to this, symmetric division involves equal distribution of both young and old mitochondria between the two daughter cells. Thus, metabolism and mitochondrial biology play significant roles in deciding the fate of stem cells. Since tissue homeostasis is directly affected by these decisions, it is essential to understand the regulatory mechanisms which govern cellular metabolism. A correlation between the survival and CSC population has been reported in highly aggressive cancers like glioblastoma [107]. In fact, self-renewal property of CSCs (i) ensures maintenance of CSC pool and the metabolic defects can shift cell symmetric division, thereby leading to stem cell exhaustion [108], and (ii) generates the heterogeneous population of non-stem cancer cells that comprise the tumor mass. This information signifies that CSCs play a pivotal role in survival and growth of the tumor mass. Recent reports demonstrate that the matrix degradation mediates the cell growth and survival by different protease-mediated mechanisms regulating bioavailability of growth factors. A variety of critical growth factors are released as part of a protein complex which prolongs their half-life. However, upon association with these binding proteins, their availability to the cells is limited [109]. Proteolytic modification of these binding proteins is therefore necessary to release the growth factors and permit their interactions with the corresponding receptors to induce cellular proliferation (Fig. 4). As an example, insulin-like growth factors (IGFs) are released upon cleavage of IGF binding proteins (IGFBPs) by MMP7 and ADAM 28 [110–112]. MMP7 cleaves pro-HB EGF to produce the mature HB EGF. ADAM17 mediates the shedding of ectodomain of TGF-α [113], while MMP9, MMP2, and cathepsin B are involved in the release of TGF-β [114]. Thus, proteolytic processing is required by various growth factors in order to gain receptor specificity and activity. Apart from that, pericellular proteases are involved in the regulation of tumor cell survival by processing ECM, and induction of apoptosis. An interesting example in this regard can be the proteolytic processing of the Fas ligand [115]. Contrariwise, proteases have the ability to minimize tumor cell proliferation and survival by cleaving the ecto-domain of the cytokine receptors, ultimately leading to its inactivation.

Moreover, MMP-3 is reported to act as a regulator of Wnt-signaling, whereas MMP-7 regulates Notch signaling—two important signaling pathways leading to maintenance of pluripotency of embryonic as well as cancer stem cells [116]. Furthermore, MMP9 localizes to the cell surface utilizing the surface receptor CD44 to proteolytically activate TGFβ. On the other hand, protease-activated receptor PAR2/GSK3β pathway has been demonstrated to play a crucial role in regulating proliferation and survival of colon cancer stem/progenitor cells [117]. According to Amoury et al. [118], Granzyme B-based cytolytic fusion protein targeting EpCAM, overexpression of which is associated with CSC phenotype, has been found to eliminate triple negative breast cancer cells in vitro and also to inhibit tumor growth in vivo.

These reports on the whole indicate the possibility of these proteases to regulate the fate of survival of tumor by orchestrating regulation of CSCs.

9 Tumor Resistance and Recurrence

The phenomenon of drug resistance exhibited by tumors over the years is an emerging problem. The efficacy of the treatment is hampered, and the resistance to the chemotherapeutic agents ultimately results in tumor recurrence [119]. Despite the tremendous effort for targeted therapies against cancer, development of resistance to genotoxic drugs is a predominant hurdle in successfully eliminating the aggressive tumors [120]. The resistance phenomenon that is exhibited in response to chemotherapeutics is normally subdivided into two broad classes: (1) intrinsic or innate resistance, which mainly refers to the resistance potential that exists in the tumor cells before any exposure to chemotherapy, and (2) acquired or adaptive resistance, which normally develops during the treatment procedure of tumors. Acquired resistance may occur due to mutations emerging during the treatment or alteration of signaling pathways [119]. Intensive research in the recent past has identified that the CSCs are predominantly responsible for tumor resistance and relapse. The conventional chemotherapeutics fail to eliminate the CSCs residing in the tumor bulk which ultimately results in cancer relapse [121]. In this context, Liu et al. [122] observed that the CSCs associated with glioblastoma exhibit immense resistance to chemotherapy. The drug resistance gene BCRP1 along with MGMT (DNA mismatch repair gene) was found to be significantly up-regulated. Apart from this, aberrant expression of ATP-binding cassette (ABC) drug transporters in CSCs is essentially responsible for drug resistance [121]. ABCG2 and multidrug resistance-associated protein-1 (MRP1) have been implicated in protecting the CSCs from chemotherapy [123, 124]. Recently, research work from our laboratory has reported that the CSCs, upon treatment with chemotherapeutic agents, foster an inflammatory environment dependent on NFκB-IL6-pathway that enhances multidrug resistance and initiates stemness in the non-stem cancer cells [39]. Therefore, it is clearly evident that the CSCs contribute significantly in drug resistance and relapse. In this context, we would like to highlight the role of proteases in modulating the phenomenon of drug resistance in cancer via CSCs. The aberrant activation of the Notch pathway is found to be crucial in maintaining CSC self-renewal [125–127]. Further, investigations have revealed that Notch activity is essentially involved in imparting radiation resistance in cancer [128], and hence, this pathway is gaining attention by representing a critical target for overcoming radiation resistance [129, 130]. Interestingly, γ-secretase is involved in the processing of Notch-1. The intracellular domain of Notch-1 is activated by the proteolytic activity of this protease which assists its translocation to the nucleus in order to exhibit its function. Further research has highlighted the role of γ-secretase inhibitors in negatively regulating the Notch activity. The processing of Notch was impeded by these protease inhibitors, thereby affecting the Notch pathway activation. Lagadec et al. [131] demonstrated that the expression of Notch ligands and receptors was significantly down-regulated, upon induction of radiation, when the mammospheres were treated with a γ-secretase inhibitor. Also, Mamaeva et al. [132] have depicted a novel delivery vehicle using nanoparticles that accommodates γ-secretase

inhibitors for targeting CSCs. Thus, γ-secretase represents a potential target for essentially enhancing the effectiveness of radiation therapy. Apart from this, MMPs are significantly involved in activating protease-activated receptor 1 (PAR1) [133]. Further, Fujimoto et al. [134] highlighted the role of PAR1 in inducing multidrug resistance in gastric CSCs. Therefore, the above discussion showcases that proteases notably help the CSCs to become drug resistant which further assists in tumor recurrence (Fig. 4).

10 Apoptosis

Apoptosis or programmed cell death is a process that leads to changes in cell characteristics, thereby inducing cell death in multicellular organisms. However, a certain degree of resistance is observed in this intrinsic process during oncogenesis. Apoptosis is triggered via two pathways: (i) extrinsic pathway that involves the death receptor cascade and (ii) intrinsic pathway involving the mitochondria [135]. The two apoptotic pathways ultimately trigger the activation of cysteine proteases called caspases. Since the caspases are usually found in an inactive form, mild proteolysis of these proenzymes, either autoproteolysis or by other caspases, is essential for their enzymatic activation [136]. The crucial role of caspases in regulating apoptosis is evident from the investigations which concluded that the deficiency of FADD and Apaf-1, which assist the activation of caspases, results in impaired apoptosis in mice. Similarly, knockout of Apaf-1, which activates caspase-9, resulted in malformations in multiple tissues further characterized by developmental cell death in mice [137, 138]. Reports indicate that elevated levels of survivin mediate multidrug resistance and reduced apoptosis in breast CSCs [139]. The outcome of the WW domain containing oxidoreductase (WWOX) gene on apoptosis as well as cell cycle regulations in human ovarian CSCs was explored [140]. Results conferred that the WWOX protein was expressed stably in ovarian CSCs which inhibited its proliferation. The WWOX gene imparted its effects by down-regulating the expression levels of essential cell cycle regulators like cyclin D1-CDK4 and cyclin E-CDK2. In addition to this, WWOX gene can lead to the overexpression of JNK, Wnt-5α, and caspase-3 which contributes to the ovarian CSC apoptosis. The WWOX gene may thus prove to be an essential target for ovarian cancer therapeutic treatment imminently. An interesting finding by Jinesh et al. [141] portrayed a survival mechanism by CSCs, induced upon apoptosis. The cells undergoing apoptosis fuse to form “blebbishields” which form tumorigenic spheres. Activated caspases are involved in the formation of these spheres with studies highlighting the role of caspases in protecting CSCs from apoptosis by use of inhibitors. Similarly, reports have also indicated that Salinomycin causes apoptosis of breast CSCs by down-regulating the Hedgehog signaling modulators, Smo and Gli [142].

These are just a very few examples out of hundreds that demonstrate the fact that apoptosis of CSCs is essential in order to destroy the entire tumorigenic mass completely and have a better chance of survival. Apoptosis is an essential intrinsic mechanism of cell death in multicellular organisms that balances cell proliferation and cell death. As has been discussed above, cells which escape the cues that control apoptosis can lead to many debilitating and life-threatening diseases like cancer. However, this is just one side of the story. CSCs are master manipulators of their niche and local microenvironment. Here, they reign supreme. Thus, we need to also explore the fact that CSCs might themselves regulate the apoptosis of and sacrifice non-stem cancer cells (NSCCs) and other cells of the surrounding tissue when it proves advantageous to the CSCs. The cellular debris thus produced may help to occupy the attention of the immune cells and help the CSCs to escape immunosurveillance.

11 Inflammation and Immune Surveillance

Substantial clinical evidences have identified the role of inflammation in the development of cancer. Chronic inflammation has been found to foster an environment which is conducive to tumor development. Researchers have identified that in certain types of cancers the inflammatory environment is present before the occurrence or development of tumor. On the other hand, in certain tumor types, oncogenic insults result in an inflammatory tumor microenvironment [143]. This tumor microenvironment that houses an inflammatory state enhances the infiltration of tumor-associated macrophages (TAMs), nuclear factor kappa B (NFκB), inflammatory cytokines such as interleukin (IL)-6, IL-1, tumor necrosis factor (TNF), thereby consequently facilitating carcinogenesis [144]. The inflammation associated with cancer has been termed as the 7th hallmark of cancer, owing to its significant contribution in tumor development and maintenance [145]. Therefore, the “smouldering” inflammation associated with the tumor microenvironment contributes significantly in tumor development and proliferation [143]. In this context, the links between inflammation and CSCs are also being investigated. The immune cells present in the tumor microenvironment are responsible for the secretion of various cytokines which modulate cellular networks leading to CSC growth and maintenance [146]. Recent studies have highlighted the role of chemo-resistant CSCs in modulating the inflammatory states of tumor microenvironment [147]. Therefore, we can clearly understand the critical link between inflammation and CSC development. Interestingly, the CSC niche housing inflammatory immune cells orchestrates multiple functions via proteases. Hagemann et al. [148] have experimentally confirmed that the invasiveness of tumors is enhanced due to the secretion of matrix metalloproteinases (MMPs) by the TAMs. The observed overexpression of MMPs was dependent on tumor necrosis factor-α (TNF-α). Apart from this, it was also validated that breast cancer cells, overexpressing protease cathepsin B [149], enhanced the expression of protease cathepsin

B in tumor-associated monocytes in an IL-6-dependent manner [150]. Interestingly, cathepsin B was found to be involved in regulating the self-renewal of glioblastoma CSCs by inducing the expression of Sox2 and Bmi1 [151].

During tumor development, the cells of the immune system try to avert the occurrence of malignant cells, thereby inhibiting the progression of cancer. This is achieved by a mechanism termed as immune surveillance [152–154]. The tumor microenvironment plays a pivotal role in immune surveillance. Natural killer (NK) cells, which are part of the CSC niche, depict an essential role in regulating immune surveillance in cancer by secreting serine proteases called granzymes [155, 156]. Apart from NK cells, mast cells, present in the CSC niche, are also implicated in immune surveillance and granzyme secretion [157, 158]. Recent studies have also highlighted the ability of NK cells to preferentially kill CSCs via NKGD2-dependent pathway [159]. Interestingly, ADAM 10 (a disintegrin and metalloproteinase) and ADAM 17 proteases have been found to be involved in the shedding of NKGD2 ligands (NKGD2L) in tumor cells [160]. Recent investigations have revealed the role of tenascin-C, an extracellular matrix protein, in protecting CSCs from immune surveillance. The activation of T cells was prevented by this protein [161]. Consequently, it was also observed that ADAM 9 acted as a critical modulator in tenascin-C induced invasiveness in brain CSCs. The proteolytic activity of ADAM 9 facilitated the invasiveness of brain CSCs [162]. It is evident that the CSC niche modulates the functions of the immune cells via proteases. Thus, the above discussion provides an overview of the role played by proteases in modulating inflammation and immune surveillance in CSCs (Fig. 4).

12 Therapeutic Strategies

Proteases are involved in wide-ranging activities of tumorigenesis. As discussed above, they modulate numerous steps starting from tumor initiation, angiogenesis, survival proliferation, invasion and metastasis, which facilitate carcinogenesis. Therefore, involvement of different proteases in cancer paves the way to promising therapeutic approaches using protease inhibitors for patients suffering from metastatic cancer of poor prognosis. In a report, Remacle et al. showed that MMP14 influences tumor growth by its HPX domain in a breast cancer mouse model [163]. For this purpose, Developmental Therapeutics Program (NCI/NIH) virtual ligand screening compound library was utilized in order to determine specific inhibitors of the HPX domain of MMP14. But clinical trials organized with broad-spectrum MMP inhibitors (MMPIs) resulted in outcomes with unsatisfactory results. Alternative approaches include changing the proportion of proteases and protease inhibitors or restricting the inhibitory effects of individual proteases by using engineered protease inhibitors called TIMPs. Further, modification of TIMPs with the help of strategies involving mutagenesis enhanced the binding capacity for the target protease [116, 164]. An alternative strategy for directing TIMP activity to the plasma membrane involves using constructs of TIMPs fused to GPI anchors. This

resulted in a shift in the TIMP-1 activity from ECM to the cell surface, ultimately resulting in apoptosis and decreased proliferation of renal carcinoma cells [165].

In a recent report by Darini et al. [166], it has been observed that HIV-protease inhibitors (HIV-PIs) selectively target CSCs from tumors with different origins. HIV-PIs were observed to reduce proliferation and induce cell death efficiently in a dose-dependent manner with enhanced specificity for CSCs in comparison with the entire cell population of cancer cells or embryonic stem cells. Lopinavir (LPV), the most effective HIV-PI among the studied, has been shown to impair self-renewal and induce apoptosis of CSCs, consequently inhibiting CSC-induced allograft formation in vivo. Killing of CSCs by LPV involved activation of caspase 3 (CASP3) expression and subsequent cleavage of poly (ADP-ribose) polymerase (PARP-1). Moreover, two key pharmacophores within the LPV structure were recognized, which enable LPV to selectively target CSCs. In addition to this, several protease inhibitors that are used in combination with other drugs to treat HIV infection may also be effective in targeting certain types of cancer [167]. Altogether, these reports identify protease inhibitors as compounds having selective toxicity for CSCs with a stemness signature.

13 Conclusion

With the advent of the CSC theory, the intriguing questions, relevant in the field of cancer biology, which remained unanswered for many years, have been addressed to certain extent. The CSCs have been predominantly identified as the master regulators in imparting distinct properties and characteristics to the tumors. It is implicated that the proteolytic networks are critically involved in the functioning of the CSCs. The niche of the CSC, which harbors different cell types, assists the CSCs in their activities. The proteases are involved in the regulation of distinctive cellular functions which ultimately modulates the development of cancer. The above discussions have highlighted the critical crosstalk between CSCs and proteases and how they are intricately involved in regulating the multiple steps of carcinogenesis starting from tumor initiation, angiogenesis, invasion and metastasis, proliferation, and survival. Apart from this, the CSCs also significantly modulate the process of apoptosis via proteases. The resistance exhibited by the tumors to various drugs ultimately leads to recurrence of the disease. In this context, the proteases play an essential role in drug resistance. On the other hand, the proteases have been found to display a dual role in regulating immune surveillance of CSCs. Therefore, our discussion emphasizes the widespread activities of proteases, which are majorly regulated by the CSCs and its associated niche, in determining the progress of tumor development and sustainability. Hence, antiprotease therapeutics may be regarded as a single bullet through which multifaceted proteolytic networks can be targeted.

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Role of Proteases in Tumor Immune Evasion

Abir K. Panda, Sreeparna Chakraborty, Kirti Kajal, Dia Roy,
Tania Sarkar and Gaurisankar Sa

Abstract

Proteolysis or protein degradation is an important biological function which has been attributed to a major class of enzymes called proteases. In the living system, a fine-tuning between the activity of proteases and their counterparts can be seen, and disruption of this balance leads to the occurrence of various diseases including cancer. Several studies indicate that protease activity highly correlated with cancer advancement. The hallmarks of cancer like tissue evasion and metastasis, apoptosis, angiogenesis largely depend on proteolytic degradation and protease activation. Protease also contributes to avoidance of immune system, one of the emerging hallmarks in cancer progression. The immune system can recognize intracellular pathogens including cancer and elicits an effective immune response to restrict their activity and subsequently protect the host. In cancer microenvironment, immune cells are unable to recognize tumor neoantigens as well as incapable of inducing proper immune response for the clearance of tumor cells. Recent studies found that proteases not only promote tumor cell migration and metastasis it also encourages to maintain the tolerogenic tumor microenvironment by modulation of immune cell functions. Proteases are involved in several immune responses such as antigen processing and presentation, lymphocytes and neutrophil infiltration, activation of dendritic cells. The anomalies of protease actions dampen immune cell activities and establish immune tolerance. Inappropriate protease activities are responsible for inhibiting immunosurveillance and maintaining immune tolerance that ultimately leads to tumor immune evasion. Involvement of proteases in cancer suggests the use of protease inhibitors as anticancer drugs to targets not only tumor cells as well as tolerogenic immune cells to reeducate immune responses

A.K. Panda · S. Chakraborty · K. Kajal · D. Roy · T. Sarkar · G. Sa (✉)
Division of Molecular Medicine, Bose Institute, P-1/12 CIT Scheme VII M,
Kolkata 700054, India
e-mail: gauri@jcbose.ac.in

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and reestablish immune surveillance. This chapter summarizes the current understanding of the interplay between proteases and the immune cells of the body and their involvement in cancer progression.

1 Introduction

The immune system comprises of distinct specialized cells that act in a collaborative way to recognize the presence of foreign invaders and cancer outgrowth and induces specific effective immune responses to clear them from vertebrate's body. Although, the immune system is organized and orchestrated at various cellular, molecular, and systemic levels to discriminate and stimulate effective tumor immunity, but cancer also exploits several strategies to avoid immune surveillance and established immunosuppression that subsequently leads to cancer progression [1]. Tumor microenvironment mainly consists of tumor cells, stromal cells, extracellular matrix and immune cell that allow the cancer cells to foster proliferation, survival, metastasis, and evasion. Therefore, successful, effective immune responses against cancer thus hindered by several immune escape strategies such as tumor inducing tolerogenic environment, avoidance of immune systems, activation of negative regulatory pathways, and secretion of immunosuppressive factors [1, 2]. The immunosuppression can arise either from an outgrowth of escape tumor mutants or during tumor sculpting immune responses. The involvement of immune system in cancer development has long been witnessed. In 1867, Rudolf Virchow first observed that leukocyte infiltration highly correlated with fostering tumor inflammation. In 1957, Sir Macfarlane Thomas and Lewis Burnet postulated the evidence of tumor immune surveillance mechanisms: the immunological resistance mechanisms to eradicate cancer progression relentlessly. This hypothesis has been challenged for years due to lack of experimental validation. In 2003, Schreiber and colleagues put forward cancer immune editing hypothesis which consists of three different and integrated steps (a) elimination, (b) equilibrium, and (c) escape. This three "E"s of immune editing stages defined either cancer eradication (immunosurveillance) or cancer development (immune escape). Immunosurveillance takes place during the elimination process whereas Darwinian selection ensued in equilibrium phases and the clinically apparent tumor has been found in escape stages [3].

1.1 Elimination

In the elimination or cancer immunosurveillance stage, successful eradication of tumor development takes place without progression of subsequent stages. In the

first phase of elimination, once solid tumors begin to grow invasively it requires an adequate amount of nutrients through the production of stromagenic and angiogenic factors by the formation of new blood vessels or angiogenesis [3, 4]. Invasive growth damages surrounding tissues and induces tumor-specific inflammatory signals that ultimately triggers the infiltration of innate immune cells (dendritic cells, macrophages, NK/NKT, γδ-T cells, etc.). The tumor-associated antigens displayed by transformed cells recognized by innate immune cells such as γδ-T cells, NK/NKT cells which are then stimulated to produce IFN-γ. In the second phase, IFN-γ that initially made by innate immune cells induces the limited amount of antiproliferative and apoptosis mediated tumor cell apoptosis. IFN-γ also activates to produce numerous chemokines and monokines which include CXCL-10, CXCL-11, and CXCL-9 from cancer cells and nearby surrounding normal tissues [3, 5]. Several monokines and chemokines have strong angiostatic activities, thus perturbing the formation of new blood vessels or angiogenesis process within the tumor which also induces tumor cell apoptosis. Tumor cell debris generated either direct or indirect consequences of IFN-γ. In the initial stages, tumor cells are ingested by local dendritic cells, which home to draining lymph nodes. Chemokines and monokines produced at tumor inflammation also recruit several immune cells such as macrophages, dendritic cells, NK cells at the tumor site. In the third phases, tumor-infiltrating NK cells and macrophages transactivate each other to produce IFN-γ and IL2 in a reciprocal way. Activated NK cells and macrophages also induce tumor cell killing through several mechanisms such as perforins, granzymes, reactive oxygen, nitrogen intermediates, and necrosis-related apoptosis-inducing ligand-mediated cell death. In the draining lymph nodes, newly immigrated activated dendritic cells also induce tumor-specific $CD4^+$ IFN-γ^+ T cells (Th1 cells) that in turn stimulated the development of $CD8^+$ IFN-γ^+ T cells or cytotoxic $CD8^+$ T cells (Tc cells). In the last phase of elimination, both Th1 and Tc cells infiltrate into tumor sites and destroy the remaining tumor antigen-bearing cancer cells that show enhanced immunogenicity after exposure of locally produced IFN-γ and other chemokines [3, 5, 6].

1.2 Equilibrium

In this stage, some transformed tumor cells have become resistant and survived in an elimination process that eventually enters into a dynamic equilibrium. In equilibrium-phase immune cells, chemokines and cytokines especially lymphocytes and IFN-γ exert strong selection pressure that is insufficient to complete clearance of mutant-transformed tumor cells. The tumor bed contains several genetically unstable and rapidly mutant cancer cells. The Darwinian selection process takes place during this period and many of the original preexistent tumor cells destroyed and cleared although newly transformed cells arisen that carry different mutations, eventually resistant to immune attacks and withstand to survive [3, 5, 6]. Tumor cells exploit several strategies to demolish the constant threat by immune cells and thereby maintained a quiescent, dormant state. In equilibrium phase, either immune

surveillance or immune escape decision may take place [5, 7]. Depending on the situations, this equilibrium may swing toward either the elimination of tumor cells or their escape from immune surveillance. Equilibrium phase is the longest phase among three stages and may occur over a period of years.

1.3 Escape

In the escape phase, newly transformed mutant cells that acquired insensitivity and resistant to apoptosis escape from immunological detection constant immune attacks in immune surveillance stage. Mutant cells from escape stage acquired consecutive genetic and epigenetic alterations that constantly divide in an uncontrolled way that results clinically detected malignant cancer if left unchecked may cause the death of the host [5, 7, 8]. The highly malignant cancer cells modify themselves in such a way that are not detected by the immune system as a result effective immune response to complete clearance of them not successfully generated [9]. Moreover, avoidance of immune system ensues when cancer cells exploit several immunoevasion strategies like secretion of immunosuppressive factors such as TGFβ, IL-10, VEGF-A, PGE2, IDO, galectins that converts potent immunogenic immune cells become highly tolerogenic so that they cannot discriminate and destroy cancer cells. In addition to the death of T-effector and NK/NKT cells, shifting of M1 macrophages to M2 macrophages/tumor-associated macrophages (TAMs); conversion of immunogenic dendritic cells (iDCs) to plasmacytoid DCs; development of myeloid-derived stromal cells (MDSCs), and the generation of T-regulatory cells create a grave environment. Cancer cells completely paralyze the immune system and grow continuously in an uncontrolled manner [1, 7, 10].

Proteases play indispensable roles in several biological processes and are associated with several physiological conditions including cancer. Proteases are proteolytic enzymes that perform specific catalytic cleavage of peptide bonds and largely influence the cell behavior, cell death, and survival [11]. In 1946, Fisher first proposed degradation of extracellular matrix and successive tumor invasion into the surrounding normal tissue caused due to the tumor-associated proteolytic activities. The human degradome consists of at least 569 types of proteases that are divided into five subclasses. In human; 194 Metalloproteinases, 176 serine proteases, 150 cysteine proteases, 28 threonine proteases, and 21 aspartic proteases have been characterized to date [12]. The proteases can contribute all stages of tumor progression. Both intracellular and extracellular proteases can activate signaling cascades that are essential in cancer progression [13, 14]. Proteases regulate several biological activities which include proliferation, adhesion, apoptosis, autophagy, senescence, angiogenesis as well as immune evasion. The intracellular proteases are associated with aspartyl cathepsins and lysosomal cysteine-mediated degradation and removal of undesired or damaged endocytosed proteins. Moreover, cysteine proteases are responsible for tightly controlled apoptosis [13, 15]. The cysteine autophagins carry out self-eating that is mainly observed during starvation. Another cysteine protease deubiquitinases (DUBs) involved in the removal of ubiquitin

modification or ubiquitin-related modifier (SUMO) from proteins. All of these intracellular proteases are involved in protective mechanisms. The loss of function mutation of these proteases is responsible for cancer development which clearly indicates that they serve as a tumor suppressors [15]. On the other hand, extracellular protease frequently gets overexpressed in several types of cancers by activating oncogenic transcriptional pathways. Matrix Metalloproteinases (MMPs) are the major class of extracellular proteases which are highly involved in several types of cancer progressions. Cysteine protease and serine protease also show antitumor activities. Extracellular proteases and pericellular which include MMPs, cathepsins, kallikreins, prostasin (PRSS8), neprilysin (MME), testisin (PRSS21), dipeptidyl peptidase 4 (DPP4), ADAMTSs (disintegrin-Metalloproteinases with thrombospondin domains) exert tumor-defying activities [15–18].

Both extracellular and intracellular proteases directly or indirectly involved in cancer progression as well as responsible for tumor immune suppression. Immune cells such as macrophages, neutrophils, polymorphonuclear leukocytes secret intense-level matrix Metalloproteinases (MMP-8, MMP-26) that induce cancer inflammation and metastasis. Protease executes several immunosuppressive strategies such as apoptosis, autophagy, immune cell infiltration into tumor site, immune cell dysfunction (dendritic cells, macrophages, NK, and NKT cells) antigen presentation which converts immunosurveillance to immune escape and established tumor immune evasion [19, 20].

2 Protease and Immune Systems in Cancer Immune Evasion

2.1 Apoptosis and Involvement of Protease in Immune Evasion

Apoptosis refers to an energy dependent form of programmed cell death that appears to be a crucial phenomenon of several distinct processes including the cycle of normal cell death and rebirth, hormonal regulation as well as immune system functioning. This coordinated catabolic process requires the coordinated involvement of an array of cysteine proteases namely endoproteases called "caspases." Apart from the two distinct apoptotic pathways, namely death receptor-mediated pathway called extrinsic pathway and mitochondrial pathway called intrinsic pathway, there is a third pathway involving T cell-mediated cytotoxicity and granzyme/perforin-mediated apoptosis [21]. All three modes of apoptosis converge to a common pathway involving the cleavage of caspase 3 which results in breakage of DNA and fragmentation of nuclear as well as cytoskeletal proteins. Caspase 3 is the most important effector caspase which is involved in the activation of the endonuclease CAD. CAD normally stays in its inactive form via forming a complex with ICAD. In apoptotic cells, caspase 3 releases CAD via cleavage of ICAD. CAD then causes DNA degradation and chromosomal condensation [22].

Caspases, expressed by most of the cells in their proactive forms, when get activated allows the activation of other pro-caspases, thereby initiating a caspase cascade. This chain reaction amplifies the apoptotic signal that in turn results in rapid cell death. Activation of caspases marks the irreversible commitment of the cell toward apoptosis. Till date, caspases can be categorized as initiators which include caspase 2, caspase 8, caspase 9, and caspase 10, effectors or executioner caspases, namely caspase 3, caspase 6 and caspase 7 as well as inflammatory caspases, namely caspase 1, caspase 4, and caspase 5. Apart from these three subtypes, few caspases like caspase 11 and caspase 12 play significant roles in the regulation of apoptosis during septic shock and endoplasmic–specific apoptosis, respectively.

2.1.1 Perforin-Granzyme Pathway–Potent Immunosurveillance Mechanism Against Tumor

T_c cells can exert their cytotoxic effects on the target cells via both extrinsic and intrinsic pathways. Apart from these, T_c cells secrete perforin that create pores on the target cells specifically the tumorigenic cells with the subsequent burst of cytoplasmic granules through the pores inside the target cells, thereby causing effective apoptosis of the target cells [23]. Two major players of this pathway are granzyme A and granzyme B. Apart from direct cytotoxicity, granzyme B can activate the execution-specific apoptotic caspase 3, thereby bypassing the initial step of apoptosis. Cytotoxicity mediated by granzyme B helps in the proliferation of Th2 cell types. Since Th2-type biasness is a marked feature of tumor progression, it can be fairly concluded that granzyme B-mediated apoptotic pathway can prevent Th2-biased and hence tumor progression.

Unlike granzyme B, granzyme A facilitates T cell-mediated apoptosis without the aid of caspase. Inside the cell, granzyme A cleaves the nucleosome assembly protein SET which in turn form complex with the tumor suppressor protein NM23-H1. Cleaving of SET restricts the inhibition of the tumor suppressor protein NM23-H1 which plays a major role in immunosurveillance to prevent cancer via the activation of tumor cell apoptosis mainly by causing DNA nicking [24].

2.1.2 Apoptosis in Maintaining T Cell Homeostasis

Caspase-dependent apoptotic pathway plays a significant role in numerous vital immunological processes that include lymphocyte development, positive and negative selection and homeostasis of T cells [25]. In the periphery, silencing of autoreactive lymphocytes occurs via inactivation also referred to as anergy or the autoreactive cells are destroyed by receptor-mediated apoptosis [26]. Furthermore, central tolerance relies mostly on the intrinsic mitochondria-based pathway of apoptosis stimulated by the T cell receptor-mediated pathway. Clonal deletion of the autoreactive T cells may be hampered if there occur some defects in the intrinsic mitochondria-based pathway of apoptosis. Thus, both the extrinsic and intrinsic pathways of apoptosis are vital for maintaining T cell homeostasis and both these pathways are executed via the activation of a cascade of proteolytic enzymes, as mentioned previously, called caspases [27].

In a nutshell, caspases, crucial for apoptosis, provide one of the robust mechanisms to safeguard body against cancer development. Reduced expression of caspases is validated in a variety of cancers as for example inactivating CASP8 mutations have been reported in different types of cancers. WT caspase 8 emerges out to be a potent tumor suppressor gene as it leads to death receptor-induced apoptosis. Thus, activation of caspases can act as a “safety catch” to be used in cancer therapy [28].

3 Autophagy and Role of Proteases in Tumor Immune Evasion

Autophagy emerges from Greek word “autophagy,” meaning “eating self-components” refers to an array of highly regulated complex catabolic processes that lead to the degradation of surplus macromolecules or whole organelles, mostly mitochondria, thereby maintaining cellular homeostasis. The process involves the sequestration of the organelle within the double-membrane-bound vesicles called phagosomes which in turn fuse with a lysosome to form the autolysosome resulting in lysosomal degradation of the contents within the vesicles. This process encompasses the involvement of proteolytic enzymes that breaks the peptide bonds and thereby playing a major role in the initiation and progression of autophagy. Concomitantly proteases can also dampen autophagy under few circumstances [29]. In the current section, we will talk about an important protease family involved in autophagy-autophagins-4 mammalian orthologs of yeast Atg4 and its role in shaping the proper immune response of the body.

3.1 Role of Proteases in Autophagy

The initial step of macroautophagy involves the proteolytic cleavage of the autophagy-related proteins Atg8 by Atg4, a cysteine protease which in turn results in the fusion of Atg8 with phosphatidyl-ethanolamine and subsequently forms the autophagic vessel [30]. Based on the catalytic residues present in active site, Cathepsin A type of lysosomal hydrolase can be categorized into several groups, namely aspartic, cysteine, and serine cathepsins, which along with other factors cleaves the peptide bond of autophagy substrates facilitating the outflux of the products of autophagy. Apart from being a positive regulator of autophagy, some cathepsins, such as cathepsin L, help in degradation of specific protein constituents of the lysosomal membrane, namely GABARAP-II and LC3-II. Proteolytic processing of LAMP-2A by serine protease cathepsin A inhibits chaperon-mediated autophagy. Calpain, a non-lysosomal calcium-dependent protease, cleaves and inactivates major Atg proteins, thereby negatively influencing the execution of autophagy [31].

3.1.1 Mitophagy and Immune Cell Homeostasis in Context of Tumor Microenvironment

Mitochondria also referred to as the powerhouse of the cells plays an important function in various aspects of cell-mediated functions including production of energy, controller of apoptosis as well as a regulator of immune signaling. Therefore, maintenance of the functional attributes of mitochondria is of prime concern for the cell [32]. Mitophagy can be viewed as a specialized form of autophagy by which cell maintains an overall homeostasis of the mitochondrial population by identifying and repairing or eliminating the dysfunctional mitochondria from the system [33]. Mitophagy is also involved in supporting the immune system. Quiescent naive T cells after activation enter the log phase of growth. During log phase, the primed cells increase in size, undergo proliferation and finally differentiation to other subsets. Immune-suppressive regulatory T cell depends on fatty acid oxidation occurring in mitochondria for energy. Similarly, M2-like macrophages promoting the repair of tissue rely on beta-oxidation and oxidative phosphorylation for ATP production. Both regulatory T cells and M2 type of macrophages are predominant in the tumor microenvironment. Thus, dysfunction of mitophagy results in uncontrolled proliferation of regulatory T cells and tumor-associated macrophages (M2) which in turn results in tumor progression [34].

3.1.2 Role of Protease in Coordinating Mitophagy

Proteases regulate a number of proteolytic reactions in a signaling cascade, for a number of protein components. In this context, protein cleaving enzymes (AAA protease, Lon protease) are known to regulate the process of mitophagy. Comprising the first line of cellular defense, two membrane-bound AAA proteases maintain the integrity of the inner mitochondrial membrane by clearing the mitochondrial matrix from the damaged mitochondrial proteins. The Lon proteases remove the damaged proteins which constitute the matrix of the mitochondria. Apart from this, m-AAA and Lon proteases control mitochondrial biogenesis at the transcriptional and translational level, respectively [35]. Thus, overexpressing the proteases may be a way for prompt tumor progression as they positively regulate mitochondrial biogenesis and the large energy burst that enables a rapid proliferation of tumor-promoting immune cells (T-regulatory cells and M2 macrophages).

3.2 Autophagy and Innate Immunity in the Context of Protease System

Autophagy appears to be the one of the many arms of the body's first line of defense system and macrophages play an important role as a soldier of the innate immune system. In macrophages, secretion of active IL1b upon stimulation with LPS is controlled by autophagy [36]. ProIL1b is cleaved to its active IL1b form by the pro-inflammatory protease caspase 1. Strikingly, this activation step is controlled by an important autophagic protein Atg16 L1. It has been found that loss of expression of this autophagic protein leads to uncontrolled secretion of this

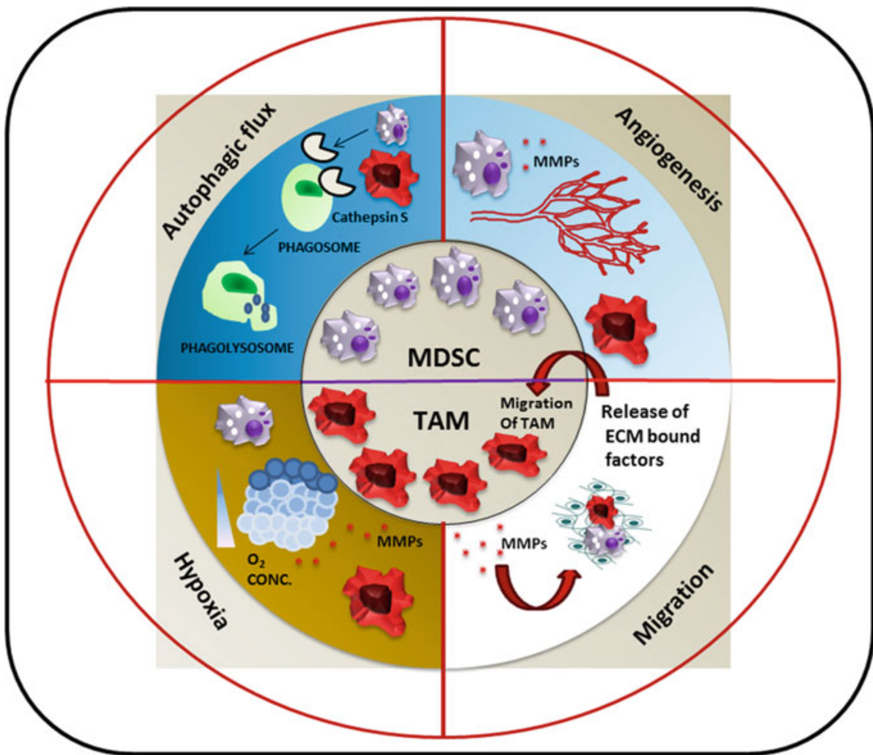

Fig. 1 **a** Autophagy and adaptive immunity: Lysosomal degradation of the antigen by cysteine protease-like cathepsin S produces small antigen peptides which are loaded onto the MHC-II molecules and are subsequently transported on to the cell surface. **b** Autophagy and innate immunity: Upon bacterial LPS stimulation, caspase 1 cleaves the pro-IL1ß to its active form. The functioning of caspase 1, in turn, is tightly controlled by an autophagy-related protein Atg16L1. Thus, the loss in activity of the autophagy-related protein leads to the uncontrolled secretion of IL1ß. **c** Tumor immunity and mitophagy: Membrane-bound AAA protease and Lon protease successfully remove the damaged misfolded proteins from the inner mitochondrial membrane and the mitochondrial matrix, respectively. This in turn positively regulates mitochondrial biogenesis and consecutive energy burst. This, in turn, leads to a rapid proliferation of regulatory T cells and tumor-associated macrophages and helps in tumor progression

inflammatory cytokine. Thus, tight regulation of autophagic process is of prime importance to prevent excessive inflammatory response which can be observed in autoimmune diseases as well as in many types of cancer (melanoma, prostate cancer) [37] (Fig. 1).

3.2.1 Autophagy: Role in MHC-Mediated Antigen Presentation in Context of Protease System

Autophagy plays a major role in modulation MHC class I and II antigen presentation which in turn regulates autoimmunity and tolerance as well as tumor

immunity. Macroautophagy and cell-mediated autophagy turn out to be the major player in mediating antigen presentation by MHC molecules. MHC class I molecules present the antigens from endogenous viruses, tumors, or self-proteins to the $CD8^+$ T cells [38]. Cytosolic proteases such as proteasome degrade these endogenous antigens which are subsequently transported to ER and bind to the nascent MHC 1 molecules. Apart from this, cysteine proteases such as cathepsin S help in the process of cross-presentation by degrading the exogenous antigens in phagosome via TAP-independent manner [39]. This process is particularly important as immunosurveillance mechanism against the tumor [40].

4 Proteases and DC Cell Inactivation

During antigen presentation, endogenous proteins are presented on MHC-I of DCs to CD8+ T-lymphocytes and exogenous proteins are presented on MHC-II and activate CD4+ T-lymphocytes. In another mechanism, exogenous proteins are presented on MHC-I and stimulate CD8+ T-lymphocytes. This procedure is called cross-presentation and it is the crucial defense mechanism against tumor cells [41, 42]. The formation of an immunological synapse between DCs and T-lymphocytes is critical for successful T cell activation and effective tumor cell elimination [43]. Matrix metalloproteinase 13 (MMP-13) regulates dendritic cells (DCs) immune biology. DCs express MMP-13, and it has an important role in the context of the tumor. MMP-13 enhances the antitumorigenic function of DCs by up-regulating MHC-I surface expression, cytokine/chemokine secretion, and T-lymphocyte activation. MMP-9 is crucial for migration of DCs in response to tumor antigens, but MMP-13 has no role in the migratory capacity of DCs. Inhibition of MMP-13 lowers the capability of DCs to activate CD8+ T cells, through reducing MHC-I surface presentation [44]. MMP-13 increases the surface expression of CD11c and maintains the DC cytokine/chemokine profile such as IL-6, IL-12, and IL-23. Pro-inflammatory cytokine IL-12 increases the expansion and survival of $CD8^+$ T cells by driving the help of CD4+ T cells toward a Th1 phenotype [45]. IL-23, another powerful pro-inflammatory cytokine, encourages the generation of IL-17-producing Th17 cells which can effectively kill tumor cells. Inhibition of MMP-13 down-regulates all the above-mentioned DC-derived pro-inflammatory cytokines with detrimental consequences on NK cells, Th17 cells, memory T cells, and $CD8^+$ cytotoxic T cells subsequently leading to decreased tumor cell apoptosis. Hence, MMP-13 may be a promising target for a therapeutic approach in malignancy.

5 Proteases in Macrophages and Myeloid-Derived Stromal Cell (MDSC) Inactivation

Tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSCs) deserve special attention. Monocytes circulating in the blood mature to form macrophages. These macrophages can be classified into M1 or classically activated macrophages and M2 or alternatively activated macrophages [46]. Tumor-associated macrophages possess the phenotype of alternatively activated macrophages (M2 types) and these TAMs help in tumor cell proliferation and neovascularization. Apart from these, TAMs dampen the adaptive immune response and incessant matrix turnover which leads to tumor progression via neoplastic transformation and tumor immune evasion. Similar to macrophages, MDSCs represents a class of myeloid cells which facilitates the progression of the tumor [47]. MDSC can be categorized into two major subgroups: monocytic and granulocytic subtypes. Although TAMs and MDSCs represents two different immune cell types, but the demarcation lines between them are fuzzy and they share many similar attributes. Herein, we will discuss the role of these two subsets in tumor progression via protease secretion.

5.1 Signaling Pathway Mediating Protease Secretion in TAMs and MDSCs

Macrophage secreting matrix metalloproteinase 9 (MMP9) acts as a major armor in the progression of tumor and thus detailed overview of their regulation in macrophage deserves special mention. It has been reported that IL6 possesses a regulatory role on MMP9 expression. IL6-mediated induction of MMP9 is modulated by two pathways (a) Cox-2 → PGE2 → MMP-9 pathway as reported in macrophages of murine origin and (b) IL6 positively regulates MMP9 expression via activation of $MAPK^{erk1/2}$ whereas JAK-dependent activation of IL10 negatively regulates MMP9 expression [48]. IL6 positively regulates MMP9 expression by facilitating co-induction of microsomal PGE synthase and Cox2 and concomitantly inhibiting the expression of 15-hydroxyprostaglandin dehydrogenase. Inhibition of the above-mentioned dehydrogenase leads to increase in the expression of prostaglandin E2 which in turn increases the expression of matrix metalloproteinase 9 [49] (Fig. 2).

5.2 Role of Proteases in TAM- and MDSC-Mediated Angiogenesis

TAMs and MDSCs exert a crucial role that enhances the dynamic extracellular matrix remodeling and degradation of the basement membrane which are important prerequisites for angiogenesis. Through the production of proteolytic enzymes and

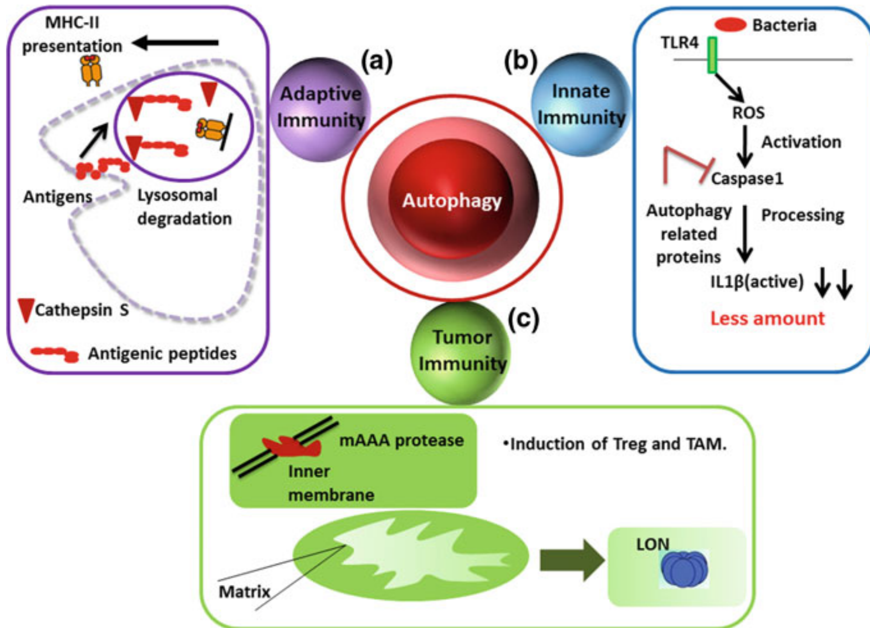

Fig. 2 Role of proteases in the context of MDSC(s) and TAM(s): Autophagic. Flux: Cathepsin S, a serine protease secreted by MDSC and TAM, facilitates the fusion of the phagosome with lysosome resulting in the formation of phagolysosome. Angiogenesis: Proteases secreted by MDSC(s) and TAM(s) help in the formation of new blood vessels, thereby playing a major role in tumor progression. Hypoxia: MMP1 and MMP7—two proteases secreted by MDSC(s) and TAM (s) create a hypoxic environment which in turn facilities tumor progression. Migration: Extracellular proteases secreted by TAM(s) and MDSC(s) play a major role in untangling the extracellular matrix, thereby facilitating the migration of these immune cells

MMPs, namely MMP-2, MMP-7, MMP-9 and MMP-12 [50], TAMs and MDSCs reorganize the extracellular matrix and degrade the basement membrane which helps in modulating angiogenesis and in turn provides a conduit for tumor cell extravasation. It has been reported that abrogation of MMP9 activity in MDSCs completely demolishes their role as tumor-promoting agent [51]. Heparin-bound growth factors like VEGF-A are released by the action of proteases like MMP-9 and cathepsin secreted by MDSCs and TAMs.

5.3 Role of Proteases in TAM and MDSC Migration

Apart from acting as angiogenesis modulating enzymes, these extracellular proteases (MMPs, cathepsins, and Urokinase-type plasminogen activator) facilitate macrophage and MDSC migration [52]. Two different molecular mechanisms involving migration exist. (a) Proteases untangle matrix proteins and/or release

cryptic chemotactic factors that help in migration. (b) Proteases target macrophage cell surface proteins to regulate adhesion sites. Few extracellular matrix molecules and their respective proteolytic fragments play a distinct role in the recruitment of innate immune components and the expression of inflammation-inducing genes [53]. Fragmented collagen I and elastin generated by MMPs play a crucial role as a chemoattractant for monocytes and macrophages [54].

5.4 Protease-Mediated Autophagic Flux in Macrophages

Proteases expressed by tumor-associated macrophages (TAM) mediate autophagic flux in TAMs, thereby accelerating tumor development. In this context, cathepsins—cysteine lysosomal proteases, play a pivotal role [55]. Cathepsin S found predominantly in lymphatic tissue, macrophages, and other APC helps in the fusion processes of autophagosomes and lysosomes followed by the degradation of the contents present within the vacuoles. Thus in the context of the tumor microenvironment, the activity of CatS plays an important role in macrophage autophagy and also mediates M2 type transition of TAMs [56]. As mentioned before, macrophages activated by IL4, IL13, and IL10 are referred as M2 macrophages which apart from having a profound anti-inflammatory property also induce angiogenesis and tissue repair [57].

5.5 Protease-Mediated Hypoxia

Proteolytic enzymes mainly MMP 1 and MMP7 released by TAMs and MDSCs play a major role in creating hypoxic tumor microenvironment [58]. Apart from facilitating angiogenesis, hypoxia facilitates the polarization of the macrophages toward the pro-angiogenic (M2) phenotype [59]. HIF-1α and HIF-2α-2 hypoxia-inducible factors play a role in regulating M1/M2 polarization with HIF-1α-regulating NOS2 expression and the M1 state and HIF-2α arginase 1 expression and the M2 state [60].

In a nutshell, few key points can be highlighted:

- TAMs and MDSCs localizing in areas of tumor invasion appears to be a potent source of proteases which in turn play a critical role at multiple stages in the metastasis cascade, including the invasion and intravasation step.
- Proteases facilitate tumor cell migration by facilitating the breakdown of components of the extracellular matrices (ECM) and basement membrane.
- Among different types of proteases, MMPs and lysosomal proteinases mostly released by macrophages received most of the attention.

These proteases help in tumor progression and invasiveness as well as in the activation of growth factors and cytokines by prodomain cleavage. These products range from anergic products like CXCL1, and CXCL4, and antagonists like CCL7,

or more potent chemoattractants like CXCL8, thereby modulating the composition of leukocyte population within a tumor.

6 Proteases in Antigen Presentation and Tumor Immune Evasion

6.1 Proteases and MHC-I-Mediated Antigen Presentation

T cell is one of the strongest arms of the adaptive immune response. Evolution of Adaptive immune system was done to recognize the products of intracellular partial proteolysis. $CD8^+$ T cells recognize peptides bonded with major histocompatibility complex (MHC) class I molecules (MHC-I); on the other hand, $CD4^+$ T cells recognize peptides bound to MHC class II molecules (MHC-II). MHC gene is composed of a large variety of polygenic stretch of which many conserved genes are there in addition to MHC-I and MHC-II molecules. Some of them express products important to MHC-I and MHC-II function. In different species, MHC encodes diverse MHC-I and MHC-II molecules. It is expected to have ascended by gene duplication. Structures of MHC-I and MHC-II have been identified which shows great variety in structures across species. The peptide-binding structure contains a membrane-distal groove. It is designed by two antiparallel α-helices overlapping an eight-strand β-sheet [61]. In the case of MHC-I, the groove resembles adjacent amino acid order designed by the N-terminal region of the single MHC-encoded subunit, α-chain, while for MHC-II it is formed by the apposition of the N-terminal regions of MHC-encoded α- and β-chains. The peptide-binding groove of MHC-II composed of conserved domain, one of the α-subunit and another of the β-subunit. For MHC-I, another protein, β2-microglobulin, a soluble product is non-covalently associated with the heavy α-chain. In the ER, assembly of the MHC class I occurs. It is assisted by chaperones like calnexin, calreticulin, the protein disulfide isomerase (PDI), and ERp57 [62].

Peptides are the products of proteolysis. The majority of proteolysis is occurring inside the proteasome of the cytosol. Proteasome is a cylindrical complex comprised of four stacked rings which form a central pore. The outer rings of the proteasome are composed of α-subunits. The middle two rings are composed of β-subunits. β1, β2, and β5 establish the active proteolytic machinery and function of α-subunits is to maintain a gateway through which proteins enter into the proteasomal barrel [63]. LMP2 and LMP7, subunits of a subset of proteasomes, are proteins encoded by MHC genes and believed to play a role in antigenic peptide formation. Interestingly, expression of LMP2 and LMP7 is enhanced by cytokine IFN-γ [64]. Precursor peptides produced from self- and nonself-proteins are then transported by heterodimeric transporter, viz TAP1 and TAP2, which are associated with antigen presentation, from the cytoplasm into the ER. Chaperone tapasin is a constituent of the multimeric peptide-loading complex (PLC), which is required for stabilization of TAP and eases peptide packing onto MHC class I molecules [65].

Another ER luminal component that is important for the correct expression of MHC-I-peptide complex is an aminopeptidase [66, 67]. These proteases are involved in the last and vital step of the production of MHC class I-binding peptides because only optimally clipped peptides from the antigen with a definite binding motif are precisely loaded onto MHC class I. Then, they are transported to the cell surface for recognition by CD8$^+$ T cells or CTLs. Hence, ER aminopeptidases are a crucial determinant of antigen processing and presentation. This aminopeptidase is named in the mouse as endoplasmic reticulum aminopeptidase associated with Ag processing (ERAAP) in the rat [66] and endoplasmic reticulum aminopeptidase 1 (ERAP1) in the human [67]. A structural change is required for the cleavage of antigenic protein by aminopeptidase which is specially induced by a longer peptide and thus prevents over the trimming of TAP-translocated peptides, to a size that would remove their skill to bind MHC-I [68]. The expression of aminopeptidase is also cordially regulated by different cytokines, viz IFN-γ, TNFα.

Distinct histology of human tumors shows that they express the very low level of MHC class I surface antigens, which may be due to variation or inhibition of the generation of numerous MHC class I-mediated antigen presentation machinery (APM) [65, 69]. Mutations and/or deletions in β2-microglobulin are noticed in colon carcinoma (21%), melanoma (15%), and other tumors (<5%) [69]. But in some neuroblastoma and melanoma mutations in TPN and LMP subunits are also reported. These mutations are either point mutations or base-pair deletion mutation, and this modifies their epigenetic, transcriptional, and posttranscriptional regulations. For example, in some esophageal squamous cell carcinoma, RCC and colon carcinoma-altered epigenetic modifications, viz methylation and histone deacetylation of APM components, are observed and could be renovated by handling with histone deacetylase inhibitors or DAC [70]. Apart from this, in some cases IFN-mediated induction of MHC class I APM is also repressed, which is caused by different anomalies in the IFN-γ signal transduction cascade [71]. Moreover, distinct expression profiles of both cytosolic and ER-resident aminopeptidases have been defined in some tumors [72], and the aberrant production of these peptidases affects MHC class I antigen presentation. In many human tumors, viz gastric cancer, thyroid cancer, renal cancer, melanoma, the distinct expression pattern of aminopeptidase N (APN) or CD13 is observed [73, 74], although the underlying molecular mechanisms associated with this differential expression pattern remains to be explored.

6.2 MHC-II-Mediated Antigen Presentation

MHC class II molecules present exogenous antigens to CD4$^+$T-lymphocytes. Three types of MHC-II molecules, viz HLA-DR, HLA-DQ, and HLA–DP, are present in human. The antigen-binding groove in MHC-II is consists of a position of the N-terminal regions of MHC-encoded two α- and β-chains with each side of the peptide can extend the binding groove. Assembly of MHC-II molecules occurs within the ER, smoothed by a specific chaperone, the invariant chain (I chain)

whereas functional activation occurs in endosomal compartments where antigenic peptides are formed by proteolysis. I chain is a type-II transmembrane glycoprotein and non-polymorphic, which accumulates in the ER as homo- or heterotrimers, involving p33, p35, p41, and p43 in humans [75]. The function of this I chain is to prevent antigenic peptide binding with MHC-II αβ-dimers in their early biosynthesis stage within ER, and this prevents cross-presentation of the antigenic peptide specific for MHC-I [76]. Next, MHC-II-I chain complexes are routed from ER to the endocytic pathway by trans-Golgi network (TGN) and endocytosis from the cell membrane (1) which is directed by I chain di-leucine motifs [77]. In acidic endosomes, progressive proteolysis releases I chain, and an unevenly extended peptide of 20 residues remains bound to MHC-II antigenic binding groove which is known as CLIP [78]. MHC-II alleles have a low affinity for CLIP and are predisposed to the advancement of autoimmunity because premature release of CLIP favors the choice of epitopes from autoantigens or self-peptides within different endosomal compartments [79, 80]. The release of CLIP from MHC-II is favored by a different MHC-encoded glycoprotein, HLA-DM [81]. In the late endosomes, HLA-DM promotes a conformational change in MHC-II which induces dissociation of CLIP, similar to tapasin-mediated MHC-1 peptide editing. HLA-DM removes low-affinity peptides from MHC-II binding groove, and this ensures the buildup of MHC-II complexes with high-affinity peptides [82]. On the other hand, the function of HLA-DM is further inhibited by HLA-DO which is also MHC-encoded MHC-II-like αβ-heterodimer [83] (Fig. 3).

Many pathogens, viz viruses, bacteria, and fungi use endocytic pathways as a gateway into cells, but this favors their antigenic presentation as well as immune recognition. In many cases, disruptions of these pathways allow immune evasion [84]. Clathrin-mediated endocytosis or phagocytosis and macropinocytosis remain common routes that are involved in antigen internalization. They are then sorted to vesicular organelles for processing in endosome and presentation by MHC-II molecules (1). All of these pathways exist in all the professional antigen presenting cells (APCs), viz macrophages, DCs, and B lymphocytes, although some differences in competence and regulation persist among them. In phagocytosis, internalized antigens enter into the phagosome and early endosome, which then converted to late endosome containing reactive oxygen species (ROS), proteases, and antimicrobial agents by a series of event, which favors protein denaturation and proteolysis. Early endosomes are converted first into late endosomes then into lysosomes by a sequential increase in luminal acidification as well as by accumulation of different proteases delivered by TGN-derived vesicles. The functional activity of lysosomal is influenced by endosomal pH [61, 85].

Many endosomal proteases, viz cathepsins B, D, E, F, K, L, and S participate in I chain degradation as well as in antigen processing [86, 87]. Cathepsins B and D are two major aspartyl and cysteine proteases, respectively, that are involved in antigen degradation, but splenocytes which do not have abundant cysteine protease unexpectedly showed normal Ii degradation. These indicate that both cathepsins B and D are dispensable for MHC-II antigen presentation [88]. Alternatively, other two

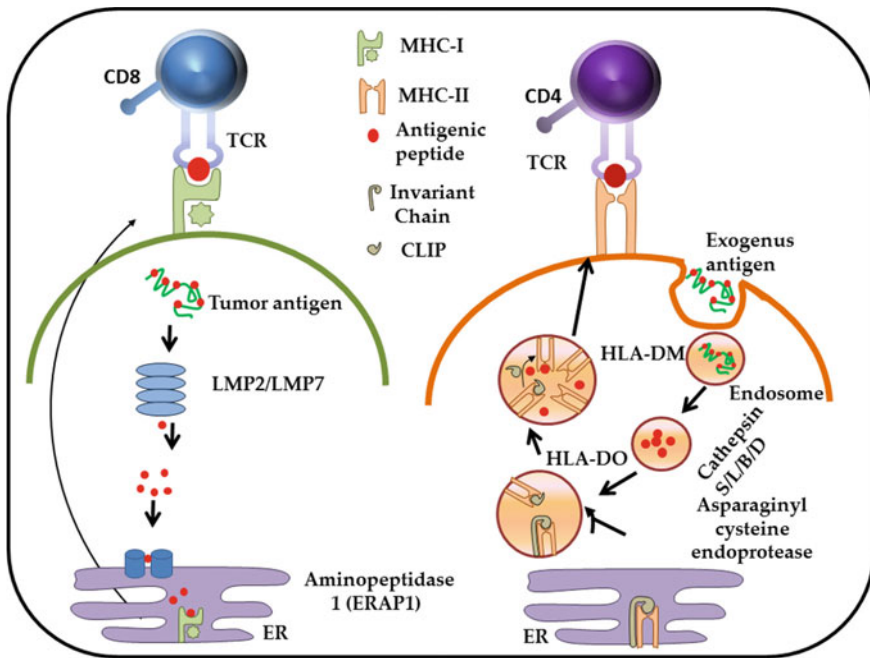

Fig. 3 Endogenous and exogenous antigen presentation pathways showing the involvement of CD8+ and CD4+ T cells, respectively. Aminopeptidase 1 (ERAP1) is involved in the processing of endogenous peptides; on the other hand, cathepsins S, L, B, and D and asparaginyl cysteine endoprotease are involved in the processing of exogenous peptides

lysosomal cysteine proteases, viz cathepsin S and cathepsin L, have distinct and indispensable roles in antigen presentation.

Cathepsin L is a papain-like cysteine protease, similar like cathepsins B, K, F, and W. These cysteine proteases contain a pro-region at their N-terminus within the active site which prevents its untimely activation during their journey through the ER and Golgi [89, 90]. The pro-region also played a role in the proper folding [90]. Mannose-6-phosphate receptor-dependent pathway is responsible for the transportation of the protease endosomes [69] and where the pro-region dissociates by the acidic environment within the endosome which finally results in the enzyme activation [91]. Cathepsin L or the human homolog, cathepsin L2 and cathepsin V, is abundantly expressed in the thymus [92]. Another member of the papain family of cysteine proteases is Cathepsin S which is mainly expressed in some professional APCs, viz DCs, macrophages, and B cells [93]. This protease is responsible for the generation of the different epitopes from the antigenic peptide. In the absence of cathepsin S germinal center, the formation is impaired [93].

Endosomal antigens are processed by many other exopeptidases and endopeptidases which directly affect the construction or destruction of epitopes that are recognized by $CD4^+$ T cells. Asparaginyl cysteine endoprotease (AEP) is first

discovered in human B cells [94, 95]. This Asparaginyl cysteine protease induces the first cuts in the protein, which further makes the additional processing sites accessible to other cysteine proteases [95]. But this protease plays negative effect in the processing of some antigenic peptides, i.e., destructs some of the immunodominant epitopes of myelin basic protein (MBP) [96]. Apart from these, closely related to caspases and separases is AEP. It is capable of degrading I chain [97]. AEP is insensitive to leupeptin [95, 96] and plays a role in the first couple of steps of I chain cleavage and AEP-cleavage sites mutation in I chain inhibit the generation of intermediate fragments and radically curtailed the rate of MHC class II expression [95].

Lysosomal cysteine proteases which are the critical dictator of the multiple steps in the endosomal pathway are very precisely regulated. These cysteine proteases are appointed to the maturing phagosome in a very ordered fashion. For example, cathepsin S, cathepsin L, and cathepsin B are stable and active within a span of wide pH and they are incorporated specifically into late endosomes. Conversely, ap41 fragment of I chains binds keenly to the active site of cathepsin L [98, 99] and regulates the activity of this cathepsin in a negative feedback loop. Along with these, also the inflammatory mediators, viz IFN-γ, up-regulate the expression of cathepsins in different cells [100]. In macrophages, IFN-γ increased cathepsin S activity to mediate I chain degradation.

Different endogenous TAAs, viz transmembrane proteins or cytoplasmic and nuclear antigens, are endocytosed and sent to lysosomes for degradation and finally presented by MHC class II molecules [101]. But, this MHC class II antigen processing and presentation pathway is portentously altered as a part of tumorigenesis process. Impairments in the presentation of tumor-associated antigens (TAAs) to MHC class II molecules certainly favor the evasion of tumor immune “escape.” Although MHC class II does not express by several solid tumors and infiltrating APCs engulf tumor cells and present the antigenic peptide to $CD4^+$ T cells. Some of the tumor cells can present antigens, but the absence of co-stimulatory molecules promotes tolerance. MHC class II molecules are often expressed in tumor cells in breast and colorectal cancer [102]. The MHC class II molecules do not typically express in normal breast epithelium, but MHC expression phenotype is induced in the presence of different hormones or cytokines [103]. However, diverse important constituents of the MHC class II pathway is often lost in MHC-II-expressing tumor cells. Despite high expression of surface MHC class II, B cells from B-cell chronic lymphocytic leukemia (B-CLL) patients have limited capacity to present a soluble antigen [104]. In different adenomas and carcinomas, I chain expression is very high which certainly indicates the malfunctioning in the degradation of I chain by different proteases, and this leads to the inability of MHC-II to present the associated TAAs [105]. While considering tumor vaccines, genetically engineered tumor cells express MHC class II, but they do not express Ii [106], and thus, the binding of a palette of antigens (including TAAs) to MHC class II molecules increases considerably over an extensive variety of compartments. Moreover, nowadays, milatuzumab, the MHC-I chain-specific monoclonal antibody is used as immunotherapeutic agent [107].

In various tumor cells, different cysteine proteases, viz cathepsins S, B, L, H and D, are highly expressed and predominantly involved metastasis by the degradation of ECM. In lung and prostate tumors, the cathepsin S level is elevated [108, 109]. But interestingly, apart from tumor cells, increased cathepsin S expression has been observed in alveolar macrophages, tumor-infiltrated macrophages and B lymphocytes, and a higher risk of death is associated with patients with low levels of cathepsin S [110]. These indicate that in lung tumors, cathepsin S fuels the defense mechanisms probably by enhancing the MHC-II-mediated antigen presentation.

7 Proteases in T Cell and Neutrophil Infiltration

The extracellular matrix (ECM) represents a structural support as well as an obstacle for moving cells, such as T-lymphocytes. T cell migration through ECM involves adhesive and proteolytic interactions with components of the ECM, including degradation of ECM by proteolytic enzymes such as matrix Metalloproteinases, serine proteases, and cathepsins. But rapidly moving T cells use non-proteolytic migration which depends on gliding and squeezing through the ECM which is independent of collagenase activity. ECM-degrading enzymes are expressed at mRNA level in rapidly moving T cells, but they do not express at the cell surface. Consequently, non-proteolytic T cell migration is insensitive to treatment with therapeutic protease inhibitors. All the information provides an interesting approach to MMP inhibitor therapy in cancer patients [111].

Polymorphonuclear neutrophils (PMNs) play an important role in the progression of pancreatic ductal adenocarcinoma (PDAC). PMNs have attracted to the tumor site by tumor-secreted chemokines which correlate with poor prognosis. PMN can kill tumor cells directly or indirectly by antibody-dependent cell-mediated cytotoxicity (ADCC), but on the other side, PMN has various detrimental effects, such as secretion of matrix-degrading proteases [111]. Once activated, these proteases promote turnover of the ECM components, thus facilitating angiogenesis and tumor cell migration. Neutrophil elastase, cathepsin G, proteinase 3, and ADAMs are the most powerful serine proteases that are secreted by neutrophils [112]. Neutrophil elastase degrades a variety of substrates such as elastin, collagen, cadherins, proteoglycan, fibronectin, platelet receptors, complement receptors, thrombomodulin, growth factors. Imbalance of elastase and its inhibitors plays a crucial role in liver, lung, or colorectal cancer advancement [113]. Infiltrated neutrophils are the major source of elastase in the pancreatic tumor, and an increased concentration of this protease is associated with poor prognosis [113]. Neutrophil elastase cleaves the tumor cell adhesion molecule E-cadherin, allowing tumor cell to separate from the primary tumor, followed by epithelial to mesenchymal transition, migration, and invasion [114]. On the other hand, tumorigenic activities of cathepsin G-included ECM degradation [115], receptor processing, or shedding [116–118], and increased tumor cell invasion through ECM. ADAM8 and ADAM10 expression by PMN is associated with increased invasiveness and

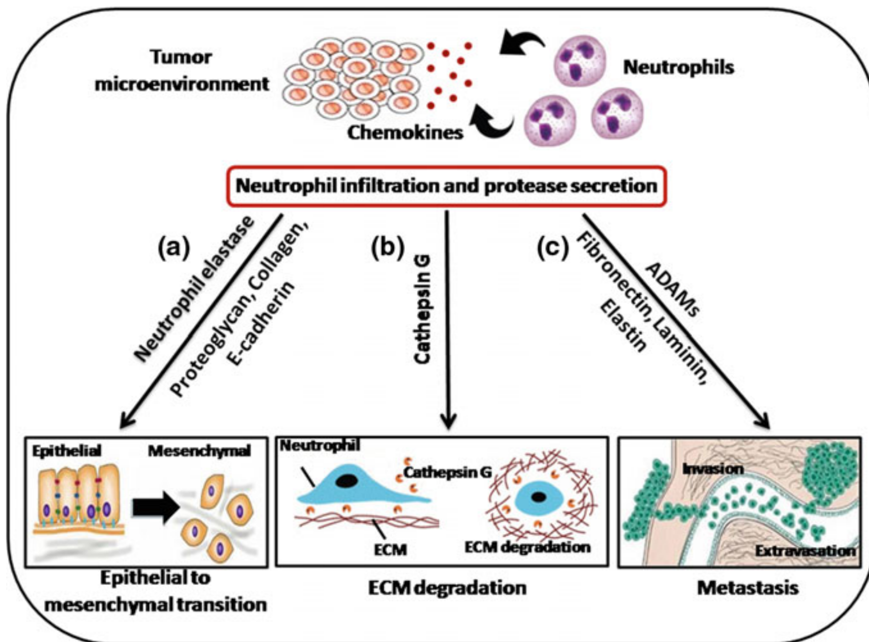

Fig. 4 Polymorphonuclear neutrophils are attracted to the tumor microenvironment by tumor cell-secreted chemokines, and these infiltrated neutrophils secrete varieties of proteases. Neutrophil elastase, cathepsin G, and ADAMs are the most important among these cytokines. **a** Neutrophil elastase cleaves proteoglycan, collagen, and E-cadherin subsequently leading to epithelial to mesenchymal transition or EMT. **b** Cathepsin G promotes ECM degradation. **c** ADAM family proteases cleave fibronectin, laminin, and elastin leading to metastasis

reduced patient survival [119]. ADAM9 degrades several extracellular matrix proteins, including fibronectin, entactin, laminin, and elastin. Zinc-endopeptidases ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) are also released by neutrophils that cleave heparan sulfate proteoglycans syndecan-1, and glypican-1, ultimately leading to cancer progression [120, 121] (Fig. 4).

Cysteine cathepsins mostly act as lysosomal endopeptidases, but cathepsins B, C, H, and X are lysosomal exopeptidases. They are involved in tumor progression and consequent innate and adaptive immune responses. Cathepsins B, H, and L are expressed constitutively at the surface of most innate immune cells and play a significant role in cancer cell phagocytosis [122]. Other cysteine cathepsins (such as cathepsin X) are expressed more precisely on limited cells.

Cathepsin X has carboxypeptidase activity, and its expression is limited to monocytes, macrophages, T cells, and dendritic cells. It regulates dendritic cell maturation and T cell activation by its synergy with β2 integrins and heparan sulfate proteoglycans [123]. Integrins connect the cells with ECM proteins. Following adhesion, integrins recruit various cytoskeletal proteins, and anchor this protein complex to the actin filament. These sequentially guide the renovation of actin

filament and the formation of typical adhesive complex, labeled as focal adhesions. Besides forming a structural connection between the actin cytoskeleton and ECM proteins, focal adhesions are extensive junctions of signal transduction that control cell migration, proliferation, and differentiation [124]. Active cathepsin X regulates β2-integrin-dependent adhesion and activation of T cells by interacting with lymphocyte function-associated antigen 1 (ITAL or LFA-1) and macrophage antigen 1 (Mac-1) which induces homotypic aggregation and cytoskeletal changes in T cells [125]. Likewise, in 2D and 3D in vitro Matrigel models that simulate the ECM, cathepsin X intensifies T-lymphocyte migration, invasiveness. On the other hand, Cathepsin S is involved in MHC class II-mediated antigen presentation. Cathepsin C is expressed by CD8+ cytotoxic T cells and NK cells where it is involved in converting pro-granzymes into proteolytically active granzymes, which elicit apoptosis in malignant cells. All these activities of cysteine cathepsins are restrained by endogenous cysteine protease inhibitors, cystatins.

8 Proteases and NK/NKT Cell Dysfunction

Cytolytic and cytokine-producing natural killer (NK) cells are one of the key components of the innate immune system, constituting the first line of defense against tumor cells. NK cells have a cytotoxic function which gets activated without any prior activation, something which T cells cannot do; so NK cells were initially named so. NK cells release varieties of cytokines and chemokines, viz IFN-γ, TNFα, IL-3, IL-10, GM-CSF, G-CSF, CXCL8, CCL2, CCL3, CCL4, CCL5. IL-2, IL-12, IL-15, IL-18, and IFNs are all powerful catalysts of NK cells activity. On the other hand, natural killer T (NKT) cells which originate in thymus show a combination of NK cell and T cell aspects. They express various NK cell markers and T cell receptor complex on their cell surfaces.

NK and NKT cells negotiate a strong pro-inflammatory response and are linked with progression of various cancers. NK cells terminate cancer cells without prior activation by binding to MHC class I molecules expressed on the surface of cancer cells. The efficacy of NK and NKT cells against cancer cells depends on the number of activated NK and NKT cells. It also depends on the expression of cytotoxic granules, inhibitory receptors, activating receptors and on the fine harmony between activating and inhibitory signals sent by surface receptors. For example, if the activating signal is robust, then NK cell will be activated; similarly, if the inhibitory signal is stronger, then NK cell activity will be inhibited. Activating receptors of NK and NKT cells are C-type lectin family receptors (Ly49, NKG2), NCR (natural cytotoxicity receptor) and CD16 (FcγIIIA) that can recognize self-molecules encoded by host's genome whose expression is up-regulated during cancer. On the other hand, inhibitory receptors (Killer-cell immunoglobulin-like receptor (KIR), Leukocyte inhibitory receptor (LIR)) are broadly recognized by MHC class I molecules.

Flawed NK and NKT cell activity can be seen in gastric cancer, colorectal cancer, and pancreatic cancer. Cancer cells by immunoediting cause NK cell modulation by up-regulation of inhibitory receptors (KIR3DL1, KIR2DL1/DS1) and down-regulation of cytotoxic granules (Perforin-Granzyme B), activating receptors (NKG2D, NCR), and decreased secretion of cytokines (TNFα, IFN-γ). The underlying mechanisms behind this are mostly unknown.

NK cell activating receptor NKG2D and its ligand MHC-I chain-related molecule (MIC) are one of the key players of tumor immunoediting. NKG2D is expressed by NK cells, NKT cells, $CD8^+$ T cells, and $\gamma\delta$ T cells. Ligands for NKG2D receptor are poorly expressed on the majority of normal cell surfaces but are up-regulated on the tumor cell surface [126]. These ligands can be released from the cell surface by proteolytic cleavage. Ligation of NKG2D receptor with its tumor-associated NKG2D ligands (NKG2DL) sends an activating signal to NK cells and co-stimulatory signal to CD8+ T cells and causes recognition of tumor cells by cytotoxic lymphocytes, resulting in cellular or genotoxic stress. But cancer cells evade immune attack by shedding membrane ligands for the NKG2D receptor; leading to desensitization of NK cells and it is a major countermechanism of cancer cells to overturn NKG2D-mediated immunosurveillance. NKG2DL MICA (MHC-I chain-related molecule A) is released by proteolytic cleavage in the stalk of the MICA ectodomain, and it involves the participation of “a disintegrin and metalloproteinase” (ADAM) family. ADAM10 and ADAM17 are crucially involved in the proteolytic release of MICA, thus helping tumor immune escape [127]. Thus, MICA shedding by tumor cells can be restricted by blocking ADAM10 and ADAM17 proteases. But MICA shedding by membrane-type matrix metalloproteinase (MT-MMP) MMP14 is independent of ADAMs. Silencing of MMP14 expression prevents MICA shedding and overexpression of MMP14 augments MICA shedding. Hence, therapeutic blockade of ADAM10, ADAM17, and MMP-14 is a promising treatment for cancer.

Matrix metalloproteinase 9 (MMP-9) is a 92-kDa type-IV collagenase and is released by mesenchymal stem cells (MSC). MMP-9 significantly down-regulates the cytotoxicity of NK and NKT cells [128, 129]. Indoleamine 2,3-dioxygenase (IDO) plays a major role in MMP-9-mediated immunosuppression by impeding NK cell accumulation in tumor milieu [130]. Hence, tumor-induced NK cell dysfunction is mainly carried out by IDO and MMP-9. Decreased infiltration of NK cells in tumor milieu is associated with increased COX-2 expression, which promotes tumor growth by producing prostaglandin E2 (PGE2) in NK cell-dependent manner [131–133]. Together, IDO, MMP-9, and PGE2 are potent factors in the synergy between NK cells and cancer cells in the tumor microenvironment [134].

NK and NKT cell cytolytic function against tumor cells can be induced directly by various receptor expressions on the cell surface. It can also be triggered through Fc receptors resolving antibody-dependent cellular cytotoxicity (ADCC) by antibodies. The efficacy of ADCC is directly parallel to CD16 receptor (FcγRIIIa) expression on NK cells. The CD16 expression is negatively altered by upsurged expression of MMPs. Inhibition of MMPs causes elevated CD16 expression by NK cells and strengthened ADCC activity against antibody-coated tumor cells [135].

Hence, MMP inhibitors enhance NK cell-mediated ADCC against tumor cells and can be exploited therapeutically.

9 Proteases and Ubiquitin System in Tumor Immune Evasion

Ubiquitin system is an integral part of immune tolerance, immune cell development, T cell differentiation, antigen or cytokine-induced signaling pathways, and hematopoiesis [136]. Ubiquitination is a three-step enzymatic reaction. E1, E2, and E3 ubiquitin ligases are enzymes which perform catalysis of different proteins. E1 enzyme forms thiol ester bond, and this leads to activation of ubiquitin. E1-activated ubiquitin gets transferred to an E2 enzyme. E2 complex interacts with the E3 enzyme. This interaction leads to isopeptide bond formation between glycine (C-terminal) of ubiquitin and an amino group of a lysine on substrate protein or ubiquitin [136, 137]. E1, E2, E3 ubiquitin ligase and deubiquitinases (DUBs) build, edit and remove ubiquitin chains [138]. E1, E2, E3, and DUBs play a central role in posttranslational modification of proteins. E3 ligases which play an important role in immune regulation are GRAIL (gene related to anergy in lymphocytes), CBL-B (Casitas B-cell lymphoma-B), and ITCH (AIP4). GRAIL and CBL-B contribute to T cell tolerance and peripheral T cell tolerance, respectively. ITCH plays an important role in T cell tolerance and TNF signaling. A20 (TNFAIP3) has a pivotal role in B-cell tolerance, germinal center selection and CD40 signaling, TNF, TLR, and NLR signaling [136, 138]. N-terminal domain of A20 acts as DUB while its C-terminal end acts as E3 ligase [138]. Ubiquitin system plays a pivotal role in antigen presentation, and this establishes proper coordination between innate and adaptive immune system. Innate immune system (dendritic cell, NK cells, and macrophages) interacts with the adaptive immune system so as to have prolonged protection against distinct microbes. The adaptive immune response evoked by pathogen leads to the formation of TCR (T cell receptor) and BCR (B-cell receptor) which are programmed to eradicate infectious pathogens [138].

Dys-regulation in proteasomal machinery will lead to malfunctioning of both adaptive and innate immune cells. These will create "danger-free zone" for cancer cells as there will be no checkpoint, and hence, tumor cells will grow profusely in the host system. Therefore, a glitch in Ubiquitin system leads to tumor immune evasion.

9.1 Ubiquitin system and Treg-Mediated Immunosuppression

Suppressive action of Treg cell is a characteristic marker of various types of cancers such as myeloma, sarcoma, melanoma. Transcription factor Foxp3 plays a pivotal role in imparting suppressive property to T-regulatory cells. Deubiquitinase USP7

regulates FOXP3 protein expression in Treg cells by deubiquitination of Foxp3 protein. These stabilize FOXP3 protein amounts. USP7 knockdown leads to impaired FOXP3 protein expression which affects Treg cell-suppressive property [139]. P5091 is a chemical inhibitor of USP7 and has been found to be effective against multiple myelomas [140].

9.2 Ubiquitination, Impairment of NFκβ Activation, and Antitumor Immune Responses

NFκβ/Rel is transcription factor that regulates both innate and adaptive immune system in eukaryotes. DUBs play an important role in NFκβ regulation. TNFAIP3 or A20 is one such DUB which regulates NFκβ. In both T and B lymphocytes, A20 acts as negative regulator of NFκβ signaling [139]. NFκβ activation in T and B lymphocytes is regulated by paracaspase activity of MALT1 and deubiquitinase action of A20. MALT1 cleaves A20 to weaken its NFκβ inhibitory function, and this activates NFκβ signaling in T cells [140]. Interactions between ubiquitinated MALT1 and IKK complex are impaired by deubiquitinase A20 [141]. These show remarkable equilibrium between MALT1 and DUB A20 whose coordination regulates NFκβ signaling. This balance gets dys-regulated during B-cell lymphomas. In B-cell lymphoma, paracaspase activity of MALT1 is constitutive in B-cell lymphomas and catalytic activity of MALT1 gets inhibited; this keeps A20 intact leading to inhibition of NFκβ [142]. TNFAIP3 might act as a tumor suppressor in various forms of B-cell lymphomas. Point and deletion mutation in A20 leads to DLBCL (diffuse large B-cell lymphoma), marginal zone lymphoma, MALT lymphoma, and Hodgkin lymphoma [142–144].

CYLD belongs to DUB family and acts as tumor suppressor and is an NFκβ regulator. An inactivating mutation in CYLD leads to multiple myelomas and T-ALL (T cell acute lymphoblastic leukemia). In T-ALL, Notch/Hes 1 pathway represses CYLD expression and these results constitutive IKK activation and prolonged cell survival [145]. A20 also plays a crucial role in MDSCs and negatively regulates apoptosis in many cell types. Silencing of A20 not only inhibited tumor growth but also decreased infiltration of MDSCs at the tumor site. Inhibition of A20 activates JNK pathway which subsequently induced caspase 3- and caspase 8-mediated apoptosis [146–148]. Moreover, another ubiquitination enzyme CBL-B negatively regulates IL2 production and T cell proliferation. CBL-B also controls TCR and CD28 signaling. Hence, knockdown of CBL-B in effector T cells may lead to CD28 expression and enhanced autocrine production of IL2. These will increase survival and proliferation in CD8+ T cells which will make them responsive to tumor antigen. Adoptive transfer of CBL-B$^{-/-}$ CD8 T cells proved to be effective against leukemia. Therefore, abrogation of CBL-B in CD8 T cells can become an immunotherapy modality to treat human malignancy [147–149].

10 Conclusion

Summing up the evidence discussed so forth, it can be concluded that proteases emerge out to be a crucial target for cancer therapeutics. Therefore, tight regulation of proteases emerges out to be a critical step in developing a successful antitumor response. Thinking about the potent role of proteases in tumor prognosis, pharmaceutical companies started developing MMP inhibitors (MMPI). Till date, two protease inhibitors, namely DX2400 and REGA 3G-12, have been tested. Both the inhibitors successfully reduce the tumor burden in a murine model. Another intriguing fact about these two inhibitor(s) is their substrate specificity. DX-2400 inhibits MMP14 whereas the latter inhibits MMP9. Apart from this, several clinical trials have been done to use doxycycline as MMP inhibitor in combinatorial cancer therapy. But the majority of current therapeutic approach targets nonspecifically wide array of any dividing cells. These lead to adverse immune response associated with chemotherapy. Thus, targeted therapy encompassing MMP inhibition is the new challenge to be faced. Apart from liposome-mediated drug delivery, targeted genome editing of the MMP gene can be explored as a successful anticancer therapeutic approach.

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Protease, an Advance Therapeutic Target in Cancer

Animesh Chowdhury, Donatella Romaniello, Soma Ghosh, Tapati Chakraborti, Soumitra Roy and Sajal Chakraborti

Abstract

Proteases are known to be associated with cancer development because of their aptitude to degrade extracellular matrices, which enables invasion and metastasis. Recent studies have demonstrated that a variety of substrates are the main target of these important enzymes and favour all steps of tumour evolution. An extensive number of reports have been available which shows a positive correlation between the activity of several proteases and tumour progression suggesting the usefulness of protease inhibitors as anticancer drugs. Nowadays, the cure for metastatic diseases is still a utopia and many efforts are focused on finding new sensitive biomarkers for a precise diagnostic as well as prognostic and therapy. In this scenario, understanding of the proteases, how they are involved in early to the end point in cancer progression is needed. In this review, we will focus on the role of proteases as prognostic and therapeutic targets in various types of cancers.

Keywords

Protease · Metalloprotease · Serine protease · Cystein protease
Aspartate protease · Cancer

A. Chowdhury (✉) · D. Romaniello · S. Ghosh
Department of Biological Regulation, Weizmann Institute of Science,
Rehovot 76100, Israel
e-mail: animeshchowdhury9@gmail.com

T. Chakraborti · S. Chakraborti
Department of Biochemistry and Biophysics, University of Kalyani, Kalyani,
West Bengal, India

S. Roy
Department of Physiology & Cell Biology, Faculty of Health Sciences,
Goldman Medical School, Ben Gurion University of the Negev, Beer Sheva, Israel

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1 Introduction

Cancer is a major burden of disease with millions getting affected worldwide each year. It is associated with abnormal cell growth that has potential to invade and/or spread to other parts of the body. The cancer cells grow and divide to create more cells which results in tumour formation. However, not all tumours are cancerous and are referred as benign tumours. The benign tumours are not malignant and so neither do they invade nearby tissue nor spread to other parts of the body. The molecular mechanisms of the complex interplay between the tumour cells and its microenvironment play a critical role in the formation of tumorigenesis leading to cancer. At a stage of cancer invasion and metastasis, interstitial connective tissue and basement membranes get degraded and molecular events that take place during tumorigenesis and signalling molecules related with the progression of cancer development are being studied over the past decades.

Proteolysis is one of the vital processes among the biological reactions. It is an irreversible regulatory mechanism and facilitated by the selectively cleavage of specific substrate (s). Proteolytic activity has been attributed to a class of enzymes called proteases. These enzymes are of wide variety and play a significant role in biological and pathological processes. In addition, multimeric and multicatalytic proteases are known to degrade multiple intracellular proteins, called proteasomes, essential for biological processes [1]. Proteases in normal cells are very worth in executing crucial biological processes; however, recent studies proved the fact that these enzymes are also essential in the development of tumour growth and metastasis [2]. The recent accessibility of the genome sequence of different organisms makes it easy for the recognition of the entire protease repertoire, which has been defined as degradome. The human degradome is made up of at least 569 proteases that are divided into five broadly classified groups: metalloproteases (194 in number), serine proteases (176 in number), cysteine (150 in number), threonine proteases (28 in number), and aspartic proteases (21 in number) [3]. Serine, cysteine, and threonine proteases are associated with covalent catalysis. In contrast, metalloproteases and aspartic proteases are involved in non-covalent catalysis [4]. Serine, cysteine, and threonine act directly as nucleophiles that attack an amide carbonyl C, whereas aspartate, glutamate, and metalloproteases activate a water molecule that then acts as a nucleophile. These enzymes are also classified into exopeptidases and endopeptidases depending upon the position of the peptide bond in a protein they cleave. Exopeptidases truncate one or several amino acids from either the N- or the C-terminus of a peptide, whereas endopeptidases cleave an internal peptide bond. A positive correlation between the aggressiveness of tumour and the secretion of various proteases has been found, yet the secretion of some specific proteases in tumour cells make prognosis complicated. The ability to degrade extracellular matrices and proteins, proteases are strongly linked with cancer progression including invasion and metastasis. Nevertheless, proteases are not completely expressed by cancer cells and in several cases tumour cells induce the expression of proteolytic enzymes in non-neoplastic neighbouring cells, taking

over their activity to favour tumour development [5]. The aim of this article is to critically review the current status of proteases as prognostic markers in cancers mainly focusing on metallo, cysteine, serine, and aspartic proteases. The present review will give the reader a strong milieu in the field of cancer research associated with proteases and an outlook of how this field can advance further.

2 Metalloproteases in Cancer Development

The extracellular matrix (ECM) is composed of highly variable and dynamic components that regulate cell behaviour. ECM is a major component of the local microenvironment or niche of cancer cells that plays important roles in cancer development. It is a complex network of macromolecules with distinctive physical, biochemical, and biomechanical properties and tightly controlled during embryonic development and organ homeostasis; however, the ECM is usually deregulated and becomes messed up during cancer progression. Deregulated ECM metabolism affects cancer progression by directly promoting cellular transformation and metastasis. ECM remodelling (synthesis and degradation) is mainly controlled by matrix metalloproteases (MMPs), which are zinc-dependent endopeptidases and synthesized as inactive form and activated during pathophysiological condition and also secreted from the cells. To date, 28 members of MMP have been identified and 23 of them are found in humans. MMPs are classified according to their structure and/or ECM substrate specificity [6]. Collagenases, including MMP-1, MMP-8, MMP-13, and MMP-18 (Xenopus) can degrade native collagen-like collagens I, II, and III. MMPs also cleave other ECM and non-ECM molecules. Although stromelysins including stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and stromelysin-3 (MMP-11) share structural similarity with the collagenases but are not able to cleave native collagen, gelatinases such as gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are well known to cleave mostly denatured collagens (gelatins) as well as type IV collagen in basement membranes. They have 3 structural repeats of a type II fibronectin domain inserted in the catalytic domain, which bind to gelatin, collagens, and laminin. Matrilysins, which lack the hemopexin domain, include matrilysin-1 (MMP-7) and matrilysin-2 (MMP-26, endometase). They mainly degrade ECM constituents such as laminin and entactin. Membrane-Type MMPs (MT-MMPs) are entrenched in the plasma membrane of the cells via type I or II domains or glycosylphosphatidylinositol (GPI) anchors. Their family includes MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), and MT4-MMP (MMP-24), and the GPI-anchored proteins MT5- and MT6-MMP (MMP-17 and MMP-25). MT1-MMP can cleave type I, type II, and type III collagen, and other components of ECM and can activate proMMP-2 to MMP-2. Other MMPs such as MMP-11, MMP-12, MMP-19, MMP-20, MMP-22, MMP-23, and MMP-28 do not conform easily to this classification system.

Regulation of MMPs' activity is a complex process including three different levels of activation: (a) regulation of MMPs gene expression; (b) regulation of MMP enzyme activity by cystein switch mechanism; and (c) inhibition of MMPs by its endogenous inhibitors, called tissue inhibitor of metalloproteases (TIMPs). High expression of a variety of MMPs has been observed in almost all type of human cancer and correlates with advanced stage of invasion and metastasis [7–11]. Among the MMPs, MMP-2 and MMP-9 have been implicated as the most important prognostic factor in various types of cancer model [12]. A previous study examined the correlation between MMP-2 and breast tumour progression in parallel with the clinicopathological parameters such as tumour size, histological type, lymph node status [13]. Immunoreactivity of MMP-2 was noticed in the cytoplasm in both tumour and tumour stromal cells and significantly associated with tumour size [13]. Another study suggested that MMP-2 overexpression is present in the malignant breast tumours induced by paracrine stimulation of soluble factors [14]. In the same manner, also MMP-9 expression and activity were investigated in 81 malignant breast tumours and correlated with known pathological parameters in which MMP-9 was seen to be overexpressed in node-positive tumours and the preoperative blood serum of patients [15]. Using immunoperoxidase staining, the prognostic value of MMPs was investigated in a case study of 210 breast cancer tissues: mainly MMP-9 staining was detected in cancer cells and slightly in surrounding stromal cells; however, MMP-9 expression was not noticed in normal breast tissue [16]. Thus, to find out the optimal treatment, the overexpression of MMP-2 and MMP-9 in breast cancer could be useful as a prognosis marker to subdivide node-negative breast cancer patients. Besides these studies, the impact of targeting the expression of MT1-MMP in breast cancer and its clinical relevance was also investigated. Jiang et al. showed that MT1-MMP staining was present in both membrane and cytoplasm in breast cancer cells [17]. Tumour cells exhibited stronger staining in comparison with normal mammary epithelial cells showing slightly higher levels of the MT1-MMP transcript compared with normal tissues [17].

In a recent work, cytoplasmic and stromal expression of MMP-2 was investigated in 39 (54.2%) lung cancer patients and the overexpression of MMP-2 was correlated with tumour size, lymph node and distant metastasis [18]. Other studies also correlated the high expression of MMP-2 and MMP-9 with lung cancer invasion, metastasis, and progression [19–23].

Recent studies have also demonstrated the importance of MMP-2 and MMP-9 in ovarian cancer progression and metastasis. A new study specified the role of MMP-2 in ovarian cancer progression presenting that knockdown of C3G suppressed cell invasion, intravasation, and extravasation in parallel with the reduction of Rap1 (Ras-proximate-1 or Ras-related protein 1) activity and secretion of MMP-2 and MMP-9 [24]. Therefore, C3G-mediated activation of Rap1 could direct the tumour pattern of human ovarian cancer by promoting the secretion of MMP-2 and MMP-9 [24]. Hilary et al., using organotypic 3D culture of the omentum, showed increased MMP-2 proteolytic activity during adherence to ovarian cancer cells [25]. The activated MMP-2 further cleaves the matrix proteins fibronectin,

vitronectin, and collagen-I into smaller fragments, which then promotes cancer cell adhesion and invasion by binding to their cognate integrin receptors [25]. Research has shown that in vivo inhibition of MMP-2 using a siRNA or a blocking antibody before adhesion decreases the number of metastasis and tumour weight in a significant manner in a xenograft mouse model [25]. Another novel study proposed that inhibitor of DNA binding/differentiation 1 (Id1) could enhance endothelial progenitor cell (EPC) angiogenesis in ovarian cancer involving PI3 K/Akt and NF-κB/MMP-2 signalling pathways [26]. Therefore, Id1 and its downstream effectors MMP-2 could be possible targets for the treatment of ovarian cancer due to their role in angiogenesis.

For colorectal cancer (CRC), several research studies have revealed an interrelation between increased MMP-2 and MMP-9 expression and their outcome. Compared to without lymph node metastasis, an increased level of plasma MMP-2 expression has been noticed in lymph node-positive patients with CRC [15, 27]. Many studies have suggested the effectiveness of serum MMPs as markers for CRC invasion, as they show higher expression levels of MMP-2 and MMP-9 proteins in the sera of the patients compared to normal controls. These results have greater diagnostic sensitivity than two other biomarkers currently used in clinical practice, CEA and CA19-9 [16, 28]. Another study observed the interaction of β1-integrins with MMP-2 in colon cancer cells. Here, the MMP-2 was highly expressed in invasive colon cancer cells leading to CRC progression [17, 29]. Considering other MMPs, MMP-1 genetic polymorphisms are also linked with increased CRC susceptibility [30]. Also, possible role for MMP-13 too as a prognostic marker for CRC was evaluated where it indicated that there was increased MMP-13 expression with advanced cancer stage, leading to nearly eightfold increased risk of post-operative relapse compared to those without MMP-13 overexpression [31, 32].

3 Cysteine Proteases in Cancer Development

Like MMPs, other proteases, e.g. cathepsin cysteine proteases are also required for ECM degradation. Cathepsins of the cysteine protease family are mainly localized in lysosomes and endosomes, and digest intracellular or endocytosed proteins. Among the members of the cysteine protease enzyme family, the catalytic site of CA-clan papain-like cysteine proteases possesses cysteine (Cys), histidine (His), and aspartate (Asp) residues and is highly conserved [33]. Cysteine proteases are acceptably validated targets for the treatment of many human diseases. In cancer, several cysteine proteases endow with specific targets for enzyme inhibitors, especially cathepsins and calpains. The active-site cysteine offers specificity to design many inhibitors over other families of proteases, such as aspartate and serine; however, the strategy for inhibition often (a) employs covalent enzyme modification and (b) achieves selectivity within families of cysteine proteases. The cysteine proteases can be subgrouped into several families involving interleukin-1-converting enzyme (ICE), the calpain family, and the papain family [34, 35].

The papain group of the cysteine proteases family are most abundant among all cysteine proteases. This family consists of papain, related plant proteases, and lysosomal cathepsins B, C, F, H, K, L, O, S, V, W, and X [35, 36]. Cathepsins N and T have also been included in the family; albeit a detailed characterization is still waiting to be explained [35]. Nearly all of the cathepsins are endopeptidases [35, 37], but cathepsins B and H could be functional as a dipeptidyl carboxypeptidase [38] and as an aminopeptidase [39], respectively. Cathepsin C is an amino dipeptidase [35, 36] and cathepsin X is a carboxy-mono or carboxy-dipeptidase [40]. Turk et al. disclosed the activation process of cathepsins [36], and according to their concept, cathepsins are produced as preproenzymes and after removal of the prepeptide during the passage to the endoplasmic reticulum, procathepsin is formed. Finally, the active cathepsin is synthesized after proteolytic removal of the propeptide in the acidic environments of late endosomes or lysosomes. This final proteolytic process is mediated by the involvement of several proteases, such as pepsin, neutrophil elastase. Most cathepsins like cathepsins B, F, H, K, L, and V are usually active optimally in acidic environments and are merely weakly active at neutral pH. In contrast, cathepsin S is generally active at neutral pH optimally [36].

There are numerous studies published which shows a correlation between cysteine protease and invasion, proliferation, or metastasis of tumour cells. Cysteinyl cathepsins were invented to be associated with the regulation of cell proliferation involving signal transduction pathway. Inhibition of cathepsins by a synthetic broad-spectrum cysteine protease inhibitor has been shown to markedly reduce tumour cell proliferation in a mouse model of pancreatic islet cells [41]. Gocheva et al. demonstrated that lack of cathepsins B or L reduced tumour cell proliferation [42]. Although cathepsin X has been found to suppress proliferation of mononuclear cells by activation of Macrophage-1 antigen (MAC-1) (CD11b/CD18), yet cathepsin X induces the proliferation of T lymphocytes by activation of lymphocyte function-associated antigen 1 (LFA-1) (CD11a/CD18) [43]. A recent study has shown that the decline of Cux1 progression by cathepsin L deletion results in the accumulation of Cux1, downregulation of p21/p27 leading to induce cell proliferation [44].

Early reports indicated a correlation between cathepsin B and cancer implying that cathepsin B is released from malignant human breast tumour explants and is found in the blood serum of patients with neoplastic vaginal lesions. Cathepsin B has also been shown to be associated with the dissolution and remodelling of connective tissue and basement membrane in the processes of tumour growth, invasion, and metastasis [45–47]. Cathepsin B among all of the cysteine protease was also identified as the most efficient lysosomal protease to be associated with breast cancer [48]. A recent study established that while cathepsins V and D expression levels were noticed to be connected with breast cancer metastasis, the expression levels of cathepsins B and D were associated with poor disease-free survival in breast cancer patients [49]. In addition, univariate analysis displayed that the expression of cathepsin B enzyme and metastasis to the bone in breast cancer model were also associated with poor disease-free survival [49]. Moreover, Cathepsin B was recognized as a potential prognostic and therapeutic marker for

human lung squamous cell carcinoma and non-small cell lung cancer (NSCLC) [50, 51]. One previous study investigated the role of cathepsin B contributing in the mechanisms of invasion by ovarian cancer [52]. This study showed that expression of cathepsin B is closely related to the invasion of ovarian cancer and suppression of cathepsin B activity using cathepsin B inhibitors markedly blocked cancer cells growth [52]. Kawasaki et al. demonstrated that the level of cathepsins D and B was also associated with tumour growth and invasion in oral squamous cell carcinoma [53]. While cathepsins B and L have been seen more frequently overexpressed in chronic atrophic gastritis with dysplasia, only cathepsin B has been significantly overexpressed in laryngeal carcinoma [54]. Additionally, contribution in regulation of angiogenesis revealed another distinct role of cathepsin in tumour progression [47, 55]. Considering another cysteine proteases, a very recent report implicated the role of cathepsin K in breast cancer cells indicating that cathepsin K induces platelet dysfunction involving cell-signalling cascade [56]. This study demonstrated that platelets were aggregated by cathepsin K but not by other cysteine cathepsins in a dose-dependent manner and PAR-3 and PAR-4 are the main targets of cathepsin K in this scenario. Moreover, studying co-culture experiments, this study showed that platelets activated by cathepsin K induce the upregulation of SHH (Sonic hedgehog), PTHrP (Parathyroid hormone-related protein), OPN (Osteopontin), and TGFβ in epithelial-mesenchymal-like cells from patients with luminal B breast cancer and concluded how cathepsin K induces platelet dysfunction with the involvement of cellular signalling affected by cathepsin K in breast cancer cells [56]. Duong et al. confirmed that increase in cathepsin K protein and mRNA levels is associated with the induction of human breast cancer primary and metastatic tumour growth [57]. These data evidenced the role of cathepsin K in breast cancer skeletal growth and metastasis and inhibition of cathepsin K activity using pharmacological or genetic inhibitors may stand for a novel oral therapy for the treatment of metastatic breast cancer [57]. Chen and Platt [58], using multiplex zymography, verified that cathepsin K was unique compared to cathepsins L and S and the level of cathepsin K was significantly higher for all cancers (lung, cervical, breast) even at the earlier stage. A large number of studies indicated the role of cathepsin K in osteoclast-mediated bone degradation, which has been assumed to be produced by cancer cells that metastasize to bone where cathepsin K functions in the proteolytic pathways leading to cancer cell invasion. Highly selective and potent cathepsin K inhibitors have been recently developed and shown to be positive antiresorptive agents in the treatment of osteoporosis. Furthermore, preclinical studies revealed that inhibition of cathepsin K using specific inhibitors reduced the chance of breast cancer-induced osteolysis and skeletal tumour burden. The possible reason in the depletion of skeletal tumour burden is recommended to be due to the antiresorptive activity of cathepsin K inhibitors that leading to deprive cancer cells of bone-derived growth factors which are essentially required for tumour growth [59]. The cysteine protease cathepsin L is also often thought to act as a tumour promoter by enhancing tumour progression and metastasis. This happens due to the increase of cathepsin L activity in various tumour tissues. A recent study demonstrated that cathepsin L expression is linked with the progression of breast cancer invasion and metastasis [60].

Cathepsin L has also been observed to be involved in prostate cancer and bone metastasis, and cathepsin L inactivation has been found to inhibit prostate cancer cell dissemination in a preclinical bone metastasis model [61].

Beside cathepsin, calpains are also cysteine proteases which are calcium-dependent [62, 63]. Two major isoforms of calpain, μ-calpain or calpain-1, which entails micromolar Ca^{2+} concentrations for activity, and m-calpain or calpain-2, which requires millimolar Ca^{2+} concentrations, are ubiquitously expressed; however, other tissue-specific forms of calpains are also exist. For example, calpain3 (CAPN3) is a skeletal muscle-specific protease, but its expression also appears transiently in the human embryonic heart [64]. Both μ- and m-calpains form heterodimers with a common regulatory subunit, calpain 4/CAPNS1 (calpain small-1). Binding of Ca^{2+} to μ- or m-calpain leading to the release of constraints that are imposed by domain interactions results an activation process [65]. On the other side, in the absence of cytosolic calcium flux, calpains are also activated through the direct phosphorylation at serine 50 by extracellular signal-regulated kinases (ERK). Calpains (specifically the ubiquitous μ- and m-calpain) play the important role in numerous physiological and pathological phenomena. Several studies indicated the importance of calpains in cancer development and progression, including cell transformation, migration and tumour invasion, apoptosis/survival, as well as angiogenesis [66]. Therefore, calpains could be considered as a potential anticancer targets.

A large number of data have been demonstrated indicating the role played by the two ubiquitous calpains (μ- and m) during malignant transformation. Carragher et al. first revealed that the balance of calpain system was modified by the oncoprotein v-Src [67]. They showed that v-Src induces an increase in the expression of m-calpain and also the degradation of its endogenous inhibitor calpastatin leading to a significant increase in the calpain activity [67]. They also suggested that the m-calpain activity is also significantly augmented by several other oncoproteins, such as v-Myc, v-Jun, k-Ras, and v-Fos during the cell transformation [68]. The increased calpain activity is responsible for the anchorage-independent growth of the v-Src-transformed cells. Niapour et al. also demonstrated that c-Myc stimulates calpain activity through the suppression of calpastatin expression and promotes the transformation of cells [69]. These different studies strongly demonstrate that calpains could be major effectors of malignant transformation.

A recent study indicated the role of calpain in breast cancer cell invasion and metastasis [70]. This study revealed that altering calpain activity, ezrin controls focal adhesion (FA) and invadopodia dynamics; two key processes essentially entailed for efficient invasion of cancer cells to induce breast cancer cell invasion. This study also demonstrated that ezrin-depleted cells show reduced calpain-mediated cleavage of the FA and invadopodia-associated proteins such as talin, focal adhesion kinase (FAK), and cortactin and also reduced calpain-1-specific membrane localization, suggesting a requirement for calpain-dependent ezrin in breast cancer invasion and metastasis [70]. Another recent study revealed the correlation between the expression of calpain-2 and breast cancer-specific overall survival in both pragmatically defined basal-like and triple-negative phenotype subgroups. Furthermore, the importance of calpain-2 expression was verified in a

separate, defined cohort of invasive breast cancer patients. This provides strong evidence for the prognostic significance of calpain-2 expression [71]. However, Lau et al. recently established that calpains are responsible for the apoptosis in human small cell lung cancer [72]. They showed that capsaicin, which is a known agonist of the TRPV receptor that induces apoptosis in human SCLC cells, was mediated via the TRPV receptor family member: TRPV6. Inhibition of TRPV6 receptor using genetic inhibitor eliminated the capsaicin apoptotic activity in SCLC cells. Immunostaining and ELISA study confirmed that TRPV6 receptor was expressed significantly higher in human small cell lung cancer (SCLC) tissues (from patients) and SCLC cell lines but almost absent in normal lung tissues. This study also indicated that pro-apoptotic activity of capsaicin was mediated by the intracellular calcium-dependent calpain-mediated pathway. Capsaicin treatment to human SCLC cells increased the activity of both ubiquitous calpain (μ and m) by threefold relative to untreated SCLC cells, and the calpain is downstream of the TRPV6 receptor in capsaicin-induced SCLC cells [72]. In contrast, a recent past study suggested that calpains are overactivated in alveolar rhabdomyosarcoma (ARMS) [73]. This study showed that compared to non-malignant myoblasts, peroxiredoxins (Prx) family member, Prx IV is overexpressed by five times in ARMS cells. Prxs are known to be overexpressed and associated with the progression of several tumours. Moreover, the inhibition of calpains using pharmacological inhibitors resulted in a decrease in Prx IV abundance. Thus, calpains could be accepted as the tumour phenotype of ARMS cells especially through Prx IV regulation and, thus, might represent a potential therapeutic target to inhibit progression of ARMS tumour [73]. Considering lung cancer, two studies have shown that the migration of the tumour cells induced by nicotine and NNK (Nicotine-derived nitrosamine ketone, formed by the nitrosation of nicotine) was associated with the activation of both μ- and m-calpains [74, 75]. These two cigarette smoke components induce an increase in the phosphorylation of the two ubiquitous calpains (μ and m), which promote invasion and migration. However, the signalling pathways responsible for the activation of these enzymes appear different for the two components of cigarette smoke. While the phosphorylation of calpains induced by nicotine was observed to be mediated by PKC iota, the NNK-induced calpain phosphorylation is dependent on ERK/MAPK pathway [74, 75]. Another study has highlighted the association of m-calpain with the invasion and migration in lung cancer cells. According to this study, fibronectin stimulates the invasion and migration of lung cancer cells through the activation of FAK-ERK/MAPK signalling pathway, resulting augmentation of m-calpain activity [76]. The overexpression of calpain-1 and calpain-2 is also connected with the progression of ovarian cancer. Storr et al. in their study indicated that the expression of m-calpain is closely associated with response to platinum-based chemotherapy, progression-free and overall survival in ovarian cancer [77]. Taking into account the various cellular functions of m-calpain, unusual overexpression and high activity could also play an important role in prostate cancer progression including migration, invasion, and metastasis. It is realistic that m-calpain may be acceptable as a target to restrict tumour progression. In fact, inhibition or downregulation of m-calpain by its pharmacological or genetic

inhibitors significantly reduces migration and invasion of DU-145 prostate cancer cells in vitro and in vivo [78]. Furthermore, m-calpain results in cancer cell alteration towards androgen-independent growth and cancer cell proliferation during androgen deprivation treatment. This is due to the cleavage of androgen receptor (AR) resulting in a truncated, functional AR without ligand-binding domain in androgen-sensitive prostate cancer cells [79]. Prolonged reduction in androgen levels promotes overexpression of m-calpain and increased its activity, leading to an increase in the fragmental cleavage of AR and filamin A (FlnA). Enhanced expression of m-calpain followed by FlnA may result in the development of an aggressive phenotype of prostate cancer. Therefore, it is assumed that a drug combination, wherein androgen deprivation with m-calpain inhibition, could result in new therapeutic strategy to prevent prostate cancer [80]

On the other hand, Lal et al. demonstrated that m-calpain expression is required for the metastasis of glioblastoma cells within the dynamic microenvironment of the brain, in a zebrafish model and thus m-calpain could be an important therapeutic target to ameliorate the progression of brain tumorigenesis [81]. Calpains are also appearing to be strongly implicated in angiogenesis through the regulation of migration and survival of endothelial cells [82]. A previous study suggested that calpains are essential for the growth factor-stimulated migration of microvascular endothelial cells highlighting particularly the role of m-calpain during angiogenesis. Indeed, VEGF induces the migration of endothelial cells by augmenting an increase of m-calpain expression and activity to enable tail retraction [83]. Thus, inhibition of calpains with pharmacological or genetic (siRNA) inhibitors or by overexpression of calpastatin could prevent VEGF-induced angiogenesis.

4 Serine Proteases in Cancer Development

The serine proteases are the most flexible and widely studied proteolytic enzymes till date, and trypsin among the all serine proteases grips the most valuable functions responsible for digestion, blood coagulation, fibrinolysis, development, fertilization, apoptosis, and immunity. Serine proteases are grouped into 13 clans and 40 families. The family name stems from the nucleophilic serine (Ser) in the enzyme's active site, which attacks the carbonyl moiety of the substrate peptide bond forming an acyl-enzyme intermediate [84]. Catalytic triad of Asp, His, and Ser residues is the major object for nucleophilicity of the catalytic Ser and commonly referred to as the charge relay system [85]. An inactive trypsin-like serine protease zymogen precursor is mainly activated by the proteolytic processing involving cleavage of the proprotein precursor between residues 15 and 16 (chymotrypsinogen numbering) [86]. The nascent N-terminus induces conformational change in the enzyme through the formation of an ion-pair with the highly conserved D194 that organizes both the oxy anion hole and substrate-binding site. On the other hand, proteases like tissue-type plasminogen activator possesses Lys at position 156 that connects D194 with an ion-pair that confers a catalytically competent fold without proteolytic cleavage at residue 15 [87].

Kallikreins are a subgroup of the serine protease enzyme family. The human kallikrein family includes 15 kallikrein genes. Kallikreins are expressed differentially in almost all tissues, including prostate, breast, ovary, and testis, and are regulated by steroid hormones in cancer cell lines. There is growing evidence connecting kallikreins and cancer. Prostate-specific antigen (PSA: hK3) and human glandular kallikrein (hK2) are widely accepted tumour prognosis markers for prostate cancer nowadays and are expressed highly in the prostate. hK6, hK10, and hK11 are also promising new serum biomarkers for ovarian and prostate cancer diagnosis and prognosis. Some other kallikreins are differentially expressed in various endocrine-related malignancies, and they have strong predictive value. Kallikrein became ideal markers for prostatic diseases due to their restricted tissue expression and secretion into biological fluids. Several studies presented detailed explanation on hK2 and hK3 as a biomarker in the progression of cancer [88]. Furthermore, hK3, mainly a promising marker for prostate cancer diagnosis and prognosis, recently accepted as a biomarker for breast cancer prognosis [89–91]. hK4 is also another candidate showing role in inducing prostate cancer and acts as a prognostic biomarker for prostate cancer [92].

Human kallikreins possibly contributes in many stages of the metastatic cascade by advancing tumour cell detachment and by facilitating invasion through multiple ECM barriers, independently or together with other extracellular proteases, and, potentially, by contributing to the metastatic spread of prostate cancer cells to bone. In line with this, one study showed that the invasion of MDA-MB-231 breast cancer cells into Matrigel was suppressed by a synthetic hK1 inhibitor [93] whereas other study demonstrated that invasion of LNCaP prostate cancer cells (androgen-sensitive human prostate adenocarcinoma cells) through Matrigel was shown to be reduced by hK3-neutralizing antibodies [94, 95]. Several lines of evidence suggested the role of kallikreins in the formation of osteoblastic (bone-forming) metastases that occur in $\sim 90\%$ of prostate cancer cases. A previous study demonstrated that hK3 is an important mediator of prostate cancer cell–bone endothelium interactions. An hK3 antibody treatment prevented prostate cancer cell adhesion to BMECs and knockdown of KLK3 mRNA by RNA interference reduced in the production of hK3 in prostate cancer cells leading to lower adhesive ability [96]. Kallikreins could be crucial to induce angiogenesis also. Several in vitro studies proved that kallikreins are responsible for angiogenesis by directly and/or indirectly disrupting ECM barriers. It has been reported previously that kallikreins, such as hK2, hK3, hK6, hK7, and hK14, directly catalyse the hydrolysis of a distinct and partially overlapping set of ECM proteins, which might promote endothelial-cell as well as tumour cell migration and invasion [97–102]. Several hKs might also indirectly facilitate ECM degradation. For example, hK1 indirectly stimulates the activation of pro-MMP2 and pro-MMP9, which are type IV collagenases that degrade collagen IV and other constituents of basement membranes [103, 104]. Alternatively, hK2 and hK4 also activates the uPA–uPAR system leading to the degradation of a wide spectrum of ECM components through plasmin, accompanied by the activation of sequestered pro-angiogenic growth factors, e.g. vascular endothelial growth factor (VEGF) and pro-MMPs [105, 106]. On the

other hand, hK3 might directly induce angiogenesis through the activation of the pro-angiogenic growth factor TGFβ [107]. Moreover, hK1 is exceedingly expressed in angiogenic endothelial cells [108] and involves in a proteolytic cascade-regulating tumour angiogenesis. hKs has also been suggested to play a critical role to induce tumour cells growth through the activation of PAR [109].

Urokinase plasminogen activator (uPA) is a serine protease that shows a crucial role in cellular migration, tissue remodelling, and cancer metastasis. A number of groups have suggested uPA as a marker of disease outcome in node-negative breast cancer patients [110–112]. However, it is also prognostic in node-positive patients, premenopausal patients, post-menopausal patients, and the ER-positive subgroup [113, 114]. uPA has also been shown to be a prognostic marker in cancers other than that of the breast. A study with 76 completely resected gastric cancers using univariate analysis indicated that high levels of uPA were significantly associated with decreased survival [115]. Although using multivariate analysis with variables such as tumour size, nodal status, the presence of distant metastases, grade, and PAI-1, it has been suggested that uPA was not an independent prognostic marker. However, if PAI-l was removed from the calculation, uPA became an independent factor. Several preliminary studies also suggested that uPA is an effective factor of disease outcome in patients with bladder, lung, cervix, and ovary cancer [116–119]. However, in these cancers, uPA by itself has not yet been shown to act as an independent prognostic factor.

5 Aspartate Proteases in Cancer Development

Aspartic proteases include several important enzymes, such as pepsin, chymosin, renin, cathepsin D, and these are isolated from numerous fungi. Among the aspartic proteases, cathepsin–D (Cath-D) has a great importance in pathophysiology. Cath-D is ubiquitously distributed in lysosomes and its main function is to degrade proteins in lysosomes at an acidic pH [120]. Besides its classical role as a major protein-degrading enzyme in lysosomes and phagosomes, Cath-D can also activate precursors of biologically active proteins in pre-lysosomal compartments of specialized cells [121]. However, Cath-D has been recognized as an important prognosis marker in cancer development due to its role in cancer progression and development. The direct role of Cath-D in cancer metastasis was first demonstrated in rat tumour cells in which Cath-D overexpression increased the metastatic potential in vivo [122, 123]. Several reports demonstrated that Cath-D induces cancer cell proliferation, fibroblast outgrowth, angiogenesis, and metastasis [124–126]. Berchem et al. [127] revealed that Cath-D is responsible for the stimulation of metastasis which has a positive effect on cell proliferation, favouring the growth of micrometastases, rather than increasing the invasive potential in a rat tumour model. Its expression has also been observed to be increased and secreted at high levels by human epithelial breast cancer cells [124, 128, 129], suggesting Cath-D could be a therapeutic marker of breast cancer [130, 131].

6 Conclusion and Future Direction

Proteases are among the first group of molecules causally involved in metastasis which act as prognostic markers in cancer. Numerous studies around cancer research involving proteases made us to be thoughtful that proteases are fuel in the cancer research and worth therapeutic targets for drug design. The evidence discussed above illustrates that overexpression of proteases is synonymous with tumour progression and poor clinical prognosis. Some of the proteases could positively regulate several aspects of cancer and are summarized in Table 1. The functional exploration of the proteolytic systems that are associated with cancer has led to a change in the way we view in this complex field. We have described

Table 1 Proteases in different types of cancers

Family	Name	Location	Cancer
MMPs	General	Extracellular	Most
	MMP-1, MMP-8, MMP-13		Breast
	MMP-2, MMP-9		Breast, colorectal, lung, malignant gliomas, ovarian
	MMP-14	Membrane	Breast
Cysteine protease	General	Intracellular, lysosome	Most
	Cathepsin K	Extracellular	Breast, bone
	Cathepsin B	Extracellular and pericellular under pathological conditions	Breast, cervix, colon, colorectal, gastric, head and neck, liver, lung, melanoma, ovarian, pancreatic, prostate, thyroid
	Cathepsin L	Lysosome	Breast, colorectal, lung, prostate, ovarian
	Calpains	Intracellular, non-lysosome	Breast, lung
Serine protease	uPA, uPAR	Membrane, pericellular	Cervical, colorectal, gastric, prostate, lung, breast
	General	Intracellular, secreted	Most
	hK1		Possibly in ovarian, breast, and prostate
	hK2		Prostate and breast cancer
	PSA (hK 3)		Prostate, ovarian, breast
	hK6		Ovarian cancer
	hK10		Colon, ovarian, pancreatic, head and neck
	hK11		Ovarian and prostate cancer
	hK15		Ovarian, prostate
Aspartate protease	Cathepsin E	Endosomal structures, ER, Golgi	Cervical, gastric, lung, pancreas, breast
	Cathepsin D	Lysosome	Breast, colorectal, ovarian, prostate, lung

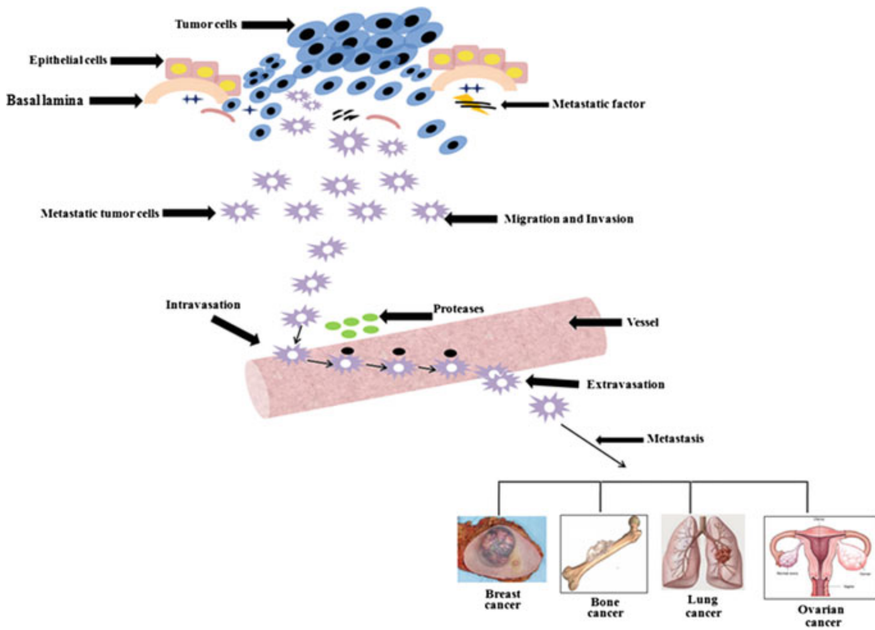

Fig. 1 Role of proteases in different types of cancers

tumour-associated proteases as non-specific and late-acting pro-metastatic enzymes and discussed as key proteins involved in early stages of cancer. Finally, we depicted the role of these pro-tumorigenic enzymes in various types of cancer metastasis. There are many reports published before this into clinical benefits for cancer patients but, hopefully, this attempt would help to ascertain the building of a conceptual framework regarding the antiprotease therapeutic drug and also facilitate to design anticancerous drug in our war on cancer. Figure 1 represents the involvement of proteases in tumour progression and metastasis leading to various types of cancer.

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Part II
Role of Proteases in Different Diseases

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Matrix Metalloproteinases in Parasitic Infections

Fabrizio Bruschi and Barbara Pinto

Abstract

Matrix metalloproteinases (MMPs) constitute a wide family of more than 20 distinct endopeptidases, either secreted or membrane-bound. They are involved in many physiological (embryogenesis, precursor or stem cell mobilization, tissue remodeling during wound healing, etc.) as well as pathological (inflammation, tumor progression and metastasis in cancer, vascular pathology, etc.) situations. These proteinases have been considered in the past exclusively for their degradation ability of molecules of extracellular matrix (ECM) (e.g., collagen, laminin, fibronectin) as well as that to release hidden epitopes from the ECM. In more recent years, it has been completely clarified that these enzymes are also involved in the immune response, acting on cytokines, hormones, and chemokines. Among many others, the so-called gelatinases, MMP-2, and MMP-9 are produced by neutrophils, macrophages, and monocytes. When infection is associated with leukocyte influx into specific organs, immunopathology and following tissue damage may occur, facilitated by gelatinases. The focus of this chapter will be on the participation of MMPs and in particular of gelatinases in either protozoan or helminth infections. A well-studied model is represented by cerebral malaria, for example, where MMPs play a crucial role in the pathogenesis of such disease. Also trypanosomosis and toxoplasmosis will be considered for protozoan infections, as well as neurocysticercosis, angiostrongyloidosis and trichinellosis, for helminth infections.

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F. Bruschi (✉) · B. Pinto
Department of Translational Research, N.T.M.S., School of Medicine,
University of Pisa, Via Roma, 55, 56126 Pisa, Italy
e-mail: fabrizio.bruschi@med.unipi.it

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1 Introduction

Matrix metalloproteinases (MMPs) consist of a large family of proteolytic multi-domain calcium/zinc dependent endopeptidases, encoded by 24 distinct genes in human [1]. These proteinases can break down most of the components of the extracellular matrix (ECM), and even non-matrix proteins [2].

In this chapter, we will focus our attention on the role carried out by these enzymes in the inflammatory response which occurs during infections caused by several parasites, in particular by protozoa or helminths, with the aim to show that many mechanisms involved are similar in the different situations.

Before reporting on the data accumulated in the literature during the last years, dealing with the different parasitic infections, we think it necessary to briefly discuss about the structure and function of the extracellular matrix, which represents the main target of the endopeptidases, and then to proceed with the description of the different MMPs currently known.

2 The Matrix

The extracellular matrix (ECM) is a complex non-cellular component surrounding cells in connective tissues that provide them biochemical and structural support. It exists in two main forms, the interstitial extracellular matrix, in which collagen is the most abundant fibrous protein, and the epithelial-cell associated basal membrane (BM), the latter representing a thin fibrous network that underlies epithelial layers and separates cells from the connective tissue [3, 4]. In most tissues, the ECM is made up of three major types of interwoven proteins, i.e., structural fibrous proteins (collagen, elastin) glycoproteins (fibrillin, fibronectin, tenascins, and laminins) [5], and several classes of proteoglycans (such as aggrecans) [6, 7]. These consists of a "core protein" containing one or more large glysoaminoglycan (GAG) chains (chondrohitin sulfate, heparan sulfate and keratan sulfate) with the exception of hyaluronic acid.

ECM not only joins the cells together providing mechanical adhesion and protection, but also regulates cell–cell interaction in both normal and pathological conditions including cell differentiation, angiogenesis, and apoptosis [8] and influences their survival, development, shape, polarity, and behavior [7]. It may

further provide a support for cell–cell and cell–matrix cohesion in tissues. This adhesion is regulated by adhesive molecules (CAM), such as those acting, for example in wound healing [4, 9]. Cell-ECM adhesion is mediated by ECM receptors mainly integrins, discoidin domain receptors, and syndecans [10]. The ECM is also involved in the transmission of extracellular signals to cells, thus influencing cell survival and proliferation, but also affecting cell differentiation and death [7, 11–13].

In addition to proteins, the major components of the ECM are water, and polysaccharides. However, each tissue has a specific ECM which may differ in composition and topology, and that is produced during tissue development [10]. Its amount varies depending on tissues. Indeed, it is scant in the muscle and nervous tissue, whereas it is abounding in blood and in cartilage and bone [14] tissue [15]. Components of the ECM are synthesized intracellularly by fibroblasts, chondrocytes, and osteoblasts, and only successively secreted into the ECM by exocytosis [16]. Once secreted, they gather with the matrix.

The ECM is a highly dynamic entity that undergoes continuous remodeling, through the action of enzymes or non-enzymatic molecules [10] during development, morphogenesis, tissue repair, and remodeling. Proteolysis certainly constitutes one of the main processes leading to structural and biochemical changes in the ECM [17]. Many different classes of proteolytic enzymes are involved in ECM degradation. The proteolytic system present in human tissues is quite complex, since more than 500 genes coding for proteases or *protease-like* molecules are present in the human genome. Within this heterogeneous enzyme system, the major enzymes degrading the ECM are represented by serine-proteases, cysteine-proteases as well as members of the family of MMPs [18].

3 The MMP Family

MMPs are members of a large superfamily of Ca^{2+}-dependent endopeptidases, called Metzincins, strictly related, containing an active site Zn^{2+} [2]. Currently, the MMP family consists of a group of at least 25 related, but distinct, soluble and membrane-bound proteases, 24 of which are present in mammals [19]. They are produced by a wide range of cell types, in particular inflammatory cells, and are secreted into the extracellular space in an inactive form, called zymogen or proenzyme, or pro-MMP that is activated by proteolytic cleavage [20, 21]. Fibroblasts [22], monocytes [23], neutrophils and macrophages [24, 25], T cells [26], smooth muscle cells [27], astrocytes [28], glial cells [29], eosinophils [30–32], and endothelial cells [33] can synthesize MMPs, which after their synthesis, can be accumulated into inflammatory cell granules. More frequently, they are secreted and found anchored to the cell surface or tethered to other proteins on the cell surface or within the ECM [34].

These proteinases are evolutionarily conserved and rigorously regulated [35, 36]. As shown in Fig. 1, all members of this MMPs share a primary highly conserved organization called “minimal domain” consisting of three common major functional regions: an amino-terminal (N-terminal) hydrophobic signal sequence called pre-domain (Pre) or signal peptide (SP), a propeptide domain (Pro) containing a thiol group (–SH) and a furin-cleavage site (Fu), the catalytic domain containing the zinc-binding site (Zn^{2+}) (Fig. 1).

As previously stated, the majority of MMPs have an amino-terminal signal peptide containing 18–30 residues rich in hydrophobic amino acids [37]. The signal peptide manages their synthesis to the endoplasmic reticulum [38], inside which the signal is removed, and to their secretion into the extracellular space [39].

The cleavable propeptide domains (about 80 amino acids) contain a highly conserved consensus sequence PRCXXPD next to the C-terminal end of these segments.

Prodomain is essential for maintaining enzymatic latency through a mechanism identified as “cysteine switch,” where the unpaired cysteine in a highly conserved amino acid sequence (Pro-Arg-Cys-Gly-X-Pro-Asp) forms a bridge with the catalytic zinc [39]. The cleavage of the propeptide domain produces a conformational change that modifies the proenzyme to its active form.

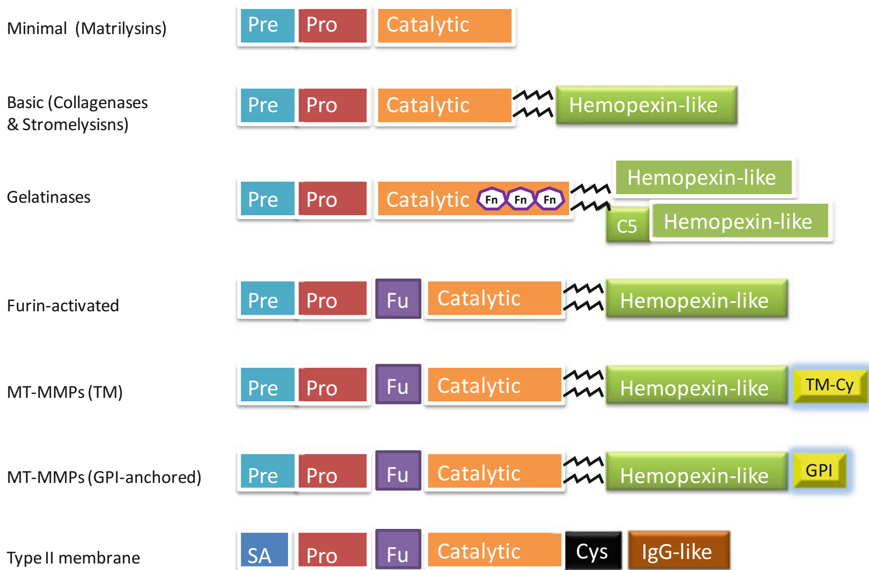

Fig. 1 Domain structure of the MMP family. The mammalian MMP family encompasses 25 members identified by different numbers or depending on their matrix substrates. The MMP motifs are indicated. *C5* type V collagen-like domain; *Col* collagenase-like protein; *Cs* cytosolic; *Cys* cysteine array; *Fn* fibronectin repeat; *Fr* furin-cleavage site; *Pro* prodomain; *SH* thiol group; *SP* signal peptide; *Zn* zinc. From Parks et al. [113], modified

The furin recognition site is a domain containing about 9 amino acids that are present in many MMPs including all membrane-bound MMPs. Its consensus sequence RXKR between their procatalytic and catalytic domains leads to intracellular activation cleavage by furin, a serine proteinase belonging to the convertase family [37].

The catalytic domain is characterized by a 3-histidine zinc-binding motif (HEXXHXXGXXH) to which the active site Zn^{2+} is bound [19] and a conserved methionine-turn motif following the active site. Indeed, this domain comprises two zinc ions and one or more calcium ions coordinated to different residues [40]. One Zn^{2+} ion (namely, the catalytic zinc) is embedded into the active site and is needed for the proteolytic activity of the MMPs. The other Zn^{2+} ion (also known as structural zinc) and the Ca^{++} ion are located in the catalytic domain [40]. The three histidine residues bound to the catalytic Zn^{2+} are conserved among all the MMPs.

Overall, the 3D conformation of the MMPs catalytic domain is similar. Therefore, substrate specificity is not simply explained by the structural features within the enzymes themselves [19].

With the exception of MMP-7, MMP-23, and MMP-26, a carboxy C-terminal hemopexin domain (Hpx) is present, linked to a short proline-rich hinge region, which likely has a critical role in substrate binding specificity [41–43] and in interactions of these enzymes with their tissue inhibitors [44]. The Hpx region (about 200 residues) is composed of four repeats with a Cys residue at either end that resemble plasma hemopexin and contain a disulfide bond (S–S) between the first and the last subdomain [39]. In matrilysins, this domain is absent, probably because of a deletion rather than of an evolutionary origin prior to the addition of Hpx [37, 45].

Other MMP motifs consist of a gelatin-binding fibronectin-like domain intercalated within their catalytic domain (gelatinases MMP-2 and MMP-9), a serine-, threonine-, and proline-rich collagen type V-like (C5) domain (MMP-9), a C-terminal transmembrane (TM) domain, a glycosylphosphatidylinositol (GPI) anchor, an N-terminal signal anchor (SA) and, in some cases, a cytoplasmic (Cs) domain [36, 46, 47].

One major distinction of MMPs is between secreted and membrane-anchored proteinases. MMPs may be anchored to the cell surface by their TM (MMP-14, MMP-15, MMP-16, and MMP-24), GPI (MMP-17 and MMP-25), or SA (MMP-23) motif. The TM domains and the GPI anchors are attached to the Hpx-like domain by a short linker peptide [42].

The MMPs have been further divided into several clades, at least five or six subgroups, according to substrate specificity and amino acid sequence homology (Table 1): collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs [48, 49] (Table 2).

The smaller MMPs actually known are the matrilysins 1 and 2 (MMP-7, MMP-26) [50, 51]. These enzymes break down several ECM components, including gelatin and fibronectin [52, 53]. As previously stated, matrilysins do not contain the C-terminal Hpx-like domain and are consequently called the “minimal-domain MMPs” [43, 54].

Table 1 MMP family subgroups

Common name	MMP	Chromosomal location (human)	M.W. (kDa)	Collagen substrates	Some additional substrates*
Collagenases					
Collagenase-1	MMP-1	11q22-q23	55/45	I, II, III, VII, VIII, X	Aggrecan, gelatin
Collagenase-2	MMP-8	11q21-q22	75/58	I, II, III, VII, VIII, X	Aggrecan, gelatin, fibronectin
Collagenase-3	MP-13	11q22.3	60/48	I, II, III, IV, IX, X, XIV	
Collagenase-4	MMP-18	(*Xenopus*)	70/53		Aggrecan, gelatin, fibronectin
Gelatinases					
Gelatinasi A	MMP-2	16q13	72/66	I, II, III, IV, VII, X	Gelatin, fibronectin, fibrillin
Gelatinasi B	MMP-9	20q11.2-q13.1	92/86	IV, V	Gelatin, elastin, fibrillin
Stromelysins					
Stromelysin-1	MMP-3	11q23	57/45	II, III, IV, V, IX, X, XI	Gelatin, plasminogen
Stromelysin-2	MMP-10	11q22.3-q23	57/44	IV	Laminin, fibronectin elastin,
Stromelysin-3	MMP-11	22q11.2	51/44	IV	Fibronectin, laminin, aggrecan
Matrilysins					
Matrilysin-1	MMP-7	11q21-q22	28/19	IV	Fibronectin, laminin, gelatin
Matrilysin-2	MMP-26	11p-15	28/19	IV	Fibrinogen, fibronectin, gelatin
Metalloelastase	MMP-12	11q22.2-q22.3	54/45	IV	Elastin, fibronectin, latent TNF
MT-MMP Tm-type I					
MT1-MMP	MMP-14	14q11-q12	66/56	I, II, III	Gelatin, fibronectin, laminin
MT2-MMP	MMP-15	15q13-q21	72/60		Gelatin, fibronectin, laminin
MT3-MMP	MMP-16	8q21	64/52	III	Gelatin, fibronectin, laminin
MT5-MMP	MMP-24	20q11.2	–/52		Gelatin, fibronectin, laminin
GPI-anchored					Fibrinogen, fibrin
MT4-MMP	MMP-17	12q24.3	57/63		Fibrin, gelatin
MT6-MMP	MMP-25	16p13.3		IV	Fibronectin Gelatin, laminin

(continued)

Table 1 (continued)

Common name	MMP	Chromosomal location (human)	M.W. (kDa)	Collagen substrates	Some additional substrates*
Other MMPs					
	MMP-19	12q14	54/45	IV	Aggrecan, elastin, fibrillin
Enamelysin	MMP-20	11q22.3	54/22		Gelatin
	MMP-21	ND	70/53		Aggrecan
CA-MMP	MMP-23	1p36.3			Aggrecan
	MMP-27	11q24			Gelatin, casein, fibronectin
Epylisin	MMP-28	17q21.1	56/45		Casein

ND not determined. The list of substrates is not exhaustive

Table 2 Functional relevance of USPs in signaling pathways

Signaling pathway	Function	USPs
p53	Activators	USP7, USP10, USP24, USP29, USP42
	Repressors	USP2, USP4, USP5, USP7, USP15, USP22
NF-κB	Activators	USP2, USP6, USP7, USP17, USP21, USP25
	Repressors	CYLD, USP4, USP7, USP10, USP11, USP15, USP18, USP21, USP25, USP34
RTK	Activators	USP2, USP8, USP18
	Repressors	USP8
Wnt	Activators	USP4, USP5, USP14, USP34, USP47
	Repressors	CYLD, USP4, USP15
TGF-β	Activators	USP4, USP9X, USP11, USP15
	Repressors	CYLD

Collagenases can generally cleave the interstitial collagens I, II, and III and to digest other ECM as well as non-ECM proteins [2, 54].

The gelatinase group, (MMP-2 and MMP-9), mainly digests gelatin, the denatured form of collagen [2, 54]. Gelatinases contain three repeats homologous to the type II module of fibronectin incorporated into the catalytic domain that has been shown to be involved in binding to denatured collagen or gelatin [49]. This domain, known as the gelatin-binding domain or fibronectin type II-like domain, is unique to the gelatinases.

ECM components such as collagen IV and fibronectin are digested by the stromelysins MMP-3 and MMP-10. Another stromelysin (MMP-11 or stromelysin-3) has a different sequence and substrate specificity compared to MMP-3 and MMP-10. For this reason, some authors consider MMP-11 in the heterogeneous subgroup [2, 55].

MMP-12, or human macrophage elastase, is another member of the stromelysin subgroup initially found in alveolar macrophages of cigarette smokers [56].

Six isoforms of the membrane-type MMPs (MT-MMP) are currently known. They can digest ECM proteins such as gelatin, fibronectin, and laminin [57]. Differently from secreted MMPs, the MT-MMPs are membrane associated with cytoplasmic domains important in cellular signaling [58]. MT-MMPs in mammals include four type I transmembrane proteins (MT-1, MT-2, MT-3, MT-5-MMP) and two glycophosphatidylinositol-(GPI-) anchored proteins (MT4-MMP and MT6-MMP) [59, 60]. Moreover, most MT-MMPs activate pro-MMP-2 [2, 61]. Besides the highly conserved MMP functional domains, the MT-MMPs have also additional insertion sequences (IS) controlling the insertion of these enzymes into the cell membrane and conferring unique functional roles [58].

Finally, a heterogeneous subgroup includes the remaining MMPs which are characterized by different substrate specificity, amino acid sequence or domain organization [1, 65, 77]. To this subgroup belong MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, and MMP-28, all able to cleave elastin and aggrecan [2, 62, 63].

4 The Role of MMPs in Normal and Pathological Conditions

MMPs have been considered for a long time as the principal enzymes responsible for the turnover and degradation of extracellular matrix [19]. Indeed, MMPs participate in remodeling and degradation of ECM and basement membranes (BM) occurring throughout the life in a number of physiological processes such as embryogenesis, cell proliferation, migration and differentiation, ovulation, mammary gland involution, as well as in epidermal wound healing, and tissue repair after injury such as myocardial infarction [48, 60, 64]. Some members of this family, including several MT-MMPs, likely play a role in homeostasis since they are expressed in resting tissues or in the regulation of several functions including cell survival, angiogenesis, inflammation, and signaling [65, 66], as well as in neuronal physiology of the adult brain [67].

However, recent findings have clearly demonstrated that MMPs also act on a wide range of non-matrix extracellular proteins, such as cytokines, chemokines, several receptors and membrane proteins, as well as antimicrobial peptides [19] and can also degrade proteins inside the cell, in the cytoplasm, mitochondria, and nucleus [68, 69]. Their targets include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor-binding proteins, cell surface receptors, cell–cell adhesion molecules, and theoretically all structural extracellular matrix proteins [38].

The gelatinases are type IV collagenases which specifically degrade type IV collagen, a component of the basal membrane [64]. These proteinases have been associated by many authors with inflammatory diseases. Indeed, MMP-9, which is

present in very low levels in brain, is markedly up-regulated during an inflammatory response [70].

It is well established that MMP-9 plays a crucial role in chronic inflammation when neutrophil accumulation occurs [71]. MMP-9 is also present in neutrophil extracellular traps, which contain also a mixture of nucleic acids and histones, once released by these cells after activation by infectious agents such as for example *Candida albicans* [72] or *Schistosoma japonicum* [73].

Increase of MMP expression and activity is implicated in a number of acute and chronic pathological conditions, such as arthritis [74, 75], cardiovascular disease including acute myocardial infarction [76], chronic heart failure [77], chronic obstructive pulmonary disease [78–80], asthma [81], inflammatory bowel disease [42, 67] neuroinflammation [65], diabetes [82], tumor growth and metastasis [83–85], multiple sclerosis [86, 87] systemic sclerosis [47], Alzheimer's disease [88], brain injury and disease [89–91]. MMPs are also important in the etiopathogenesis of endometriosis, being involved in the ECM invasion of the endometriotic cells in the ectopic site [39].

Cell types express distinct patterns of MMPs under different stimuli [19]. Similarly, the same proteinase expressed by each cell type may act on different substrates and likely impacts different processes.

5 Regulation of MMP Activity

Several authors have drawn recently their attention on the regulation of MMPs [21, 38, 65]. In vivo, the function of MMPs is controlled at various levels: biosynthesis which include both gene transcription and protein translation, zymogen activation, compartmentalization (ie., the intracellular and pericellular distribution of enzymes), secretion, cell surface expression, activation and inhibition by protein inhibitors, ECM or cell surface localization, oxidative modification.

Physiologically, the expression of many MMPs is accurately and mostly regulated during the transcription of the gene and promoter elements, by specific signals [92]. In normal tissues, MMP expression is usually low, but during tissue remodeling, for example, their synthesis is rapidly induced. Transcription of MMPs is up- or down-regulated by various molecules such as growth factors (EGF, HGF, and TGF-ß), cytokines like interleukins (IL-1, IL-4, and IL-6), or tumor necrosis factor alpha (TNF-α), and chemokines [1, 38, 42, 93, 94].

Post-translational modifications also can occur in fact, activation of pro-MMP precursor zymogens and acetylation [38, 93, 94], represent another level of MMP regulation. As previously stated, most MMPs are generally produced by cells as pre-proenzymes in a latent state and then activated either pericellularly or outside the cell [48]. In contrast, the MT-MMP isoforms can be activated just inside the cell by furin. The signal peptide is cleaved during translation, and pro-MMPs are then obtained [21]. Activation of zymogens is an essential regulatory step of MMP activation and activity. The interaction between the thiol group of a conserved

cysteine residue (Cys[73]) in the prodomain and the Zn^{2+} of the catalytic site maintains the latent state of the pro-MMPs [42, 95]. Proenzymes are transformed to active enzymes by disruption of this interaction, a process known as the cysteine-switch mechanism [96], achievable by two main mechanisms: proteolytic cleavage of the prodomain, including autolysis, or by allosteric change of the thiol group present in the cysteine [42, 61]. A relevant side of the cysteine-switch mechanism activation is that the only step which is really crucial is the disruption of the interaction between the Zn^{2+} and the thiol group. The prodomain can remain attached, and the MMP can be active. Indeed, the thiol-Zn^{2+} interaction can also be disrupted by non-proteolytic mechanisms such as allosteric perturbation by non-substrate macromolecules like integrins and glysaminoglycans (GAGs) or in conditions reproduced in the laboratory using organomurcials, such as APMA, or SDS [19, 92]. In a gelatin zymography, for example, SDS in the running buffer is able to disrupt the Zn^{2+}-thiol binding thus making fully active the proenzyme. The reader is addressed for more details to reviews focusing on regulation of MMPs activity [19, 61, 92].

The extracelluar proteolytic activation of the proenzyme is regulated by several steps which are involving also other MMPs as well as serine proteinases like plasmin [38, 59]. Glycosilation may provide an additional level of control [97].

The intracellular and pericellular distribution of enzymes (compartmentalization) is another noteworthy mechanism for regulating the specificity of MMP proteolytic activity. Indeed, both secreted and membrane-bound MMPs are localized inside the cell to, the nucleus, cytosol, and organelles [39].

After being activated, MMPs are further regulated by endogenous inhibitors, autodegradation, and selective endocytosis. MMP-2, MMP-9, and MMP-13, for example, are internalized by a low density lipoprotein receptor-related protein (LRP) pathway [98]. Control over MMP activity is exerted by specific endogenous inhibitors, among them α2-macroglobulin and tissue inhibitors of MMPs (TIMPs) are the most important [42, 65].

TIMPs constitute a family of proteins which are secreted and are able to inhibit MMP activity outside the cells [48]. In mammals, four TIMPs have been identified at a gene level, namely TIMP-1, TIMP-2, TIMP-3 and TIMP-4 [99]. Inside normal cells, TIMPs rigidly regulate MMPs [49, 100]. TIMPs are synthesized in many tissues and by different cell populations. Their expression is regulated during several processes like, for example, development and tissue remodeling [99]. TIMPs inhibit almost all MMPs which have been evaluated. However, they differ in their affinity for specific MMPs, but their binding does not always mean inhibition [42]. Their amino-terminal domain is required for the inhibitory ability since this domain binds to the active site of the MMPs [49], whereas the other domain (C-terminal) interacts with the Hpx-like domain present in the proenzyme [60]. The balance between MMPs and their corresponding TIMPs is a key factor in normal and pathological tissue remodeling [49, 101]. TIMPs are also involved in MMP-independent TIMP regulation of cell behavior [102]. Furthermore, some TIMPs also may act as anti-angiogenetic [103] and anti-apoptotic factors [104].

A further control of MMPs regulation is exerted by the availability and affinity of substrates, but also by compartmentalization of the proteinases. Reviews of this topic were recently published [61, 92].

6 MMPs and Protozoan Infections

6.1 Malaria

Malaria is one of the major public health problems worldwide; in fact, it causes 300–500 million clinical cases and about 1 million fatalities annually. Five *Plasmodium* spp. may cause malaria in humans, but the most pathogenic species is certainly *Plasmodium falciparum*, particularly in Sub-Saharan Africa [105], although also *Plasmodium vivax* is responsible of a significant burden of morbidity and associated mortality [106]. Among the different pictures characterizing severe malaria, one of those potentially fatal is cerebral malaria (CM). Three mechanisms are envisaged as responsible for this complication: (i) the accumulation of red cells in the capillaries, facilitated by adhesion of infected erythrocytes to endothelial cell surface; (ii) the increase of Blood Brain Barrier (BBB) permeability; (iii) as a consequence of the latter point, the passage of toxic products or proinflammatory molecules like TNF-α, interleukin (IL)-1 and (IL)-6, but also chemokines and other molecules into the brain tissue [36, 107, 108]. Proinflammatory cytokines, like those produced by the Th1 cells activated by the parasite, up-regulate adhesion molecules present on the surface of the endothelial cells. This results in the erythrocyte accumulation in the different organs [107, 109].

The same above mechanisms are responsible also for tissue damage affecting other organs (lungs, kidney, and placenta in pregnant women) during severe malaria [107, 110, 111].

Different strategies are used by the parasite to evade the host immune response, for instance, *Plasmodium* spp. codes and produce antigenic proteins, underlying variation, localized on the surface of the red cells. That is the case of the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) which allows the parasite binding to the molecules localized on the endothelial cell membrane [107].

Between the blood and the nervous tissue or fluid spaces, there are different barrier layers which regulate molecular exchange. The most important of these is represented by the BBB, constituted by the endothelium which is located between blood and brain interstitial fluid. This barrier is permeable in a selective way and regulates ion and nutrient transport into the brain, representing a filter between the CNS and the blood and limiting the passage of molecules from the blood circulation to the nervous tissue.

The blood vessels in the CNS are lined by specialized endothelial cells which are strictly joined by tight junctions and surrounded by a basal lamina along with astrocyte end-foot protuberances [112]. The choroid plexus epithelium between

blood and the ventricular CSF and the arachnoid epithelium between blood and the subarachnoid CSF represent other barriers [112].

When BBB integrity is lost for any reason the transit of potentially toxic molecules into the nervous tissue is facilitated, leading to possible tissue damage. During CM, vascular structure alteration may damage seriously the BBB, and this process is enhanced by the inflammatory cascade previously cited [110].

MMPs, produced by infiltrating leukocytes or by cells of the CNS, mediate in many neurological diseases the disruption of the BBB [113] by cleaving matrix proteins which play a fundamental role in maintaining the integrity of BBB as well as in neuronal survival [114]. Experimental studies show that when MMPs are injected intraparenchymally a rupture of the BBB and the subsequent permeabilization of capillaries is obtained. Even MMP inhibitors, like TIMP-2, are involved in this process, by reducing the degradation of extracellular matrix, caused for example, by type IV collagenase, resulting in protection of the BBB [115].

At least two mechanisms can be envisaged to explain the role of either MMPs or TIMPs in CM pathogenesis: (i) modification of BBB structure and (ii) their participation as effectors and regulators of the immune response, since MMPs can cleave different molecules which participate in such response, like cytokines, chemokines, etc. [36]. TIMPs are family of proteins which are involved in many functions such as the turnover of extracellular matrix, the tissue remodeling, etc. [116].

In vitro, studies have clearly shown that human monocytes incubated with free hemozoin (Hz), a catabolic product of heme component of hemoglobin or Hz-containing trophozoites and endothelial cells, increase MMP-9 expression, production, and activity [36, 117]. The same does not occur for MMP-2 [118].

Data on the possible pathogenetic role of MMPs in CM, occurring in humans, derived from autopsy studies or from evaluations in blood or serum from malaria patients (either uncomplicated or severe) are not conclusive and consistent [36, 119].

In the brains of patients with fatal CM, pro-angiogenic uPAR resulted particularly concentrated [120], as well as MMP-1 and VEGF [121] in Durck granulomas, but anti-angiogenic endostatin/collagen XVIII was not observed in ring hemorrhage areas [122]. All these data suggest that the pro-MMP-9 underlies activation in those areas where parasite sequestration and vascular damage are more evident [36].

Activation of the human MMP-9 gene was observed using microarray analysis carried out on blood from Kenyan children affected by severe *P. falciparum* malaria, with a contemporary increase in neutrophils [123].

However, serum MMP-9 levels were not modified in Ghanaian and Gabonese children, independently on the severity of clinical picture [119, 124]. In the study carried out in Gabon, TIMP-2 levels resulted even lower in malaria patients, compared to healthy people. Vice versa, TIMP-1 resulted associated to the severity of malaria. As regards serum MMP-8 levels, these resulted increased in Gabonese children independently on the severity of disease [124], but not in Nigerian children suffering of uncomplicated malaria [125].

According to all these data, it is possible to conclude that TIMP-1 and MMP-8 levels may preconize the severity of malaria. In particular, TIMP-1 seems to prevent further the damage induced by MMPs by blocking the enzymatic activity of MMP-9, and to a lesser extent, that of MMP-8.

As in other inflammatory conditions involving the brain, such as the experimental model of lipopolysaccharide (LPS)-injured brain or in multiple sclerosis [126, 127], MMP-9 might also be involved significantly in CM pathogenesis, by facilitating the BBB permeability and infiltration of leukocytes. In particular, this enzyme might be induced by the catabolic product of hemoglobin, the hemozoin, in the endothelials, as observed by in vitro studies. Furthermore, this molecule, a side product of parasite invasion of red cells, increases the expression of MMP-1, MMP-3, and also of TIMP-2 [128].

In malaria, caused by *P. falciparum*, according to the analysis of post-mortem brain samples, MMP-1 resulted accumulated in astrocytes of the BBB and in macrophages/microglial cells, which are present in Dürck's granulomas [121], represented by microglial-astroglial nodules surrounding the damaged vessels.

From all these data, we can argue that in *P. falciparum* malaria, particularly when complicated by CM, there is an unbalance between MMP and TIMP, in favor of the former. However, the conclusive demonstration of the role played by the MMPs in disrupting the BBB during CM has to be obtained, and we need more research to highlight this issue [113].

7 Artesunate and MMPs

During *Plasmodium* infection, the blood levels of TNF-α increase because of the activation of monocyte-macrophages, caused either by infected erythrocytes or parasite molecules, such as the malaria pigment hemozoin. As previously said, this induces an up-regulation of MMP-9 either in monocytes or macrophages with a following proteolytical effect not only on the ECM but also on precursors of inflammatory cytokines, amplifying the inflammatory response [36].

Artemisinin and its derivatives such as artemisone, artesunate, and dihydroartemisinin (DHA) can immunomodulate the host response, in fact, they inhibit the mRNA synthesis and the production of MMP-9 in human monocyte-like cells, after stimulation with hemozoin or TNF-α. Artesunate, artemisone, and DHA were capable to antagonize MMP-9 secretion as well as its expression, stimulated by hemozoin, up to 50%.

The derivatives significantly down-regulated both TNF-α-induced MMP-9 secretion and mRNA levels of the enzyme, in a more active way than hemozoin itself. Both hemozoin and TNF-α increased the transcription dependent on NF-kB by 11 and 7.7 fold, respectively. Artesunate, artemisone, and DHA inhibited the NF-kB driven transcription, induced by hemozoin, by 28, 34, and 49%, respectively. In a similar manner, the derivatives, differently from artemisinin, prevented TNF-α-induced NF-kB driven transcription by 47–51%. Furthermore, contrary to

artemisinin, its derivatives such as artemisone, artesunate, and DHA, modulated MMP-9, and also other genes which depend on NF-kB, like TNF-α.

Artemisinins may turn off the inflammatory response of monocytes in vivo. Thus, the beneficial clinical effects of artemisinins for the treatment of malaria would include not only the direct anti-parasitic activities but also the ability to attenuate the inflammatory response, thus reducing the risk of progression to the more severe form of the disease, like the CM [129].

8 The Role of MMPs in the Malaria Vector

A link between MMP and vector competence in mosquito-*Plasmodium* interactions has been observed recently. In fact MMPs resulted involved in the process of tissue invasion within the mosquitoes by malaria stages (ookinetes and sporozoites). In the genome of the malaria vector *Anopheles gambiae* there are three genes encoding MMPs, among them the *A. gambiae*, MMP-1 (*Ag*MMP-1) gene has resulted expressed during the blood digestion, midgut epithelium invasion by *Plasmodium* ookinetes, and the production of oocysts. The two isoforms of *Ag*MMP-1 derive from alternative splicing: the secreted (S-MMP-1), which is associated with hemocytes, and the membrane-type MMPs (MT-MMP-1). This latter is particularly abundant in the cell attachment sites of the midgut epithelium. During ookinete midgut invasion, in particular, MT-MMP-1 resulted increased in expression, in zymogen maturation, and subcellular redistribution. All these modifications suggest an implication of such protease in the midgut epithelial healing which follows ookinete invasion.

Furthermore, during oocyst development, using the RNA interference-mediated silencing of the *Ag*MMP-1 gene, a post-invasion protective function of this gene was observed [130].

9 African Trypanosomosis

This disease is caused by *Trypanosoma* (*T. brucei*), of which two species affect humans (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*) and represents one of the most important infection, caused by parasites, in Sub-Saharan Africa: the human African trypanosomiasis (HAT), also called the human sleeping sickness, and Nagana which affects the cattle. In humans, the disease can progress in two stages: the hemolymphatic one and later on the meningo-encephalitic stage which occurs when parasites succeed in crossing the BBB and invading the CNS [131].

After introduction during the blood meal by the tsetse fly bite, metacyclic trypomastigotes underlie morphological and behavioral modifications in the circulation from which only later they can migrate through the BBB [132]. After passing

through the choroid plexus, the parasites enter into the CSF, to finally reach the nervous tissue. At that time, it is possible to observe an increased expression of receptors, present on the host endothelium, like ICAM-1. Then parasites accumulate in the brain, and this is followed by dramatic leukocyte arrival and activation of astrocytes and microglia, leading to chronic encephalopathy, possibly fatal, when not treated [133].

The mechanisms which cause the disruption of BBB, during African trypanosomosis, are not very well clarified; for example [134], the parasite can transit through the barrier using a cysteine proteinase, partially in a Ca^{++}-dependent way. However, the passage of inflammatory cells and trypomastigotes through the basal lamina into the brain during the meningo-encephalitic stage of HAT may have mechanisms involving certainly the MMPs, in both cases.

It was therefore elucidated that MMP-9 and ICAM-1 represent reliable staging markers for *T. b. gambiense* HAT, and that MMPs as well as ICAMs, alone or in combination, can predict the meningo-encephalitic stage of HAT [135]. Both MMP-2 and MMP-9 create a localized temporary opening of the *glia limitans* (a component of the BBB) by selectively cleaving the β-dystroglycan subunit which anchors the astrocyte end-feet to the parenchymal membrane [136]. In this way, the gelatinases MMP-2 and MMP-9 allow the penetration of inflammatory cells through the outer parenchymal basal membrane into the brain.

MMP-3, MMP-8, and MMP-12, in the brain of *T. b. brucei*-infected mice, at thirty days post-infection, underwent an up-regulation of mRNA expression, concomitantly with a massive accumulation in parasites and leukocytes. On the contrary, the levels of MMP-1b, MMP-2, MMP-7, MMP-9, MMP-11, MMP-13, MMP-14 and MMP-19 as well as TIMP-1 and TIMP-2 mRNA were unmodified. MMP-10 mRNA expression resulted even undetectable [137].

A neutral *T. brucei*-MMP with a marked proteolytic activity on either gelatin or casein, and with a molecular mass of approximately 40 kDa, was partially characterized from the biochemical point of view. Classical inhibitors of MMPs resulted able to inhibit its activity [138].

The MMPs present in the *T. brucei brucei* extract depend strictly on the surrounding environment, with a stability of the proteolytic activity at pH7 but not pH5 or 9 and at 37 °C, as regards the temperature [138].

The levels of MMP-2, MMP-9, ICAM-1, VCAM-1, and E-selectin in the CSF of patients infected by *T. b. gambiense* in the two stages of the disease.

Furthermore, by the evaluation of ICAM-1 and MMP-9, alone or in combination, it was possible to distinguish between stage 1 and stage 2 patients with HAT with very high sensitivity and specificity, and a better performance compared to that obtained with other known markers such as the chemokine CXCL10 [135].

In conclusion, also in trypanosomosis, as it is well established in CM, increased MMP levels play a crucial role in facilitating the passage not only of the parasites, but also of leukocytes, through the BBB into the nervous tissue, where they may induce pathological modifications.

10 Toxoplasmosis

Toxoplasma gondii is an obligate intracellular parasite able to infect virtually any homeoterm animal species [139].

In humans, *Toxoplasma* is widespread and in individuals with an immature or suppressed immune response the infection can cause severe disease, an example is represented by toxoplasmic encephalitis [140].

In *Toxoplasma* encephalitis in mice, CD4+ and CD8+ T cells are mainly recruited to the brain, where they can prevent the reactivation of latent infections [141]. This process is mediated by the Th1-dependent IFN-γ which stimulates anti-parasitic effector mechanisms and regulates chemokine expression and the consequent leukocyte recruitment [142].

Different molecules such as IL-1, IL-23, TNF-α, and COX-2 stimulate, during toxoplasmosis, MMP production in the brain [143].

For example, an increase in CD4+ and CD8+ T cells producing both MMP-8 and MMP-10, has been observed in the brain tissue of *T. gondii*-infected mice [144].

Furthermore, in invading T cells as well as in CNS-resident astrocytes, during infection, TIMP-1 is expressed [143]. In wild type mice, changes in tissue morphology and signs of astrocyte activation occur, contrary to what happens in infected TIMP-1 KO mice where an increase in CD4+ T cells along with a significantly reduced parasite burden in the brain was observed, differently from the peripheral amount of parasites. This was not accompanied by any substantial pathological change in the brain according to histological analyses.

The up-regulation of TIMP-1 during infection might inhibit the pathogen clearance by reducing lymphocyte penetration into the CNS, driven by MMPs. The resulting inhibition of MMPs by an increased expression of TIMP-1 may represent an evasion mechanism of the parasite; in this way, it would try to control the arrival of immune cells or a host response to down-regulate immune-mediated damage.

When the TIMP-1 gene is disrupted in infected mice, in fact, perivascular inflammation is reduced or even absent, but a higher number of CD4+ T cells infiltrating the brain parenchyma is observed, probably reflecting a failure of control of the degradation processes of the basal lamina, carried out by MMPs. This further suggests that these proteinases are essential for the parasite clearance from the brain.

In congenital toxoplasmosis, the parasite arrives to the fetus after crossing the placental barrier.

MMP-12 might participate to elastin degradation which occurs when toxoplasmosis affects pregnant women where serum levels of this proteinase resulted increased along with elastin degradation products [144].

Furthermore, in *T. gondii*-infected pregnant women, it has been recently observed that *T. gondii* induces an increase in MMP-2 and MMP-9 serum and umbilical cord levels.

By co-immunoprecipitation analyses, it was possible in fact to show that MMP-2 and MMP-9 can interact with fibronectin in pregnant women, where the fibronectin monomer of 220 kDa resulted higher than in healthy controls. It is possible to envisage that MMP-2 and MMP-9 may be involved in extracellular matrix degradation and placental barrier dysfunction, which facilitates *T. gondii* transmission to the fetus [145].

11 MMPs and Helminth Infections

11.1 Neurocysticercosis

Neurocysticercosis (NCC) is the infection of the CNS caused by the larval stage of *Taenia solium*, which is acquired after parasite egg ingestion (NCC without taeniasis), or when the parasite cycle is completed, after raw or poorly cooked pork consumption (NCC with taeniasis) [146]. NCC is the most frequent cause of acquired epilepsy globally [147].

The clinical picture of NCC is characterized more frequently by active seizures. The disease is slowly progressive, and multiple factors are involved in determining the severity of the symptoms; among them, the degree of inflammatory reaction in the brain tissue is certainly relevant. It may also happen frequently; however, that NCC patients could remain asymptomatic for long periods, but we do not know the reason [148].

According to studies carried out in experimental animals, MMP expression plays a crucial role in the differential breakdown of the BBB, where distinct populations of immune cells play a role, and different MMPs can cleave cytokines, chemokines, and adhesion molecules [149]. In NCC patients, serum levels of MMP-2 and MMP-9 correlated with clinical manifestations of the disease (presence of seizures).

Mean serum MMP-2 levels were higher independently of the presence of symptoms in NCC cases in comparison with healthy controls, with no difference between the two patient groups.

On the contrary, MMP-9 serum levels were significantly increased in symptomatic NCC patients in comparison with asymptomatic NCC cases or healthy individuals [150].

Recently, higher levels of MMP-9 were found to correlate with epilepsy [151, 152], suggesting that in symptomatic NCC patients the seizures might be facilitated by increased levels of this gelatinase.

In a study conducted in India, some Toll-like receptor (TLR-4) gene polymorphisms in patients with solitary cysticercus granuloma, which is the most frequently observed type of NCC in that country, resulted particularly frequent, suggesting a possible association to the susceptibility to infection of individuals with such genetic traits [153].

The TLR-4 gene polymorphisms Asp/Gly and Thr/Ile, Gly (Asp/Gly plus Gly/Gly) and Ile (Thr/Ile plus Ile/Ile) resulted significantly associated with calcified

neurocysticercosis. Vice versa, Gly/Gly and Ile/Ile genotypes were not significantly associated with either group. Furthermore, the levels of MMP-9 resulted higher in calcified neurocysticercosis, particularly when calcifications were multiple.

The heterozygous forms Asp/Gly and Thr/Ile, on the contrary, were significantly associated with seizure recurrence. The Gly (Asp/Gly plus Gly/Gly) and Ile (Thr/Ile plus Ile/Ile) genotypes were also significantly associated with seizure recurrence, to which also serum MMP-9 levels were significantly associated. The Authors suggested that The TLR-4 gene abnormalities may facilitate the inflammatory response around calcified neurocysticercosis leading to an increase in perilesional edema and following risk of seizures [154].

12 Nematode Infections

MMPs have been described in several parasitic nematodes (*Brugia malayi*, *Toxocara canis*, *Strongyloides stercoralis*, *Nippostrongylus brasiliensis*, *Dirofilaria immitis*, *Trichuris suis*, *Ancylostoma caninum*, *Caenorhabditis elegans*, and *Gnathostoma spinigerum*), but their sole in the life cycle is not always well clarified [155].

It is supposed that in parasitic nematodes, as in the case of hookworms, TIMPs play crucial roles in the host-parasite relations, which include the invasion process and establishment in the vertebrate animal hosts [116].

By a large-scale investigation of TIMP proteins in several human parasites (nematodes such as *Necator americanus*, *Ascaris suum*, but also the liver flukes *Clonorchis sinensis* and *Opisthorchis viverrini*, as well as the schistosome blood flukes), 15 protein sequences with a high homology to known eukaryotic TIMPs were predicted from the sequence data available for parasitic helminths and subjected to in-depth bioinformatic analyses [116].

A very well-studied nematode model in vivo as regards MMP involvement in the mechanisms responsible for organ damage (mainly the brain) mediated by MMPs is infection caused by *Angiostrongylus cantonensis*, a parasitic nematode which causes an eosinophilic meningitis which can occur mainly in the southeast Asia [156].

This rat lung worm is obligate to migrate inside the cerebral parenchyma in its hosts [157]. When non-permissive hosts such as humans and mice are infected with this parasite, the worms pass to the brain and mature into young adults; however, contrary to rats, they fail to reach maturity within the heart and lungs [157]. This nematode has a particular tropism for the nervous tissue; in fact, it requires a sejour in the CNS of mammalian hosts to grow [158].

The infective L_3 larvae orally infect the final host and by the general circulation arrive to the CNS, here they molt twice to transform in immature adults and enter the subarachnoid space. In a permissive host (like rats), immature adults migrate from the brain to the lungs, where they fulfill the maturation process [157].

In non-permissive hosts, however, the immature adults remain in the CNS of the host, causing eosinophilic meningitis or meningoencephalitis [159]. What we know about this nematode infection derives most from experimental studies in rodents. In fact, mice infected with *A. cantonensis* undergo eosinophilic meningitis, with a peak at around three weeks of eosinophilia in the CSF [160, 161]. The presence of MMP-9 in CSF was revealed 10 days after inoculation (a.i.) with a peak between day 15 and 25 a.i.

The MMP-9 is localized within inflammatory cells such as eosinophils and macrophages, accumulated in the subarachnoid space of experimentally infected mice, according to immunohistochemical results, suggesting that infiltrating leukocytes are the relevant source of MMP-9 in this type of meningitis [162].

The increase of MMP-9 activity was paralleled by that, even more rapid, of CSF eosinophils and the inflammatory reaction of the subarachnoid space. Differently from MMP-9, MMP-2 activity did not change during infection [162].

In *A. cantonensis*-infected patients, affected by, eosinophilic meningitis, the amounts of MMP-2, MMP-9, and TIMP-1 resulted significantly increased in the CSF, compared to healthy controls, whereas TIMP-4 levels resulted significantly lower. MMP-9 in CSF increases in patients in parallel with CSF leukocyte counts and CSF/serum albumin ratio (QAlb) values. When patients are treated with mebendazole and dexamethasone, during recovery from eosinophilic meningitis, a gradual decrease in MMP-9 levels, QAlb and TIMP-1, and a contemporary increase in those of TIMP-4, was observed. During the acute stage of infection, TIMP-4 levels were lower, even when MMP-9 and TIMP-1 have already decreased, until eosinophil meningitis was not definitely declined. This suggests that in these patients TIMP-4 has an important role in regulating the proteolytic-derived BBB damage, [155]. These results in humans further supported what we knew from experimental studies.

The activity of MMP-9 in the CSF of patients was not completely suppressed, since simultaneously TIMP-4 decreases, leading to BBB dysfunction, as indicated by the higher QAlb values detected in these patients. Changes in the cytokine milieu have a strong impact on modulation of TIMP expression and may be responsible for modifications of the levels of these proteins (MMPs/TIMPs) in this kind of meningitis [155].

Also, MMP-12 along with its substrate, elastin (present in the meningeal vessel of the subarachnoid space), is involved in the inflammatory response. MMP-12/TIMP-1 ratio, in fact, is significantly increased in the CSF of *A. cantonensis*-infected mice from day 10 post-infection (p.i.) and reached the peak on days 20 and 25 p.i. The production of MMP-12 is correlated with several parameters (elastin degradation, number of eosinophils, blood–CSF barrier permeability, and pathological modifications in the subarachnoid space).

When the antihelmintic albendazole is associated with doxycycline (used in this case as a non-selective MMP inhibitor), the amounts of MMP-12, elastin, and Evans blue accumulation in the CSF in mice affected by meningitis were significantly reduced, indicating that MMP-12 participates to the degradation of elastin [163].

The invasion by the parasite of the host meninges or nervous parenchima occurs with a concomitant increase of molecules such as tissue-type plasminogen activator (tPA), urokinase-type PA (uPA), MMP-9, and MMP-12 in the CSF [163, 164]. As a result of the action of proteolytic enzyme increase, the brain barrier is disrupted, facilitating the eosinophil infiltration, as well as larval diffusion.

During *A. cantonensis* infection, leukocytes can pass the blood–CSF barrier, then entering the subarachnoid space by crossing the wall of meningeal vessels, formed by endothelial cells and elastic tissue which is degraded by proteinases such as MMP-12, released by infiltrating leukocytes [163].

After MMP-9 and -12 activation, the BBB becomes more permeable because tight junction proteins are destroyed. It has been shown that MMP-9 through the NF-κB/MMP-9 signaling pathway in angiostrongyloidosis mediates the degradation of claudin-5, followed by blood–CSF barrier impairment in the brain [165] (Fig. 2).

13 MMPs and Trichinellosis

Trichinellosis is a foodborne parasitic disease caused by nematodes of the genus *Trichinella*. We presently distinguish eight species in this genus (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi*, *Trichinella murrelli*, *Trichinella patagoniensis*, *Trichinella pseudospiralis*, *Trichinella papuae*, and *Trichinella zimbabwensis*) and three genotypes distributed in different geographical areas [166]. The first above five species may be grouped in the clade of encapsulating species, whereas the three remaining, in that of non-encapsulating species [166].

Infection is acquired by ingestion of raw or undercooked meat from infected (i.e., containing the L_1 infective larva) mammals, birds, and reptiles. This larva, inside the nurse cell, may be surrounded or not by a collagen capsule (see above), is released by gastric juice action in the stomach and after arriving to the intestine molts in adult male or female worms. After mating, the females, localized inside the epithelial intestinal cells, begin to shed the newborn larvae which enter the blood and lymph circulation to arrive to the final destination, the skeletal muscle cell, the only tissue where they can survive and develop [167]. Encapsulated species such as *T. spiralis* and non-encapsulated species like *T. pseudospiralis* behave differently in the host-parasite relationship as regards, for example the inflammatory response at muscle level which is stronger in the former [168].

In experimental murine infection with the nematode *Trichinella* spp. MMP-9 and MMP-2 underlie significant level changes during infection [169].

In mice infected with *T. spiralis*, the total MMP-9 serum level increased 6 days p.i., whereas that of total MMP-2 started to increase later. A similar result was obtained in mice infected with *T. pseudospiralis*, but the level of MMP-9 resulted

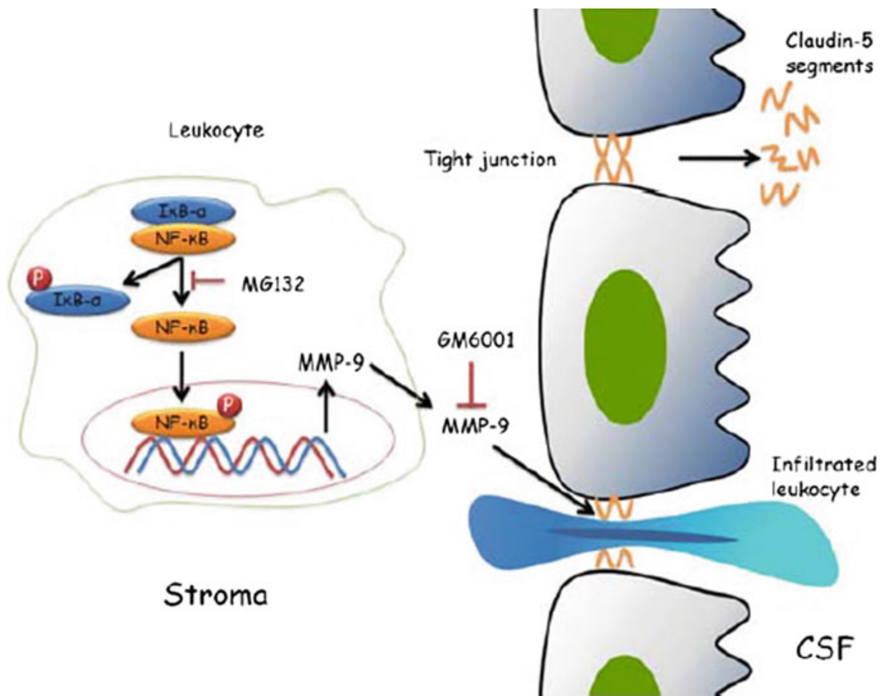

Fig. 2 Mechanisms of matrix metalloproteinase (MMP)-9 leading to claudin-5 degradation via the NF-κB pathway. The activation of NF-κB up-regulates MMP-9 production in *Angiostrongylus cantonensis*-induced leukocytes. Blocking of MMP-9 activity can reduce claudin-5 degradation and blood–CSF barrier permeability during angiostrongyloidosis meningoencephalitis. Therefore, MMP-9 is suggested to cause claudin-5 degradation and that it promotes leukocyte infiltration into the CSF by the paracellular route during *A. cantonensis* infection in the mouse choroid plexus. From Lai [76] with permission

lower than that found in *T. spiralis*-infected mice. As regards MMP-2 levels, significant differences were also observed between the two experimental groups of mice (infected either with *T. spiralis* or *T. pseudospiralis*).

Furthermore, serum TIMP-1 mean levels in *T. spiralis* or *T. pseudospiralis*-infected animals resulted significantly higher than in control mice at 21 days p.i. However, the TIMP-1 level was significantly higher in *T. spiralis* infected than in *T. pseudospiralis* infected animals, only at two weeks of infection. The kinetics of TIMP-1 paralleled that of TNF-α. On the contrary, IL-1β did not change during the whole period of observation in both groups of animals infected with the two *Trichinella* species.

In conclusion, MMP-9 and MMP-2 have resulted reliable markers of inflammation in both *T. spiralis* and *T. pseudospiralis* infections [169].

Serum levels of these MMPs were also analyzed in patients affected by trichinellosis during an outbreak caused by *T. britovi*. A representative zymography carried out in patient sera is shown in Fig. 3.

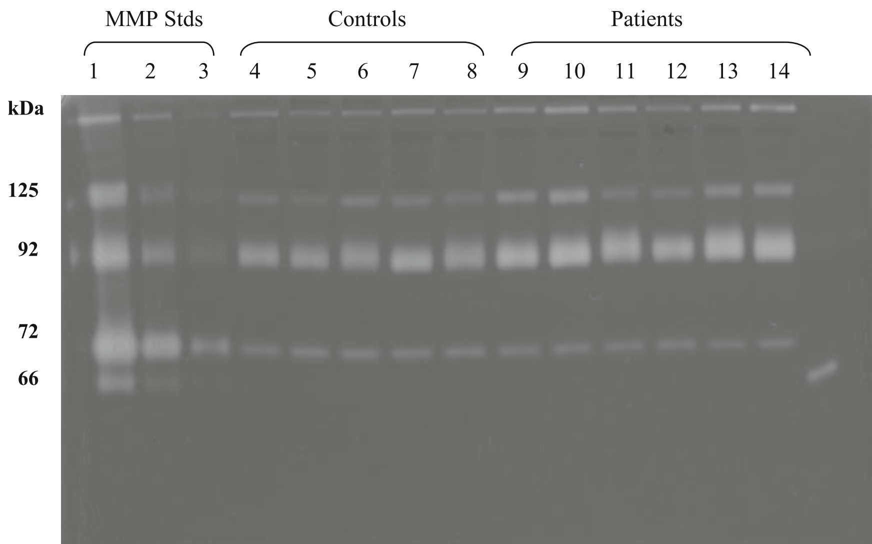

Fig. 3 Zymographic analysis of sera from *T. britovi*-infected patients and healthy individuals. Lane 1–3 and lane 15, Molecular standards; lane 4–8, healthy controls; lane 9–14 *T. britovi*-infected patients. The number on the left show the molecular weights of respectively: MMP-9/NGAL complex (125-kDa), Pro-MMP-9 (92-kDa), Pro-MMP-2 (72-kDa), Active-MMP-2 (66-kDa)

Gelatinolytic activity, corresponding to pro-MMP-9 resulted significantly increased in most of the patients in comparison with the healthy group. The same did not occur for pro-MMP-2 activity. The zymographic analysis of the gels showed the presence in serum samples of gelatinase bands at approximately 125, 92, and 72-kDa, corresponding to the MMP-9/Neutrophil gelatinase-associated lipocalin (NGAL) complex and proenzyme forms of MMP-9 and MMP-2, respectively. MMP-9/NGAL serum level was significantly augmented in patients, compared to healthy controls.

The MMP-9 levels resulted increased also by ELISA in the patient sera (Fig. 4a), with a significant correlation with zymographic data, as shown in Fig. 4b.

Furthermore, they resulted higher in more affected patients (suffering diarrhea, facial edemas, and myalgia). On the contrary, no difference was observed in patients with or without eosinophilia and muscle enzyme increase. In the light of these results, MMP-9 might be considered as a marker of inflammation in *T. britovi* patients, differently from MMP-2 which did not result significantly changed in patient sera, in comparison with controls (Fig. 4).

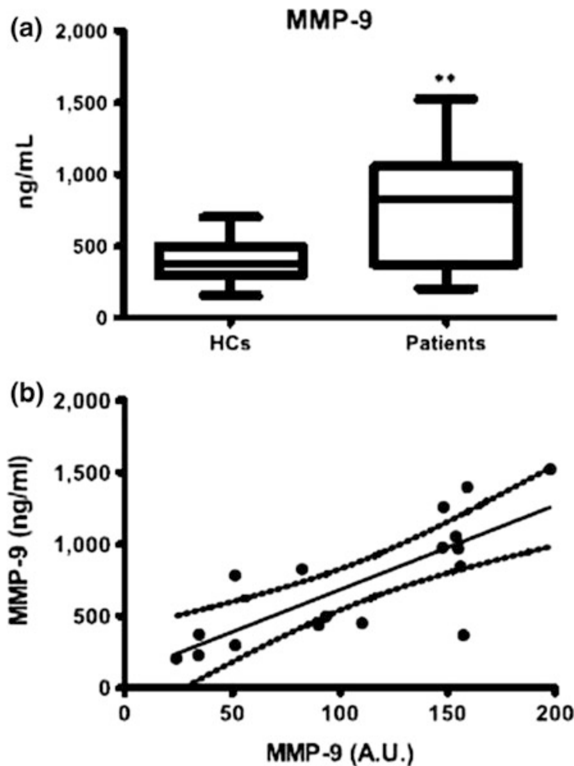

Fig. 4 **a** MMP-9 serum levels in trichinellosis patients. ** indicates the significant difference compared to healthy controls ($p = 0.0037$). **b** in a simple regression analysis, MMP-9 activity and MMP-9 serum levels were highly significantly related to each other ($r = 0.77$, $p < 0.001$) in patients. Solid line indicates the linear regression line. Dot lines indicate the 95% Confidence Intervals. MMP-9 levels are expressed as concentration (ng/ml) in ELISA test and as arbitrary units of gelatinolytic activity (A.U.) × 106 in the gelatin zymography. From Bruschi et al. [18] with permission

14 Concluding Remarks

Parasitic infections (caused by protozoa such as *Plasmodium*, African *Trypanosoma*, *T. gondii* or helminths such as *T. solium*, *A. cantonensis*, *Trichinella*), have in common an increase in levels of several MMPs, which can be induced either directly or indirectly by regulating cytokine levels, therefore, with an imbalance between such enzymes and TIMPs. The result of this unbalance is the amplified inflammation mediated damage which may occur in several organs.

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Roles of *Candida albicans* Aspartic Proteases in Host-Pathogen Interactions

Mariusz Gogol, Oliwia Bochenska, Marcin Zawrotniak, Justyna Karkowska-Kuleta, Dorota Zajac and Maria Rapala-Kozik

Abstract

Candida albicans—a common opportunistic fungal pathogen of humans—causes serious, disseminated invasive infections (candidiases) executed due to the action of several groups of virulence factors. One of the most critical is a family of secreted aspartic proteases involved in the destruction of host proteins and tissues. This chapter aims to characterize biochemical and structural properties of these enzymes that determine their functions and summarize their specific roles in the development and propagation of fungal infections. Candidal aspartic proteases deregulate the host biochemical homeostasis, by impairing the major proteolytic cascades such as the blood coagulation, the kallikrein-kinin system, and the complement system, by unleashing the activity of host proteases due to the degradation of specific endogenous inhibitors and by the inactivation of antimicrobial peptides and proteins produced by host cells. The degradation of important host proteins influences the fungal adhesion to the host cell surfaces, promotes the subsequent tissue damages, and enables the further dissemination of the pathogen. Confirmed multiple roles of candidal aspartic proteases in the host-pathogen interactions during candidiasis qualify these enzymes as promising potential targets for novel antifungal therapies.

M. Gogol · M. Zawrotniak · J. Karkowska-Kuleta · M. Rapala-Kozik (✉)
Department of Comparative Biochemistry and Bioanalytics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland
e-mail: maria.rapala-kozik@uj.edu.pl

M. Gogol · O. Bochenska · D. Zajac
Department of Analytical Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Gronostajowa 7, 30-387 Krakow, Poland

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Candida albicans · Fungal infection · Aspartic proteases (Saps)
Protein degradation · Host cell interaction · Antibacterial peptides · Kinins

1 Introduction

The current progress in the development of advanced health technologies, rescuing critically ill patients, has inevitably led to a considerable increase in the number of immunocompromised individuals who are particularly vulnerable to infections caused by opportunistic microorganisms including pathogenic fungi [1]. The catheterization and application of parenteral nutrition, hematologic malignancy, surgical treatments, including those related to cancer therapy and organ transplantation, HIV infection, the use of immunosuppressive therapies and broad-spectrum antibiotic treatment, as well as inherited immunodeficiency, old age and prematurity, are the main risk factors contributing to the development of opportunistic fungal infections [2–5].

The *Candida* spp. yeasts belongs to the most commonly identified fungal opportunistic pathogens of humans, responsible for serious, disseminated invasive infections [6, 7]. These fungi are also considered to be some of the major pathogens responsible for nosocomial bloodstream infections associated with high mortality rates in the range of 40–70% of infected patients [1, 2, 8]. *Candida albicans*—the most prevalent species from the *Candida* genus—is a part of normal human microbiota as a commensal microorganism that colonizes the skin and mucous membranes of the oral cavity, gastrointestinal tract, or genitourinary system [9, 10]. The genital or oral carriage of these yeasts is reported to be present in 20–65% of healthy individuals. However, when the delicate balance between microbial colonizers and the host is disturbed, *Candida* yeasts can cause annoying and painful superficial infections including thrush, oral candidosis, or candidal vulvovaginitis that can affect considerably large number of individuals colonized by these fungi [11–14].

The significant changes in the global distribution of particular *Candida* species and diversified prevalence among different groups of patients have been noticed over the last few decades [3, 15]. Despite the fact that *C. albicans* is still the major infectious agent from the genus *Candida* responsible for approximately 50% of all candidiases worldwide, other, so-called non-albicans *Candida* species have been emerging as fungal pathogens of humans, attributed to an increasing share in the overall number of candidal infections [16, 17]. This group includes mainly four species—*C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*—that greatly differ in terms of the mechanisms of their pathogenicity [18].

Candida yeasts display a broad range of virulence attributes (Fig. 1) that allow them to successfully colonize and invade the host organism. During the infection, several mechanical, physical, and chemical protective barriers and biochemical defenses have to be affected by pathogens [19].

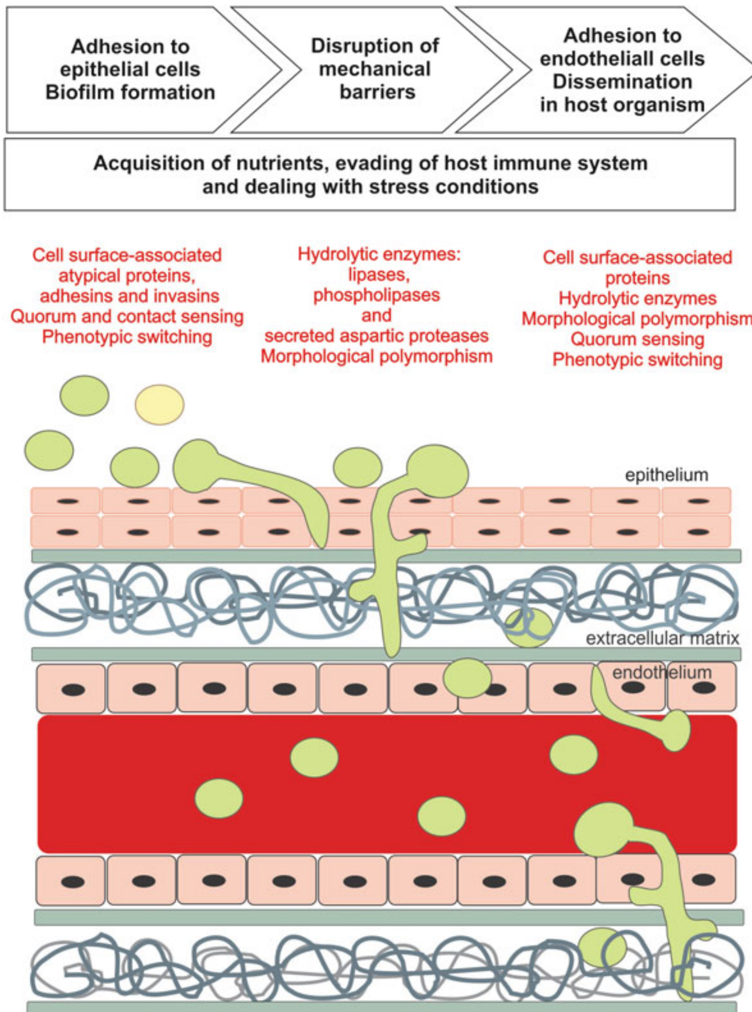

Fig. 1 Involvement of *C. albicans* virulence factors and traits related to its pathogenicity in the sequential stages of interaction with the human host during infection (based on [20, 21])

The candidal virulence factors primarily include two large groups of proteins: (i) an abundant set of cell surface-exposed proteins (adhesins and invasins) [22–24] and (ii) secreted hydrolytic enzymes such as lipases, phospholipases, and aspartic proteases [25–27]. The presence of such molecules, in a combination with other virulence-related fungal traits like a morphological polymorphism—i.e., the ability of the fungus to grow as unicellular, ovoid yeast-like forms or as filamentous forms (true hyphae or pseudohyphae)—the contact- and quorum-sensing, the phenotypic switching (“white” → “opaque”) and the biofilm formation greatly facilitates the initiation of the first contact of fungal cells with the host, followed by further

dissemination within the human body and the subsequent development of infection [28]. All of these features and abilities contributing to the fungal pathogenicity work together to successfully combat or evade the host immune system and take control of the processes involved in maintaining physiological homeostasis of the host. In particular, the role of proteolytic enzymes in this phenomenon can hardly be overestimated [21, 26].

2 The Family of *C. albicans* Secreted Aspartic Proteases (Saps)

The proteolytic activity of *C. albicans* was first described in 1965 [29] and has more recently been assigned to 10 secreted aspartic proteases (Saps). The open reading frames (ORFs) for their genes, located on five different chromosomes, vary between 1173 and 1764 bp in length [26]. The products of their expression are preproenzymes longer by about 60–200 amino acids than active proteins due to the presence of the N-terminal signal peptide and the propeptide, which are proteolytically removed by the signal peptidase and Kex2 protease, respectively, during the classical secretory pathway in order to form the final products with a molecular mass within the range of 35–50 kDa [30, 31].

Although all *C. albicans* aspartic proteases are directed to the secretory pathway, only Sap1–Sap8 are secreted in the form of soluble enzymes. The other two, Sap9 and Sap10, are equipped with glycosylphosphatidylinositol (GPI) anchor that attaches them to the fungal cell wall or both, the cell wall and cell membrane [25, 32]. These enzymes are structurally similar to yapsins, proteases involved in maintaining the cell wall integrity in *Saccharomyces cerevisiae* [32].

Within the *C. albicans* Sap family, three separate groups can be distinguished according to the degree of amino acid sequence similarity [25]. Among them, Sap4–Sap6 and Sap1–Sap3 represent the highest similarity degree (Fig. 2). The expression of these proteases seems to be dependent on the specific morphological form of the yeast, because Sap1–Sap3 are expressed primarily by the yeast-like forms, whereas Sap4–Sap6 are characteristic for hyphal forms. Saps that belong to these groups have a high sequence similarity to Sap8 which constitutes the third group together with Sap7 that shares only 20–27% sequence identity with other proteases and with Sap9 and Sap10 which are slightly closer homologs [30, 33].

3 Biochemical Properties of *C. albicans* Aspartic Proteases

So far, for four out of ten Saps of *C. albicans*, the crystal structures have been solved, including Sap1 in an inhibitor-free form [34], the complexes of Sap2 with inhibitor A70450 [35, 36] and benzamidine [37], Sap3 in the free form and bound with pepstatin A [38], and the complex of Sap5 with pepstatin A [34]. The latter structure

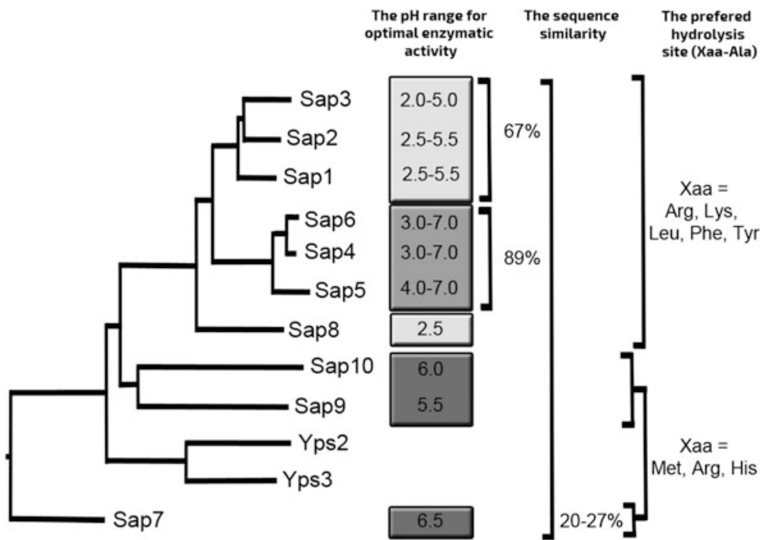

Fig. 2 Dendrogram presenting the amino acid sequence similarity between the members of *C. albicans* aspartic protease family (Sap1–Sap10). A close relationship of Saps to *S. cerevisiae* yapsins (Yps) is also emphasized. The optimal pH for Sap action and the preferential hydrolysis sites are also specified [25, 30]

is presented in Fig. 3. All structurally characterized Sap isoenzymes show similar features that classify them as pepsin-like aspartic proteases. These kidney-shaped bilobed globular proteins predominantly consisting of β-sheets are clearly divided into N-terminal and C-terminal domains, each providing one catalytic Asp residue, belonging to highly conserved regions in aspartic proteases with the motifs Asp-Ser-Gly or Asp-Thr-Gly and the disulfide bridge. Another conserved region that overlaps the active center is a β-hairpin loop, commonly known as the active site flap, which contains a catalytically essential Tyr residue. The disulfide bridges tie together the N-terminal and C-terminal entrance loops. The presence of the N-terminal entrance loop, consisting of 11 amino acids and specified as the second site flap [39], distinguishes Saps from the other aspartic proteases [34, 38, 40].

In addition, the structural and conformational differences allow the Sap isoenzyme structures to be divided into distinct subgroups. The most significant differences between Sap1–Sap3 and Sap5 regard the substrate binding site pockets, which have different characteristics, shapes, and sizes. On one hand, the S3 and S4 pockets, relatively large and with negative polar character in Sap1–Sap3, in Sap5 have a reduced size and the polar character is turned highly positive due to the substitutions of Leu297 (Sap1–Sap3), Asp299 (Sap2), Asp120 (Sap2, Sap3), and Gly299 (Sap1, Sap3) residues with Arg residues. On the other hand, the S2 pocket is enlarged in Sap5 because of the replacement of Asn131 (Sap1, Sap2) with Gly. The S1 and S2 substrate binding pockets in the central region of the enzymes show

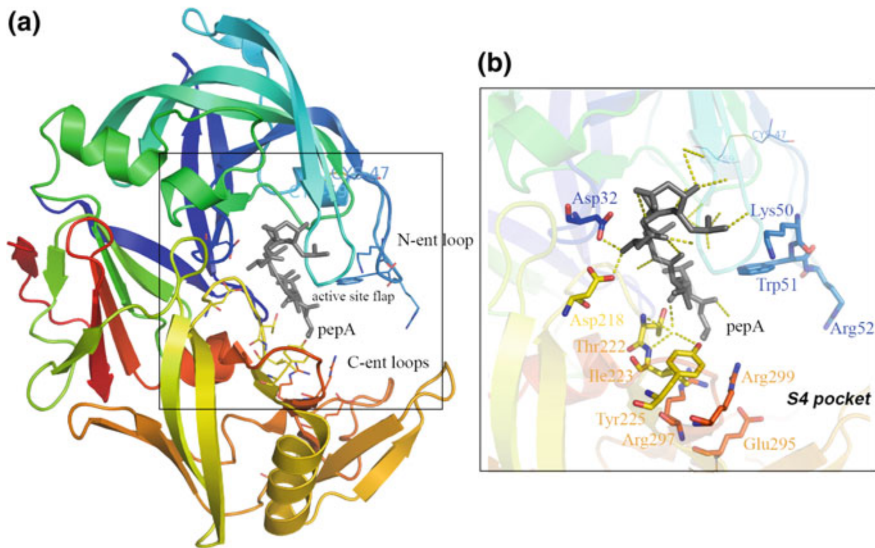

Fig. 3 Structure of the complex of Sap5 with pepstatin A (pepA). **a** The overall structure of the Sap molecule, showing its kidney-shaped appearance and the predominance of β-sheets. The loops tied together by the disulfide bridges are specified as the N-terminal entrance loop (cysteine residues 47 and 59 showed as blue sticks) (N-ent loop) and the C-terminal entrance loops (cysteine residues 256 and 294) (C-ent loops). **b** A close-up view of the active site with marked conserved Asp32 and Asp218 residues and the disulfide bridge (Cys47 and Cys 59), Lys50, Trp51, and Arg52 residues forming the N-ent loop directed to the active site cleft and the residues Thr222, Ile223, Tyr225, Glu295, Arg 297, and Arg299 involved in substrate binding in the S4 pocket. Yellow dashed lines indicate hydrogen bonds between pepA and residues that form the substrate binding pocket. The figure was made with Pymol [41], PDB ID:2QZX

only a few differences, of which the formation of an extra cavity within the S1 pocket in Sap5 due to the substitution of Arg195 and Glu193 with Thr195 and Lys193 is the most important. Another significant difference is a narrowed entrance to the active site cleft in Sap5 relative to Sap1–Sap3 because the N-terminal loop in the former has larger residues, such as Lys50, Trp51, and Arg52, pointing down into the substrate binding cleft. Although the active site is strongly negative in all isoenzymes, the overall electrostatic charge of Sap5 is positive while Sap1, Sap2 and Sap3 are negatively charged. The difference in overall electrostatic charge of the molecule can underlie a rise of the optimal pH for Sap5 activity, compared to Sap1–Sap3 [34].

The pH dependence of Sap enzymatic activity was characterized for recombinant proteins [30, 42–44] as well as for Sap1–Sap3 purified from *C. albicans* culture supernatants [45] and determined using resorufin-labeled casein [30, 42], FRETS-25Ala library [30], bovine hemoglobin [43], bovine serum albumin [45], and a peptide, histatin 5 (His5) [44]. The analysis performed in the broad range of pH between 2.0 and 7.5 indicated that most of Saps displayed the optimum for

Table 1 Biochemical properties of recombinant Saps [30]

Enzyme	Sap1	Sap2	Sap3	Sap4	Sap5	Sap6	Sap7	Sap8	Sap9	Sap10
Molecular mass (kDa)	36	36	37	37	37	37	47	35	53	45
Optimal pH for hydrolytic activity	5	4	3	5	5	5	6.5	2.5	5.5	6
pH range for activity	2.5–6.5	2.5–5.5	2–5	2.5–7	4.5–6.5	2.5–6.5	4–7.5	2–6.5	2.5–7	3–7
Pepstatin A inhibition	+	+	+	+	+	+	–	+	+	+
N-glycosylation	–	–	–	+	–	+	+	+	+	+

proteolytic activities at pH 3.0–5.0 (Table 1), a feature typical for aspartic proteases; however, slightly variable results were obtained by different research groups. The pH optimal for Sap4–Sap6, Sap7, Sap9–Sap10 is less acidic, and the enzymes are still active at neutral pH. Sap3 and Sap8 differ from other Saps in showing substantial activity at pH 2.0. Interestingly, Sap8 shows the lowest pH optimum of 2.5, whereas Sap7, which is the less related with other Saps, shows the highest (6.5). The activity of all Saps, except Sap7, is inhibited by the classic aspartic protease inhibitor, pepstatin A. It has been suggested that pepstatin A insensitivity is due to the presence of Met242 and Thr467 residues which restrict the accessibility of pepstatin A to the binding site [46]. The biochemical characteristics of Sap isozymes are summarized in Table 1 [30].

Substrate specificities of all ten Sap isoenzymes (briefly summarized in Fig. 2) were determined by using FRETS-25Xaa libraries [30] or distinct peptide substrates [32, 43, 47]. A study of the substrate specificities at the P1 and P1′ sites for Sap1–Sap3 and Sap6 [43] showed that P1′ specificities are generally broader than those observed for P1. In general, Sap1–Sap6 and Sap8 have a broad substrate specificity and, like other aspartic proteases, prefer to hydrolyze peptide bonds after hydrophobic residues such as Leu, Phe, and Tyr, but also after positively charged residues such as Arg and Lys. In contrast, Sap7, Sap9, and Sap10 have narrower substrate specificities and prefer at the P1 site residues such as Met, Arg, and His Sap9 and Sap10 perform hydrolysis after dibasic (LysArg, LysLys) or monobasic (Lys, Arg) residues [32, 47], and almost all Saps hydrolyze peptide bonds before Ala [30]. Based on the similarities and differences in substrate specificities, Sap isozymes can be categorized into three groups [30]. Group 1 comprises Sap7 and Sap10, and most notably differs from other Saps in terms of substrate specificity, which here is narrowest. Sap4–Sap6 are categorized into group 2, and group 3 comprises Sap1–Sap3 and Sap8–Sap9, with a very similar, broad substrate specificity.

4 Functions of Saps in *C. albicans* Virulence

C. albicans exploits its proteolytic enzymes for host tissue invasion and inactivation of the host's immune defense, to establish fungal infection. These factors are mandatory for the degradation of tissue barriers and acquiring nutrition at different host niches [48]. The adaptation to the host environment and the propagation of infections as well as further dissemination demand the involvement of the proteases in, for instance, combating the host immune cells like neutrophils or mononuclear phagocytes [49] as well as inactivating proteins of the complement system [50]. The roles of Saps during host infection, in terms of interactions of these pathogen proteases with major cellular, proteinaceous and peptide targets of the host, and the further consequences of these interactions, are briefly summarized in Fig. 4, and will be discussed in detail in the following subsections.

4.1 Expression of *C. albicans* Aspartic Protease Genes During Candidal Infection

As the individual members of *C. albicans* Sap family play diverse roles during both commensal and pathogenic interactions with the host, their genes are differentially expressed at various body sites, depending on the type, phase, and site of the infection [25, 51–53]. *SAP1–SAP3* gene expression was detected in both yeast and hyphal cells. *SAP1–SAP3* genes, predominantly expressed during mucosal infections [51, 54], were suggested to be significant for infection process in general, whereas the gene encoding Sap4, which belongs to the Sap4–Sap6 subfamily, was expressed in the hyphal phase during the adhesion to and penetration of epithelial cells [33, 52]. This Sap isoenzyme was suggested to be essential for the development of systemic infections and to be involved in avoiding the immune response [55]. In vivo, analysis of the expression of *SAP1–SAP8* genes in oral candidiasis [25, 51, 52] showed the highest frequency of *SAP2* expression both in colonized and infected patients. During oral infections, the expression of *SAP1*, *SAP4*, *SAP7*, and *SAP8* genes was also detected at a significant level [25]. In the oral reconstituted human epithelium (RHE) model, *SAP1* and *SAP3* expression was detected, followed by *SAP2* and *SAP8* expression [57]. In contrast, in the RHE model of vaginal candidiasis, *SAP2* and *SAP9* expression preceded *SAP1* expression [58]. Nevertheless, in both oral and vaginal RHE models, *SAP1–SAP3* and *SAP9* contributed to tissue damage [25, 58, 59]. In addition, an extensive expression of *SAP1–SAP3* and *SAP7–SAP8* genes was observed in a model that mimicked bloodstream infections [55, 60]. An investigation of *SAP7* expression during *C. albicans* adhesion to the intestinal human cells indicated a meaningful role of Sap7 during the initial adaptation of *C. albicans* to intestinal tract which decreased over time [61]. Sap8 correlates mostly with oral or vaginal infections, with the expression of its gene detected in a RHE model together with *SAP1–SAP3* [33]. *SAP9* was one of the most expressed genes in vivo during human mucosal infections and in

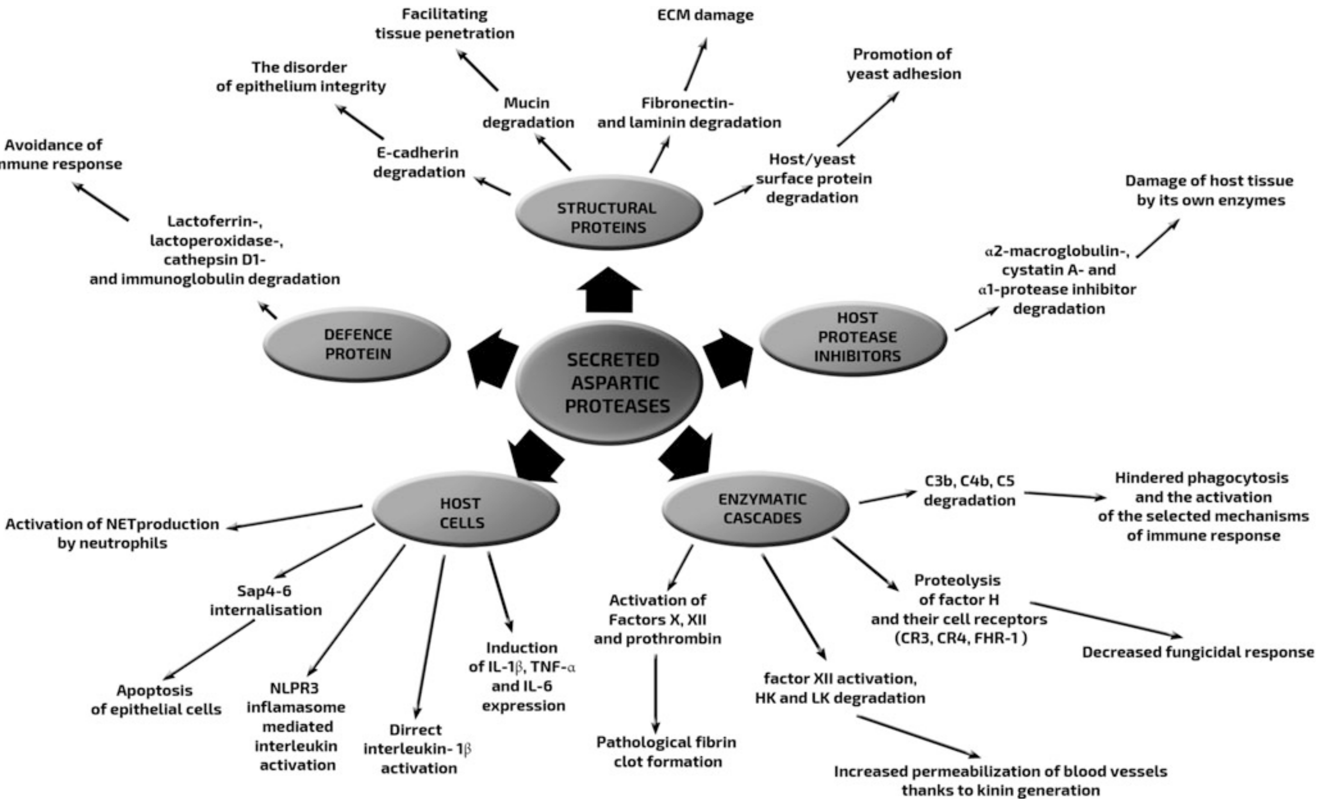

Fig. 4 Roles of *C. albicans* Saps in the course of candidal infections: interactions of Saps with major targets in the host, with further consequences for the development and progress of infection

Table 2 Expression of Sap-encoding genes in humans and in animal models

Model	Infection	Proteases involved	References
Mouse and guinea pig	Systemic infections	Sap4–Sap6 are involved in the progression of systemic infection	[62]
Mouse and guinea pig	Disseminated infection	Sap1–Sap3 presumably play an important role during disseminated candidiasis	[63]
Mouse	Gastrointestinal infection	Expression of *SAP1–SAP3* is lower than that of *SAP4–SAP6*	[64]
Mouse	Disseminated candidiasis	*SAP1*, *SAP2*, *SAP4, SAP5*, *SAP6,* and *SAP9* are the most commonly expressed Sap-encoding genes within 72 h after infection	[65]
Mouse	Disseminated candidiasis	Sap1–Sap6 do not play a significant role in the murine model of disseminated candidiasis	[66]
Mouse	Keratitis infection model	*SAP6* is associated with corneal pathogenicity and appears to be associated with morphological transformation into invasive hyphae	[67]
Rat	Vaginal candidiasis	Proteases are actively secreted during rat vaginitis and localized in the cell wall of *C. albicans* during infection	[68]
Human	Oral and cutaneous candidiasis	Expression of *SAP1–SAP3* is higher than *SAP4–SAP6* in oral and cutaneous candidiasis	[52]
Human	Vaginal candidiasis	Expression of *SAP2*, *SAP9*, and *SAP10* occurs in the early stages of infection. Transcripts of *SAP1*, *SAP4*, and *SAP5* appear after 12 h, while *SAP6*- and *SAP7*-transcripts were found in the late stages of infection (after 24 h); *SAP1–SAP2* play a key role during infection	[58]
Human	Oral candidiasis	At least one of *SAP1-SAP3* genes is expressed during oral candidiasis	[59]
Human	Oral and vaginal candidiasis	*SAP5* and *SAP9* are the most highly expressed Sap-encoding genes in patients with oral and vaginal candidiasis	[54]
Human	Oral candidiasis	The presence of *SAP1-*, *SAP3-* and *SAP6*-encoding genes in the case of the 29-year-old female patient suffering from acute oral candidiasis and *SAP2* gene obtained from an HIV-infected patient from a lesion of chronic oral candidosis (the clinical specimens)	[57]

oral RHE models [54] and detected in both infectious and commensal forms of *C. albicans*. The Sap9 enzyme was suggested to play a role in the cell wall integrity, as well as the efficiency of yeast cells in contact with the mucosal surface of the host [32]. The clinical specimens of patients suffering from acute oral candidosis and from a lesion of chronic oral candidosis showed the expression of *SAP1*, *SAP2*, *SAP3*, and *SAP6* genes [57]. The data regarding the expression of Sap-encoding genes, involving human samples and animal models, are summarized in Table 2.

4.2 Degradation of Main Functional Proteins of the Host

A broad spectrum of host substrates hydrolyzed by Saps, identified in early studies, included lactoferrin, lactoperoxidase, cathepsin D, albumin, hemoglobin, and the extracellular matrix components such as keratin, collagen, and vimentin [25, 48]. Sap2, produced in high amounts by yeast-like forms of *C. albicans,* was shown to contribute to the damage of mucin, the main component of protective layer for the mucous membrane [69]. The degradation of mucin can facilitate not only the penetration of mucous barrier enabling the further invasion of tissues, but can also provide carbon and nitrogen required for fungal growth [70].

The activity of Saps also affects the structural integrity of the epithelium. During the contact of *C. albicans* with the human oral mucosa, *C. albicans* uses its proteolytic potential to degrade E-cadherin in epithelial adherent junctions. Studies with protease mutant strains indicated that Sap5 is the major enzyme responsible for this process [71].

The invasion of host tissue can also be facilitated due to the degradation of proteinaceous components of subendothelial extracellular matrix, mainly laminin and fibronectin. Their proteolysis could presumably be important in the process of bloodstream penetration by the yeast [72]. Evidence suggested that strains that possessed a higher proteolytic activity strongly adhered to epidermal keratinocytes [73], corneocytes [74], and cells of the oral mucosa [75]. Although the role of Sap activity in this process was partly confirmed with the use of the specific inhibitor, pepstatin A, the mechanism of this effect was not fully elucidated. It was proposed that the increased adhesion to host cells resulted from the Sap-dependent degradation of certain host surface proteins which revealed additional potential binding sites for *C. albicans* [56].

Presumably, Sap9 and Sap10 can also participate in the regulation of adhesion due to the degradation of chitin synthases that participate in the process of cell wall formation, and a number of yeast surface proteins such as yeast-form cell wall protein 1 (Ywp1), agglutinin-like sequence protein 2 (Als2), and the protein repressed during hyphae development 3 (Rhd3) [32, 47].

4.3 The Interaction of Saps with the Complement System and the Antibodies

After invading host tissues, *C. albicans* encounters the innate immune system that acts against the pathogen through numerous antimicrobial peptides, the complement system, and specialized immune proteins and cells [50]. *C. albicans* aspartic proteases can prevent or modulate the functionality of immunoglobulins, in particular immunoglobulin A, resistant to the majority of bacterial proteases, which influences the attachment of *C. albic*ans to buccal epithelial cells [76, 77].

The components of the complement system involved in pathogen removal were also shown to be targets of Saps [78, 79]. Sap1, Sap2, and Sap3 degraded complement proteins C3b, C4b, and C5, preventing both phagocytosis and the final

formation of the terminal complement complex (TCC) and thus the activation of the selected mechanisms of the immune response [78]. Furthermore, Sap2 was able to degrade factor H, a complement system controller whose binding to the surface of *C. albicans* cells can increase the fungicidal response through bridging yeast and host immune cells. The same protease could also degrade CR3 and CR4, and FHR-1 receptor involved in the recognition of pathogens by the cells of the immune system [80].

4.4 Propelling the Host Proteolytic Cascades: The Kinin Production and Clot Formation

During fungal cell proliferation and further dissemination, Saps are able to degrade important components of proteolytic cascades involved in maintaining the biochemical homeostasis of the host organism, including proteins that comprise the contact system (i.e., the surface-activated kinin-generating system in plasma) and the blood clotting pathways [78, 80–83].

Primarily aimed at effectively defending against microbial infections, the activation of the contact system that results in the production of kinins can also, to some extent, be beneficial for pathogens [84]. The kinins are vasoactive peptides [85] and play an important role of inflammation mediators [86]. The increased permeabilization of blood vessels caused by kinins can not only support the migration of immune cells but can also increase the availability of nutrients for the pathogens and the possibility of disseminating the infection [87]. The contact system consists of two initially inactive serine proteases—factor XII and plasma prekallikrein—and a non-enzymatic protein, high-molecular-mass kininogen (HK). The factor XII is activated on a contact with negatively charged cell surfaces and subsequently activates prekallikrein. The active kallikrein releases a kinin—the nonapeptide bradykinin—from HK [88]. Kinins can also be released from the low-molecular-mass kininogen (LK), which does not belong to the contact system, but serves as the substrate for tissue kallikrein [89].

During the infection, *C. albicans* can trigger the release of kinins indirectly, by the activation of factor XII [90] or by direct action of Saps on HK molecule [81]. It was reported that mixtures of proteases secreted into a growth medium by both morphological forms of *C. albicans* released kinins from both HK and LK [81]. Studies with purified Sap2 confirmed its ability to release kinins from kininogens and also clearly indicated that LK is a protein much more susceptible to the direct proteolysis than HK [91]. The detailed analysis of LK degradation by all Saps showed that the majority of proteases, except Sap7, are able to produce kinins [92]. Sap3 released Met-Lys-bradykinin—a peptide able to exert the kinin-like biological effects through the activation of cellular kinin receptors—to a very high yield. Other Saps (except Sap9) could produce small amounts of the same kinin and bradykinin but primarily generated peptides comprising a kinin sequence extended at N- and/or C-termini. These peptides could be further processed by the action of Sap9, resulting in an efficient production of Met-Lys-bradykinin. The cooperative

degradation of kininogens by several Saps can be exploited by *C. albicans* to produce an optimal amount of kinins at the site of the infection. However, Sap9 alone released des-Arg^{1}-bradykinin, a peptide devoid of kinin-like biological activity [92].

The activation of coagulation factor XII by Saps can also initiate the process of blood clotting; however, this protein is not the only component of the cascade that is susceptible to a limited proteolysis by Saps. The process of fibrin clot formation can also be assisted by the Sap-dependent activation of factor X and prothrombin. A contribution of Saps to the development of the fibrin clot may underlie the septic coagulation and an insufficient peripheral circulation during infections [83, 93].

4.5 Deregulation of Host Proteases by Degradation of Their Inhibitors

Saps are able to degrade proteinaceous inhibitors that control the activity of main proteases of the host. Sap2 was shown to cleave α2-macroglobulin [94], cystatin A [95], and α1-protease inhibitor (A1PI), the latter also being susceptible to the action of Sap1, Sap3, Sap 4, and Sap9 [96]. By impairing the enzyme-inhibitor balance through the inactivation of the inhibitor molecule, Saps can indirectly contribute to the destruction of host tissues, supporting the process of infection. For instance, it was shown that the proteolytic cleavage of A1PI may assist in damaging epithelial and endothelial cells, caused by neutrophil extracellular traps (NETs). NETs are defense structures composed of DNA and microbicidal molecules released from neutrophil after a contact with a variety of microorganisms and molecules, which appear in response to infection, such as interleukin 8 (IL-8) [97]. Pathogens located within the NETs are exposed to direct contact with microbicidal molecules including neutrophil elastase (NE). The degradation of A1PI contributes to unrestrained NE activity, which might lead to the damage of host tissues, thereby supporting the colonization of the host by *C. albicans* cells that survived despite the killing properties of NETs. Furthermore, the degraded inhibitor had a reduced ability to complexation of IL-8 which can contribute to the increased influx of neutrophils to the site of infection and the progression of NET formation [96].

4.6 Interaction with Host Cells

The first host cells that come into contact with *C. albicans* but play an important function in preventing the fungal invasion are epithelial cells. Saps probably assist the adhesion of fungal cells to epithelium, acting as a ligand for the host cell receptors or modifying the surface components of both types of cells to allow for better interaction [25]. The E-cadherin cleavage by Saps resulted in epithelial integrity destruction and an increase of monolayer permeability [71, 98].

Sap4-Sap6 have the ability to bind to epithelial surface integrins. This interaction, mediated by Sap amino acid motif RGD/KGD, enables the internalization of

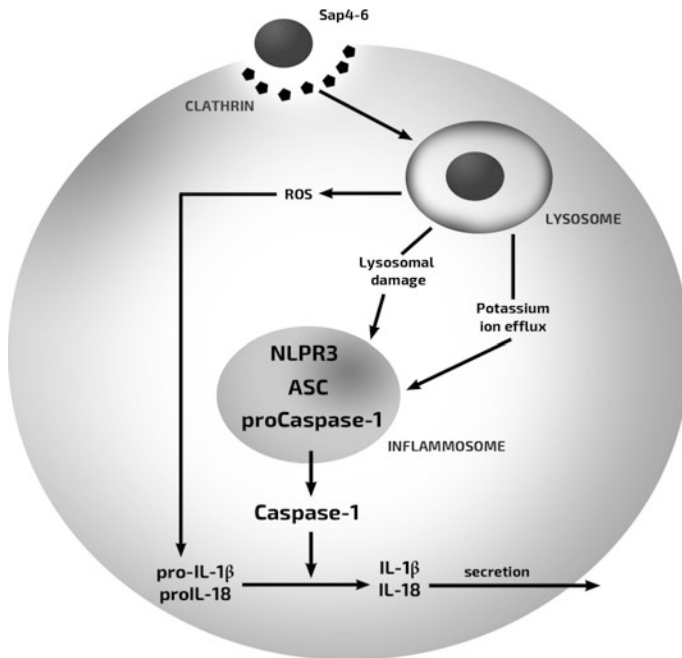

Fig. 5 NLRP3 inflammasome activation by Sap2 and Sap6 that leads to IL-1β and IL-18 production [103 modified]

proteases to endosomes and lysosomes by a still unknown mechanism. The location of these proteases in acidic interior of lysosomes leads to their activation, resulting in a partial permeabilization of the lysosome membrane and a subsequent caspase activation [99]. This kind of apoptotic pathway has been well established and occurs in many pathological conditions [100].

Proteases of *C. albicans* have the ability to induce cytokine expression by host cells. An increase in the production of interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) was observed in the macrophages after stimulation with Sap1–Sap3 and Sap6. Additionally, all these proteases except Sap3 increased the secretion of IL-6. The induction of cytokine secretion appeared to be regulated by the Akt/NF-κB activation and was independent of the Sap enzymatic activity [101]. It was also shown that aspartic proteases expressed by both morphological forms of *C. albicans* were able to produce IL-1β by direct, limited proteolysis of its precursor [102].

The formation of mature interleukin requires the involvement of apoptosis-associated speck-like protein (ASC), pro-caspase-1, and activated NLRP3 inflammasome, which is a pro-inflammatory complex presented in the monocytes, monocyte-derived macrophages, and dendritic cells (Fig. 5). It was shown that Sap2 and Sap6 were able to stimulate this inflammatory process, undergoing an internalization via the clathrin-dependent mechanism [103].

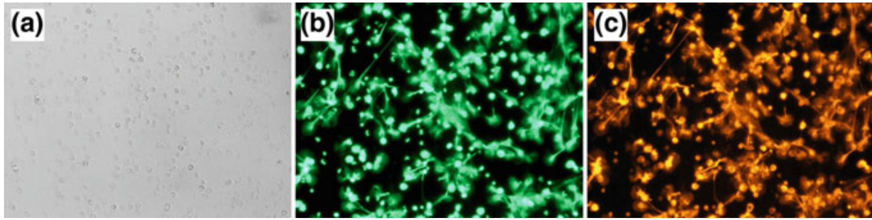

Fig. 6 Sap treatment of neutrophils (**a**) activated the release of NETs with antifungal properties. The visualization of NETs was performed with detection of DNA (Sytox staining, **b**) and histones (staining with antibody against histone 3, **c**). Based on the authors' unpublished data

At the place of infection, the fungal pathogens are faced with locally presented or attracted phagocytes, mainly neutrophils and macrophages. Regardless of their efficiency, *C. albicans* cells have developed several mechanisms to evade phagocyte control, with the engagement of proteolytic enzymes (see the reviews: [104, 105]).

Neutrophils, equipped with many receptors sensitive to fungal pathogen-associated molecular patterns (PAMP), are the main immune cells involved in the host defense against *C. albicans.* These phagocytes migrate to the place of the fungal infection across Sap concentration gradient, in a dose-dependent manner [106]. The chemoattractant properties of those proteases, confirmed in a study of *Candida* mutant strains with selective deletion of Sap genes, were attributed to Sap9 [107]. The influx of neutrophils to the place of infection was also confirmed in vivo, in a mice model, in response to Sap2 [108].

Saps can also influence the ROS production by the activation of neutrophil oxidative burst, as was deduced from an observed decrease of ROS formation during the contact of neutrophils with *C. albicans* mutant strains with deleted Sap9-encoding gene [107]. Both processes seemed to depend on Sap proteolytic activity, because enzymatically inactive Saps did not cause neutrophil activation [108]. A further stimulation of neutrophils with Sap9 also triggered the apoptosis of these cells [107]. On the other hand, independently of their enzymatic activity, Saps induce interleukin release by epithelial cells. Interleukins, especially IL-8, are chemoattracting agents for neutrophils; therefore, inactive Saps can also indirectly modulate the influx of neutrophils to the infection foci [109].

The formation of NETs, composed of decondensed chromatin incrusted with a subset of granular proteins, is a relatively recently recognized mechanism used by neutrophils to capture and kill the microbial pathogens outside of the phagocyte cells [110]. We recently found *SAP* genes to be overexpressed in the yeast during the contact with NET-forming neutrophils [111]. On the other hand, the *SAP*-encoded proteins triggered the NET release in a dose-dependent manner, engaging two different mechanisms, which depended on fungal morphology and correlated with preference to secreted Sap type (Fig. 6). For Sap1–Sap2 and Sap8–Sap10, we observed the release of NETs in a ROS-dependent way. Sap5 and Sap7 acted similarly, but with lower efficiency. A ROS-independent mechanism of NETosis

was observed for neutrophils treated with Sap4 and Sap6, for which the NADPH oxidase inhibition only partially lowered the NET release (unpublished data).

4.7 Sap Impact on the Action of Antimicrobial Peptides Produced by the Host

Besides degrading a vast number of the host proteins, the arsenal of ten *C. albicans* Saps effectively degrades and neutralizes some of human antimicrobial peptides (AMPs) which are key components of the innate immune system of the host, and represent a first, primitive line of defense against attacks of a wide range of microorganisms and are often called natural antibiotics.

It has been shown that *C. albicans* cells use Saps to hydrolytically inactivate His5, a histidine-rich cationic salivary component that possesses potent antimicrobial activities, in particular against *C. albicans* [112]. The investigation of four *C. albicans* aspartic proteases—Sap2, Sap5, Sap9, and Sap10—showed that His5 is effectively cleaved by all these enzymes except Sap5 and that the main protease responsible for His5 degradation is Sap9. A recent study expanded the known characteristic of His5 degradation on all ten *C. albicans* proteases using peptide chemistry methods [44] and indicated that seven Sap family members (Sap1–Sap4 and Sap7–Sap9, used as recombinant proteins) could rapidly degrade this salivary AMP under conditions corresponding to the oral cavity environment.

Human cathelicidin LL-37 was another human AMP shown to be prone to Sap-dependent degradation and inactivation [111]. This cationic α-helical AMP with antimicrobial and immunomodulatory properties [113], constitutively expressed in epithelial cells and the cells of the innate immune system such as human neutrophils, was cleaved into multiple products by six Sap enzymes, Sap1–Sap4 and Sap8–Sap9. The progress of degradation deprived this peptide from its fungicidal activity, thus enabling the pathogen to survive and propagate despite presence of AMP.

Nonetheless, at the initial stages of Sap treatment of two peptides, LL-37 and His5, truncated derivatives—LL-25, LL8-37, His-21, His-17, and His-13—that still possessed some antifungal activity were produced and, therefore, the body's first line of defense against the infection was initially sustained [44, 111]. However, the LL-25 peptide, despite possessing antifungal properties, was devoid of the immunomodulatory properties of full-length LL-37, i.e., did not affect the generation of ROS by neutrophils, lowered the chemoattractant activity toward neutrophils by significantly decreased calcium flux and IL-8 production after neutrophil stimulation, and also lost the function of an inhibitor of neutrophil apoptosis [111].

It was recently demonstrated [114] that Saps can degrade and inactivate two antimicrobial peptides—designated NAT26 and HKH20—that can potentially be excised from human kininogens by host proteases such as NE [115, 116]. The NAT26, a helical and positively charged peptide responsible for the antimicrobial properties of the domain 3 of LK and HK, was effectively cleaved by all Saps except Sap10. In contrast, the HKH20, a histidine- and lysine-rich, positively

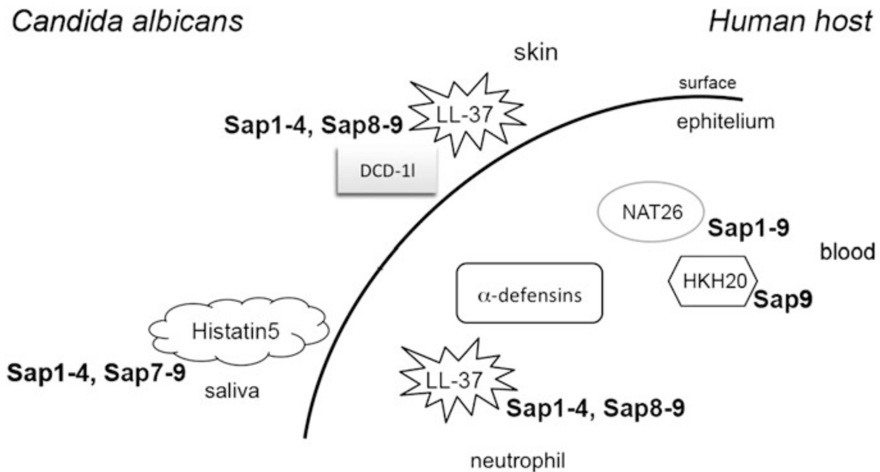

Fig. 7 Scheme presenting the places where AMP are prevalent in the human cells/tissues, with the indication of *C. albicans* aspartic proteases capable of their degradation

charged peptide derived from domain 5 of HK, was completely cleaved only by Sap9.

A susceptibility of human AMPs to Sap action is schematically summarized in Fig. 7.

A detailed kinetic analysis of Sap-catalyzed degradation of LL-37 [111], His5 [44], and NAT26 and HKH20 [114] revealed that, despite the high hydrolytic activity of these proteases in an acidic environment, most of them were able to process the peptides over a broad pH range, with the highest activity at a neutral pH for Sap3, Sap4, and Sap9. These findings suggest that proteolytic processing of AMP can possibly proceed in the various niches of the body where *C. albicans* reside.

4.8 Involvement of Saps in the Formation and Resistance of Polymicrobial Biofilm

On mucosal or artificial surfaces, *C. albicans* forms three-dimensional polymicrobial communities with extracellular matrix layers also containing host immune cells. Such a complex biofilm is highly resistant to the host's immune activity and antifungal drugs [117].

The role of Saps in biofilm formation is poorly recognized. In the simple artificial models, it was demonstrated that *C. albicans* biofilm secreted more Saps than the planktonic counterparts [118]. In the oral mucosal epithelia model coinfected with *C. albicans* and *Streptococcus oralis*, the microorganisms synergized to activate the host enzyme, calpain 1, involved in the cleavage of epithelial junction proteins and increased fungal invasion but the fungal protease activity was not

required for this affect [119]. In the study of a biofilm, formed by *C. albicans* and oral streptococci, the cell wall-associated Sap9 was found to be required for the control of hyphal filamentation of *C. albicans*, and for the regulation of mixed species biofilm formation [120].

5 Secreted Aspartic Proteases of Other *Candida* Species and Their Roles in Candidal Infections

Apart from *C. albicans,* three non-albicans *Candida* species possess in their genome the genes that encode secreted aspartic proteases: *C. parapsilosis* (*SAPP1–SAPP3*), *C. tropicalis* (*SAPT1–SAPT4*), and *C. dubliniensis* (*SAPCD1–SAPCD4*, *SAPCD7–SAPCD10*).

C. parapsilosis possesses three *SAPP* genes (*SAPP1–SAPP3*) and two *SAPP2* homologs that demonstrate 91.5% amino acid sequence identity. To date, only the products of *SAPP1* and *SAPP2* genes have been isolated and purified [121, 122]. Both Sapp1 and Sapp2 are extracellular enzymes; however, Sapp1 was also reported to occur in a cell wall-attached form [123]. They have an identical molecular mass of 37 kDa, and their amino acid sequence is 53% identical [124]. While the secretion of Sapp2 does not depend on the type of nitrogen source, the production of Sapp1 is induced by the presence of protein in the growth medium [121]. The optimal pH for Sapp1 and Sapp2 hydrolytic activity is in the acidic pH range, and Sapp1 possesses a broad substrate specificity [121, 125].

Currently, the tertiary structures of Sapp1 and of one of the Sapp2 homologs are known [122, 125]. During the maturation of proenzymes, they can be activated autocatalytically or by the action of endoproteinase Kex2, and only removed peptides possess potential glycosylation sites [124, 126].

It was reported that *C. parapsilosis* aspartic proteases demonstrated the ability to degrade several host proteins, i.e., Sapp1 hydrolyzed IgA and activated prothrombin and coagulation factor X, while Sapp2 degraded keratin and trypsinogen [121, 127]; both proteases had the ability to generate biologically active kinins from human kininogens [81, 128].

Sapt1 is the only secreted aspartic protease of the Sapt family that could be successfully isolated from *C. tropicalis* cultures. The amino acid sequence similarities between particular Sapts do not exceed 63% but they are closely related to the enzyme produced by *C. albicans,* with Sapt1 showing a large similarity to Sap8, while Sapt4 showed similarities to the Sap1–Sap3 subfamily [129]. A tertiary structure of Sapt1 is already known [130]. The production of this enzyme can be induced by the presence of an exogenous protein, such as bovine serum albumin, in the culturing medium [131]. *C. tropicalis* proteases might be directly involved in fungal invasion, with the destruction of the host tissues and degradation of the host proteins, e.g., of the human kininogens with generating of kinin-related peptides [81], and their activity may be also important for fungal cell adhesion and development of the disseminated candidiasis in patients with leukemia and neutropenia

[132]. However, the expression of the *SAPT* genes during the colonization of the oral epithelium was not strictly correlated with the invasion [133].

C. tropicalis also produces tropiase, a protease that does not belong to the Sapt family. In contrast to the previously described enzymes, tropiase demonstrates the proteolytic activity in a broader pH range of 7–9 and is stable at pH between 3 and 12 and at high temperatures [134]. Tropiase is involved in the degradation of casein, keratin, and collagen. Moreover, this enzyme also hydrolyzes α and β chains of fibrinogen, but without demonstrated clotting activity and fibrin formation. Interestingly, the purified tropiase possesses hemorrhagic and capillary permeability-increasing activities which may highly contribute to the development of candidiasis [134, 135].

Another non-albicans *Candida* species, *C. glabrata,* which is more closely related to baker's yeast *S. cerevisiae* than to other pathogenic species of *Candida genus,* possesses in its genome at least 11 *YPS* genes which are similar in structure to *SAP* genes [136]. The *YPS*-encoded proteins (yapsins) constitute a family of aspartic proteinases with a GPI-anchor, involved in the maintenance of cell wall integrity and cell–cell interactions. *C. glabrata* yapsins are considered to have a strong structural similarity to Sap9 and Sap10 [32].

6 Fungal Aspartic Proteases as Drug Targets—Future Application

From the perspective of Saps' contribution to the fungal pathogenicity, they are ideal drug targets, especially in the context of increasing resistance of *C. albicans* strains against the commonly used antifungal agents. The inactivation of Saps could successfully stop the infections on different levels correlated with the facets of the infection process. They can be the targets for the process of tissue barrier degradation, destruction of the host's defense molecules, acquiring nutrients for pathogen propagation, the adhesion, and biofilm formation on the host tissues or abiotic surfaces [39, 137–140].

However, due to their wide substrate specificity and broad range of pH operation [30], it is difficult to find a universal inhibitor, working with high efficiency. Pepstatin A, the most popular inhibitor of aspartic proteases like pepsin and cathepsin D, is also effective toward *C. albicans* Saps as was presented in many in vitro studies [141]. In the model of human oral candidiasis, it was shown that the inhibition of Saps with pepstatin A can influence the fungal adhesion and invasion, associated with a reduction of tissue damages [142]. However, its inhibition profile toward Saps is not universal. The activity of Sap9 and Sap10, GPI-anchored to the fungal cell membrane, was only partially blocked by pepstatin A [47], and Sap7 was proven to be insensitive to pepstatin A [46]. Some researchers also suggested that the effectiveness of pepstatin A action can be restricted to the used models or conditions [143]. Moreover, the possible therapeutic application of pepstatin A in

mice model failed due to its metabolism in the liver and rapid clearance from the blood [144].

An interesting opportunity has opened for finding Saps inhibitors since an observation of declined *Candida* infections in AIDS patients after the application of antiretroviral therapy that included HIV protease inhibitors (HIV PIs). Since *Candida* Saps and HIV proteinase belong to the same class of aspartic proteases, it was postulated that HIV PIs can be also effective against Saps in vivo [36, 145]. Among analyzed compounds, ritonavir was the most potent inhibitor of Sap2, the main candidal protease active in fungal infection, while saquinavir, indinavir, and nelfinavir inhibited Sap2 activity with lower efficiency [72, 146]. The inhibitory activity of these compounds toward Sap1–Sap3 and the attenuation of *Candida* cell adhesion were observed in RHE model of oral candidiasis and in a model of experimental rat vaginitis [147]. However, it is not clear whether the modulation of *Candida* adhesion to epithelial cells, observed in vitro, results only from proteolytic activity of Saps, that can affect some proteinaceous targets on the surface of epithelial cells, allowing for better adherence of fungus to them. The current results also pointed out a possible interruption of some specific interactions of aspartic proteases, not connected with their enzymatic activity, with the host proteins located on the surface of these cells. Such drugs, blocking the adhesion of *Candida* cells, would be particularly attractive. However, this issue was addressed by only one report for oral candidiasis [148].

Currently used antifungal drugs, which take advantage of their inhibitory activity toward *Candida* aspartic proteases, do not satisfy the medical standards, due to their potency, pharmacokinetic properties, and increased toxicity at higher concentration [149]. Therefore, new sources of Sap inhibitors are being sought including programmed, variable domain antibodies, produced against Sap2, whose protective properties were observed in experimental rat vaginal candidiasis [150]. Many peptidomimetic inhibitors have been developed that are derived from the structure of pepstatin A, which inhibited proteases of different *Candida* species [148]. Also the molecular modeling and new key structural information about Saps' active centers have been adopted for a design and synthesis of new Sap inhibitors [151–153]. Research was also focused on natural sources of inhibitors, including the extracts of plants [154], bacteria [155], and marine organisms [156] or artificial materials such as triangular gold nanoparticles [157].

The current progressive increase in fungal resistance to the available drugs, a noticeable shift of the infection profiles toward non-albicans *Candida* species, as well as the problems with toxicity and delivery of existing drugs to the place of infection should prompt the search for various fungal targets. At this point, candidal aspartic proteases, with their broad spectrum of engagement in physiological and pathological processes, seem to be among the best candidates.

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Pseudomonas aeruginosa and Its Arsenal of Proteases: Weapons to Battle the Host

Anna Clara M. Galdino, Marta H. Branquinha, André L.S. Santos and Lívia Viganor

Abstract

Pseudomonas aeruginosa is a ubiquitous and opportunistic human pathogen that represents a critical problem to the clinician due to the increased number of resistant strains isolated from hospital settings. In addition, there is a great variety of pathologies associated with this versatile Gram-negative bacterium. *P. aeruginosa* cells are able to produce an incredible arsenal of virulence factors, especially secreted molecules that act singly or together to ensure the establishment, maintenance, and persistence of a successful infection in susceptible hosts. In this context, pseudomonal proteases' roles are highlighted due to their ability to cleave key host proteinaceous substrates as well as to modulate several biological processes, for example, escaping and modulating the host immune responses in the bacterial own favor. Proteases secreted by *P. aeruginosa* include elastase A (LasA), elastase B (LasB), alkaline protease (AP), protease IV (PIV), *Pseudomonas* small protease (PASP), large protease A (LepA), MucD, and *P. aeruginosa* aminopeptidase (PAAP). In the present review, we discuss the role of each of these relevant proteases produced by

A.C.M. Galdino · A.L.S. Santos
Programa de Pós-Graduação em Bioquímica (PPGBq), Instituto de Química (IQ), Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

A.C.M. Galdino · M.H. Branquinha · A.L.S. Santos (✉) · L. Viganor
Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes (IMPG), Centro de Ciências da Saúde (CCS), Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: andre@micro.ufrj.br

L. Viganor (✉)
The Centre for Biomimetic & Therapeutic Research, Focas Research Institute, Dublin Institute of Technology, Camden Row, Dublin 8, Dublin, Ireland
e-mail: liviaviganor@gmail.com

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P. aeruginosa taking into consideration their main biological functions in the bacterium–host interaction that favors the establishment of the infectious process.

Keywords
Pseudomonas aeruginosa · Proteases · Virulence factors

1 Introduction

Pseudomonads are bacteria well known for their metabolic versatility and widespread spatiotemporal distribution [1]. One of the most important species of pseudomonads is, with no doubt, *Pseudomonas aeruginosa*, which is a fascinating ubiquitous Gram-negative bacterium with rod shape measuring 0.5–0.8 μm × 1.5–3.0 μm (Fig. 1a) [1, 2]. *P. aeruginosa* presents the following metabolic features: non-fermentative, catalase positive, oxidase positive, ammonia producer, and usually aerobic, but it also can grow in an anaerobic environment if nitrate, citrate, and arginine are available [3]. The production of 2-aminoacetophenone by the bacterial cells generates the fruity grape-like odor that is characteristic of this pseudomonad species. On blood agar plates, colonies of *P. aeruginosa* often display beta-hemolysis and a greenish metallic sheen due to the production of pigments [2]. The characteristic that most distinguishes *P. aeruginosa* from the other pseudomonads, and from the other species of Gram-negative non-fermenting bacteria, is its ability to produce pyocyanin, a blue-green phenazine pigment that gives the green color to the bacterial colony (Fig. 1b) and also to the pus observed in *P. aeruginosa-infected* tissues. This pigment and several others, such as pyochelin

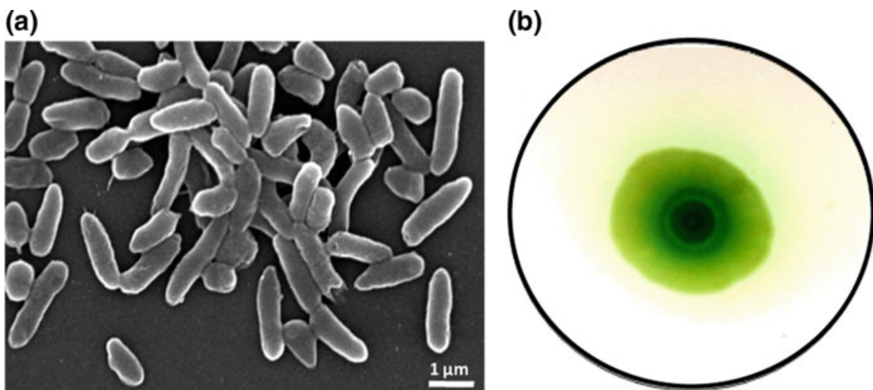

Fig. 1 Scanning electron microscopy (**a**), showing the characteristic bacterial rod shape, and colony morphology (**b**), evidencing the pyocyanin pigment, of *Pseudomonas aeruginosa*

(purple-cyan), pyoverdin (yellow, green and fluorescent), pyomelanin (light-brown), and pyorubin (red-brown), are secondary metabolites of *P. aeruginosa*, which play an important role in bacterial nutrition, such as iron acquisition and pathogenesis [2, 3]. Almost all *P. aeruginosa* strains are motile due to the presence of a single polar flagellum that facilitates the locomotion and colonization of a wide range of environmental niches [2]. This microorganism can grow within the temperature range from 4 to 42 °C in terrestrial (soil) and aquatic habitats (polluted, salt, and freshwater) as well as on the surface of animate hosts (insects, plants, animals, and humans) and inanimate surroundings, mainly in the hospital environment (distilled water, disinfectants, sinks, medical devices, and equipment), being an important causative agent of nosocomial infections, particularly in intensive care units (ICUs) [1–4]. One of the interesting characteristics of *P. aeruginosa* is its pan-genome, which presents a larger genetic repertoire than the human genome. This intriguing feature explains the broad metabolic capabilities of *P. aeruginosa* and its distribution and adaptability in diverse environments [5].

P. aeruginosa is one of the most important bacterial species for public health considerations due to its high resistance to different classes of antibiotics and its capability to cause serious health care-associated as well as nosocomial infections [6, 7]. Results reported from an International Nosocomial Infection Control Consortium (INICC) surveillance study, performed between 2007 and 2012, in Latin America, Asia, Africa, and Europe, in which prospective data were collected from 605,310 patients hospitalized in 503 ICUs, displayed frequencies of 42.8% of *Pseudomonas* isolates resistant to amikacin and 42.4% to imipenem [8]. In the USA, an estimated 51,000 health care-associated *P. aeruginosa* infections occur each year, in which more than 6,000 (13%) of these are multidrug-resistant and 400 deaths per year are attributed to these infections [9]. The analyses based on data extracted from the Public Health England (PHE) voluntary surveillance database in the period 2008–2012 showed that 92% of *Pseudomonas* spp. isolates identified from bacteremia in 3,457 reports were *P. aeruginosa* [10]. In Brazil, the National Health Surveillance Agency (ANVISA), through the National Monitoring Microbial Resistance Network Health Services (RM Network), published a report that shows the main etiologic agents and the resistance phenotypes responsible for causing primary bloodstream infections associated with the use of central venous catheter in adult patients interned at ICUs from Brazilian hospitals between January and December 2013. According to that study, 18,233 notifications were reported, of which 1,850 (10.1%) were caused by *P. aeruginosa*, being the fifth pathogen most often reported as the etiologic agent. The resistance rate to the carbapenems reached 37.4% (692 *P. aeruginosa* isolates) [11]. Additionally, the Infectious Diseases Society of America has highlighted *P. aeruginosa* as part of a faction of antibiotic-resistant bacteria, called 'the ESKAPE pathogens'—*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp., capable of 'escaping' the bactericidal action of antibiotics and mutually representing new paradigms in pathogenesis, transmission, and resistance [12].

P. aeruginosa is extensively resistant to multiple drugs and is increasingly resistant to most available antibiotics, being a great emergency problem in the hospital settings worldwide [13]. Interestingly, *P. aeruginosa* has evolved over time in its ability to find new ways to be resistant to different classes of chemical compounds as well as to build strategies to exchange genetic materials, allowing that other bacteria also become drug-resistant [5]. Generally, resistance usually occurs due to a combination of factors acting synergistically: (i) *P. aeruginosa* is intrinsically resistant to antimicrobial agents due to its outer membrane/cell envelope composition that reduces the permeability of several drugs; and (ii) *P. aeruginosa* expresses a powerful repertoire of resistance mechanisms that can be developed through mutations in the genomic content that regulates resistance genes, and also acquired from other organisms via plasmids, transposons, or bacteriophages [14].

As a major opportunistic pathogen for humans, *P. aeruginosa* causes a plenty variety of acute and chronic infections and presents significant levels of morbidity and mortality [15, 16]. *P. aeruginosa* typically infects through airways, wounds, urinary tract, ear canal, via ocular and implanted medical devices (e.g., catheters or ventilators). Thereby, it is the main cause of eschars, conjunctivitis, keratitis, corneal ulcer, osteomyelitis, otitis, urinary infections, surgical site infections, bloodstream infections in ICUs and hospital-acquired pneumonia in immunocompromised individuals, mainly in patients with severe burn wounds, AIDS, lung cancer, chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis [16–18].

It is known that Gram-negative bacteria are common causes of a huge diversity of infections including, intra-abdominal infections (IAIs), urinary tract infections (UTIs), ventilator-associated pneumonia (VAP), and bacteremia [19]. In particular, *P. aeruginosa* is one of the most important pathogens in the hospital setting, being responsible for 27% of all pathogens and 70% of all Gram-negative bacteria causing health care-associated infections in the USA, and it is the most common Gram-negative organism causing VAP and the second most common organism causing catheter-associated UTIs [7, 19]. The Centers for Disease Control and Prevention found that *P. aeruginosa* totalized 7.1% of health care-associated infection in the USA in 2011, being the second most common cause of pneumonia in hospital settings and the third most common Gram-negative bacterium to cause bloodstream infections [20]. *P. aeruginosa* is also a major cause of concern in the cystic fibrosis setting, being the most common pathogen isolated from cystic fibrosis sputum, and approximately 70% of adult cystic fibrosis patients are chronically colonized by this microorganism [21, 22].

The pathogenic potential of *P. aeruginosa* is not only due to its metabolic/genetic versatility and both intrinsic and acquired antibiotic resistance. Its ability to form biofilm and to produce an arsenal of virulence attributes, including cell-associated determinants (e.g., lipopolysaccharide, pili, and flagellum) and soluble secreted factors (e.g., extracellular polysaccharides, exotoxins, pigments, and proteases), is very important for the survival and adaptation of this pathogen in distinct environments [17, 22, 23].

2 *Pseudomonas aeruginosa*: Establishing and Maintaining an Infection

In order to establish an infection, *P. aeruginosa* cells count on a suite of virulence factors (Fig. 2) [17, 24]. These factors act together not only causing injuries on the host epithelial cell lining but also inducing dysfunctions in physiology and function, such as host cell shape, membrane permeability, and protein synthesis, as well as manipulating/overcoming host defenses, down-modulating the immune responses and preventing *P. aeruginosa* endocytosis and obstructing clearance mechanisms, thereby allowing this microbe to persist in cells/tissues and to establish an infection in the host [25, 26]. The virulence of *P. aeruginosa* is mediated by multiple mechanisms, but the major contributor is the production of extracellular proteases. In general, these enzymes regulate multiple cellular and physiological processes

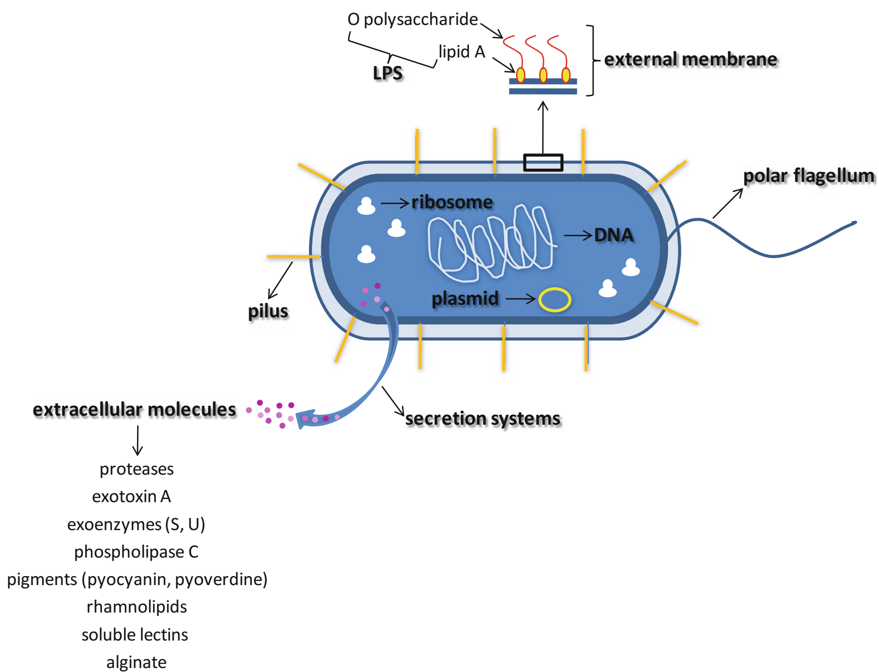

Fig. 2 Virulence factors expressed/produced by *P. aeruginosa* cells: (i) lipopolysaccharide (LPS) that induces cytokine production, (ii) pili that help bacterial adherence to the respiratory epithelial cells, (iii) flagellum that participates in mobility, adherence, and internalization events, (iv) extracellularly released molecules like proteases (responsible for the cleavage of key host proteins), exotoxin A (inhibition of host protein synthesis), exoenzyme S (induces cytotoxic effect), exoenzyme U (antiphagocytic effect), phospholipase C (cleavage of membrane phospholipids), pigments (many biological effects, like pyocyanin that induces free radicals in host cells), rhamnolipids (detergent action), soluble lectins (inhibition of beating of lung cells), and alginate (phagocytosis inhibition, antifungal action, and host immune responses)

and are essential to the success of the infection. They degrade a wide array of host proteins, impairing host defenses and destroying physical barriers that normally prevent attachment and penetration of the bacteria [26–28].

3 Proteolytic Enzymes Produced by *Pseudomonas aeruginosa*

P. aeruginosa is able to extracellularly release different kinds of proteases (Fig. 3), which together are responsible for invasion and destruction of host tissues. Because of the relevant roles played by proteases on the physiopathology of *P. aeruginosa,* it has been shown that the majority of environmental and clinical strains of *P. aeruginosa* exhibited proteolytic activity, particularly elastase activity [29–31]. According to Stover and co-workers [32], approximately 3% of the whole *P. aeruginosa* genome is composed by open reading frames that encode proteases [32]. Thus, the high genomic variability allows the bacterium to adapt its virulence arsenal machinery to support the variations of environment conditions, and for that, protease production in *P. aeruginosa* can vary greatly (Fig. 4) [32].

The expression of extracellular proteolytic enzymes in *P. aeruginosa* is directly influenced by environmental factors and changes in the physicochemical properties of culture medium (e.g., nutrients, temperature, pH, and aeration), which significantly modulate the production of these crucial virulence factors [26, 33]. In addition, the amount of protease produced depends on the cell cycle moment (e.g., lag, exponential, or stationary growth phase) and on the growing lifestyle (e.g., planktonic or biofilm). For instance, the total protease production (Fig. 5a) as well as the specific elastase secretion increases along the first 48 h of in vitro cultivation

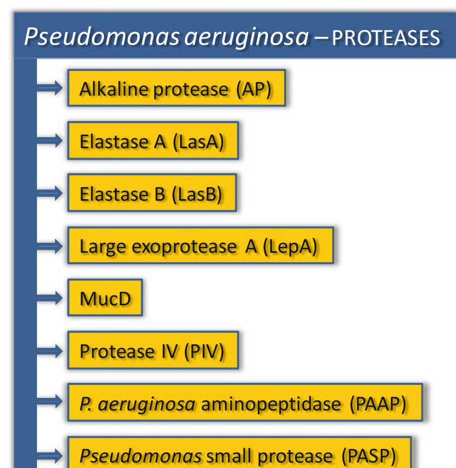

Fig. 3 Proteases secreted by *P. aeruginosa* cells

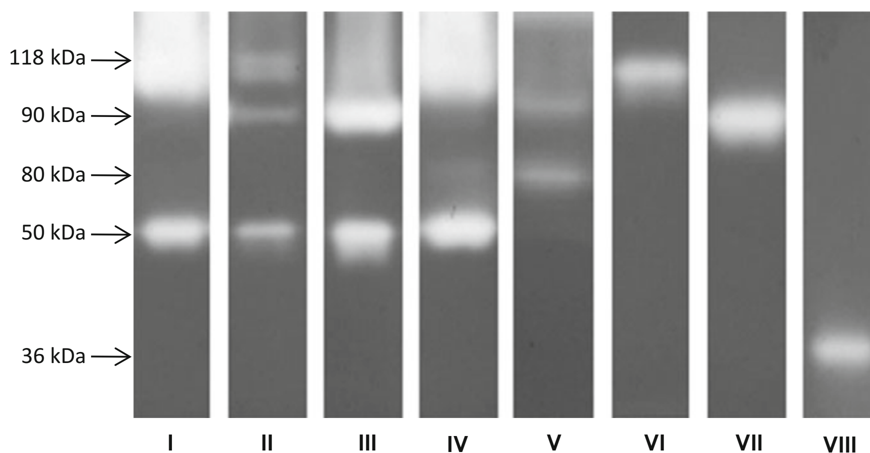

Fig. 4 Production of extracellular proteases in clinical isolates of *P. aeruginosa* recovered from different anatomical sites. The proteolytic profiles were characterized by sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin as the protein substrate. Profile I—118 + 50 kDa; Profile II—118 + 90 + 50 kDa; Profile III—90 + 50 kDa; Profile IV—118 + 80 + 50 kDa; Profile V—90 + 80 kDa; Profile VI—118 kDa; Profile VII—90 kDa, and Profile VIII—36 kDa

of *P. aeruginosa* planktonic cells (Fig. 5b). Further, according to Hastie and co-workers [34], after 85 h of bacterial growth, the elastase production dropped off.

3.1 Elastase B

One of the best proteases characterized in *Pseudomonas* is elastase B (LasB), also known as pseudolysin. This 33-kDa enzyme belongs to the M4 thermolysin-like family of neutral, Zn-dependent metallo-endopeptidases (Fig. 6). This enzyme is encoded by *lasB* gene as a pre-pro-protein, containing at the N-terminal region a signal peptide of 23 amino acids that transport the enzyme through the inner membrane to periplasmic place by bacterial secretory system [35].

The first and the most studied substrate of elastase B is bovine and human elastin [36–38]. Some reports correlate the elastinolytic activity of elastase B to *Pseudomonas* infections in cystic fibrosis patients [39–43]. Histological studies have detected altered elastin fibers in lung alveoli of cystic fibrosis patients on autopsy, indicating a probable elastase activity on cystic fibrosis lung [39]. In addition, the elastase activity is associated with vascular inflammation during *P. aeruginosa* infection, since the disorganization of elastin fiber in vascular tissue caused by protease degradation was observed [44]. Previously, our group analyzed the production of virulence attributes in 96 clinical strains of *P. aeruginosa* recovered from patients attended at hospitals located in three States of Brazil (Espírito Santo, Minas Gerais, and Rio de Janeiro), and it was shown that all bacterial strains exhibited a

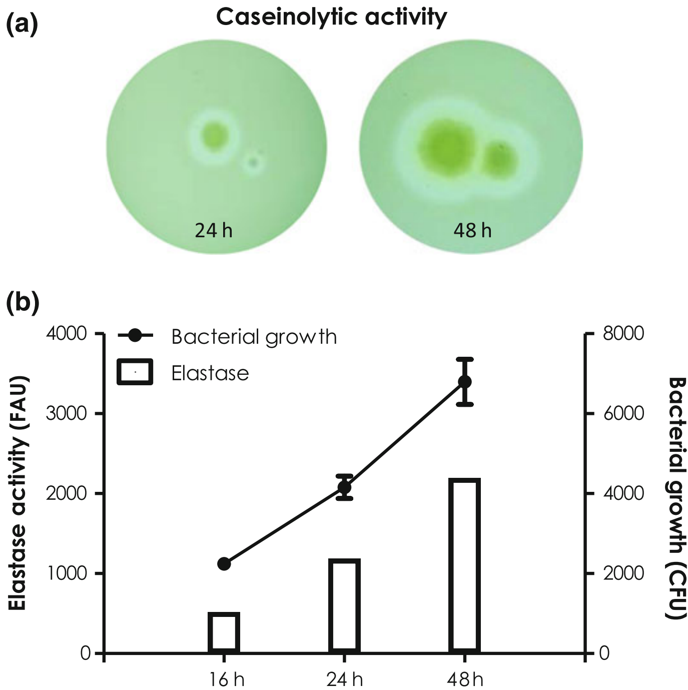

Fig. 5 Protease detection in *P. aeruginosa*. **a** Total extracellular protease production was analyzed by the degradation of casein (1%) incorporated into Luria Bertani agar medium up to 48 h at 37 °C. **b** The elastase activity was measured in the cell-free culture supernatant obtained from *P. aeruginosa* cells grown in tryptic soy broth up to 48 h at 37 °C, using the fluorogenic peptide substrate Abz-Ala-Gly-Leu-Ala-*p*-Nitro-Benzyl-Amide. Results were expressed as fluorescence arbitrary units (FAU). In parallel, the number of bacterial cells along each time point was evaluated by plating cells onto agar medium and expressed as colony-forming units (CFU)

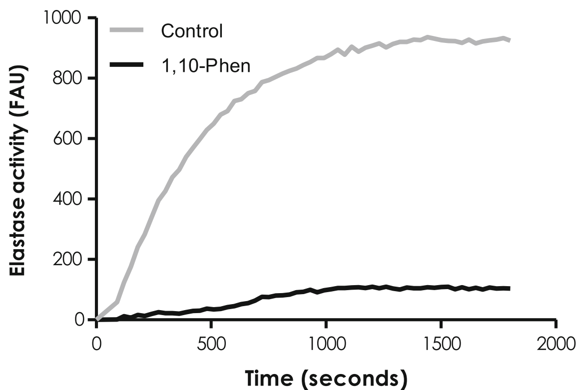

Fig. 6 Elastase of *P. aeruginosa* is a typical zinc-metalloprotease. The purified elastase B is able to cleave the fluorogenic peptide substrate Abz-Ala-Gly-Leu-Ala-*p*-Nitro-Benzyl-Amide along the time. Conversely, 1,10-phenanthroline (1,10-Phen), a metalloprotease inhibitor, at 10 μM was able to block the substrate cleavage. FAU, fluorescence arbitrary units

homogeneous elastase activity, with an average of 1069.28 ± 213.95 fluorogenic arbitrary units (FAU) with no correlation with the original anatomical site of isolation [16]. On the other hand, *P. aeruginosa* strains recovered from trachea, urinary tract, and wounds of patients attended at University Medical Center/Texas Tech Health Sciences Center were able to produce different amounts of elastase [45]. Woods and co-workers [46] showed that Canadian *P. aeruginosa* strains isolated from acute lung infections showed the highest production of elastase (0.053 ± 0.021 mg/ml) compared with elastase activity of strains isolated from burns, wounds, cystic fibrosis lung, and blood.

LasB is also able to cleave other host extracellular matrix proteins, such as collagen type III and IV. Interestingly, after subcutaneous injection of purified elastase B into mice, an intense degradation of basement membranes was observed, and elastase B was responsible for severe hemorrhage and tissue damage [47]. Several studies have demonstrated that LasB-associated epithelial disruption is mediated by the attack to intracellular tight junctions and cytoskeleton reorganization via inhibition of protein kinase C and activation of EGFR, ERK1/2 and NFκB, urokinase, and protease-activated receptor 2 (PAR-2) [48–53]. Elastase B can also interfere with the host bacterial clearance by degrading several components of innate and adaptive immune defenses, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-2 (IL-2), monocyte chemotactic protein-1 (MCP-1), and epithelial neutrophil activating protein-78 (ENA-78) [52–57]. In addition, it was shown that elastase B was efficient in the inactivation of key components of the complement system such as fluid-phase and cell-bound C1 and C3 and fluid-phase C5, C8, and C9 [44]. This multifunctional enzyme is also able to cleave surfactant protein A and D (SP-A and SP-D), also known as collectin. SP-A and SP-D are synthesized by alveolar type II epithelial cells and are responsible for the recognition and binding to oligosaccharides present on the cell surface of many bacteria to be phagocytized by host macrophages [58]. Previously, Meyer and co-workers [59] have reported that a decrease on the SP-A and SP-D levels in bronchoalveolar lavage (BAL) was observed in the lung of cystic fibrosis individuals. Also, SP-D knockout mice were more sensible to *P. aeruginosa* corneal infections when compared to wild-type animals, and only the wild-type mice recovered completely of the infection [60]. Based on this, elastase B was suggested to be responsible for the SP-D degradation in the eye [25, 26]. Furthermore, pseudomonal elastase can interact with host adaptive immune system by degrading immunoglobulins [61–63]. Bainbrigde and Flick [61] showed that elastase B was able to cleave IgG molecules recovered from cystic fibrosis patients and the degradation products bound to IgG-receptors of human neutrophils, thereby inhibiting the opsonization of bacterial invaders. Lomholt and Kilian [63] reported the IgA degradation in tears from patients infected with *P. aeruginosa.* They also observed that isogenic mutants of *P. aeruginosa* knockout to either elastase or alkaline protease were not able to completely inhibit the IgA degradation, indicating that several proteases were working in concert to cleave IgA.

Furthermore, elastase B plays a key role in the differentiation of pseudomonal biofilms. Tielen and co-workers [64] showed that strains that overexpress *lasB* gene

were not able to form robust biofilms, and they observed the formation of few microcolonies after 72 h of contact with glass surface. Those authors also assigned that *lasb*-overexpressed strain shifted the composition of its extracellular polymeric substances, reducing the alginate content as well as enhancing the rhamnolipids concentration [64]. However, Yu and co-workers [65] demonstrated that elastase B is crucial for biofilm formation. They observed that Δ*lasB* mutant decreased the biofilm formation through down-regulation of rhamnolipids synthesis.

3.2 Elastase A

Another extracellular protease produced by *P. aeruginosa* is elastase A (LasA), a metalloprotease that belongs to the subgroup A of M23 family of staphylolytic or β-lytic zinc metallo-endopeptidases. LasA is codified as an elastase A pre-pro-protein with molecular mass of 40 kDa [66, 67]. After its synthesis in intracellular bacterial environment, LasA is secreted via type II secretion machinery and when it is secreted to the extracellular space, LasA is immediately converted to its mature and active form of 27 kDa due to the cleavage by other pseudomonal-secreted endopeptidases, such as LasB, LysC, and protease IV [68, 69].

Elastase A is also called as staphylolysin, because it is able to cleave the pentaglycine bonds in the peptidoglycan of *Staphylococcus aureus* [70]. As well, LasA degrades several glycine-rich synthetic peptides [71]. However, LasA exhibited a limited elastinolytic activity [72]. Kessler and co-workers [71] showed that LasA prefers cleaving Gly–Ala peptide bonds within the Gly-Gly-Ala sequences surrounded by apolar sequences. Such sequences are uncommon in elastin, resulting in low elastinolytic activity [26, 73]. Besides its own intrinsic elastinolytic activity, LasA enhances significantly the elastinolytic activity of other proteases, including LasB in *P. aeruginosa*, but also human leukocyte elastase and human neutrophil elastase [74, 75]. Moreover, LasA is responsible for inducing shedding of the host cell surface proteoglycan syndecan-1 (co-receptor proteins), which has been shown to be important for *P. aeruginosa* survival [25, 26].

3.3 Alkaline Protease

Another pseudomonal protein shown to be important for phagocytic evasion is alkaline protease (AprA), which is also known as aeruginolysin. Alkaline protease is a 50-kDa zinc-metalloprotease, member of subfamily B of the M10 peptidase family and metzincin superfamily. AprA, encoded by *aprA* gene, has a C-terminal secretion signal located within the last 50 amino acid residues necessary to be translocated and secreted by AprD, APrE, and AprF membrane proteins, which form the bacterial type I secretory machinery [35].

It was reported that alkaline protease is able to degrade a large number of host proteins, including fibronectin and laminin, important components of basal lamina and endothelium. Therefore, alkaline protease develops an important function in

invasion and hemorrhagic tissue necrosis in infections caused by *P. aeruginosa* [76]. Furthermore, this protease was found in many isolates of *P. aeruginosa* recovered from different human anatomical sites with especial elevated expression in clinical isolates from eyes, gastrointestinal tract, and mucoid wounds exacerbated in cystic fibrosis patients [25, 61]. AprA is important to bacterial escape from the host immunological defenses, degrading complement proteins (C1q, C2, and C3) and cytokines (IFN-γ, TNF-α and IL-6) [76]. Also, alkaline protease and elastase B are able to inhibit chemotaxis of neutrophils and block efficiently the phagocytosis, which gives the pathogen an advantage in escaping from phagocyte cells that are one of the first lines of host defense mechanisms [25, 31, 77, 78]. Moreover, alkaline protease is able to inhibit flagellin recognition by TLR5 due to the degradation of free flagellin monomers, helping *P. aeruginosa* cells to avoid the immune detection [79]. This enzyme has also been shown to aid *P. aeruginosa* survival in iron limitations conditions during human infections by cleaving transferrin that increase the siderophore-mediated iron uptake [80]. Gupta and co-workers [81] also reported that treatment of mouse corneal tissue with alkaline protease (50 ng) increases the binding of *P. aeruginosa* to the epithelial surface.

3.4 Protease IV

P. aeruginosa secretes a serine-type protease designated as protease IV (PIV) or lysyl endopeptidase (PrpL), a 26-kDa protease belonging to the chymotrypsin family S1 that has been demonstrated to be an important virulence factor in the rabbit cornea, but is found in clinical isolates recovered from all the anatomical sites analyzed [35, 82]. Its catalytic domain is formed by the triad His_{72}, Asp_{122}, and Ser_{198}. Moreover, it was demonstrated that the residue Ser_{197} adjacent to Ser_{198} is critical to the catalytic activity [83]. Protease IV is encoded by *piv* gene (PA4175), with a full length of 48 kDa, which is initially expressed in the cytoplasm in a pre-pro-enzyme form and then processed to the 26-kDa mature protease after its secretion into the extracellular milieu [83].

PIV participates in the tissue invasion/damage processes and hemorrhagic events due to the cleavage of fibrinogen. It is well known that fibrinogen is required after vascular damage, but the degradation of fibrinogen by PIV leads to hemorrhage during *P. aeruginosa* infection [84]. PIV is also important to evade host immune defenses because it is able to degrade plasminogen, immunoglobulin, C1q and C3, and host antimicrobial peptide LL-37 [25, 68]. Furthermore, Malloy and co-workers [82] observed that PIV degrades the surfactant proteins, SP-A, SP-D, and SP-B, by a time- and dose-depended way in cell-free bronchoalveolar lavage fluid. Those authors reported that degradation of SPs by protease IV reduced the association among bacteria and alveolar macrophage. Interestingly, the incubation of pulmonary surfactant with pseudomonal protease IV reduced the ability of the surfactant to diminish the superficial tension within the lung [82]. Protease IV has been shown to be an iron-regulated protein, suggesting that its expression is regulated irrespective of *quorum sensing* system, which is distinct from other pseudomonal

proteases [69]. Protease IV has also been correlated to ring abscess lesions present in pseudomonal keratitis [68]. Corroborating this finding, Engel and co-workers [85] showed that protease IV-deficient mutants exhibited lower ocular virulence in rabbits when intrastromally infected.

3.5 Pseudomonas Small Protease

P. aeruginosa small protease (PASP) is described as a 18.5-kDa secreted zinc-dependent leucine aminopeptidase. *PASP* gene has been found in a large number of *P. aeruginosa* clinical strains, but its higher expression is found during the ocular infection [86]. Previous reports showed that PASP is found only in the bacterial supernatant culture. According to Tang and co-workers [86], the sequence of *PASP* gene appears to have a signal peptide consistent with that needed for type II secretion system.

Direct inoculation of purified PASP into the rabbit cornea causes severe ocular pathology, including epithelial erosion and ulcer in stroma, edema, and neutrophil infiltration into the corneal stroma [87]. PASP has also been demonstrated to cleavage host proteins required for maintaining structure of cornea, such as collagens, fibrinogen (but not fibrin), complement C3, and antimicrobial peptide LL-37. Studies of PASP, coupled with those of PIV, strongly support the hypothesis that *Pseudomonas* proteases play a major role in keratitis [87].

3.6 Large Exoprotease A

Large exoprotease A (LepA) is an exoprotease with molecular mass of ~ 100 kDa produced by *P. aeruginosa*. LepA, as well as thrombin and trypsin, cleaves human protease-activated receptors (PARs) 1, 2, and 4 in order to activate the critical transcription factor NF-κB, which is associated with host inflammatory and immune responses [49, 88].

3.7 MucD

MucD was reported to be a serine endoprotease that is localized within the periplasmic space. Data suggest that MucD induced a significant reduction on the levels of IL-1β, neutrophil-chemoattractant chemokines KC, and macrophage-inflammatory protein-2 (MIP-2) in the early stages of bacterial infection as well as it inhibited the recruitment of polymorphonuclear (PMN) cells into the cornea. Furthermore, a decrease in PMN cells recruited to infection site favored the establishment of infection by *P. aeruginosa*. MucD may be secreted to the extra-cellular space, interfering with the biological functions of cytokines and chemokines, but further investigation is needed to understand the mechanisms underlying the role of MucD in keratitis [89, 90].

3.8 Aminopeptidase

The *P. aeruginosa* aminopeptidase (PAAP) or leucine aminopeptidase has been speculated as complementary enzyme to the activity of other endopeptidases. PAAP has an important function in bacterial physiology; it acts releasing free amino acids/small peptides from protein fragments produced by the others *P. aeruginosa* endopeptidases, thereby providing low molecular mass nutrients that can be taken up by the bacterium, which in turn may promote bacterial growth and proliferation [26].

4 Conclusions

P. aeruginosa is a metabolically versatile bacterium that can cause a wide range of severe opportunistic infections in hospitalized patients. To cause this huge variety of infections, *P. aeruginosa* has an arsenal of proteases that are involved in critical events of bacterial pathogenicity and virulence, which are important for survival in the host, tissue invasion, and evasion of host immune defenses. Therefore, this review has highlighted the importance of each pseudomonal protease in bacterial physiology and/or in infectious events. In this context, inhibitors able to block the proteases produced by *P. aeruginosa* cells would represent a new drug class quite promising to combat this widespread bacterial pathogen.

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Serine Proteases as Metabolic Regulators in Yeast

Alexis N. Campetelli and Noelia E. Monesterolo

Abstract

Serine proteases are enzymes that break peptidic bonds in proteins, being serine the nucleophilic amino acid at the enzyme's active site. These proteases are found ubiquitously in both eukaryotes and prokaryotes. Based on their structure, serine proteases may be classified into two super families: chymotrypsin-like (trypsin-like) or subtilisin-like. This chapter focuses on aspects related to the molecular structure, subcellular localization, mechanism of action, and physiological role, among others, of Kexin, Ynm3p, Prb1p, Ssy5p, Lpx1p, and Pcp1p, the best characterized serine proteases found in yeast, taking as a reference the almost fully characterized yeast model *Saccharomyces cerevisiae*. Table 1 summarizes the general information about the proteases mentioned above, and such general information is illustrated in Fig. 1.

1 Introduction

Serine proteases are widely distributed in nature and found in all kingdoms of cellular life as well as many viral genomes. Above 30% of all known protein degrading enzymes are serine proteases, highlighting its importance in evolution. They are grouped into 13 major clans and 40 families. This way to classify segregates proteases into clans based on their action mechanism and families on evolutionary basis. The family name has its origin based in the nucleophilic Ser in the enzyme active site. This serine attacks the carbonyl group of the substrate

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A.N. Campetelli (✉) · N.E. Monesterolo
Departmento de Biología Molecular, Facultad de Ciencias Exactas,
Físico-Químicas y Naturales, Univesidad Nacional de Río Cuarto, Río Cuarto, Argentina
e-mail: acampetelli@exa.unrc.edu.ar

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Table 1 General information about serine proteases that will be discussed throughout this chapter

Standard name	Pcp1p	Ssy5p	Prb1p	Kex2p	Ynm3p	Lpx1p
Systematic name	YGR101w	YJL156c	YEL060c	YNL238w	YNL123w	YOR084w
Enzyme class N°	E.C. 3.4.21.105	E.C. 3.4.21.–	E.C. 3.4.21.48	E.C. 3.4.21.61	E.C. 3.4.21.–	E.C. 3.1.1.– E.C. 3.4.21.–?
Aliases	Rhomboid protease Rbd1, MDM37	RAA3, SPS-sensor component serine protease SSY5	proteinase B (PrB) CVT1	Kexin QDS1, VMA4, yscF, SRB1	Ynm3 or Nma111 (nuclear mediator of apoptosis)	Peroxisomal matrix-localized lipase
Description						
Length (a.a.)	346	699	635	814	997	387
Mol. weight (Da)	38839.9	77518.4	69606.1	89957.0	110860.3	43730.5
Isoelectric point	10.78	6.42	6.36	4.59	5.80	8.29
Location	Mitochondrial inner membrane	Plasma membrane	Vacuoles	Secretory pathway	Nucleus	Peroxisomal/cytosol
Tipe of serine protease	Rhomboid	Chymotrypsin-like	Subtilisin-like	Subtilisin-like	Chymotrypsin-like	?
Substrate	Mgm1p, Ccp1p	Stp1p, Stp2p	Histone H3	α factor, Kex2p, etc. (S. cerevisiae), Sap2 (C. albicans), Lip2 (Y. lipolítica)	Bir1p	p-nitrophenyl butyrate DPG, Glycerophosphocholine, Tubulin

(continued)

Table 1 (continued)

Standard name	Pcp1p	Ssy5p	Prb1p	Kex2p	Ynm3p	Lpx1p
Function	Pcp1 converts L-Mgm1 into S-Mgm. S-Mgm is crucial for wild-type mitochondrial morphology and maintenance of mitochondrial DNA Pcp1 participates in the second processing step, yielding the mature Ccp1, protein involved in degradation of reactive oxygen species	Ssy5p is responsible for the endoproteolytic processing of transcription factors Stp1 and Stp2, allowing them to enter the nucleus and activate the transcription of amino acid permease genes	PrB activity increases significantly and participates in degradation events. Prb1 cleaves histone H3 tail, under nutritional stress and during the sporulation process	Participates in several cellular processes including mating pheromone processing, hyphal development, cell wall remodeling, and activation of preprohormons, neuropeptides, and extracellular enzymes	Ynm3 exhibits its proapoptotic activity because of Bir1p degradation (Bir1p acts as an inhibitor of apoptosis) Regulator of fatty acid metabolism Chaperone activity in vitro	Shows acyl esterase, lipase, and phospholipase A activity toward PNB, DPG and BPC Lpx1p hydrolyzes acetilated tubulin, inducing AcTub/H^+-ATPase complex dissociation, which results in H^+-ATPase activation
Reference	Urban et al. [75], Herlan et al. [80]	Forsberg et al. [85], Poulsen et al. [87], Pfirrmann et al. [91]	Moehle et al. [62], Pérez et al. [72], Xue et al. [65]	Rockwell et al. [18, 33]	Padmanabhan, et al. [45], Belanger et al. [36], Walter et al. [47], Fahrenkrog et al. [44]	Thoms et al. [50, 58], Campetelli et al. [51]

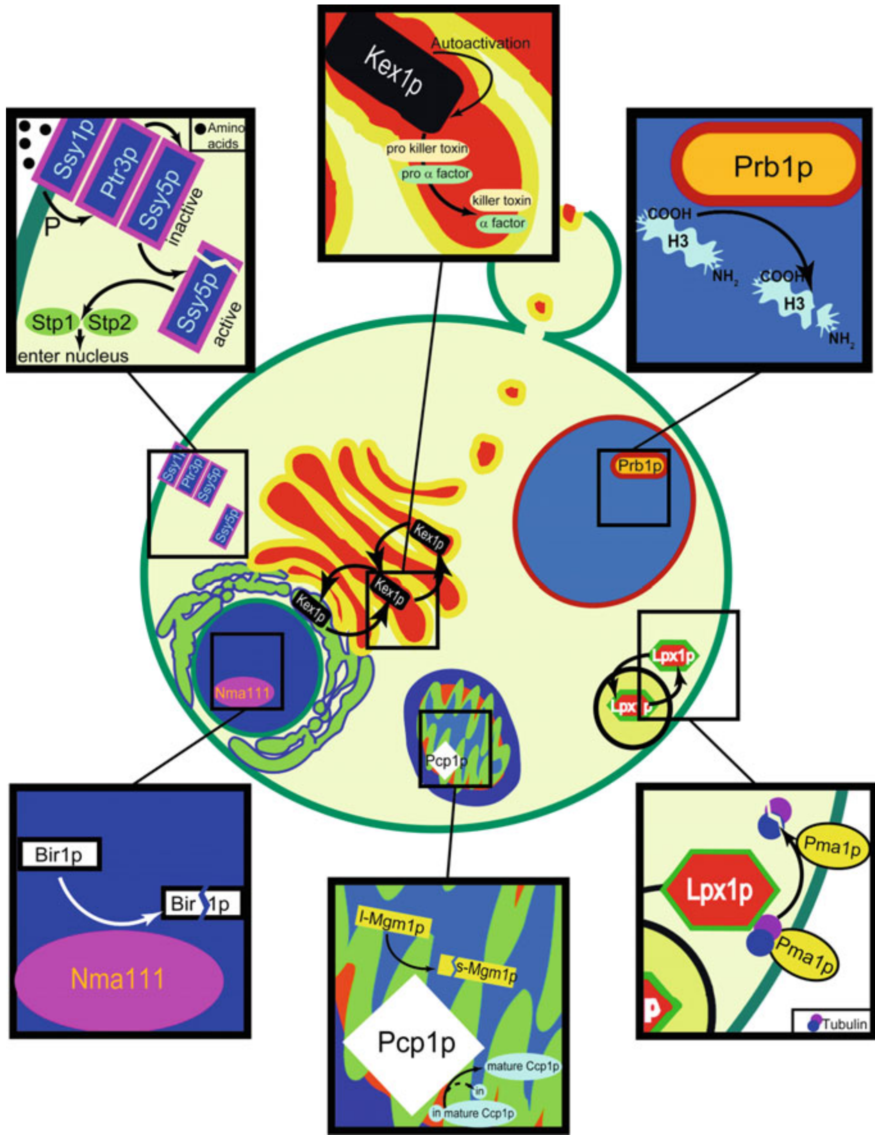

Fig. 1 Several metabolics and cell signalling pathways are regulated by serine proteases in yeasts

peptide bond to form an acyl-enzyme intermediate [1]. Nucleophilicity of this serine depends generally on a catalytic sequence of Asp, His, and Ser residues, commonly termed as the charge relay system [2]. The success of the catalytic break depends on this triad, which is found in four distinct evolutionary pathways. Serine proteases are normally endoproteases, and their substrates are generally cleaved close to the middle of their primary sequence. However, some families of proteases

that remove one or more amino acids from the ends of target polypeptide chains have been described (Table 1 and Fig. 1).

In the yeast *S. cerevisiae*, a group of serine proteases composed by Kexin, Ynm3p, Prb1p, Ssy5p, Lpx1p, and Pcp1p, play key roles in the cell cycle progress. The aim of this chapter is to review the aspects related to the molecular structure, subcellular localization, mechanism of action, and physiological role of these serine proteases.

2 Kexin

Kex2p was first described in *S. cerevisiae*. Wickner and colleagues identified this as a genetic locus for killer toxin expression [3], but it was immediately related to the production of α-factor, the mating pheromone secreted by MATα haploid cells [4]. Further structural characterization of these two effectors showed that both are excised from larger precursors by specific endoproteolysis [5]. Later, it was showed that Kex2 encodes a Ca^{2+}-dependent serine protease, with a single transmembrane domain, followed by a cytosolic tail which is responsible for Kex2p partitioning at the late compartments secretory pathway of yeasts.

2.1 Homologs

While Kex2p has its respective homologs in other fungi (krp1 in *Schizosaccharomyces pombe* [6], KEX1 in *Kluyveromyces lactis* [7], XPR6 in *Yarrowia lipolytica* [8], and Kex2 in *Candida albicans*, *Candida glabrata* and *Aspergillus niger* [9–11]), and other organisms like *Drosophila melanogaster*, the mammalian homologs are numerous, including the prohormone convertases PC1/3 and PC2, which clivates proinsulin, prohormones, and neuropeptides. These enzymes lack the transmembrane domain and are mainly expressed in neuroendocrine cells and localized at the regulated secretory pathway. Another Kex2p-related mammalian homolog is furin, which is found more ubiquitously, and has a transmembrane domain that, as in the case of Kex2p, allows it to cycle between late compartments of the constitutive secretory pathway. Other homologs in mammals include PC4, PC5/6, PC7, and PACE4, with some of them displaying several isoforms.

In mammalian, the family members are classified into two distinguishable subfamilies, according to whether they harbor a transmembrane domain or not, and therefore, its delivery to the constitutive or regulated secretory pathway.

2.2 Subcellular Localization

These two arms of this family are needed in significantly different localizations. Kex2p, as well as furin, display a fast cycling between different compartments in

the secretory pathway. There, they are temporally exposed to their target substrates, which are mixed with an excess of incorrect substrates, on a timescale of minutes. In the other hand, PC1/3 and PC2 localize into the dense-core secretory granule, where most precursors predominate, during a longer period of time.

2.3 Molecular Structure

Characteristics of the primary structures of Kexin-related enzymes are visibly conserved. In general, most of them harbor an N-terminal signal sequence responsible for entering the secretory pathway. This N-terminal sequence is followed by a prodomain, which once the protein reach maturity is autocatalytically removed [12, 13], a catalytic domain (with the catalytic triad playing a pivotal role) [14], and an exclusive domain called the P-domain in PC-type serine proteases [15] —which is necessary for substrate recognition and activity. PCs harbor C-terminal domains that are important for correct subcellular localization since they carry sorting signals. In these enzymes, the single transmembrane domain is followed by a cytosolic tail containing several signaling sequences that allow them to cycle between the trans-Golgi network and endosomal compartments [16]. Structural studies have shown that regions C-terminal to the P-domain could work as flexible harms in vivo. The catalytic site of Kex2p and furin display similar fold and topology as those of the other subclass, subtilisin, and proteinase K; however, some of the loops at the core surface differ in length, and some α-helix and β-sheet are slightly shifted. These differences build the platform that interacts with the P-domain and alter the substrate-binding pocket. Other conserved residue is the well-known “oxyanion hole” Asn, which, at least in subtilisin, has been proposed as a stabilizer of the transition state.

2.4 Action Mechanism

In general, kexins cleaves amide or ester substrates, and the action mechanism is described by acyl transfer reactions carried out by the classical serine protease mechanism, which uses the catalytic triad of serine, histidine, and aspartate. In the case of proteases of the subtilase superfamily, a fourth conserved catalytic residue, the oxyanion hole Asn is also an active residue in the action mechanism. But let explain more exactly the role of these group of residues on the action mechanism. The Ser provide the hydroxyl nucleophile for attack of the scissile bond; in the other hand, His acts as the catalytic base for de-protonation of the nucleophilic Ser, and finally, Asp stabilizes the assembling of positive charge on the protonated His [17]. During the nucleophilic attack on the scissile bond, the negative charge of the carbonyl oxygen is stabilized by the “oxyanion hole,” which further contributes a pair of hydrogen bond donors to the carbonyl oxygen atom. Once a substrate binds a serine protease, the Ser attacks the scissile bond, giving rise to the first step of the reaction, the acylation. Then, the separated C-terminal fragment of the substrate is

released, while the nucleophilic Ser remains covalently linked as an ester to the remaining N-terminal fragment, being the second step of the reaction. Finally, a solvent water molecule hydrolyzates this acyl-enzyme intermediate (deacylation), thereby releasing the protease-N-terminal fragment complex.

Unlike degradative proteases, processing proteases and therefore kexins must be exceptionally accurate and very efficient, because they are often only exposed to their substrates for extremely short time periods.

How these proteases exploit the serine protease mechanism to achieve the required efficiency and specificity can be partially explained, at least partially, on the specificity of P sites. In the case of P4, either positive charge or a large aliphatic side chain is sufficient to satisfy the enzyme–substrate interaction [18]. Kex2 recognizes basic residues like Arg, Lys, and ornithine at P2 [19] with no differences in catalytic parameters. Consequently, the contribution of the aliphatic region of the Lys or Arg to catalysis is poor at this position. However, when these interactions are omitted by replacement of Arg or Lys by acidic or aromatic residues, larger defects are observed. At P1, Kex2 displays high specificity for Arg. Substitution by Lys or ornithine at this site resulted in a serious *k*cat/*K*M defect of at least 70-fold despite the positive charge is conserved with both residues, demonstrating the overriding importance of electrostatic interaction with Arg at the P1 position as a determinant of Kex2 specificity.

In the structure of Kex2p [20], three Ca^{2+} ions can be found. However, in subtilisin, only two Ca^{2+} ions are found. While two of these ions occupy sites equivalent both in kexins and subtilisins, the third Ca^{2+} in the Kex2p structure is absent in the crystal structures of any subtilisin or proteinase K, albeit the third Ca^{2+} binding site is conserved. In kexins, this bound Ca^{2+} is placed at the base of the S1 pocket, where interacts with two conserved Asp, one conserved Glu and some water molecules. A key role in substrate recognition has been conferred to this ion because one of the conserved Asp residues contacted also directly contacts the side chain of the substrate P1 Arg.

2.5 Physiological Role

In yeast, kexins display a broad range of activities including the processing of cell wall building and repairing proteins [16], key proteins for the aerial hyphae development [21], lipases [22], α-type mating prepropheromones and killer toxins [23], polysaccharide-degrading enzymes [24, 25], zymogens of extracellular proteinases, and themselves as well [8, 11, 26].

While in *S. cerevisiae*, kex2-null mutants are viable, some conditional morphological abnormalities are observed [27]. Some of the found defects include the following: poor vacuolar pH control [28], cold-sensitive growth [27], impaired meiosis, and a probable impairment of the RNA polymerase II complex formation [29]. In the opportunistic fungal pathogen *C. albicans*, Kex2p participates in pathways involved in the expression of virulence factors such as the secretion of proteinases and hyphal formation. When the growth conditions allow hyphae

production, the double mutant kex2/kex2 cells become larger than the wild-type, multiple buds and nuclei are often observed, abnormal hyphae are produced, and secreted aspartyl proteinase (SAP) are misprocessed, leading to diminished secretion of the enzyme [11]. In a mouse model of systemic infection, the *C. albicans* double mutant *kex2/kex2* is markedly impaired, lacking its ability to invade the kidney, which is one of the main virulence factors in this condition. Also, the null mutants lose the ability to produce hyphae either when grown embedded in agar, when ingested by macrophages, or when disseminated in mice [30].

2.6 Inhibitors

As mentioned above, Kex2p requires Ca^{2+} ions for activity, and thus is inhibited by chelators such as EDTA. It can be additionally inhibited by reducing agents such as DTT and p-chloromercuribenzoate [31], by peptidyl chloromethyl ketones and short peptides with the same sequence than the recognition site of the substrate [32], by the serine protease inhibitor DFP and provably by PMSF, and by heavy metals such as Cu^{2+} and Zn^{2+} that could be competing with Ca^{2+} ions. On the other hand, K^{+} and other monovalent metal cations have been shown to activate Kex2, probably due to a specific allosteric interaction [33].

An extra system to control the activity of proteases is carried out at the level of compartmentalization. Many proteases localize in degradative compartments such as the yeast vacuole. In the case of protease leakage from these organelles, some cytosolic proteins act as inhibitors, presumably as insurance against massive proteolysis. Furthermore, some sequences that can be found naturally, including the prodomains of the proteases themselves, are heavy inhibitors.

3 Ynm3p

Ynm3p or Nma111 (from **n**uclear **m**ediator of **a**poptosis) is a member of evolutionarily conserved apoptosis-regulatory proteins. In *S. cerevisiae,* it is an HtrA (high-temperature requirement A)-like serine protease of about 111 KDa. In this yeast, Ynm3p break Bir1p, the only known IAP (**i**nhibitor-of-**a**poptosis **p**rotein) in yeast. Ynm3p induces apoptosis in a serine protease-dependent manner and exhibits molecular chaperone function under high temperature stress, and its activity occurs exclusively in the nucleus.

3.1 Homologs

Homologs of HtrA/DegP are ubiquitously disseminated in all kingdoms. Normally, these proteins are constituted by two conserved core domains, a chymotrypsin-like protease domain, and at least one PDZ domain found always in the C-terminal side.

While most of the protease-chaperone systems require ATP, the HtrA represents the first well-known protein quality control factor that does not use this energetic molecule [34, 35].

3.2 Subcellular Localization

Under steady-state conditions, Ynm3p is a nuclear protein. It was shown, by a heterokaryon assay, that Ynm3p cannot shuttle between the nucleus and the cytoplasm, remaining in the nucleus, when apoptosis is induced by H_2O_2.

The nuclear localization of Ynm3p is given by a nuclear localization signal (NLS) localized in the N-terminal end of the mature protein. The protein Kap95p, which recognizes this NLS sequence, has been proposed as the nuclear import receptor for Ynm3p [36].

In mammalian cells, HtrA2 is mostly localized in the mitochondria and translocated to the cytosol during apoptosis. Once in the cytosol, it interacts with IAP, which allows the activation of caspases [37–39]. In the case of yeast, although Ynm3p is mainly nuclear, a subpopulation remains associated with mitochondria suggesting a putative role in mitochondrial homeostasis during aging, since Ynm3p null mutants displayed poor oxidative growth during prolonged incubation.

3.3 Molecular Structure

As mentioned above, an exclusive feature of the HtrA protein family is presence of two conserved domains. A catalytic serine protease domain followed by one or two PDZ domains. PDZ domains are very usual protein interaction motives normally involved in signaling pathways and structurally consist of small globular structures [34, 40–42]. In *S. cerevisiae*, Ynm3p harbors an internal duplication of the HtrA-like sequence. While an active domain retaining the complete catalytic triad is placed in the N-terminal end, the C-terminal end harbors the other incomplete serine protease site, which is inactive [34, 40, 43].

3.4 Action Mechanism

The yeast Ynm3p null mutant can grow normally at elevated temperatures and apoptotic signals as ROS accumulation and chromatin condensation are absent [44]. In the other hand, overexpression of Ynm3p induces apoptosis. When the Ser235 residue is replaced by cysteine (S235C mutant), a notorious reduction of proapoptotic properties is observed. Surprisingly, the other main function of this protein, it is its chaperone activity, would depend on Ser236 [45].

In mammalians, the homolog Omi/HtrA2 induces apoptosis by binding and degrading cellular IAPs [37, 39]. The hallmark characteristic of IAPs is the presence of BIR (baculovirus IAP repeat) domains [46]. In *S. cervisiae*, only one BIR

protein has been determined, Bir1p, which is a substrate for Ynm3p, and therefore the only IAP in this organism. Recombinant purified Bir1p interacts physically with Ynm3p in vitro, and this interaction depends on the N-terminal HtrA repeat [47]. How Bir1p is degraded by Ynm3p is not clear; however, it was structurally demonstrated in the *E. coli* HtrA homolog DegP that substrate binding to the PDZ domain of DegP results in oligomerization of hexameric DegP giving as a results an active enzyme. In HtrA proteases, the specific loop 3 (L3) that works as a conserved molecular switch senses the activation signal and transmits this information to the active site which acts as canonical serine protease [48].

3.5 Physiological Role

The main role of Ynm3p in yeast is related to induction of apoptosis. Bir1p is a substrate of Ynm3p and prevents apoptosis. Its degradation in hands of Ynm3p, instead, results in apoptosis induction. Since both proteins localize in the nucleus, the process takes place in this organelle.

By two hybrid analyses, it could be shown that Ynm3p interacts with the acyl-CoA synthetase Faa1p and Faa4p, and therefore regulating their activities. Ynm3p null mutants display increased import of fatty acids, misregulated expression of Δ^9-acyl-CoA desaturase, and accumulation of free fatty acids and triglycerides. So, an important role of Ynm3p in fatty acid metabolism must be considered [49].

Ynm3 exhibits general chaperone activity in vitro. Ynm3p inhibited the aggregation of citrate synthase (which is not degraded by Ynm3p) in in vitro thermal induced aggregation assay. This inhibition was achieved in the absence of ATP, suggesting a no conventional chaperone activity. This additional function would involve the PDZ domain, since recombinant proteins lacking these domains lose their ability to confer thermos protection [45].

4 Lpx1p

The green fluorescent protein (GFP) tagged form of Lpx1p was found in peroxisomes in *S. cerevisiae*. Its localization was confirmed by sedimentation assays [50]. Its transport into the peroxisome depends on the QKL motif present in its PTS1 (peroxisomal targeting signal) domain.

The first functions attributed to Lpx1p were in vitro acyl hydrolase and phospholipase A activities. However, it was shown later that Lpx1p would participate in events that lead to Pma1p (the yeast plasma membrane proton pump) activation by hydrolyzing its natural inhibitor tubulin [51].

4.1 Homologs

Its global structure has many similarities to E-2AMS hydrolase from *Mesorhizobium loti*, an α/β hydrolase from *Novosphingobium aromaticivorans* and of *E. coli* YbfF. The function of the *Novosphingobium* protein is unknown. *E. coli* YbfF [52, 53] has been described as an esterase with activity toward p-nitrophenyl butyrate [54].

4.2 Subcellular Localization

The PTS1 domain is the responsible of the delivery of most matrix proteins to the peroxisome. Pex5p, the peroxisomal receptor which recognizes the PTS1 localized at the C-terminus of Lpx1p, is involved in the peroxisomal delivery of Lpx1p [55]. However, some remaining Lpx1p is observed in the cytosol, and other yeast proteins that contain the QKL motif present in the PTS1 are not targeted to peroxisomes, like the elongation factor EFB1 [56] or RPT4, an ATPase of the 19S regulatory particle of the 26S proteasome required for spindle pole body duplication which is mainly nuclear [57].

4.3 Molecular Structure

The molecular mass of Lpx1p is about 44 kDa. Its structure is composed of a catalytic triad with an unusual location of the acid residue after strand β6 of the canonical α/β-hydrolase fold. The active site is covered by a four-helix cap domain, and the interface between the α/β-hydrolase core and this domain would be the potential substrate-binding site. This interface would also form the tunnel connecting with the protein interior and widens into a cavity. Other two tunnels that connect the protein surface with the active site have been found, and a role in substrate accessibility was given. Lpx1p is a homodimer that results from the binding between the cap domain of one monomer and the C-terminal helix of the other, giving as a result a complex of about 6000 $Å^2$. The tunnel and the cavity are located at the interface between the α/β hydrolase core fold and the cap. The N-terminal loop of the Lpx1p cap domain (residues 174–190) has been proposed as lid that would regulate substrate access to the active site, because it displays high flexibility [58].

4.4 Action Mechanism

In Lpx1p, residues Ser145, Glu169, and His323 located after strands β5, β6, and β8, respectively, conform the typical catalytic triad. In Lpx1p, Ser295 occupies the position after strand β7 where the acidic residue of the triad is generally located.

Some authors suggest that due to the presence of this additional serine, the term catalytic triad should be changed to “catalytic tetrad” [53, 58].

Regarding its enzymatic activity, Lpx1p hydrolyzed the test substrate p-nitrophenyl butyrate (PNB) with a K_M of 6.3 μM and Vmax of 0.17 μmol s^{-1} suggesting esterase activity. Lpx1p also exerts lipase activity toward DPG of 5.6 pmol h^{-1} μg^{-1} and phospholipase A activity of 7.9 pmol h^{-1} μg^{-1} when the BODIPY dye-labeled glycerophosphocholine (BPC) is used as substrate [50].

There is also biochemical and genetic evidence indicating that Lpx1p would exert serine protease activity on tubulin; however, the protease activity of Lpx1p was not reported in vitro with purified Lpx1p and tubulin [51].

4.5 Physiological Role

Oleic acid strongly induced Lpx1p expression, which is also regulated by stress-associated transcription factors [59, 60]. In Lpx1p null mutants, peroxisomes display abnormal morphology, being mainly vesiculated. This drastic phenotype is similar to the peroxisomal morphology found in some peroxisomal disorders [50].

In *S. cerevisiae*, the plasma membrane H^+-ATPase (Pma1p) is inhibited by acetylated tubulin. Glucose, the natural activator of Pma1p, dissociates tubulin from the pump, restoring activity [61]. It was demonstrated that glucose-induced dissociation of the Pma1p-tubulin complex happens when a membrane tubulin-specific protease is activated. This protease has similar biochemical characteristics than Lpx1p. Membrane tubulin degradation was not observed in Lpx1p null mutants treated with glucose, the Pma1p-tubulin complex remained assembled, and no changes in Pma1p activity were observed. The proposed mechanism of H^+-ATPase activation by glucose involves a decrease in the cytosolic pH, generated by tubulin inhibition of Pma1p, a concomitant fall in intracellular pH that results in Lpx1p activation that hydrolyzes AcTub, accelerating the process of the AcTub/H^+-ATPase complex dissociation and the activation of the enzyme. All these data postulate Lpx1p as an endogenous regulator of intracellular pH in *S. cerevisiae* [51].

5 Prb1p

The Prb1p (also proteinase B or PrB) is a soluble serine protease of the subtilisin family that resides in the *S. cerevisiae* vacuoles. To reach the mature form, Prb1p is proteolytically processed four times. Prb1p activity is mostly detected under nutritional stress and during the sporulation process. As many serine proteases, it is completely inhibited by phenylmethylsulphonylfluoride (PMSF) and diisopropylphosphofluoride (DPF). Prb1p is responsible of the degradation of the histone H3 N-terminal tail in *S. cerevisiae.*

5.1 Homologs

After maturation, Prb1p is a 33 kD glycoprotein whose primary sequence is highly homolog to subtilisins, proteinase K, and thermitase. This serine protease harbors a free cysteine residue near the active site, and this residue is conserved in proteinase K and thermitase, but not in subtilisins.

The mouse embryonic stem cells lysosomal protein Cathepsin L also cuts the histone H3 N-terminus, and this activity has been associated with gene expression regulation during mouse embryogenesis. Other studies reported Glutamate dehydrogenase (GDH) as another histone H3-specific protease in chicken liver, and its role has been connected to gene expression during aging.

The alkaline proteases of *Aspergillus* species, such as protease B of *C. albicans* and thermomycolin from *Malbranchea pulchella,* display also functional homology with Prb1p. Protease B of *C. albicans* catalyzes a subtilisin-like cleavage of the oxidized β chain of insulin [62].

5.2 Subcellular Localization

The vacuole has been proposed as the lysosome for yeast. In *S. cerevisiae,* a variety of hydrolases were found in vacuole, among them two endopeptidases, the proteinase A and B, and five exopeptidases, carboxypeptidases Y and S, aminopeptidases I and Co, as well as dipeptidyl aminopeptidase V [63].

5.3 Molecular Structure

The PRB1 gene encodes a 73 kDa protein precursor (preproPrb1) that needs four proteolytic cleavage steps for maturation. First, the signal peptide (SS) is removed; later, in autocatalytic manner, a fragment of 260 amino acids (P1) localized at the N-Terminus is removed. The third cleavage, catalyzed by Protease A, eliminates a small region named P2 in the C-terminus. Finally, the segment P3 (about 6 KDa, in the C-Terminus) is auto catalytically removed to give the 31 kDa mature enzyme (mPrb1) [64].

5.4 Action Mechanism

In vitro experiments made by Xue et al. [65] with purified Prb1p from yeast demonstrated that the cleavage site in H3 is in the N-terminus between Lys23 and Ala24. Nevertheless, the authors mention the possibility that Prb1p might be required for the activity of yet another unidentified nuclear serine protease which cleaves H3 at Ala21/Ser22 [65].

5.5 Physiological Role

Prb1p participates in the degradation events that occur under nutritional stress and during the sporulation process [62, 65]. The proteolytic cleavage in the histone H3 N-terminus is a modification conserved in *S. cerevisiae*. The eukaryotic nucleosome contains two copies each of the core histones H2A, H2B, H3, and H4 [66]. These histones are highly conserved in evolution and suffer several modifications, both at the N-terminus that extends from their globular cores and at the globular domains themselves.

Many modifications in the histone H3 N-terminal tail are involved in gene regulation and silencing of transcription [67], in the assembly and remodeling of heterochromatin [68, 69], replication [70], nucleosome assembly [71], both in sporulation and stationary phase.

When yeast cells are grown exponentially in YEPD, a commonly used rich medium, the levels of protease B activity are low, because glucose is a repressor of PRB1 gene expression. Prb1p activity is regulated not only at the transcriptional level, but some posttranslational events have been also stablished [72].

5.6 Inhibitors

PrB1, like most serine proteases, is completely inhibited by the subtilisin-like protease inhibitors PMSF, DPF, $HgCl_2$, chymostatin, and antipain. However, under growth conditions where PRB1 expression is induced, higher concentration of these inhibitors is necessary. A potent natural endogenous inhibitor is I2B, which is encoded by the PBI2 gene and is localized in the cytoplasm [73].

6 Pcp1p Rhomboid Protease

Pcp1p rhomboid protease is a mitochondrial serine protease. Its main function is the processing of several mitochondrial proteins and the maintenance of mitochondrial DNA and mitochondrial morphology. It belongs to the superfamily of intramembrane rhomboid-peptidase GlpG [74].

6.1 Homologs

Rhomboid proteins reside in the internal membrane of mitochondria. Its serine proteases activity is involved in epidermal growth factor receptor (EGFR) activation in *Drosophila* [75]. Rhomboids serine proteases are conserved throughout evolution [76] and are present even in eukaryotes with no EGFRs, suggesting additional roles. At least five active rhomboids have been found throughout the mammalian genome, including the presenilin-associated rhomboid-like (PARL)

which also localize at the inner mitochondrial membrane [77]. PCP1 gene homologs have been found in *Kluyveromyces lactis*, and *Eremothecium gossypii*.

6.2 Subcellular Localization

Rbd1p–GFP colocalized with the yeast mitochondrial protein porin, indicating its mitochondrial localization. More precisely, Rbd1p localizes in the mitochondrial inner membrane as an integral membrane protein with six predicted transmembrane domains (TMDs) [78].

6.3 Molecular Structure

The PCP1 gene from *S. cerevisiae* encodes a protein of 38.8 kDa. Its primary structure contains a mitochondrial targeting sequence, six or seven transmembrane helices, a weak ABC3 transport family pattern, and a rhomboid-GlpG motif as predicted by the program MitoProt II (MIPS) [74].

During log-phase growth, wild-type yeast cells display tubular mitochondrial structures around the cell cortex; however, the mitochondria of Rbd1 null mutants appear as small fragments and aggregated masses throughout the cell and nucleoid structures, which represent mitochondrial DNA are not observed upon DAPI staining [79].

6.4 Action Mechanism

Pcp1p is an endoprotease whose catalytic mechanism involves the common catalytic triad found in most serine proteases, consisting of a serine nucleophile that is activated by a proton relay from an acidic residue and a basic residue which is usually histidine.

6.5 Physiological Role

S. cerevisiae has two rhomboids: Rbd1p and Rbd2p. Rbd1p main substrates are cytochrome c peroxidase (Ccp1p); and Mgm1p (a dynamin-like GTPase).

I. Removal of a Mitochondrial Signal Sequence

The yeast protein cytochrome c peroxidase (Ccp1p) is involved in ROS (reactive oxygen species) degradation. This protein is imported from the nucleus into the mitochondrial intermembrane space. It is synthesized as an immature precursor with an N-terminal pre-sequence, which is proteolytically removed in two steps during the delivery of the protein. The mAAA protease (which functions in the degradation

of incorrectly folded or unassembled mitochondrial proteins) was identified as essential for the first processing step. The second step is carried out by the PCP1 gene product, giving the mature Ccp1 protein [74].

II. Processing of Proteins for Mitochondrial Morphology and Mitochondrial DNA Preservation

The structure of mitochondria is very dynamic, and this dynamism is mainly controlled by fusion and fission processes. In addition to regulating these processes, Mgm1p also remodels the mitochondrial inner membrane. Mgm1p exists in two forms of different lengths; the large isoform (l-Mgm1) harbors an N-terminal transmembrane domain that is absent in the short isoform (s-Mgm1). Both isoforms are necessary for complete functioning. The conversion of l-Mgm1 into s-Mgm1 depends on Pcp1p. Therefore, the processing of l-Mgm1 by Pcp1 is crucial for wild-type mitochondrial morphology and maintenance of mitochondrial DNA [80].

6.6 Inhibitors

Like other serine proteases, Prb1p is inhibited by different inhibitors, among which are synthetic chemical inhibitors and natural inhibitors. There is a family of natural inhibitors called “serpins” (abbreviation of serine proteases inhibitors), which form a covalent bond with serine proteases, thereby inhibiting their function, and also artificial small molecules that are irreversible inhibitors called AEBSF and PMSF [81].

7 Ssy5p

SPS complex is formed by Ssy1p, Ptr3p, and Ssy5p and is localized at the yeast plasma membrane. This system involved in amino acid metabolism detects the presence of amino acids in the extracellular medium. Upon amino acid sensing, this system triggers metabolic signals that regulate the functional expression of a battery of amino acid-related enzymes that includes amino acid transporters and amino acid metabolizing enzymes [82].

7.1 Homologs

Ssy5p shares structural homology with a lytic protease from *Lysobacter enzymogenes*. The Ssy5p protease domain sequence was modeled using PHYRE [83], and this region was predicted to have predominantly α structure, while the N-terminal proregion displayed a high helical content [84]. The regions where more homology was detected include the domains that flank the Ser and Asp of the catalytic triad.

7.2 Subcellular Localization

Ssy1p, Ptr3p, and Ssy5p are sequentially ordered at the plasma membrane. There, they adopt different conformations and modifications that will depend on the amino acids in the extracellular side and the presence of the other two partners [85].

7.3 Molecular Structure

We will focus on Ssy5 which acts as a chymotrypsin-like serine proteases. It is expressed as a zymogen that by autocatalysis cleaves the peptide bond between residues 381 and 382, yielding a C-terminal catalytic (Cat) domain that contains the conserved catalytic triad. The deletion of Ser640 results in loss of function, confirming its triad membership [86, 87].

7.4 Action Mechanism

The first event is the sensing of amino acids in the extracellular medium by the primary amino acid receptor Ssy1p [88], which triggers the signal resulting in hyperphosphorylation of Ptr3p [89]. As a result of this hyperphosphorylation, Ssy5 becomes activated and cleaves Stp1 and Stp2, which now are allowed to enter the nucleus which activates the transcription of amino acid permease genes [90].

As mentioned before, Ssy5p is synthesized with a large N-terminal prodomain. After auto processing, the N-terminal prodomain and Cat domain remain associated. The prodomain displays a potent inhibitory effect against the Cat domain yielding a yet inactive protease that will be only activated via Ssy1p receptor, ensuring its metabolic function [91]. The prodomain removal is mediated by a conserved phosphodegron. Upon amino acid induction, the phosphodegron is hyperphosphorylated by casein kinase I. As a consequence, the prodomain becomes polyubiquitylated by the action of Skp1/Cullin/Grr1 E3 ubiquitin ligase complex (SCFGrr1). Finally, the polyubiquitylated prodomain is irreparably degraded by the 26S proteasome [82].

7.5 Physiological Role

In yeast, the transcription factors that regulate the expression of genes related to amino acid transport and metabolism are Stp1p and Stp2p. These transcription factors are synthetized as inactive precursors and localize at the cytoplasm. When the Ssy1p-Ptr3p-Ssy5p complex sense amino acids in the extracellular milieu, the activated Ssy5p processes Stp1p and Stp2p and activates them. The active transcription factors can now move to the nucleus where they induce the expression of amino acid-related genes.

8 Conclusions

In summary, serine proteases play several important roles for normal yeast cell function.

S. cerevisiae mating is a key process that takes place when two haploid cells of different mating type (a and α) are close enough to form a diploid a/α cell through shmoo formation. Diploid cells are able to enter sporulation when complete nutrient deprivation occurs, allowing cells to wait "alive" for better growing conditions. Kex2p is crucial for mating, since it maturates the α-factor, one of the mating pheromones. Kexins also participate in the maturation of enzymes involved in hyphae production. *C. albicans* ability to form hyphae has been proposed as a virulence factor, as these structures are often observed invading tissues.

In mammalians, HtrA proteins function as potential modulators of apoptosis and chemotherapy-induced cytotoxicity. The yeast homolog Ynm3p shares some of these functions as its proapoptotic role has been probed. The expression level of some HtrA serine proteases have been documented to be low in some specific cancer types, making them an attractive candidate for future study [92]. It has been postulated that HtrA serine proteases potentially interact with caspase-mediated cell death pathways, postulating them as potential targets of therapeutic treatment. Future studies unraveling the connection between caspases and HtrA serine proteases will be indispensable to fully explore the therapeutic potential of this novel serine protease pathway. Similarly, further screenings to identify the substrates of these proteases will also be essential to better understand their function and for the successful design of therapeutic drugs.

Similar to kexins, Prb1p participates in cellular events related to nutrient deprivation and following sporulation, degrading key proteins in the vacuolar environment. In the case of Ssy5p, the sensing of extracellular aminoacids by the plasma membrane Ssy1p-Ptr3p-Ssy5p sensor results in the cleavage of the transcription factors Stp1p and Stp2p, which are activated and translocated to the nucleus, where induction of genes related to the metabolism of aminoacids is launched.

Mitochondrial morphology and the processing of reactive oxygen species (ROS) that take place in this organelle depend on the activity of rhomboid proteases. It is noteworthy that these two important cellular processes rely on the activity of rhomboid proteases.

In summary, all the serine proteases described in this chapter participate in cellular events of crucial relevance, ranging from sporulation and apoptosis induction to cell breathing and nutrient processing and sensing. All these physiological processes may be accurately regulated in order to maintain normal cell function. Acquiring more in-depth knowledge about these enzymes will be of invaluable importance, since addressing questions that are still unanswered will open up new possibilities to understand pathophysiological processes where these enzymes are involved and to design new drugs and therapies.

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Caspases: Regulatory Mechanisms and Their Implications in Pathogenesis and Therapeutics

Aasna L. Parui and Kakoli Bose

Abstract

Caspases (**c**ysteine-**asp**artic prote**ases**) belong to a family of endoproteases that regulate the cellular networks governing distinct physiological functions, which mainly include apoptotic cell death and inflammation. Few decades of research have generated a plethora of information on human caspases, their homologues, substrates and role in apoptosis. Programmed cell death (*aka* apoptosis) is an energy-dependent natural physiological process that is adapted by multicellular organisms for maintenance of tissue homeostasis. It involves a complex network of cellular proteases primarily caspases, which regulate selective removal of ageing and unwanted cells, thereby maintaining a precise balance between cell survival and death. Disruption of this stasis often leads to various pathophysiological conditions including inflammation, neurodegenerative disorders and cancer. Thus, being promoter of apoptosis, several caspases are prime targets of numerous research endeavours that aim to encounter and impede the advancement of these diseases. The main objective of this chapter is to provide a comprehensive overview of the structural and mechanistic aspects of caspases as well as the pathways involved in their activation and regulation. Besides, it also elaborates on the role of this protease family in different diseases and the current therapeutic strategies that are being devised to modulate their functions with desired characteristics.

Keywords

Caspase · Programmed cell death · Proteases · Regulation
Cancer · Neurodegenerative diseases · Therapeutics

A.L. Parui · K. Bose (✉)
Integrated Biophysics and Structural Biology Lab,
Advanced Centre for Treatment, Research and Education in Cancer (ACTREC),
Tata Memorial Centre, Navi Mumbai 410210, Maharashtra, India
e-mail: kbose@actrec.gov.in

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1 Introduction

Apoptosis is an evolutionarily conserved phenomenon of **p**rogrammed **c**ell **d**eath (PCD) that is observed in a wide variety of multicellular biological systems. It is primarily needed for the selective removal of ageing, unwanted and impaired cells. It is also essentially involved in chemical-induced cell death and maintenance of several processes such as normal cell turnover, immune system, embryonic development, metamorphosis and hormone-dependent atrophy [1]. During apoptosis, a series of distinct morphological and biochemical alterations lead to the disintegration of cellular components into smaller apoptotic bodies. Thus, in contrast to necrosis, a process wherein cell death results into an inflammatory response, apoptosis is a more controlled process where the contents are strictly maintained within the cell membrane [2]. Deregulated apoptosis has been implicated in many human diseases, including neurodegenerative disorders such as Alzheimer's and Huntington's disease, ischaemic damage, autoimmune disorders and several forms of cancer [3, 4]. Therefore, tight regulation of PCD is critical for proper maintenance of cellular homeostasis, i.e. fine balance between cell proliferation and death.

Since its first revelation by Carl Vogt way back in the early nineteenth century, a plethora of research endeavours on this vital cellular process improved much of our understanding towards the complexities of the biochemical pathways involved [5]. This includes the interaction and crosstalk of critical molecules with myriads of other cellular components to form specialized machinery responsible for this form of cell suicide. The central component of this machinery is an intricate proteolytic system involving a family of proteases. These proteases upon activation via a specific proapoptotic signal result in the cleavage of several essential cellular proteins such as cytoskeletal proteins, DNA repair enzymes and nuclear proteins. The apoptotic machinery is majorly regulated by a class of cysteine proteases called Caspases (**C**ysteinyl **asp**artate-specific prote**ase**), although several other less prominent proteases such as granzymes, calpains, cathepsins and HtrAs are also involved [6]. Well-studied proteolytic systems reveal an intriguing fact that proteolytic reactions are specific and irreversible unlike post-translational modifications [7]. Hence, these enzymes are found at the apex of irreversible processes such as development, cell cycle and, most importantly, cell death.

Apart from being mostly synthesized as precursors called zymogens that have little catalytic activity, proteases are known to regulate their own activation through positive and negative feedbacks. In case of caspases, the active protease can directly or indirectly activate its own precursor and result in an exponential rate of activation that ensures the protease accomplishes its goal more efficiently. The effective concentration of active proteases is also accompanied by the presence of endogenous inhibitors that regulate this crucial process by establishing a significant concentration threshold [7]. This prevents the consequences of an undesirable event of spontaneous cell death upon enzyme activation. Since caspases are the key proteases involved in apoptosis, deregulation of this intricate process can lead to a

variety of human pathologies. Therefore, understanding caspase regulation becomes extremely important so as to rationally manipulate apoptosis for therapeutic gain.

In this chapter, we attempt to describe the properties of caspases, the cellular pathways controlling their activation and their modes of regulation, and also discuss the potential therapeutic strategies involving caspase modulation.

2 The Mediators of Apoptosis: Caspases

For a considerably long period of time, apoptosis has remained a descriptive cell biology phenomenon, until Sulston and Horvitz initiated cell lineage study in 1977 and elaborated more on programmed cell death in the nematode *Caenorhabditis elegans* (*C. elegans*). The group demonstrated that 131 out of 1090 somatic cells of *C. elegans* invariantly underwent the cell death process during normal development [8, 9]. This information was consequently followed up by genetic and molecular studies, wherein the discovery of cell lineage-dependent apoptosis paved the way for genetic characterization of its molecular machinery in *C. elegans* [10, 11]. During this investigation, a cysteine protease CED-3 (**Ce**ll **D**eath Abnormality-**3**) that is required for cell death in *C. elegans* was found to be related to mammalian ICE (**I**nterleukin-1β–**C**onverting **E**nzyme or caspase-1). The central components of the apoptotic machinery in *C. elegans* additionally included CED-4 and CED-9. In mammals, the homologues of CED-9, CED-4 and CED-3 were found to be present in the Bcl-2 (**B C**ell **l**ymphoma 2) family, Apaf-1/NOD-like (**N**ucleotide-binding **O**ligomerization **D**omain-like) receptor family and caspase family, respectively [12–15]. Since the mammalian homologues of CED-3, CED-4 and CED-9 have been found to regulate apoptosis, it is likely that the cellular machinery that modulates programmed cell death in *C. elegans* shares its evolutionary origin with that of mammalian apoptosis. This seminal finding, together with the observation that overexpression of ICE also induces apoptosis, suggested that it possibly plays a key role in promoting cell death. Thus, programmed cell death in *C. elegans* represents a primordial type of apoptosis that implicated the role of caspases in this vital cellular process. Expression of a caspase inhibitor protein, CrmA (**C**ytokine **r**esponse **m**odifier gene product encoded by cowpox virus), in sensory neurons prevented neuronal cell death induced by trophic factor deprivation [16]. This also provided the first evidence for understanding the functional role of caspases in neuronal cell death. Finally, caspase-3 was shown to be a critical mediator of the endogenous apoptotic pathway in mammalian cells [17], and in this way, the journey towards understanding caspases continued.

2.1 General Features of Caspases

The caspases comprise a family of cysteine proteases that use a cysteine residue as the catalytic nucleophile and share an impeccable specificity for cleaving their

target peptide substrates after specific aspartate residues. This concerted action of caspases is essentially responsible for apoptosis; however, a subgroup of the caspase family is also involved in inflammation, where they act as procytokine activators [18]. Further identification of caspases in *C. elegans*, Drosophila and other vertebrate species demonstrated the evolutionary conservation of the death machinery. Till date, 14 distinct mammalian caspases have been identified, of which 11 caspases are from humans: caspases-1–10 and caspase-14 [19]. Caspase-11 and Caspase-12 are murine enzymes that are homologues of human caspase-4 and caspase-5, while caspase-13 was later found to represent a bovine homologue of caspase-4. Thus, the number of caspases discovered increased over the phylogenetic time, with four caspases being identified in *C. elegans*, seven in Drosophila, as well as 11 in mice and humans [20].

Overall, the caspases share similarities in a number of distinct features that include their amino acid sequence, structure and substrate specificity [21]. They are generally expressed as proenzymes (30–50 kD) that contain three domains: an amino (NH_2) terminal domain, a large subunit p20 ($\sim$20 kD) and a small subunit p10 ($\sim$10 kD). In some cases, these subunits are separated by a linker region that has unknown function. The NH_2-terminal domain is found to be highly variable in sequence as well as length and is involved in regulating the activation of proenzyme. Moreover, all these domains are derived from the proenzyme by cleavage at caspase consensus sites. This implies that the caspases can be activated either autocatalytically or in a cascade by enzymes with similar specificity. Generally, the activation is brought about by proteolytic processing between domains, which is then followed by heterodimerization caused due to the association of the large and small subunits. Crystal structures of two active caspases (caspase-1 and caspase-3) further reveal the presence of two heterodimers that associate to form a tetramer with two independent catalytic sites [22–24]. The large and small subunits are closely associated with each catalytic domain, and both these subunits contribute residues necessary for substrate binding and catalysis (described in Sects. 3.1 and 3.2).

As per research reports, caspases have an unusual and absolute requirement for cleavage after aspartic acid and hence, belong to C14 family of the clan CD of cysteine proteases. However, the substrate specificities of the individual caspases are distinct, being determined by the residues present in the pockets of P2, P3 and P4 of the substrate. Proteases are generally known to possess the substrate-binding pockets S1–S2–S3 ... S*n* (where '*n*' represents the number of substrate-binding sites). Amino acids on the amino- and carboxy-terminal of the scissile bond in the substrate are called the P and P′ sites, respectively. For example, if S2, S1 and S1′ are the three well-defined substrate-binding sites, which involve both the main and side chain contacts between substrate and enzyme residues, P2, P1 and P1′ become the corresponding substrate residues that bind to the enzyme's respective subsites. Apart from that, recognition of an active-site pentapeptide QACXG (where X is R, Q or G) is also found to be necessary for efficient catalysis [1]. This preferred pentapeptide recognition motif, that lies NH_2-terminal to the cleavage site, differs significantly among caspases, and hence, varied diversity exists in the biological

functions of caspases [25]. Since not all proteins containing the optimal tetrapeptide or pentapeptide sequence are cleaved, the tertiary structural elements can be said to influence the event of substrate recognition.

The '*oxyanion hole*' in caspases is known to be important for polarizing and stabilizing the scissile P1 carbonyl group of the substrate. It is a pocket in the active site of an enzyme that hydrogen bonds the carbonyl oxygen of P1 residue of the substrate and stabilizes the negative charge on a deprotonated oxygen or alkoxide in the transition state. Thus, it lowers the activation energy required for the reaction and in turn promotes catalysis [26]. The concept of an oxyanion hole was first proposed in trypsin-like serine proteases. In caspases, it is generally formed by the backbone nitrogens of a strictly conserved Gly and the catalytic dyad Cys residue [27]. Along with a His residue, the catalytic Cys is primarily involved in forming the crucial catalytic dyad. Thus, in general, the active-site residues in caspases are in the order His and Cys forming the catalytic dyad. Both active-site residues are preceded by blocks of hydrophobic residues. The catalytic residues are found in the motifs His-Gly and Ala-Cys. These motifs are conserved throughout clan CD. For example, in caspase-1, amino acids such as Arg, Gln, Arg and Ser are known to be involved in shaping the P1 carboxylate-binding pocket. These amino acids are together found to be conserved in all the other caspases, except for a conservative substitution of Thr for Ser in caspase-8. Thus, this justifies the unconditional requirement of an Asp in the P1 position. However, the residues forming the P2–P4 binding pocket are not found to be well conserved, and therefore, they may be involved in determining the substrate specificities of different caspases (Table 1). In consensus with this, the cleavage of proteins by caspases is not only found to be specific, but also highly efficient ($k_{cat}/K_m > 10^6\ M^{-1}\ s^{-1}$) [7]. This strict specificity governs the fact that apoptosis is not accompanied by indiscriminate protein digestion; rather, a selective set of proteins is cleaved usually at a single site and in a coordinated manner leading to programmed cell death.

2.2 Classification of Caspases

Despite multiple rubrics that have been used to classify caspases, the tight structure–function correlations existing within the caspase family mainly govern the classification. Depending upon the two majorly known functions, the caspases are grouped into proapoptotic and proinflammatory subfamilies. Proapoptotic caspases (caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9 and caspase-10) are generally known to be involved in mediating cell death signalling transduction, whereas proinflammatory caspases (caspase-1, caspase-4, caspase-5, caspase-11 and caspase-12) are important in maturation of cytokines and promoting inflammation. Among the inflammatory caspases, mouse caspase-11 was found to be a

Table 1 Human caspases and their substrate specificities

Group	Human caspases	Alternative names	UniProtKB accession identifier	Substrate specificities P4–P3–P2–P1↓	P4 Site Preference
I	Caspase-1	ICE	P29466	W-E-H-D	Large P4
	Caspase-4	ICH-2, ICE(rel)-II, Mih1, Protease TX	P49662	(W/L)-E-H-D	
	Caspase-5	ICH-3, ICE(rel)-III, Mih1, Protease TY	P51878	(W/L)-E-H-D	
II	Caspase-2	ICH-1, NEDD2	P42575	D-E-H-D	Small charged P4
	Caspase-3	Apopain, CPP-32, Protein Yama, SCA-1	P42574	D-E-V-D	
	Caspase-7	CMH-1, ICE-LAP3, Mch-3	P55210	D-E-V-D	
III	Caspase-6	Mch-2	P55212	V-E-H-D	Intermediate P4
	Caspase-8	CAP4, FLICE, MACH, Mch-5	Q14790	L-E-T-D	
	Caspase-9	APAF-3, ICE-LAP6, Mch-6	P55211	L-E-H-D	
	Caspase-10	FLICE2, Mch-4, ICE-like apoptotic protease 4	Q92851	(I/L/V) E-X-D	–
–	Caspase-12	–	Q6UXS9	–	–
–	Caspase-14	–	P31944	–	–

The table represents the preferred tetrapeptide substrate sequences (P4–P1) for various human caspases that have been obtained from a positional-scanning combinatorial substrate library. On the basis of the size of S4 subsite and P4 residue, the caspases can be divided into three groups exhibiting distinct cleavage specificity. The terms P1–P4 and S4 have been described in the text

homologue of human caspase-4, while caspase-13 is a bovine ortholog of human caspase-4. In humans, caspase-12 is generally truncated due to the presence of a premature stop codon, but in African descendants, a read-through mutation causes expression of the full-length protein and increases the risk of sepsis [28]. Overall, this form of caspase classification is incomprehensive, as there are growing evidences that indicate the role of caspases in multiple cellular processes other than

just being proapoptotic or proinflammatory (as described below). Moreover, apoptosis can also be induced through the activation of 'proinflammatory' caspases.

An alternative method of caspase classification is based on their positions in the apoptotic signalling cascades. Herein, they are categorized as initiator caspases (caspase-1, caspase-2, caspase-4, caspase-5, caspase-8, caspase-9, caspase-10, caspase-11 and caspase-12) and effector/executioner caspases (caspase-3, caspase-6 and caspase-7). This form of classification also categorizes caspases according to the length of their recruitment domains (or prodomains), which are placed at their N-termini. The initiator caspases that act upstream in cell death pathways share long, structurally similar prodomains. These prodomains comprise one of the two characteristic protein–protein interaction modules: DED (**D**eath **E**ffector **D**omain) or CARD (**C**aspase **A**ctivation and **R**ecruitment **D**omain) domains. The prodomain is separated from the catalytic domain by a flexible interdomain linker that is cleaved during the activation and maturation process.

Although poor solution behaviour and tendency to aggregate have limited the scope of structural studies on procaspases, recent advances in structural biology and protein engineering have helped circumvent the problem. As a result, few procaspases and several prodomain structures are currently available that revealed a classical hallmark structure of a **D**eath-fold **D**omain (DD) superfamily exhibited by these caspases [29, 30]. Generally, the group of proenzymes exists as stable monomers in the cell until they are activated by dimerization. Once dimerized, the initiator caspases achieve sufficient activity to autoprocess and subsequently cleave between their prodomain and intersubunit linker. The prodomains also allow initiator caspases to bind to the activation complexes by interacting with other adaptor proteins. Functionally, the initiator caspase-2 and caspase-9 are involved in the intrinsic pathway that is activated either by mitochondrial damage, cytotoxic stress, chemotherapeutic drugs or through certain developmental signals. On the other hand, the initiator caspase-8 and caspase-10 are activated by the extrinsic pathway, upon ligand binding to a death receptor at the membrane, which belongs to TNFR (**T**umour **N**ecrosis **F**actor **R**eceptor) superfamily (as described in Sects. 4.1 and 4.2). In contrast to the initiator caspases, the effector caspases do not require death scaffolds for dimer formation [31, 32]. These caspases possess short prodomains that are typically processed and activated by upstream caspases, thus performing the downstream execution steps of apoptosis by cleaving multiple cellular substrates. As the executioner caspases exist in the cell as preformed but inactive homodimers that must be processed by initiator caspases, their inappropriate activation is usually prevented. Upon activation, a single executioner caspase can cleave and activate other executioner caspases, resulting in an accelerated feedback loop of caspase activation. However, the described upstream and downstream relationship is not absolute and may only exist transitorily during very early phases of apoptosis [33]. The catalytic processing and different prodomain types in caspases have been illustrated in Fig. 1.

The protease families can also be classified according to their substrate preferences, despite the absolute requirement for an aspartate residue at the P1 site of the caspase substrates. A positional-scanning synthetic combinatorial library approach

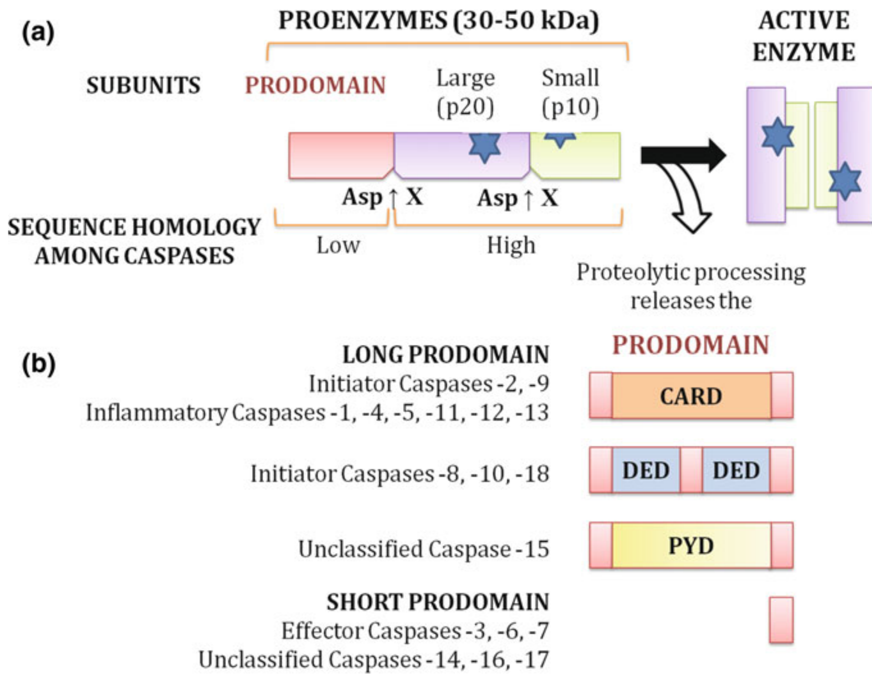


Fig. 1 Domain organization of the caspase protein family. **a** Caspase zymogen (proenzyme) comprises NH_2-terminal prodomain as well as large and small subunits separated by short linker region that harbours the cleavage site (represented by Asp↑X). Proteolytic processing releases the prodomain, and subsequent association of the large and small subunits leads to heterodimerization. Two such heterodimers come together to form the mature active caspase that possesses two independent catalytic sites (indicated by *star*). **b** Based on the size and length of prodomains, procaspases are functionally categorized into inflammatory, initiator and effector caspases

was employed to delineate the cleavage specificities of different caspases in vitro using the general structure of peptide-aminomethylcoumarin (Ac-X-X-X-Asp-AMC) substrates [34]. With this approach, the preferred tetrapeptide substrate sequences (P4–P1) for caspases-1–11 were determined [25] (Table 1), and based on that, these caspases were separated into three classes. The first group (class I) includes caspase-1, caspase-4 and caspase-5, which prefer bulky, hydrophobic side chains in the P4 substrate position, best described by the sequence —WEHD. The second class (class II) consists of caspase-2, caspase-3, caspase-7 and CED-3 that show a preference for DEXD (where X is V or H). Finally, the third class (class III), comprising caspase-6, caspase-8 and caspase-9, displays preference for the optimal sequence (L/V)EXD (where X is V, T or H) (Table 1). The P4 site (i.e. fourth amino acid N-terminal to the cleavage site) has also been found to be a critical determinant of substrate specificity. Those in Group II require Asp for efficient catalysis, while the Group III caspases although have broader specificity at P4

position prefer those with larger aliphatic side chains [25] (Table 1). The optimal cleavage site of caspase-11 has been determined as (I/L/V/P)EHD [35], while substrate recognition sequence for caspase-12 and caspase-14 is yet be to determined [36]. Overall, this form of substrate specificity classification is also in coherence with the aforementioned functional subdivisions of the family as shown in Fig. 1. The substrate preferences match well with the sites found in the known in vivo substrates, such as IL-1β and IL-18, laminin A, PARP and DNA-PK [25]. The processing sites between the large and small subunits in all initiator caspases resemble their own substrate preference, which explains the self-activation ability of initiator caspases. Furthermore, the processing sites in effector caspases fit the preferences of group II caspases, and therefore, they act as downstream activation targets of the initiator caspases. Interestingly, while the presence of a CARD domain in caspase-2 allows it to play the initiator role, both its substrate preference and the cleavage site between the large and small subunits are found to be strikingly similar to that of the executioner caspases (DXXD). These characteristics suggest that caspase-2 is a proximal responder to autoactivation in apoptosis signalling under certain conditions [37–39]. Apart from that, it can also function as an executioner caspase by cleaving cellular targets [19]. An interesting amount of data has been generated through the substrate specificity studies that may further help understand the differences in the physiological roles of individual caspases. However, since these data are derived using a short synthetic substrate in vitro, they need to be interpreted cautiously before being considered conclusive. So far, more than 700 substrates for mammalian caspases have been reported and compiled into a searchable online database known as 'CASBAH' (http://bioinf.gen.tcd.ie/casbah/) [40].

The other caspases that still remain unclassified include caspase-14, whose expression is known to be restricted to epidermal keratinocytes and is specifically involved in differentiation [41]. Similar to the effector caspases, caspase-14 possesses a short prodomain with no adaptor regions. The list of unclassified caspases further includes caspase-15, caspase-16 and caspase-17. Caspase-15 contains a **Py**rin-like region in its pro**d**omain (PYD), like those found in zebrafish counterparts Caspy and Caspy2 [42]. It is also known to be expressed in several other mammalian species including pigs, dogs and cattle [43]. Like caspase-14, caspase-16 and caspase-17 also contain a short prodomain with no adaptor regions [44]. Caspase-16 has been conserved in marsupials and placental mammals, including humans, while caspase-17, which is most similar to caspase-3, has been conserved among fish, frog, chicken, lizard and the platypus but is absent from marsupials and placental mammals. On the contrary, caspase-18 is found in opossums as well as in chickens and is known to contain two tandem DED regions in its prodomain like caspase-8 and caspase-10. It is most likely thought to act as an initiator apoptotic caspase [44]. Further studies on these newly identified caspases will help understand their physiological roles as well as complementarities and redundancy in this protein family.

3 Caspase Structure, Specificity and Catalysis

With the structural determination of caspase-1, elucidation of the structures of several other caspases, their inhibitors and caspase–inhibitor complexes came to the forefront. This focused endeavour provided tremendous information on caspase structure, mode of activation, specificity and regulation. In the following section, we discuss the major characteristics of the '*caspase fold*' and its significance in the various cellular processes.

3.1 Structure of Active Caspases

All caspase structures exhibit the fundamental catalytic domain that comprises a large (17–20 kDa; p20) subunit and a small (10–12 kDa; p10) subunit, which are tightly packed together into a compact ellipsoid of approximate dimensions of 25 Å × 50 Å × 30 Å [45]. A twofold crystallographic axis relates the pairs of catalytic domains, each of which arises from a single procaspase molecule.

During activation and/or maturation, each of the catalytic domains is cleaved between the large (p20) and small (p10) subunits, which in turn interact intimately with each other to form a heterodimer (p20–p10). The secondary structure of these mature caspases is composed of a slightly twisted, but mostly parallel, six core β-strands. These strands are further sandwiched between two layers of α-helices, with two main helices on one face of the protein and three helices on the other. Thus, while the large subunit comprises the first four core β-strands and helices 1–3, the last two core β-strands and helices 4–5 form the small subunit. The quaternary structures of caspases are finally formed when two such heterodimers associate with each other through antiparallel alignment of their C-terminal β6 strands, generating a continuous 12-stranded β-sheet [46]. Therefore, the active form of a caspase is generally referred to as '*homodimers of heterodimers*' [22, 23], where two active sites are positioned at opposite ends of each molecule (Fig. 2). However, this topology is quite intriguingly different for caspase-9, where only one '*active centre*' is formed [19, 47].

Generally, the '*active centre*' of caspases is exclusively formed by flexible loops (L1–L4) [19], where these loops are involved in shaping the substrate-binding groove. While loop L1 constitutes one side of the groove, L4 represents the other side. Loop L3, along with a β-hairpin, is collectively referred to as L3 and is located at the base of the groove. Loop L2, which harbours the catalytic residue cysteine, is positioned at one end of the groove with its side chain primed for binding and catalysis [48]. The catalytic histidine (another catalytic dyad residue) is part of a loop extending from the C-terminal end of β3. Loop L1 and a portion of L2 constitute a part of the large subunit, whereas L3 and L4 come from its smaller counterpart (Fig. 2; only L3 in case of caspase-3). Among all the caspases, the size and topology of the L1 and L3 loops are found to be highly conserved, whereas the L2 and L4 loops display a greater degree of variation.

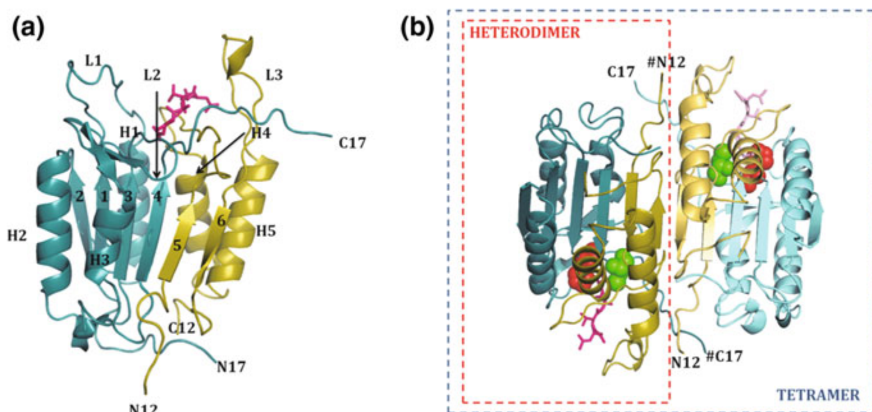


Fig. 2 Crystal structure of mature caspase-3 in complex with a tetrapeptide aldehyde inhibitor (PDB ID: 1CP3). **a** Cartoon representing mature caspase-3 heterodimer comprising the large subunit (p17) and the small subunit (p12). The 17 kDa large subunit (*deep teal*) comprises the first four core β-strands and helices 1–3 (present on one face of the protein), while the last two cores β-strands and helices 4–5 (present on the other face) form the 12 kDa small subunit (*olive*). The N- and C-termini of the large (p17) and small (p12) subunits are represented as N17-C17 and N12-C12, respectively. The β-strands are numbered from 1 to 6, the α-helices are represented by *upper-case letters* (H1–H5), and the loops are shown as L1–L3. **b** Quaternary structure of the active form of caspase-3. Cartoon representing each heterodimer (shown as *A* and *B*) comprising large and small subunits undergoes processing at the intersubunit linker (indicated by *dotted arrow*). Upon activation, two such heterodimers (*dark and light shades,* respectively) associate to form an active tetramer (*blue dotted box* comprising molecules *A* and *B*), with a continuous 12-stranded β-sheet that is sandwiched by α-helices. The #N17 and #C12 represent the N- and C-termini of one of the heterodimers (*dark-coloured dimer*). His-121 and Cys-163 of the catalytic dyad of caspase-3 are shown in *red* and *green,* respectively. In the tetramer, the two active sites are positioned at opposite ends of each molecule. The bound inhibitor Ac-DVAD-fmk is shown as stick model (*pink*). The figures are generated using PyMOL (DeLano Scientific, USA)

Typically, a caspase is dimeric and the intersubunit linker needs to be essentially cleaved for its activation. For effector caspases, equilibrium favours the inactive dimer, while for the activation of initiator caspases, dimerization seems to be the main challenge to overcome. Moreover, since the initiator caspases have longer intersubunit linkers than effector caspases, their cleavage in effector caspases is not necessary for activation. It would rather stabilize the active conformation of these caspases. In general, cleavage of the intersubunit linker occurs first soon after dimerization and is further followed by cleavage of the prodomain. Prior to cleavage, the intersubunit linker from one monomer occupies the dimer interface. Upon activation, cleavage of the intersubunit linker, i.e. loop L2, liberates the C terminus of large subunit (referred to as L2′) and the N terminus of small subunit (Fig. 2). The C-terminal portion of the linker L2′ now vacates the central cavity and rotates about 180° towards the active site. Herein, L2′ forms contact with L2, L3 and L4 from the opposite monomer and develop the so-called ‘*loop bundle contacts*’, which are known to stabilize the active site. This movement of L2′ out of the

dimer interface allows L3 to slide in towards the interface and form the substrate-binding pocket. Rotation of a key arginine on L2 from a solvent-exposed position into the interface allows its neighbouring residue, the catalytic cysteine, to assume its proper position for catalysis. Thus, for the construction of substrate-binding groove of the catalytic domain, a total of five loops seem to be important: loops L1 to L4 that come from one monomer and loop L2′ that is a part of the other monomer.

As mentioned earlier in the text, in addition to the catalytic domain, inflammatory and initiator caspases carry at least one or two copies of prodomains. These death domains have a globular architecture, wherein six amphipathic α-helices are arranged in an antiparallel α-helical bundle, with helices α1–α5 forming a conserved α-helical Greek key topology [45]. Despite the lack of obvious sequence similarity, these prodomains are found to share a common fold. However, few structural changes are also observed such as variation in lengths of α-helices and surface distribution of charged as well as hydrophobic residues. While the structural similarity strongly suggests a common evolutionary origin for the prodomains [49, 50], these subtle structural differences define their functional specificity. The primary function of prodomains is the recruitment of caspases to adaptor proteins and subsequent formation of intricate higher-order oligomers that are prerequisites for caspase activation in vivo. The mechanism for the same has been further elaborated in Sects. 4.1 and 4.2.

3.2 Active-Site Architecture

As described earlier, the binding pockets for P4–P3–P2–P1 positions in the substrate are known as S4–S3–S2–S1 subsites, respectively. Since caspases exhibit a very stringent specificity for P1 residue, their S1 pockets are found to be almost identical. S1 pocket is a deep, highly basic groove that is constructed of side chains of strictly conserved residues Arg (placed in the β1–α1 region of the L1 loop), Gln (positioned at the beginning of L2 loop) and Arg (placed at the end of L3 loop). In accordance to this, enzymes that exhibit open α/β structures generally have their active sites placed at positions within or close to the C-terminal end of central β-sheet in which loops originating in two adjacent β-strands lead to the opposite helical layers [45]. This general rule is shown to be valid for caspases, wherein the catalytic dyad residue His is located in the loop that connects strand β3 to the 'front' helix α3, while the neighbouring β1 strand is followed by the 'back' helix α1. Here, 'back' and 'front' refer to the standard caspase orientation. The loop comprising the β4–β5 linker contains the second catalytic dyad residue Cys. It protrudes from strand β4 (terminating in Gln) towards the front helical layer, while the one coming from the adjacent strand β5 leads to the back helix α4 (Fig. 2). Finally, the β5–α4 loop contains Arg (placed at the end of L3 loop) that is involved in substrate fixation. These series of interactions and coordinated movements lead to the effective interaction of S1 pocket residues with the catalytic dyad residues.

The S1 pocket is ideally designed to accommodate an aspartate side chain, and therefore, replacement of a glutamate residue at P1 leads to up to fourfold reduction in catalytic efficiency for cleavage of peptides [51]. Notably, mouse caspase-12 is a less catalytically efficient caspase, as it contains Lys at the position equivalent to the residue Arg [45]. S2 pocket is the only site to show a considerable alteration during substrate binding. In caspase-3 and caspase-7, it is formed by side chains of aromatic residues Tyr, Trp and Phe and can favourably accommodate small aliphatic residues such as Ala and Val. However, due to the substitution of Tyr → Val (Ala in caspase-2), the subsite S2 is found to be larger in inflammatory and initiator caspases [45]. This Val forms a large hydrophobic S2 pocket that is structurally tuned to accommodate residues with bulkier side chains such as His or Thr as P2 residues. Besides having a role in the S1 pocket, the Arg residue placed at the end of L3 loop also mediates main-chain–main-chain hydrogen bond interaction, through its guanidinium group, with the carboxylate of a P3 glutamate residue [45]. This justifies the preference for the glutamate residue in small peptide substrates and inhibitors [25, 52, 53]. Moreover, tighter binding of inhibitors containing a glutamic acid residue at position P3 is promoted by adjacent basic residues of the S3 pocket like Arg (placed in the β1–α1 region of the L1 loop) [45]. Although S1 and S3 subsites exhibit similar character, the chemical nature and geometry of S4 pocket do not appear to be conserved among all caspases [54]. This provides a rationale for the distinct macromolecular specificities and hence functions of the different caspase subclasses.

In the inflammatory caspase-1 as well as in caspase-4 and caspase-5, the S4 subsite is found to be an extended, shallow hydrophobic depression that prefers to accommodate indole moiety of large aromatic side chains such as Trp residue [34]. On the contrary, caspase-2, caspase-3 and caspase-7 possess a narrow pocket containing several side chains that serve to stabilize the aspartic acid residue present in P4 position. In caspase-3, this P4 aspartic acid seems to be required for efficient hydrolysis, as the replacement of this residue with any other amino acid results in >100-fold decrease in k_{cat}/K_m [54]. The S4 subsites of caspase-8 and caspase-9 occupy intermediate positions and show preference for small hydrophobic Val or Leu residue [33]. However, caspase-8 is shown to equally tolerate both small hydrophobic [55] and acidic residues like Ile and Asp, respectively, at P4 position [56]. An occupied S5 pocket is an exceptional and additional requirement in caspase-2 so as to bring about efficient substrate cleavage. The presence of a P5 residue confers 35-fold increase in catalytic efficiency, and this may be due to a better burial of the P4 aspartate [57]. This extended specificity has also been well understood from the high-resolution structure of caspase-2 in complex with acetyl-Leu-Asp-Glu-Ser-Asp-CHO [57]. The P5 Leu side chain occupies a small groove formed by two residues of the hydrophobic S5 pocket, Pro and the phenyl moiety of Tyr. This explains the preferential accommodation of small hydrophobic residues at this position in caspase-2. The primed subsites (P1′–P4′), however, seem to be less restrictive than the S1–S4 pockets and have been recently characterized crystallographically [58]. Using fluorescent peptidyl substrates, an apparent degree of discrimination for small residues (Gly, Ala, Ser) at position P1′ has been

observed in several caspases. The large aromatic side chains (Phe/Tyr) were also found to be well tolerated, whereas polar residues or proline were prohibited [51]. Thus, these investigations verified and extended the observations done on the P1′ substrate specificity of caspase-1 and caspase-4 [52, 59].

In summary, a consensus caspase substrate sequence can be defined as (W/Y/V/L/D)-E-X-D↓-Φ with P4-P3-P2-P1↓-P1′. Herein, X represents any amino acid, ↓ represents the scissile bond, and Φ stands for a small uncharged residue. Knowledge of this inherent specificity that is exhibited by caspases has led many investigators to construct peptide-based substrate reporters for measuring caspase activity, both in academic and in commercial settings. Such reporter-based substrates typically constitute a tetrapeptide sequence matching a preferred caspase consensus coupled to a fluorogenic probe at P1 position. The probe is released upon cleavage and produces a fluorescent signal. Although these methods are useful to individually characterize purified caspases in vitro, they cannot distinguish between caspases in a complex milieu, as significant overlaps also exist in the specificity of caspases.

3.3 Proposed Catalytic Mechanism

Experimental investigations using time-resolved crystallography or any detailed theoretical calculations of substrate cleavage have not yet been reported for caspases. However, the similarity of chemical groups involved in catalysis and crystal structures of active caspases bound to substrate-like inhibitors have provided valuable information [45]. It has been found that caspases have a course of catalytic reaction that is similar to what is generally observed in serine proteases and other families of cysteine proteases. As described earlier, caspases contain a catalytic dyad comprising Cys and His. On the basis of the accepted catalytic mechanism for cysteine proteases, a mechanism of catalysis has been proposed for caspases [60]. Herein, the catalytic histidine first abstracts a proton from the nucleophilic catalytic cysteine. The anionic sulphur group of this deprotonated Cys now attacks the carbonyl oxygen of P1 residue of the substrate and forms a covalent tetrahedral intermediate with the peptide substrate. This intermediate state is stabilized by imidazole moiety of His, which in turn protonates α-amino moiety of the peptide leaving group and triggers release of the C-terminal part of the peptide. The deprotonated Nδ atom of His now abstracts a proton from water molecule that further attacks the thioester carbonyl, resulting in the formation of a second tetrahedral intermediate. This is followed by the cleavage of the covalent bond formed between carbonyl carbon and sulphur of Cys. Finally, hydrolysis frees the N-terminal part of the peptide and reprotonates catalytic histidine, and this regenerated enzyme can now start up a new proteolytic cycle. The proposed mechanism thus practically reverses roles of the '*classic oxyanion hole*' and His [45].

However, there arise some controversies with the proposed mechanism. Unlike most proteases, a 6–7 Å distance between the catalytic dyad residues is found to be larger in caspases and this makes the occurrence of a direct hydrogen transfer

slightly unlikely [26]. Molecular dynamics simulation studies show that these residues cannot exist as a charged pair prior to catalysis [61]. This clearly indicates that at such an unusual Nδ1/Nε2–Sγ distance, His cannot accept a thiol proton of Cys. It also implies that Cys may not be pre-polarized, but the nucleophile may develop along the reaction coordinate [26]. The step takes place in unity with the pH optima of all caspases lying in a narrow range of 6.8–7.4 [62]. Further, several line of evidences raise an intriguing question on the presence of a third catalytic residue in caspases. Many investigators, including Wilson and colleagues, proposed a possible participation of the carbonyl oxygen of Arg residue to be an important element of the caspase-1 catalytic machinery [23, 24, 55]. Herein, the oxygen atom accepts a hydrogen bond from the Nε atom of His and might either affect the basicity of imidazole moiety and/or orientate it similarly to the side chains of catalytic Asn/Asp residues in clan CA cysteine peptidases and the carboxylate of Asp in trypsin-like proteases. However, the participation of this third catalytic residue in caspase-mediated catalysis still remains as a disputed topic [45]. Many other investigators, including Miscione and co-workers as well as Sulpizi and his group, have also proposed reaction schemes that differ from the classical one in some or the other step [27, 61]. Thus, rigorous quantum mechanics or molecular dynamics calculations along with structural and mutagenesis studies are required to establish a universal caspase catalytic mechanism.

4 Cellular Pathways Controlling Caspase Activation

There arises a need to control active caspases via stringent mechanisms as they are responsible for causing rapid cell death. These mechanisms primarily include synthesis of caspases as inactive zymogens, involvement of highly evolved upstream regulatory pathways that control their activation, and presence of endogenous inhibitors. The apoptotic signalling pathways that lead to the processing of caspase zymogens are subdivided into two major categories: extrinsic and intrinsic pathways of apoptosis.

4.1 The Extrinsic Pathway of Apoptosis

As stated earlier, this pathway includes the cell surface sensor-directed apoptotic signals involving the **D**eath-**I**nducing **S**ignalling **C**omplex (DISC) and is usually exemplified by the signalling of the death receptor family. Belonging to a family of type I transmembrane proteins, the **D**eath **R**eceptors (DRs) are characterized by the presence of multiple cysteine-rich repeats in their extracellular region that is required for recognition of their specific ligands (Table 2). Few examples include TNF-R1 (**T**umour **N**ecrosis **F**actor **R**eceptor **1**), Fas (also known as CD95 or APO-1), DR3, DR4 (TRAIL-R1), DR5 (TRAIL-R2) and DR6. These DRs are also known to have the protein–protein interaction module known as **D**eath **D**omain (DD)

Table 2 Extrinsic pathway molecules and their functions

Group	Protein	Acronym	Alternate names
Receptors	Fatty acid synthetase receptor	FasR	Fas receptor, TNFRSF6, APT1, CD95
	Tumor necrosis factor receptor 1	TNFR1	TNF receptor, TNFRSF1A, p55 TNFR, CD120a
	Death receptor 3	DR3	TNFRSF12, Apo3, WSL-1, TRAMP, LARD, DDR3
	Death receptor 4	DR4	TNFRSF10A, TRAILR1, APO2, KILLER
	Death receptor 5	DR5	TNFRS10B, TRAIL-R2, TRICK2, CHE1
Extracellular ligands	Fatty acid synthetase ligand	FasL	Fas ligand, TNFSF6, Apo1, apoptosis antigen ligand 1, CD95L, CD178, APT1LG1
	Tumor necrosis factor alpha	TNF-α	TNF ligand, TNFA
	Apo2 ligand	Apo2L	TRAIL/TNFSF10
Adaptor proteins	Fas-associated death domain	FADD	MORT1
	TNF receptor-associated death domain	TRADD	TNFRSF1A associated via death domain
	Receptor-interacting protein	RIP	RIPK1
Decoy protein	FLICE-inhibitory protein	c-FLIP	Casper, I-FLICE, FLAME-1, CLARP, MRIT
Initiator protein	Cysteinyl aspartic acid protease-8	Caspase-8	MACH-1, MCH5, ICE-LAP6, Mch6, Apaf-3

Adapted from [63]

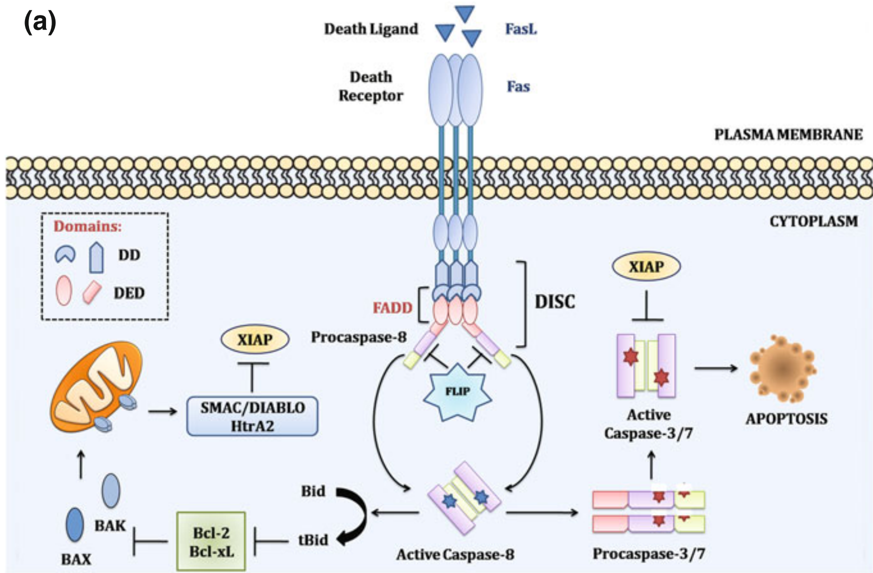
in their cytoplasmic tails. Similar to the prodomains in caspases, these DDs belong to the Death Domain superfamily and are involved in initiating assembly of large macromolecular complexes through a series of homotypic interactions.

When the cell surface sensors detect extracellular danger signals through ligand binding, clustering of the receptors recruits a DD-containing adaptor molecule, FADD (**F**as-**A**ssociated protein with **D**eath **D**omain) through DD–DD interactions (Fig. 3). For Fas and TRAIL (**T**umour necrosis factor-**R**elated **A**poptosis-**I**nducing **L**igand) receptors, the adaptor protein FADD is recruited to DISC through its C-terminal DD. TRAIL-mediated extrinsic pathway involves five TRAIL receptors, TRAIL R1-R4 and osteoprotegerin (OPG) [63]. The first two promote cell death in different tumour cells, while TRAIL R3 and TRAIL R4 are devoid of cytoplasmic death domains (DD) and act as '*decoy*' proteins with significantly reduced proapoptotic activities. OPG, however, with a lower binding affinity for TRAIL 1 and 2 ligands has limited effect at physiological temperature [64]. Generally, the binding of the ligand induces receptor multimerization, and this exposes another

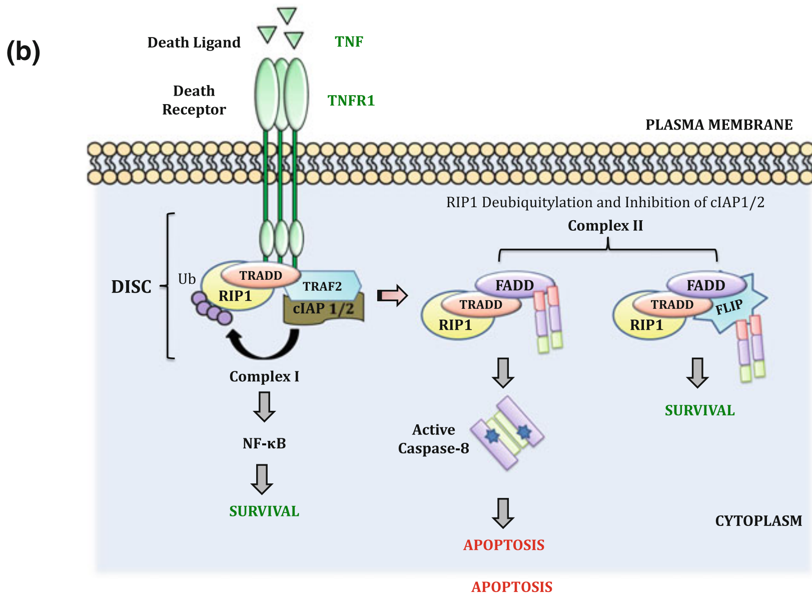
death-fold domain (DED) in FADD. The exposed DEDs of FADD in turn interact with the N-terminal DEDs of monomeric procaspase-8 molecules (Fig. 3a). The complex, so formed at the cytoplasmic tail of engaged DR, now represents a caspase activation platform called DISC, and it may include multiple adaptor molecules. Further, recruitment and oligomerization of caspase-8 in DISC cause its autocatalytic cleavage in between the large and small subunits as well as between large subunit and prodomain. The first cleavage stabilizes the active dimer that is required for homodimer activity in this pathway, while the second cleavage releases it from DISC [65]. The formation of DISC increases the local concentration of monomeric initiator caspase zymogens through proximity induced by the adaptor molecules [66]. The active caspase-8, so formed, further cleaves and activates the downstream or executioner procaspases, mainly procaspase-3 and procaspase-7. The activated caspase-3 cleaves **X**-linked **I**nhibitor of **A**poptosis **P**rotein (XIAP), which is the most widely expressed endogenous inhibitor that plays an important role in regulating cell survival (Fig. 3a). This event also creates a positive feedback loop of self-activation, thereby synergistically enhancing the cell death process [67, 68]. Apart from sharing similarities in their physiological role, active caspase-7 differs in significant ways from caspase-3. It specifically cleaves several substrates that are not cleaved by caspase-3, and its activation requires caspase-1 inflammasomes under inflammatory conditions [69]. Subsequently, these effector caspases bring about proteolytic cleavage of important cellular proteins, thus culminating into apoptosis [70, 71]. TRAIL is also capable of inducing apoptosis through an intrinsic pathway, which gets activated when mitochondrial proapoptotic molecules Smac/DIABLO or HtrA2 restore caspase-3 activity by relieving inhibitory effect of XIAP [72, 73]. Smac/DIABLO (**S**econd **m**itochondria-derived **a**ctivator of **c**aspases/**D**irect **IA**P-**B**inding protein with **Lo**w pI) and HtrA2 (**H**igh-**t**emperature **r**equirement protease **A2**) both are mitochondrial molecules that are released into the cytosol upon apoptotic induction. In addition to this, TRAIL weakly induces NF-κB pathway with the help of adaptor proteins Rip and TRAF-2 [74].

In case of TNFR1 (**T**umour **N**ecrosis **F**actor **R**eceptor-1) receptor, the signalling pathways engaged downstream are more complex and its activation does not necessarily lead to cell death but may potentially lead to different outcomes (Fig. 3b). Herein, a different DD-containing adaptor TRADD (**TNFR**-**A**ssociated **D**eath **D**omain) is first recruited upon the interaction of ligand TNFα with TNFR1 and induces TNFR1 ligation. Subsequently, this leads to the formation of two distinct signalling complexes [75].

(1) *Complex I formation*: Since TRADD does not directly bind to or activates caspase-8, the ligand instead acts as a membrane-bound scaffold that recruits additional signalling molecules such as a serine/threonine-protein kinase RIP1 (**R**eceptor-**I**nteracting **P**rotein-1) and ubiquitin ligases, TRAF2 and cIAP1/2 (**c**ellular **I**nhibitor of **A**poptosis **P**rotein) (Fig. 3b). This event leads to the formation of a signalling platform known as Complex I. The assembly and non-degradative ubiquitylation of its components by molecules such as cIAPs and LUBAC (**L**inear **Ub**iquitin **A**ssembly **C**omplex) culminate in the activation



(a)
Death Ligand
FasL
Death Receptor
Fas
PLASMA MEMBRANE
CYTOPLASM
Domains:
DD
DED
FADD
DISC
Procaspase-8
XIAP
SMAC/DIABLO
HtrA2
FLIP
XIAP
Active Caspase-3/7
APOPTOSIS
Bid
tBid
BAK
BAX
Bcl-2
Bcl-xL
Active Caspase-8
Procaspase-3/7



(b)
Death Ligand
TNF
Death Receptor
TNFR1
PLASMA MEMBRANE
RIP1 Deubiquitylation and Inhibition of cIAP1/2
Complex II
DISC
Ub
TRADD
RIP1
TRAF2
cIAP 1/2
FADD
TRADD
RIP1
FADD
TRADD
FLIP
RIP1
Complex I
NF-κB
SURVIVAL
Active Caspase-8
SURVIVAL
APOPTOSIS
CYTOPLASM
APOPTOSIS

◀ **Fig. 3** Receptor-mediated extrinsic cell death pathway. The death receptor signalling pathway is triggered when death ligands such as FasL, TNF or TRAIL engage with their cognate receptors—Fas, TNFR, or DR5, respectively. **a** Ligation initiates the recruitment of adaptor proteins, FADD or TRADD (through DD–DD interactions), towards Fas and TRAIL receptors, respectively. This further recruits zymogenic initiator procaspase-8 (through DED–DED interactions) and forms the caspase activation platform called DISC. Activation of caspase-8 promotes maturation of executioner caspase-3, caspase-6 and caspase-7 that further bring about substrate proteolysis and cell death. On the other hand, it crosstalks with the mitochondrial cell death pathway by cleaving the proapoptotic Bid protein. The truncated Bid (tBid) inhibits the antiapoptotic Bcl-2 and Bcl-xL proteins, which prevent the oligomerization of proapoptotic Bax and Bak proteins. Smac/DIABLO and HtrA2 are the mitochondrial proteins that act as XIAP antagonists and promote apoptosis. **b** The TNFR receptor ligation with TNF-α leads to further recruitment of the adaptor protein, TRADD, and the kinase, RIP1. The death-inducing complex (DISC), so formed, further attracts additional partners such as the ubiquitin ligases, TRAF2 and cIAP1/2, which catalyse the non-degradative ubiquitylation of RIP1. Furthermore, it stabilizes a prosurvival and proinflammatory signalling platform called complex I. However, inhibition of cIAP1/2 or activation of deubiquitylases destabilizes complex I and allows subsequent release of TRADD and RIP1 into the cytosol. These proteins, in turn, recruit FADD as well as caspase-8 and promote caspase activation. Expression of FLIP inhibits the activity of both complexes, as it heterodimerizes with caspase-8 and simultaneously blocks apoptosis

of NF-κB signalling pathway followed by an inflammatory response rather than causing cell death [76].

(2) *Complex II formation*: TNFR1 ligation can lead to cell death only when ubiquitylation of RIP1 is compromised by inhibition of ubiquitin ligases cIAP1/2 or through direct de-ubiquitylation of RIP1. TRADD and RIP1 are then released from TNFR1 to form a series of dynamic cytosolic signalling platforms that is collectively known as complex II. This complex leads to apoptosis when cytosolic TRADD recruits FADD (via DD–DD interaction), which in turn binds to and activates caspase-8 similarly to the DISC (Fig. 3b) [77, 78]. However, when activation of NF-κB induces the expression of caspase-8-like protein FLIP (**F**LICE-**L**ike **I**nhibitory **P**rotein) [79], apoptosis does not proceed upon TNFR1 ligation. FLIP is a molecule that inhibits caspase-8 activation. It possesses a domain structure similar to that of caspase-8 and preferentially binds to FADD (via DED–DED interactions) as well as a monomer of caspase-8. Thus, the caspase-8–FLIP heterodimer is catalytically active [80, 81], but does not promote apoptosis probably due to rapid degradation of the complex [82]. The FLIP expression and assembly of complex II under conditions of active NF-κB signalling therefore lead to a survival outcome.

This overall balance of complexes I and II ultimately decides the fate of life or death; however, the process still remains elusive.

The outcome of DR-mediated activation of caspase-8 also depends on cell types, which are categorized on the basis of their dependency on the mitochondria for the induction of Fas/TRAIL death receptor-mediated apoptosis. In certain cell types, known as type I cells, it initiates apoptosis directly by cleaving and thereby

activating downstream executioner caspase-3 and caspase-7 [83]. The apoptotic signal amplification through the existence of such caspase cascades becomes essential, as initiator caspases are unable to complete the execution of cell death on their own. In addition, such pathways may enable cells to maintain a stronger control over the cellular suicide mechanism at multiple points. In type II cells, where apoptotic signalling is initiated by an insufficient amount of active caspase-8 or downstream caspases, death receptor signalling must be amplified by a mitochondrial amplification step to induce efficient cell death [84]. In this case, caspase-8 cleaves cytosolic BH3-only proapoptotic Bcl-2 family member Bid, and the truncated Bid (tBid) then translocates to mitochondria, thus promoting the mitochondrial cell death pathway (described in Sect. 4.2.1) [85, 86]. The event subsequently promotes **M**itochondrial **O**uter **M**embrane **P**ermeabilization (MOMP), which results in the release of intracellular **I**nhibitor of **A**poptosis **Pr**oteins (IAPs) antagonists—Smac and HtrA2. The IAPs are known to block executioner caspase function unless they are suppressed by proteins released from mitochondria. Smac and HtrA2 neutralize XIAP and allow apoptosis to proceed. Through the knockout studies in mice, thymocytes were reported to be type I, while liver cells were type II cells [87], and these cells differ well in their IAPs content [88, 89].

4.2 Intracellular Sensor-Mediated Pathway (Intrinsic Pathway)

Being another major category of apoptotic signalling, this pathway is activated by stimuli such as DNA damage and cytotoxic drugs which act inside the cell. It is very similar to type II cell death receptor mechanism in terms of its requirement for signal amplification through mitochondrial damage. However, since cells possess multiple means of targeting mitochondria for the release of apoptogenic factors, this category could be further subdivided into following discrete signalling pathways that influence the mechanism of caspase activation in their distinct ways.

4.2.1 Mitochondrial Pathway Activation Through Apoptosome

This form of intrinsic pathway depends on various factors released from the mitochondria upon its activation by a number of stimuli. These include growth factor deprivation, cytoskeletal disruption and build up of unfolded proteins, DNA damage and so forth. The apoptotic stimuli consequently trigger **M**itochondrial **O**uter **M**embrane **P**ermeabilization (MOMP), a phenomenon that induces release of all soluble proteins present in the mitochondrial intermembrane space into the cytosol. MOMP is tightly regulated by myriads of Bcl-2 family proteins. These include proapoptotic effector proteins, Bax and Bak, that are necessary and sufficient for MOMP; the antiapoptotic Bcl-2, Bcl-xL and Mcl1 proteins that block MOMP; and the BH3 (**B**cL-2 **H**omology domain)-only proteins, Bid, Bim, Bad and Noxa, which activate the proapoptotic effectors and/or neutralize antiapoptotic Bcl-2 proteins [90]. The activation of one or more members of the BH3-only

protein family above a certain critical threshold helps in overcoming the inhibitory effect of antiapoptotic Bcl-2 family. On the other hand, proapoptotic effectors—Bax (**B**cl-2 **a**ssociated **X** protein) and BAK (**B**cl-2 homologous **A**ntagonist **K**iller)—are directly responsible for the loss of mitochondrial outer membrane integrity (Fig. 4). Upon activation, they form large oligomers that insert into the mitochondrial outer membrane, disrupt it [91–94] and initiate the release of cytochrome c [95]. Apart from cytochrome c, the other key players of this intrinsic pathway include Apaf-1 (**A**poptotic **p**rotease-**a**ctivating **f**actor-1) and initiator caspase, caspase-9. Both of these proteins exist in a resting cell as cytosolic, inactive monomers. Apaf-1 contains an N-terminal CARD, an expanded nucleotide-binding domain NACHT and a C-terminal WD40 repeat domain (Trp-Asp sequence repeating 40 times). The binding of cytochrome c to WD40 domain of Apaf-1 monomer triggers hydrolysis of the Apaf-1 cofactor, dATP (deoxy-adenosine triphosphate) to dADP (deoxy-adenosine diphosphate) [96]. A conformational change subsequent to this interaction exposes a nucleotide-binding site in the NACHT domain of Apaf-1 where dATP binds. This induces a second conformational change in Apaf-1 that exposes both its oligomerization and CARD domains. Seven such activated Apaf-1 monomers then assemble into an oligomeric complex that contains the CARDs in the centre. These CARDs together then recruit and interact with the prodomain (CARD) of caspase-9 (Fig. 4) [97, 98]. The wheel-shaped multimeric protein complex, so formed, is termed as '*apoptosome*', which comprises Apaf-1, cytochrome c as well as the cofactor dATP/ATP. At the centre of the apoptosome, interaction of the exposed CARDs brings inactive caspase-9 monomers into close proximity for activation and autoprocessing [99]. The lifetime of an active apoptosome is directly proportional to the amount of unprocessed caspase-9 present [100], since caspase-9 sustains the catalytic activity in this bound state [26, 101, 102]. This activated initiator protease further processes other downstream caspases, caspase-3 and caspase-7, to carry out apoptosis (Fig. 4).

4.2.2 Caspase-2 Activation Through PIDDosome

Being one of the earliest caspases to be discovered, caspase-2 is the most highly conserved initiator caspase with a long prodomain. Upon treatment with chemotherapeutic drugs, caspase-2-deficient germ cells and oocytes have been found to become resistant to cell death [103], which implicates it to be involved in the process of oocyte apoptosis [104] and degenerating neurons [105–107].

In response to apoptotic stresses like heat shock, cytoskeletal disruption, metabolic perturbation and DNA damage, inactive procaspase-2 monomers are known to induce oligomerization and get activated by induced proximity (mechanism described in Sect. 5.2) [108]. Procaspase-2 oligomerization is primarily mediated by the adapter protein RAIDD (**R**IP-**A**ssociated **I**CH-1 homologous protein with a **D**eath **D**omain). RAIDD was originally identified as a protein associated with RIP, the DD-containing Ser/Thr kinase involved in TNF signalling. It has both an N-terminal CARD and a C-terminal DD and acts as a bridge between caspase-2 and the adapter molecule, PIDD (**p**53-**I**nduced **D**eath **D**omain) (Fig. 5a). PIDD is a death domain-containing protein that is involved in p53-dependent apoptosis. The

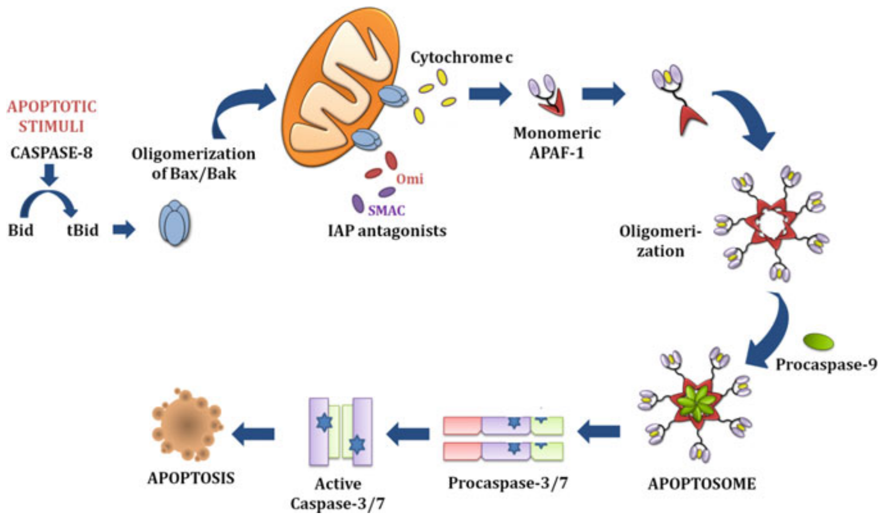


Fig. 4 Mitochondria-mediated intrinsic cell death pathway. Activation of caspase-8 through the extrinsic pathway promotes the truncation of proapoptotic molecule, Bid. The truncated version of this protein (tBid) induces oligomerization of proapoptotic effector proteins such as Bax and Bak. The large oligomers, so formed, then get inserted into the mitochondrial outer membrane, and the subsequent membrane permeabilization induces the efflux of cytochrome c and other antiapoptotic proteins such as HtrA2 and Smac/DIABLO. The released cytochrome c binds to the inactive monomer of Apaf-1, which induces a conformational change and promotes oligomerization of the latter to further form a heptameric wheel-like structure called '*apoptosome*'. This complex later recruits and activates initiator procaspase-9 through the interaction of exposed CARDs. Activated caspase-9 then propagates a proteolytic cascade by cleaving effector caspase-3 and caspase-7 to trigger apoptosis

activation platform is formed when RAIDD binds to procaspase-2 via CARD–CARD interaction that is being regulated through phosphorylation of the caspase (elaborated in Sect. 7.5). Procaspase-2 bound RAIDD molecules further form a complex with the adapter protein PIDD through adapter-mediated homotypic interaction.

PIDD is a 100 kDa protein that is composed of seven N-terminal LRRs (**L**eucine-**R**ich **R**epeats), two ZU-5 (**ZO**-1 and **U**nc**5**-like) domains, a putative oligomerization domain UPA (uncharacterized protein domain in **U**NC5, **P**IDD and **A**nkyrin) and a C-terminal death domain (DD). It contains two intein regions that promote its autoproteolytic processing in cells to generate three fragments. PIDD-N (48 kDa) contains the N-terminal LRRs and the proximal ZU-5 domain; PIDD-C (51 kDa) comprises the distal ZU-5 domain, UPA and DD; and PIDD-CC (37 kDa) lacks the ZU-5 domain but retains an intact UPA and DD [109]. The first cleavage produces PIDD-C that forms a complex with RIP1 and NEMO (a component of NF-κB-activating kinase complex), which in turn leads to NF-κB activation [109, 110]. The second cleavage generates PIDD-CC that binds to RAIDD via DD–DD interaction [109, 111]. The PIDD–RAIDD–procaspase-2 large protein complex, so

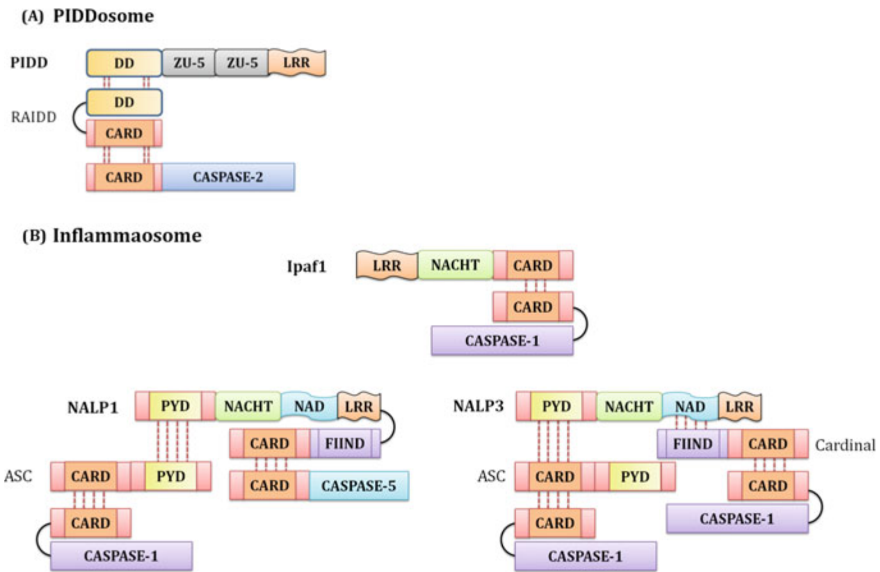


Fig. 5 Caspase activation through PIDDosome and inflammasome. **a** PIDDosome structure. **b** Inflammasome structure. The *red dotted lines* indicate the interaction between the domains of different protein molecules involved in forming the PIDDosome and inflammasome. The acronyms for the domains involved have been described in the text

formed, has been shown to mediate caspase-2 activation in response to genotoxic stimuli and is together termed as PIDDosome. The crystal structure of PIDDosome has revealed the presence of multiple PIDD and RAIDD subunits [112, 113], and the structure resembles the CD95–FADD complex that is involved in procaspase-8 activation.

Caspase-2 activation can also occur through a mechanism that involves p53-dependent CD95 upregulation and recruitment of caspase-8 to the DISC complex. This coordination of caspase-2 and caspase-8 cleaves Bid and subsequently activates mitochondrial apoptosis [114, 115]. However, caspase-2 is also involved in other divergent functions such as protection against DNA damage [116] or cancer development [117], and its activation has been found to occur even in the absence of PIDD in some cases [118, 119].

4.2.3 Proinflammatory Caspase Activation Through Inflammasome

Apart from being proapoptotic factors, several caspases also act as critical mediators of innate immune responses as mentioned earlier. These proinflammatory caspases are known to regulate both cytokine processing and inflammatory ignalling. In humans, caspase-1, caspase-4, caspase-5, and caspase-12 are included in the inflammatory subset, whereas the same function is served by caspase-1, caspase-11, and caspase-12 in mice. The genes encoding the human

proinflammatory caspases are located in close proximity on chromosome 11q22, while the murine proinflammatory caspases are clustered on chromosome 9A1 in the following order from the telomere: caspase-1, caspase-11 and caspase-12. The organization of the syntenic region in humans is similar, except that the location of murine caspase-11 gene is replaced by the genes of human caspase-5 and caspase-4 [120]. Similar to their proapoptotic counterparts, these caspases have large prodomains containing CARDs and are produced as inactive zymogens in resting cells. The procaspases are activated only after cellular stimulation through infectious agents (e.g. viruses, bacteria and fungi) or inert substances (e.g. uric acid crystals, calcium phosphate crystals, alum and asbestos) [121]. These stimuli induce inflammation through the formation of a cytosolic caspase activation platform known as inflammasome.

The external stimuli are initially recognized by a family of **P**attern-**R**ecognition **R**eceptors (PRRs), including TLRs (**T**oll-**L**ike **R**eceptors) and NLRs (**N**ucleotide-binding domain, **L**eucine-rich repeat-containing **R**eceptors) proteins. PRRs recognize the conserved PAMPs (**P**athogen **A**ssociated **M**olecular **P**atterns), which are unique to each pathogen, and therefore, are essential molecular structures for pathogen survival and to regulate innate immune responses. In a resting cell, the NLRs exist as monomers and are held in an inactive conformation. Different NLR-driven inflammasomes contain several other NLR members [33], which mainly include three subfamilies of proteins:

(1) *IPAF (or NLRC4) subfamily*: IPAF contains domains like CARD, NACHT and the C-terminal LRR domain. It associates specifically with CARD of caspase-1 through CARD–CARD interaction, while the NACHT domain induces oligomerization and promotes proximity of the caspases. The LRR domain is probably involved in ligand sensing (Fig. 5b).
(2) *NAIP subfamily*: This NLR subfamily of proteins shares highest sequence similarity with IPAF in its NACHT and LRR domains. However, NAIP harbours three N-terminal-BIR domains (**B**aculovirus **I**nhibitor of apoptosis **R**epeats) instead of CARD, and the protein was originally proposed to interact with IPAF. This suggests that it may be a part of the same caspase-1-activating complex [122, 123] that includes both IPAF and NAIP.
(3) *NALP subfamily*: NLRs like NALP1, NALP2 and NALP3 were shown to be the central scaffold of inflammasomes. These proteins harbour NACHT and LRR like those present in IPAF and NAIP, but they are additionally characterized by an N-terminal **Py**rin **D**omain (PYD). The PYD of NALPs is known to interact with the N-terminal PYD of the adapter protein ASC (**A**poptosis associated **S**peck-like protein containing **C**ARD). ASC also contains a C-terminal CARD that binds and further recruits the inactive inflammatory procaspase, typically procaspase-1. The inflammasome, so formed, may also further recruit caspases such as caspase-5 via the C-terminal CARD of NALP1 or another caspase-1 molecule via the C-terminal CARD domain of protein CARDINAL that is another component of the NALP2/3 inflammasome (Fig. 5b).

Towards the end of the pathway, activated caspase-1 processes proinflammatory cytokines such as pro-IL-1β, pro-IL-18 and pro-IL-33 that eventually leads to inflammation. The repertoire of caspase-1-activating molecular machines thus seems to be potentially very complex, as there exists a differential requirement of IPAF, ASC and NALP3 in sensing diverse stimuli activating caspase-1 [124, 125].

Besides the aforementioned caspase-dependent pathways, multiple non-caspase proteases are also shown to process and activate caspases directly. For example, upon exposure to endoplasmic reticulum stress, the Ca^{2+}-activated protease, m-calpain can process caspase-12 [126], whereas the lysosomal protease cathepsin B was shown to process caspase-1 and caspase-11 in vitro [127, 128]. The serine protease granzyme B directly activates caspase-3 and is critical for the killing of virally infected cells by **c**yto**t**oxic **l**ymphocytes (CTL) [129].

In conclusion, cells possess a vast array of caspase activation cascades that respond differently and specifically to a variety of cytotoxic and inflammatory stimuli.

5 Molecular Basis of Caspase Activation

Apart from a few special cases, caspase processing is usually mediated by caspases themselves. This was supported by early observations made in bacteria, wherein simple overexpression of wild-type caspases, but not active-site mutant zymogens, resulted in spontaneous activation of these active caspases [62, 130]. Likewise, in the highly purified samples of caspase-1, cleavage between its large and small subunits was assisted with a concomitant increase in activity [131–133]. The concept that the processing sites of individual initiator caspases often conform to their own substrate specificities also provided a mechanistic explanation for their autoactivation [25]. This observation in turn correlated with the presence and importance of residual activity in caspase zymogens [133]. Initiator caspases such as caspase-8 and caspase-9 are known to be quite active as zymogens, whereas the executioner caspase-3 is the one that acts as a 'true' zymogen, since it is much less active [66]. Thus, such differences in caspase zymogen activity highlight the ability of initiator caspases to serve as proximal responders to proapoptotic signals due to their high propensity towards autoactivation. It also necessitates the activation of executioner caspases by their predecessors.

5.1 Activation of Effector Caspases: Transactivation Mechanism

The process of executioner caspase activation is generally termed as '*transactivation*', since it is mediated *in trans* by another heterologous caspase. As demonstrated through in vitro experiments, the upstream or apical initiator caspase-8 and caspase-10 that possess long prodomains can efficiently cleave and activate

executioner procaspase-3 and procaspase-7, which either have or lack short prodomains. These downstream caspases further serve in amplifying the death signal and cause cell demise. As discussed in the mitochondrial intrinsic pathway, caspase-9 cleaves and activates caspase-3 and caspase-7. Activated caspase-3 further cleaves caspase-2 and caspase-6 as well as provides a feed-forward amplification loop by cleaving procaspase-9 [134]. Caspase-7 has no significant role in sensitivity to intrinsic cell death. In contrast, caspase-3 can inhibit ROS production and is the effector caspase necessary for efficient cell killing [135]. According to a model proposed for the maturation of procaspase-3 dimer, it is suggested that the zymogen exists as a dimer in vivo and the removal of the N-terminal propeptide is neither required nor beneficial for its activation [32]. Thus, in contrast to the caspases containing long prodomains, the propeptide of caspase-3 does not appear to be involved in dimerization. However, a research work suggested that apart from propeptide cleavage, additional unknown structural determinants may also play a critical role in the activation process of caspases such as caspase-6 [136].

On the basis of biochemical and structural characterization, active and zymogenic forms of caspase-7 were found to be homodimers [137, 138]. Upon activation, the overall structure of the enzyme undergoes very minor changes, except for the rearrangement of crucial catalytic loops L2, L3 and L4, which undergo dramatic conformational transitions. In procaspase-7, these three critical loops stay away from each other in a conformation prohibitive to substrate binding and this provides a molecular mechanism that explains its zymogenicity [19]. During the activation process, cleavage at Asp rescues L2′ loop from the neighbouring small subunits in caspase-7 homodimer and allows it to form a tight ‘active bundle’ with L2 and L4 loops. This initial cleavage in turn produces 90° rotation of L2 and makes catalytic Cys accessible to the solvent. The subsequent processing steps thus become dispensable for the full activation of caspases. However, this activation mechanism just primes caspase-7 for substrate binding. The comparison of the active caspase-7 with its inhibitor-bound structure suggests that the substrate-binding groove in the former is still not properly formed, as L2′ loop remains in the closed conformation [137]. Upon substrate/inhibitor binding, the formation of the ‘*active bundle*’ is completed and it triggers 180° flipping of L2′ loop. This subsequently results in its upward movement towards L1–L4, which completes the active bundle formation. Therefore, binding of the substrate by caspases is a dynamic ‘induced-fit’ process [48]. This information signifies that caspase processing and substrate binding lead to continuous sequence of conformational changes that eventually guide the ‘loop bundle’ formation and proper arrangement of the active centre [137, 138]. This unique mechanism thus emphasizes the importance of dimerization for executioner caspase activity.

5.2 Activation of Initiator Caspases: Allosteric Regulation and Induced Proximity Mechanism

On the basis of all crystal structures available for caspases, it was believed that they are activated by proteolytic cleavage within their linker region [134]. However, the initiator caspase-8 and caspase-9 are unique among all the other caspases, as studies reveal that proteolytic cleavage is neither sufficient nor necessary for their activation [26, 47, 101]. In contrast to the zymogens of effector caspase-3 and caspase-7 that are already present as dimers in their latent forms, the initiator caspases reside as monomers and require dimerization to assume an active conformation. The precise detail of initiator caspase activation, however, still remains elusive. Since there is no proteolytic enzyme upstream of these caspases, the zymogens tend to exhibit high intrinsic residual activities and low zymogenicity values (i.e. ratio of activity of the cleaved to the uncleaved form). The zymogenicity for caspase-8 and caspase-9 were found to be of at most 100 or 10, respectively, in contrast to the values of >10,000 for caspase-3 [134]. This also raises an intriguing question as to how such activity is regulated in living cells.

This intrinsic zymogenicity feature of initiator caspases is very much similar to the mechanistically unrelated serine proteases of the blood clotting and fibrinolytic cascades. However, the initiator caspases vary from other serine proteases due to the cofactor induced oligomerization that result in the activation of these upstream proteases [134]. Unlike effector caspases, cleavage at the intersubunit linker in initiator molecules is dispensable for its activity; however, this structural rearrangement might provide stability to the protease. This oligomerization is adequate enough to trigger the processing of inflammatory and initiator procaspases, thus establishing the central postulate of the induced proximity model.

The *Induced Proximity Model* was first proposed in 1998, which states that the initiator caspases autoprocess themselves when brought into close proximity of each other [66]. Being the most upstream caspase in the extrinsic pathway, activation of caspase-8 is brought about by the adaptor-mediated multimerization. Even though this zymogen possesses significant activity, subsequent proteolytic cleavage is still required for its full activation [139] and an '*induced proximity*' mechanism has been suggested for the same. In this model, FADD-mediated trimerization increases the local concentration of caspase-8 zymogen and brings about intermolecular processing of the protease [19]. The model has been further refined by Salvesen and colleagues to a '*proximity-induced dimerization*' model. Herein, biochemical studies show that dimerization of initiator caspases drives their activation. Thus, internal proteolysis is a secondary event that does not activate these apical caspases but results in partial stabilization of activated dimers [140]. Moreover, studies of FLIP-mediated caspase-8 activation have provided additional inputs on the allosteric aspect of this model [80, 141]. As described earlier, FLIP is a close homologue of caspase-8 and is catalytically inactive. It generally exists in two splice variant forms: short FLIP(S) and long FLIP(L) [142]. The former is a homologue of caspase-8 prodomain and can directly block caspase-8 recruitment into the DISC [143]. However, the long form contains a pseudo-catalytic domain

that lacks the active-site Cys. This form has been known to induce a reordering of the catalytic centre of caspase-8 in a manner similar to dimerization-driven caspase-9 activation [80]. Also, the L2 loop of this protease might be long and flexible that supports its autocleavage and results in the generation of a partially processed, but fully active, heterodimer of FLIP(L) and full-length caspase-8. Thus, the activation of caspase-8 may include both allosteric and processing-based mechanisms.

Caspase-9 is unique among all the other caspases. The X-ray structural studies reveal the active site of monomer to be in the inactive conformation, and its activation is driven by a dimerization-dependent conformational change. Upon activation, the interaction surface of the neighbouring monomer provides an acceptor site for the activation loop and results into a rearrangement in the substrate-binding pocket and reorientation of the catalytic Cys, thus forming a functional active site. Interestingly, caspase-9 dimer possesses only one active site, but the significance of this finding is currently unknown [47]. Allosteric regulation of this protease is served by the apoptosome complex (as earlier described in Sect. 4.2.1), which promotes its homodimerization and activation [144]. Therefore, the apoptosome complex can be considered as a holoenzyme, in which the caspase is 1000-fold more active than its monomeric form [101].

In contrast to caspase-8, L2 loop of caspase-9 might be extended enough to eliminate the need for proteolytic activation completely [47]. Therefore, it exhibits a much higher level of catalytic activity in the apoptosome through '*induced conformation model*'. This model proposes that the apoptosome may directly activate monomeric caspase-9 through allosteric modification, as described earlier. Alternatively, the apoptosome may assemble dimeric caspase-9 into a higher-order complex that results in the modification of active-site conformation for its enhanced activity. This form of allosteric mechanism may also contribute to the activation of caspase-1 and caspase-5 in '*inflammasome*' complex involving NALP1 [145].

6 Caspase Cross-Activation

As previously described, the established activation mechanisms precisely explain the initiator–executioner dichotomy of caspase family. However, this segmentation primarily applies only to the initial stages of apoptotic signalling. A complex network of caspase cross-activation supposedly exists in the downstream steps of apoptosis [19]. Recombinant caspase-1, caspase-2, caspase-3, caspase-6, caspase-7 and caspase-8 are known to process numerous in vitro-translated caspase zymogens [146]. For instance, caspase pair caspase-6/caspase-3, caspase-6/caspase-7 and caspase-8/caspase-3 form reciprocal feedback loops. Caspase-8 has been shown to process all caspases efficiently, whereas caspase-1 and caspase-11 are able to activate executioner caspases, but not vice versa. It has been demonstrated that caspase-2 can be activated by a host of caspases, but it failed to process any tested caspase.

As executioner caspases are also capable of processing the normally upstream activator molecules in vitro, these findings largely highlight the complexity of caspase transactivation patterns. An intricate network of one such caspase transactivation event has been ably demonstrated in a cell-free apoptosis system [147]. The addition of cytochrome c to post-nuclear Jurkat (JK) cell lysates resulted in the initiation of caspase-9/caspase-3 partnership, with caspase-3 subsequently processing caspase-2 and caspase-6. These, in turn, activate apical caspase-8 and caspase-10. On the other hand, besides processing caspase-3, caspase-9 also cleaves caspase-7. Caspase-3 simultaneously forms a feedback loop to cleave caspase-9. The primary function of the latter event is to remove binding site for XIAP from the L20 loop of caspase-9, thus changing its direction towards inhibition [148].

These in vitro observations briefly elaborate the potential complexity of putative downstream caspase cross-activation networks induced inside cells. Although upstream activation may initially involve particular caspases, there also resides the probability that a vast array of additional family members may eventually get activated. Such transactivation processes blur one thin line of distinction existing between initiator and executioner caspases. This may serve to expand the repertoire of accessible caspase substrates and stimulate diverse downstream signalling processes, for example, cytokine processing versus apoptosis [19]. Thus, in the event of inhibition or malfunctioning of a specific pathway, the transactivation and cross-activation processes can subsequently provide multiple redundant pathways that ensure smooth execution of apoptotic programme. Subsequently, it can also accelerate the same programme through massive caspase activation, trigger specific apoptotic execution subroutines and generate sustained signalling through positive feedback loops.

7 Regulation of Caspases

Since proteolysis is an irreversible process, activation of most of the constitutively expressed caspases is tightly regulated in multiple cell types. The activation mechanisms described above represent by far the most important ways to regulate caspase activity. Caspases with long prodomains play a regulatory role in apoptosis by activating those effector caspases with shorter prodomains, as they may undergo structural rearrangements required for their autoactivation. However, to prevent unwanted physiological responses, cells may also employ additional regulatory mechanisms that have been well established and are elaborated in this section.

7.1 Transcriptional Regulation of Caspases

The existence of an endogenous suicide mechanism was first deduced from the fact that new protein synthesis is required in certain apoptotic paradigms [149]. Although this is not applicable in most cases, subsequent analysis of the murine

caspase-11 demonstrated the importance of this regulatory mechanism and its critical role in controlling the caspase activity before or during an apoptotic stimulus. Usually, the basal expression level of caspase-11 in healthy mice and resting cells is found to be very low. However, in vivo treatment with lipopolysaccharide (LPS) or other pathological stimuli led to a remarkable upregulation of caspase-11 expression [35, 150]. The transcription of caspase-5, which is the putative human caspase-11 homologue, has also been found to be upregulated upon LPS treatment [151]. On the translational front, mRNA synthesis, protein expression and catalytic activation of caspase-5 were highly regulated in response to various proinflammatory stimuli (such as LPS), ATP and endoplasmic reticulum (ER) stress inducers [152]. The caspase-2 transcriptional regulation appears to be coordinated with transcriptional induction of its activators PIDD and RAIDD upon DNA damage [153]. A bit counter-intuitive study showed a p53/p21-dependent pathway for downregulating caspase-2 mRNA expression occurs in resting cells as well as in response to DNA damage [154] and thus reduces unwanted extraneous cell death.

Caspase-2 is known to have two isoforms: the proapoptotic long isoform, caspase-2L, and the antiapoptotic short isoform, caspase-2S. Through RNAi-mediated loss-of-function approach, it has been demonstrated that the RNA-binding protein HuR negatively regulates both caspase-2 mRNA and the protein levels [155]. Attenuation of HuR sensitizes adenocarcinoma cells by increasing the translation of caspase-2L and enhancing intrinsic apoptosis. On the other hand, the nucleotide excision repair factor XPC (genetic deficiency of which increases predisposition to cancer) is known to enhance DNA damage-induced apoptosis by downregulating caspase-2S [156]. In case of caspase-9, the alternative splicing results in two different caspase-9 transcripts that differ by four exons. The splice variant caspase-9a is found to be proapoptotic, while the shorter isoform, caspase-9b, is antiapoptotic [157, 158]. However, this splicing mechanism has been found to be defective in **N**on-**S**mall-**C**ell **L**ung **C**arcinoma (NSCLC), a type of epithelial lung cancer that accounts for about 85% of all lung cancers [159]. Moreover, caspase-9a to caspase-9b ratio increases with K-Ras^{12V} overexpression as caspase-9a promotes apoptosis, while **E**pidermal **G**rowth **F**actor **R**eceptor (EGFR) overexpression lowers this ratio. K-Ras is known to be a downstream component of EGFR signalling network and associates the growth-promoting signals from cell surface to the nucleus. On the other hand, the EGFR signalling network regulates cancer cell proliferation, apoptosis and tumour-induced neo-angiogenesis.

RfCasp10, a homologue of caspase-10 from black rockfish (*Sebastes schlegelii*), has been found to be upregulated upon pathogenic stimuli elicited by *Streptococcus iniae* and mediates apoptosis in immune-relevant tissues such as blood, liver and spleen [160]. Caspase-10 mRNA level is also negatively regulated in Jurkat cells expressing HIV tat gene when compared with control-treated Jurkat cells [161]. The tat protein (HIV-1-trans-activating protein) is a nuclear transcriptional activator of viral gene expression that enhances the efficiency of viral transcription. The reduction of caspase-10 mRNA, coupled with a concomitant increase in the expression of c-FLIP, is sufficient enough to enhance the resistance of

Tat-expressing T cells to TRAIL-induced apoptosis. Elaborating on caspase-14, it is known to be transcriptionally induced during the terminal differentiation of keratinocytes [162].

Deregulation of oncogenic E2F (E2 factor) transcription factor by adenovirus E2A oncogene overexpression or through loss of the tumour suppressor Rb gene results in the accumulation of zymogen forms of multiple caspases through a direct transcriptional mechanism [163]. The E2F proteins belong to a family of transcription factors that are downstream effectors of the retinoblastoma (RB) protein pathway and are believed to play a critical role in cell division control. Increase in zymogen concentration, thus, appears to potentiate p53-mediated apoptotic signals, and this ably explains why oncogene-expressing cells exhibit an increased sensitivity to multiple apoptotic stimuli. This area of research has now been exploited for anticancer therapy. The adapter protein, Apaf-1, has also been found to be transcriptionally regulated by E2F1 and p53 [164].

7.2 Metabolic Regulation of Caspases

Numerous signalling pathways, linking cell survival and metabolism, have been shown to directly influence the activation of caspase-dependent cell death [165] and regulate apoptosis. For example, Akt (protein kinase B) is a serine/threonine-specific protein kinase that plays a crucial role in regulating multiple cellular processes such as metabolism, cell proliferation, apoptosis, transcription, cell migration and angiogenesis. Cell survival mediated by this oncogenic protein is dependent on glucose metabolism to inhibit Bax activation and cytochrome c release [33]. The addition of glucose-6-phosphate in Xenopus oocyte system has been shown to delay the activation of caspases. NADPH (**N**icotinamide **A**denine **D**inucleotide **P**hosphate), which gets generated through the pentose phosphate pathway, is important in inhibiting caspase-2 activation through the inhibitory phosphorylation at Ser-135 present in the CARD domain of the zymogen. Phosphorylation is also mediated by calcium/calmodulin-dependent protein kinase II (CaMKII) [104], which allows the binding of 14-3-3ζ that prevents caspase-2 association with the PIDDosome [166].

The genome-wide screen for genes required for apoptosis revealed multiple genes directly involved in cellular metabolism of Drosophila cells. These included the genes encoding **c**itrate **s**ynthase (CS) and 3-ketoacyl-acyl carrier protein synthase, which protects against apoptosis that is induced by the removal of DIAP1, a direct inhibitor of Drosophila caspases [167]. Further studies reveal that the cellular NADPH levels also modulate the Drosophila initiator caspase, Dronc, through Ser-130 phosphorylation. Depletion of NADPH removes this inhibitory phosphorylation and results in the activation of Dronc, which is then followed by cell death [168]. Further studies, thus, become essential in identifying the precise metabolic changes mediated by the up-/downregulation of the constitutively expressed genes that safeguard cells against apoptosis.

7.3 Nitrosylation

Nitric oxide (NO) is a short-lived gaseous free radical that regulates apoptosis through **c**yclic **G**uanosine **M**ono**p**hosphate (cGMP)-dependent and cGMP-independent pathways. NO donors are known to directly inhibit caspase activity through active-site nitrosylation [169], as the catalytic cysteine of caspases is very active and susceptible to such modifications. This mechanism has been implicated in the NO-mediated inhibition of apoptosis or inflammatory cytokine production.

Denitrosylation of caspase-3 occurs in cells following treatment with Fas ligand, and this increases the caspase activity with subsequent cell death in response to Fas. It has also been shown that only a subset of caspase-3 gets localized to the mitochondria and is nitrosylated [170]. Moreover, the primary adhesion fibroblasts isolated from patients showed diminished apoptotic responsiveness upon this nitrosylation event [171]. The crucial role of nitrosylation in regulating caspase-3 has been well established in other models, including treatment of cardiac muscle cells with pharmacological NO donor, S-Nitroso-*N*-acetyl-DL-penicillamine (SNAP) and doxorubicin [164]. Conversely, it has been demonstrated that a single cysteine in thioredoxin (Trx), a class of small redox proteins, is capable of bringing about a targeted, reversible trans-nitrosylation reaction with active-site cysteine of caspase-3 in response to intracellular NO levels [172].

Both in vitro and in vivo studies have shown that abnormal S-nitrosylation contributes to the pathogenesis of Parkinson's disease (PD) by impairing anticaspase-3 and thus, the antiapoptotic function of endogenous caspase inhibitor, XIAP [173, 174]. Procaspase-9 is also known to induce its cleavage by trans-nitrosylating XIAP via Trx system [175]. NO induces S-nitrosylation of caspase-8, which in turn reduces the sensitivity of hepatocytes towards TNF-α/Actinomycin D-induced apoptosis [176]. While carrying out in vivo studies in rat livers treated with TNF-α and D-galactosamine, the liver-specific NO donor 'V-PYRRO/NO' was found to block caspase-8 activity, Bid cleavage and mitochondrial cytochrome c release [176]. Caspase-9 has also been shown to be modified by nitrosylation when intracellular NO levels increase [177], and this event preferentially occurs in the mitochondrial compartment [170]. On the other hand, treatment of cell lines with SNAP does not block mitochondrial cytochrome c release but reduces caspase-9 activation in a cell-free system.

Cerebral ischaemia is a condition, wherein there is insufficient blood flow to the brain. It is promoted by the activation of neuronal nitric oxide synthase (nNOS) that significantly increases S-nitrosylation of Fas. S-nitrosoglutathione (GSNO) pre-treatment has been known to protect against this condition, as the compound inhibits post-ischaemic nNOS activation and NO release. This subsequently disrupts the activation of proapoptotic caspase-8, Bid as well as caspase-9 and caspase-3 [178]. Thus, it downregulates Fas S-nitrosylation and the consequent Fas signalling cascade. On the contrary, KRGE (**K**orean **R**ed **G**inseng **E**xtract) is another traditional herbal medicine that is known to prevent endothelium dysfunction in the cardiovascular system, increase nitric oxide (NO) production via Akt-dependent activation of endothelial NO synthase (eNOS) and thus, inhibit

caspase-9/caspase-3 activities [179]. IL-15 is also known to differentially regulate caspase activity and influence susceptibility to T cell death. It was been reported that this interleukin molecule, with reduced glycolysis, increases the reactive nitrogen as well as oxygen species and promotes T cell survival via S-nitrosylation-mediated inhibition of caspase-3 [180]. Thus, the outcome of the NO generation might ultimately depend on the level of its production in the cellular context.

7.4 Oxidation

Similar to nitrosylation, excessive generation of reactive oxygen species (ROS) may also lead to inhibition of caspases. In the intrinsic mitochondrial apoptotic pathway, it is the oxidized cytochrome c that is capable of inducing the caspase cascade, and therefore, it is suggested that the redox state of cytochrome c might regulate apoptosis initiation by controlling the cellular ROS level [181]. XIAP is also known to regulate apoptosis by modulating the intracellular ROS levels and by enhancing the expression of antioxidant genes such as SOD-1/-2 and Txn2 [182]. Melatonin, a pineal hormone that regulates circadian and seasonal rhythms, promotes human platelet apoptosis by significantly increasing the generation of intracellular ROS and Ca^{2+}, thus facilitating events that lead to mitochondrial damage [183]. The NOD-like receptors (NLRs), NOD2 and the downstream adaptor protein, RIP2, are known to regulate ROS production. Increase in the intracellular ROS levels amplifies caspase-11 expression and non-canonical NLRP3 (**N**ucleotide-binding domain, **L**eucine-**R**ich-containing family, **P**yrin domain-containing-3) inflammasome activation by enhancing the c-Jun N-terminal kinase (JNK) signalling pathway [184]. This inflammasome complex plays a critical role in the pathogenesis of various acute or chronic inflammatory diseases.

Recent in vivo studies have revealed that caspase-2 confers protection against oxidative stress [185]. Stimulation of IL-1β secretion from the macrophages increases the activity of xanthine oxidase (XO). This enzyme is known to increase ROS generation and brings about NLRP3 inflammasome activation [186]. On the other hand, carbon monoxide and the transcription activator Nrf2 (**N**uclear factor E2-**r**elated **f**actor-2) have been shown to negatively regulate this inflammasome activation event and prevented subsequent mitochondrial dysfunction by inhibiting caspase-1 activation and secretion of IL-1β [187, 188]. Although ROS possesses high intrinsic toxicity, inhibition of caspases by such stimuli might not result in the alleviation of death. On the contrary, it may lead to a diversion from apoptosis to necrosis that is a non-specific caspase-independent death caused by overwhelming stress [189, 190]. For example, CHOP (**C**/EBP **Ho**mologous **P**rotein) is a transcription factor that is activated at multiple levels during various extracellular or endoplasmic reticulum (ER) stresses and plays a critical role in ER stress-induced apoptosis. LGH00168 is a novel activator of the CHOP promoter that is found to decrease the antiapoptotic Bcl-2 expression. However, it does not induce apoptosis

but rather leads to RIP1-dependent necroptosis via caspase-8 inhibition and mito-ROS production [191].

7.5 Phosphorylation

Phosphorylation is an extensively utilized cellular regulatory mechanism that is best in bringing about rapid and reversible modulation of multiple signalling networks. As activation of caspases often initiates an irreversible process, direct phosphorylation-dependent caspase modulation might not be appropriate. However, phosphorylation plays an important role in regulating various other steps in apoptosis signalling. In response to prosurvival stimuli through growth factor stimulation, Akt and ERK (**E**xtracellular signal **R**egulated **K**inase) or MAPK (**M**itogen-**A**ctivated **P**rotein **K**inase) have been shown to phosphorylate caspase-9 at Ser-196 or Thr-125 residue, respectively [192, 193]. These phosphorylation events lead to inhibition of caspase-9 activity and therefore prevent apoptosis. Later studies have demonstrated that during hyperosmotic stress, phosphorylation on Ser-144 of caspase-9 by a serine/threonine kinase, PKC ζ (**P**rotein **K**inase **C zeta**), causes inhibition of procaspase activation and holds back the intrinsic apoptotic pathway [194]. The kinase is known to be involved in a variety of cellular processes such as proliferation, differentiation and secretion. Moreover, during mitosis, caspase-9 is phosphorylated at Thr-125 by the mitosis-promoting kinase complex, CDK1–cyclin-B1 (cell cycle regulator **C**yclin-**d**ependent **k**inase 1 (CDK1) bound to cyclin B1) [195]. During cell cycle, this process is thought to dampen the threshold for intrinsic apoptosis signals, especially upon prolonged mitotic arrest. However, this phosphorylation event does not affect binding of caspase-9 to Apaf-1, and its mechanism still remains obscure.

It has also been demonstrated that when NADPH levels decrease in cells, the enzyme PP1 (**P**rotein **P**hosphatase 1) dephosphorylates caspase-2 and permits its activation by the PIDDosome [166]. CDK1–cyclin-B1 also phosphorylates caspase-2 [196] in the interdomain region between the apparently large and small subunits of active caspases. This event directly inhibits the generation of mature, stable enzyme and thus, suppresses apoptosis. Conversely, mimicking the CDK-2 phosphorylation of full-length Bcl-xL (an antiapoptotic member of Bcl-2 protein family), at Ser-73, results into its cleavage and caspase activation [197]. Recently, it has been demonstrated that in tumour cells exhibiting high expression of Plk-3 (a **P**olo-**l**ike **k**inase that acts as an important regulator of cell cycle progression), ligand stimulation of the death receptor CD95 brings about increase in activity of Plk-3. This event further leads to stimulation of extrinsic death pathway through Thr-273 phosphorylation of caspase-8 by the kinase [198].

7.6 Ubiquitination and Degradation

Since activated caspases are short-lived species inside cells and exhibit a more dynamic turnover than the inactive zymogens, another mechanism of caspase regulation involves degradation via the proteasomal pathway [199]. Apparently, it has been suggested that proteins responsible for the rapid removal of caspases include IAPs, and therefore, an increasing attention has been paid in recent years to understand the ubiquitination/ubiquitylation of caspases by IAPs. As later elaborated in Sect. 7.7.2, many IAPs are known to contain RING domain and ubiquitin-associated domains that are involved in ubiquitin ligation of caspases [200, 201]. For example, XIAP contains a carboxy-terminal RING domain critical for its E3 ubiquitin ligase activity. E3 ubiquitin ligases are essential components of the proteasomal degradation process. They are known to recruit an E2 ubiquitin-conjugating enzyme and assist or directly catalyse the transfer of ubiquitin from E2 to its required protein substrate. XIAP is shown to polyubiquitylate the large subunit of active caspase-9 in vitro through its E3 ubiquitin ligase activity, but it cannot perform the same with the inactive procaspase-9 [164]. Conversely, GIDE (**G**rowth **I**nhibition and **D**eath **E**3 Ligase) is another mitochondrially located E3 ubiquitin ligase that induces apoptosis via activating caspases and inhibiting XIAP function [202]. Hence, additional studies become requisite to fully understand the channel by which XIAP-mediated caspase-9 ubiquitination can be controlled, as well as the significance of this activity in controlling apoptotic progression.

IAPs other than XIAP, such as cIAP1 and cIAP2, are known to monoubiquitylate caspase-3 in vitro [203]. The physiological relevance of cIAP2 has been demonstrated in neutrophil granulocytes, wherein induction of these IAPs by endotoxins has been found to accelerate the degradation of activated caspase-3 and subsequently reduce apoptosis during sepsis [204]. A recent study highlights the role of cIAP1 in ubiquitination of an intermediate processed form of caspase-3, which subsequently leads to proteasome-dependent degradation of the effector caspase as well as increases resistance to TRAIL-induced apoptosis [205]. The study is supported by a work that demonstrates the stabilization of cleaved caspase-3 (active subunits) and enhancement of apoptosis upon treatment of cells with the proteasome inhibitor, lactacystin [206]. Another research work has established that XIAP can polyubiquitylate active caspase-3 (not procaspase-3) and lead to its proteasome-dependent degradation [207]. However, further validation of these observations in animal models is required for harnessing their therapeutic potential [164]. Similar to caspase-3, it has been reported that cIAP1 can only ubiquitylate the mature protease, rather than the partially processed form as seen in caspase-3 [205].

In case of caspase-8, a cullin3 (CUL3)-based E3 ligase performs the polyubiquitination event and enhances the enzymatic activity of this apical protease [208]. After recruitment of caspase-8 to the DISC, this ubiquitination allows binding of active caspase-8 to p62. p62, being a poly-Ub-binding protein (Fig. 6), facilitates an association between CUL3-modified caspase-8 and similar other complexes in the cell that together lead to the formation of an aggregate of active

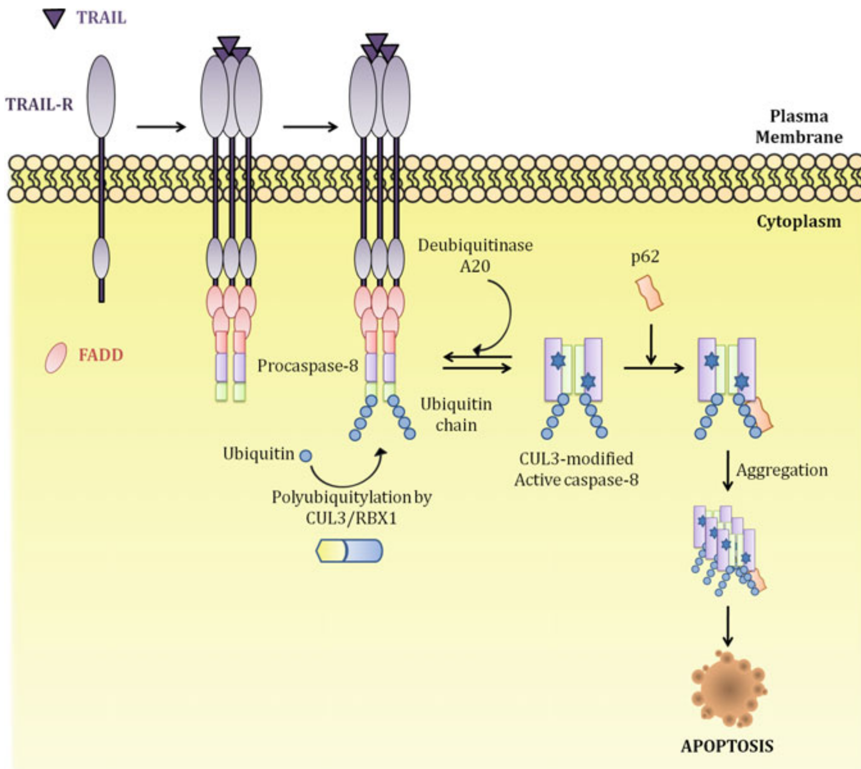


Fig. 6 Regulation of caspases through ubiquitination. Initiation of the extrinsic apoptotic pathway via the death receptor, TRAIL-R, occurs upon binding of TRAIL ligand and subsequent receptor multimerization at the plasma membrane. This leads to the formation of DISC and further activation of procaspase-8 through autoprocessing and dimerization. Upon activation, a cullin3 (CUL3)-based E3 ligase promotes ubiquitination of caspase-8, which could be reversed by deubiquitinase, A20. The ubiquitination process further leads to the interaction of active caspase-8 with a poly-Ub-binding protein, p62. Interaction of the CUL3-modified caspase-8 with p62 results into aggregation of this complex in the cytosol. These aggregated foci are supposed to have enhanced caspase-8 activity due to stabilization of the active dimer and thus trigger apoptosis

caspase-8 and p62. Interestingly, unlike other caspases, caspase-8 activity appears to be enhanced within these aggregated foci due to an increased stability of cleaved caspase-8. However, the deubiquitinase A20 has been reported to reverse the CUL3-mediated caspase-8 polyubiquitination [208].

7.7 Protein–Protein Interactions

Since programmed cell death involves a network of proteins that work in a coordinated manner, protein–protein interactions involving caspases play pivotal roles

in regulating these proteases and maintenance of cellular homeostasis. Caspases exhibit the presence of well-conserved recruitment domains such as CARD or DED that mediate their homotypic interactions with adaptor proteins or other proteins of the apoptotic pathway often forming multiprotein complexes. Mature caspases also interact with substrate and inhibitor molecules through different binding sites as well. Therefore, the nature of these protein–protein interactions impacts the apoptotic pathway either positively or in a negative manner.

As discussed in the above subsections, there exist a variety of proteins that directly or indirectly bring about specific modifications in the caspases and promote their activation. For example, the serine protease granzyme B has been shown to catalyse activation of several effector caspases in vitro, thus leading to the assumption that this is one of its primary roles in cytotoxic lymphocyte-mediated cell death [209]. Moreover, proteins such as CAS (**C**ellular **A**poptosis **S**usceptibility protein) and Hsp70 (**H**eat **s**hock **p**rotein 70) act as mediators of a tumour suppressor protein PHAPI and together enhance caspase-9 activation. This is accomplished by facilitating the nucleotide exchange on Apaf-1, which is the backbone of caspase-9-activating apoptosome [210]. Thus, in addition to the protein–protein interactions, the levels of other (non-protein) cellular components, such as nucleotides and ions, also appear to play critical roles in regulating certain steps in caspase activation and promoting apoptosis.

On the contrary, the ability of inhibitors to mimic the binding site of a good substrate and take advantage of the substrate-binding cleft makes them suitable candidates in regulating complex proteolytic processes. In the apoptotic systems, these inhibitors may establish thresholds that determine the concentration of active cell death proteases required to initiate cell disassembly. Thus, it also prevents the consequences of accidental or spontaneous proenzyme activation and is known to confine the activity of these enzymes to specific cellular locations. The direct caspase inhibitors are some of the first proteins to be discovered that regulate caspase activity. These viral caspase inhibitors include CrmA (**C**ytokine **r**esponse **m**odifier A) from the cowpox virus and p35/p49 proteins produced by baculoviruses. Both of these are active-site-directed suicide inhibitors and do not appear to have direct cellular counterparts.

CrmA is a member of the serpin family and is a potent inhibitor (K_i < 1 nM) of some active initiator and inflammatory caspases such as caspase-1, caspase-8, and caspase-10. Expression of CrmA prevents the activation of both the executioner caspases (caspase-3 and caspase-7) as observed in a model of Fas-induced apoptosis [211]. On the other hand, the baculovirus protein p35 has no known homologues, and its selectivity for caspases is not clearly defined. Thus, these viral inhibitors are known to achieve rapid inhibition of caspases in a relatively non-selective manner, as a consequence of their requirement to present substrate-like sequences to the caspase.

Apart from these, there exist many proteins that affect caspase activity directly or indirectly by modulating the activation platform of these caspases. The inhibitory mechanism employed by decoy molecules involves proteins that are structurally related to caspase prodomains and would therefore compete for the same adaptors

within activation platforms. For example, caspase-1-related CARD-only decoy proteins such as COP (**C**ARD-**O**nly **P**rotein), INCA (**In**hibitory **CA**RD) and ICEBERG (a novel inhibitor of IL-1β generation) bind to caspase-1 prodomain via CARD–CARD interactions and prevent its recruitment to inflammasomes [212]. A few examples of significant proteins that interact directly with caspases and alter either their enzymatic activities are briefly elaborated below.

7.7.1 FLIP

The FLIP (**F**ADD-**l**ike **I**CE inhibitory **P**rotein) family of proteins was first discovered in a viral context, while its mammalian and other homologues were discovered concurrently [213]. As earlier mentioned, these proteins are similar to procaspase-8 in their primary sequence but lack essential catalytic residues. This structural identity helps them compete with procaspase-8 for binding to its cofactor, FADD, and prevent caspase activation. The long form of the mammalian cellular FLIP (c-FLIP(L)) is usually recruited to the DISC, where it is partially processed, while a portion of this protein is still retained at the DISC. This interaction with the caspase activation platform inhibits any subsequent recruitment of caspase-8 [164]. However, c-FLIP(L) induces a conformation of procaspase-8 that allows partial but not complete proteolytic processing, whereas the shorter splice variant, c-FLIP(S), can completely prevent partial processing of DISC-bound caspase-8 [214].

Interestingly, the FLIP proteins have also been shown to function as activators of the apoptotic process [80, 141]. In this case, c-FLIP(L) appears to heterodimerize with caspase-8, thus activating the caspase dimer. This hypothesis has been proven with the use of kosmotropes that induce the formation of FLIP(L)/caspase-8 heterodimers. In contrast to the proapoptotic caspase-8 homodimer, these heterodimers have been shown to have a lower kinetic barrier to activation [215] and can promote cell survival [216, 217]. Thus, it is suggested that it may be the local concentration of c-FLIP at the DISC that ultimately determines its role as an inhibitor or activator of caspase-8.

7.7.2 IAPs

The inhibitor of apoptosis proteins (IAPs) includes a conserved family of proteins that contain at least one **B**aculovirus **I**AP **R**epeat (BIR) domain that is a novel zinc-binding fold of about 70 amino acids. As discussed in Sect. 7.6, these proteins may also contain a RING domain that confers E3 ubiquitin ligase function [218]. Mammalian IAPs, mainly **X**-linked **IAP** (XIAP) and **c**ellular **IAPs** (cIAPs) are the best-characterized vertebrate members of the IAP family, which are able to convert the survival signal into a cell death-inducing signal or vice versa. For example, XIAP has been shown to be involved in the potent as well as selective inhibition of caspase-3 and caspase-7 in vitro. This multidomain protein efficiently and selectively inhibits the executioner caspases by binding to their active sites via its BIR2 domain. It also inhibits the initiator caspase-9 by preventing or reversing dimerization via its BIR3 domain [199]. These domains of XIAP combine two specific but relatively weak interactions with their target caspases for achieving specific

tight inhibition via a two-site mechanism, and therefore, they are mechanistically distinct from the viral inhibitors mentioned earlier in this section [219].

The inhibitory action of XIAP can be antagonized by mitochondrial proapoptotic proteins Smac/DIABLO and HtrA2, which are released during an apoptotic stimulus along with cytochrome c [220–222]. As a direct inhibitor of these caspases, XIAP has been detected on the apoptosome [102, 223]. XIAP inhibits caspase-9 by a mechanism where the third BIR binds to the processed amino terminus of monomeric caspase-9 and prevents its dimerization [224]. A recent study in melanoma cell lines revealed caspase-mediated cleavage of XIAP via a positive feedback loop. This feedback event reduces caspase inhibition and XIAP levels through proteasomal degradation [225].

Other IAPs related to XIAP include cIAP1, cIAP2, ILP-2, ML-IAP as well as survivin; however, these do not directly inhibit caspases [219]. Like XIAP, cIAP1 and cIAP2 have three BIRs and a RING domain. Although they are incapable of inhibiting caspases under physiological circumstances, they are known to cause an indirect abrogation of caspase activity. This is accomplished by binding to IAP antagonists such as Smac/Diablo, thus allowing more XIAP molecules to be free to inhibit caspases [224]. As discussed in the previous subsections, they may also influence signalling via NF-κB and MAPK, or target caspases for ubiquitylation and proteasomal degradation. cIAP1 also specifically blocks apoptosis downstream of cytochrome c release by binding to and inhibiting active caspase-9 within the apoptosome [226]. In addition, XIAP and DIAP proteins have been shown to act as direct stoichiometric inhibitors of caspases. Apart from that, these IAPs also bring about specific modifications or interact with other proteins and indirectly assist in the regulation of caspase activity. For example, human oncoembryonic protein AFP (**A**lpha-**F**eto**p**rotein) is known to induce apoptosis in tumour cells by positively regulating cytochrome c/dATP-mediated apoptosome complex formation. It accelerates the release of active caspase-9 and caspase-3 as well as displaces cIAP-2 from the apoptosome [227]. Through abrogation of the inhibitory signalling, AFP affects XIAP–caspase interaction and rescues caspase-3 from inhibition [228]. Thus, IAPs are key players that are majorly involved in regulating caspase activity and in determining the cellular fate.

8 Caspase Regulation in Non-apoptotic Cellular Processes

Apart from their indispensable role in apoptosis, several active caspases have been found to be involved in various non-apoptotic cellular functions such as inflammation, protein secretion and differentiation. In the absence of a cell death signal, caspase activity may exhibit deadly consequences due to the dearth of a fine-tuned regulatory process. Therefore, tight control of caspases becomes particularly critical under circumstances in which the proteases are activated for short, well-defined periods of time or at isolated subcellular locations. For example, in hippocampal neurons, the transient activation of caspase-3 has been shown to play a role in

synaptic LTD (**L**ong-**t**erm **D**epression: an activity-dependent reduction in the efficacy of neuronal synapses) and the internalization of the glutamate receptor, AMPA (α-**a**mino-3-hydroxy-5-**m**ethyl-4-isoxazole-**p**ropionic **a**cid receptor) [229]. In fact, treatment with NMDA (**N**-**m**ethyl-**D**-**a**spartate), a specific agonist at the NMDA receptor that mimics the action of glutamate, facilitates mitochondrial release of cytochrome c and caspase-9 activation, which are subsequently necessary for caspase-3 activation.

Further studies reveal that overexpression of a non-cleavable Akt1 (Protein kinase B) in hippocampal slice cultures inhibited LTD. Thus, the cleavage and inactivation of Akt1 is considered as a part of the link between caspase-3 activity and LTD [164]. Recent studies have shown the involvement of XIAP in regulating LTD and learning rate. Herein, XIAP deletion causes an increase in the caspase-3 activity within central neurons. This enhances AMPA receptor internalization, sharply increases LTD and thus, plays an important physiological role [230]. Another study using **O**lfactory **S**ensory **N**eurons (OSNs) has demonstrated the non-apoptotic role for Apaf-1 and caspase-9 signalling in development [231]. This non-apoptotic caspase activity leads to cleavage of the membrane-bound protein, Semaphorin 7A, which is critical for appropriate formation of axonal projections [232]. A variety of problems exist in mice lacking Apaf-1 or caspase-9 expression, and these include immature as well as erroneously routed OSN axons. However, Apaf-1 and caspase-9 do not impact the generation of OSNs, but rather affect specific formation of axonal projections. Thus, apart from being involved in cell death processes, caspases perform a plethora of cellular functions that are as diverse as signal transduction, cytoskeletal remodelling, and are also known to direct the behaviour of cells nearby the apoptotic cell through the caspase-dependent secretion of paracrine signalling factors [233].

9 Caspases in Pathophysiology of Diseases

Cells adopt different ways to regulate the normal functioning of its intricate apoptotic machinery by maintaining a precise balance between different agonists and antagonists. A slight imbalance in this homeostatic process can, therefore, have detrimental effects on the normal functioning of a healthy cell. Moreover, caspase activation can be considered as a double-edged sword, wherein diseases either associated with or that emanate from its low or high activity might vary from neurodegenerative disorders to cancer. Defective caspase activation that leads to inadequate cell death can promote tumourigenesis, whereas tremendous caspase activity and the resulting excessive cell death can initiate neurodegenerative processes. Caspases are also known to be involved in inflammation, where insufficient activation can lead to increased susceptibility towards infection, while hyperactivation promotes inflammatory conditions. In that light, restoring normal apoptosis in these different diseased conditions seems to be one of the major challenges of current biomedical research.

9.1 Caspases in Cancer

Cancer, the disease that involves rapid creation of abnormal cells, is a leading cause of death worldwide, and according to WHO (**W**orld **H**ealth **O**rganization), it accounted for 8.2 million deaths alone in 2012. To encounter cellular events such as mutations, altered gene expressions, variable occurrence of several apoptosis-related genes such as p53 tumour suppressor gene, alteration in the epigenetic profile and mRNA instability, etc., there exist several inherent sophisticated mechanisms that recognize and repair DNA mutations before they can become oncogenic. However, since evasion of apoptosis is one of the crucial hallmarks of human cancers and caspases are known to be the primary molecules that execute this cell death modality, deregulation of caspase activity and the pathways in which they participate might promote tumourigenesis. Also, through genetic and inhibitor studies, it has been demonstrated that the inactivation of individual caspases is not usually sufficient to either prevent progression of caspase cascade or disrupt the alternative non-apoptotic mechanisms. The malignant cells, instead, seem to gain a survival advantage by inactivating signalling mediators upstream of caspase activation [234].

In correlation to this, many cancers have shown to exhibit either reduced expression of proapoptotic caspases [235], or specific inactivating mutations. The absence of the inflammatory caspase-1 caused an increase in the proliferation of colonic epithelial cells in early-stage tumours, whereas it reduced apoptosis at the advanced stage [236]. The prevalence of mutations in another inflammatory caspase, caspase-5, was found to be highest in microsatellite instability (MSI)-positive gastric carcinomas [108]. Caspase-6 mutations have been found in 2% of 150 human cancers of colonic or gastric origin [237], and it is also suggested that decreased expression of this protease might assist in gastric cancer development [238].

Somatic mutations in caspase-7 have been found to affect the executioner function and result in pathogenesis of some human solid cancers, including colon, oesophageal and head/neck carcinomas. These tumour-derived caspase-7 mutants caused reduced apoptosis upon their overexpression in 293T human kidney cells [239]. Wild-type caspase-8 is known to act as a tumour suppressor in childhood neuroblastomas by exhibiting amplification of the proto-oncogene, N-myc [240]. N-myc is a nuclear transcription factor that is highly expressed in the foetal brain, where it is critical for normal brain development and its overexpression can lead to tumourigenesis. Inactivating caspase-8 mutations leads to loss of caspase-8 function and renders neuroblastoma cell lines to become resistant to death receptor-induced apoptosis [108]. In a study of 180 human colorectal tumours, the somatic caspase-8 mutations were detected in 5% of invasive carcinomas. Three of these mutations acted in a dominant-negative fashion and caused decrease in the caspase-8-mediated apoptosis [241]. In a similar case study of hepatocellular carcinomas (HCCs), a single somatic caspase-8 frameshift mutation resulted in a two-base-pair deletion and caused premature termination of translation as well as subsequent loss of caspase-8 function [242]. On the other hand, inactivating

caspase-10 mutations have been found in **n**on-**H**odgkin's **l**ymphoma (NHL) samples [243], while rare caspase-10 mutations have also been detected in T cell acute lymphoblastic leukaemia, multiple myeloma [244] as well as in colon, breast, lung, hepatocellular [245] and gastric cancers [246].

Certain caspase polymorphisms also seem to affect caspase abundance or activity and therefore, have variable tumorigenic outcome. Polymorphism is a type of discontinuous genetic variation that results from evolutionary processes and leads to the occurrence of several different forms or types of individuals among the members of a single species. **S**ingle **N**ucleotide **P**olymorphisms (SNPs) are one of the most common types of genetic variation in a single nucleotide that occurs at a specific position in the genome, once in every 300 nucleotides on an average. Inheritance of such polymorphisms includes examples such as D302H variant in caspase-8 which substitutes histidine for an aspartic acid residue. In addition, a six-nucleotide deletion (-652 6N del) polymorphism has been found to be associated with a significantly reduced overall risk of breast cancer [247, 248] and other cancers [249]. Here, these alterations code the mutated caspase-8 protein which supposedly enhances its proapoptotic effects and thus prevents tumour cell persistence, although this remains to be proven under in vivo conditions [108].

Significant alterations of caspase-8, caspase-9 or caspase-1 have also been found to be associated with NHL [250]. In a study including 36 apoptosis pathway genes, germline variation in the caspase-9 gene as well as alterations of caspase-9 at SNP levels has been shown to be associated with NHL risk [251]. In lung cancer patients, there exist various polymorphisms that significantly decreased the risk of lung cancer. For example, as compared to the other haplotypes, increased promoter activity of the GC haplotype enhances caspase-9 expression, which is perhaps responsible for granting the protective effect [252]. A haplotype usually refers to the genetic make-up of an organism that is inherited together from a single parent because of the genetic linkage, and therefore, it can also refer to the inheritance of a cluster of SNPs. Thus, if a haplotype is associated with a certain disease, one can identify patterns of genetic variation and recognize the gene/s responsible for causing the disease.

Many studies have analysed the role that caspase-3 gene alterations play in promoting tumourigenesis. Compared with the GG genotype, the TT polymorphic variant was found to increase the risk of squamous cell carcinomas of the head and neck (SCCHN) in certain subgroups that included younger (<56 year) subjects, males and non-smokers [253]. In a stretch of DNA sequence in the chromosome, the description of a sequence change starts with a number referring to the first nucleotide affected (e.g. 76A > T), substitutions are designated by a '>' character, and the letters indicate the nucleotides involved in the substitution (A to T substitution). In lung cancer patients bearing at least one allele with a −928A > G, 77G > A or 17532A > C polymorphism, a significantly decreased risk of lung cancer as compared with those homozygous for the wild-type caspase-3 allele was observed [254]. Similarly, certain caspase-3 variants were found to reduce multiple myeloma risk and NHL [255, 256]. Of the 35 selected caspase-7 SNPs in the endometrial cancer patients, four were found to be in high linkage disequilibrium

and caused an increased risk, while two SNPs were associated with reduced risk. The two other caspase-7 SNPs caused an increased risk as compared to individuals who are homozygous for the major caspase-7 alleles. Thus, in all, the polymorphisms existing in the crucial caspases and their haplotypes distinctly define an individual's genetic susceptibility towards cancer development.

9.2 Caspases in Inflammatory Diseases

A wide variety of inflammatory and autoimmune diseases have been attributed to the production of IL-1 and subsequent activation of caspase-1 [257]. Gout and pseudogout are the two most common crystal-induced arthropathies and autoinflammatory disorders whose pathogenesis has been linked to inflammatory responses in joints that are activated by the deposition of crystals. **M**ono**s**odium **U**rate (MSU) crystals are deposited in case of gout, while calcium pyrophosphate dihydrate crystals are present in pseudogout. Apparently, the deposition of crystals is mediated by the NLRP3 inflammasome [108]. Moreover, mutations in NLRP3 component of the inflammasome cause three rare inherited autoinflammatory diseases that are collectively known as **C**ryopyrin-**A**ssociated **P**eriodic **S**yndromes (CAPS). These mutations bring about alterations in the NLRP3-encoded protein, cryopyrin, and result into hyperactive NLRP3 inflammasome activity along with elevated IL-1β levels [258, 259].

Excessive caspase-1 activity has also been implicated to play a role in type 2 diabetes (T2D), as the elevated IL-1β levels are known to be involved in T2D development [260] that ultimately contributes to insulin resistance [261]. Familial Mediterranean fever (FMF) is another autoinflammatory disease that arises from missense mutations affecting the carboxy-terminal domain of pyrin, which in turn influences its interaction with procaspase-1 [262]. The C-terminal domain of pyrin is necessary and sufficient for the interaction, and binding is reduced by these FMF-associated mutations. On the other hand, a rare autosomal-dominant genetic disorder PAPA (**P**yogenic sterile **A**rthritis, **P**yoderma gangrenosum and **A**cne syndrome) is caused by mutations in the CD2-binding protein 1 (CD2BP1) gene. These mutations result in increased binding of CD2BP1 to pyrin and consecutively reduce its ability to inhibit inappropriate inflammasome activation [263].

Non-sterol isoprenoid deficiency, especially geranylgeranyl groups, is characteristic of an autosomal recessive disorder known as Mevalonate kinase deficiency (MKD) [264]. It is an autosomal recessive metabolic disorder that disrupts the biosynthesis of cholesterol and isoprenoids. It is characterized by recurrent episodes of fever, which usually begin during infancy. This isoprenoid deficiency is known to induce PI3K pathway-dependent procaspase-1 activation that leads to increased IL-1β production [265]. Thus, abnormality in the functioning and homeostasis maintenance by caspase-1 and IL-1β levels can considerably lead to severe inflammatory diseases.

9.3 Caspases in Other Diseases

Apart from playing a significant role in various physiological processes, caspases are also involved in the pathophysiology of several degenerative disorders and autoimmune diseases. From studies of post-mortem human brain samples and animal models, caspase activation has been demonstrated to play a key role in multiple neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and **A**myotrophic **L**ateral **S**clerosis (ALS) [266]. Even though apoptosis is not the solitary player of cell demise in these disorders, it acts as a key component in partnership with other detrimental events such as inflammation, mitochondrial dysfunction, oxidative stress or protein misfolding and aggregation.

Rohn and co-workers have reported caspase-mediated cleavage of β-amyloid precursor protein (APP) [267]. In a murine AD model, caspase activation associated with this disease onset occurred earlier than the induction of neuronal apoptosis [268]. Likewise, caspase activation has also been noted before the development of neurofibrillary tangles of Tau in the brain of tau transgenic mice [269]. A nucleotide substitution from G to A in a particular SNP placed in the 5′ untranslated region of caspase-3 influenced the susceptibility towards Kawasaki disease (KD). Kawasaki disease is a rare childhood illness that brings about inflammation in medium-sized blood vessels throughout the body and is largely seen in children under the age of five years. The described substitution eliminates the binding of the NFAT (**N**uclear **F**actor of **A**ctivated **T** cells) transcription factor to the DNA sequence surrounding the SNP and alters caspase-3 expression in immune effector cells [270]. **A**utoimmune **L**ympho**p**roliferative **S**yndrome (ALPS) is another diseased condition that exhibits dominant mutations in CD95, CD95L or caspase-10 [108] and is thought to be caused by insufficient apoptosis of autoreactive T cells during negative thymic selection [271].

10 Targeting Caspases for Treatment of Various Diseases

As described in the previous section, deregulation of apoptosis can either cause damage to normal tissues through excessive apoptosis or may contribute to tumourigenesis, wherein apoptosis is prevented. The mechanistic significance of either inhibiting caspases to prevent excessive apoptosis in several diseases including neurodegeneration or reinstating apoptotic signalling towards selective targeting of malignant cells immensely exploits the caspase family of death-inducing molecules as a powerful therapeutic platform.

10.1 Caspase Activation and Treatment of Cancer

Chemotherapeutic drugs and irradiation are toxic to both normal and cancerous cells, and the damage is then translated through multiple steps of caspase activation, which ultimately leads to cell death. However, the therapy fails when these steps are compromised, and therefore, employment of alternative therapeutic strategies become essential. Within the last decade, several attempts have been made to develop molecules that directly activate caspases for use in cancer therapy. Justicidin A, a novel derivative obtained from the plant *J. procumbens*, is known to induce apoptosis in hepatocellular carcinoma through the activation of both intrinsic and extrinsic apoptosis pathways [272]. For example, through the activation of caspase-8 in the intrinsic pathway, justicidin A increases intracellular Bid that modulates $\Delta\psi_m$ (change in mitochondrial membrane potential) and causes the release of cytochrome c, but in turn it also decreases antiapoptotic Bcl-xL. Subsequently, the release of proapoptotic Smac/DIABLO and HtrA2 from the mitochondria activates caspase-9 and caspase-3 that initiate the intrinsic pathway. 6′-Hydroxy justicidin A (JR6) is another derivative that shares similarity with the lead antitumour drug. Podophyllotoxin has been shown to have promising apoptosis-induction efficacy in human bladder cancer cells [273]. Retinoic acid (RA) and its derivatives such as 13-cis RA, 9-cis RA and 4HPR (fenretinide) are also known to induce paracrine release of TRAIL in promyelocytic leukaemia cells as well as upregulate caspase-8 expression in neuroblastoma, medulloblastoma and small cell lung cancer cell lines [274]. This induced expression in turn sensitizes these cancer cells to TNF-α and other chemotherapeutics [275].

Since loss of caspase expression has also laid the grounds for many human cancers including neuroblastomas and small cell carcinomas, reinstating the expression and activation of caspases, especially caspase-8, might be of significant therapeutic advantage. This would also be particularly helpful in tumours caused due to gene dosage effect or hypermethylation of caspase-8 promoters. For example, 5-aza-2′ deoxycytidine (decitabine) is a cytosine nucleoside analogue that promotes genome-wide demethylation by inhibiting DNA methyl transferase covalent binding, which upregulates the expression of transactivators [276]. Therefore, rather than mediating caspase-8 promoter-specific demethylation, the compound increases caspase-8 promoter availability by allowing the binding of SP1 and ETS-like transcription factors [277, 278]. These transcription factors bind with high affinity to GC-rich motifs and to DNA sites with a central GGA(A/T) DNA sequence, respectively, and thus, regulate a variety of processes such as cell growth, apoptosis, differentiation and immune responses. The compound has been demonstrated to restore caspase-8 in a number of cancers including breast cancer, neuroblastoma, medulloblastoma, Ewing's sarcoma, lung carcinoma and gastric carcinoma [279].

Histone deacetylase inhibitors have also been known to assure adequate acetylation and transcriptional availability of caspase-3 promoter. These inhibitors have been demonstrated to activate apoptosis and increase sensitization to TRAIL-induced, radiation-induced and chemotherapy-induced apoptosis in prostate

cancer, lung cancer, Ewing's sarcoma and medulloblastoma [279]. Similar to caspase-8 expression, approximately 30% of melanoma and lung cancer cell lines harbour inactivating mutations in the IFN-γ signalling pathway [280]. Therefore, it has been found that IFN-γ ably sensitizes the caspase-8-deficient neuroblastoma, medulloblastoma and Ewing's sarcoma cells by inducing apoptosis through death receptor ligand, cytotoxic drugs and radiotherapy in vitro [281, 282].

One of the challenging strategies involves selective activation of death receptor complexes that are directly linked to initiator caspases. High-throughput screening (HTS) studies have identified a series of molecules that include a-(trichloromethyl)-4-pyridineethanol (PETCM), gambonic acid and its derivative MX-2060, which can efficiently activate the executioner caspase-3 in vitro [283–285], and induce apoptosis in cancer cell lines. **P**rocaspase-**A**ctivating **C**ompound-1 (PAC-1) is another promising caspase-3 activator that contains a zinc-chelating motif [286] and activates the proenzyme through this motif [287]. However, the mechanism put forth by the research group seems to be controversial. Another independent group has strongly counter suggested that PAC-1 cannot directly activate executioner caspases, but uses an indirect and less effective activation mechanism instead [288]; however, further investigation is required to shed light into this ambiguity. As shown in recent in vivo canine studies, a '*next-generation*' compound (S-PAC-1) has been proved to be efficacious in inducing partial tumour regression [289].

Alternatively, active research is also underway in identifying compounds that activate caspases indirectly, which are currently under clinical trials. Some of these agents are known to block endogenous caspase inhibitors such as the Bcl-2 and IAP proteins [290], while some act as analogues of the endogenous IAP inhibitor Smac/DIABLO [291]. In addition, there are activators and antibodies that engage death receptors such as Fas, TNFR and TRAIL [292]. Survivin, the smallest protein in the IAP family, is found to be the fourth most upregulated gene in several cancers as compared to normal tissue [293]. Its increased expression is associated with loss of apoptosis, enhanced survival, angiogenesis [294] and resistance to therapy [295]. Therapeutic targeting of survivin is currently in various clinical trials, including the use of antisense oligonucleotides. LY2181308 is one such antisense molecule that induces caspase-3 proteolytic activity, cell cycle arrest and inhibition of cytokinesis in human tumour cells.

Various research laboratories and pharmaceutical companies have also attempted to develop recombinant forms of TRAIL or TRAIL receptor agonists (e.g. receptor-specific mAb) that are under various stages of clinical trials [296]. Apart from these, caspase activators (both peptidomimetics and small molecules) are being designed to specifically target cancer cells. Inducible caspases such as caspase-9 under the control of prostate-specific promoter specifically target prostate cancer cells [297]. Gene therapeutic targeting of caspases to cancer cells has been another popular approach in combating tumourigenesis [298].

It is important to note that the apoptosis-inducing agents for cancer treatment also encounter the same limitations of delivery and specificity as traditional chemotherapeutics. Exceptions such as certain caspase activators that inhibit anti-apoptotic molecules like Bcl-2 seem to have an enhanced therapeutic index when

used to treat cancer cells that rely mainly on antiapoptotic proteins to stave off cell death [299]. However, a lot more needs to be done in this area of research to bring caspases in the forefront of cancer therapeutics. Substantial research endeavours using interdisciplinary approach have been currently set forth to rationally understand the structural and catalytic properties of active caspases and thus, consecutively manipulate the intricate apoptotic machinery for therapeutic interventions.

10.2 Caspase Therapy in Inflammatory Diseases

Advances in understanding the role of caspases in promoting various forms of inflammatory diseases have resulted in the development of various therapeutic targets. An inducible caspase-9 '*safety switch*' agent has been currently under phase I/II clinical trial and is used for the elimination of autoreactive lymphocytes in **g**raft-**v**ersus-**h**ost **d**isease (GVHD). The agent consists of a truncated caspase-9 protein that lacks the CARD domain and is fused to a forkhead protein-binding sequence. When used as a therapy, this caspase-9 safety switch protein is virally transduced into allo-depleted T cells, which are then administered to patients who have received a T cell depleted stem cell transplant [300]. The transduced protein agent then dimerizes in the presence of a particular small molecule, activates the hydrolytic function of enzyme and in turn, triggers apoptosis [301]. Since caspase-1 activation and subsequent generation of active IL-1β have also been found to be responsible for many inflammatory disorders, clinical trials are currently under way to assess the efficacy of antagonizing inhibitors and related molecules. These agents can antagonize either the generation or function of IL-1β or its receptor (IL-1R). For example, IL-1β antagonists have been found to be effective in the treatment of type 2 diabetes [302], confirming the important role of the NLRP3 inflammasome containing caspase-1 as a sensor of metabolic stress [303]. Patients with gout, pseudogout or pulmonary silicosis have also shown great improvement after treatment with such antagonists [108].

10.3 Caspase Inhibition and Prevention of Cell Death in Neurodegeneration

As described earlier, excessive apoptosis has been found to be responsible for several serious pathologies including neurodegenerative diseases, ischaemia-reperfusion injury, graft-versus-host disease and autoimmune disorders. Though there have been less striking therapeutic effects obtained by inhibiting caspase activity than those achieved through caspase activation, there still exist several instances, where caspase inhibition has ameliorated the symptoms of these diseased conditions that are affected by inappropriate apoptotic cell death. For example, preventing apoptosis through p35 expression prevents blindness in Drosophila mutants with retinal degeneration, which indicates that inhibition of caspases can functionally rescue cells from death [304].

A recent clinical trial has examined the therapeutic potential of a caspase inhibitor used for curing chronic hepatitis virus C infection, a condition that is accompanied by detrimental hepatocyte apoptosis [108]. Similarly, peptidyl caspase inhibitors have been found to be effective in animal models for stroke, myocardial ischaemia-reperfusion injury, liver disease and traumatic brain injury. Caspase inhibitors have also shown promising results in mouse models of neurodegenerative diseases such as Alzheimer's disease [305]. However, to prevent the risk of autoimmune disease or tumour progression to occur by chance, tissue-specific delivery of selective inhibitors becomes essential. Also, there are difficulties involved in generating small molecule, non-peptide inhibitors of proteolytic enzymes that would be selective, stable and would penetrate membranes effectively. Despite these, there has been an appreciable amount of work done on cysteine protease inhibition that provides a lead for the identification of several classes of potent, reversible and irreversible caspase inhibitors [306].

10.4 Current Scenario and Future Perspectives

Although a huge deluge of information on apoptosis has been made available from the current literature, translation of that knowledge into therapeutics still remains mostly unrealized due to several relevant concerns. For example, application of caspase inhibitors has been limited by their side effects, ineffectiveness due to redundancy in the apoptotic pathway and lack of an appropriate animal model system. However, a precise amalgamation of different aspects of various disease management strategies, including early detection, diagnosis and combination therapy, might circumvent the problem and will also provide a wider applicability of caspase mimetics/modulators in modern clinics.

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Gastric Pathology and Metalloproteinases

Snehasikta Swarnakar, Anirban Roy, Satyabrata Ghosh, Rajib Majumder and Sandip Paul

Abstract

The spectrum of gastric pathologies involves heterogeneity with respect to biochemical mechanisms and clinical outcome and is globally common. Each year, 5–6 million people worldwide are affected by gastric ulcer, gastric cancer and inflammatory bowel diseases, and mortality rate being >50% shows steep increase in incidence. Hence, understanding the underlying pathogenesis and better therapeutic strategies remain the major challenges in gastroenterology field. Current knowledge of gastric pathology reveals that extracellular proteases vastly influence functional irregularities of cells along with their responses to microenvironment. Based on studies on metalloproteinases and their inhibitors, it is well accepted about their important roles in physiological developmental processes as well as pathological conditions. From past several years of extensive research on matrix, metalloproteinases (MMPs) establish their critical role in several cellular functions including proliferation, apoptosis and angiogenesis. MMPs are a family of "molecular scissors" with ambivalent actions and ability to cleave extracellular matrix (ECM) proteins that in turn facilitate tissue remodelling. Approximately, 27 subtypes of MMPs are there having mutual interaction among each of them in gastrointestinal disorders. Functional overlap between the MMPs leads to non-specificity, which makes designing MMP inhibitors more difficult. Thus, specific MMP inhibitors would be promising therapeutic tool against inflammatory diseases including gastric diseases. This chapter illustrates the new insights into mechanism of MMP regulation in

S. Swarnakar (✉) · A. Roy · S. Ghosh · R. Majumder
Cancer Biology and Inflammatory Disorder Division, CSIR-Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700032, India
e-mail: snehasiktas@hotmail.com

S. Paul
Structural Biology and Bioinformatics Division, CSIR-Indian Institute of Chemical Biology, Kolkata 700032, India

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gastrointestinal inflammatory disorders encompassing clinical trials for MMP inhibitors and new therapeutic strategies by targeting specific MMP(s) to control gastrointestinal pathologies.

Keywords
Gastric ulcer · Cancer · Matrix metalloproteinase
Inhibitor · *Helicobacter pylori*

1 Introduction

Metalloproteinases degrade extracellular matrix (ECM) proteins and regulate both cell–cell and cell–ECM interactions, which influence cell differentiation, migration, proliferation and survival. They belong to metzincin group of proteases, characterized by the presence of zinc in the catalytic domain, that includes bacterial serralysins and astacins, adamalysins (a disintegrin and metalloproteinase domain or ADAMs) and matrixins (matrix metalloproteinases or MMPs) [1–3]. Metzincins use three histidine (H) residues to bind the zinc ion at their active site [4, 5]. A water molecule that is essential for hydrolysis of the peptide bond also coordinates with the metal ion as a fourth ligand in the active form of metallopeptidase. Members of this superfamily of enzymes are involved in diverse physiological processes as embryonic development, morphogenesis, bone formation, reproduction, cell adhesion and migration. Aberrant activities of metalloproteases have been implicated in various pathological conditions like arthritis, cancer, cardiovascular diseases, nephritis, central nervous system disorders and fibrosis [2, 6, 7]. There are ample literatures which state that ECM degradation plays pivotal role in gastrointestinal diseases, thus role of MMPs are evident [2, 3, 8]. Collectively, these enzymes are capable of degrading collagens, elastins, gelatin, matrix glycoproteins and proteoglycan as well as number of bioactive molecules.

According to the classification of proteases, based on their 3-D structure in the MEROPS database (http://merops.sanger.ac.uk), metallopeptidases may be classified into forty-six families. The families are further grouped into fourteen different clans based on metal ion binding motifs and 3-D structure similarities [9, 10]. MMPs are calcium-dependent, zinc containing endopeptidases [11]. The name is derived from consensus sequence and structural features, specifically a “HExxH” zinc-binding motif (zincin) and a C-terminal conserved methionine residue, which forms a conserved structure, called “met turn” [8]. MMP family comprises $\sim$27 member proteases characterized in humans, rodents and amphibians [12–14]. They were first described in vertebrates (1962), including humans, but are also found in invertebrates and plants. MMPs are secreted by a variety of connective tissues and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes. These enzymes are expressed as zymogens,

which are subsequently processed by other proteolytic enzymes (such as serine proteases, furin, plasmin and others) to generate the active forms through a cysteine switch mechanism.

MMPs are classified into collagenases, gelatinases, stromelysins and matrilysins depending on their specificity as depicted in Fig. 1. Another subclass of MMPs is membrane-type MMPs (MT-MMPs) that additionally contain a transmembrane and cytoplasmic domain [12]. The activities of most MMPs are very low or negligible in the normal steady-state tissues, and their expression is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell–cell and cell–matrix interactions [15] (Fig. 2).

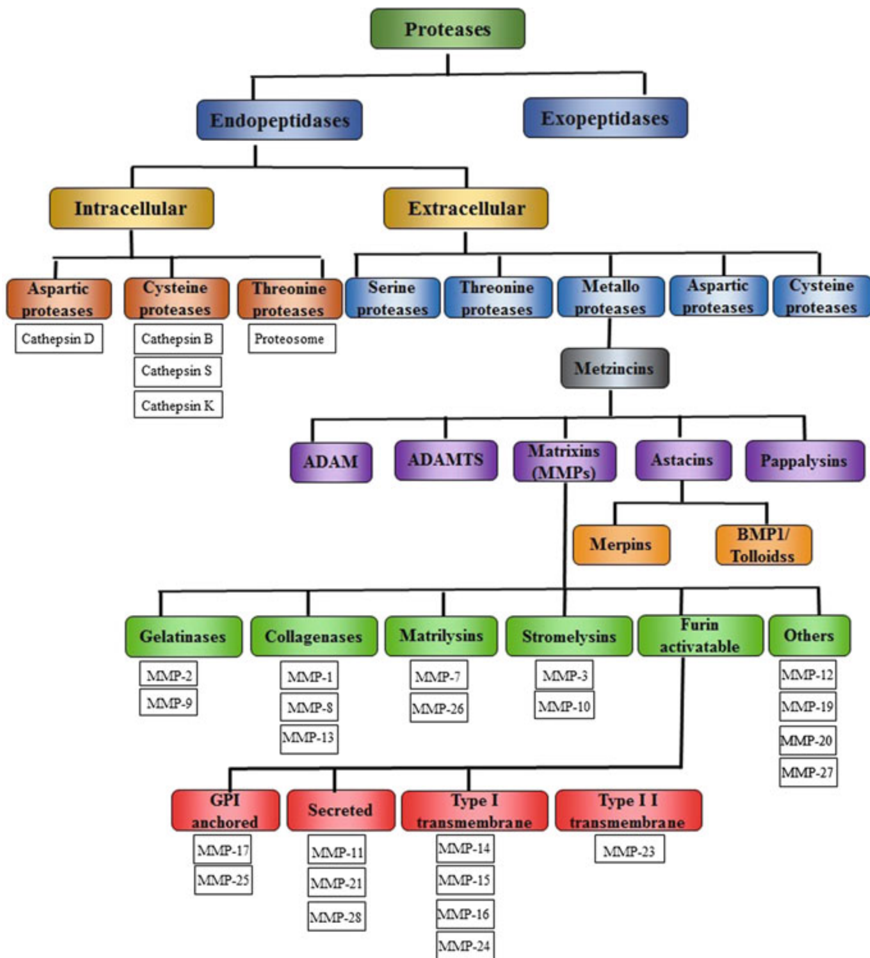

Fig. 1 Overview of the MMP family members and their evolutionary connection with other metzincin superfamily members

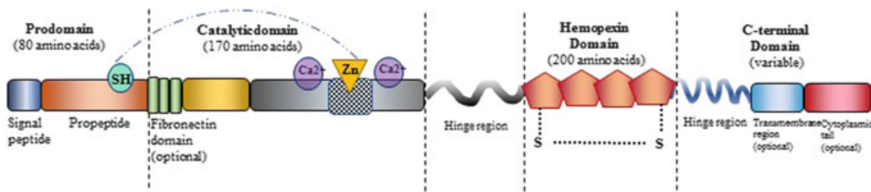

Fig. 2 Generalized domain structure and amino acid length of mammalian MMPs. MMPs contain a signal peptide followed by a propeptide which constitutes the pro-domain. It contains the conserved cysteine switch sequence, which makes a complex with the Zn^{2+} ion in the zymogen form of it. In case of only gelatinases (MMP-2 and MMP-9), the catalytic domain has a gelatin binding domain. Except for few MMPs (MMP-9, MMP-26, MMP-7), all other members contain a proline-rich hinge region followed by a hemopexin-like C-terminal domain, which helps in substrate recognition and its interaction with endogenous inhibitors. A major difference between secreted and cell surface anchored MMPs (MMP-14, MMP-15, MMP-16 and MMP-24) consists of intrinsic motif called transmembrane region and cytoplasmic tail

From the structural point of view, a typical MMP consists of approximately 80 amino acid long propeptide, about 170 amino acids catalytic metalloproteinase domain, followed by a linker peptide of variable length and a 200 amino acid long hemopexin (Hpx) domain (Fig. 3).

Among all the members of MMP family, MMP-7, MMP-26 and MMP-23 are the exceptions as they lack the Hpx domain along with the linker peptide, and MMP-23 has an additional cysteine-rich domain followed by an immunoglobulin-like domain after the metalloproteinase domain [16–19]. The signal peptide is removed during translation, and proMMPs are generated [20].

The activity of MMPs is very tightly regulated in the cell under normal physiological conditions. This regulation occurs at different levels; gene expression, proteolytic cleavage of the zymogens, transcription and inhibition of the active forms by various non-specific endogenous inhibitors such as α2-macroglobulin and specific tissue inhibitors of metalloproteinases (TIMPs) [1, 12, 13]. TIMPs inhibit active MMPs by forming 1:1 stoichiometric enzyme-inhibitor complexes leading to inhibition of their proteolytic activity [14, 15, 21]. TIMP-1, -2 and -4 are secreted, while TIMP-3 is sequestered to the ECM. The substrate specificity of TIMPs varies. A critical balance between MMPs and their endogenous inhibitors plays a pivotal role in vivo. Similar to MMPs, the proteolytic ADAM and ADAMTS family members are inhibited by specific TIMPs [18, 22, 23].

Reactive oxygen species (ROS) produced at the site of inflammation produced by activated neutrophils and macrophages has also a great influence on the function of MMPs [24]. These oxidants initially activate MMPs via oxidation of the pro-domain cysteine [25]. Eventually, MMPs may be inactivated by the enzyme myeloperoxidase secreted from inflammatory cells or by modification of catalytic domain amino acids by hypochlorous acid [26].

Detailed genetic and proteomic studies in experimental animals as well as in humans have provided insights into the involvement of MMPs in various disorders. The first human degenerative disease identified where MMPs were found to be

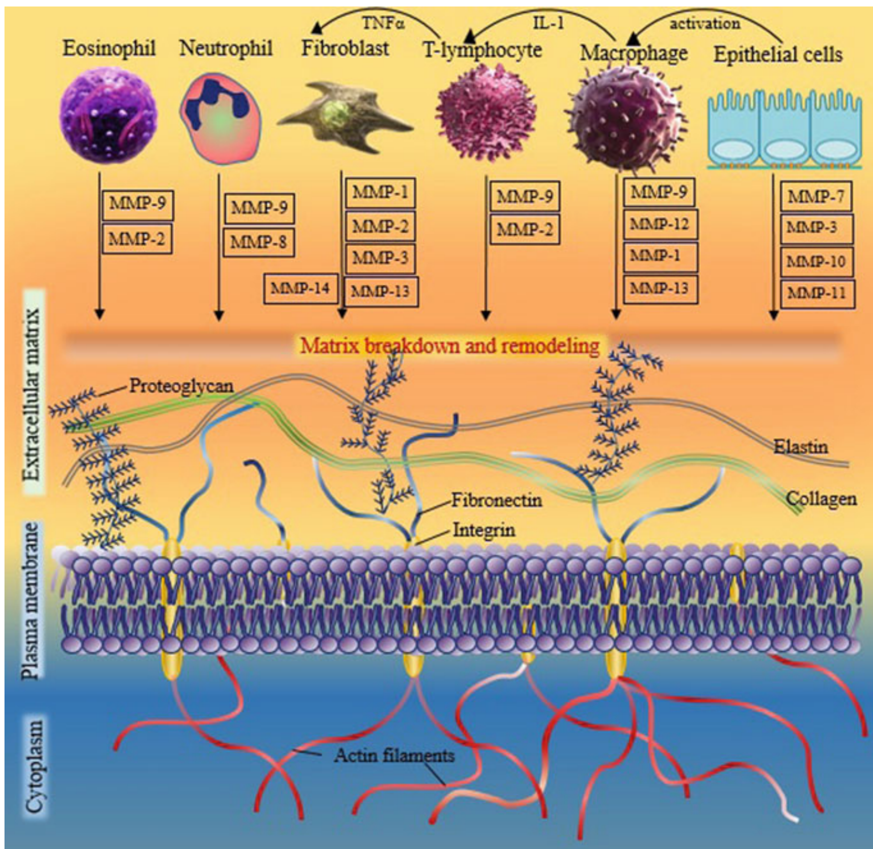

Fig. 3 Schematic diagram of intercellular signalling events that drive secretion of MMPs towards ECM during physiological and pathological conditions. Infiltrating immune cells secrete cytokines and MMPs simultaneously, which either activate other cells or degrade the ECM. These fragments of ECM components can mount the inflammatory response by a variety of events including immune cell chemotaxis, activation of receptors or chemokine ligands

linked was Sorsby's fundus dystrophy [27]. Stromelysin-1 knockout mice showed increased occurrence of collagen-induced arthritis. Several studies on MMP-null mice demonstrated impaired responses to pathological conditions. MMP-2, -7, -9 and -11 showed considerable [28, 29] influences on tumour progression and carcinogenesis in null mice. High expression levels of several MMPs have been correlated with tumour aggressiveness, stage and poor prognosis of various human cancers, but not always [29, 30]. MMPs are known to contribute to angiogenesis by degrading basement membranes, allowing for endothelial cell invasion, thus metastasis [25, 31, 32]. Abnormalities in ECM glycosaminoglycans and loss of glycosaminoglycans in epithelial basal lamina are detected in gastrointestinal inflammation like ulcerative colitis, peptic ulcers and Crohn disease [33, 34]. There

is also increased expression of stromelysins, matrilysins and collagenases, which suggests a strong correlation among inflammation and tissue injury. Literatures also suggest that mucosal immune system triggers the response through MMP-dependent pathways [35]. The extent of damage in gastric tissues due to breakdown of ECM by MMPs not only depends on the high expression of MMPs but also on the relative ratio of MMPs and TIMPs [23]. However, in some diseases like inflammatory bowel disease (IBD), there is evidence for overproduction of few MMPs [35].

2 Various Gastrointestinal Pathologies and Role of MMPs Therein

Gastrointestinal pathology is the subspecialty of surgical pathology that deals with the diagnosis and characterization of malignant, non-malignant, acute and chronic diseases of the digestive tract along with the accessory organs such as the pancreas, gallbladder, liver and intestine. MMPs play pivotal role in many gastrointestinal ailments like gastrointestinal mucositis, gastric ulcer, gastric cancer, colon cancer, pancreatic cancer, gallbladder cancer, hepatic cancer, colorectal cancer, etc. Detailed discussions have been provided in the following section.

2.1 Involvement of MMPs in Gastric Ulcer

Non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, stress, alcohol consumption, smoking and family history are considered as risk factors in the pathogenesis of gastric ulcer [36]. Mucosal tissue injury may lead to gastric ulcer, which is triggered primarily by ischaemia, along with depletion of nutrient delivery [37]. There are many endogenous aggressive factors (gastric hydrochloric acid, pepsin, reactive free radicals and oxidants, leukotrienes, refluxed bile and endothelins) which actually counterbalanced by the protective factors like gastric mucosal barrier, bicarbonate, mucosal blood flow, surface active phospholipids, prostaglandins (PG), nitric oxide (NO) and antioxidants [38, 39].

MMPs, especially proMMP-2 (72-kDa gelatinase A) and proMMP-9 (92-kDa gelatinase B) as well as their active forms, are associated with gastric injury [40]. Although MMP-2 appears to be constitutively expressed by many cell types in culture, MMP-9 expression is induced during gastric ulcer development [41]. In addition, MMP-1 and 3 are also upregulated in gastric and duodenal ulcers [42]. Other studies reported that NSAIDs increase MMP-9 activity and suppress MMP-2 activity during gastric ulcer. In chronic conditions, MMP-2 activity also gets upregulated with MMP-9. This suggests that MMP-9 expression is crucial for the development of gastric ulcers, but MMP-2 may be involved in the turnover of gastric ECM. Menges et al. reported the upregulation of MMP-1 and MMP-9 during *Helicobacter pylori* (*H. pylori*) infection in cultured cells [43]. Acetic

acid-induced experimental ulcer also showed the upregulation of MMP-9, but there were no significant change in the expression of MMP-2 [44]. In addition, infection can also influence the upregulation of MMP-1, -2, -3 and -7, but the mechanisms and pathways are not yet well understood. In contrast, in H_2O_2-mediated ulcers, MMP-2 activity and expression get downregulated [45]. Witzum et al. demonstrated that H_2O_2 alters the structure of MMP-2 by oxidation and catalytic domain inhibition [46, 47]. Singh et al. demonstrated that proMMP-9 along with pro and active MMP-2 gets upregulated in ethanol-induced gastric ulcer in experimental rats [48, 49]. Thus, the critical balance of MMP-9 and MMP-2 activities may be a determinant in the- progression as well as healing of gastric ulcer.

2.2 Role of MMPs in Gastric Cancer

Cancer is a multistage process, which requires various genetic and epigenetic changes in the tissue microenvironment. Alterations that occur during the malignant transformation are regulated by MMPs and their endogenous inhibitors TIMPs. Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide [50].

Different factors are having role and influence gastric cancer development and progression. Chronic inflammation or ulcer might be a linker for gastric cancer to many other types of malignancies for the future [51]. Studies of different surgical specimens showed chronic gastritis were more advanced in individuals with gastric cancer than in individuals with duodenal ulceration. It is now known that *H. pylori* is a major factor in both the induction of atrophic gastritis and histological progression to gastric cancer [52]. Literatures suggest that MMPs modulate the function of cytokines and chemokines and the consequences of functions of immunoregulatory cells during progression of gastrointestinal cancers.

Several knockout animal-based studies and case-control studies have confirmed that MMP-7 is an important member of MMP family that is upregulated in gastric carcinoma. Histology and immunohistochemistry revealed that it promotes tissue invasion and metastasis. Studies from our laboratory showed that single nucleotide polymorphism (SNP) in MMP-1 promoter at -519 A/G and MMP-3 promoter -375 C/G increases the risk of gastric cancer in Indian population [53]. Recently described downstream signalling molecules of MMP-7 include E-cadherin, Fas ligand and pro TNFα. E-cadherin is a cell adhesion molecule, which is responsible for the epithelial to mesenchymal (EMT) transition during cancer progression. Witty et al. reported that the colon cancer cells gained significant invasive potential when MMP-7 was transfected to them. Studies also emphasized the involvement of MMP-7 to the tumourigenicity and disease progression in malignant colorectal tumours [54]. Reverse transcription-polymerase chain reaction (RT-PCR) data revealed high expression of MMP-7 mRNA in the sentinel node lesions in patients with gastric carcinoma. In addition, SNP in MMP-7 promoter at -181 A/G increases the gastric cancer risk as reported from our laboratory [53, 55, 56].

In vitro studies on gastric cancer, cell lines demonstrated that the gene and protein levels of human epidermal growth factor 2 (HER 2) and MMP-9 are very tightly associated in the pathogenesis of gastric cancer [57]. Knocking down of HER 2 gene by shRNA significantly inhibited the invasion and metastasis of gastric cancer by downregulating the expression of MMP-9 while HER 2 overexpression again improved the MMP-9 transcription.

Yoo et al. found that signalling through sonic hedgehog pathway promotes the invasiveness of gastric tumours through activation of PI3 k/Akt pathway leading to EMT followed by MMP-9 activation [58]. Alakus et al. found that expression of MMP-2 was linked with the clinicopathological parameters in gastric cancer. High expression of MMP-2 from epithelial cells was associated with tumour stage and poor survival [59].

2.3 Specific Role of MMPs in Colorectal Cancer

Colorectal cancer (CRC) is a complex, multistage process, starts from neoplasia, followed by tissue invasion, vascular intra and extravasation and distant metastasis. The stromal cells of colon interact with the ECM and breakdown of ECM components is important for a cell to migrate from the primary site of tumour. Research on CRC has elucidated the role of distinct immune cells, cytokines and other immune mediators in virtually all steps of colon tumourigenesis, including initiation, promotion, progression and metastasis [60].

All groups of MMPs play role in the development as well as progression of CRC. The collagenases, i.e. MMP-1 and MMP-13 expressions were observed in the advanced stages of CRC with the lymph node involvement and poor prognosis. Huang et al. reported an approximately eightfold increased risk of post-operative recurrence in those patients who had MMP-13 overexpression [61]. There are studies on correlation of MMP-2 and -9 expressions with CRC and worse outcome. Patients having lymph node metastasis with CRC had an elevated level of plasma MMP-2 compared to the patients of early stage. Some reports also suggested serum MMPs as candidate biomarkers for CRC metastasis, as researchers have found higher ratio of expressions of MMP-2 and MMP-9 in CRC patients compared to normal subjects, and TGFβ is the key transcription factor responsible for MMP-9 expression [62, 63]. Elevated level of p38 gamma MAPK induces c-Jun synthesis, which in turn, increases the transcription of MMP-9, thus invasion in CRC [64]. TGFβ receptor kinase blocker was found effective to reduce MMP-9 expression and block CRC metastasis [65, 66]. In addition, MMP-7 was also found to activate proMMP-2 and proMMP-9 to promote lymph node metastasis, and upregulation of MMP-7 was found in ~80% of advanced stage of CRC [64]. MMP-7 knockout mice models of CRC demonstrated decreased tumour burden and reduced colon cancer multiplicity. Interestingly, MMP-12, also called the metalloelastase, was found to be protective in CRC and its inhibition was lethal in experimental animal models. Higher expression of MMP-12 inhibits distant metastasis by downregulating VEGF expression and angiogenesis in CRC [64].

2.4 MMPs in Inflammatory Bowel Disease

Ulcerative colitis and Crohn's disease both together called as inflammatory bowel disease IBD, which can affect any segment of the gastrointestinal tract. From the epidemiological survey, it was seen that genetic predisposition for IBD might play a role towards development of malignancy from IBD [67]. Epidemiological studies estimated the occurrence of IBD is 1 in 1000 individuals in western countries, but the rate is rising globally because of the lifestyle and diet [68]. Histologically, the disease is characterized by presence of granulomas, fibrosis in the tissue space along with fistulae [69].

The levels of several MMPs including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, MMP-13 are modulated during IBD pathogenesis in the inflamed colon mucosa or serum [70, 71]. Gene expression profiling demonstrated the transcriptional upregulation of MMP-1 with the severity of the disease and is linked with hypoxia inducing factor-1. Most importantly, the critical ratio of MMP-1: TIMP-1 gets altered with the severity of the disease [70]. The secretory MMP-9 mucosal expression level as well as serum antigen level was found significantly higher in ulcerative colitis patients compared to healthy subjects [72]. In vivo gelatinases double knockout mice model showed resistance from DSS, TNBS and *Salmonella typhimurium*-induced colitis [73]. In addition, MMP-12$^{-/-}$ mice were protected from TNBS-induced colitis [74]. Beside gelatinases, stromelysins (MMP-3 and MMP-10) were also found upregulated in the inflamed areas of IBD patients. SiRNA mediated silencing of MMP-3 confers protection from DSS-induced colitis [75]. A study in New Zealand patient pool on SNP showed that genes of MMP-3, MMP-8, MMP-10 and MMP-14 were associated with IBD [76].

Accumulating data from several studies indicated that IL-17A and IL-17F can act as inducers for the secretion of MMP- 1 and -3 in subepithelial myofibroblasts and also promote the actions of IL-1 and TNF- on these MMPs via MAPK mediated pathway [77]. The disruption in the protease–antiprotease balance of MMP: TIMP may also promote fibrosis in the intestine during the disease progression. In humans, the fibrosis in the gut is inhibited by TGFβ/Smad pathway where MMPs are downregulated and TIMP expression gets upregulated. MMPs regulate both pro- and anti-angiogenic factors which may contribute to the pathogenesis of IBD or mucosal healing. While new mechanisms are emerging for the IBD pathogenesis, it is crucial to understand the scenario where MMPs play significant role in mucosal healing, ECM remodelling, regulation of angiogenesis or immune response during disease pathogenesis.

2.5 Implication of MMPs in Crohn's Disease

Crohn's disease is a chronic inflammatory disease of the digestive tract and also falls under IBD. It affects the end of the small intestine, i.e. the ileum, but it may also affect other parts of the gastrointestinal tract and the entire thickness of the intestinal wall [78]. Epidemiology states that Crohn's disease affects approximately

3 per 1000 individual in western countries, and it is less common in Asia and Africa [68].

MMPs have been strongly implicated in the tissue injury in Crohn's disease. Recently, elevated expressions of MMPs have been found in the inflamed tissues of patients having Crohn's disease [79], which implies that there is a role of MMPs in the increased proteolysis in the mucosa, ulceration followed by inflammation and fistula formation. MMP-9 gets upregulated in the inflamed tissues, and MMP-9 transcripts were found only in the highly inflamed regions of the tissues [80]. MMP-3 levels were also found elevated in mononuclear macrophage-like cells and fibroblasts in patients [81]. There were no significant differences in MMP-2 expression reported. Downregulation of TIMPs is also very significant as TIMP-1, TIMP-2 and TIMP-3 level goes down during acute stage of the disease [82]; thus, disrupts the protease–antiprotease homeostasis. In addition, high MMP-3 expression was consistently found in fistulae in patients suffering from Crohn's disease. Microarray analysis of the inflamed tissue lysates showed that MMP-3 transcripts and proteins were localised particularly in large mononuclear cells as well as macrophages. Although MMP-10 falls under the same stromelysin group of MMP family with MMP-3, transcripts as well as expression of MMP-10 were found negative. Moreover, SNP in the promoter region of MMP-3 gene in 5A/6A position confers higher rate of promoter activity and increases the susceptibility of the disease [83] (Table 1).

3 Role of Microbiome in Gastrointestinal Ailments

Human beings are inhabited by a complex array of microorganisms that interact with each other and with the host. They, as a whole, represent an integrated and functional ecosystem (microbiota) that have important role in human health and disease. The composition and diversity of the microbiota vary among different normal individuals [84]. Herein, the latest findings on gastrointestinal microbiota, in relation to their composition and prevalence in the presence or absence of *H. pylori* infection are highlighted. It was a notion that stomach is a sterile organ due to several innate defences including acid secretion, migrating motor complexes, enterosalivary circulation of nitrate. However, there is a significant influence of microorganisms on stomach and intestinal microenvironment, both in physiological and pathological conditions (Fig. 4).

3.1 *Helicobacter pylori* Mediated Gastric Ulcer and Involvement of MMPs

Nobel laureate Robin Warren and Barry Marshall discovered the role of *H. pylori* in gastric ulcers in the year 1982 and considerable literature documented the presence of many acid-resistant strains, such as *Streptococcus*, *Neisseria*, *Lactobacillus* etc.

Table 1 Involvement of different MMPs with their activating cytokine/chemokine in different gastrointestinal pathologies and expressing cell types

Disease	MMP involvement	Activating factor	Expressing cell
Gastric ulcer	MMP-1, MMP-2, MMP-3, MMP-7, MMP-10, MMP-12 MMP-14	Cytokine factor	Migrating epithelial cell, connective tissue
H. pylori induced gastritis	MMP-2 MMP-9	NF κβ, IL-12 TNR	Fibroblasts, keratinocytes, endothelial cells, monocytes and macrophages express
Gastric cancer	MMP-3 MMP-8 MMP-9	NF-κβ	Gastric epithelial cells
Colon cancer	MMP-1 MMP-2 MMP-7 MMP-9 MMP-13	TGF-β, SMAD	Colon epithelial cells
IBD	MMP-1, MMP-2 MMP-3, MMP-7 MMP-9, MMP-10 MMP-13	IL-37, IL-1, NF-κβ	Colonic epithelia
Crohn's disease	MMP-3 MMP-9	IL 12, TNF-α	Granulated epithelial cell

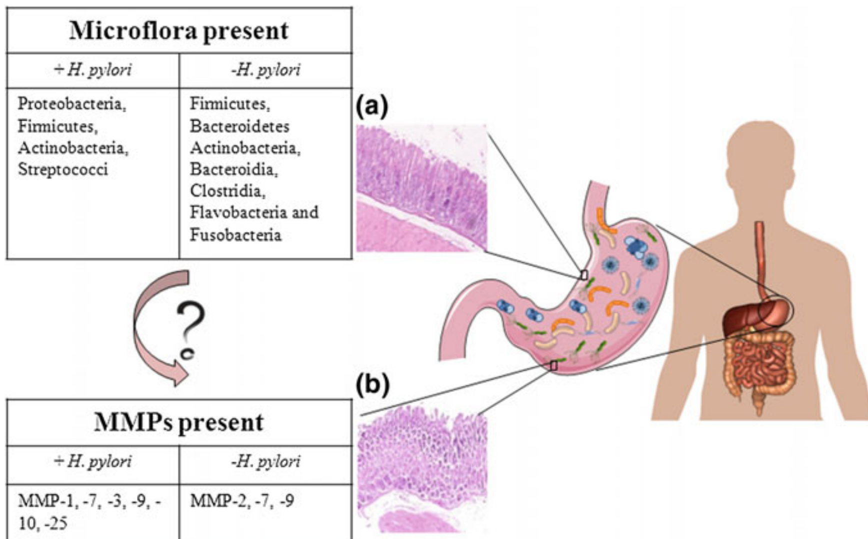

Fig. 4 Microbiome in human gut and MMPs expressed. Histology of the epithelium of stomach infested with pathogenic bacteria shows denudation and exfoliation of the layers in comparison with the control. Infiltration of inflammatory cells in the connective tissue is the benchmark of inflammation due to infection. The bacterial population and MMP expressed in presence and absence of *H. pylori* are shown in tables, but there is a gap in the information about the MMPs expressed in particular infection of the gastric system

[85–87]. *H. pylori* has been recognised as a Class I carcinogen [88]. There is enormous heterogeneity in the consequences of *H. pylori* infections. People acquire the infection early in life and are followed by a long quiescent phase when there is a chronic gastritis of variable intensity but with minimal symptoms [89]. Infection with *H. pylori* is not sufficient to induce gastric cancer, some other factors, i.e. bacterial and host cofactors are required to establish the disease [90, 91]. Only 10–15% of individuals infected with *H. pylori* develop ulcerative lesions in stomach, and the risk of gastric cancer is estimated to be approximately 1–3% [85, 92, 93].

Vitro studies suggest that *H. pylori* induce apoptosis of gastric epithelial cells and stimulate epithelial cells to secrete several chemoattractants [94]. Moreover, there is a marked increase in Th1-type cytokines, including IFN-γ, IL-12 and TNF-α in *H. pylori*-infected mucosa, all of which have been reported to be involved in tissue degradation in other systems [95, 96].

H. pylori infection induces the secretion of MMPs from a variety of gastric cells in vivo as well as in cultured cells, which in turn contribute to the pathogenesis of gastric ulcer and gastric cancer [97–99]. Gastric epithelial cells appear to be the major source of MMPs in *H. Pylori*-infected gastric tissues [100, 101]. A recent hospital-based study on gastric cancer patients with *H. pylori* infection revealed that the infection upregulated the expression of MMP-1 and MMP-10 [102, 103]. MMP-1 predominantly degrades the stroma, which is linked with invasion and metastasis [23]. In addition, microarray analysis of uninfected human gastric epithelial cell line (AGS) and *H. Pylori*-infected co-cultures demonstrated that along with MMP-10, several other MMPs, such as *MMP-1*, *MMP-7*, *MMP-25* genes were also upregulated [104, 105]. Among them, MMP-10 showed significant increase in expression in comparison to other MMPs [103].

3.2 Human Gastric Microbiota and Gastric Disorders

The gastrointestinal tract is the most populated organ in human body. Different sites of the GI tract are inhabited by different microbiota, including the stomach [106]. The very low pH value (median pH 1.4) of stomach makes it a very harsh and hostile environment for bacterial growth. Thus, the microbial colonization in stomach is very less (10^2–10^4 colony forming units (CFU)/g) compared to colon (10^{10}–10^{12} colony forming units (CFU)/g) [107]. The major constituent of human gastric microbiota is the Proteobacteria *H. pylori* although many other bacteria can also survive in this hostile environment, making it more diverse and complex. Along with the host physiology various other factors, including diet, *H. pylori* infection, enteral feeding, proton pump inhibitors, antibiotics and diseases shown to contribute in shaping the gastric microbiota [108]. With the advances of the DNA-based sequencing technologies, the culture-independent survey of human gastric microbiota is possible based on the analysis of a gastric biopsy sample. In 2013, Sheh and Fox summarized in their study that in stomach the most commonly found phyla are Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria. The most abundant phyla are Proteobacteria, Firmicutes and

Actinobacteria for *H. pylori* positive samples, while Firmicutes, Bacteroidetes and Actinobacteria are most abundant phyla for *H. pylori*-negative samples [109]. Interestingly, *H. pylori* being the most dominant bacteria comprise of 72–99% of sequencing reads in the stomach [110, 111]. Other commonly identified genera are of Streptococcus, Prevotella, Veillonella and Rothia species [112]. Surprisingly, the correlation was found only between the presence of Streptococci and peptic ulcer disease [113]. Another study compared gastric microbiota profile according to *H. pylori* status in chronic gastritis patients using high-throughput 16S rRNA sequencing. The microbiota of *H. Pylori*-negative patient sample was represented by the member of bacterial class alpha-, beta-, gamma-proteobacteria, bacilli, bacteroidia, clostridia, flavobacteria and fusobacteria [114].

The model proposed by Correa postulated that the chronic *H. pylori* infection of the gastric mucosa progresses through different stages like chronic active gastritis, intestinal metaplasia, dysplasia to subsequent development of gastric cancer [115]. Nonetheless, it has been clearly identified that *H. pylori* is the major risk factor in gastric cancer development [116, 117].

Other studies suggested that there is a gradual shift in the composition of gastric microbiota, which might plays a key role in the progression of pre-malignant lesions to gastric carcinoma. Additionally, gastric cancer samples showed decrease in the relative abundances in bacteria belonging to the phyla Proteobacteria, namely *Neisseria* spp, *Haemophilus* spp, *Bergeriella denitrificans*, *Epsilonproteo*bacteria and Helicobacteriaceae [114, 115] as well as Bacteroidetes (*Porphyromonas* spp and *Prevotella pallens*). The bacteria within the phyla Firmicutes are increased (Streptococcaceae, Lachnospiraceae and *Lactobacillus coleohominis*) [114, 115] or decreased *Streptococcus sinensis* [115].

4 Targeting MMPs as Therapeutic Strategy

Protease inhibitors are essential tools for the investigations of MMPs activities. They are useful not only for assessing the activity but also for inhibition of unwanted proteolysis in an experimental system. People started working on MMPs after proving its role on cancer stage, patient prognosis and death. Almost every pharmaceutical company started manufacturing MMP inhibitor (MMPI) to block MMP-mediated angiogenesis and metastasis. The programme started about 25 years ago and led to a number of small-molecule inhibitors in phase III clinical trials [25, 118, 119].

Chelating agents such as EDTA and 1,10-phenanthroline are routinely used in the laboratories to block MMP activities in in vitro experiments. Synthetic inhibitors commonly contain a chelating moiety, such as a carboxyl, a thiol, a phosphorous or a hydroxamic acid group. The chelating group is attached to a series of other groups that fit the specificity pocket of a particular metallopeptidase [120].

4.1 Endogenous MMP Inhibitors: TIMPs

TIMPs contain an N- and C-terminal domain of $\sim$125 and 65 amino acids, respectively, with each containing three conserved disulfide bonds. The N-terminal domain folds as a separate unit and is capable of inhibiting MMPs. However, their range of activities is broader as it inhibits several disintegrin-metalloproteinases, namely ADAMs and ADAMTSs. Pathological conditions are associated with imbalanced MMP activities due to altered TIMP levels as important factor. Structural studies of TIMP-MMP complexes have allowed the generation of TIMP variants that selectively inhibit different groups of metalloproteinases. Engineering such variants is complicated by the fact that TIMPs can undergo changes in molecular dynamics induced by their interactions with MMPs. TIMPs are involved in cell growth and differentiation, cell migration, anti-angiogenesis, anti- and pro-apoptosis and synaptic plasticity [120, 121].

4.2 Antibody-Based Inhibition Targeting Catalytic Domain

Reports are available on the use of functional blocking antibodies, which have high potency for MMPs. Several functional blocking antibodies have been developed that selectively target the membrane-anchored MMPs. Combining a human antibody phage display library with automated selection and screening strategies resulted in the identification of a highly selective antibody-based MMP-14 inhibitor called DX-2400. It displayed anti-invasive, antitumour and anti-angiogenic properties and blocked MMP-14 mediated pro-MMP-2 processing [122]. To date, at least two monoclonal antibodies have been tested which bind to the catalytic domain, without interacting with the catalytic zinc. DX-2400 is a MMP-14 specific inhibitor, which binds to the catalytic domain with a K_i in the sub-nanomolar range.

Thus far, preclinical studies of DX-2400 indicated that the antibody is capable of inhibiting all of these activities while there was no measurable effect on other MMPs. In mouse studies, the drug was observed to decrease tumour burden significantly and decreased metastases in lung and liver. Further, DX-2400 was effective against HER2-positive xenografts both when used as a single agent or in combination with paclitaxel. This marks DX-2400 as an attractive candidate for patients diagnosed with triple-negative breast cancer, although clinical trials for this therapeutic have not yet been initiated [118, 122].

Other groups developed selective MMP-14 inhibitory antibodies that were successfully tested in vitro and in vivo. The neutralizing monoclonal antibody REGA-3G12 acts as a selective inhibitor of MMP-9 by binding against catalytic domain but not against the fibronectin or zinc-binding domains. A murine monoclonal antibody, termed REGA-3G12, has also been generated by hybridoma technology against the catalytic domain of human MMP-9. This inhibits MMP-9 without affecting activity of MMP-2, which shares high homology. Therefore, a therapeutic that can differentiate between these two highly similar enzymes may prove very useful [120]. In this regard, blocking antibodies were found to act on

specific functions of the MMP rather than the general proteolytic activity. For example, 9E8 monoclonal antibody targets the MMP-2 activation capacity of MT1–MMP rather than the general proteolytic activity [118, 121].

The mechanism for TIMPs to inhibit MMPs can be utilized to develop different antibody-based strategies for effectively targeting the in vivo activity of MMP. A neutralizing antibody was developed based on the three-dimensional structure and amino acid sequence of MMP-13, which bind only to the active form of MMP-13. Monoclonal antibody against MMP-2 exhibited inhibition over MMP-2 activity but did not affect the structurally similar MMP-9 activity [121].

4.2.1 Hemopexin Domain Inhibitor and Small Molecule Inhibitor of MMPs

The interaction of inhibitor with the hemopexin-like domain prevents binding of endogenous partners that can promote angiogenesis or cancer cell migration. The hemopexin domain among different MMPs exhibits significantly less sequence and structural homology compared to the catalytic domain. This domain comprises a succession of four structurally similar hemopexin-like repeats to create a central funnel-like tunnel. Each hemopexin-like repeat is made up of four β-strands; the first three β-strands bear the highest homology across the MMP family whereas the b4 strands bear the least. As many as four structural ions have been found to be coordinated within this tunnel, and it has been proposed that these ions confer a stabilizing function for the whole domain. MMP-9 has only a sodium ion and displays a flexible architecture and considerable deviation from the structure of hemopexin domains reported for other MMPs. The first ion binding position, which is closest to the linker region connecting the hemopexin domain to the catalytic domain, is generally either a sodium or calcium ion [122].

In silico analysis of the MMP-14 hemopexin domain identified a druggable pocket-like site in the centre of the hemopexin structure. Binding of small molecule compounds in this site should, in theory, allosterically block dimerization. Compounds which bind to the hemopexin domains and prevent dimerization have been shown to significantly decrease tumour size, reduce MMP-mediated cell scattering/invasion, angiogenesis, and tumour metastasis both in vitro and in animal models. A subsequent docking study of small-molecule compounds led to identification of a compound which is selective for MMP-14 compared to MMP-2 and was not cytotoxic and did not affect catalytic activity (including MMP-14-mediated activation of MMP-2). Report is there for inhibition of hemopexin domain that was effective in attenuating cancer cell migration and in vivo-reduced tumour volume. In another study, an inhibitor was made with no proteolytic or cytotoxic effects while significantly decreased cancer cell migration and invasion and significantly decreased tumour size as well as the number of metastases in vivo [120, 123, 124].

Bimodal approach confers increased selectivity for MMP-2 as compared to individual subunit. A fusion protein has been designed which links the ten amino acid sequences of a MMP-2 selective inhibitory peptide (APP-IP, a β-amyloid precursor protein) to the N-terminus of TIMP-2. This macromolecular protein,

which binds with a K_i in the sub-picomolar range, is designed to interact with both the active site and the hemopexin-like domain of MMP-2 [125–127].

In addition, small peptides have been used successfully to block dimer-induced functions of MMPs. Owing to induced intracellular cytoskeleton rearrangements necessary for processing of migration and invasion machinery, MMP-14 homodimerizes and also heterodimerizes with CD44. This includes proteolysis of proMMP-2 by MMP-14. A number of peptides generated that mimic the sequence of the residues of hemopexin-like domains required for dimerization were able to reduce tumourigenic effects in vitro and in vivo [128] (Table 2).

Table 2 Synthetic MMP inhibitors and their application in different diseases

Inhibitor name	MMPs inhibited	Disease model	Clinical trial	Reference
Batimastat (BB-94)	Broad-spectrum MMPs	Various tumours	Yet to be approved	[129]
Neovastat (AE-941)	MMPs 2, 9, 12; VEGFR-2	Renal cell carcinoma, non-small cell lymphoma	Phase III of clinical trial	[130]
Prinomastat (AG-3340)	MMPs 2, 3, 9, 13, and 14	Renal cell carcinoma	Phase III of clinical trial completed	[131]
Rebimastat (BMS-275291)	MMPs 1, 2, 8, 9, and 14	Advanced non-small cell lung cancer	Phase III of clinical trial	[132]
Marimastat (BB-2516)	Broad-spectrum MMPs	–	Development terminated due to poor performance in clinical trial	[133]
Ilomastat	MMPs (1–3, 8, 9)	–	–	[118]
Doxycycline hyclate (Dermostat, Periostat) [CollaGenex Pharmaceuticals]	Collagenase	Periodontal disorders	Launched	[127]
		Rosacea	Phase III	
		Acne	Phase II	
AZD 8955 [Astra Zeneca]	Collagenase	Osteoarthritis	Phase II	
PCK 3145 [Ambrilia Biopharma]	MMP9	Prostate cancer	Phase II	
Apratastat [Amgen/Wyeth]	MMP1, MMP9, MMP13, TACE	Rheumatoid arthritis	Phase II	
Incyclinide [CollaGenex Pharmaceuticals]	MMP2	Acne	Phase II	
		Brain cancer	Phase II	
		Kaposi's sarcoma	Phase II	
		Cancer metastases	Phase I	
		Solid tumours	Phase I	

(continued)

Table 2 (continued)

Inhibitor name	MMPs inhibited	Disease model	Clinical trial	Reference
ABT 518 [Abbott Laboratories]	Unknown	Solid tumours	Phase I	
MPC 2130 [Myriad Pharmaceuticals]	Unknown	Cancer	Phase I	
		Haematological malignancies	Phase I	
MMP12 inhibitor [Merck]	MMP12	Multiple sclerosis	Phase I	
AS111793 [Serono Pharmaceutical Research Institute (Geneva, Switzerland)]	MMP12	Reduces airway inflammation in mice exposed to cigarette smoke	–	[134]

5 Limitations/Challenges for MMPs Inhibition

Although several preclinical research that supported the importance of MMPs in cancer, all Phase III cancer trials using different inhibitors of MMPs are failed unfortunately. The major reason is lack of specificity of inhibitors and insufficient knowledge on the complexity of cancers [118]. The information of preclinical studies in the mouse models and the clinical trials in patients varied much that might be the reason behind the failure of the clinical trials as well as adverse effect on patients. The adverse effects were mostly due to their broad-spectrum inhibition of MMPs and the cross-inhibition among other family of proteins, e.g. ADAM (a disintegrin and metalloproteinase) and ADAMTS (ADAMs with thrombospondin motifs). In addition, several MMP inhibitors were neither metabolically stable, nor orally bio-available and toxic as well. In mammals, MMP genes are conserved indeed and are essential for normal functioning of the organism. It is worth mentioning that MMPs activities are not always harmful but becomes detrimental with its anti-targets actions in other physiological conditions. In contrast to other proteases (like caspases), most MMPs contain conserved amino acid sequences having high homology in the substrate binding domain which hinders the fabrication of specific substrate-based inhibitors. Interestingly, the evolution of many MMPs occurred by gene duplication in the mammalian genome, that leads to the formation of MMP genes clusters on particular chromosomes (for example, the chromosome-9 in the proximal mouse harbours ten MMP genes in less than 500 kb) and possess widespread homology in their amino acid sequence. As a consequence, translating in vitro research work with in vivo applications remains a difficult task. In vitro studies with active MMP and any protein are resulted in cleavage at particular sites. However, this cleavage might not essentially occur in an in vivo condition in physiological system. Scientists had attempted in knocking out many MMP-coding genes in mouse models (as in vivo systems) to investigate the consequences of the absence of these genes. However, not much has been evaluated

in tissue-specific knockout mice as there is no obvious phenotypic abnormality in unstimulated conditions in most MMP-deficient mice (except for MMP-14 and MMP-20 deficient mice). Moreover, insufficient knowledge on the spatiotemporal activities of MMPs in pathological conditions adds to the unsuccessful attempts for clinical trial of MMP inhibitors.

6 Future Directions

A plethora of literature as well as supporting data revealed that MMPs play crucial roles in both physiological and pathological processes. They could be exploited as independent prognostic factors in gastrointestinal inflammation and malignancies. MMPs are associated with multiple diseases; hence they can be considered as drug targets to treat those diseases. A number of studies from knockout mice and in vitro cultured cells have shown that their involvement as integral part in acute as well as chronic inflammation. The major task for the future is to design specific MMP inhibitors and to elucidate the crosstalk among the members of MMP family. Newer activity-based imaging probes specific for MMPs will facilitate the elucidation of the structural role of inhibitors in gastrointestinal disorders. Although, clinical trials with the therapeutic MMP inhibitors encountered several challenges, studies in both in vivo and in vitro are in progress to target the specific MMP in gastrointestinal pathologies. Interaction between different transcription factors and different MMP promoters provides valuable insights into the mechanism of disease progression. Inhibition to specific MMP in gastrointestinal disorders and its effect at multiple cellular pathways become formidable task for therapeutic use. Although, monoclonal antibody-based therapy is promising for the prognosis and therapy of gastrointestinal cancers, however, its validation in experimental knockout animals and cancer models is prerequisite. Tailor-made therapies and drugs based on set of specific MMP in gastric disorders of different individual could be useful to develop good quality drug. Moreover, development of assay tool against a set of MMPs may lead to formulate commercially viable kits for early prognosis of gastrointestinal diseases using patient serum. Nonetheless, a role for MMPs in pathology of gastrointestinal tract could be related to tissue-specific expression and function of MMPs and be exploited as target for therapies.

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Role of Proteases in Diabetes Mellitus

Shatadal Ghosh, Bhawna Pandey and Parames C. Sil

Abstract

Dipeptidyl peptidase-4 (DPP-4), a 110 kDa exopeptidase, selectively cleaves N-terminal dipeptides from a vast array of substrates. DPP-4 is expressed on the surface of many cell types and plays various important roles in diseases like cancer, inflammation, diabetes, obesity. In type 2 diabetes mellitus (T2DM), incretin hormones, glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP) play major roles in the regulation of insulin secretion. Both GLP-1 and GIP are the substrates of DPP-4. That is why DPP-4 inhibitors have gained significantly increasing interest in treating T2DM recently. In addition to some general information on DPP-4, this chapter mainly describes its effects on relevant organs associated with T2DM and recent clinical trials. Besides, roles of some other proteases in diabetes mellitus have also been briefly discussed.

Keywords

Dipeptidyl peptidase-4 (DPP-4) · Incretin hormones
Glucagon-like peptide-1 (GLP-1) · Glucose-dependent insulinotropic polypeptide (GIP) · Clinical trials

S. Ghosh · B. Pandey · P.C. Sil (✉)
Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M,
Kolkata 700054, West Bengal, India
e-mail: parames@jcbose.ac.in; parames_95@yahoo.co.in

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1 Introduction

Diabetes mellitus (DM) becomes one of the most common chronic, non-communicating diseases nowadays. DM is developed when insulin, responsible for the regulation of blood glucose, is either not produced, produced in insufficient amounts, or when the body cannot use insulin properly. Diabetes is mainly of three types: type 1 (T1DM), type 2 (T2DM), and gestational diabetes (GD). However, hyperglycaemia is common in all three types. If left untreated, sometimes even treated, DM generally accounts for long-term tissue damage throughout the body that explains the numerous complications associated with this disorder [1]. Acute complications include diabetic ketoacidosis (DKA) and nonketotic hyperosmolar state (NKHS). Vascular and nonvascular disorders are the main chronic symptoms [2–4]. In a global study in 2013, it is revealed that almost 382 million people are suffering from diabetes for a prevalence of 8.3%.

A major problem in the health care of diabetic patients is diabetic foot syndrome. Growth factors and proteases regulate the essential processes of normal wound healing [5, 6]. The diabetic foot syndrome occurs as a severe complication in the late stages of diabetes and is considered to be one of the most important complications of this disease. Diagnosis, classification, and proper therapy can help in reducing this problem. The rational development of treatments could be achieved only with the understanding of the cellular and molecular abnormalities that contribute to the diabetic foot syndrome.

As such to develop therapeutics and better understanding of the role of proteases, their detection becomes very important. Chromogenic and fluorometric substrates can be used for detection of protease activity. Immunoblotting or ELISA is also used for their detection. The least common methods for detection of these proteases are immunonephelometry, radial immunodiffusion, and immunoelectrophoresis. Also, degradomics is being used as an approach for screening the activity of proteases. The degradome represents the complete set of proteases expressed in certain medium at a specific time. Zymography and mass spectrometry or both are the approaches taken to serve the goal of degradomics.

Proteases not only play roles in catabolism of amino acids, but also perform vital roles involving the fate, functions, and interaction of proteins, leading to the regulation of different important cellular phenomenon including cellular signalling, transcription, and translation [7, 8]. Abnormalities in specific protease activity manifest in many pathologic conditions including DM. Proteases play vital roles in various biological processes including cell proliferation and differentiation, wound healing, tissue remodelling, angiogenesis, inflammation, immunity, autophagy, and cell death; all of which can be impaired, decreased, or augmented in DM [4, 6, 9–15]. So, these proteases might play a role in diabetic pathogenesis and associated complications. The present understanding, however, is limited due to poor data availability, although dipeptidyl peptidase-4 (DPP-4) inhibitors have been studied in some details [10, 11, 16–19]. DPP-4, also known as CD26, is a 110 kDa type II transmembrane glycoprotein [1–3]. Its inhibition has been shown to prolong

biological activity of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) which are incretins by nature and can stimulate a decrease in blood glucose levels. The approval of DPP-4 inhibitors for the treatment of type 2 diabetes mellitus (T2DM) has raised its importance in the pharmaceutical world. The role of proteases in diabetes also includes the association of single nucleotide polymorphisms (SNP)/genes with increased risk and protease profiling. Protease profiling, however, becomes important because of diagnostic and/or prognostic values.

The first part of this chapter basically deals with some general information about DPP-4 and its numerous biological functions, mainly protease activity, in regard to T2DM and its treatment. The other part describes an up-to-date understanding of the roles of DPP-4 and its inhibitors on the pathophysiology of T2DM. Some recent clinical studies are also discussed briefly.

2 Dipeptidyl Peptidase-4

A multifunctional protein, dipeptidyl peptidase-4 (DPP-4), shows a vast array of biological activities including: protease activity [1], binding with adenosine deaminase (ADA) [2] controlling viral entry through cell surface co-receptor [3], interaction with the extracellular matrix [4], and cell migration and proliferation [5]. The pleotropic DPP-4 action is further amplified by the variety of DPP-4 substrates that act as important biochemical messengers in many tissues.

2.1 Discovery, Genomic Organization, and Superfamily of related Enzymes

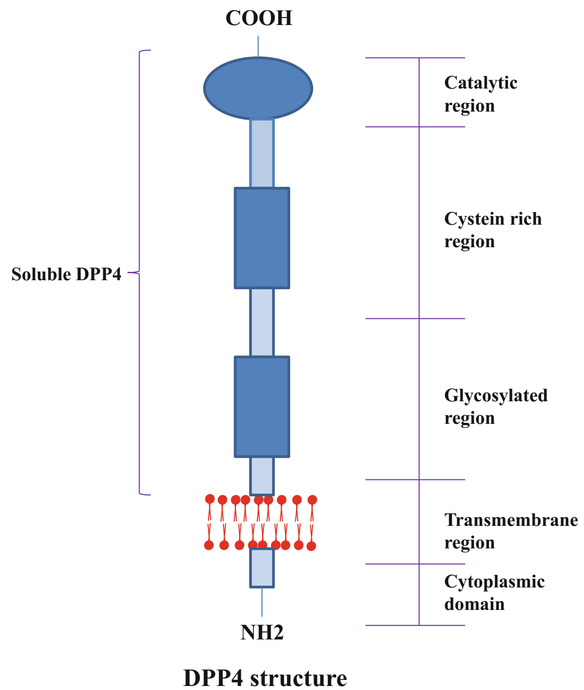
DPP-4 was discovered in 1966. This aminopeptidase was observed to have unique substrate characteristics [16]. In 1992 [20, 21], 70-kb human gene of DPP-4 was identified. This gene is situated on the long arm of chromosome 2 (2q24.3) comprising of 26 exons that encodes a 766-amino acid protein. The serine protease catalytic site is encoded by two exons (exons 21 and 22).

DPP-4 is a member of the serine peptidase/prolyl oligopeptidase gene family. This can be alternatively subclassified as fibroblast activation protein (FAP)/seprase, the membrane-boundpeptidases, etc. The catalytic activity site within the C-terminal region of these related enzymes is highly conserved both in prokaryotes and eukaryotes. But the other sequences may differ significantly [22].

2.2 Molecular Function

DPP-4 transmits signals across cell membranes and interacts with other membrane proteins (Fig. 1). Interestingly, most of the protein is extracellular, including the

Fig. 1 DPP4 structure



C-terminal catalytic domain, a cysteine-rich area, and a large glycosylated region linked by a flexible region to the transmembrane segment. Only six N-terminal amino acids of DPP-4 are anchored into the cytoplasm. The active site was found to be Ser 630 which is flanked by the serine peptidase motif Gly-Trp-Ser630-Tyr-Gly-Gly-Tyr-Val [23, 24]. It was found that dimers are the predominant species [25]. Further studies confirm that monomeric form shows less activity and homodimerization significantly increases DPP-4 activity [26]. Crystal structure also demonstrated that two soluble homodimer can also form tetramers.

Membrane-bound DPP-4 can interact with several signalling molecules including ADA [27], CXCR4 receptor, caveolin-1 [28], the Na^+/H^+ exchanger, and the T cell antigen CD45 [29], and initiate intracellular signalling. The soluble form of DPP-4 (sDPP-4) was first identified in serum and saliva [30]. Later, it was also found in cerebrospinal and seminal fluid and bile. sDPP-4 can also activate intracellular signalling pathways and increases the proliferation of human lymphocytes.

2.3 Regulation of DPP-4 Expression

Regulation of DPP-4 expression is a complex phenomenon. It includes control at the level of both transcription and translation. The *DPP-4* gene lacks conventional TATAA or CCAAT promoter sequences rather contains a cytosine/guanine-rich

promoter region [31]. Cell surface and intracellular DPP-4 expression is highly regulated though the control of DPP-4 shedding, which in turn increases the levels of the circulating DPP-4 (sDPP-4) form. Studies showed high activity of sDPP-4 in spleen, lung, thymus, and liver; and medium activity in the intestine, aorta as well as in bone marrow.

2.4 Post-translational Modifications of DPP-4

DPP-4 may go through N-glycosylation or N-terminal sialylatio. There are 8–11 potential N-glycosylation sites available on DPP-4 that contributes to its folding and stability [32] but not any other activity. N-terminal sialylation, on the other hand, facilitates trafficking of DPP-4 to the apical membrane [33].

2.5 DPP-4 Substrates

DPP-4 activity was first investigated in hydrolysis of dietary prolyl peptides. Numerous studies confirm that DPP-4 can cleave dozens of peptides (such as neuropeptides, chemokines, and regulatory peptides) containing a proline or alanine residue at position 2 from the amino-terminal region. Moreover, peptides having position 2 replaced with alternate residues (such as hydroxyproline, threonine, valine, dehydroproline > alanine > glycine, or leucine) at the penultimate position are also cleaved by DPP-4. This phenomenon indicates to a stereochemical preference [34].

The following are some important substrates for DPP-4 peptidase:

I. Physiological peptide substrates: These are intact peptides and/or DPP-4 cleavage products detected at different levels in experiments with wild type versus *DPP-4* knockout animals or human subjects treated with a selective DPP-4 inhibitor.
II. Pharmacological substrates: These are substrates shown to be cleaved at penultimate residue, predominantly in vitro.

2.5.1 Glucagon-Like Peptide-1 (GLP-1)

GLP-1 is secreted from L cells in the distal portion of the intestinal tract. It is derived from proglucagon after post-translational cleavage by prohormone convertase (PC)1/3. It is a neuropeptide and an incretin. There are two biologically active forms of GLP-1: GLP-1-(7-37) and GLP-1-(7-36) NH2. These are produced as a result of selective cleavage of proglucagon. Intact GLP-1 functions as an enhancer of glucose-stimulated insulin secretion. Moreover, it suppresses glucagon secretion, appetite, and gastric emptying [35]. DPP-4 cleavages this incretin and thereby destroys its above-said activities.

2.5.2 Glucagon-Like Peptide-2 (GLP-2)

GLP-2 is a 33-amino acid incretin co-secreted with GLP-1 from L cells in the distal bowel. HPLC analysis has detected both GLP-2 (1-33) and its DPP-4 cleavage product GLP-2 (3-33) in rat ileal [36]. A DPP-4 inhibitor (Val-Pyr) completely abolished formation of GLP-2 (3-33) in human plasma in vitro [37].

2.5.3 Glucose-Dependent Insulinotropic Polypeptide (GIP)

GIP is a 42-amino acid peptide. It is derived mainly within K cells in the duodenum and proximal jejunum from preproGIP after post-translational processing by PC1/3. GIP is also expressed in α-cells of the islet via PC2 processing. It stimulates insulin secretion. It is a physiological substrate for DPP-4 that cleaves GIP at specific site producing a dipeptide (Tyr-Ala) [35, 38].

Both GLP-1 and GIP increase the level of insulin. Moreover, they can also promote β-cell proliferation and suppress β-cell death [39, 40].Studies showed the blood glucose lowering effect of exogenously administered GLP-1 in many other organs like liver, adipose tissue, brain, and intestine [41]. It can also enhance glucose sensitivity of β-cell and induce glucose competence in previously unresponsive β-cells [42]. Besides, GLP-1 can also destroy hepatic glucose production [43]. In spite of many beneficial effects, GLP-1/GIP treatment is limited mainly because of its short half-life. The half-life of GLP-1 and GIP is less than 2 min and 7 min in human, respectively [39]. This is because DPP-4 acts on and thereby converts them into their inactive forms. This DPP-4 activity was first reported in early 1990s [17] and later confirmed in vivo (Fig. 2) [18].

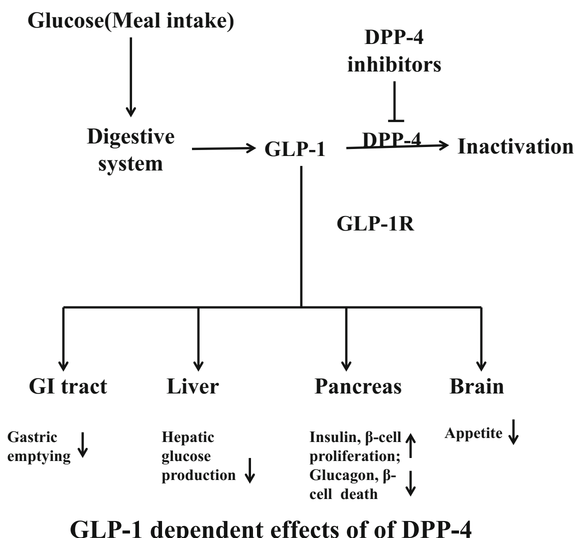


Fig. 2 GLP-1 dependent effects of DPP-4

2.5.4 Glucagon

Glucagon is a peptide hormone produced by α-cells of the pancreas and stimulates hepatic glucose production. Mass spectrometry analysis revealed that incubation of purified porcine DPP-4 with glucagon in vitro resulted in the formation of glucagon (3-29) and glucagon (5-29). In human serum glucagon can also be metabolized to glucagon (3-29) ex vivo. However, Ile-thiazolidide, a DPP-4 inhibitor, prevents the metabolization [19]. In spite of this, the metabolization of glucagon in porcine plasma is not affected by another DPP-4 inhibitor valine-pyrrolidide [44]. Overall, the data available so far lead to the conclusion that glucagon is a pharmacological but not a physiological substrate.

2.5.5 Gastrin-Releasing Peptide (GRP)

GRP is a 27-amino acid peptide and a substrate for DPP-4 which produces GRP (3-27) and GRP (5-27) [45]. It has been observed that DPP-4 inhibitor valine-pyrrolidide when administered orally before infusion of GRP causes a 25% increase in the acute insulin response to i.v glucose in anesthetized mice [46].

2.5.6 Brain Natriuretic Peptide (BNP)

BNP is synthesized as a 134-amino acid precursor (preproBNP). It is then processed to a 108-residue proBNP (1-108) that is cleaved by furin or corin to BNP (1-32). BNP (1-32) promotes natriuresis through engagement of natriuretic peptide receptor type A. BNP (1-32) is cleaved by DPP-4 to a number of related metabolites. However, there are limited data on how DPP-4 inhibitors react on BNP [47].

2.5.7 Erythropoietin

Erythropoietin is a 166-amino acid protein. It has a proline at position 2 that makes it an ideal substrate for DPP-4. It was observed that hematopoiesis in response to radiation or chemotherapy was induced by genetic or pharmacological reduction of DPP-4 activity in mice. However, present understanding cannot confirm whether DPP-4 inhibition induces erythropoietin-dependent hematopoiesis in humans, and available data only indicate that erythropoietin may be a physiological DPP-4 substrate [50].

2.5.8 Eotaxin

Eotaxin is a chemokine, i.e. small peptides with chemoattractant properties. CC motif chemokine (CCL) 11, a 74-amino acid chemokine, is secreted by Th2 cells to attract eosinophils. CC chemokine ligand 11 (CCL11) is the member of all CC chemokine receptor 3 (CCR3) ligands and is shown to be selective for this receptor. It has been observed to be cleaved by DPP-4 into eotaxin (3-74). The potency for eosinophil chemotaxis is also reduced [51]. CCL11 also activates CCR5 but only in the micromolar range. Inactivating chemokine receptors in allergic disease models,

it is tempting to study similar compounds derived from CCL11 as demonstrated for DP4-resistant n-nonanoyl (NNY)-CCL14 and for amino-oxypentane (AOP)-CCL5 [52].

3 DPP-4 Inhibitors and Diabetes

Incretin response is impaired in patients with type 2 diabetes, and DPP-4-mediated incretin degradation is thought to be responsible for that pathogenesis of type 2 diabetes. DPP-4 inhibitors are newly available oral drugs for type 2 diabetes used either solo or in combination with other oral medications (such as metformin, glitazones, and sulphonylurea) for type 2 diabetes [35]. DPP-4 inhibitors are used to inhibit the activity of DPP-4 and consequently to increase the half-life of incretins, which in turn lowers glucose level post-prandially [53].

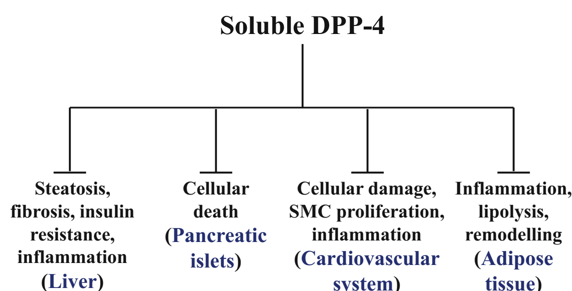
It was observed that DPP-4 inhibitors are only moderately effective for the treatment of T2DM when used solo. It can only lower HbA1C level by 0.6–0.8% in patients with a starting level around 8% [54]. However, they are used increasingly now in clinic due to the safety and low side effect [55, 56]. Side effects of DPP-4 inhibitors in clinical trials include mainly respiratory tract and urinary tract infection as well as headache [57].

Currently, FDA or EU approved several DPP-4 inhibitors. These DPP-4 inhibitors may be divided into two classes depending on structure: DPP-4 dipeptide structures mimics and non-peptidomimetics. β-aminoacid-based sitagliptin, vildagliptin, and saxagliptin belong to the class one, whereas non-peptidomimetics inhibitors include alogliptin (modified pyrimidinedione) and xanthine-based linagliptin. Some of the DPP-4 inhibitors approved by FDA or EU are currently available in the market. FDA already approved sitagliptin (2006), alogliptin (2013), linagliptin (2011), and saxagliptin (2009).Vildagliptin was approved by EU in 2007. Both teneligliptin and anagliptin were approved in Japan in 2012. Apart from these, some other DPP-4 inhibitors like dutogliptin and gemigliptin are also going through clinical trials and awaiting FDA approval [58].

4 Impact of DPP-4 on T2DM-Relevant Organs and Associated Comorbidities

DPP-4 inhibitors maintain glucose metabolism by prolonging the effects of GLP and GIP-1, which in turn increases glucose-mediated insulin secretion and suppresses glucagon secretion [59]. Apart from the hypoglycaemic actions of DPP-4 inhibitors, newly acquired data indicate that incretin-based medications may also have an important role on the physiology of many organs. Thus, then next section of this chapter focuses on the role of DPP-4 action in the organs affected in T2DM and various complications associated with it (Fig. 3).

Fig. 3 Schematic diagram of the effects of soluble DPP-4 on various T2DM relevant organs



4.1 Adipose Tissue

Recent study showed that DPP-4 is highly expressed in human primary adipocytes and DPP-4 expression is increased in obese individuals [60, 61]. Visceral fat of obese persons showed the highest DPP-4 level. From this data, sDPP-4 may be taken as a novel adipokine released from primary human adipocytes. DPP-4 is also involved in adipose tissue lipolysis. It has been revealed that DPP-4-bound ADA, a monomeric enzyme catalyzing deamination of adenosine to inosine and ammonia [62, 63], has a 1000-fold greater activity than free ADA [64], thus reducing the antilipolytic effects of adenosine. In addition, DPP-4 takes part in adipose tissue remodelling and cell plasticity [65]. DPP-4 can also impair insulin signalling at the level of Akt in primary human adipocytes and induce inflammation [66].

4.2 Cardiovascular System

Many studies have shown the protective ability of DPP-4 inhibitors on the cardiovascular system. DPP-4 inhibitors have been shown to decrease ischaemia/reperfusion injury, reduce myocardial infarct size, and protect against myocardial ischaemia [67]. In SAVOR-TIMI53, EXAMINE, or VIVIDD, clinical trials conducted among cardiovascular patients failed to confirm cardioprotective effect of the used DPP-4 inhibitors [68–70]. Studies on GLP-1 receptor (GLP-1R) signalling indicated ameliorative cardiovascular effects GLP-1 itself. In fact, GLP-1R mRNA has been detected in the heart of rodents [71] and humans [72]. Pilot studies in patients with heart failure showed cardioprotection by GLP-1 infusion [73]. In another study, exenatide (GLP-1 analog) showed a significant (20%) reduction of CVD events compared with patients on other hypoglycaemic drugs [74].Apart from GLP-1, two other substrates of DPP-4, SDF-1α, and brain natriuretic peptide (BNP) showed cardioprotective effects. DPP-4 inhibitor sitagliptin increased circulating EPC levels and SDF-1α plasma concentrations [75] in T2DM patients. DPP-4 cleaves the physiologically active BNP (1–32) to BNP (3–

32) lowering plasma cGMP levels. Elevated BNP is a sensitive marker of heart failure and helps in cardiac remodelling and healing after acute MI [76, 77].

4.3 Liver

Diabetes and obesity are major risk factors for non-alcoholic fatty liver disease (NAFLd) [78] which is one of the main causes for chronic liver disease [79]. In the obese persons, elevated triglyceride degradation in adipose tissue causes an increased hepatic uptake of fatty acids leading to fat accumulation within the tissue. Moreover, reactive oxygen species (ROS), produced during lipid oxidation, also induce hepatocyte death. In human liver, DPP-4 was found only in hepatic acinar zones 2 and 3. This heterolobular distribution indicates that DPP-4 might be involved in the hepatic metabolism [80]. Moreover, mRNA level of DPP-4 has been observed to increase markedly in NAFLD livers compared to control [81]. Kaji et al. showed that sitagliptin significantly inhibits the development of liver fibrosis in rats by the suppression of collagen synthesis and hepatic stellate cell proliferation [82]. The mechanism of action of sitagliptin includes dephosphorylation of ERK1/2, p38, and Smad2/3 in the hepatic stellate cells. Moreover, hepatic steatosis was also prevented in several animal studies by DPP-4 inhibition [83]. Though clinical data are limited, some of them revealed the efficacy of DPP-4 inhibitors to improve the levels of liver transaminases and liver fat in diabetic patients [84, 85].

4.4 Pancreas

It has been shown that DPP-4 localization within the pancreatic islets differs between species [86, 87]. A recent study revealed DPP-4 activity in the conditioned medium of human islets indicating that DPP-4 is released from human islets as well [88]. Emerging in vitro and pre-clinical data demonstrated that DPP-4 inhibition may have beneficial effects on T2DM-induced β-cell dysfunction and apoptosis. Omar et al. showed the existence of DPP-4 in mouse and human islets. Their study also revealed that inhibition of islet DPP-4 activity directly stimulates insulin secretion GLP-1 dependently [87]. Moreover, DPP-4 inhibition has been shown to be associated with increased β-cell mass and function in various models of T2DM [89, 90]. These ameliorative effects, however, were revealed to be due to the transcriptional activation of pro-survival and anti-apoptotic genes, and the suppression of pro-apoptotic genes in β cells [91]. Clinical data of DPP-4 inhibitors on β cells are still limited. Both the short- and long-term treatments with vildagliptin increased β-cell function in humans [92, 93]. Interestingly, the SAVOR-TIMI53 trial, originally done to evaluate the cardiovascular safety of saxagliptin, revealed that DPP-4 inhibition may attenuate the progression of diabetes [94]. The exact mechanism how inhibition of DPP-4 activity increases insulin secretion and β-cell

mass is still not fully clear. This is because prolonged GLP-1 activity or improved glycemic control could not explain the above-said phenomenon sufficiently.

5 Clinical Trials

A xanthine-based DPP-4 inhibitor, BI 1356, has reached phase III trials. This compound has shown potential to inhibit DPP-4 in vitro and in vivo. Pre-clinical trials confirmed that a once-daily administration of 5 mg to be efficient. This particular dose was also long lasting and without known side effects [95].

In an open label, multicentre phase I trial, the efficacy of DPP-4 inhibitor linagliptin was evaluated in African American patients with type 2 diabetes mellitus (T2DM). Forty-one patients were included in this study. 5 mg linagliptin, once daily for 7 days, was administered to them, followed by 7 days of out patient evaluation. It is to be mentioned that the exposure range and overall pharmacokinetic/pharmacodynamic profile of the drug in this study of that particular population with T2DM was very much comparable with that in other populations. Results showed that the geometric mean of DPP-4 inhibition at steady state was 84.2% at trough and 91.9% at maximum, and urinary excretion was low (0.5 and 4.4% of the dose excreted over 24 h, days 1 and 7) [96].

T2DM medication sometimes requires combination therapy. In this trial, scientists have assessed the effects of the DPP-4 inhibitor alogliptin (ALO) combined with the pioglitazone (PIO) on β-cell function and glycemic control in T2DM. Patients were treated with combined ALO at 25 mg and PIO at 30 mg daily or ALO 25 mg daily monotherapy or PBO. Results showed that short-term usage of ALO/PIO or ALO improved glycemic control in T2DM patients. β-cell function, however, was improved only by combined ALO/PIO treatment [61].

Gemigliptina is a selective DPP-4 inhibitor used to treat type 2 diabetes. In a combination therapy, the pharmacokinetics (PKs) of combination and gemigliptin, rosuvastatin, and irbesartan mono-therapies was evaluated. Randomized, open-label, 3-treatment, 6-sequence, 3-period, crossover studies were performed. Healthy male volunteers were given 50 mg gemigliptin alone, 20 mg rosuvastatin (part A) or 300 mg irbesartan alone (part B), with concomitant gemigliptin, and each drug was administered as part of once-daily, 7-day, repeated dosing regimens with a 14-day washout period. Results showed that gemigliptin have no effect on the PK properties of rosuvastatin or irbesartan and vice versa [97].

Another phase 2, multicentre, randomized, double-blind, placebo-controlled, 12-week dose-ranging study was conducted to evaluate the efficacy and safety of the DPP-IV inhibitor, PF-734200, in type 2 diabetic patients (men and women) with metformin as their sole diabetes medication. Results revealed that PF-734200 is safe and well tolerated at all the four doses. PF-734200 also significantly lowered HbA1c and 20 mg dose provided the most effective outcome in post-prandial glucose [98].

To determine efficacy of dutogliptin, a DPP-4 inhibitor in T2DM patients, a 12-week multicentre, randomized, double-blind, placebo-controlled trial in 423 patients was conducted. Results showed that dutogliptin treatment for 12 weeks improved glycaemic control in patients with T2DM who were primarily on a background medication of metformin. Results also showed well tolerability of dutogliptin at both the doses tested. The higher dose (400 mg), however, showed better outcome in terms of changes of HbA1c and FPG and subject number [99].

The aim of another study was to quantitatively evaluate the incretin effect after treatment with the DPP-4 inhibitor vildagliptin in patients with T2DM. Twenty-one patients (three women, 18 men) with T2DM primarily treated with metformin were studied in a two-period crossover design. They received 100 mg vildagliptin once daily or placebo for 13 days randomly. The incretin effect was measured on day 12 and 13. Results indicated that DPP-4 inhibition augmented insulin secretory responses both after oral glucose and during isoglycemic i.v. glucose infusions. But no net change in the incretin effect was observed [100].

The effect of GLP-1 in the human vasculature and how it is regulated by DPP-4 inhibition is still unknown. Besides, DPP-4 also degrades the vasodilator brain natriuretic peptide (BNP) to a less potent metabolite. This study was conducted with seventeen healthy subjects participated in this randomized, double-blinded, placebo-controlled crossover. Results showed that GLP-1 does not act as a direct vasodilator in humans, and sitagliptin does not regulate vascular function in healthy humans by degrading GLP-1 and BNP [101].

6 Some Other Proteases Associated with DM

DPP-4 is the most extensively studied proteases and has been developed as a major target for therapeutic purposes via the use of DPP inhibitors. However, a number of other proteases reported recently can serve as biomarkers as well as therapeutic targets for the treatment of DM. Strategies to lower the activity of calpain, cathepsin S, and neutral endopeptidase have been recently studied.

Cathepsin S (Cat S), a cysteine protease, is responsible for cleavage of invariant chain of MHC for proper antigen presentation. Increased activity of Cat S was reported in many pathological conditions. The adipose tissues of obese individuals over-express Cat S, and it has been found to promote neoplastic progression via matrix-derived angiogenic factors [102]. In addition to the cardiovascular and malignant diseases to which enhanced Cat S activity is expected to contribute, type 2 diabetes is one in which this enzyme is expected to play some important role as this disease is considered to be the most devastating comorbidities of obesity. Lafarge et al. [103] reported that anti-cathepsin therapy lowered blood glucose level to normal, comparable to that in cathepsin knockout mice. At present, Cat S inhibitors have been proposed for treatment of autoimmune diseases with the expectation that they could help to lower hepatic glucose output at risk for type 2 diabetes in obese individuals.

Neutral endopeptidase (NEP) is a protease and is reported to be involved in the degradation of vasoactive peptides (natriuretic peptides, calcitonin gene-related peptide, adrenomedullin, bradykinin as well as endothelin). Its levels increased in diabetic patients which facilitate the degradation of those vasoactive peptides. AVE7688, a vasopeptidase inhibitor, improves neurovascular and neural function in streptozotocin-induced type 1 diabetes animals as well as in other type 2 diabetes models [104].

Calpains, a superfamily of Ca^{2+}-activated proteases, are reported to be associated with various physiological and pathophysiological events including diabetes. Recently, increased activity of these proteases has been reported to be linked to reduced endothelium-derived nitric-oxide-mediated vasodilatation in diabetes [105]. Studies suggest that the calpain inhibitor, A-705253, *N*-(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[E-2-(4-diethyl-aminomethylphenyl) ethen-1-yl]benzamide, improved penile nitrergic neurovascular function in streptozotocin-induced diabetic mice [106].

The majority of the serine protease inhibitors linked to diabetes constitute the family of serpins. The level of these proteins is found to be decreased in both T1D and T2D. Hyperglycaemia promotes pro-inflammatory conditions and results in impaired wound healing, a common secondary complication in diabetes. The release of pro-inflammatory cytokines activates the metalloproteases (MMPs). These MMPs have a role in wound repair, cell migration, and tissue remodelling. The use of protease inhibitors for therapeutic purposes for improved wound healing, especially in diabetic conditions, is prevalent.

Doxycycline, an antibiotic of tetracycline family, is an unusual metalloprotease inhibitor. It is reported to improve healing of chronic diabetic foot ulcers. Gelatin, a substrate for MMPs, can be used for healing of chronic wounds [106]. The temporal and spatial features of the activity of MMPs and TIMPs can be used as biomarkers for determining the progress of the diabetic conditions. Alterations in the level of metalloproteases (MMPs) have also been linked to diabetic conditions. MMP-2 is reported to increase in both T1D and T2D, diabetic nephropathy, retinopathy and lesions. MMP-8 is associated with diabetic nephropathy and retinopathy, whereas MMP-9 is associated with T1D, diabetic nephropathy, retinopathy, and macrovascular diseases. Metalloprotease aminopeptidase N is an important marker associated with diabetic nephropathy. Out of the four TIMPs, only TIMP1 and TIMP2 are linked with diabetes. Muller et al. [107] conducted a study in 16 patients with neuropathic diabetic foot ulcers. He proposed a correlation between high ratio of MMP-1/TIMP1 and good healing. Also, they reported that there is a decrease in the level of MMP-8 and MMP-9 in good healers. So, this study showed that the ratio of MMP-1/TIMP1 can be a predictor of healing, and thus their therapy will be designed accordingly. Also, those inhibitors of MMP-8 and MMP-9 can be used for therapeutics development for chronic wound healing purposes [107].

Caseiro et al. [108] performed protease profiling for type 1 diabetes (T1D) in serum, urine, and saliva. An increased activity of MMP-2 and MMP-9 was observed in serum. In urine and saliva, besides MMPs, increased activity of a number of proteases was found in type 1 diabetic patients with nephropathy and/or

retinopathy like MMP-9/neutrophil gelatinase-associated lipocalin, aminopeptidase N, azurocidin, and kallikrein. This report suggests that these proteases found in biofluids can be potential biomarkers or screening targets for monitoring proteolytic activity in T1D patients [108].

Nowak et al. [109] has shown cathepsin D to be an important biomarker for insulin resistance (IR) in T2DM. Using a high throughput 92 protein as say, along with cathepsin D, they identified six other proteins as circulating IR markers in two large community cohorts [109].

7 Conclusion

After the approval of the first DPP-4 inhibitor on 17 October 2006, numerous patients with T2DM have been treated with DPP-4 inhibitors. Though DPP-4 inhibitors are generally well tolerated, adverse effects, especially in case of cardiovascular patients is not uncommon. The result of a recent study drives the scientists further to investigate the mechanism of action of DPP-4 inhibitor saxagliptin that has been shown to increase the rates of hospitalization for heart failure in few patients under trial. Moreover, many DPP-4 substrates are cleaved by multiple enzymes leading to renal or hepatic clearance. It challenges the dominant role of DPP-4 in maintaining peptide bioactivity. However, modern liquid chromatography mass spectrometry-based peptide profiling technology with the help of biochemistry could be a promising approach for revealing the clearer picture of DPP-4 action and would lead to a better and more accurate use of DPP-4 inhibitors pharmaceutically.

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Role of Proteases in Regulating Cell Death Pathways

Thomas Divya, Sekar Vasudevan and Ganapasam Sudhandiran

Abstract
Cell death is a critical process involved during development, tissue homeostasis, and aging. Multiple forms of cell death exist such as apoptosis (type I cell death), necrosis, and autophagy (type II cell death). Recently, other selective forms of cell death such as pyroptosis, eryptosis, entosis, mitophagy, and oncosis are also reported. These cell death pathways collaborate with each other, and regulation of such mechanisms is crucial for maintaining cellular homeostasis. Interestingly, proteases are the one that mediate the cell death programs, and immense research is focused on elucidating the mechanisms through which protease regulates cell death program. In this chapter, we focus on various cell death pathways and how protease regulates these pathways.

Keywords
Apoptosis · Autophagy · Proteases · Necroptosis · Cell death

1 Introduction

Organisms live in an environment that constantly copes up with extrinsic and intrinsic damaging agents. In every second of our life, millions of new cells are born and millions of damaged cells die. Fate of a cell to death or survival is accomplished by a complex signaling network with several mediators. Cell death is a

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T. Divya · S. Vasudevan · G. Sudhandiran (✉)
Cell Biology Laboratory, Department of Biochemistry, University of Madras,
Guindy Campus, Chennai, India
e-mail: sudhandiran@yahoo.com

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decisive phenomenon that maintains tissue homeostasis and abolishes potentially damaged cells and organelles. Damages to the structural integrity of a cell lead to passive cell death. In another way, a cell itself can decide to die owing to more constrained biological processes. This passive type of cell death is uncontrolled which is harmful to the organism. In contrast, firmly coordinated active cell death supports to maintain homeostasis and a healthy cellular environment [1, 2]. The cellular proteolytic machinery includes numerous proteases localized in different intracellular compartments that play a crucial role in various cell death mechanisms [3]. In order to know how such proteases can regulate dismantling of a cell, one has to be aware of what protease signaling is in this context.

Initially, proteases which regulate a great variety of physiological processes were considered mainly as protein-degrading enzymes only. However, nowadays this view has totally changed, and proteases are treated as most important signaling molecules that control several critical processes including cell cycle, cell death, wound healing, protein and organelle recycling, and blood coagulation [4–6]. In addition with these crucial roles of proteases in maintaining normal cell functions, alterations in proteolytic cascade stimulate several pathological conditions such as cancer cell metastasis [7], neurodegenerative and neuroinflammatory diseases [8], inflammatory and cardiovascular diseases [9, 10]. The protease functions by cleaving and thereby modulating the properties of its substrate irreversibly [11]. Since the protease cleavage is an irreversible reaction, protease signaling is one of the effective methods for cell death, as once started the death mechanism cannot be reversed or prevented. One interesting fact about protease is that, for most proteases, it is uncertain how many physiologically significant substrates they have, how a given protease is activated within particular tissue, and how the characteristics of protease differ in disease conditions. The principal focus of this chapter is to discuss the potential role of proteases in regulating various cell death mechanisms which decide the fate of a cell under conditions of stress.

2 Protease Family

Five mechanistic classes of proteases are recognized according to the key catalytic group in the active site: cysteine (Cys), serine (Ser), threonine (Thr), aspartate (Asp), and metalloproteases. Yet another family of protease also has been identified, glutamic protease; the only type of protease that does not found in mammals. Cys, Ser, and Thr act directly as nucleophiles that attack an amide carbonyl C where histidines function as a base. Whereas, Asp and metalloproteases activate a water molecule that acts as a nucleophile which is non-covalent catalysis in which Asp residue and zinc serve as acids and bases [12, 13].

2.1 Cysteine Proteases

The major function of cysteine proteases is intracellular protein catabolism. In addition, they specifically function in the activation of selective signaling molecules such as interleukin, protein kinase C. Cysteine proteases also function in the degradation of extracellular protein. They are secreted by specific cell types under pathological conditions. Cathepsins are cysteine proteases localized in the lysosome, and calpains are the cysteine proteases localized in the cytosol [14, 15]. The catalytic thiol groups at the active site of cysteine proteases are highly oxidized, and hence, they are constrained to the reducing environment.

2.2 Serine Proteases

These are the group of proteases that closely associated with growth and differentiation. Serine protease activities are essential for normal physiological processes of the cell. However, abnormal regulation of serine proteases results in pathological conditions such as tumor development and metastasis [16].

2.3 Threonine Proteases

Threonine proteases are primarily involved in the process of polyubiquitination [17]. The major function of threonine proteases is intracellular protein turnover in cells, i.e., processing and degradation of major proteins that required for regulation of various cellular activities [18, 19].

2.4 Aspartate Proteases

Major function of aspartate proteases is the protein degradation especially in lysosomes and phagosomes. The classical example for aspartate protease is cathepsin D [20].

2.5 Metalloproteases

Matrix metalloproteases (MMPs) are a family of eighteen or more Zn-dependant endopeptidase family of enzymes. They specifically function in cleavage of the components of extracellular matrix [21]. These groups of enzymes are either secreted type or membrane bound type of enzymes.

3 Protease Signaling

To understand the role of protease in a biological process, the important task is to identify the protease's physiological substrates and its downstream effects. Identifying the downstream targets of protease cascade is challenging but very crucial as it is important in controlling various biological processes. There is significant difference between protease signaling and the other types of signaling cascades such as receptor signaling. Protease signaling is an irreversible pathway, and the signal is transmitted through the dismantling of substrates which results either activation or inactivation of the protein function [22]. Unlike other cellular signaling, the criterion for protein degradation by protease signaling is not necessary that the physiological substrate possesses the recognition sequence for a protease. On the other hand, the substrate has to be co-localized with the active protease in the same cellular compartment and so that the substrate can subsequently be processed [23]. Protease signaling can either be a simplest mechanism by which direct cleavage of the substrate leads to the conformational change to the protein, eg. processing of Interleukin-1β by cysteine proteases [24]. Alternatively, the protease signaling can be complex which involves sequential modification of the substrate. For example, the mechanism of protein degradation by ubiquitin/proteasome pathway involves two consecutive steps: (1) binding of various ubiquitin moieties to the protein substrate and (2) specific degradation of the targeted protein by proteasome complex [25].

4 Roles of Protease in Disease

In spite of the essential role of proteases in maintaining cell behavior and cellular turn over, the protease signaling may also play major role in disease progression. Studies have pointed out that the events in cancer progression, invasion and metastasis in which proteases are involved are not only late events but can occur early stages itself [26, 27]. Moreover, studies show that various other stages of cancer such as cell proliferation, apoptosis, angiogenesis are also protease dependant [28, 29]. Recently, mitochondrial proteases in the inner mitochondrial membrane have emerged as culprits in several human neurodegenerative diseases [30, 31]. Available reports suggest that coagulant and fibrinolytic proteases evoke pro-inflammatory and remodeling actions in inflammatory and fibro-proliferative diseases. Coagulants, plasminogen activators, plasmin, and plasmin-activated MMPs provoke cell-mediated responses via receptors (e.g., protease activated receptors) and co-receptors (e.g., integrins). Plasmin also indirectly contributes to inflammatory processes by growth factor receptor trans-activation [32–34]. Collectively, these reports indicate the importance of protease signaling in healthy as well as diseased conditions.

5 Proteases and Cell Death Signaling

Any external or internal triggers such as physical or chemical insult, hormonal activation, specific cell-derived signals that activate various cellular mediators can induce cell death [35]. Various types of cell deaths have been identified: necrosis, apoptosis, autophagy, and more specific death mechanisms such as mitophagy, pyroptosis, eryptosis, NETosis, autosis etc. In all the cell death mechanisms, proteases possess a key role by cleaving the protein substrates. Hence, the nature of substrate and the site of proteolytic cleavage are very much decisive in cell death signaling.

5.1 Protease Signaling in Apoptosis

Apoptosis is one of the most illustrated forms of programmed cell death which plays crucial role in embryogenesis, tissue homeostasis, and aging [36, 37]. The mechanism of apoptosis highly relies upon the regulated activation of proteases and hydrolases which rapidly cleave the cellular structures. The events of apoptosis that lead to the biological and morphological changes include shrinkage of the cell, plasma membrane blebbing, condensation of chromatin (pyknosis), nuclear fragmentation (karyorrhexis), and eventually cell death [38]. In concept, apoptosis occurs within the plasma membrane of the cell, it is an immunologically silent mechanism of cell death without affecting neighboring cells and eliciting any inflammatory response. Therefore, a network of controlled actions is needed to pack the cells for cleavage. This network of events absolutely requires the involvement of caspases [39, 40].

Caspases (cysteine-dependent aspartate-specific protease) are intracellular proteases that initiate a series of events to trigger cell death and an essential mediator of apoptosis. On the basis of the presence or absence of protein interaction domain at N-terminus, apoptotic caspases are majorly distinguished into two: initiator (caspase-8, -9, and -10) and effector (caspase-3, -6, and -7) caspases [41]. The importance of caspases in cell death mechanism is well established, and its function during apoptotic cell death was identified three decades ago itself [42]. There are two major pathways to initiate apoptotic cell death: mitochondrial-mediated intrinsic pathway and death receptor-mediated extrinsic pathway. Cellular stress such as cytotoxic drugs and DNA damage activates the intrinsic cell death pathway, and Bcl-2 family proteins are the major regulator of intrinsic pathway of cell death. The activation of Bcl-2 family proapoptotic mediators such as bcl-2 associated X (BAX) and bcl-2 homologous antagonist/killer (BAK) induces mitochondrial outer membrane permeabilization that results in release of cytochrome c to the cytosol. Cytochrome c then associated with Apaf-1 (apoptotic protease-activating factor 1) into a multimeric complex apoptosome which further activates caspase-9 [43]. Activation of extrinsic apoptosis pathway is mediated by death receptors and is initiated upon binding of ligands to tumor necrosis receptor super family (e.g., TNFR,

Fas, TRAIL) [44]. This activates the initiator caspase-8 or -10, through FAS-associated death domain protein (FADD) or TNFR-associated death domain protein (TRADD). Activation of initiator caspases by either intrinsic or extrinsic pathways mediates the activation of effector caspase-3, -6, and -7 [45]. The role of caspases is well known in apoptotic cell death. Several other proteases such as calpains and cathepsin are also involved in apoptotic machinery.

Calpains are Ca^{2+}-activated non-lysosomal cysteine proteases found in the mitochondria and cytosol. These cysteine proteases are crucial for a number of cellular processes such as cytoskeletal remodeling, attachment of proteins to the membrane, and several signaling pathways [46]. Since calpains are calcium-dependant proteases, loss of Ca^{2+} homeostasis deregulates its activity which results in tissue damage. Available reports provide evidences for the role of calpains in apoptotic cell death. When there is Ca^{2+} overload in endothelial cells, it induces the cleavage of mitochondrial calpain 1 that leads to accumulation of Ca^{2+} in mitochondria. Cleaved calpain 1 further cleaves Bcl-2 family protein BH3 interacting-domain (Bid), inducing cytochrome c release that leads to apoptosis [47]. Yet another protease calpain 10 specifically cleaves electron transport chain proteins that result in decreased mitochondrial respiration. Covington et al. have demonstrated that glucose-induced loss of calpain 10 leads to mitochondrial dysfunction and apoptosis in streptozotocin-induced diabetic rats with kidney dysfunction [48]. Caspase-12 and calpain-mediated extrinsic apoptotic cell death has been reported in HL-1 cardiomyocytes [49].

Cathepsins are being referred as ‘suicide bags’ because of their high degradation potential and of the presence of high level of hydrolases [50]. The role of lysosomal cathepsin was well known for a long time during autophagy and necrosis cell death, and it was totally unclear about their involvement in apoptosis. Two decades before, Deiss et al. have reported the possible role of cathepsin D in apoptosis and hence cathepsin D also considered as the proteases that positively mediates apoptosis [51]. Caspases can initiate apoptosis only upon its activation. In contrast, cathepsins that released to the cytosol following lysosomal membrane permeabilization are active and can initiate apoptotic machinery [52]. As discussed earlier, identification of substrate is an important task in protease signaling. For caspases, several hundred substrates are known [53]. However, very few substrates have been identified which mediates apoptosis. The key identified cathepsin substrate for apoptosis is Bid [54, 55]. Bid was initially known as substrate for caspase-8, the caspase which links intrinsic and extrinsic pathways of apoptosis [56]. In addition, Bcl-2, Bcl-XL, and Bax were also found to be as substrate of cathepsin D [57]. Cathepsin-mediated apoptosis is further confirmed as it can degrade X-chromosome-linked inhibitor of apoptosis (XIAP) protein which suggests that cathepsin mediates apoptosis downstream of mitochondria [58].

5.2 Relevance of Proteases in Autophagy

Autophagy, type II cell death, is a self-degradative process occurring in all eukaryotic cells which is crucial for maintaining tissue homeostasis. It is a

well-organized cellular mechanism in response to external or internal stress [59]. The cellular components or damaged organelles of cytosol to be digested are sequestered into a double-membrane vesicle called autophagosomes. This vesicle fuses with lysosome, and the degradation of cellular components takes place at lysosome which contains hydrolases [60]. Autophagy is a cell survival mechanism which provides protection to the cells upon external or internal stress conditions such as starvation, endoplasmic reticulum (ER) stress, growth factor deprivation, and pathogen infection [61]. Multiple steps are required for the sequestration of cytoplasm through autophagy. These steps include induction of autophagy; packaging up of cargo to the vesicle; nucleation of autophagosome formation; expansion and completion of autophagosome; docking and fusion of the completed autophagosome with the lysosome; and breakdown of the damaged organelles and other cargos [62]. Several autophagy-related (Atg) genes are involved in autophagy. Five different complexes of Atg protein that function in autophagy have been identified: (1) the ULK1 complex, (2) Beclin-1/PI3KIII complex, (3) LC3/Atg8 lipid conjugation ubiquitin-like complex, (4) Atg5-Atg512-Atg16 complex, and (5) Atg9 protein and its cycling system [62].

The kinase complex mammalian target of rapamycin (mTOR) is a major checkpoint in autophagy signaling, which allows base-level formation of autophagosome; however, its inhibition triggers autophagy [63]. Impaired autophagic cell death has recently been identified in numerous pathological conditions including neurodegeneration, inflammation, and cancer [64–66]. Identifying the importance of autophagy in these pathological conditions considered autophagy as an emerging therapeutic target for the prevention of diseases through either its activation or inhibition. In this regard, protease signaling gains much attention recently in the regulation of autophagic cell death, as several proteolytic enzymes are recognized as indirect modulators of the autophagy signaling. Since autophagy is a lysosomal-degradative mechanism, a number of lysosomal proteases such as calpains, caspase, cathepsins are concerned to be participating in several stages of the autophagic pathway. Interestingly, yet another protease, the cysteine protease Atg4 also plays a crucial role in the execution of autophagic cell death.

The central role of Atg4 is well identified in the LC3/atg8 lipid conjugation complex, which is essential protein complex for the elongation and complete formation of autophagosome membrane. Lang et al. have reported for the first time the physical interaction of Atg4 with Atg8 in the yeast *Saccharomyces cerevisiae* [67]. During the formation of autophagosome, Atg8 covalently binds to phosphatidyl ethanolamine (PE). The function of protease Atg4 in the complex is that it removes C-terminal arginine of Atg8 and felicitates its binding with PE [68]. Additionally, Atg8 orthologs are differentially cleaved by the different Atg4 family members, with Atg4B representing the most potent of all of them in terms of substrate specificity. In fact, Atg4B is able to process a wide range of Atg8 orthologs [69], and the analysis of their structures has been very useful to understand the interaction between both enzyme and substrate [70]. Moreover, apart from their central role in the Atg8/LC3 lipid conjugation system, recent reports have pointed out a potential link between Atg4D and apoptosis, indicating a reputed role for Atg4D at

the interface between autophagy and apoptosis [71, 72]. Dysfunctional autophagic responses with regard to Atg4 have been reported in several pathological conditions. The essential role of ATG4A in regulating the tumorigenicity of breast cancer stem cells has been reported in vivo [73]. The tumor suppressor role of Atg4C is reported in murine fibrosarcoma model [74]. These reports suggest the crucial role of this cysteine protease in regulating autophagic cell death.

The close connection of apoptosis and autophagy cell death is well illustrated. Available reports suggest that induction of either of these cell death mechanisms is depending on the cellular context. Blockade of apoptosis in cells results in the activation of autophagy, or inhibition of autophagy may trigger apoptotic cell death [75, 76]. Cysteine protease caspases are the major molecules that link apoptosis and autophagy. The role of proteases that links apoptosis and autophagy pathways is depicted in Fig. 1.

The proteases calpain and caspases can cleave human ATG proteins. hATG3 has been reported to be lysed by caspase-3, -6, and -8 [77]. Cho et al. have suggested that caspase-mediated cleavage of ATG6 links the apoptotic and autophagic signaling pathways and have reported Beclin-1-Atg 6 complex as caspase-3 target

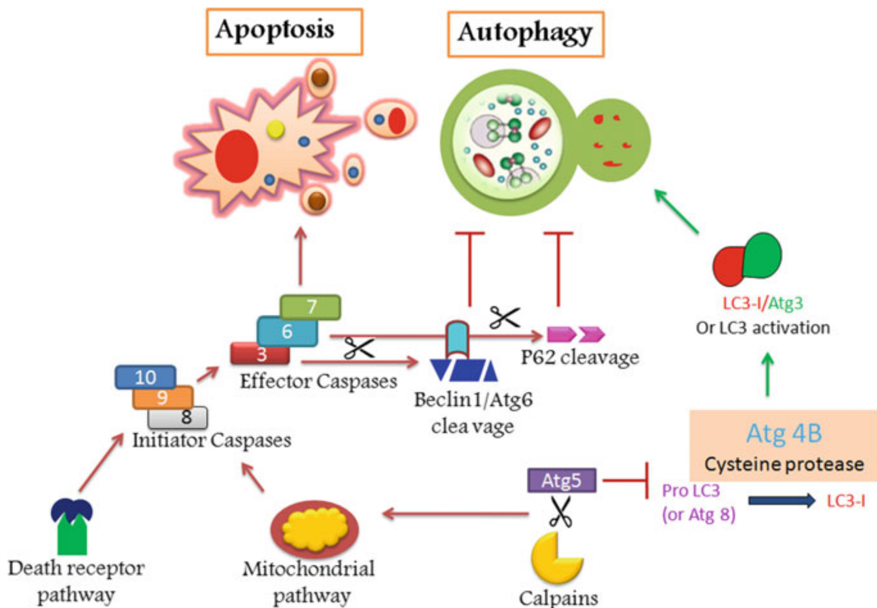

Fig. 1 Role of proteases in apoptosis and autophagic cell death. Mitochondrial and death receptor pathways initiate apoptosis through the activation of initiator and effector caspases. In addition, caspase-mediated cleavage of autophagy proteins Beclin1/Atg6 and p62 inhibits autophagy. The cysteine protease calpain mediates cleavage of Atg5 results in the liberation of a proapoptotic fragment of Atg5 that leads to mitochondrial outer membrane permeabilization and cell death. The cysteine protease Atg4B activates autophagy through the activation of LC3-mediated autophagosome formation

[78]. Caspase-6 and -8 cleave p62, an autophagy adaptor protein that leads to inhibition of autophagy [79]. Apart from caspases, cathepsins being major protease in the lysosome also function in the regulation of autophagy. Boland et al. have reported that inhibition of cathepsin-mediated proteolysis in Alzheimer's model results in accumulation of incompleted autophagosomes with partially degraded LC3II which suggests the importance of cathepsins along with caspases during autophagy [80]. In support to this view, a vital role of cathepsins in the degradation of damaged organelles and associated cargos within lysosome upon the fusion of autophagosomes with lysosome to form autolysosomes has been reported [81]. These reports suggest the autophagy regulatory function of cathepsins. Hence, it is obvious that protease signaling is significant in the regulation of autophagic cell death.

5.3 Proteases in Necrotic Cell Death

While apoptosis is a programmed type of cell death, necrosis, type III modality of cell death is not a controlled mechanism but is characterized by dysregulation of cellular activities and collapse of cellular environment under stress conditions. The morphological characteristic features of necrotic cell death include plasma membrane rupture, swelling of mitochondria, dissolution of the endoplasmic reticulum, and extensive vacuolation of the cytoplasm. During necrotic cell death, the damaged cellular contents are released into the intracellular space, and hence, it affects neighboring cells by inducing an inflammatory response [82]. The ligand–receptor interactions in necrotic cell death are highly regulated by the serine/threonine kinase receptor-interacting protein 1 (RIP1) which is calcium dependant. During necrosis, cytosolic calcium level significantly increases which typically lead to calcium overload in mitochondria that activates proteases and phospholipases [83]. Since calcium is the notorious factor in necrotic cell death, calcium-dependant proteases: calpains and cathepsins play major role in the regulation of necrosis. Because of the enormous destructive potential of cathepsins, massive lysosomal rupture leads to necrotic cell death. Jacobson et al. have reported cathepsin-mediated lysosome disruption and necrotic cell death in myeloid leukocytes [84]. Increased number of evidences show the interrelation between apoptosis and necrosis. For example, low concentration of an external stress insult induces apoptosis in cells, while the same stimuli at higher concentration induce necrosis in the same cells [85–87]. The necrotic cell death can be partially mediated by apoptotic cascade such as caspase 8/Bid strongly highlights the interplay of these two cell death mechanisms [88]. In necrotic cell death, the non-caspase protease cathepsin-induced morphological changes resemble the caspase-induced morphological changes in apoptosis [89]. However, the caspase-mediated cell death is highly regulated, while the cathepsin activity is not specific and is less precise, and hence, it mediates more disastrous necrotic cell death.

5.4 Protease in Necroptosis Signaling

The concept of uncontrolled and unregulated necrotic cell death has been changed nowadays, and evidences revealed the regulated mechanism of necrotic cell death. This programmed necrosis is termed as 'necroptosis' which has important functions in the organogenesis and in the maintenance of tissue homeostasis [90]. Tumor necrosis factor receptor 1 (TNFR1) initiates the necroptosis signaling in cells. However, TNF function in cell is dependent on cell type and its surroundings, and based on this, TNF administration can result in cell survival, apoptosis, or necroptosis [91]. In necroptosis signaling, the protease caspase 8 modulates the molecular events in response to TNFR1 activation. The RIP1, RIP3, and mixed lineage kinase domain-like (MLKL) proteins are the major regulators of necroptosis signaling. Under condition of blockade of apoptosis, necroptosis cell death serves as critical mechanism to remove damaged cells [92]. Necroptosis is also closely linked with apoptosis. Cysteine protease caspases interlink these two pathways. In response to external or internal stimuli, TNF induces a TRADD (Tumor necrosis factor receptor type 1-associated death domain)-dependant complex 1 by recruiting RIP1, TRADD, TRAFs (TNF-receptor-associated factors), etc. Formation of this complex ensures the activation of canonical nuclear factor-κB (NF-κB) pathway [93]. Role of caspases is significant here to decide whether to undergo apoptosis or necroptosis. The cleavage of RIP1 and RIP3 by caspase 8 initiates apoptosis. The inhibition of caspase activity leads to the auto-phosphorylation of RIP1 and RIP3 which recruit MLKL protein to form a complex called necrosome that initiates necroptosis [94]. Thus, RIP1 is the master switch protein that decides the fate of cell whether to undergo caspase-8-mediated apoptosis or MLKL-dependant necroptosis.

5.5 Proteases in Pyroptosis

Pyroptosis is a proteolytic cell death mechanism that first described in *Shigella flexneri*-infected macrophages [95]. It shares some of the features with apoptosis including DNA fragmentation and nuclear condensation. Pyroptosis is recognized as caspase-1-dependant modality of cell death [96]. In pyroptosis, caspases are directly activated via a caspase activation and recruitment domain (CARD)-containing inflammasome. Inflammatory response in cell induces caspase activation that results in initiation of cell death signaling. Caspases induce permeabilization of the plasma membrane that results in flushing of water and ions into the cell causing cell rupture and the release of cytosolic contents [97]. Along with caspase-1, caspase-11 is also gaining much attention in pyroptosis research [98, 99]. A research group recently reported that cleavage of pannexin-1 channel is essential for pyroptosis which is catalyzed by caspase 11 [100]. In contrast, yet another research group has reported that caspase-1 but not caspase-11 was required for inflammasome formation during pyroptosis in Legionella species as well as in mice [101].

5.6 Possible Role of Proteases in Eryptosis

The life span of circulating erythrocytes is normally 100–120 days [102]. How these cells are cleared? Prior to cellular senescence, erythrocytes may be removed by suicidal death, termed as eryptosis upon external stimuli or cell injury. Eryptosis is characterized by the similar morphological features of apoptosis as cell shrinkage and rupture of cell membrane. The breakdown of plasma membrane leads to the translocation of phosphatidylserine (PS) to the cell surface, a hallmark of suicidal cell death [103]. The translocation of PS to the cell surface is calcium dependent, and increased Ca^{2+} concentration inside the cell further facilitates the membrane rupture and translocation of PS [103]. Though erythrocytes express caspases and these caspases function in stimulating the expression of PS at outer membrane [104], the calcium-mediated membrane rupture is independent of caspase activity [105].

5.7 Proteases in ER-Stress-Mediated Cell Death

We have discussed the role of proteases in various modes of cell death. What exactly prompts a cell to die? Several direct and indirect factors cause cell death. In this context, endoplasmic reticulum (ER)-stress-mediated cell death is very much emerging and gaining attention of researchers recently. The preliminary observations of our laboratory demonstrate that ER stress plays an important role in inducing caspase-mediated apoptotic cell death in bleomycin-induced pulmonary fibrosis model. In this regard, it would be logic to discuss the potential role of protease in ER-stress-mediated cell death.

Since protein folding is taking place in ER, intracellular stress by a wide variety stressors disturbs the normal environment of the ER which leads to unfolded protein accumulation [106]. Normally, proper protein processing and folding occur in ER using molecular chaperones, lectins, and foldases [107]. However, any disturbance to normal protein folding results in ER-associated degradation (ERAD) pathways of mis-folded proteins [108]. Prolonged ER stress triggers apoptosis as a last choice of eukaryotes to dish out the dysfunctional cells [109]. As in other cell death mechanisms, caspases are the major proteases involved in ER-stress-mediated cell death. Available reports show that caspase-12 associates with activated inositol-requiring enzyme 1 (Ire1) which is a key mediator of ER stress pathway [110]. High concentration of calcium in ER under stress condition activates calpains which further stimulate the proteolytic activity of caspase-12 [111]. But caspase 12 is absent in most humans [112], and therefore, the relevance of caspase-12 in ER-stress-mediated apoptosis is questionable. In this point of view, human caspase-4 which is a close paralog of rodent caspase-12 may be assumed to participate in ER-stress-mediated cell death in humans [113]. However, further studies are warranted to validate the role of protease as therapeutic target in context of ER-stress-mediated cell death.

6 Conclusion

For long time, apoptosis was considered as the only mechanism for the removal of unwanted cells. However, it has become clear that along with apoptosis, various other cell death mechanisms such as autophagy, necroptosis, pyroptosis, NETosis, mitophagy play an important role in maintaining tissue homeostasis. These different modalities of cell death are being intensely studied in context of various diseases in order to elucidate the mechanism that how cell death contributes for disease progression. Since proteases play a decisive role in the regulation of cell death programs, elucidation of cell death pathways and the mechanisms through which they are regulated by proteases will lead to development of novel drugs for life-threatening diseases.

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Role of Proteases in Idiopathic Pulmonary Fibrosis

Soumya Chatterjee, Kaustav Chakraborty, Subhabrata Moitra and Arindam Bhattacharyya

Abstract

Idiopathic pulmonary fibrosis may be described as the debilitating condition of lung where excessive collagen-rich extracellular matrix (ECM) gets deposited. From the chemical and biological properties, it can be assumed that the activities of proteases can degrade matrix. Though, some of the proteases are anti-fibrotic, whereas most of them have profibrotic functions. Proteases perform important functions in a range of biological processes, like tissue repairing, remodeling, and providing immunity. However, the exact mechanism is yet to be known how these enzymes work during fibrosis; i.e., the proteins that the proteases target to perform a specific process and its effect on ECM turnover is still opaque. However, experimental models and clinical studies have identified some crucial steps that could help understanding the disease mechanism and also herald a ground for future therapy.

Keywords

Pulmonary fibrosis · Proteases · Lung disease · MMP

S. Chatterjee · K. Chakraborty · A. Bhattacharyya (✉)
Immunology Laboratory, Department of Zoology, University of Calcutta,
35, Ballygunge Circular Road, Kolkata, India
e-mail: arindam19@yahoo.com

S. Chatterjee
e-mail: soumyac.il15@gmail.com

K. Chakraborty
e-mail: kaustavc17@gmail.com

S. Moitra
Department of Pneumology, Allergy and Asthma Research Centre, Kolkata, India
e-mail: subhabrata2207@gmail.com

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Abbreviations

α-SMA	α A smooth muscle actin
AHR	Airway hyper reactivity
ALI	Acute lung injury
AR	Amphiregulin
ARDS	Acute respiratory distress syndrome
BALf	Broncho alveolar lavage fluid
Cat K	Cathepsin K
COPD	Chronic obstructive pulmonary disease
ECM	Extracellular matrix
EGFR	Epithelial growth factor receptor
EMT	Epithelial mesenchymal transition
IPF	Idiopathic pulmonary fibrosis
IGFBP-3	Insulin growth factor binding protein-3
MMP	Matrix metalloprotease
NE	Neutrophil elastase
PFT	Pulmonary function test
PTGS2	Prostaglandin G/H synthase 2
ROC	Receiver operating characteristic curve
TGF β	Transforming growth factor β

1 Introduction

Idiopathic pulmonary fibrosis (IPF) is the commonest form age-related diffuse lung disease that is known to be persistent with progressive collagen production in alveolar septa leading to the damage of the lungs affecting the gas-exchange mechanism [1–3]. With a prevalence of approximately 20 per 100,000 people, the burden of this disease is alarmingly increasing with an estimated rate of 4.6–16.3 cases per 100,000 population, each year [4, 5]. Patients with IPF suffer mostly from cough and dyspnea, thus lead a compromised quality of life. This disease is often misdiagnosed in the initial stage, and therefore, the prognosis becomes extremely marginal [6]. The pathologic mechanisms of IPF are not fully understood; hence, targeted treatment regime has been extremely limited. Risk factors, such as chronic or repetitive exposure to cigarette smoking, occupational and environmental pollutants, chronic gastroesophageal reflux and chronic lower airway infection, are trivial in the manifestation of the disease. The progressive and irreversible disruption of alveolar bed induces an accelerated accumulation of fibroblasts in the disrupted area where the cells are transformed into myofibroblasts and deploy collagen and other extracellular matrix molecules, the milieu which distorts

pulmonary architecture leading to impaired lung function [7]. The immediate response due to tissue injury, shown during inflammation, is usually caused by stimuli like pathogens, different chemicals, etc. [8]. Lung inflammation is a kind of innate immune response that is directed in a coordinated expression of different inflammatory cytokines and immune cells aiming to clear the pathogenic agents [9]. Different proteases in lung regulate the activities of these inflammatory cytokines. Several studies have explored the role of proteases in different diseases [10]. Inflammatory cells like lymphocytes, neutrophils, macrophages, mast cells, as well as lung native cells like epithelial, endothelial, and fibroblasts act as the major protease source in lung [11–15]. Most of these proteases show profibrotic properties whereas some responses as anti-fibrotic components. The detailed properties of the proteases in regulation of IPF are described here.

2 Idiopathic Pulmonary Fibrosis (IPF): Overview of the Disease

Idiopathic pulmonary fibrosis (IPF) is a type of interstitial lung disease with progressive fibrotic development which has been studied from ancient time [16–18]. Modern-day science diagnoses this by surgical lung biopsy [19, 20], and the presence of patchy inflammatory cells, myofibroblasts, foci of proliferating fibroblasts, deposited collagen, and Pulmonary Function Test (PFT) [21, 22]. Recently, through some unique pathologic analysis, different types of pulmonary fibrosis have been described [23, 24]. Currently, IPF is one of the most common and severe forms of pulmonary fibrosis, with a very low rate of survival [5]. The two main biological processes, apoptosis of fibroblasts, proliferation and accumulation, breakdown of ECM are the cause behind the progression of this disease. Imbalance of ECM deposition, turn over and fibroblast proliferation, apoptosis may lead to pulmonary fibrosis. Reports have said that by circulating fibrocytes, monocytes, the bone marrow derived cells may produce the ECM-producing cells [25–28], or epithelial-mesenchymal transition (EMT) [29]. In pulmonary fibrosis, myofibroblast is the ultimate effector cell. In association with the lung fibroblasts, alveolar epitheliums also have been found to be involved in disease pathogenesis [30, 31]. The epithelial-mesenchymal interplay plays the pivotal role in pulmonary fibrosis, in which ECM gets accumulated due to coupling of the lung mesenchymal cells with the injured alveolar epithelium cells, and lung architecture alters.

Extensive studies have proved that among the cytokines, TGF-β plays a key role in stimulation of fibroblast proliferation and IPF progression [29]. Apart from that, the Epithelial Growth Factor Receptor (EGFR) and its ligand Amphiregulin (AR) have shown to induce the ECM deposition and myofibroblast proliferation [32]. It has been postulated that among the proteases, Neutrophil Elastase (NE) may be regulating the early stages of lung inflammation during the developmental stage of pulmonary fibrosis [33]. NE acts as a promising link between emphysema and

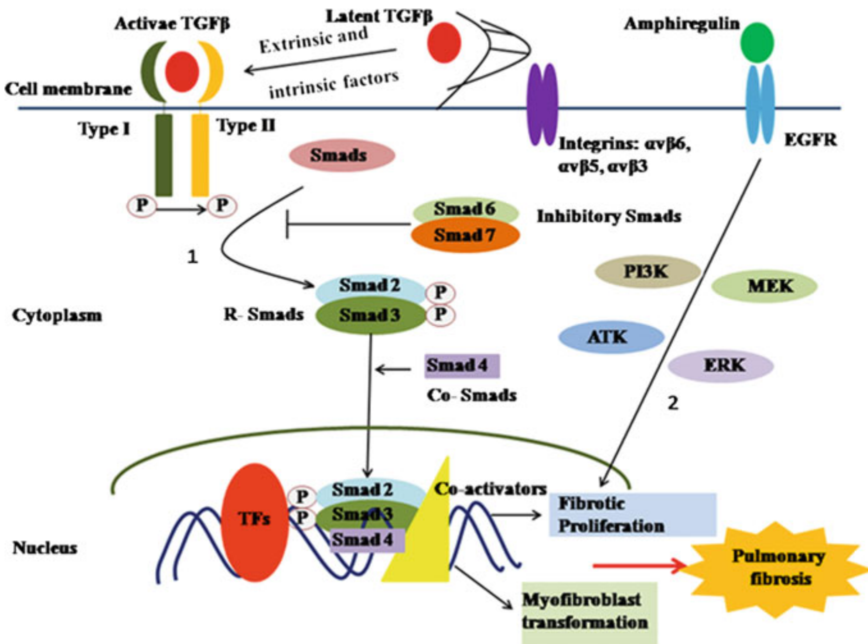

Fig. 1 Role of different extrinsic, intrinsic factors, cytokines, cell surface receptors, and different molecular pathways in regulation of pulmonary fibrosis. *1* TGFβ is the key molecule behind the fibrotic proliferation in lung that leads to IPF. TGFβ is an immunosuppressive cytokine that upon activation attaches to its receptor and activates the Smad-dependent pathway (canonical pathway) which results in activation of the profibrotic genes that later leads to pulmonary fibrosis. *2* Role of EGFR signaling on pulmonary fibrosis. Apart from TGFβ, EGFR is another important receptor that has shown impressive impact as a probable cause and therapeutic target of IPF. Amphiregulin, the EGFR ligand upon activation, phosphorylates the downstream proteins. This phosphorylation leads to activation of several Transcription factors that induce the fibrotic proliferation [32]

fibrosis [34]. But metalloproteases have established themselves as the most potent proteases that have pivotal role in the disease etiology (Fig. 1).

3 Role of Proteases in IPF

As already discussed, inflammatory cells, fibrotic foci, myofibroblasts, and collagen deposition are the notable histopathological features of IPF. These cells either secret or help secreting proteases that cause the irreversible figrogenic changes in the lungs. Tissue growth factor-β (TGF-β) stimulates fibroblast proliferation, and it has been observed that lung cells treated with (TGF-β) exhibit an increased activity of matrix metalloproteinase-2 (MMP-2) leading to progressive changes in the lungs. Another proteolytic enzyme, neutrophil elastase (NE), has also been demonstrated to be involved in the development of inflammation during pulmonary fibrosis. The

Table 1 Different types of proteases involved in Idiopathic Pulmonary Fibrosis (IPF)

Type of protease	Subtype	References
Serine protease	Neutrophil Elastase	Belaaouaj et al. [35]
Cysteine protease	Cathepsin	Lecaille et al. [77]
	ATG4B protease	Cabrera et al. [37]
Aspertic protease	Pepsin	Davis et al. [38]
	Napsin A	Samukawa et al. [39]
Metalloprotease	MMP-3	Peters et al. [41]
	MMP-7	Li et al. [46]
	MMP-8	Garcia-Verdugo et al. [48]
	MMP-9	Lee et al. [53]
	MMP-12	Kang et al. [62]
	MMP 13	McQuibban et al. [68]
	MMP 19	Yu and Stamenkovic [54]

recent understanding of the role of NE in emphysema highlights a link between emphysema and IPF and also indicates NE as a common possible inducer of these two diseases. Amphiregulin has also been found to induce IPF by regulating myofibroblast proliferation in the lungs. Proteases are now classified into seven families based on the nature of the catalytic residues. These are aspartic- cysteine-, serine-, metallo-, threonine-, glutamic-, and asparagines peptidase. Among these seven types of proteases, mainly four classes of proteases have been found in lungs that are predominantly supposed to impact in IPF (Table 1). Below we discuss the roles of different proteases in IPF.

I. Serine Protease

A. Neutrophil Elastase (NE)

Polymorphonuclear (PMN) cells have been found to contribute to the pathogenesis of IPF. NE is a neutrophil-derived serine protease that acts on a wide variety of substrates and has been found crucial in protecting against pulmonary infection [35]. NE promotes the proliferation of lung fibroblasts. In an experiment, it was found that at modest concentrations of NE fibroblast got proliferated. There are also some reports that show that NE also promotes myofibroblast differentiation.

II. Cysteine protease

A. Cathepsin

Cathepsin K (Cat K) expression gets enhanced in response to different fibrogenic particles The co-relation between Cat K response and the development of lung fibrosis was tested in an experiment. After one

month of the experimental pulmonary fibrosis condition, Cathepsin K showed significant high level of elevation than the control group [36]. The expression level of Cat K was inversely associated with the fibrotic development in murine strains, suggesting the contribution of Cat K to limit lung fibrosis. Several other in vivo and in vitro studies prove the role of TGF-β in repressing the expression of Cat K in lung fibroblasts resulting in fibrosis development. The findings thus help to conclude Cat K as a potential collagenolytic mammalian protease.

B. **ATG4B protease**

ATG4B is another class of cysteine protease that has been recently investigated to modulate cellular events in pulmonary fibrosis. In a group of *Atg4b*-deficient mice treated with bleomycin, Cabrera and colleagues (2015) observed significantly higher inflammatory response, increased neutrophilic infiltration, and significant alterations in proinflammatory cytokines [37]. They also observed that *Atg4b* unsettlement resulted in increased apoptosis of the alveolar and bronchiolar epithelial cells leading to a progressive and irreversible damage to the lungs. Deregulated extracellular matrix-related gene expression and increased collagen accumulation are associated with these changes [37]. Therefore, ATG4B protease could have a protective role against bleomycin-induced fibrotic changes of the lungs.

III. **Aspertic protease**

A. **Pepsin**

Pepsin has been established as a novel biomarker for the diagnosis of IPF. Gastroesophageal reflux has been reported to jeopardize respiratory conditions. Pepsin, a proteolytic enzyme secreted by the gastric cells, has been found in BALF in patients with GERD and lung transplantation and asthma. Recently, higher BALF pepsin level has been found to associate with higher rate of exacerbation of the IPF patients [38].

B. **Napsin A**

Napsin A, aspartic protease, is mostly found in alveolar type-II cells and has been observed to impact significantly in conditions like lung cancer. In a study, it was observed that levels of napsin A in serum were higher among the IPF patients than the controls, and such elevated level also contributed to the severity of disease [39] (Fig. 2).

IV. **Metalloproteases**

A. **MMP-3**

Increased expression of MMP-1, 9, and 13 in IPF patients indicates collagenase activity of the proteins in different conditions [40]. MMP-3 (stromelysin-1) has been shown to have profibrotic activity as fibrotic change is reduced after bleomycin challenge in *Mmp*-$^{-/-}$ mice [41, 42].

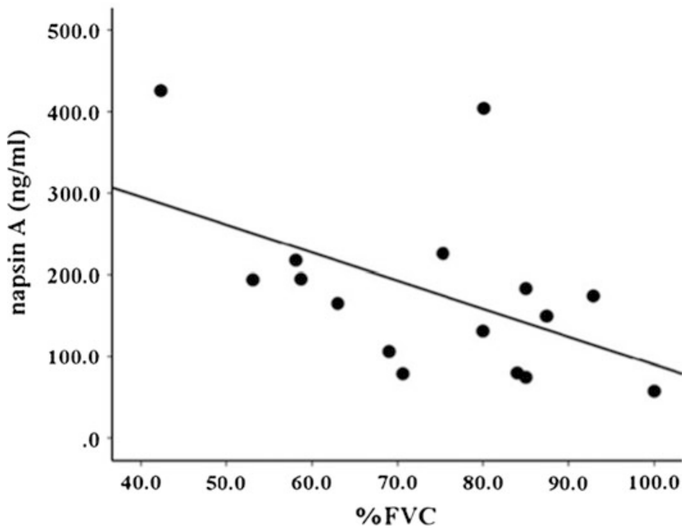

Fig. 2 Inverse correlation between napsin levels and lung function as measured by percent-predicted FVC (% FVC) (n = 16, $p < 0.05$) [reproduced from 39]

It is believed that the epithelial cells are pushed to an epithelial-mesenchymal transition by MMP-3, leading to the formation of myofibroblast-like cells in IPF. However, the exact role of MMP-3 has not been fully understood. Thus, the exact role of MMP-3 in promoting fibrosis through EMT remains an open question [43, 44].

B. **MMP-7**

MMP-7 or matrilysin is over-expressed in fibrosis than in healthy lung tissues [45, 46]. Alike MMP-3, MMP-7 has also been identified as profibrotic protease MMPs have been found to release chemokines [47]. According to a newly identified pathway, MMP-7 breaks down the carrier molecule of chemokine CXCL1, syndecan-1, and this cleavage in the glycosaminoglycan chain promotes the transepithelial migration of neutrophils [46]. Therefore, a possible neutrophil influx mechanism has been proposed in the development of IPF which is manifested by an up-regulated activity of MMP-7. Apart from its profibrotic nature, MMP-7 can have anti-fibrotic effects too [48].

C. **MMP-8**

MMP-8 has also been reported as profibrotic; however, its activity is opposite in liver tissues where MMP-8 serves as anti-fibrotic [49–52]. Although inactivation of interleukin IL-10 by MMP-8 was observed as a crucial step in IPF [49, 50], another contradictory report suggests that lung fibrosis is initiated by over-expression of IL-10 [53].

D. **MMP-9**

MMP-9 has also been shown to contribute to the development of IPF through a pathway involving the activation of TGFβ1 [54, 55]. In

experimental models, although the occurrence of bleomycin-induced IPF was not substantially different between wild-type and MMP-9$^{-/-}$ mice [56], probability of bleomycin-induced fibrosis was lesser in transgenic mice in which MMP-9 expression in macrophages was regulated by a scavenger receptor class A promoter [57]. Still, the role of MMP-9 in fibrosis is still not clear. While MMP-9 was associated with mild fibrotic changes in one study [57], another study confirmed it as profibrotic [54], while others were unsure about its role [56, 58]. MMP-9 expression has been linked to chemokine-level modulation and leukocyte influx in allergic lung disease [59–62] indicating that more studies are warranted to elucidate its function in disease mechanism.

E. **MMP-12**

Most of the experimental fibrosis models have demonstrated MMP-12 as a profibrotic protease [63–66]; however, the role of this protein in augmenting IPF has remained contradictory. While one study reported reduced fibrotic changes in MMP-12 null mice after bleomycin challenge than the wild type [63], other study did not find any conclusive evidence of such change between the two genotypes [57, 67]. Although such contradictions can be explained by their study design and measurement time points, experimental results indicate that an MMP-12-driven effect in IPF may only be seen visible in an advanced stage. MMP-12 might have a role in the activation of fibroblast or collagen production. MMP-12 was also established as a profibrotic in another study where pulmonary fibrosis was induced by anti-Fas antibody (Jo2) [64]. The investigators observed that targeted deletion of MMP-12 significantly reduced inflammation and protected the animals against Fas-induced fibrosis. The investigators also observed that the expression of two other profibrotic genes, egr1 and cyr61, reduced in MMP-12 transgenics than the wild-type animals [64]. In another study, Madala and colleagues (2010) suggested that MMP-12 could suppress other anti-fibrotic genes, thus manifests its profibrotic activity. However, results of two studies were contradictory where the investigators reported no effect or anti-fibrotic or no effect of MMP-12 [68, 69]. Therefore, more investigation is required to confirm the role of MMP-12 in disease manifestation. Though it has been proposed as a profibrotic protease, there are contrasting findings proposing MMP-12 as anti-fibrotic protease [68]. However, a direct link is still missing between fibrotic mechanism and role of MMP-12.

F. **MMP-13**

The role of MMP-13 in fibrosis has been investigated over a long time. Flechsig and colleagues (2010) reported reduced inflammation in radiation exposed MMP-13 null mice. However, this observation was contradicted by the findings of Sen and colleagues (2010). They reported that fibrotic changes were almost similar in MMP-13 null and wild-type mice in induced-hyperoxia; however, they found an elevated

inflammation in the MMP-13 null animals. MMP-13 also has roles in the metabolism of various chemokines; thus, it can induce the activity of the chemokines [70–73], and such altered activities of the chemokines could play pivotal role in the development of fibrosis and also to the severity of the disease.

G. **MMP-19**

Up-regulation of MMP-19 expression has been observed in the epithelial cells of IPF patients [55]. Fibrosis in MMP-19 null mice exhibited aggravated hydroxyproline and αSMA staining than wild-type mice which may indicate that MMP-19 could modulate disease progression. MMP-19 has been reported to exert an anti-fibrotic effect by modulating the cyclooxygenase (COX)-2 pathways that produce prostaglandin E_2 (PGE_2). PGE_2 has been found to minimize fibroblast proliferation, migration, collagen synthesis, and differentiation into myofibroblasts, thus suppress fibrosis [74–76] (Fig. 3).

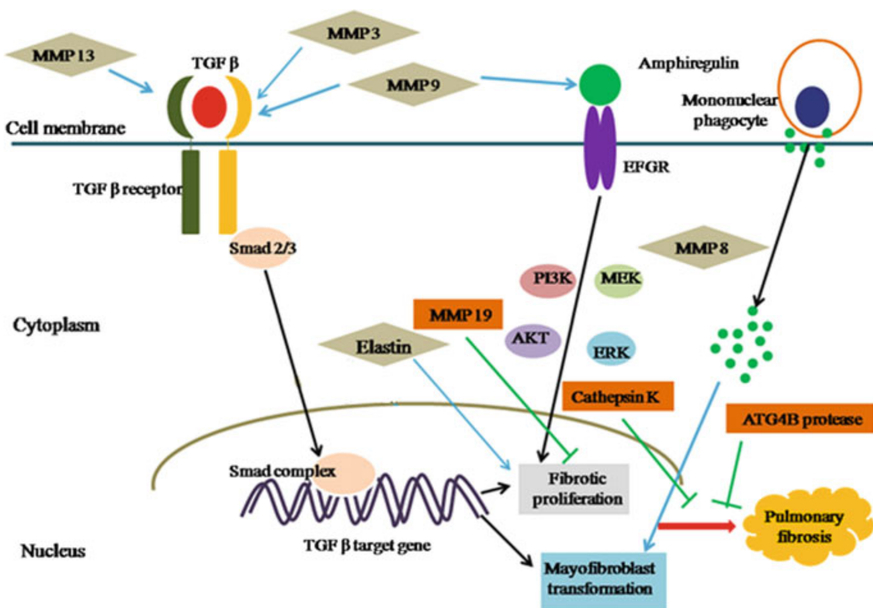

Fig. 3 Role of different proteases in IPF. MMP 9 has proved itself as a potent activator of Amphiregulin that activates the EGFR pathway whereas MMP 9 and MMP 13 and MMP 3 have shown to activate TGFβ. Mononuclear phagocyte releases MMP 8 and has positive regulatory effect on myofibroblast proliferation and ECM deposition. Increased Elastin level in lung during fibrotic condition has established itself as a novel biomarker for the disease. MMP 19 has shown to induce fibrotic proliferation of cell. Among all these profibrotic proteases, Cathepsin K and ATG4B protease negatively regulate pulmonary fibrosis

4 Protease Inhibitors: A Potent Target for Therapeutic Approach

Proteases are the potent profibrotic molecules, whereas some of the results show anti-fibrotic properties. The profibrotic proteases mostly show their effect by activating the latent TGFβ or IL-13 or by inducing the EGFR signaling cascade. They have also shown to block the anti-fibrotic protease functions resulting in imbalance of pro-/anti-fibrotic proteases in lung. Thus, targeting the profibrotic proteases may be beneficial for the attenuation of pulmonary fibrosis. There are some protease inhibitors that have been shown to exert anti-fibrotic response.

A. **ONO-5046**

As earlier discussed, NE is a potent profibrotic protease. ONO-5046 is known to be a potent antagonist of mouse and human NE. In an experiment, the later was used to see whether it could reduce pulmonary fibrosis or not. What was found is that ONO-5046 reduced lung fibrosis significantly. The results from hydroxyproline content and histological analysis proved that blocking NE attenuates experimental pulmonary fibrosis.

5 Conclusion

Pulmonary fibrosis is a diffused parenchymal lung disease with distinct radiographic, clinical, pathologic, and physiologic signs. However, till date, there is no proven and effective therapy to manage these patients. The recent discoveries of various protease-mediated pathways have been quite helpful in understanding the pathological conditions and mechanism of the disease. Modification of ECM, which is an inevitable and crucial aspect of identifying cases of IPF, has provided a ground zero as an effective treatment modality. Apart from that, molecules propagating anti-fibrotic proteases and protease inhibitors also seem to be a promising future therapy for the management of IPF and other lung diseases.

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Protease-Activated Receptor Signaling in Lung Pathology

Ganapasam Sudhandiran, Srinivasan Kalayarasan, Thomas Divya and Bakthavatchalam Velavan

Abstract

Protease-activated receptors (PARs) are self-activated G-protein-coupled receptors that have diverse roles in several disease paradigms including neurodegeneration, cancer, cardiovascular diseases, and others. Recently, extensive research on PAR family and its effect in modulating signaling pathways have gained attention. There is evidence that PARs are expressed in the airways in a variety of cell types that are relevant to inflammatory lung diseases, and activation of these receptors might be linked to significant pathological changes. Thus, PARs are exciting targets in lung disease research. This chapter mainly focuses on the role of PAR family members in several lung diseases. In this context, modulation of PAR signaling might open novel avenues in the treatment interventions in a number of respiratory conditions at least in part to reduce the burden of the diseases.

Keywords

Proteases · Protease-activated receptor · Lung
Pulmonary fibrosis · Inflammation

1 Introduction

The lungs are one of the most affected organs during infection and oxidative stress due to its large surface area and frequent exposure to oxygen. Since lungs are highly vascular organ, the functions of lungs are greatly influenced by blood coagulation

G. Sudhandiran (✉) · S. Kalayarasan · T. Divya · B. Velavan
Cell Biology Laboratory, Department of Biochemistry, University of Madras,
Guindy Campus, Chennai, India
e-mail: sudhandiran@yahoo.com

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cascade. In addition to the exchange of gases between lungs and blood, they also function in filtering the venous blood which prevents blood clots and other fibers from entering the arterial circulation [1]. Proteases play an important role in the systemic coagulation cascade as the series of events during blood coagulation end up in the formation of thrombin, a serine protease. The formation of thrombin is critical during coagulation, and it catalyzes the conversion of fibrinogen to fibrin to form a clot. Hence, the serine protease thrombin is considered as the central enzyme which regulates the entire coagulation system [2]. Thrombin is generated early during acute lung injury and is thought to contribute to increased lung vascular permeability [3].

While the involvement of thrombin is crucial for the progression of lung disorders, it is interesting to know how it communicates to cells. Protease-activated receptors (PARs) provide an answer that how thrombin produces signals. PARs were first identified in the year 1991 as a class of seven trans-membrane domain G-protein-coupled receptors (GPCRs). The inactive GPCRs are activated by ligand receptor interactions which are furnished by proteolytic cleavage [4, 5]. Almost all eukaryotic cell types including epithelial cells, endothelial cells, fibroblast express PARs which regulate important physiological processes, and hence PARs are emerging as novel therapeutic target for various diseases [6–8]. Moreover, PARs are reported to play major role in airway inflammation as it is distributed throughout the airways. Endogenous or exogenous airway proteases highly regulate the activities of airway PARs by either activating or inhibiting the receptors. Elevated levels of PAR levels are associated with pathological conditions in the lung, and its role in developing lung diseases is extensively being investigated [9–11]. A thorough understanding of PAR-mediated cellular events is considered as one of the novel therapeutic approach targeted to prevent lung diseases and is the principal focus of this chapter.

2 The PAR Family Members

Till now, four different types of PARs have been identified named PAR-1 to PAR-4. Among these PAR1, PAR3, and PAR4 are thrombin receptors. PAR2 is an exceptional; serine proteases such as trypsin, tryptase as well as coagulation factors VIIa and Xa activate PAR2 [12, 13]. PAR1 is detected in fibroblasts, T cells, monocytes, hematopoietic progenitor cells, natural killer cells, smooth muscle cells, mast cells, neurons, glial cells, and epithelial cells [14]. Thrombin activates PAR1 by cleaving its extracellular amino terminus domain. Other proteases such as coagulation factor Xa, matrix metalloprotease-1 (MMP-1), and activated protein C (APC) can also activate PAR1. Out of these four proteases, PAR1 has been recognized as the major receptor that mediates pro-fibrotic and pro-inflammatory effects of thrombin [15, 16]. Initially trypsin was identified as the enzyme to

activate PAR2 [17]. Since, gastrointestinal (GI) tract highly expresses PAR2 as well as trypsin; early research focus was on investigating the role of the PAR2 in diseases associated with GI tract only [18]. However, several further studies identified that mast cell tryptase and other proteases activate PAR-2, which elaborate research studies on the role of PAR2 in a wide range of other adult tissues, especially, kidney, heart, vascular endothelium, and lung [19]. The discovery of PAR3 was quite interesting. A research group has suggested the presence of further receptors for thrombin other than PAR1. They have derived platelets from PAR1-deficient mice and have analyzed its response to thrombin and it was found that the platelets were responsive to thrombin [20]. This report strongly recommends the existence of PAR3 as receptors for thrombin. The occurrence of PAR3 in mouse bone marrow, spleen, and human tissues has been reported [21]. PAR4 was identified and cloned in the year 1998 by Kahn et al. They have reported that, while PAR3 is essential for normal thrombin responses in rat platelets, still another PAR4-mediated pathway also exists for thrombin signaling [22]. Though PARs are differing in their mechanism of activation, all PAR genes are reported to have the same structure with two exons and two introns. The similar gene arrangements in PARs propose the theory that these protease receptors rose from a common ancestral gene and suggest the existence of a common gene family [23].

3 Mechanism of Activation and Signaling of PARs- General Aspects

The mechanism behind the activation of PARs was primarily recognized for PAR1 [4]. The similar paradigm was found to be associated with the activation of other PARs also. The irreversible proteolytic activation of PARs initiate by the cleavage of the amino-terminal domain of the receptor. This proteolytic cleavage produces a new amino terminus end that functions as a ligand for the receptors to initiate further trans-membrane signaling [24]. However, the mechanism of PAR3 activation differs from this model. Ishihara et al. have demonstrated the mechanism of activation of PAR3 in COS-7, kidney fibroblast cells and suggest that initial cleavage at ectodomain of PAR3 alone is sufficient to activate it [21]. Interestingly, Nakanishi et al. have reported that murine PAR3 act as a co-factor for thrombin-mediated cleavage and activation of PAR4 [25]. Studies show that when compared with PAR1 or PAR3; PAR4 requires increased thrombin level for its activation. The reason is, thrombin highly binds to the carboxyl-terminal sequence of PAR1 or PAR3 receptors which is absent in PAR4 exodomain [26]. Apart from these, the interesting fact is that activation of PARs is an irreversible mechanism and thus the regulatory mechanisms of PAR signaling are critical determinants of the protease response in cells.

4 PAR Activation and Signaling Is Tissue Specific?

An important question that could arise is whether PAR signaling is tissue specific or not. Different proteases can activate PARs under various conditions. The hypothesis behind this mechanism is that, when proteases cleave PARs at the same sites it activates same signaling cascades with common patho-physiological outcomes. However, when proteases cleave PARs at distinct sites, the consequences of PAR cleavage could be varying. In this way, the activation of PARs by proteases may differ in normal and pathological conditions depending on the site of protease cleavage. The outcome of PAR signaling or activation of PARs by same protease is also tissue specific and can vary between tissues. For example, Ku et al. have described a distinct outcome of PAR1 activation in endothelial as well as vascular smooth muscle cells. They have demonstrated that activation of PAR1 by thrombin results in relaxation of the coronary artery. However, removal of endothelium results in contraction. This could possibly due to different signaling mechanisms by endothelial versus vascular smooth muscle cells [27].

5 PAR Signaling in the Lung

All four subtypes of PARs appear to be expressed in human respiratory tract. PARs are present in alveoli, fibroblasts, smooth muscle cells, epithelial cells, endothelial cells, goblet cells, as well as nerves within the lungs [6, 9]. Any internal or external inflammatory stimuli may enhance the expression of PARs, as well as proteases that activate PARs. Among the four PARs, PARs1, and 2 are reported to have a major role in inflammatory and fibro proliferative processes in the lungs. This hypothesis is supported by a number of evidences that increased expressions of PARs1 and 2 are observed during the development of inflammatory-mediated pulmonary diseases such as asthma, bronchitis, and pulmonary fibrosis [10, 11, 28]. Various proteases that present in the airways activate PARs. For example, PAR2 is activated by mast cell tryptase; PAR1, 2, and 4 are activated by trypsin; PAR1 is specifically activated by chymase; Cathepsin G activates PAR4. Active research is currently progressing on the regulation of PAR activity which helps to define potential therapeutic approaches for the prevention of respiratory diseases.

6 'Double Edged Sword' Nature of PARs in the Airways

An increased expression of PARs 1 and 2 has been well documented in a number of inflammatory and fibrotic lung diseases. However, the argument on the bronchoprotective nature of PARs still exists. There are evidences for protective role of PAR2 as it possesses anti-inflammatory effect following activation. A research group has reported that PAR2-activating peptide protects against

bronchoconstriction induced by histamine in guinea-pig model [29]. Another research group has demonstrated that activation of PAR2 provides protection against bronchoconstriction induced by 5-Hydroxytryptamine in rats [30].

On the other hand, evidences show that PAR2 stimulation results in pro-inflammatory responses, bronchial hyper-responsiveness, and contribute to allergic inflammation of the airway in mice over expressing PAR-2 [31]. Activation of PARs (PAR1, 2, and 4) in human respiratory epithelial cells is associated with Interleukin-6 (IL-6), IL-8, and prostaglandin E2 release [32]. Vliagoftis et al. have reported the important role of PAR2 in the regulation of matrix metalloproteinase-9 (MMP-9) in airway epithelial cells and demonstrated as critical elements in tissue reorganization during asthma and other inflammatory conditions in the respiratory system [33]. In addition, activation of PAR2 can directly produce airway contraction and may facilitate hyper-responsiveness in guinea pig airways [34]. PAR activation may also play a role in remodeling through the stimulated release of MMP-9 and procollagen from bronchial epithelial cells and lung fibroblasts, respectively [33]. Activation of PARs 1 and 2 in epithelial cells and fibroblasts has also been reported to stimulate the secretion of chemokines including IL-8, eosinophil survival promoting factors, granulocyte-macrophage colony-stimulating factor, as well as other pro-inflammatory and fibrotic mediators [35–37]. Taken as a whole, these observations point out that PAR activation may alternately stimulate inflammatory cell activity and play an important role in the progression of inflammatory and fibrotic lung diseases. The possible role of PARs in various lung diseases is discussed below.

7 PAR Signaling in Lung Diseases

7.1 Potential Role of PARs in Asthma

Asthma is a chronic inflammatory airway disease, characterized by inflammation of the airway epithelium which results in airway obstruction and bronchial hyper-reactivity reactions [38]. This disease has been postulated as a consequence of repeated injury to the mucosal surfaces of the lung. Both endogenous as well as exogenous proteases play major role in the development of asthma. Endogenous proteases such as thrombin, trypsin, tryptase, specific allergens, and serine proteases (Der p 3, 6, and 9), have been reported to regulate airway epithelial cell function during the progression of asthma [39–41]. Indeed, Terada et al. have found an increased level of thrombin in bronchoalveolar lavage (BAL) fluid along with other inflammatory mediators such as IL-5 and growth factors such as transforming growth factor-β (TGF-β) in asthmatic patients [42]. Since PAR1 can be activated by thrombin and increased concentration of thrombin in airway of asthmatic patients indicate that PAR1 plays a major role in the development of asthma. Similarly, increased expression of PAR2 has also been reported in bronchial epithelium of asthma patients [43]. A research group has detected the presence of PAR2 in airway

epithelial cells and administration of selective PAR2 agonist through nasal cavity stimulated macrophage infiltration into BALF in ovalbumin challenged mice [31]. Moreover, it was found that inhibition of PAR2 suppresses airway hyper-responsiveness induced by ovalbumin and over expression of PAR2 worsening this conditions [31]. The possible reason could be that the allergens which express several proteases can activate PAR2 and thereby facilitate inflammatory reactions. This was supported by an interesting study in which cockroach extract used as a challenging antigen, and the investigators revealed that the allergic airway sensitization to cockroach extract induces eosinophilic airway inflammation which depends on the ability of cockroach extract to activate PAR2 [44, 45]. Yet another group has reported the expression of all the four PARs (PAR1–4) on epithelium as well as smooth muscle in human bronchial biopsy samples from patients with asthma. PAR1 and 3 were predominately present within the columnar epithelial cells, whereas PAR2 and 4 were more widely diffused throughout airways. The investigators have further concluded an increased expression of PAR2 in bronchial epithelium and airways that might play a major role in airway inflammatory response [46].

Taken together, these reports suggest that activation of PARs especially PAR2 may enhance airway hyper-responsiveness and airway inflammation in allergic reactions and asthma. Various proteases such as mast cell tryptase and proteases present in the allergens are able to cleave PAR2 and represent one of the mechanisms for the aggravation of the disease.

7.2 Role of PARs in Chronic Obstructive Pulmonary Disease

A growing attention on the role of PAR signaling exists in chronic obstructive pulmonary disease (COPD) because, like asthma, COPD also associated with airway remodeling and activation of the coagulation cascade. Cigarette smoking is certainly the predominant risk factor for COPD [47]. One interesting fact is that PAR1 signaling promotes goblet cell metaplasia and excessive mucus production which is a hallmark feature of COPD and sheds light on the involvement of PAR signaling in COPD. In addition, PAR1 activation by thrombin has been shown to endorse airway remodeling by enhancing the expression of TGF-β1 in ovalbumin-allergic rats [48]. Another group has reported that there is no difference in the expression of PAR2 in bronchial biopsy of patients with COPD [49]. In contrast, Miotto et al. have reported an increased expression of PAR2 in the respiratory tract of COPD patients [50]. BAL from COPD patients contains increased level of trypsin [51]. Though COPD patients have enhanced coagulant activity, increased thrombin levels have not been reported in BAL from patients with COPD [52]. These studies indirectly help to find out the hypothetical involvement of PAR2-activating enzymes in COPD. However, further studies are warranted to elucidate the mechanisms involved in detail.

7.3 Potential Role of PARs in Pulmonary Fibrosis

Pulmonary fibrosis (PF) is a chronic obstructive pulmonary disease characterized by the excessive accumulation of extracellular matrix components within the pulmonary interstitium [53]. Several processes such as injury to the alveolar epithelial and endothelial cells, activation and proliferation of fibroblasts, epithelial-to-mesenchymal transition, inflammatory cell influx, hypercoagulation, collagen accumulation, angiogenesis, and aberrant repair process leading to fibrogenesis of lungs [54–56]. Following tissue injury, one of the initial events occur in lungs is the activation of the coagulation cascade. Activation of coagulation cascade during the development of several fibrotic lung diseases such as systemic sclerosis [57], idiopathic pulmonary fibrosis (IPF) [58], and pneumonitis [59] have been reported. The incidence of increased thrombin levels in BALF from patients with PF associated with systemic sclerosis was illustrated nearly two decades ago itself [60]. The occurrence of high thrombin level in premature infants with chronic lung diseases was also reported [61]. The biological effect of thrombin during obstructive pulmonary diseases might be an indirect mechanism through the production of inflammatory mediators and fibroproliferative growth factors, which further activate PAR1 fibroblasts. In support of this, Mercer et al. have reported that PAR1 knockout mice are protected from the pathological consequences of bleomycin (BLM)-induced lung inflammation and fibrosis [62]. Studies involving primary human lung fibroblasts revealed that PAR1 exerts potent fibrogenic effects, including promoting platelet derived growth factor (PDGF)-mediated fibroblast proliferation and increased collagen synthesis. Furthermore, in animal models of fibrotic lung injury, the same research group has shown that BLM-induced inflammatory cell infiltration and lung collagen accumulation were reduced in PAR1 knockout mice [28]. TGF-β1 is considered as a notorious fibrotic mediator in multiple fibrotic conditions by promoting myofibroblast differentiation and extracellular matrix deposition [63]. Studies show that PAR1 contribute to fibrogenesis through the activation of TGF-β1 in fibroblasts and alveolar epithelial cells [64].

The crucial role of PAR1 in the progression of PF is well studied. However, limited reports are available on the involvement of other PARs in this disease model. In this context, we have assessed the role of PAR2 in BLM-induced experimental fibrosis model. RT-PCR, Confocal microscopic analysis, and Western blotting were performed to examine the expression of PAR2 in rat lungs administered with BLM. Normal rat exhibits little expression of PAR2. However, after BLM instillation the expression of PAR2 was markedly increased in rat lungs. Our findings suggested that PAR2 is involved in two different ways in the progression of PF. One mechanism is the MMP2/9 and cytokine-mediated fibroblast proliferation [11]. The second mechanism is PAR2-mediated epithelial cell apoptosis which involves p38 MAPK, c-JNK, and caspase-3. We showed evidences that organosulfur compound diallyl sulfide provide protection against BLM-induced lung fibrosis through the attenuation of PAR2 [11]. Further, we have shown evidence that PAR2 induces inflammation and apoptosis in lungs of rats administered with BLM [65]. Schematic representation of the PAR-mediated events taking place

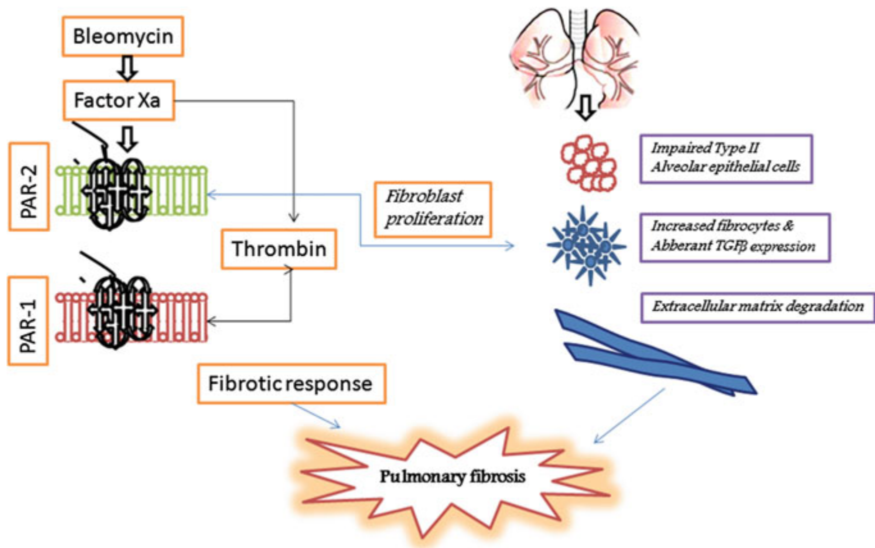

Fig. 1 PAR-mediated events in the progression of pulmonary fibrosis

in PF is depicted in Fig. 1. Available reports suggest that PAR4 may also have a fibroproliferative role. Stimulation of PAR with thrombin or a synthetic PAR4 agonist peptide induces morphological changes in primary cultured alveolar epithelial cells and A549 cells. PAR4 stimulation decreases E-cadherin expression and increases the expression of mesenchymal markers. This report indicates that PAR4 induces epithelial-to-mesenchymal transition which is a key mechanism in the progression of PF [66]. Together, PAR signaling is a promising mechanism in PF research and could be a better target for the regulation of this disease.

7.4 Role of PARs in Acute Lung Injury

Acute lung injury (ALI) is a disease condition occurs as a result of various diseases such as pneumonia, acute pancreatitis, sepsis, and trauma. The alveolar damage during ALI leads to the formation of lung edema. Injury to the alveolar cells and damage of the epithelial barrier enhance the fluid accumulation in the alveolar and interstitial space of the lungs [67]. Though the inflammatory and coagulation responses can be considered as protective mechanism to the host especially in the context of infection, the excessive coagulation and inflammation can lead to extreme tissue injury with disruption of the alveolar-endothelial capillary barrier which leads to the accumulation of protein-rich fluid in the alveolar spaces [68]. Available report suggests that PAR2 does not have a role in the development of acid-induced experimental acute lung injury in mice [69]. However, one report shows that mast cell tryptase mediates ALI through the activation of PAR2 to

produce IL-8 in experimental animals [70]. In contrast, reports recommend that PAR1 signaling influences several key features of ALI including neutrophil infiltration, alveolar leakage in BLM-induced lung injury in mice through the activation of TGF-β1 [71].

7.5 Role of PARs in Bronchitis

Bronchitis is a respiratory disease in which the mucus membrane in the lungs' bronchial passages becomes inflamed. This airway inflammation is one of the three elementary components of all airway diseases, the other two are airflow obstruction and airway hyper-responsiveness [72]. The very first event of the inflammatory reaction starts upon the activation of resident cells in the damaged tissue. Among these, mast cells and macrophages play a predominant role by releasing inflammatory mediators, such as cytokines, nitric oxide (NO), prostaglandins, histamine, and serotonins. Since mast cells express PAR1 and PAR2, these two gained much attention in inflammatory lung diseases where the activation of these two receptors leads to mast cell activation and subsequent release of inflammatory mediators [73]. Several research studies have illustrated the pro-inflammatory role of PARs in the respiratory system. For instance, increased secretion of eosinophils and eotaxin content were observed in BALF from PAR2 deficient mice even after sensitization with ovalbumin, whereas wild-type mice exhibited increased infiltration of eosinophils in BALF after ovalbumin induction [31, 74]. Another report suggests a neuropeptide dependant mechanism of PAR2-mediated inflammation, which shown evidences that removal of sensory neurons significantly decreased the PAR2-mediated airway constriction, pulmonary inflammation, and edema [75]. In contrast to the pro-inflammatory role, anti-inflammatory effect of PARs has also been reported. PAR1-activating peptides (APs) cause prostaglandin E2 (PGE2) release which has a role in limiting the immune/inflammatory response especially in the lungs [76], from human bronchial epithelial cells [32] and human lung fibroblasts [77], suggest anti-inflammatory role of PARs in the lungs. In support with these findings, D'Agostino et al. have reported that PAR2-APs significantly inhibit bronchoconstriction, airway hyper-responsive reactions, and immune response induced by allergens in rabbits [78]. Together, more research on the possible role of PARs whether pro-inflammatory or anti-inflammatory, in airway inflammation and bronchitis helps to tone down the severity of the disease condition.

7.6 Possible Role of PARs in Lung Cancer

Lung cancer is one of the leading cause of cancer death worldwide and is the second most common cancer overall [79]. The important role of PARs in tumor development and metastasis has been suggested in late 1990s. Among four, PAR1 has been reported to play major role in cancer metastasis. A research group has reported that inhibition of PAR1 ligation at the site of tumor helps to regulate tumor metastasis [80].

Another group has reported the importance of thrombin receptor in cancer invasiveness [81]. Subsequently, Boire et al. have found that, MMP-1 cleaves PAR1 at the proper site to generate PAR1-dependent Ca^{2+} signals to enhance migration and tumor invasion [82]. Cisowski et al. have reported that lung cancer cell lines express PAR1 at high concentration than other group of PARs. Primary cell cultures isolated from lung cancer patients shown 40-fold higher expressions of PAR1 and exhibit increased migratory capacity than epithelial cells cultured from nonmalignant lungs. Experiments performed with cells silencing PAR1 expression revealed PAR1 as a possible target for therapy in lung cancer [83]. In continuation, another research group has reported that activation of PAR2 prevents apoptosis of lung cancer cells. They showed evidences that tryptase activates PAR2 to regulate the expression of epidermal growth factor receptor (EGFR) and to prevent apoptotic cell death of cancer cells [84]. Yet another group has identified the mechanism behind the pro-invasive property of PAR1 and has reported that the cooperation between PAR1 and integrins via its cytoplasmic tail mediates tumor invasion, therefore, PAR1-integrin complex may represent an important therapeutic target to prevent tumor invasion during lung cancer [85].

8 Conclusion

In summary, PAR family of receptors are emerging mediators of a wide range of cellular and molecular events during the development and progression of several lung diseases. The development of antagonists for PAR activation still remains to be established. Modulation of PAR signaling pathways may represent a novel therapeutic approach for the prevention of number of respiratory diseases to reduce the burden of disease condition.

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Unfolding the Mechanism of Proteases in Pathophysiology of Gastrointestinal Diseases

Sharmistha Banerjee, Sumit Ghosh, Krishnendu Sinha
and Parames C. Sil

Abstract

The intestinal epithelial biology is controlled by the presence of the gastrointestinal protease pool and their inhibitors. Enteric bacterial proteases and their signalling mechanisms contribute to the pathogenesis of IBD. Proteases derived from *Helicobacter pylori* (*H. pylori*) are mainly responsible for ulceration, low stomach acid and gastritis. *H. pylori* infection initiates the inflammatory and apoptotic pathways. The apoptotic pathways involve the proteolytic activities of caspases, i.e. cysteine aspartate proteases. However, some types of matrix metalloproteinases restrain such infection while other types act as the causal agent for gastric ulceration. Serine proteases and PAR-2 have been found to elicit signalling pathways related to induction of pain in patients suffering from irritable bowel syndrome. The prognostic role of tumour-associated proteases in colorectal cancer has also been investigated. The increased levels of cathepsin in Gaucher storage cells point towards their role in pathogenesis of the disease. On the other hand, excessive trypsin and chymotrypsin activity has been observed in case of chronic pancreatitis. This chapter focusses on the role of proteases on the pathology of various diseases.

Keywords

Gastritis · Gastropathy · Matrix metalloprotease
Inflammatory bowel disease · Tumour necrosis factor-α

S. Banerjee · S. Ghosh · P.C. Sil (✉)
Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M,
Kolkata 700054, West Bengal, India
e-mail: parames@jcbose.ac.in; parames_95@yahoo.co.in

K. Sinha
Jhargram Raj College, Government of West Bengal, Jhargram, India

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1 Introduction

Proteases are proteolytic enzymes that cleave the peptide bonds formed between the amino and carboxyl groups of adjacent amino acid residues of a polypeptide chain for the purpose of protein catabolism. About, 550 different types of proteases have been identified from the human genome, and more than 100 have been predicted. Proteases account for about 5% of the genome of infectious organisms such as bacteria and viruses, thereby establishing themselves as potential drug targets.

Proteases can be classified into seven groups: serine proteases, aspartate proteases, cysteine proteases, glutamic acid proteases, threonine proteases, metalloproteases and asparagine peptide lyases [1]. Of these, the mechanism of action of serine proteases has been extensively studied till date. Aspartic, glutamic and metalloproteases activate water molecules for nucleophilic attack on the peptide bonds for their hydrolysis. On the other hand, the serine, threonine and cysteine proteases use a catalytic triad for nucleophilic attack. A catalytic triad refers to three amino acids which function together at the active centre of transferase or hydrolase enzymes. Such triads point towards the convergent evolution in the proteolytic activity of such proteases [2].

The broad spectrum of protease activity is attributable to their wide range of applications as important biological tools in industrial and therapeutic research. Nowadays, the gastrointestinal diseases are found to be quite common in both developing and developed countries. Lifestyle, personal preferences for food, rampant exposure to pollutants, etc. are important causes for the prevalence of gastric diseases. The role of gastric proteases in the progression of these diseases has been elaborated in this chapter. Diseases like gastritis, sepsis, Gaucher disease, colorectal cancer, inflammatory bowel disease, acute and chronic gastropathy. Figure 1 has been discussed in this chapter, mainly focussing on the role of

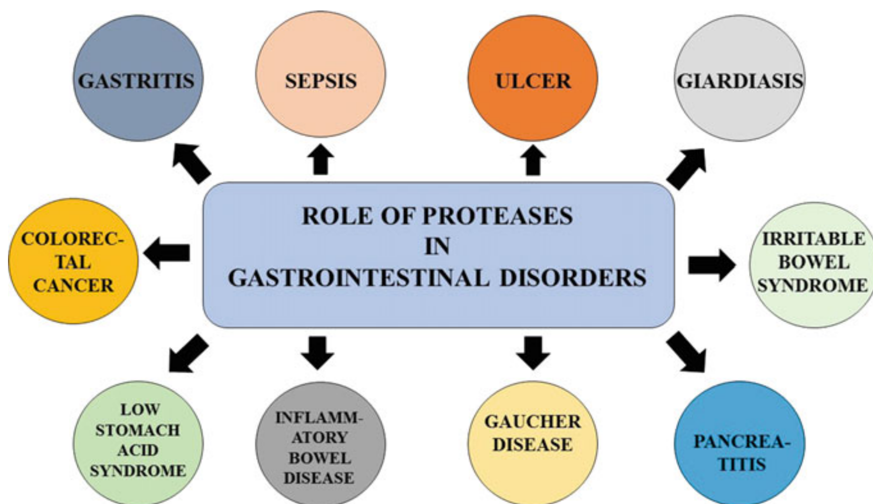

Fig. 1 Depicts the association of protease in various gastrointestinal diseases

proteases and the associated molecular mechanisms responsible for the regulation of concerned pathogenicity.

The cosmopolitan occurrence of proteases in lower to higher groups of organisms impart immense significance to these enzymes as they are being the important sources for the study of evolutionary convergence or divergence in structure–activity relationships. Their ability to adapt to the mechanisms of 'limited' to 'unlimited' proteolysis and vice versa (depending on the substrate or other micro-environmental factors) has increased their domain of functional activities.

Modern molecular reductionism has enlightened us on the role of enzymatic activities in the onset and maintenance of different diseases. The major theme of this chapter is centred on the holistic overview of the potential role of proteases in different gastrointestinal disorders. This approach has been adopted with the intention of providing an essence of the various mechanisms associated with the protease-mediated pathogenicity of gut-associated diseases.

2 Mechanisms of Gastrointestinal Protease Activity: A Brief Overview

Though proteases are holistically considered as enzymes for degradation, a much wider aspect of their functions is chiefly attributable to the emergence of the concept of 'limited proteolysis'. Proteolytic cleavage is considered to be an ideal multicomponent irreversible signalling event. Such events are regulated by different enzymes, growth factors, surface receptors, etc. On the other hand, 'limited proteolysis', a term coined by Lang and Linderstrom, refers to non-specific protein degradation. It plays a vital role in maintaining the function of intestinal epithelial cells (IEC).

The gastric functions are dependent on pericellular proteolysis. Intestinal proteases induce the pathogenesis of gastrointestinal inflammatory diseases [3]. Most of these proteases are synthesized as concealed zymogens which undergo proteolytic cleavage for activation [4, 5]. Multiple families of proteases function together to form networks [6]. Matrix metalloproteinase (MMP) zymogens which degrade the ECM are cleaved to active enzymatic forms by serine proteases associated with the plasminogen activator system and [7] also by furin-like prohormone convertases [8]. The functional specificity of enterocyte proteases to suitable substrates is controlled through spatial and chronological compartmentalization. Mast cell proteases are catalogued within intracellular granules and undergo intestinal inflammation-mediated retortion [9]. Proteases are localized in the pericellular environment through various mechanisms. For example, matriptase, a type II epithelial transmembrane serine protease, is reported to get fastened directly to the plasma membrane by its membrane-spanning domain [10, 11]. Termination of protease activity is accomplished by several mechanisms. Serpins, a family of inhibitors of serine protease, target different stages of the serine protease signalling cascades by functioning as irreversible 'suicide substrates' [12]. Proteolysis-mediated activation of PARs is reported to exhibit

gastrointestinal and physiological activities [13]. Serine proteases of the intestinal lumen activate PARs to exert their functions [14]. Proteases are essential for wound repair and inflammation [15]. Components of the ECM, such as proteoglycans, fibronectin, collagen undergo MMP-10-mediated degradation while different protease inhibitors are regulated by cytokines released in response to repair of wound [16].

3 Gastritis

Helicobacter pylori (*H. pylori*), a gram-negative microaerophilic bacteria colonizing the human gastric mucosa, is the causative agent of gastritis and is approximated to affect 50% of the world population. Gastritis is associated with inflammation of the gastric mucosa. In 1984, Warren and Marshall discovered the role of *H. pylori* in the pathogenesis of gastritis and consequently received the Nobel Prize in 2005. Different types of matrix metalloproteases (MMPs), tissue inhibitors of MMPs (TIMPs) and a disintegrin and metalloproteases (ADAMs) are involved in progression of the disease [17].

H. pylori infection is acquired mainly through the faecal–oral route during infancy and such bacterial colonisations may persist throughout lifetime thereby offering a range of clinical manifestations [18]. The severity and progression of the disease depend on interactions of different factors related to both the host (genetic make-up, immunological state, etc.) and the bacteria (adaptation capacity, genomic plasticity, generation of virulence factors like vacuolating cytotoxin (VacA) and cytotoxin-associated antigen A (CagA)). CagA and VacA are coded by a pathogenicity island, called cag PAI and are present in the genome of *H. pylori* [19].

The extracellular matrix metalloproteases (MMPs) are a family of zinc-dependent proteases which vitiate the machinery of the extracellular matrix (ECM) and also participate in various physiological and pathological processes like morphogenesis, tumoural angiogenesis, metastatic invasions [20].

MMP-1 degrades interstitial collagen types I, II and III and thereby participates in the pathogenesis of gastritis [21]. MMP-2 and MMP-9 (i.e., Gelatinase A and B, respectively) degrade the major structural unit of the basement membrane, i.e. collagen type IV. In gastric tissues of gastritis patients, IL-21-mediated increase in expression of MMP-9 and MMP-2 compared to that in normal tissue has been reported [22]. Increased expression of MMP-8 has also been reported in case of gastritis patients [23].

MMP-7 can degrade fibronectin, proteoglygans, elastin, etc. and is reported to exhibit a vital function in the progression of the disease. MMP-7 is associated with induction of epithelial to mesenchymal transition of human gastric cells and is considered to play an important role in the development of gastric cancer [24]. It induces IGF-II (insulin-like growth factor binding protein)-mediated metastatic invasion of neoplastic cells from affected gastric tissues to other parts of the body [25, 26]. MMP-7-181G allele induces the expression of MMP-7, thereby stimulating the degradation of the extracellular matrix in gastric tissues of gastritis patients [27].

The cag PAI-mediated expression of MMP-7 is regulated by the phosphorylation and intracellular redistribution of p120-catenin. On being translocated to the nucleus of gastric cells, p120-catenin releases a repressor of MMP7, thereby controlling its expression [28].

The MMPs are inhibited non-covalently by the tissue inhibitors of MMPs (TIMPs). Increased mRNA levels of TIMP-1, -2, -3 and -4 are observed in case of *H. pylori* infected patients [29].

The A disintegrin and metalloproteinase (ADAM) family of proteases is associated with the activation of signalling pathways related to cellular adhesion and fusion and the release of growth factors and cytokines [30]. High levels of ADAM10 and ADAM17 in the gastric antrum of *H. pylori* infected individuals activate an array of signalling molecules such as tumour necrosis factor, c-Met, E-cadherin, thereby leading to the progression of gastric cancer [31, 32].

Neprilysin, a zinc-dependent metalloprotease, is also reported to play an important role in the prevention of gastritis. *H. pylori* infection reduces the level of expression of substance P, vasoactive intestinal peptide (VIP), neprilysin, etc. [33].

A subfamily of G-protein-coupled receptors, i.e. the protease-activated receptors (PARs), regulates cellular functions through the proteolysis of a receptor fastened peptide domain. PAR-1 and PAR-2 are ubiquitously present along the entire gastrointestinal (GI) tract. Thrombin and trypsin-like proteases activate PAR-2 and PAR-1 on the neurones of the vagus nerve, thereby increasing the intracellular levels of calcium via activation of phospholipase C and inositol triphosphate (IP3) signalling pathway. Increased expression of PAR-2 and PAR-1 transcripts is observed with the progression of inflammation. Such deviations from the normal physiological conditions are also reported to cause changes in gastric motility and induction of gastritis or inflammatory bowel disease. The activation of vagal efferent DMV neurons, displaying functional PAR-2 and PAR-1, leads to robust gastric stasis [34].

4 Gaucher Disease

Gaucher's disease or Gaucher disease (GD) is characterized by the accumulation of glucocerebroside (a sphingolipid) in white blood cells (mainly tissue resident macrophages i.e. Gaucher cells) and different organs like the kidneys, lungs, brain, bone marrow, etc. due to deficiency of glucocerebrosidase activity. The symptoms include anaemia, low platelet count and enlargement of the spleen and liver.

Gaucher disease is of three clinical subtypes:

GD type I (non-neuropathic) is the most common form of the disease and is associated with hepatosplenomegaly, anaemia, leukopenia and thrombocytopenia.

GD type II (acute neuropathic) is characterized by progressive damage of the brain, disorders of optic movements, limb rigidity and a poor ability to suck and swallow in infants.

GD type III (chronic neuropathic) is characterized by skeletal defects, anaemia, respiratory problems, etc.

In GD type I, massive accretion of glucosylceramide occurs in the lysosomes of tissue resident macrophages, called Gaucher cells. These cells are mainly found in the spleen, liver and bone marrow and are associated with hepatosplenomegaly, avascular necrosis, pancytopenia, etc. [35]. Substrate reduction therapy and enzyme replacement therapy are associated with treatment of the disease. The enzyme replacement therapy is based on the oral administration of N-butyl-deoxynojirimycin, an inhibitor of glucosylceramide biosynthesis [36]. Substrate reduction therapy is based on the oral administration of miglustat, an inhibitor of glucosylceramide synthase, the key enzyme in glucosylceramide biosynthesis [37]. The high cost of such therapies led to the quest for detecting different plasma biomarkers related to Gaucher disease. It is a cost-effective approach for personalized treatment. Two specific Gaucher cell markers that have been identified include chitotriosidase and CCL18, both of whose levels get elevated in Gaucher patients [38, 39]. Investigation on the plasma protein expression patterns in Gaucher patients using the conventional two-dimensional gel electrophoresis (2DGE) within the acidic range led to the observation, that in the case of plasma samples of patients, there was lack of common high molecular weight proteins while novel low molecular weight proteins (degradation products) were present. Such a unique observation indicated the involvement of proteases functioning preferably in an acidic environment. The responsible protease(s) in Gaucher plasma have not been identified yet. The involvement of MMPs and Cathepsin B, D, K, S has been elucidated through extensive research. Increased concentrations of different cathepsins [36] and angiotensin-converting enzyme (ACE) [25] have been observed in the plasma of symptomatic Gaucher patients.

5 Sepsis

Sepsis is a serious pathophysiological condition, generated when the body's internal response to infection harms its own tissues and organs. The common symptoms include fever, increased breathing rate, increased heart rate and confusion [40].

PARs are activated through proteolysis, which is triggered by extracellular proteases. PARs can convert events of extracellular proteolytic cleavage into an intracellular signal. These receptors carry their own fastened ligands, which remain ambiguous until unveiled by receptor N-terminal cleavage. An intramolecular rearrangement follows which then allows the receptor moieties and the ligand to interact with each other [41].

PAR-1 plays an important role in coordinating the interaction between inflammation and coagulation, and thus, extensive research in this field may lead to documentation of novel therapeutic targets for the treatment of sepsis [42]. PAR-1 is associated with thrombin-mediated aggregation of platelets in humans. It can trigger various cellular responses through different signalling mechanisms by switching from one conformational state to another. PAR-1 activated by thrombin

increases endothelial permeability [43]. It is responsible for *Streptococcus pneumonia*-induced sepsis through an interaction with platelet-activating factor receptor (PAFR). It also induces the influenza virus infection-associated pathogenicity and aggravates pulmonary oedema [44]. PAR-1 is reported to amplify PAFR dependent pneumococcal dissemination thereby inducing severe pneumonia. It opposes the progression of sepsis, and so, PAR-1 activation or blockade proves to be alternatively favourable at different stages of various sepsis models [45].

The proteasome degrades damaged proteins by proteolysis, a chemical reaction catalysed by proteases. Experiments on rabbit (as the working model) have shown that Toll-like receptor 4, the IκB kinase and the proteasome can be potential therapeutic targets in the treatment of lipopolysaccharide (LPS)-induced intestinal sepsis. It is reported that systemic blockade of Toll-like receptor 4, IκB kinase or the proteasome ameliorates the effect of LPS on the mRNA expressions of acetylcholine, substance P, prostaglandin E2, IL-8 and IL-6 mRNA expression in the rabbit intestine [46].

6 Giardiasis

Giardia duodenalis is the major aetiological agent of waterborne diarrhoeal diseases throughout the world. It is estimated to taint about 280 million individuals annually. The parasite has been included in the neglected diseases initiative of the World Health Organization (WHO) since 2006 [47]. Giardiasis is characterized by stunted growth in children, anion hypersecretion, shortening of microvilli of the intestinal brush border, enterocyte apoptosis, etc. [48]. When the infection reaches its peak, the trophozoites of *G. duodenalis* induce various pathological processes within the epithelial cells of the intestine thereby leading to the development of diarrhoeal disease.

Cathepsin cysteine proteases of *G. duodenalis* play a vital role in the induction of Giardiasis. The trophozoites produce cathepsin B and L in various different strains. The cathepsin cysteine proteases help in trophozoite encystation and excystation [49]. These proteases contain cysteine and histidine residue bearing active site [50]. The genome of *G. duodenalis* contains genes for different catL- and catB-like cysteine proteases [51]. The catB-like cysteine proteases degrade CXCL8, thereby attenuating neutrophil chemotaxis [52]. Giardia contributes to diarrhoea by disrupting the organization of the epithelial brush border microvilli. Giardia is also reported to induce epithelial apoptosis [53]. Villin is a pro-survival factor whose overexpression delays epithelial apoptosis [54]. It also helps in cellular migration and wound healing [55]. Thus, villin maintains homeostasis at the cellular level. The Giardial cathepsin cysteine proteases are reported to degrade host intestinal villin via activation of the myosin light chain kinase (MLCK). This helps in progression of the disease. *G. duodenalis* uses various mechanisms for the disruption of villin and the intestinal epithelial brush border.

G. duodenalis cathepsin cysteine proteases act as potential parasitic virulence factors having the capacity to induce different pathological responses within the host intestinal epithelial cells [56].

7 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is an inflammatory disorder which affects the gastrointestinal tract and colon mucosa. IBD is primarily manifested as ulcerative colitis (UC) and Crohn's disease (CD). One of the characteristic features of this disease is that relapse and remissions occur frequently. Environmental factors, immune response-mediated tissue injury, and genetic factor play major role in the pathophysiology of IBD [57]. Cytokines play a significant role in the regulation of this disease [58]. Gastrointestinal tract (GI) of human contains more than 500 species of bacteria. These bacteria secrete various compounds which have beneficial as well as adverse effects. These bacteria colonizing the GI tract helps to maintain gut homeostasis. The intestinal immune system has evolved to protect against luminal bacteria. Under normal condition, gastrointestinal injury and tissue repair are tightly regulated by interaction and interplay between array of defensive and aggressive factors in the lumen and wall of intestine. Inflammosomes are multiprotein complexes that regulate various mechanisms during homeostasis and inflammation. These complexes consist of different NLR proteins like NLRP1, 2, 3, 6 and others. NLR6 has been reported to play crucial roles in protection against infection and inflammation [59, 60]. This molecule performs crucial function in the maintenance of intestinal homeostasis and healthy intestinal microbiota. NLRP6 is also required for mucosal self-renewal and proliferation. Secretion of mucus by goblet cell of large intestine serves for a protective mechanism at the interface between eukaryotic and prokaryotic cells of the intestinal ecosystem.

NLRP6 deficiency leads to microbial dysbiosis by favouring the development of colitogenic microbiota. This microbial population is associated at the base of the crypt and stimulates the release of pro-inflammatory cytokines ultimately leading to chronic inflammation paving a road for inflammatory bowel disease [61]. It can be concluded that microbial dysbiosis in the intestine is one of the major causes of IBD [62].

The complex system of intestinal homeostasis is also modulated by environmental stress. In animal models, stress has been found to reactivate colitis. The central nervous system responds to environmental stress and links with the intestine through dorsal root nuclei, spinal cord and intestinal neurons on one hand and via neuroendocrine system on the other hand. This coordination leads to activation or inhibition of specific neurons in the intestine and stimulates the release of certain molecules which help to aggravate the disease [63, 64].

Proteases are associated with the progression of the disease. Metalloprotease (MMP) is one family of protease. MMPs are zinc-dependent endoproteases which help in degradation and remodelling of extracellular matrix (ECM). These proteases

are secreted as inactive precursor known as zymogens, and they are converted to active enzyme in the extracellular space. Regulation of MMPs occurs in four ways: at the level of their transcription from gene, through interaction with specific ECM components, at the level of activation from precursor enzyme and by TIMP inhibition [65, 66].

MMPs can proteolytically cleave and activate various non-matrix substances like cytokines, chemokines and growth factors.

MMP13 is a matrix metalloprotease which belongs to the class collagenase. Literature suggests that MMP13 is involved in ulcerative colitis and Crohn's disease. mRNA expression of this MMP was significantly increased in IBD biopsy specimens [67]. There was a positive association between MMP13 expression and histological inflammation scores in mucosal samples taken from IBD patients [68]. MMP13 deficiency protected mice from LPS-induced inflammation, intestinal permeability, mucus depletion and endoplasmic reticulum stress (ER stress). This protease converted pro-TNF-α into mature bioactive TNF which aided in augmenting inflammation and mucus depletion MMP13 deficiency also resulted in reduced symptoms of clinical colitis in DSS treated mice [69] (Fig. 2). Increased mRNA expression of MMP-13, MMP-7, and MMP-2 mRNA were observed in biopsy specimens of Crohn's disease and ulcerative colitis. Expression of MMP-7 and MMP-2 was enhanced also at protein level [70]. Serum level of MMP-3 and MMP-9 has been co-related with the disease symptoms and activity in children.

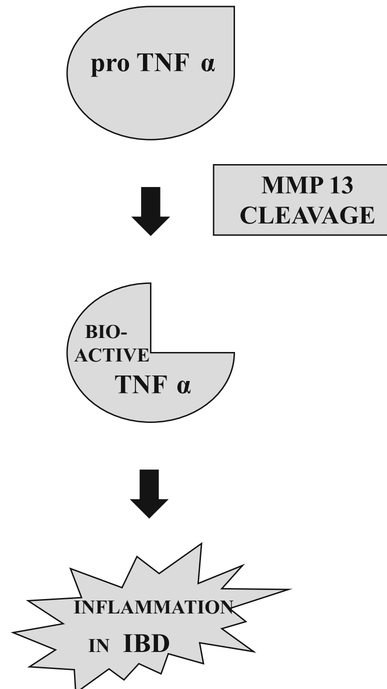

Fig. 2 Depicts the activation of TNF-α by MMP13

In a study involving, 31 children aged between 3 and 18 years with ulcerative colitis had higher level of serum MMP-3 and 9 as compared with children without ulcerative colitis. Elevation of these two proteins suggests the possibility of their use as clinical biomarkers of IBD [71]. A positive correlation was found between elevated expression of MMP-13 and histological evidence of inflammation in patients suffering from Crohn's disease (CD) and ulcerative colitis (UC). Expression of this protease was absent in non-inflamed colonic mucosa [72]. Enhanced expression of interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) transcripts was observed in granular tissue around ulceration site in patients with Crohn's disease or ulcerative colitis (UC). Elevated expression of MMP-13 and TIMP-3 is observed in fibroblasts of colonic ulcer [73]. MMP-3 activation has been implicated in colonic inflammation. Stromal myofibroblasts play a role in directing extracellular matrix components through metalloproteinases secretion. Exposure of the human colonic myofibroblast cell line 18 Co to TNF-α and bradykinin stimulated expression of MMP-3 mRNA. This TNF-α and bradykinin-induced MMP-3 expression are regulated through signalling mechanism involving PKC, PKD1 and MEK [74]. MMP-9, MMP-8 and prolyl endopeptidase (PEP) degrade collagen to form proline–glycine–proline (PGP). This degradation product of collagen has chemotactic effects on neutrophil. This proteolytic cascade has been found in intestine of patients with IBD [75]. Neutrophil infiltration into ulcer site can be partially prevented by reducing collagen degradation.

Macrophage metalloelastase MMP-12 and membrane type 1-MMP (MMP-14) were over expressed in lamina propria and macrophages of gut [76]. It is frequently observed that the gut is massively infiltrated with plasma cells and B cells in the pathogenesis of both UC and CD. Human B cells secrete granzyme B (GrB) in the presence of IL-21. GrB producing CD19+ and IgA+cell numbers were significantly higher in intestinal mucosa of both CD and UC than that of normal mucosa. Pro-inflammatory cytokine, IL-21 enhanced the expression of GrB in CD19+B cells and ultimately lead to enhanced cytotoxic activities. IBD related pathophysiology is marked by the accumulation of GrB-expressing CD19+ and IgA+cells [77]. Recent evidence suggests that patients suffering from IBD fail to respond and are not operative for the treatment of Crohn's disease. Activated matrix metalloproteinase 3 (MMP3) and MMP12 whose levels are elevated in inflamed mucosa of patients with IBD, have a wide range of substrates, including IgG1. Proteolytic degradation helps to contribute to non-responsiveness to anti-TNF agents in IBD patients [78]. MMPs are also associated with *Helicobacter pylori*-associated gastritis, and gastric and duodenal ulcer. Gastrointestinal mucosal inflammatory responses to *Helicobacter pylori* are characterized by increased production of MMP-9 and induction of mitogen-activated protein kinase (MAPK) and Rac1 activation. This H. pylori-induced MMP-9 also requires the ERK-mediated phosphorylation of cPLA2 on serine 505 and is necessary for membrane translocation of Rac1.

8 Pancreatitis

Pancreatitis is an inflammatory disease which affects pancreas. The main events which lead to the initiation of acute pancreatitis are protease activation and the inhibition of zymogen secretion [79]. In cerulein-induced acute pancreatitis, zymogens are missorted and co-localized with lysosomal cathepsin B in large cytoplasmic vacuoles, the sites of trypsinogen activation [80]. Premature activation of trypsinogen in pancreas triggers the activation of the cascade of all pancreatic enzymes. Chymotrypsin C, a protease is reported to limit proteolytic activities of trypsinogen and trypsin. Hereditary pancreatitis is concomitant with CRTC mutants (p.A73T and p.G61R) which precipitate in ER, resulting in ER stress. These mutants, on getting expressed, reduce the secretion of amylase from the pancreatic acinar cells AR42 J of carbachol-stimulated rat and the pancreatic acini isolated from mice. This also resulted in acetylated tubulin level through increased phosphorylation of deacetylase, SIRT2. Inhibition of SIRT2 restored amylase secretion as well as tubulin acetylation in isolated acini and pancreatic acinar cells [81]. Cationic trypsinogen (PRSS1) and anionic trypsinogen (PRSS2) are the major trypsinogen isoforms found in human pancreas. Mutation in PRSS1 causes hereditary pancreatitis. This mutation alters cleavage of regulatory nick sites by chymotrypsin C (CTRC) and resulted in reduced degradation of trypsinogen leading to its increased autoactivation. CTRC aided to promote degradation and suppressed autoactivation of human anionic trypsinogen. This increased sensitivity to CTRC-mediated degradation was the result of an additional cleavage site at Leu148 in the autolysis loop and the absence of the conserved Cys139–Cys206 disulphide bond. Single mutations of either Leu81 or Arg122 of cationic trypsinogen resulted in total resistance to chymotrypsin-mediated degradation. This shows that CTRC suppressed autoactivation of human anionic trypsinogen more effectively than the cationic trypsinogen which explains the lack of association of anionic trypsinogen with hereditary pancreatitis [82].

Cathepsin D (CD) is an aspartic protease found in lysosomes. This protease participates in various physiological functions like regulation of apoptosis, aids in activation of enzyme precursor and macroautophagy. CD remains localized to lysosomes as a 52 kDa precursor [83] and is converted to intermediate form by cleavage of 44 amino acids from amino terminus thus producing a 48 kDa single chain. This proteolytic degradation is executed by lysosomal cysteine proteases or by autolysis of CD [84, 85]. Further, cleavage of this intermediate 48 kDa into double chain enzyme composed of heavy (34 kDa) and light (14 kDa) chains by lysosomal cysteine protease yields mature CD [86]. CD-deficient mice exhibited autophagic activity and normal pancreatic development. Accumulation of LC3II is observed in both physiological and pancreatitis conditions. CD deficiency also leads to the accretion of matured cathepsin B (CB) and cathepsin L (CL) [87]. Acute renal failure is associated with severe acute pancreatitis (SAP).

Levels of creatinine, serum amylase and blood urea nitrogen were higher in rats of SAP group after 36 h. Expression of MMP-9 and vasodilator-stimulated phosphoprotein was significantly increased in the kidney of SAP animals. Capillary endothelial cells in the renal interstitium were weakened. This sheds some light on the association of MMP-9 in renal damage in SAP [88]. In early stages of SAP, the balance between TIMP-1 and MMP-9 get disturbed. This indicates that endogenous TIMP-1 is unable to prevent excess activation and release of MMP-9. This matrix metalloprotease may be regarded as marker of severity of pancreatitis in patients [89]. Gene conversion between pseudogene trypsinogen 6 (PRSS3P2) and cationic trypsinogen (PRSS1) is observed in patients suffering from chronic pancreatitis [90].

9 Colorectal Cancer

It is reported that colorectal cancer (CRC) is the second most diagnostic cancer in females and third most in males worldwide [91]. The most prognostic factors are tumour size, grade and stage. These factors do not present the clinical outcome of a patient completely. So, it is essential to have a biomarker to predict the outcome of the patients. A meta-analysis comprising of 2985 patients from 17 studies concluded that MMP-7 can be used prognostic indicator and target for treatment of CRC [92]. Capthepsin B is a lysosomal cysteine protease which is responsible for progression of cancer. The expression of this protease is upregulated in adenomas and carcinomas with respect to normal colorectal mucosa. Expression of capthepsin B was moderate in stroma and variable in epithelium in normal mucosa. Stroma of adenoma and carcinoma expresses moderate-to-high level of the protein. The activity of the protease is very high in areas of angiogenesis, inflammation and necrosis. The activity of capthepsin B is found in granular form of the epithelium in adenoma and carcinoma. Activity of this protease is associated with invasion of cancer cells, inflammatory cells, endothelial cells and cell death in human colon [93]. MMP-9 and MMP-2 are intensely associated with metastatic progression of CRC in animal model as well as in human patients [94]. MMP-9 inhibition has been found to inhibit progression of CRC [95].

It is well established that tumour derived trypsin plays a vital role in the growth and invasion of cancer cells. In colon cancer cell, trypsin acts as a growth factor through activation of the G-protein-coupled receptor protease-activated receptor-2 (PAR-2). The activation leads to matrix metalloprotease-dependent release of transforming growth factor β (TGF-β), and this, TGF-β activates epidermal growth factor receptor (EGFR) and ERK1/2 [96] (Fig. 3). Proteases play a pivotal role in the breakdown and reconstitution of basement membrane and extracellular matrix components (ECM), angiogenesis and metastasis of CRC [97]. The disruption of fine balance between MMP and TIMP is a crucial event in CRC. TIMP-1 inhibits MMP-9 facilitated release of vascular endothelial growth factor (VEGF) from matrix thereby affecting angiogenesis [98]. The spatial and temporal dysregulation of protease activity play a vital role in the progression of malignant disease. Regular

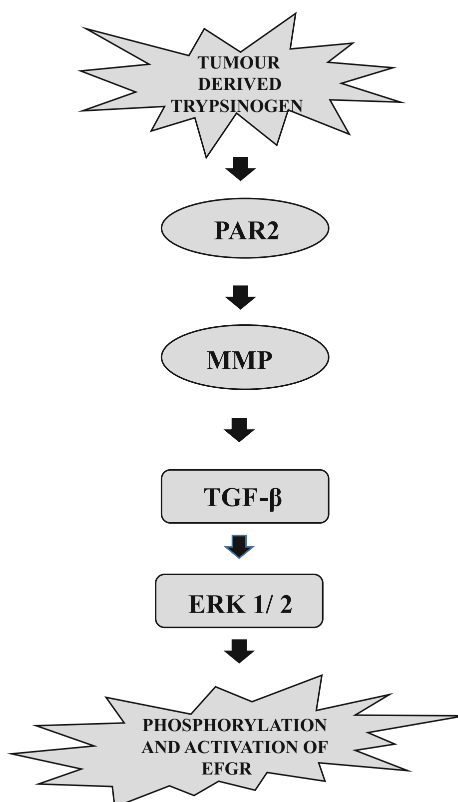

Fig. 3 Depicts the mechanism of tumour derived trypsinogen in activation of EGFR

monitoring of tumour-associated protease activity in blood sample of patients might help to improve the diagnosis of cancer and prediction of various stages. Reporter peptide is spiking and the quantification of proteolytic fragments with LC–MS showed good imitation in case of colorectal cancer patients. Functional protease profiling will improve the prognosis of colorectal cancer in future [99].

10 Gastropathy

One of the major causative agents of different gastropathies is the spiral-shaped, gram-negative, microaerophilic bacterium *Helicobactor pylori* (*H. pylori*). It has the distinctive characteristic of inhabiting in the gastric epithelium. Several studies have established that *H. pylori* are the causative agent of chronic gastritis and peptic ulcer disease (PUD). It is also highly correlated with gastric carcinoma and mucosa-associated lymphatic tissue (MALT) lymphoma [100, 101]. Its routes of transmission of this cosmopolitan pathogen may include oral–oral and faecal–oral route of transmission [100, 102]. Scientists confirmed a number of virulence factors

which are related to *H. pylori* with respect to its special ecological niche and its aetiology. These are comprised of cytotoxins, several adhesins, neutrophil-activating protein and expression of laminin, collagen, extracellular matrix proteins, type IV and vitronectin. *H. pylori* strains usually express binding of soluble plasminogen whose binding was optimal at pH 7.0 [100]. Tissue plasminogen activator converted the plasminogen to plasmin. But in case of *H. pylori* infection, the activity of tissue plasminogen activator is diminished. But in contrast, the activity of urokinase increased. For enhance tissue penetration by *H. pylori*, plasminogen binding and conversion to plasmin are very important. It is the only proteolytic activity of plasminogen involved in the aetiology of the pathogen. This activity also associated with carcinogenesis by *H. pylori*, whereas *H. pylori* eradication therapy reversed these effects. Though *H. pylori* can survive routing through the gastric juice's acid milieu, it quickly drifts through the mucus layer towards the epithelial surface which has a higher pH value. Plasminogen helps this migration [100]. Conversion of plasminogen to plasmin on the cell surface potentiate the bacterium with enhance tissue breakdown ability and thus assist the invasion of *H. pylori* through the gastric epithelium [100]. The role of plasminogen in healing of epithelial wounds still remains unclear but Bugge et al. reported that homozygous deficiency of plasminogen led to multiple abnormalities including gastrointestinal tract ulceration [103]. Studies showed that, plasminogen is also involved in invasion of tumour cells by proteolytic degradation of ECM and tissue remodelling or by matrix metalloproteases activation [100, 104]. There is also a possibility in relation to gastric carcinoma and MALT-lymphoma that plasminogen helps in progression of these diseases by binding and activation [100].

Another protease, the metalloproteinase-2 (MMP-2) has controversial roles in the treatment of gastric cancer [105]. Shen et al. systematically reviewed the indication for assessment of MMP-2 expression in gastric cancer to clarify this unclear issue [105]. They found that MMP-2 overexpression was significantly correlated with the overall decrease in the survival of gastric cancer patients [105]. They also showed, by subgroup analysis, that MMP-2 overexpression had a disparaging impact on overall survival in Asian and European countries, and that the MMP-2 overexpression was expressively related with the depth of invasion, lymph node metastasis and distant metastasis [105]. Overall, the study by Shen et al. showed that MMP-2 overexpression might be a predictive factor for poor prognosis for gastric cancer [105].

Now, coming to an important intracellular proteases, the calpains (CAPN), which play a very important role in gastric health. It constitutes a varied group of intracellular cysteine proteases that are found in nearly all eukaryotes. Calpains are cytosolic proteases, which at a neutral pH, exhibit Ca^{2+}-dependent proteolytic activity. Its activity is under strict cellular regulations. One interesting thing to know about calpain is that, unlike proteasomes and lysosomal proteases, calpain proteolyse their substrates and thus are involved in proteolytic processing, instead of for degradation. Calpain-mediated proteolysis converts the substrate into new functional states, and thus differentially affects various cellular functions. Hence, it is also referred as 'modulator protease'. The importance of the calpains in gastropathy is

exhibited by a variety of defects caused by compromised calpain function. CAPN8 and CAPN9 are gastrointestinal tract-specific calpains. They are being expressed mostly by the pit cells (surface mucus-secreting cells) in the stomach. Researchers have shown that endogenous CAPN8 and CAPN9 form a co-dependent heterodimer [CAPN8/9], G-calpain, in the stomach of mouse. Hata et al. showed that, though Capn8$^{-/-}$ and Capn9$^{-/-}$ mice appear healthy under normal circumstances, they are very much prone to ethanol-induced gastric ulcers [106]. They also showed that, CAPN8 protease-inactive knock-in mutant shows stress-induced gastropathy. These studies indicate that, CAPN8 and CAPN9 can be regarded as key components of gastric mucosal defence [106]. A single nucleotide polymorphism (SNP) database search has been adopted to studying the physiological functions of G-calpain. This alternative approach showed that human CAPN8 and CAPN9 contain several SNPs which remain responsible for resulting in amino acid substitutions. Besides, a study of in vitro expression suggested that the G-calpain variants, created because of these SNPs, have compromised proteolytic activity. Combining, it can be said that expression of certain CAPN8 and CAPN9 variants in individuals might cause gastrointestinal dysfunction. Now the question comes, 'How does calpain regulate gastric mucosal health?' Basically, there are two suggested pathways. Gastric mucosal defence is a complex process involving, mucus secretion, migration and differentiation of pit cells from their stem cell progenitors. Here, it is interesting to note that a subunit of the COPI concatamer complex involved in ER-Golgi retrograde transportation (major cellular phenomenon in the mucous secretion), the b-COP, is a substrate for CAPN8 [107, 108]. This suggests the probable link between G-calpain and the regulation of mucus secretion. Cousin et al. also showed that the expression of CAPN8 in cranial neural crest cells is involved in the cellular motility. This study in turn suggests that the G-calpain may be involved in the pit cell migration. Another class of ubiquitously present cysteine proteases, caspases plays significant roles in the gastropathy. In our study, we have shown that caspase-3-mediated cell death plays a pivotal role in indomethacin-induced gastropathy [109]. In that study, we have proposed a feedback loop triggered by the interplay between inflammation and oxidative stress which exaggerated the indomethacin-induced gastric damage executed by caspase-3 [109].

11 Conclusion

Digestive enzymes constitute a major portion of the protease pool within the human body. Therefore, the course of evolution of these proteases, in turn, reflects the changes in the feeding habits and the evolution of the digestive system, as a whole, from lower to higher groups of organisms. Within the digestive tract, the proteases are mainly concerned with metabolic processes. Acid proteases are found in the stomach and serine proteases in the duodenum.

The gastrointestinal tract is the largest reservoir of exogenous and endogenous proteases. Thus, proteases play a significant role in the progression of disorders of the digestive tract. Enteric microbes and parasites can seize the proteolytic pathways and this is referred to as 'pathogen host mimicry'. Therefore, the protease balance in the enteric milieu is necessary for the physiological maintenance of the gut and disruption of this balance leads to pathological outcomes [110].

Proteases play a pivotal role in the commencement and advancement of various diseases. Transcriptomic and proteomic profiling of proteases in blood samples can help in opening new avenues for detecting possible biomarkers associated with various gastrointestinal diseases.

Thus, this chapter provides a holistic approach towards the potent role of different proteases in the pathogenesis of a wide array of gastrointestinal disorders.

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Matrix Metalloproteases: Potential Role in Type 2 Diabetic Nephropathy

Gantala Srilatha Reddy and Hanumanth Surekha Rani

Abstract

Type 2 diabetes mellitus is the most common form and constitutes a major diabetic population in all countries. The complications of diabetes mellitus (DM) include nephropathy, neuropathy, retinopathy, and cardiovascular disease. Type 2 diabetic nephropathy (DN) is a devastating complication of DM and a main cause of end-stage renal failure. Evidences show that susceptibility to Type 2 DN has a significant genetic component in addition to environmental factors. In Type 2 DN, hyperglycemia-induced changes include extracellular matrix (ECM) deposition, basement membrane (BM) thickening, as well as vascular smooth muscle and mesangial cell growth. ECM proteins are degraded by zinc-dependent endopeptidases called matrix metalloproteases (MMPs) which in turn are regulated by tissue inhibitors of metalloproteases (TIMPs). The proteases (MMPs) and antiproteases (TIMP) offer the opportunity to identify the determinants of the disease that are very likely to be causative and might lead to new therapeutics with strong molecular underpinning.

Keywords

Diabetes mellitus · Type 2 diabetic nephropathy · Glomerular basement membrane · Matrix metalloproteases · Tissue inhibitors of metalloproteases Polymorphism

G. Srilatha Reddy · H. Surekha Rani (✉)
Department of Genetics & Biotechnology, Osmania University,
Hyderabad 500007, Telangana, India
e-mail: surekharanih@gmail.com

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1 Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is a chronic multifactorial disorder associated with a relative or absolute deficiency of insulin or its function and presently reconciles as the twenty-first century's most common chronic disease worldwide. Globally the incidence of DM is increasing in all age-groups and the prevalence was estimated to be 2.8% (175 million people) in 2000 while recent trends show that it might increase to 4.4% (366 million people) in 2030 [1, 2].

DM is the most incessant disease and is the cause of death throughout the world and now ranks the fifth, succeeding communicable diseases, cancer, cardiovascular disease, and chronic respiratory diseases [3]. The long-term effects of DM include gradual development of specific complications of retinopathy (potential blindness), nephropathy (leading to kidney failure), and/or neuropathy, etc. Renal failure in diabetes, particularly in Type 2 DM, has become "a medical catastrophe of worldwide dimension."

1.2 Diabetic Nephropathy

One of the extreme complications of DM is diabetic nephropathy (DN) and develops in 25–40% of patients with Type 1 or Type 2 DM and an essential cause of increased morbidity and mortality in these patients [4]. It is the common cause of end-stage renal disease (ESRD) requiring dialysis. The classical definition of Type 2 DN is a progressive rise in urine albumin excretion, copulates with increasing blood pressure, and leads to declining glomerular filtration and eventually ESRD.

The risk factors for Type 2 DN include reduced glycemic control, prolonged duration of diabetes, insulin resistance, high blood pressure (BP), advanced age, smoking, race, genetic propensity, etc.

The existence of microalbuminuria is the earliest clinical evidence of nephropathy. Microalbuminuria progresses result in proteinuria. Once overt proteinuria develops, renal function progressively declines and ESRD attains [5]. Both environmental and genetic factors accord to the development and outcome of Type 2 DN.

The functional unit of the kidney is the glomerulus and glomerular mesangial cells together with resident monocytes/macrophages, and surrounding matrix material constitutes the mesangium. It is involved in regulation of glomerular circulation and filtration [6]. In addition to regulating glomerular filtration, it is also involved in basement membrane (BM) remodeling. Hyperglycemia induces hemodynamic and metabolic stimuli mediators for kidney injury. These activate ischemic, pro-oxidant, fibrotic, and inflammatory pathways leading to mesangial matrix accumulation.

Prolonged duration of hyperglycemia in Type 2 DN leads to noticeable changes like: diffuse glomerulosclerosis, thickened glomerular basement membrane (GBM), nodular glomerulosclerosis, podocyte loss, exudative lesions in the Bowman's capsule, glomerular hyperperfusion, and hyperfiltration. Many factors have been divulged to be involved in this defective regulation, including vascular endothelial growth factor (VEGF), nitric oxide, and the renin-angiotensin system, especially angiotensin II [7].

Alteration in the morphology occurs in mesangial cells due to continuous cycle of stretch/relaxation and leads to enhanced proliferation and increased production of extra cellular matrix (ECM) components. The effect of stretch occurs partially as a result of increase in expression of ECM components, fibronectin, collagen I, III, and IV, and laminin. The hemodynamic changes increase the production of mesangial cell matrix, promotes the leakage of albumin from the glomerular capillaries, expands the GBM, and causes injury to podocytes. It can also induce the release of localized growth factors and cytokines which in turn activates some of the matrix metalloproteases (MMPs) which further degrade the ECM present on GBM.

2 Matrix-Metalloproteases: General Aspects

MMPs are tightly regulated enzymes having the ability to degrade the ECM and BM constituents. They are a family of endopeptidases that share structural domains but diverge in cellular sources, substrate specificity, and inducibility. All MMPs have several prevailing characteristics which are as follows:

1. MMP family members within their organization have a conserved domain pattern.
2. They contain Zn^{2+} at their active site and require calcium a cofactor for their stability.
3. They are secreted as inactive zymogens and require activation for further ECM degradation.
4. The proteins which build up the BM and the ECM are the common substrates for all MMPs.
5. The MMP's enzymatic activity is optimal at physiological pH.
6. The MMP's proteolytic activity is inhibited by TIMPs.

The MMPs have been classified into six subgroups, based on the sequence homology and substrate specificity, Collagenases (MMP-1, 8, 13 and 18), Gelatinases (MMP-2 and 9), Stromelysins (MMP-3, 10 and 11), Matrilysins (MMP-7 and 26), Membrane-type matrix metalloproteases (MT-MMP-14, 15, 16, 17, 24 and 25), and other MMPs (MMP-12, 19, 20, 21, 23, 27 and 28). Except for the MT-MMPs, most of the MMPs are secreted out and have extracellular distribution; however, recent

evidence suggests that some MMPs like MMP-1, 2 and 11 have intracellular expression where they may merge with cytosolic proteins to modulate various biological processes [8].

3 The Biological Roles of the MMPs

Numerous physiological and developmental events are regulated by ECM. MMPs degrade ECM, and the main function is assumed to be the remodeling of the ECM. Morphogenesis and tissue growth is a critical process of remodeling of ECM and MMPs control angiogenesis by releasing pro-angiogenic factors such as basic fibroblast growth factor (bFGF) or VEGF [9]. MMPs uphold cell proliferation by augmenting the release of insulin-like growth factor (IGF) and the transforming growth factor α (TGF-α). Regulation of apoptosis is the main biological role of MMPs as an increase in apoptotic cell death is substantiated by the over expression of certain MMPs like MMP-3, 7, 9, and 11.

4 Regulation of MMPs

MMPs are neutral proteases maintain the equity between synthesis and degradation of matrix proteins. The MMP proteolytic actions are controlled at three levels involving, proenzyme activation, transcription, and inhibition by the TIMPs. The MMPs are synthesized as pro-MMPs (latent zymogens), and their enzymatic activation requires prodomain removal. The serine proteases or the other active MMPs also extracellularly activate most MMPs, for example, MMP-2 requires active MT1-MMP and TIMP-2 which binds to C-terminus of pro-MMP-2 which further undergoes a complex activation pathway.

The MMP transcriptional regulation mechanism in kidney disease is quite complex and in turn induced by various signals, such as cytokines, oncogene products, growth factors and also the activation of many other signal transduction pathways [10]. Metabolic pathways are the major arbitrators of Type 2 DN involving the activation of the immune system and chronic inflammation. Several studies suggest that the meager raise in monocytes/macrophages noticed in glomeruli contributes significantly to the development of Type 2 DN. Inherent renal cells, in conjunction with mesangial, glomerular endothelial, dendritic, and renal tubular cells, are able to upregulate the inflammatory factors and cytokines, mainly interleukin 1 (IL-1), IL-6, and IL-18, VEGF, tumor necrosis factor a (TNF-a), and TGF-β, which all have been implicated in transcription regulation of MMPs in Type 2 DN progression.

Lastly, the MMP's activity is controlled tightly by the action of endogenous inhibitors (TIMPs). They have an N-terminal and C-terminal domain of 125 and 65 amino acids with each comprising three conserved disulfide bonds. The N-terminal

domain folds as a separate unit and is capable of inhibiting most of the MMPs. In addition to TIMPs, another important inhibitor of MMPs is α-2 macroglobulin which binds to MMP receptors generating an MMP-macroglobulin complex which is inactive.

5 High Glucose (HG) and MMPs

There are several mechanisms by which dysregulation in renal MMPs and TIMPs ratio or activity in kidney could commit to the development of progressive Type 2 DN. As previously mentioned, MMP's expression is firmly controlled by diverse mechanisms that include transcription and posttranscription. Evidence illustrates that increase in glucose levels may also regulate MMP gene expression via varied transcription factors NF-κB and AP-1 or relies upon growth factors like connective tissue growth factor (CTGF) and transforming growth factor (TGF-β) [11, 12].

6 AGEs and MMPs

Glycol-oxidation end products or advanced glycation end products are known as AGEs are one of the major aberrantly synthesized molecules that can influence the progression of Type 2 DN. Hyperglycemia leads to the generation of AGEs which is primarily as a consequence of a condensation of free amino group and sugar with the development of a labile Schiff base which undergoes complicated intramolecular modification to generate complex toxic AGEs. Such derivatization can occur between sugar and lipids as well, and their production can be initiated in both intra and extracellular compartments [13, 14].

Several forms of AGE derivatives have been described in renal injury related to diabetes, and morphologic changes that are often related with it encompass glomerular and tubular BM thickening, capillary aneurismal delay, mesangial expansion with production of Kimmelstiel-Wilson nodules, arteriolar thickening, and hyalinosis. AGE formation can modify the function of certain important ECM molecules, such as collagen Type 1, 3, 4, fibronectin, laminin, etc. Furthermore, AGEs can modulate the intracellular signaling pathways, gene and protein expression by interacting with their receptors, i.e., RAGE.

The AGEs via AGE: RAGE synergy can stimulate PKC, MAPK, and NF-kB, which can, in turn, modulate the expression of TGF-β and subsequently MMPs. Such ligand: receptor synergy can also generate reactive oxygen species (ROS), which then can regulate the MMPs expression via articulation of various transcription factors [15].

7 MMPs in Type 2 Diabetic Nephropathy

Type 2 diabetic nephropathy is a feature of renal and interstitial fibrosis, which ultimately progresses toward ESRD. Normal growth and development is the main physiologic feature of ECM remodeling. At several levels of ECM turnover MMPs are involved, playing a crucial role in glomerular ECM synthesis and degradation [16]. MMPs are redox sensitive, as high levels of oxidative stress and inflammatory markers activate them from their inactive latent form to active form. The amount and durational activity of MMPs determine the extent of glomerular structural damage as demonstrated by higher MMP levels in various glomerulonephritis forms [17].

The observations of Johnson et al. [18] could relate redox stress, inflammation, and MMP in glomerular failure. The presence of elevated levels of MMPs within inflammatory glomeruloscelerosis has led to the indication that increased inflammatory response coupled with increased MMP levels may cause the glomerular damage in Type 2 DN [19].

With a robust activation of MMPs, the BM and supporting ECM could be degraded allowing a complete detachment of the renal cells (mesangial cell, podocyte, and endothelial cell) within the glomerulus and the proximal renal tubule ensuing in ECM remodeling, apoptosis, atrophy, dysfunction, and cytoskeleton rearrangement. MMPs produced in various cell types in the kidney suggest their involvement in ECM degradation in glomerulus, renal morphogenesis, and remodeling [20, 21]. The dysregulated remodeling and accumulation of ECM in renal fibrosis affect all main compartments of the kidney being termed glomerulosclerosis in the glomeruli, tubulointerstitial fibrosis in the tubulointerstitium, and arterio- and arteriolosclerosis in the vasculature, where all renal cells are involved in fibrosis [22].

8 Polymorphic Studies of MMPs in Type 2 DN

Single nucleotide polymorphism (SNPs) can be used to identify the DNA sequence variation, susceptibility to disease and to understand an individual response to certain drugs.

8.1 Collagenases

Collagenases are enzymes that cleave the peptide bonds of collagen, the main component of ECM, includes collagenase-1, 8 and 13. MMP-1 (Interstitial Collagenase) which is localized on chromosome 11q22 is the most ubiquitously considered interstitial collagenase and it's over expression is associated with several diseases. MMP-1 having collagen as the main substrate is a key determinant of

ECM degradation, degrades the interstitial collagens, collagen Type 1, 2, and 3 [23]. A SNP at −1607 bp promoter region in human MMP-1 gene has been found to be associated with elevated risk of various diseases including Type 2 DN. The MMP-1 promoter region contains consensus sequences for DNA-binding proteins such as Ets/PEA-3, AP-1, and AP-2 [24].

The transcription is augmented by creating an Ets binding site at SNP (−1607) promoter region. The transcriptional start site of the MMP-1 gene at −1607 position is proportionate by two alleles (1G) or (2G) guanine nucleotides [25]. A 20 fold higher transcriptional activity seen in 2G allelic promoter of MMP-1 gene than 1G allele and is associated with higher MMP-1 levels.

MMP-8 (Matrix metalloproteinase-8) plays a major role in degradation of ECM components, collagens Type 1, 2 and 3, modifies the immune responses and regulates the cytokine activity. The gene coding for MMP-8 is located on chromosome 11q22.3. The three polymorphisms at −381A/G, −799 C > T and +17C/G in the MMP-8 gene are known to modify the gene transcription [26].

Human collagenase-3 (MMP-13), a protease, plays a role in ECM degradation and cleaves collagen Type 1, 2, 3, 4, 14 and Type 10 and also activates or degrades key regulatory proteins such as CTGF and TGFB1. The gene coding for MMP-13 is located on chromosome 11q22.2. A SNP at 77A → G in the promoter region of the MMP-13 gene has been detected. The SNP at this position leads to alterations in the gene expression and plays a modulatory role on transcriptional activity in the pathogenesis of various diseases [27].

8.2 Gelatinases

Gelatinases are proteolytic enzymes that hydrolyze gelatin into polypeptides, peptides, and amino acids. In humans, gelatinases are MMP-2 and MMP-9. MMP-2 is notably interesting because of its multiple functions and ubiquitous expression [28]. MMP-2, also known as gelatinase A, is a 72 kDa that degrades Type 4 collagen and is located on chromosome 16q12.2. It is a fundamental component of the BM. Increased expression of MMP-2 may accelerate the degeneration of gap junction protein and Type 4 collagen, leading to the vascular complications of diabetes and Type 2 DN.

Several SNPs have been described in the promoter region of MMP-2 and of these two SNPs C-1306T and C-735T prevailing upstream from the transcriptional start site effect MMP-2 transcriptional activity. MMP-2 is likely to be regulated by transcription factors; among these controlling elements, Sp1 binding site is important.

Price et al. [29] suggested that the MMP-2 transition at promoter region C-1306T could remarkably alter the promoter activity, due to disruption of the Sp1 binding site (CCACC box). MMP-2 is expressed in mesangial cells, and as a result of proinflammatory signaling, the dramatical expression of MMP-2 is elevated in various glomerulopathies [30].

MMP-9 (gelatinase B) degrades the ECM components exclusively Type 4 collagen, gelatin, and laminin, and the gene coding for MMP-9 is located on chromosome 20q11.2-q13.1 [31]. A number of SNPs found in the promoter region particularly an SNP at −1562C > T has distinct implication and has an allele-specific effect on MMP-9 transcription suggesting that MMP-9 may play a critical role in the turnover of the mesangial matrix in Type 2 DN [32]. This promoter contains the 9-bp sequence (GCGCAC/TGCC) an important gene expression regulatory element. The increased levels of MMP-9 results are due to the loss of DNA–protein interaction due to a C to T substitution.

In addition, a recent study has shown that Type 2 DN patients has elevated levels of MMP-9 and had a substantial association with age, hyperglycemia, blood pressure, body mass index, glycated hemoglobin (HbA1c), and progression of diabetes [33].

8.3 Stromelysins

Stromelysin, the enzymes of MMP family, plays a major role in the degradation of proteoglycans, gelatin, and other ECM constituents. It includes MMP-3 (stromelysin-1), MMP-10 (stromelysin-2) and MMP-11 (stromelysin-3). MMP-3 (stromelysin-1), a member of MMP-family degrades collagen Types 3, 4, 9 and 10, proteoglycans, elastin, fibronectin and laminin, and also involved in the activation of other MMPs (e.g. pro-MMP-1, -8, -9 and -13), and auto activation of pro-MMP-3. The gene is located on the chromosome 11q22.2-22.3, and the expression level of this gene was found to be altered by SNPs.

At the upstream of the MMP-3 transcription start site, the SNP identified in the promoter region at −1171 bp has one allele having five adenosines (5A) and other having run of six adenosines (6A) and was found the allele having five adenosines has higher transcriptional activity [34]. MMP-3 elevated levels have been observed in sera from patients with a number of diseases like active lupus nephritis, mesangial proliferative glomerulonephritis, IgA nephropathy, etc.

Stromelysin-2 (MMP-10) is another important Zn-dependent endopeptidase cleaves laminin, fibronectin, proteoglycan core protein, elastin, gelatins of Type 1, 3, 4, and 5. MMP-10 also activates pro-MMP-1, -7, -8 and -9, which degrades extracellular collagen in different pathological conditions along with other MMPs. The gene is part of a cluster of MMP genes and located on chromosome 11q22.3. The two polymorphisms rs17435959 (G > C) at position 102780582 and rs17293607 (C > T) at position 102779658 are identified. These two polymorphisms lead to the substitution of specific amino acids and may have functional effects. MMP-10 serum levels have a close relationship with some risk factors for ischemic stroke, such as carotid intima-media thickness, presence of carotid plaques, inflammatory markers and smoking and its role in the pathogenesis of Type 2 DN are still unexplored.

Stromelysin-3 (MMP-11), the enzyme encoded by this gene is intracellularly activated by furin, in contrast to other MMP's, and cleaves alpha 1-proteinase

inhibitor but weakly degrades structural proteins of the ECM. The gene for MMP-11 is located on chromosome 22q11.23. MMP-11 associated diseases mostly include ophthalmomyiasis and colorectal cancer.

8.4 Tissue Inhibitors Metalloproteases (TIMPs)

MMPs are inhibited by TIMPs consisting of 184–194 amino acids. Four structurally related TIMP family members include TIMP-1, -2, -3 and -4. Inhibition is accomplished by their ability to interact with the zinc-binding site within the catalytic domain of active MMPs [35]. TIMPs have a certain degree of specificity toward MMP family members. To maintain the sustainability of healthy tissues, the balance between MMPs and TIMPs is important. The balance disrupted by the polymorphic variants within TIMP gene is associated with development of various diseases.

The biological activities of TIMPs involve anti-angiogenesis, cell migration, effects on cell growth and differentiation, synaptic plasticity, anti- and pro-apoptosis. Type 2 DN certainly associate with altered activity of the MMP and TIMP which in turn leads to glomerulosclerosis [36].

8.4.1 Metallopeptidase Inhibitor 1/TIMP-1

The best-identified gene is the TIMP-1, present on X chromosome (Xp11.3-p11.23). TIMPs bind with active MMPs with high affinity and have complicated roles in pathological and physiological tissue remodeling. TIMPs inhibit all MMPs, but TIMP-1 is an indigent inhibitor of MT1-MMP, MT3-MMP, MT5-MMP, MMP-19 and, ADAM-10. At exon 6 of TIMP-1 gene located 536C/T polymorphism capable of binding and preventing the activation of most MMPs [37].

8.4.2 Metallopeptidase Inhibitor 2/TIMP-2

TIMP-2 is a secretory protein located at 17q25, inhibits the proteolytic activity of MMP-2 involved in the ECM degradation. Additionally, Apoptosis and cell growth are regulated by TIMPs [38]. An SNP identified at G > C −418 position in the promoter region of TIMP-2 may influence the binding of Sp1 transcription factor and downregulates the TIMP-2 transcriptional activity [39].

8.4.3 Metallopeptidase Inhibitor 3/TIMP-3

TIMP-3, a member of the TIMP family, inhibits the action of MMPs which are involved in ECM degradation. It has also been shown to have inhibitory effects on angiogenesis and tumor growth [12, 13]. ADAM-10, 12, 17 and ADAMTS-1, 4 and 5 are inhibited by TIMP-3, and the gene is located on chromosome 22q12.3. Recently, three novel polymorphisms in the promoter region of TIMP-3 gene (−899T/A, −915A/G and −1296T/C) have been identified. TIMP-3 polymorphism has an impact on various complex diseases such as spontaneous abortion, cancer, diabetic nephropathy, macular degeneration, and hypertension [40].

8.4.4 Metallopeptidase Inhibitor 4/TIMP-4

TIMP-4 belongs to the TIMP gene family and is expressed predominantly in the heart, which inhibits the action of MMP-2 by binding strongly to MMP-2 carboxyl hemopexin domain. The gene is located on chromosome 3p25.2. Two SNPs (rs3755724, −55C/T, promoter; rs17035945, 3′-untranslated region) were genotyped in TIMP-4 gene [41].

9 Conclusion

Type 2 end-stage renal disease is a devastating condition, MMPs are the major regulators of ECM degradation in Type 2 DN pathology and progression. The proteases (MMPs) and antiproteases (TIMP) offer the opportunity to identify the determinants of the disease that are very likely to be causative and may also lead to new therapeutics with strong molecular underpinning.

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Antihypertensive Role of Kidney: Focus on Tissue Kallikreins

Amritlal Mandal, Tapati Chakraborti and Sajal Chakraborti

Abstract

Regulation of blood pressure (BP) depends mostly on genetic and environmental factors. Among different physiological mechanisms responsible for a discernible increase in BP (hypertension), two major mechanisms are important: (a) matrix remodeling associated with arterial wall thickening with apparent reduction in blood flow and (b) activation of renin–angiotensin system (RAS). To counteract the BP rise beyond physiological limit and to ameliorate BP-rise-associated complications, nature has designed an important endogenous regulatory mechanism operative especially in kidney, the kallikrein–kinin system (KKS). In cortical collecting duct of the kidney, KKS plays a significant role in BP regulation under salt excess condition. This protective phenomenon potentially acts as a hypotensive mechanism to regulate abnormal BP increase. The functional components of the KKS are the kallikrein, a serine protease, and kinin. Proteolytic action of kallikrein on precursor kininogen forms vasoactive peptide kinin by enzymatic cleavage. Upon release, kinin binds to the B2 receptor on cell surface and exerts antihypertensive effect. Though KKS operates in both cardiovascular and renal systems, for the sake of simplicity and to have a focused but detailed understanding, only the regulatory mechanisms of renal KKS on BP homeostasis has been considered for discussion in this review.

Keywords

Hypertension · Kallikrein–kinin system · Renin–angiotensin system
Epithelial sodium channel · Kallikrein gene delivery

A. Mandal (✉)
Department of Physiology, University of Arizona, Tucson, AZ 85724, USA
e-mail: mandal@email.arizona.edu

T. Chakraborti · S. Chakraborti
Department of Biochemistry and Biophysics, University of Kalyani,
Kalyani 741235, West Bengal, India

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1 Introduction

High blood pressure (BP), commonly known as hypertension, is a manifestation of physiological conditions due to many complex factors including genetic, drug, and environmental components. Excess salt intake is a potential threat in developing BP rise [1]. Recent development of new information elucidates the basic mechanisms of BP regulation in salt excess condition.

Kallikrein, a family of serine proteases, has been implicated in BP regulation. Kallikrein-mediated blood pressure regulation is controlled by fine-tuning of the kallikrein–kinin system (KKS) in the kidney. The KKS consists of tissue kallikrein, kininogen, kinin, kininases and kinin receptor B2. Though endogenous tissue kallikrein (TK) inhibitor, kallistatin, is not considered to be an integral part of the KKS, kallistatin plays an important role in regulating kallikrein's action. These components are mainly expressed along the collecting duct of the distal nephron. The KKS maintains blood pressure homeostasis in salt excess condition by virtue of controlled electrolyte and salt absorption mechanisms in kidney. Thus, an impaired or faulty KKS could potentially generate a threat of blood pressure increase and hypertension.

The recent advances in understanding physiological and functional role of kallikrein as one of the significant protective mechanisms in hypertension and BP regulation in mineralocorticoid and salt excess conditions have established kallikrein as a potential therapeutic target to control hypertension and BP regulation. Though renin–angiotensin system (RAS), potentially a cardiovascular mechanism, is responsible for hypertensive regulation of BP, it works as an opposing mechanism of renal KKS-mediated hypotensive effect in kidney. This chapter is aimed at understanding the physiological functions of kallikrein in regulating hypertension.

In an earlier studies, participation of KKS in the intra-hepatic blood flow of patients with secondary pulmonary hypertension has been reported [2]. A separate endotoxin-mediated study in experimental ovine model demonstrated a marked decrease in PK level upon chronic exposure to endotoxin [3]. The model demonstrated a link between elevated pulmonary microvascular permeability and a tendency toward hyperdynamic circulation [4]. Urinary kallikrein excretion in salt-sensitive hypertensive subjects is found less in comparison with the salt-resistant hypertensive counterparts [5, 6]. These earlier studies have provided a strong foundation for a separate area of research on kallikreins and its role in hypertension.

2 Proteases Involved in Blood Pressure Regulation

Two main classes of neutral proteases based on their active sites are widely studied in the context of BP homesostasis. These are as follows: (i) serine proteases, including endogenous vascular elastase (EVE), the plasminogen activator/plasmin system, kallikreins, and (ii) the matrix metalloproteinases (MMPs) [7].

Kallikreins are divided into two groups: (1) plasma kallikreins (PKs) and (2) tissue kallikreins (TKs). They both differ by molecular weights, biological functions, physiochemical, and immunological properties and on their distribution in the body. Tissue kallikrein 1 (KLK1), kallikrein-related peptidases 2-15 (KLK2-15), and plasma kallikreins are a family of secreted serine protease that forms kinins. Tissue kallikreins, KLK 1–15 genes, are located on chromosomes 19q13.3-q13.4. Plasma kallikrein, KLKB1 gene, is located on chromosome 4q35.

3 Kallikrein–Kinin System (KKS)

The kallikrein/kinin system (KKS) is a very important mechanism in a wide range of physiological functions and in the development of many pathological conditions including blood pressure (BP) regulation [8]. The plasma KKS forms a cell-bound protease activation cascade with an ultimate releases of the inflammatory peptide bradykinin (BK) from high molecular weight kinininogen (H-kininogen) [9]. This highly orchestrated sequential event occurs by binding a complex of H-kininogen and plasma pre-kallikrein (PPK) to a multiprotein receptor on the cell surface requiring an optimal Zn^{2+} concentration [10–12]. Formation of HK-PPK is critical, which relies on a stoichiometric interaction between PPK and prolylcarboxypeptidase (PRCP) [13] or heat shock protein 90 (HSP90) [14]. Factor XII-independent activation of PK has also been reported by Røjkjær and Schmaier [15]. Important steps involved in plasma and tissue KKSs, including B1- and B2-receptor-mediated initiation of downstream signaling pathways, are schematically shown in Fig. 1. The KKS consists of several components that work as a cascade of fine-tuned mechanisms for blood pressure regulation. In isolated mouse-microperfused cortical collecting ducts, luminal TK was found to activate epithelial sodium channels (ENaC) [16].

4 Plasma Kallikreins (PKs)

PKs (EC 3.4.21.34) are present in circulation as inactive pre-kallikreins or Fletcher factor. Plasma pre-kallikrein is a precursor of PK, produced in liver and circulates in blood, bound with high molecular weight kininogen (HMW kininogen). Activation of pre-kallikreins and consequent formation of kallikreins have been shown to occur by the action of Hageman factor (HF) [17]. Upon activation, PK acts on endothelial cell surface during intrinsic coagulation steps and cleaves HMW kininogen to release bradykinin (BK) [18, 19].

PK directly regulates cardiovascular system by activating different proteolytic cascades which are intrinsic to BP regulation. These include KKS, the fibrinolytic system, and the alternate complement activation pathways. PK is cardioprotective in normal physiological condition.

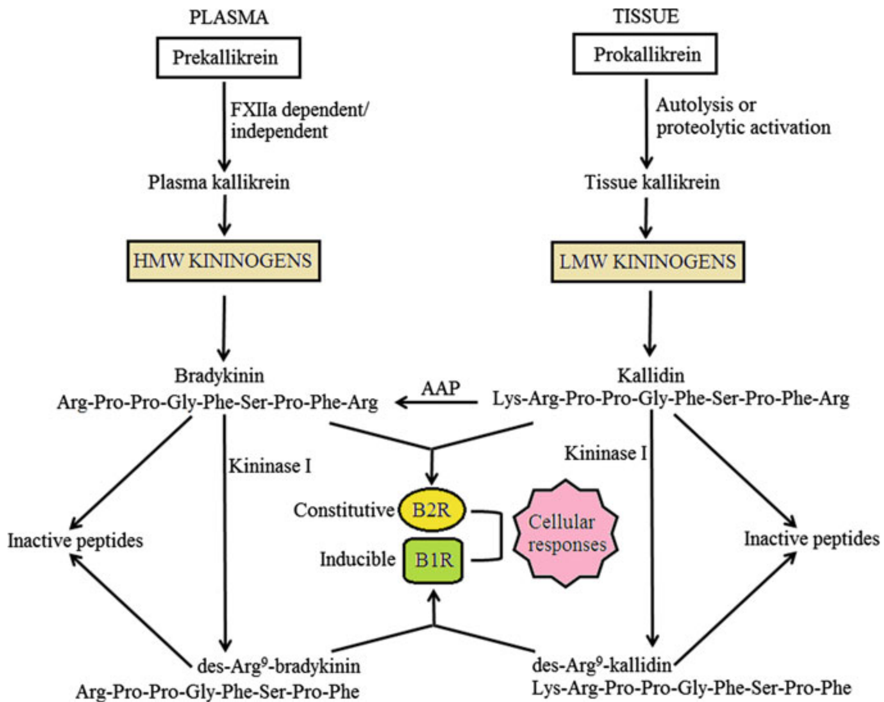

Fig. 1 Schematic diagram showing major events of the kallikrein–kinin system (KKS). Plasma pre-kallikrein and tissue pro-kallikreins secreted as zymogens and become activated by proteolytic cleavage to plasma kallikrein (PK) and tissue kallikrein (TK), respectively. PK acts on HMW kininogen, and TK acts on LMW kininogen to generate bradykinin (BK) and kallidin, which act through the constitutive B2 receptor (B2R). BK is also generated from kallidin by the action of an arginine aminopeptidase (AAP). These peptides are further processed to their des-Arg^9 (removal of Arg at position 9) metabolites by the kininases carboxypeptidases (kininases I). The Des-Arg kinins bind to the inducible B1 receptor (B1R). Kinins are rapidly degraded to the inactive peptides mainly by angiotensin-converting enzyme (ACE, kininase II). Kinin-mediated B receptor stimulation is involved in diverse physiological processes including blood pressure regulation, electrolyte balance, and inflammation

Kallikrein B1 (KLKB1), another member of kallikrein, is one of the intrinsic components of blood caoagulation cascade. KLKB1 is produced as precursor of plasma kallikrein (Pre-KLKB1) by KLKB1 gene. KLKB1 is expressed in liver, kidney, and pancreas. Low KLKB1 expression level has also been detected in heart, brain, spleen, thymus, intestine, and testis [20]. Impaired or faulty KLKB1 activity has been observed to be responsible for developing arterial dysfunction and in renal tubular defects [21, 22]. KLKB1 acts on two major substrates: coagulation factor XII and HMW kininogen [23].

5 Tissue Kallikreins (TKs)

Major source of tissue kallikrein (EC 3.4.21.35) is exocrine glands, such as the kidney, pancreas, intestine, salivary glands, bronchoalveolar lavage fluid of asthmatic patients and in synovial tissues [24–29]. TKs responsible for BP regulation are mostly synthesized in arteries and distal renal tubule, and TK is the main target of aldosterone [30]. Existence of KKS has also been reported in the vascular wall, where the presence of secreted kallikrein-like kininogenase activity has been observed [31, 32]. TKs share similar physiochemical properties and are found to be immunologically identical in the same species [33].

Expression of mRNA for TK and kininogen has been demonstrated in vascular tissue [34] and endothelial cells. TK protein expression has also been detected in human endothelial cells [35, 36]. Human umbilical vein endothelial cells (HUVEC) have been reported to be a site of synthesis and storage of TK [37].

In kidney, major quantity of TK is synthesized by connecting tubule cells. Distal convoluted tubules and the cortical collecting ducts are the minor contributing cell types for tissue kallikrein synthesis [38]. The release of TK into the tubular fluid and the peritubular interstitium eventually forms kinins. Locally available endogenous kininogens are cleaved by TK leading to the formation of kallidin (lysyl-bradykinin). The cleavage of kallidin by N-aminopeptidase produces bradykinin, which in turn activates bradykinin type 2 (B2) receptor [39]. Antihypertensive effects of ACE1 not only inhibit formation of angiotensin II, but also inhibit kinin degradation. In rodent and human, an inverse relationship exists between urinary tissue kallikrein excretion and blood pressure regulation [39].

6 Kinins

Kinins are the vasoactive polypeptides, released in the circulating bloodstream from precursor kininogens by the proteolytic action of kallikreins. Kininogens are the substrate for kallikreins. High molecular weight (HMW, ~76 kDa) and low molecular weight (LMW, ~48 kDa) kininogens have been isolated from bovine plasma [40]. Bradykinin (BK), a nonapeptide, is released in the circulation by the activation of HMW kininogen. Kallidin (KD), a decapeptide, is similarly released from LMW kinininogen by tissue kallikrein.

Kinins resembles bradykinin (BK) (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9) in structure and in pharmacological action [41]. Many of the effects of kinin are attributed to eicosanoid [42], nitric oxide (NO) [42], endothelium-derived hyperpolarizing factor (EDHF) [43, 44], or through tissue plasminogen factor (tPA) [45]. Kinins play protective role in cardiac ischemia preconditioning and in cardiovascular diseases.

Kinin's renal protective function has been a well-studied area. Renoprotective effects of kinin have been attributed due to either angiotensin-converting enzyme (ACE) inhibition [46, 47] or by angiotensin type I receptor blocker (ARB) [45, 48].

In a small family-based study, enhanced urinary kallikrein excretion has been found to be linked with a decrease of risk for essential hypertension [45]. Interestingly, human, rat, or mice with established deficiency of KKS or with a missing component of KKS did not show any discernible hypertension. In kidney, papillary blood flow largely depends on availability of kinin. Kinins play important regulatory roles in sodium and water excretion through kidney [45, 49, 50]. Indeed, B2 knockout mice show enhanced hypertension due to excess salt intake, which further established a role of kinin as antihypertensive molecule [51].

7 Kininases

Kininases act on substrate kinins in blood or tissue homogenates to rapidly degrade kinins into inactive peptides. Kininase I (carboxypeptidase N) is present in human and in animal plasma. Kininanses were first detected in kidney cortex and later in the plasma [52], which inactivates BK by cleaving C-terminal amino acids including Arg^9 [53]. Later, this enzyme has been renamed as angiotensin 1-converting enzyme (ACE). ACE inhibitors are established cardioprotective agents, which improves cardiac performance after heart failure, helps recovering from hypertensive cardiac hypertrophy, reduces myocardial infarct size, and prevents reperfusion injury [54–58]. ACE inhibitors are important clinical tools for treating hypertension and albuminuria.

8 Kallikrein Activation Mechanisms

PK in endothelial cells is activated by a membrane-associated cysteine protease. In general, autoactivation of factor XII (FXII) to factor XIIa (FXIIa) is the initial step of PK activation. FXIIa acts on inactive plasma pre-kallikrein to produce active kallikrein which in turn reciprocally activates FXII by an autocatalytic mechanism in the surface of endothelial cells. The mechanisms of plasma KKS activation in endothelial cell surface received inadequate attention due to abundant localization of FXII on the endothelial cell surface [59, 60]. This has been suggested for plasma kallekrein activation in endothelial cells. In many studies, PK activation in endothelial cells has been observed to be associated with intrinsic availability of FXII on the endothelial cell surface for autoactivation to FXIIa [61–63]. Motta et al. [64] and Rojkjaer et al. [65] have demonstrated FXII-independent activation of plasma kallikreins in cultured HUVEC cells. These studies have restricted FXII for consideration as an essential and required component of PK activation in endothelial cells. However, FXII is not an absolute requirement for endothelial PK activation and accelerated synthesis of factor XII by kallikrein [15].

Recent evidence showed that TK employs kinin-independent effects on different renal transporters [66]. The examples include epithelial Na^+ channel, transient receptor potential channel vanilloid subtype 5 (TRPV5), and the colonic H^+, K^+-ATPase.

Studies with experimental animal models demonstrated kinin-induced increase in renal blood flow, diuresis and natriuresis [67, 68]. Increased electrolyte and water excretion have been observed to be linked to the renal hemodynamic effects of kinins. Bradykinin (BK) is a very well-studied and potent inhibitor of NaCl absorption in isolated, perfused cortical collecting ducts (CCDs) [69, 70]. The BK-induced inhibition of Na^+ absorption in CCDs is possibly exerted through the activation of B2 receptors [69, 70]. Impaired B2 receptor function has been positively correlated with increased NaCl absorption, leading to increased blood volume and blood pressure increase [71].

Knockdown of TK gene in experimental mouse model has been shown to decreased kinin production [72, 73]. However, on the other hand, B2 receptor knockout mice and TK-deficient mice failed to show any changes in altered Na^+ level and BP [16], which suggests that TK has kinin-independent effects in BP regulation.

Duka et al. [74] have demonstrated that under normal physiological condition, vasoregulatory function of bradykinin occurs through B2 receptor activation. When B2 receptor expression is either selectively knocked out, B1 receptor is upregulated [75] and comes into play by overtaking the responsible functions of B2 receptors. Earlier, arachidonc acid activation cascade has been observed due to B1 receptor activation [75, 76], which directly linked to insulin-dependent metabolic controls, indicating that the B1 receptor's role as a vasoregulatory component is a novel finding.

Inactive tissue kallikreins are synthesized as pre-proserine proteases [77]. 16-30 amino acid presequence at the N-terminal in the inactive precursor is enzymatically cleaved prior to the secretion of the active kallikriens [78]. Pro-sequence containing 37 amino acids in kallikrein 5 (KLK5) and 4-9 amino acids in other kallikreins is cleaved upon activation [78]. Cleavage of arginine or lysine in the pro-domain by trypsin is believed to be the key steps in the tissue kallikrein activation. The presence of histidine and aspartic acid was found to be conserved among mature and active tissue kallikreins. Activation and physiological roles of tissue kallikreins have been reviewed by Emami et al. [79].

9 Genetic Models of TK Deficiency

Due to unavailability of potent and selective pharmacological inhibitor of TK, a need for genetically engineered animal model was felt necessary. In an effort to have this tool available, scientists have developed two important TK-deficient models. The first one is TK-deficient murine model, where the mice TK gene has been selectively inactivated by genetic engineering [16, 73]. The second is human

TK-deficient phenotype, which has been achieved by a major loss of function due to polymorphism of the TK gene [80]. Physiological studies with both models reveal its important role in arterial function, flow-induced vasodilation, and adaptation to shear stress [72, 81].

Studies on kallikrein-deficient mice provided data showing that kallikrein directly influences BP in mineralocorticoid excess and salt retention but not in normotensive animals in high renin hypertension. This critical observation sheds light on KLK as an antihypertensive agent, where Na^+ retention is the main cause of hypertension and blood pressure elevation [82].

10 TK and Blood Pressure Regulation

10.1 TK Deficiency and Impaired Blood Pressure Control

Reduced excretory level of kallikrein in urine samples has been recorded in clinical and experimental hypertension [83–86]. TK deficiency and blood pressure regulation have been extensively reviewed by Sharma et al. [87]. Role of KKS as a potent BP lowering mechanism is very well known [88]. Kinins have been observed to be effective in lowering BP, arterial vasodilatation, natriuresis, and an increase in renal blood flow with the decrease in peripheral resistance [83, 89–92]. Additionally, experimental evidence also proved a faulty or inhibited KKS could potentially cause Na^+ retention, arterial vasoconstriction, raised peripheral resistance, increased vascular or plasma volume and hypertension. A decrease of blood kinin level has been thought to be directly linked to the development of hypertension.

Arterial dysfunction is commonly observed in both capacitative and resistance vessels of TK-deficient mice [72, 93]. Non-TK-deficient wild-type mice did not show any alteration in baseline blood pressure recordings [73, 94]. The missense mutation (R53H) in human TK gene (KLK1) reduces the kinin-forming activity of TK [80, 81]. In another study, Yu et al. [94] demonstrated that multiallelic promoter variation of the TK gene is associated with hypertension. No association of this promoter variation with TK activity level has, however, been observed. Genetic model of TK deficiency has been extensively reviewed by Potier et al. [82]. KKS has been shown to play protective roles in mitigating BP increase and hypertension [95, 96].

Experimental data have provided evidence for the development of tubulointerstitial injury in kallekrein-deficient experimental models. These subjects elicit salt-sensitive hypertension. Reduced kallikrein expression has also been shown to result a reduction in kinin formation, favoring vasoconstrictive sodium retention. An impaired or faulty KKS has been shown to lead to development of renal fibrosis, which could be reverted by using pharmacological stimulus of KKS, thereby preventing progression of salt-sensitive hypertension and kidney diseases [97].

10.2 TK Deficiency in Experimental Hypertension

Blood pressure in mice with renovascular hypertension remains unaltered in TK deficiency [98]. This model of hypertension is renin dependent, and Na^+ balance has been found to be either neutral or negative, depending upon the origin and cause of hypertension. Urinary TK excretion was found to be unaltered in WT mice, where renal artery had been clipped to induce TK deficiency [98]. This study clearly shows that TK does not have any counter-regulatory hypertensive effect in renin–angiotensin model of hypertension. However, in another experimental model, where hypertension has been induced by aldosterone and salt administration, a prominent hypertension was observed [30]. This observation was consistently found to be reproducible across different mouse genetic strains.

Slight or no increase of BP has been reported in mice of different genetic background. In wild-type (WT) animals, aldosterone/salt treatment for one month elicited sustained hypertension. TK gene inactivation in experimental mice and subsequent BP control in shorter and longer time periods for both in active and inactive phases has been studied by Waeckel et al. [30]. The study showed that chronic aldosterone excess had no effect on BP in wild-type mice, whereas in TK inactivated group, severe hypertension has been observed. This animal model of hypertension is based on Na^+ retention in suppressed renin secretion. The model largely relies on renal excretion of urinary TK by aldosterone administration. Thus, a counter-regulatory mechanism exists that is dependent on increased TK synthesis in the setting of mineralocorticoid excess and prevents hypertension. Further studies in TK-deficient mice explained the antihypertensive effect of TK at the level of renal tubule by potentially inhibiting ENaC synthesis and with a resultant Na^+ retention [30, 99, 100]. No convincing mechanisms has, however, been available to link kinin-mediated effect of TK [101] to other proteolytic action of TK or both [16, 30, 102]. Azizi et al. [81] have also provided evidence supporting antihypertensive effect of TK in human under mineralocorticoid and salt excess condition. In this regard, proper evaluation of urinary and plasma TK levels could be interesting in patients with adrenal adenoma or adrenal hyperplasia to assess the degree of disease pathology in relation to hypertension.

Kinins elicit renal protective effects in diabetes [59, 103]. Incipient diabetic nephropathy has shown no significant blood pressure alteration in patients with TK deficiency. TK deficiency is usually associated with renal dysfunction and albuminurea [104]. Hypertension in association with diabetic nephropathy and the role of TK deficiency in this scenario need further investigation to understand the complex disease mechanism.

Two opposing mechanisms, the renin–angiotensin system (RAS) and KKS (the fluid homeostasis mechanism) in kidney, are crucial in maintaining normal BP regulation. KKS-mediated cardioprotective mechanisms by counteracting RAS, a potential hypertensive mechanisms, have been reviewed by Regoli et al. [105]. Concerted balancing effects of RAS-induced BP elevation in certain instances get normalized by the hypotensive effect of KKS. Renin (inactive) becomes activated by plasma or tissue kallikreins [106, 107]. Plasma KKS directly interacts with the

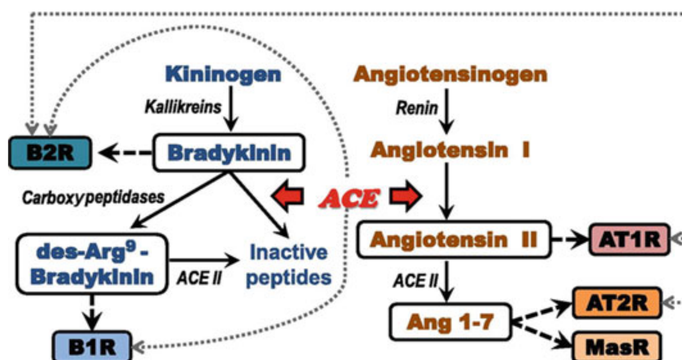

Fig. 2 Interactions between the KKS and the renin–angiotensin system (RAS). ACE represents a central physiological bridge connecting the KKS (*left side*) and the RAS (classical RAS on the right side). ACE regulates the levels of endogenous Ang II and kinins by degrading BK and converting Ang I to Ang II. A cross-talk between ACE, B1R, and/or B2R is also postulated (*broken gray arrow*) in some systems and increases the complexity of these interactions. Similarly, cross-talks between angiotensin II receptor type 1 (AT1R) and angiotensin II receptor type 2 (AT2R) with B2R have also been described (*broken black arrow*). Mas receptor (MasR), a G protein-coupled receptor, is associated with cardiac, renal, and cerebral protective responses. Taken from Ref. [129] with permission

RAS. The initial step of PK activation in the endothelial cells takes place by prolylcarboxypeptidase (PRCP) and that was recognized as the major angiotensin II-converting peptide to act as an activator of PK [106]. Increased urinary kallikrein expression has been noted in dogs upon intra-arterial infusion of angiotensin II [108]. Interaction between KKS and RAS is schematically represented in Fig. 2.

Physiological Na^+ homeostasis and BP regulation are important for healthy kidney and its capacity to appropriately reabsorb salt and water. This is achieved by proper activities of the ion transporters and channels located in the nephron [109]. The epithelial sodium channel (ENaC), located in the kidney, plays a pivotal role in extracellular volume and BP homeostasis. Cortical collecting tubule of nephron is the major site of ENaC expression which plays an important role in Na^+ homeostasis. When ENaC function is gained by mutation in β subunit, hypertension results (Liddle's syndrome) [110]. On the other hand, loss of function due to mutation leads to renal salt wasting (pseudohypoaldosterism) [111]. Recent studies demonstrated proteolytic cleavage of γ-subunit of ENaC by kallikrein and possible protection mechanisms against hypertension [112].

RAS is a well-established regulator of ENaC [109, 113]. Angiotensin I is formed from the precursor angiotensinogen by the renin-mediated proteolytic cleavage. Angiotensin-converting enzyme (ACE) acts on angiotensin I to form angiotensin II, the main regulatory component of RAS [113]. Angiotensin II causes vasoconstriction and consequent rise in BP [114]. Regulation of ENaC by bradykinin in the distal nephron in low and high salt conditions is schematically represented in Fig. 3.

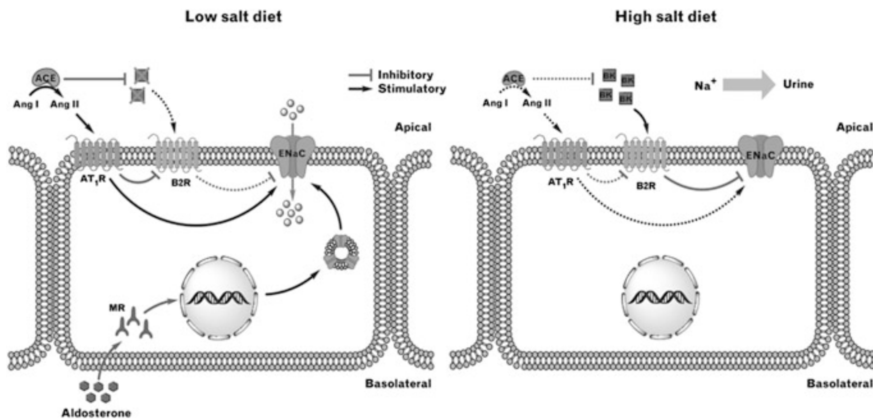

Fig. 3 Principal scheme of interaction between the kallikrein–kinin and renin–angiotensin systems in the distal nephron during different salt intakes. *ACE* angiotensin-converting enzyme; *Ang I* angiotensin I; *Ang II* angiotensin II; *AT1R* angiotensin type 1 receptor; *B2R* bradykinin type 2 receptor; *ENaC* epithelial Na^+ channel; *MR* mineralocorticoid receptor. *Dotted arrows* represent inactive mechanisms of ENaC regulation under particular sodium intake. Taken from Ref. [50] with permission

11 Kallikrein Gene Delivery

Ischemia/reperfusion (I/R) injury-induced myocardial damage is commonly observed during thrombolytic therapy for myocardial infarction and in several restorative treatment options available to the cardioplegic arrest and cardiovascular surgery. Kallikrein gene delivery using adenovirus vector to the experimental rat model of icatibant (B2 receptor antagonist)-induced hypertension was found to be effective in reducing myocardial I/R injury [115].

In a study with Dahl-salt-sensitive (Dahl-SS) hypertensive rats, human tissue kallikrein gene delivery using cytomegalovirus (CMV) vector has been found to be effective in reducing BP rise from day 2 of post-injection [116]. Adenovirus-mediated tissue kallikrein gene delivery was also found to be effective in reducing left ventricular mass, cardiomyocyte hypertrophy, attenuation of glomerular sclerotic lesions, tubular dilation, and proximal tubule brush-bordered cell damage in the Goldblatt hypertensive rat model [117] and in Dahl-SS rat model [118] of hypertension.

These findings indicated potentials of tissue kallikrein gene delivery in cardiac and renal functions. The shortcoming of adenoviral tissue kallikrein gene delivery is the eventual degradation of the DNA due to lack of viral integration factor. The host immune system plays a significant role in rapidly destroying the injected gene [119]. Modified adenovirus vectors have been shown useful for long-term transgene expression in experimental systems [120] to enable better protection against hypertension.

12 Kallistatin, the Endogenous Inhibitor of TK

Bioavailability of kallikreins depend on factors that either affect kallikrein biosynthesis or its clearance mechanisms. Posttranslational regulation of kallikreins is well studied [121]. Post-translational modification and inactivation of kallikreins by inhibitors also play regulatory roles of kallikrein's activity. Kallistatin, an endogenous serine protease inhibitor of kallikrein, has been very well investigated [122–125]. Liver synthesizes kallistatin predominantly, albeit pancreas and kidney elicit little kallistatin expression. Plasma and urine levels of kallistatin have been measured in normal and several groups of patients with a wide range of disease conditions [126, 127]. A reduced plasma kallikrein level has been observed in patients with liver diseases and sepsis [126]. The presence of higher urinary kallistatin level has been documented in urines of patients with pregnancy-related hypertension [127]. The anti-hypotensive property of endogenous kallikreins was reviewed by Chao et al. [128].

13 Conclusions and Future Direction

Kallikreins have been established as one of the intrinsic components of KKS, which plays antihypertensive role in mammalian cardiovascular and renal systems. Experimental animal models including pharmacological inhibition and conditional gene knockout for kallikreins have made it possible to study the physiological roles of the enzyme in diverse pathological conditions. Kidney tissue kallikrein plays pivotal role in renovascular blood flow regulation and epithelial salt and electrolyte excretion by employing ENaC channels located at the distal loop of the cortical collecting duct. Thus, TK is an important regulatory component in physiological salt retention and blood volume regulation. In a small family-based study, dominant kallikrein expression has been positively correlated to be cardioprotective and reduces occurrence of hypertension during the condition of excess salt intake. The subjects have also shown to increase excretion of urinary kallikreins, which was known to be renoprotective. Both plasma and tissue kallikrein-mediated complex physiological fine regulation of blood pressure usually occurs in concerted mechanisms. The activation of plasma and tissue kallikreins differs widely. Plasma kallikreins are activated on the endothelial cell surface by the action of factor XIIa. But evidence of factor XIIa independent plasma kallikrein activation has failed to prove that factor XIIa is essential for the plasma kallikrein activation. Tissue kallikrein activation has been observed to occur through proteolytic cleavage of the pre- and pro-peptide regions of the precursor molecule as posttranslational modification steps. Components responsible to limit kinin formation in kidney act toward the development of salt-induced hypertension.

In addition to the availability of endogenous kallikrein, development of kallikrein-like factors could be a potential area, where significant research effort needs to be targeted for therapeutic management of hypertension during salt excess condition.

Gene therapy is a demanding area in clinical trials. Effective design of viral vectors for a safe and successful targeting of the kallikrein gene in a tissue-specific manner could prove useful as a therapeutic approach to treat hypertension and associated complications.

The apparent decrease in excretory kallikrein levels in hypertensive subject was found to be effectively reversed by antihypertensive drug or diuretic treatment. Further studies are needed to understand the mechanism of action of hypotensive drugs and the increase in urinary kallikrein excretion.

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Proteases—The Sharp Scissors in Human Diseases

Sandipan Chakraborty and Soumalee Basu

1 Introduction

Proteases or more generally peptidases [1] are a type of enzyme that perform peptide bond hydrolysis in proteins and are found ubiquitously in eukaryotes, prokaryotes, and viruses. They cover about 2% of the total number of proteins present in an organism. All proteases share in common the general mechanism of a nucleophilic attack on the carbonyl carbon of an amide bond [2]. This results in a general acid-base hydrolytic process that disrupts the covalent bond. Different proteases utilize different strategies to generate the nucleophile and to bring together the nucleophile with the targeted bond. These distinctions form the basis for the classification of this class of enzymes. Resting on the presence of important chemical groups in their active sites, proteases were typically categorized into four major classes, namely serine, cysteine, metallo- and aspartate proteases till the end of the last century. Metallo- and aspartate proteases utilize heavy metals and aspartate residues, respectively, to immobilize and polarize a water molecule so that the oxygen atom in water becomes the nucleophile [3]. The central feature of aspartyl proteases is the presence of a pair of aspartic acid residue in different protonation states that together facilitate the attack on the peptide bond by water molecule. The deprotonated aspartic acid residue activates the water molecule by assuring its deprotonation, whereas the protonated residue polarizes the peptide carbonyl thus making it susceptible to the attack. The active site of metallo-proteases, on the other

S. Chakraborty
Department of Physical Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700032, West Bengal, India

S. Basu (✉)
Department of Microbiology, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, West Bengal, India
e-mail: soumalee@gmail.com

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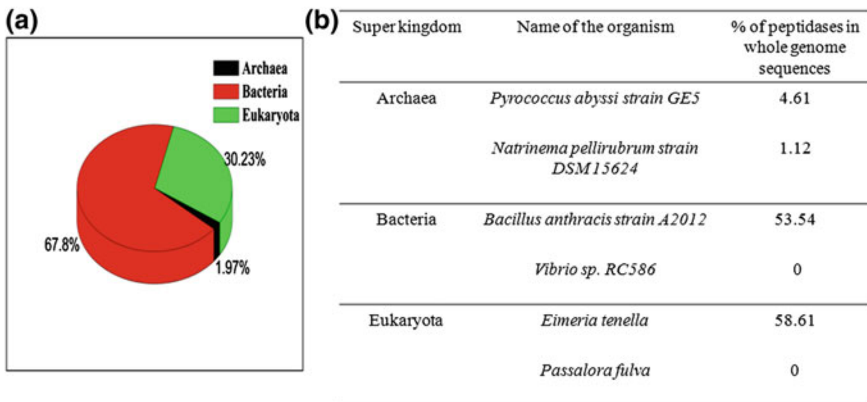
hand, is constituted of a bound metal ion, mostly zinc that activates a water molecule to attack the peptide carbonyl group as a nucleophile. Serine and cysteine proteases utilize their HO and HS side chains, respectively, directly as nucleophiles. Although not identical, the catalytic mechanisms of serine and cysteine proteases are remarkably similar. The formation of an oxyanion or thiolate anion (the nucleophile) in serine and cysteine proteases, respectively, is essential for catalysis. Thus, the presence or absence of a covalent acyl-enzyme intermediate on the reaction pathway demarcates the mechanism of catalysis by serine and cysteine proteases from aspartic and metallo-proteases. In general, these cysteine and serine proteases are folded into two relatively large globular domains surrounding a cleft containing the active site residues. Substrate entry into the cleft is a prerequisite for cleavage, and efficient entry is dictated by the structural fit between the potential substrate and the topology of the cleft, a major determinant of enzyme specificity. The formation of a spatial fit between a targeted bond of the substrate and the active site nucleophile is obviously also a critical determinant of substrate specificity.

In 1995 and 2004, threonine and glutamate proteases were included in the classification. Threonine proteases use threonine as the nucleophile, while glutamate proteases use water as nucleophiles. Inclusion of a seventh group of protease named as asparagine peptide lyase may be debatable as the proteolytic mechanism involves elimination reaction instead of hydrolysis. Proteases are also classified into acid, neutral, and basic/alkaline proteases depending on the optimal pH at which they remain active. Exopeptidases, namely aminopeptidase and carboxypeptidase A as well as endopeptidases, are also used as classes of proteases.

Protease activity can be highly specific such that hydrolysis would occur only at a particular site. It can be highly promiscuous too such that a wide range of substrates may be hydrolyzed. Specificity is dictated not only by desired interactions at the active site but more importantly by the influence of remote binding interactions on the orientation of the substrate toward the catalytic site for desired interactions to occur [4]. Nonspecific hydrolysis on the other hand occurs after the protease binds to a particular residue of the substrate and hydrolyses relatively nonspecifically at positions with preferences for certain amino acids at the P1, P2, P1′, or P2′ positions.

The human genome codes for about 560 proteases and their homologs that are distributed into 186 metallo-, 178 serine, 21 aspartic, 148 cysteine and 28 threonine proteases [5]. According to the most recent consensus, among all the known proteases, 1.97% is from archaeal origin and 67.8 and 30.23% constitute the bacterial and eukaryotic proteases (Fig. 1a). Interestingly, the percentages of proteases in whole genome sequences from different super-kingdom greatly vary. A list of percentage of the number of proteases in whole genome for the most and least abundant organism from three super-kingdoms is shown in Fig. 1b.



Super kingdom	Name of the organism	% of peptidases in whole genome sequences
Archaea	*Pyrococcus abyssi strain GE5*	4.61
	Natrinema pellirubrum strain DSM15624	1.12
Bacteria	*Bacillus anthracis strain A2012*	53.54
	Vibrio sp. RC586	0
Eukaryota	*Eimeria tenella*	58.61
	Passalora fulva	0

Fig. 1 **a** A pie chart representation of all known proteases among different super-kingdom. **b** A list of percentage of the number of proteases in whole genome for most (*top*) and least (*below*) abundant organism from three super-kingdoms (http://merops.sanger.ac.uk/)

Proteases play a very prominent role in various aspects of physiological processes such as food digestion, blood coagulation, wound healing, apoptosis, signal transduction, and immune responses to name a few. A delicate control on the activation and inactivation of protease cascades at different levels of protease gene transcription, mRNA translation, activation of zymogens, substrate specificity, enzyme kinetics, and even by means of enzyme-inhibitors needs to be maintained. A number of studies illustrate that apart from proteolysis, proteases possess a variety of regulatory functions that are mediated through intracellular signaling pathways, caspase-like enzyme activity, and/or regulation of specific cytokines and signaling receptors. Proteases are, therefore, viewed as multifunctional, hormone-like signaling molecules that are cardinal to various physiological and pathological processes [6]. Aside from their roles in the physiology of organisms, they also play crucial roles in the pathogenesis of a number of diseases, such as several parasitic infection, cardiopulmonary disease, emphysema, gingivitis, several inflammatory infections, tumor invasion, traumatic brain injury, ischemic brain injury, Alzheimer's disease, the destruction of the extracellular matrix of articular cartilage and bone in arthritic joints. Out of these, parasitic diseases represent major global health problems of immense proportion. Parasite-derived proteases have emerged as the major among the virulence factors identified. Well-characterized roles in pathogenesis played by proteases include their involvement in invasion of the host by parasite migration through tissue barriers, degradation of hemoglobin and other blood proteins, immune evasion, activation of inflammation, egression. Therefore, many of these enzymes that originate from the human genome or from the genome of the disease-causing organism form potential targets.

2 Disease-Causing Serine Proteases—*Intrinsic and Extrinsic*

Serine proteases that constitute one-third of peptidases are so named because of the presence of a nucleophilic serine residue at the active site playing important roles in mediating protein hydrolysis. The class is distinguished by the presence of novel catalytic triads and dyads with the classic catalytic His-Asp-Ser/Asp-His-Ser/Ser-Asp-His/Ser-His-Asp triad spanning over four different clans typified by chymotrypsin, subtilisin, carboxypeptidase Y, and Clp protease [4]. Other catalytic triads and dyads include Ser-His-Glu, His-Ser-His, Ser-Lys/His, and N-terminal serine [7]. Chymotrypsin, the first three-dimensional structure of protease to be determined by Blow [8], depicted the presence of catalytic, substrate recognition, and zymogen activation domain components, later found to be common to all chymotrypsin-like proteases.

Serine proteases are involved in a wide range of biological processes, such as intracellular and extracellular protein metabolism, digestion, fertilization, blood coagulation, and regulation of development. Human serine proteases like elastase, cathepsin G, PR-3, thrombin, kallikreins, tryptase, and chymase all play a role in cardiovascular disease [9]. Cystic fibrosis (CF) lung disease is referred to as chronic infection of the lung characterized by inflammation of the airway. Elevated levels of neutrophils and subsequently that of the three neutrophil serine proteases, namely neutrophil elastase, proteinase 3 (PR3), and cathepsin G, have been observed in sputum of CF patients. Excess protease activity results in the detrimental cycle of inflammation [10]. Celiac disease (CD) is triggered by the ingestion of gluten in genetically susceptible individuals and is yet another example of a disease where serine protease seems to play a significant role in the development of the disease as elafin which is a serine protease inhibitor has been found to be expressed less in CD patients. This is true for patients suffering from inflammatory bowel disease (IBD) too.

Quite a few number of serine proteases have been identified in protozoan and parasitic helminths that have putative roles in parasite development and nutrition, host tissues and cell invasion, anticoagulation, and immune evasion. Humans are constantly threatened by a wide variety of infectious diseases caused by these pathogens. The rhomboid family of serine protease, first identified in Drosophila, is conserved in prokaryotes and eukaryotes. In toxoplasma and *P. falciparum*, rhomboid proteases were identified to function as shedding of adhesion proteins during invasion [11]. The serine proteases that have been identified in parasitic helminths include nematodes or roundworms (*Trichinella spiralis*, *T. pseudospiralis*, *Onchocerca volvulus*, *O. lienalis*, *Brugia malayi*, *Ancylostoma caninum*), cestodes or tapeworm (*Spirometra mansoni, Echinococcus granulosus*) and trematodes or flatworm (*Fasciola hepatica*, *F. gigantica*, and *Schistosoma mansoni*). TspSP-1 from *T. spiralis* plays an important role in degrading cytoplasmic or intercellular proteins, thereby facilitating the movement of larvae [12]. Ts23-2 is another serine protease that is expressed after cyst formation. TspSP-1.2 has been

isolated as another member of this family that contributes to the larval invasion into the host epithelial cells and is being considered as a potential vaccine candidate against *T. spiralis* infection [13]. TsSerP is yet another member that is involved in molting process of the parasite and digestive functions. *Onchocerca volvulus* is an important filarial nematode that causes subcutaneous filariasis of humans and affects the eyes and skin. A neutral elastase of the nematode is known to degrade the elastic fibers of the host tissue, and a blisterase belonging to the subtilisin-like serine protease plays an important role in cuticle production and maintenance, neural signaling, and nematode development [14] wherefore it forms a potential drug target for controlling parasite infection. Another serine protease from *Brugia malayi* microfilariae is believed to be involved in suppressing the immune system by cleaving C5a of the complement system of the host [15]. Infection by a hematophagous nematode of the genus *Ancylostoma*, living in the small intestine of hosts, causes hookworm disease. *Ancylostoma caninum* uses an elastolytic enzyme to feed on the villous capillaries by preventing the blood from clotting. This protease is thus a potential target for chemotherapeutic or immunological intervention [16]. Serine proteases from cestodes have been found to be involved in host tissue invasion and immune evasion and have been characterized from plerocercoid larvae of *Spirometra mansoni* or from *Echinococcus granulosus* or *Taenia solium cysticercus* [17]. Serine proteases from the liver fluke disease-causing parasites *Fasciola hepatica* and *F. Gigantica* have also gained attention [18]. Schistosomiasis caused by the parasite blood fluke or *Schistosoma* spp. is a chronic disease [19] in which its serine protease "cercarial elastase" facilitates skin invasion by the infective schistosome larvae to permissive hosts including humans. The stage-specific expression of the proteases contributes to successful parasitism lasting for decades [20].

The malarial proteases are a group of molecules that are essential for parasite life cycle stages out of which serine proteases are of special interest due to their involvement in parasite-specific egress and invasion [21].

3 Disease-Causing Cysteine Proteases—*Intrinsic and Extrinsic*

Cysteine proteases are grouped into two superfamilies—the family of enzymes related to interleukin 1β converting enzyme (ICE), and the papain superfamily of cysteine proteases [22]. Other than the presence of the active site cysteine, the ICE superfamily does not share much homology with the papain family [23]. The papain superfamily is composed of the three groups, namely calpain, bleomycin hydrolase, and the papain family itself. Cathepsin from humans also belongs to this group.

Apoptosis, the physiological death of cells, is implemented by caspases. Once activated, caspases act as cysteine proteases. Enzymatic activation of initiator caspases leads to proteolytic activation of downstream executioner (effector)

caspases leading to the cleavage of a number of vital proteins, resulting in the orderly removal of the cell [24]. Thus, both reduced and increased apoptosis lead to pathology and the associated diseases include cancer, neurological disorders, cardiovascular disorders, and autoimmune diseases.

In humans, cathepsin cysteine proteases consist of a family of 11 members, namely cathepsins B, C, F, H, K, L, O, S, V, W, and Z [25]. Many cellular events in the development of atherosclerosis-based cardiovascular disease depend on the cathepsin-mediated degradation of intracellular and extracellular proteins, including cell adhesion, transmigration, differentiation, proliferation, apoptosis, neovascularization, and antigen presentation. Even though cathepsins are abundant in human, the exact role each specific cathepsin plays in heart disease development and the mechanism and significance behind their function are unknown. Cathepsins S, K, B, and L have been shown to be involved in osteoporosis and cancer. Calpain cathepsin and caspases are involved in traumatic brain injury (TBI), ischemic brain injury, and many neurodegenerative diseases. In Alzheimer's disease, cathepsin B is found in neurites and dendrites, whereas in the normal condition its activity is localized in lysosomes [26]. Neurofibrillary tangles (NFT) in brains suffering from AD contain increased levels of calpain 2 and cathepsin [27], while calpain 1 has been found to be hyperactive in brains. Hsp70, which has a dual role as a chaperone in damaged protein and also as an important factor in the maintenance of lysosomal integrity, is cleaved by calpain after modification by oxidative stress resulting in the impairment of lysosomal autophagy [28]. Cathepsin B has been shown to have multiple roles in cancer with an increased activity in lung tumor and lymph node metastases [29]. Cysteine proteases are essential for the induction and development of both innate and adaptive immune responses in humans. Their role ranges from antigen and pathogen recognition and elimination, signal processing and cell homeostasis.

There are pathogens that secrete cysteine proteases that often target the same proteins as the mammalian proteases and thereby modulate host immunity [30]. Cysteine proteases that are involved in parasitic diseases include falcipains 2, 2′, and 3 from *P. falciparum*. They are referred to as malarial hemoglobinase as they degrade hemoglobin after it has been cleaved into peptide fragments [31]. *Trypanosoma brucei* is a protozoan parasite and the causative agent of human African trypanosomiasis, a fatal disease that is transmitted by the bite of the tsetse fly. Two clan CA cysteine proteases have been identified in *T. brucei*, rhodesain, which is cathepsin L-like and most abundant and tbcatB, a cathepsin B-like enzyme. Studies show that despite being less abundant, tbcat is essential to *T. brucei* as it is implicated in iron acquisition whereby transferrin may be a natural substrate [32]. *T. brucei* cathepsin B is a logical target for the development of new anti-trypanosomal chemotherapy as *T. brucei* cathepsin B-like protease is a key enzyme in host protein turnover. It may be mentioned that the cysteine proteases staphopain A and B secreted by the human pathogen *Staphylococcus aureus* could not be inhibited by any of the seven human cystatins [33]. Cysteine proteases are also used by the worms of schistosome to digest the blood meal.

4 Disease-Causing Metallo-Proteases—*Intrinsic and Extrinsic*

Well-timed degradation of extracellular matrix (ECM) is an important feature of development, morphogenesis, tissue repair, and remodeling. Upon disruption of the precise regulation pertaining under normal physiological conditions, diseases like arthritis, nephritis, cancer, encephalomyelitis, chronic ulcers, fibrosis occur. Cardiovascular disorders such as atherosclerosis, stenosis, left ventricular hypertrophy, heart failure and aneurysm [34] result from uncontrolled ECM remodeling of the myocardium and vasculature. Major enzymes responsible for ECM degradation are the matrix metallo-proteinases (MMPs) also called matrixins. They form a large family of calcium-dependent zinc-containing endopeptidases. Humans have about 30 matrixin genes with a duplicate MMP-23 gene. Expression of matrixins is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell–cell and cell–matrix interaction, while its activity is regulated by activation of the precursor zymogens and inhibition by endogenous inhibitors, tissue inhibitors of metallo-proteinases (TIMPs) [35]. In pathological conditions, this equilibrium is shifted toward increased MMP activity leading to tissue degradation. On the basis of the specificity of MMPs, they are classified into collagenase (MMP-1, -8, and -13), gelatinase (MMP-2 and -9), stromelysin (MMP-3, -10, -11), and matrilysin (MMP-7 and -12). Another subclass is the membrane-type MMPs (MT-MMP). Most of the matrix metallo-proteinases consist of four distinct domains, which are N-terminal pro-domain, catalytic domain, hinge region, and C-terminal hemopexin-like domain. This may be responsible for the macromolecular substrate recognition and/or interaction with TIMPs. The membrane-type MMPs (MT-MMPs) contain an additional transmembrane domain that anchors them in the cell surface [36]. MMPs in the CNS are secreted by microglia, astrocytes and neurons. Under normal conditions they are either absent or present at undetectable levels in the brain and deregulation of their activity shifts the balance and induces perpetuation of chronic inflammation. This has been shown in different peripheral chronic diseases, such as atherosclerosis and rheumatoid arthritis [37, 38] and neuroinflammatory diseases, such as cerebral ischemia, stroke, and bacterial meningitis [39]. MMP-1 in synovial fluid of rheumatoid arthritis patients has shown promise as a marker of the disease, MMP-8 may be useful as a marker for plaque instability in atherosclerosis, MMP-13 has a specialized role in bone development and hence it plays important role in osteoarthritis, MMP-2, MMP-3 and MMP-9 are also important [40]. During infection of glial cells with human T-lymphotropic virus type I (HTLV-I), the causative agent of a progressive chronic myelopathy, MMP-9, increases [41]. It is worth mentioning that the role of MMPs has become well appreciated in neurodegenerative diseases with the listed diseases being Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis.

Metallo-protease falcilysin from *P. falciparum* is also involved in degradation of hemoglobin after it has been cleaved into peptide fragments [42]. The metallo-protease toxolysin 4 from *Toxoplasma gondii* localizes to the micronemes

and its secretion coincides with discharge of micronemal contents [43], thus implying its function in invasion. *H. pylori* infection increases secretion of both MMP-1 and MMP-3 by gastric epithelial cells. *M. tuberculosis* infection increases MMP-1 from human airway epithelial cells [44]. An efficient way for a pathogen to cause pathology is by operating at the top of a proteolytic cascade. An example of this is the protease of the thermolysin family secreted by *Pseudomonas aeruginosa* and *Vibrio cholera* that activate pro-MMP-1, -8, and -9 [45], while proteases from the oral pathogen *Porphyromonas gingivalis* activate MMP-1, -3, and -9 [46].

5 Disease-Causing Aspartyl Proteases—*Intrinsic and Extrinsic*

Aspartyl proteases form the smallest group of proteases in humans that has been shown to play important roles in physiological and pathological processes. Aspartic proteases such as cathepsin D, gastricin, pepsin, renin, HIV protease and others have been the subject of research in the last years. The involvement of renin in hypertension, cathepsin D in metastasis of breast cancer, BACE 1 (beta site-activated APP cleaving enzyme) in Alzheimer's disease and the protease of human immunodeficiency virus (HIV) in acquired immune deficiency syndrome (AIDS) is being vigorously studied. Multicellular organisms maintain endogenous proteins that function as protease inhibitors to regulate proteolytic activity. Most of these inhibitory proteins are directed against serine proteases, although some are known to target cysteine, aspartyl, or metallo-proteases. Indeed, inhibitors of serine, cysteine and metallo-proteases are distributed ubiquitously throughout the biological world. In sharp contrast, however, naturally occurring inhibitors of aspartic proteases are relatively uncommon and are found in only certain specialized locations.

The extended family of plasmepsin aspartyl proteases in *P. falciparum* has been the focus of intensive research mainly due to the parasite-specific nature of these enzymes. Plasmepsins are made up of four main members, two of which are thought to initiate hemoglobin degradation, while the other two function in the degradation of peptides generated by the action of upstream members of the family [47]. Plasmepsins have therefore evolved as very popular antimalarial agents and are of immense therapeutic importance. Aspartic proteases are important to the worms of schistosome as they use the protease to digest the blood meal for survival in the host and hence cause the disease.

6 Drugs as Protease Inhibitors

Deviation from the physiological role of intrinsic/endogenous proteases in humans or a spoilt balance in their amount results in severe diseases. Additionally, usage of intrinsic and/or extrinsic proteases by the human pathogens during invasion and/or egression for subsequent survival has led to the idea of employment of protease inhibitors in therapeutics. Several important drugs are protease inhibitors. For example, captopril, an inhibitor of the metallo-protease angiotensin-converting enzyme (ACE), has been used to regulate blood pressure. Crixivan, an inhibitor of the HIV protease, is used in the treatment of AIDS. This protease cleaves multidomain viral proteins into their active forms; complete blocking of this process prevents the virus from being infectious. To prevent unwanted side effects, protease inhibitors used as drugs must be specific for one enzyme without inhibiting other proteins within the body.

7 Important Databases

The MEROPS, an online database at http://merops.sanger.ac.uk, is a rich source of information on peptidases and their inhibitors. CutDB is a proteolytic event database at cutdb.burnham.org that focuses on the annotation of individual proteolytic events both actual and predicted.

The mammalian degradome database at http://degradome.uniovi.es gives complete information of the degradome of an organism whose whole genome is available. Degradome is defined as the complete set of proteases present in an organism. This Web site also provides detailed information about genetic diseases of proteolysis. Finally, the user can find additional information about protease structures, protease inhibitors, ancillary domains of proteases and differences between mammalian degradomes.

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Pathophysiological Aspects of *Aspergillus* sp.

Tapan Kumar Das

1 Introduction

Aspergillosis was firstly identified as fungal infection developed in the lungs of a jackdaw and animals [1] which was firstly focused in human in 1953 [2], and since then, the same infection increases gradually throughout European countries. Bennett examined sputum of the patients suffering from tuberculosis and identified aspergillosis in the cavities of lungs [3]. Spreading of invasive aspergillosis was reported to enhance several times day by day as described by Cawley in [4] (and by Hinson et al. in [5]).

The genus *Aspergillus* has been identified as a class of Euascomycetes of the Phylum Ascomycota [6]. This is asexual species which is supposed to be present as telomorphic forms in the ancestor of Trichocomaceae. Under the heading of the genus, *Aspergillus*, seven subgroups with containing numerous number of species are already recognized. 40 number of *Aspergillus* spp. out of approximately 184 species are found to show human or animal infections, *and* it is reported that it can multiply very rapidly with different colors of conidia like black, white, green, gray, etc. [7].

Aspergillus species living on dead or decomposing ingredients considered as saprophytic fungi are available abundantly everywhere in the world. About 1000 species have been identified in the genus *Aspergillus*. *Aspergillus flavus* and *Aspergillus fumigatus* among other species of *Aspergillus* are recognized commonly as infectious fungi to infect human severely which are about to more than 90% and about 10% are identified are invasive diseases [7]. *Aspergillus clavatus*, *A. cameus*, *A. niger*, *A. terreus*, *A. parasiticus*, *A. oryzae*, *A. wentii*, *A. versicolor*, and

T.K. Das (✉)
Department of Biochemistry and Biophysics, University of Kalyani,
Kalyani 741235, West Bengal, India
e-mail: tapndas@hotmail.com

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some other *Aspergillus* species could show infectivity character toward human and animals.

After infecting human by *A. flavus*, it causes swelling of the inner membrane in any of the hollow areas of the skull around the nose developing sinuses [8]; Otomycosis is majorly caused by *A. niger* infection to human, and in the same infection, mycelia of *A. niger* are exposed in cerumen and desquamated cells in the external auditory canal, and symptoms are found primarily as scaling, itching, and pain, but *A. niger* could not attack the tissues, and overall tissues remain intact [9]. The most dangerous pathogenic species are *A. flavus* and *A. fumigatus* which could produce alfatoxin showing as toxcin as well as carcinogen properties. Infection of *Aspergillus* causes development of a group of diseases in human and animal called aspergillosis reflecting symptoms of fever, cough, chest pain, or problem in respiration, and it may disorder the immune systems, and multiple infections may be developed in lungs.

Aspergilli also have a less reputable side in the agricultural industries; it could not favor *Aspergilli* as a few species of Aspergillus, like *A. flavus* and *A. parasiticus*, [7] are reported to pollute daily needs crops with aflatoxin secreted by the same *Aspergillus* spp. which are already examined as very toxic and carcinogen, and it can show immunosuppressive properties. In the present scenario of the developing countries in the world, after intake of such type of polluted grains products as daily food, general people are found to be infective with such type toxic materials and feeling severe life-threatening sickness, and they would be on the verge of death.

2 Aspergillosis Infection and Its Symptoms

Approximately, 40 of several number of species of *Aspergillus* are concerned in the development of infection in human or animal systems, and it can be commonly expressed as aspergillosis. *A. fumigates* and *A. flavus* are identified as a severe infectious *Aspergillus* sp. Infecting human to develop a group disease called aspergillosis in major cases and other species like *A. niger* are considered as second line of pathogenic fungi causing fungal infection to human and animal in the world. Infection of *Aspergillus* sp. could develop clinical allergies by *Aspergillus* sp. include clinical allergies, external, and confined infections, and some infections are found to develop in human degenerating tissues. In most cases, *A. fumigatus* can attack human and animals severely to develop insidious aspergillosis. Characteristics of infection are dependent on *Aspergillus* sp. and mainly on immunity of individual persons [10]. As reported, *A. flavus* along with other species of *Aspergillus* could cause severe infections in human and animals. *Aspergillus* species can be attracted at the site of sino-pulmonary tract with lung in human to initiate its infection with the symptom like high fever, pain in head, and face headache, inflammations in face, bleeding of infected nose as it is affluent in blood vessels, projection of one or both eyeball, disorders of smell, vision, eyes, taste, and

positional vertigo, ulceration and necrosis of the soft palate, and corrosion of bone. In addition to the commonly observed symptoms, like fever, cold, and cough and breathing disorder, shortness of breath (serious disease at heart and lungs, undefined symptoms are also found in vascular incursion and pleural chest pain while infected persons have been suffering from pulmonary aspergillosis for long time without treatment and extensive rate of caseating visceral tuberculosis occurs by hematogenous dissemination in any organ, and in fine, central nervous system would be arrested intensively succeeding strokes.

Most of the species of *Aspergillus* can grow on departed or putrefying substances available in the surroundings. At the first stage of the life cycle, *Aspergillus* is to generate spore which can easily spread at any places of environment through natural air circulation. The spore carried by air can enter the lungs or other organs initially through inhalation during respiration and occupy the position of alveolar spaces which would not be detached from the deposited places of alveoli by mucociliary escalator due to creation of obstruction by epithelial cells, and there is chance of killing of *Aspergillus* spores initially due to presence of alveolar macrophages by phagocytosis.

3 Aspergillosis

The reason of the infection, aspergillosis in human, is due to inhale of conidia of *Aspergillus* from the environment, and it causes lung diseases as well as immune system could be destabilized. The infection aspergillosis is classified on the basis of localization of *Aspergillus* infection. Problem in respiratory tract is found to develop after infecting with *Aspergillus* [11]. Symptoms of the various type of aspergillosis are being mentioned as below [12].

3.1 Allergic Bronchopulmonary Aspergillosis

Shortly, the same infection is called ABPA. The fungus, *Aspergillus* predominately *A. fumigatus* infects mainly at lungs after inhaling the conidia of the same fungus during respiration [13]. It could initiate the infection through vigorous response of the immune system which may be considered as one type of hypersensitivity response) to *A. fumigatus*. This is also found in a person suffering from cystic fibrosis creating very thick salty mucus and sweat; ultimately, lungs would be affected severely creating problem in respiration with allergic symptoms like generation of coughing and breathless, and at last, severe and permanent bronchitis problem are found to develop. Conidia of *A. fumigatus* would damage tissues of lungs severely if any patients suffer long time with negligence of medical treatment.

3.1.1 Symptom

(i) Reflection of allergy to coughing and wheezing
(ii) Asthma

3.2 Allergic *Aspergillus* Sinusitis

The abbreviation of allergic *Aspergillus* sinusitis is AFS. The AFS is almost similar to ABPA. The same infectious disease may be developed through the various interconnected factors and proceedings as proposed by Manning and Holman [14]. Initially, conidia of *A. fumigatus* could enter by inhalation through nasal respiration, and after entering, the host of human having inclination toward growing up an allergy in hereditary is disclosed to the fungi, *Aspergillus* showing the primary antigenic motivation, and this provocative reaction follows primarily as a consequence reaction between Gell and Coombs type I considered as an IgE mediated and type III considered as immune complex-mediated resulting tissue edema. Finally, the sinus ostium is obstructed; as a result, an accumulation of fluid occurs in the sinus which environments encourage the multiplication of *Aspergillus* creating severe allergic symptoms. Under this condition, it would produce thick, persistent, and highly dense *Aspergillus* mucin with the change of color from chocolate to shady bottle green color [15].

3.2.1 Symptoms

(i) Tissue edema due to an inflammatory reaction
(ii) Obstruction of sinus
(iii) Allergies and nasal polyps
(iv) Asthma

3.3 Aspergilloma

Aspergilloma is also acknowledged as mycetoma/fungal ball developed after assembling of fungi, *Aspergillus* sp. at hollow space of human body as at nasal cavity and also at hollow space of lungs.

In Aspergilloma, organ, lung is commonly affected in human. The patients suffering from the common aviary lung infection like tuberculosis, cystic fibrosis, bronchiectasis, any immunodeficiency, etc., can be affected generally by Aspergilloma [16]. In same, infection *A. fumigatus* is found to be common fungus to infect lung of human after inhaling the same fungal conidia through nasal route, and

then, conidia is found to deposit in a hole of lung for multiplying the same fungus as the hole is apart for any intervention since decisive components of the immune coordination are not capable of infiltrating into the hole. The fungal ball is found to constitute after assembling the growing fungus in the hole, and the same ball can associate with dead tissue around the lung, mucus, and other garbage The infection, Aspergilloma, can grow in hard organ as abscesses like in brain and kidney, generally of immune negotiated persons, and this can also be found in cavities of body in human.

3.3.1 Symptom

(i) Feeling of pain mainly in chest and bones
(ii) Visual problem
(iii) Excretion of urine with blood
(iv) Excrete decreased amount of urine
(v) Feeling of pain in head
(vi) Feeling cold
(vii) Breathing problem
(viii) Skin aching

3.4 Chronic Pulmonary Aspergillosis

This infection is also called as CPA, and it belongs to the infection aspergillosis which is considered as a long-period infection of aspergillosis found in human, while *A. fumigatus* would infect the lung of human after inhalation of conidia of the same fungus during respiration [17]. This infection is found to be very rare creating severe problem in lung resulting turmoil in respiration, but CPA is reflected generally as a widespread form considered as chronic cavitary pulmonary aspergillosis and abbreviation is CCPA. Chronic fibrosis pulmonary aspergillosis can be developed while a person has been suffering from CCPA for long time or a patient suffering from CPA remain medically untreated condition. Patients suffering from CPA may show well immune system that cures the infection created by this fungus under this condition. Accordingly, patients can not be attacked speedily by the infection; however, it would supervise to reside in the body region at which it can grip. The fungi can choose the appropriate region through which it would get access. Accordingly, fungal conidia can pass through the air route of lungs in environment, and then, it is necessary to avoid the immune system for creation of inhabitant in a cavities which are far away from the immune system like the destructed tissues which are found after tuberculosis or such type of infectious diseases, and the damaged tissues supply the garbage available after normal infectious diseases to fungus as per requirement for the footing of fungus, and the fungus initiates its growth slowly in a restricted region nearby the tissues at which

the immune system of the persons suffered by the infection can hit it which are present around the cavities. So, it is found to remain unseen for several periods with the reflection of a few symptoms of the infection; however, it can initiate to corrode the around tissues; as a result, swelling of tissues occurs in contact with the fungus. Finally, there is a formation of fungal ball in the same places of cavities with the coughing up of blood. This infection may make misconception about tuberculosis.

3.4.1 Symptoms

(i) Feeling fatigue
(ii) Loss of weight
(iii) Severe respiratory troubles
(iv) Increase amount of cough productivity
(v) Excretion of cough with blood
(vi) Presence of fungal ball/nodules in pulmonary cavity

3.5 Invasive Aspergillosis

Invasive aspergillosis is found to develop in cancer/leukemia/acquired immune syndrome disease suffered people whenever their immune system becomes destabilized, while chemotherapy and other treatment for aids are going on, and the impaired immune system can not struggle for fighting against different types infection [18]; as a result, the same aspergillosis can enter forcibly the lung tissues as well as becoming widen to the kidneys or brain, and at last, it is reported to develop severe health hazardous infectious pneumonia, while the patients suffering from invasive aspergillosis with conciliated immune organization remain untreated medically for long time [19]. In addition to these aspergillosis infections, *A. fumigatus* can affect at skin of outer surface in human to create infection called Cutaneous aspergillosis. In this case, conidia of *A. fumigatus* may be allowed to pass through the body skin around the surgery or burn wound, and the same fungal spores can make infection which may be considered as invasive aspergillosis. Cutaneous aspergillosis may also be exposed in the body skin after severely affecting lungs by invasive infection [20].

3.5.1 Symptoms

(i) Cough occasionally with blood
(ii) Chest Pain
(iii) Decreasing of breathing time
(iv) High body temperature

4 Molecular Mechanism of Aspergillosis Infection Made *Aspergillus* sp.

Aspergillus species are capable of growing on departed or decomposing materials available in the environment. Life cycle of *Aspergillus* starts with the formation of spores which can spread all over air of the environment and can move everywhere through air in combination of moisture and dust, and the same spores can easily be inhaled by human during respiration from air which is considered as a principal entrance of the *Aspergillus,* and then, spores are assembled in alveolar regions to start the infectious diseases. Macrophages found to be present in alveolar can destruct the deposited *Aspergillus* spores through phagocytosis in a well-established immune system [21]. These fungal spores can overcome the phagocytosis killing and can multiply easily with the aim of defending neutrophils. As a result, formation of invasive aspergillosis will be developing with loss of capability of host defense and allow *A. fumigatus* for survival with its growth in pulmonary surroundings. The *A. fumigatus* can grow in very fast at physiological system compared to others *Aspergillus* sp. as growth rate of *A. fumigatus* is found to go faster about 40% in physiological and pharmacological concentrations of hydrocortisone. So, growth rate is one of the important switches for reflection to the progress of the diseases, that is, of the pathogenicity. Under this condition, *A. fumigatus* can generate very small size of spores which are allowed to penetrate intensively in the lungs. The spores are able to remain survival under extreme conditions of the surroundings as the same spores are covered with special type of layer consisting of hydrophobic proteins and lipids, and these special type of spores stuck with laminin and fibrinogen are capable of defending the same spore from the attack of the host. During germination of spores of *A. fumigatus* as well as life cycle of *A. fumigatus*, different toxic materials like several proteases, ribotoxin phospholipases, a hemolysin, gliotoxin, aflatoxin, phthioic acid, etc., are reported to excrete during infection in lungs and other organs [22]. Epithelial cells are found to become loosen from pulmonary region with induction of the excreted protease with the release of pro-provocative cytokine. Host cell membrane can be damaged by phospholipases excreted by the *A. fumigatus* as well as destruction of tissues occurs. Gliotoxin excreted by the fungi is capable of reducing macrophage and neutrophils causing phagocytosis of inhaled spores of *Aspergillus* sp., and so, all of the spores remain viable. Direct role of Gliotoxin in pathogenecity is not clear till date. As reported, a rare number of *A. fumigatus* are capable producing phthioic acid. Role of phthioic acid in pathogenicity is reported to form granuloma which is also found in tuberculosis. In addition to these, supper oxide dismutase and catalases can be synthesized by *A. fumigatus* by which fungus *A. fumigatus* cannot be damaged by singlet oxygen, hydrogen peroxide, and other free radicals generated by phagocytes as well as fungal cell can attack the tissues of lungs and other organs developing aspergillosis.

5 Conclusion

Among *Aspergillus* spp., *A. fumigatus* and *A. flavus* have been recognized as general human pathogen causing development of aspergillosis in human system. Human with immune negotiated is suffering from diseases related to hematological malignancy, and transplanted patients are infected severely with *Aspergillus* sp mainly with *A. fumigatus* and *A. flavus* IA and cause of development of invasive aspergillosis. Special characteristics of such type of fungi are reflected to the formation of spores which are very small in size with the presence of a few pathogenic factors created during its germination and multiplication which are associated very deeply with the infection aspergillosis as the same fungi can grow very well at body temperature with the easy entrance of the airborne spores in the way of interior respiratory tract during respiration. Spores of *A. fumigatus* become accustomed very well to the tissues of lungs of immune-suppressed patients as well as spores can start the colony formation in affected tissues of lungs with the avoid of attack of macrophage and split up the attached tissues for required nutrients for the uncontrolled growth of same fungus as a result extreme irritation occurs with the combinations of several pathogenic factors.

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Cysteine Proteases of Parasitic Helminths

Alok K. Dixit, Pooja Dixit and Ramji L. Sharma

Abstract

Cysteine protease and/or thiol peptidase/sulphydryl peptidase/cysteinyl peptidase invariably contain cysteine at their active site and hence collectively termed as cathepsins. This proteolytic enzyme, secreted and/or regurgitated/excreted by the in situ invasive forms of metazoan parasites of men and domestic livestock, often involve in the host tissue penetration, digestion of nutrients, and their availability to the parasite for its growth and development, evasion of host defense, and in of late documented their significant role in detection of in situ parasites at an early developmental stage(s) [Immunodiagnosis] beside an important entity to develop immunoprotection strategies against the livestock diseases, so much so, to contain and curb the colossal recurring losses, incidental to widely prevalent parasitic disease, to the fast developing livestock products based food industry in the developing world. Herein, the authors discuss the historical background, molecular structure, and classification of cathepsin proteases and update advancements in exploiting the enzyme for early diagnosis and planning immunoprotection strategies against metazoan parasitic diseases with special reference to tropical fasciolosis.

A.K. Dixit (✉)
Department of Veterinary Parasitology, College of Veterinary Science & A. H., Nanaji Deshmukh Veterinary Science University, South Civil Lines, Jabalpur 482001, Madhya Pradesh, India
e-mail: alokdixit7@yahoo.com

P. Dixit
Department of Veterinary Medicine, College of Veterinary Science & A. H., Nanaji Deshmukh Veterinary Science University, South Civil Lines, Jabalpur 482001, Madhya Pradesh, India

R.L. Sharma
843-44, Ranisati Nagar, P.O. Shyam Nagar, Ajmer Road, Jaipur 302019, Rajasthan, India

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1 Introduction

Parasites invariably infect their host(s), in situ survive, and sustain metabolic activities for development and growth besides propagation. Peptidases are protein/polypeptide catabolizing enzymes which act specifically on peptide bonds [1, 2]. Proteases, proteinases, and peptide hydrolases are the synonyms of these enzymes. Peptidases are responsible for various biological activities in parasitic organisms. Immunochemical investigations revealed tissue localization to the epithelia and microvilli of the gut of the metazoans [3–5]. These enzymes help the parasite to penetrate through various barriers in the host, in addition to digestion of host proteins for their nutrition [6, 7]. Parasite peptidases also help the parasite to escape the immune response of the host [6]. In addition, these are involved in secondary protein modifications and are used in immunodiagnosis of infection [8, 9]. These enzymes also have role in triggering allergic responses in hosts [10]. Evidently, these are actively involved in penetration of tissues, parasite nutrition, and in situ protection from host immune response.

2 Classification

Depending upon the site to be cleaved by the proteases, these can be broadly divided into two groups: exopeptidases which act on terminal carboxyl or amino group and endopeptidases which act on peptide bonds. Proteases are of four types on the basis of reaction mechanism and nature of active site residue in the mechanism. Serine and cysteine proteases directly act as nucleophile, and aspartyl and metalloprotease use activated oxygen of water molecule as nucleophile. As cysteine proteases contain cysteine in their active site, these are called cysteine cathepsins, while other cathepsins may contain serine or aspartate in their active site.

2.1 Papain Family of Cathepsin Proteases

Cysteine proteases are present in almost all life-forms, even in viruses and are one of the most important enzyme groups. In 1879, the first cysteine protease was purified and characterized from *Carica papaya*, the papaya fruit, and was thus named papain. In parasites, cysteine proteases have various roles like immune

evasion, enzyme activation, cell/tissue invasion, excystment, hatching, molting, and gene encoding which help in better parasite survival. As these enzymes contribute 2% of the genomic content of the parasite and are highly immunogenic, it is used as an important vaccine candidate. Cysteine proteases elicit the immune response of the host towards either Th1 or Th2 response whichever may be insufficient for expulsion of parasite. On the basis of sequence similarity, possession of inserted peptide loops, and biochemical specificity to small peptide substrates, cysteine proteases are divided into many clans like: CA, CB, CC and CD. The main cysteine proteases are included in clan CA which is further subdivided into two families C1 and C2. Important parasitic cysteine proteases included in C1 family are cathepsin B and L like.

Though the parasites possess many types of proteases, the proteases which are released by the parasites are of sole importance in host parasite interactions. In helminths, the main source of these proteases is their gut. As these proteases are digestive proteases that too of invertebrates, these are mainly cysteine and aspartic proteases in contrast to the serine proteases secreted in intestinal digestion of vertebrates [11, 12].

2.2 The Trematode Cathepsins

Peptidases act as essential biocatalysts in various basic biological processes in trematodes too. As these proteases have their role in host parasite interactions, these can be targeted for vaccines and chemotherapeutic drug development.

Various stages of *Fasciola* express cathepsin L and B which were originally described as immunoglobulin-cleaving enzyme or hemoglobinase [13, 14]. In *Fasciola* excretory secretary (ES) cysteine proteinase group, the major component is of 26–30 kDa in size. In amino acid sequence and substrate specificity, these proteases resemble mammalian liver cathepsin L protease [15, 16]. Nowadays, these are called as the *Fasciola* cathepsin L cysteine proteinases. As these are secreted by the gastrodermis of juvenile and adult flukes, these can be in vitro isolated/purified from fluke regurgitants.

From *Fasciola hepatica* ES products, first parasitic helminth cathepsin L proteinase was described as a single chain form at 27 kDa (FhCL-1) on SDS-PAGE, and it had multiple band complexes ranging between 60 and 90 kDa in non-reducing conditions [17]. Later on a 29.5 kDa, cathepsin L-like cysteine proteinase (FhCL-2) was found in ES products of *Fasciola*, and it was also reported that cathepsin L-like cysteine proteinase alone constitutes more than 80% of ES products of the fluke [18]. From adult *Fasciola gigantica* also, cathepsin L-like cysteine proteinase of 27 kDa FgCL-1 [19], 27–28 kDa FgCL-2 [20], and 28 kDa FgCL-3 [21, 22] were isolated. In *F. hepatica* and *F. gigantica,* many cathepsin L genes were identified which help in in situ development of these parasites. The recombinant cathepsin L-like proteases like RFhCL-1 [23], RFhCL-2 [24], RFhCL-3 [25], RFhCL-5 [26, 27], RFgCL-1 [28, 29], RFgCL-1D [30], and RFgCL-2 [31] were also produced from adult *Fasciola*.

Cathepsins secreted from newly excysted juvenile (NEJ) and immature intraperitoneal flukes were different from that of adults [32–34]. The most characterized proteases in *Fasciola* spp. belong to the cathepsin L group of cysteine proteases, while cathepsin B activity is present in juvenile stages or immature flukes [35, 36]. The most abundant proteases produced by NEJs and immature *Fasciola* belong to a family of cathepsin B-like proteases [33, 35–38].

Recent analyses of the secretome of *F. hepatica* and *F. gigantica* juvenile and immature flukes have identified six types of cathepsin Bs in the ES products and even in metacercariae stage which is the infective form of the parasite [37–40]. From NEJs to 5-week-old in situ developing immature parasites FhCatB1 (CB2), one isotype of cathepsin B, was found in both somatic and ES products [33, 35, 36]. Three stage-specific isotypes of cathepsin B proteases of *F. gigantica* FgCatB1, FgCatB2, and FgCatB3 cloned from cDNA libraries were found to be orthologous to isotypes (CB1, CB2, and CB3) of *F. hepatica*. RT–PCR analysis showed that FgCatB2 and FgCatB3 were expressed in NEJs and metacercariae. FgCatB1 is mainly expressed in adult parasites though found in all stages [37, 38].

Proteomics and transcriptome analysis revealed seven additional cathepsin B sequences in *F. hepatica* [39]. The expression of these proteases is regulated by the stage of the parasite whether juvenile, immature, or adult. Six cathepsin Bs are released by invasive stages of *Fasciola* and help in the establishment of infection. FhCB2 protease is released in the initial migratory stage when the infection is asymptomatic, and this is recognized by the host immune system also. In sheep, antibody titers to FhCB2 were increased 2–5 weeks post-infection and between 8 and 10 weeks post-infection. By adult stages, FhCB6–FhCB10 are secreted which are found to be cross reactive to FhCB2. FhCB4 of adult stages is an inactive protein as it has no cysteine residue at active site. Only FhCB6 and FgCB1 are secreted by both the adult and migratoty stages of *Fasciola;* therefore, these two cathepsins are thought to play a major role in fluke biology.

2.3 The Nematode Cathepsins

In nematodes also, papain-like enzymes are common. In *Haemonchus contortus*, many cathepsin Bs have been cloned [41–44]. Cysteine protease of *H. contortus* extracts and ES products of *Necator americanus* have shown the capability of digesting haemoglobin (Hb), fibrinogen, and antibodies [45, 46].

In *Ancylostoma caninum,* cathepsin Bs have a significant role in hematophagus activity. Two cathepsin B-encoding mRNAs have been cloned from adult *A. caninum* [47]. AcCP1 cathepsin is expressed only in secretory glands and esophagus [47], having only in vitro Hb-digesting activity, while AcCP2 is produced in intestine leading to in vivo Hb-digesting activity. Optimal hemoglobinolytic activity was seen after initial cleavage of Hb by aspartic protease.

In parasitic and non-parasitic nematodes, cathepsin B-like protease genes constitute large multigene families of which the best known is from intestinal tissues of *H. contortus* [44, 48, 49]. These play a role in digestion of Hb and other nutrients.

In blood-feeder nematodes with the help of a specific sequence alignment program, hemoglobinase motif was identified in cathepsin B-like proteases [50]. As the use of gene knockout and RNA-silencing techniques are limited in helminths, therefore, it is difficult to exactly determine the role of these proteases in lysis of HB. In nematodes, tissue localization activity of recombinant enzymes, and over expression in female worms suggests a significant role of these proteases in degradation of substrate for nutrition. Cathepsin Ls are less abundant in nematodes and have comparatively more role in embryogenesis and molting than digestion of nutrients contrary to that of hematophagous trematodes [51–53].

3 Vital Role in Host's Immune Evasion

Chapman and Mitchell [13], initially postulated the role of proteinases in immune evasion. These enzymes prevent the migrating flukes from host immune attack by activating effector functions in them. In in vitro assays, it has been found that the presence of proteinases coincides with the inability of the eosinophils to maintain antibody-mediated adherence to newly excysted/migrating juveniles. Proteinases secreted by the parasites confer protection against host immune responses as proteinases cause turnover in parasite tegument, hindering the recognition of the parasite by the host immune mechanisms [32, 54].

Fasciola spp. causes a chronic liver disease in most mammalian hosts. This parasite diverts the immune response to Th2 response which helps in its longer survival and provides resistance from host immune attacks. Th2 response predominance is shown by IL4, IL5, and IL10 production but very little IFN-γ [55]. Experimentally also, it has been found that these responses are of Th2 type with progression of infection. In cattle, it was observed that in early stages of infection, mixed Th1 and Th2 responses are there which change toward Th2 response predominance as the infection progresses, but in mice, Th1 response was significantly suppressed [55, 56]. In some studies, it was found that cathepsin L1 suppresses IFN-γ leading to decrease in Th1 response and increased Th2 response [17]. This suppressive effect of FhCL-1 was further confirmed in IL4 defective mice [57].

When *Fasciola* infection occurs, all stages of parasite liberate cathepsin L which quickly subverts the immune response of the host [58]. Prowse et al. [59] reported in vitro proliferation of sheep T lymphocyte and reduction of expression of CD4 in both ovine and human cells by *F. hepatica* cathepsin L (RFhCL-5). This was further confirmed by addition of E64 (a cysteine proteinase inhibitor). CD4 is a surface marker of 64 kDa found on T cells, and it acts as a co-receptor along with TCR: CD3 complex in antigen recognition by MHC-II. This is necessary for T helper cell activation. The co-receptor functions of CD4 increase the T cell signaling activity up to 10–100 folds so that the number of molecules required for T cell activation decreases. CD4 is required for T cell activation, and cathepsin cleaves the CD4 which helps in suppression of lymphocyte proliferation. In sheep, cathepsin L of *Fasciola* induces cleavage of CD4 to suppress lymphocyte

proliferation for evasion of host immune system. In *Paragonimus westermanni*, the cysteine proteases secreted from the gut are known to degrade Hb and collagen besides suppressing immune system and inducing immune tolerance.

4 Facilitatory Role in Parasite in situ Migration and Feeding Activities

The feeding and migration of the parasite are interdependent as the degraded host tissues during migration of the parasite act as substrate for parasite feeding. Similar to mammalian host, cathepsin B enzymes have optimal activity at pH range of 5–7. This pH range indicates that the enzyme has activity inside the gut of fluke as well as host tissue pH i.e., slight acidic to neutral pH. These enzymes show their activity over a wide pH range (4–8.5) and are stable at neutral pH. It is thought that those parasitic enzymes, which show optimal activity between pH 3–4.5, does digestion in the acidic environment of the gut of the parasite, while proteases which are active at physiological pH are responsible for the digestion of the blood cells/proteins, etc., and thus help in penetration. FhCL-2 cleaving of fibrinogen produces fibrin clot so as to prevent the access of immune effecter cells to the fluke surface [60]. Role of FhCL-1 and FhCL-2 in degrading extra cellular matrix and basement membrane to facilitate in situ migration of the parasite was demonstrated by Berasain et al. [61].

As cathepsin B-like proteases are expressed in the early stages of the liver fluke, these may have important role in excystation and invasion of the host tissues [35, 36, 62]. Cathepsin B enzymes also have a role in parasite homeostasis, and this was supported by a study using RNAi to knockdown FhCB-2 expression in immature flukes [63]. In the study, RNAi treatment of NEJs was done, and this led to 78% reduction in expression of FhCB-2, thus 50% reduction in ability to penetrate rat gut in vitro. Further, prolonged incubation of these flukes led to death of the flukes too [63]. These findings are suggestive of the role of FhCB-2 in gut penetration, tissue invasion and parasite homeostasis.

5 Cysteine Protease and Fecundity of Flukes

The Mehlis glands of *F. hepatica* secrete substances, steering the formation of the egg cells [64]. The secretion contain cathepsin L, influencing in situ maturation of egg shell components and other reproductive activities of the parasite [24]. Based on an experimental study in buffaloes immunized with the cysteine proteinase, it was postulated that cathepsin L induced immunity, targeted the mehlis glands and interfered with the egg shell synthesis. Obviously, the inhibitory effects of the enzyme influences fluke fecundity and posses epizootiological impact on disease transmission from molluscan to definative mammalian hosts.

6 Immunodiagnostic Target

For control of fasciolosis, immunodiagnosis is of utmost importance as the infection can be detected in early prepatent period i.e., before the pasture contamination. This early diagnosis helps timely treatment of the infection leading to prevention of contamination of water bodies. Immunodiagnostic tests of varying sensitivity and specificity developed earlier, had restricted application incidental to cross reactivity with other in situ metazoans. This necessitated the isolation, purification and identification of specific immunodominant molecules from the complex ES antigens of the fluke [66]. For early diagnosis of *Fasciola* infections, cathepsin L cysteine proteinase has been used to develop reliable test. A single epitope was used as an antigen in peptide-based indirect ELISA to detect antibodies against *F. hepatica* in cattle. In experimental studies, *F. hepatica* specific antibodies were first detected between third and fourth week post-infection in cattle and persisted till 183rd day post-infection in peptide ELISA. However, FhCL-3 ELISA was found superior to peptide ELISA in both natural as well as experimental studies. A diagnostic ELISA with RFhCL-3 was developed to detect antibodies against *F. gigantica* in sheep [67]. The 28 kDa recombinant protein was shown to be cross reactive and detected *F. gigantica* antibodies fifth week post-infection in sheep, while 28 kDa FgCL-3 detected antibodies as early as 4 weeks post-infection (wpi) by indirect ELISA, dipstick ELISA, and western blot [25]. Dixit et al. [21] detected *F. gigantica*-specific antibodies as early as 2–4 wpi i.e., earlier than the test using RFhCL3. This study revealed the possibilities and advantages of using homologous antigen i.e., FgCL3 in the diagnosis of experimental *F. gigantica* infection. Some experimental studies have also shown 100% sensitivity of FgCL3 ELISA in bubaline fasciolosis diagnosis [68], but in natural infections, this sensitivity declined to 97% with 100% specificity [30]. Early studies with FgCL3 using serum of goats and buffaloes experimentally infected with *Paramphistomum epiclitum* evidenced no cross reactivity and encouraged its use in specific diagnosis of fasciolosis [69].

Synthetic peptides similar to *F. hepatica* cathepsin L were developed and used in immunodiagnosis of tropical ovine fasciolosis. ELISA could not detect antibodies against different peptides up to 4 weeks of infection, but these peptide constructs were of use to detect low and high degree of infection of *F. gigantica* in sheep [70].

Purified 28 kDa FgCL3 (by ion exchange chromatography) was used for immunodiagnosis of experimental *F. gigantica* infected sheep and buffaloes to further confirm earlier reports [21, 68]. The same has also been evaluated for early prepatent detection of *F. gigantica* infection (4 wpi) in experimentally infected calves. In field studies also, FgCL3 was used to detect *F. gigantica* infection using ELISA [71].

6.1 Immunoprotection

6.1.1 Fasciolosis

Immunological control of fasciolosis is preferred over chemotherapy as it is comparatively cheaper, more efficient, and provides long-term cure. A number of molecules like cathepsin L, Glutathione-S-transferase (GST), Leucine aminopeptidase (LAP), and Fatty acid binding proteins (FABP) were used for inducing immunity against *Fasciola* spp. in animals. Among all, cysteine proteinase family cathepsin L enzymes were mainly tested and given good protection if used as vaccines. In one trial, only 10 mg of cathepsin L given on three occasions induced protective response [72]. Subsequently, cathepsin L at the dose of 200 mg was also protective [73]. Cattle vaccinated with *F. gigantica* cathepsin L induced very high antibody titer, but there was no effect on in situ migration and fecundity of the parasite. There was variation in the response depending upon the adjuvant used, and the best response was found when Fruend's complete adjuvant was used with cathepsin L, while with heme-binding protein antigen, only 73% protection could be achieved [20, 72].

Vaccination trials with a combination of FhCL1 and FhCL2 in cattle and sheep produced 53 and 30–60% protection, respectively, but when these two cathepsins were combined with *F. hepatica* LAP, the protection level increased to 79% in sheep [73, 74]. The morphology of the flukes recovered from vaccinated sheep was normal indicating that vaccination has no effect on development of fluke [64], but some workers observed a reduction in the size as well as in the fecundity of recovered flukes from buffaloes [65, 72]. Evidently, the immune response depends upon the immunogen used.

Despite all these efforts, a commercialized vaccine has not yet been developed which can give sufficient protection against fasciolosis. On the basis of these studies, it was concluded that the immune response to a vaccine depends upon route of administration, immunogen type, and adjuvant used. Besides this, the commercial vaccine production should be cost-effective to compete with the chemotherapy. Various studies involving different antigens and different routes of administration induced varying levels of protection. In rats, oral inclusion bodies containing RFhCL-1-like cysteine proteinase resulted in 70–80% protection, and the acquired immunity conferred by intramuscular injection of cDNA of the enzyme, was equally comparable [75, 76]. Intranasal vaccination with the cDNA of enzyme or intramuscular injection of the recombinant enzyme with FCA, resulted in lesser protection [77]. An antigen given without adjuvant in the form of inclusion bodies, was suitable for oral vaccination, as it is protected from being digested in the gut. The immunity conferred by the intranasal vaccination with inclusion bodies of RFhCL-1 was 54 and 56.5% in calves and lambs, respectively. Besides, the flukes developed in vaccinated calves had lower reproductive potential [78].

The recombinant FhCB2 proteinas vaccine in sheep resulted in high antibody titers up to 10^6 substantiating that FhCB2 is a leading vaccine candidate [35, 79] However, it is not very clear whether the antibody titers in sheep are efficient enough to interrupt the development of the in situ flukes. Jayaraj et al. [80] have

recently validated FhCB2 as vaccine for fasciolosis in rat model. Recombinant vaccines involving FhCB2 alone as well as multivalent vaccine with recombinant proteins of proteases expressed in adult as well as in metacercariae of liver fluke were evaluated. The strategy behind this trial is multivalent vaccination (using many proteases expressed at different stages of life cycle of the parasite) will protect the host throughout the migration period as well as the patent period of the parasite thus giving long-term protection. Vaccination with FhCB2, a juvenile protease, alone was found to be highly immunogenic. It reduced 60% fluke burden and 63% reduction in size of the recovered flukes, indicating that it inhibits the development of surviving flukes by affecting the feeding behavior or migration of the parasite. Significant reduction in liver damage (61%) by the challenge dose of flukes post FhCB2 vaccination indicated that killing of parasite has occurred [80].

Multivalent vaccination with FhCB2 in combination with FhCatL5 or FhCatL1g or with all three proteins resulted in 83% protection and 76% reduction in parasite size as well as 73% reduction in liver damage. Further, FhCB2 combined with FhCatL5 had given best results indicating that if vaccination is done targeting juvenile and adult proteases, it will give comparatively better protection. As juvenile and immature *F. hepatica* express six cathepsin B cDNA sequences (FhCB1–FhCB6), there is possibility that vaccination targeting these cathepsins can add better protection [38, 39].

Only a few DNA vaccines were targeted on cysteine protease of viral and bacterial infections. DNA vaccines encoding cathepsin L-like proteases of *F. hepatica* resulted in fluke load reduction in rats [58]. Likewise, in FhCB2 DNA vaccine, the immune response was enhanced as this vaccine construct produces FhCB2 protein. In addition, these vaccines will also produce a strong memory response and antibody titers in sheep. Immune responses following FhCB2 vaccine can be augmented by the use of CpG augmentation and CTLA4-mediated targeting [79].

6.1.2 Other Metazoan Diseases

Due to problem of anthelmintic resistance and drug residues, there is a need for effective anti-nematode vaccines [81]. Vaccine against cryptic antigen of blood-feeding helminths can be a good alternative as these antigens are usually not recognized by the host in natural infections. The molecules are being employed in the vaccines against ectoparasites of domestic animals [82, 83] and *Haemonchus* in sheep [84]. Some gut membrane protease like H-gal-GP [85] are hidden from host immune system during natural infection, but host antibodies can reach these hidden antigens upon ingestion of the host blood by the parasite. Thus, these antigens can be utilized for vaccination against obligate blood-feeder parasites. In sheep, protective immunity was developed following administration of H-gal-GP antigen, and the antibodies produced could be used for passive immunization [86]. *Whereas,* vaccination studies on haemonchosis revealed that studies on individual component of the complex antigen in recombinant form are essentially required. This would be the first recombinant protein vaccine against nematode infection(s) prevalent in field.

In *Ancylostoma* parasite of dogs, immunization with recombinant Ac-APR-1 vaccine has shown a reduction in adult parasite burden [87]. In the study, the adult worm burden in terminal part of intestine increased indicating that antibodies against Ac-APR-1 forced the adult *Ancylostoma* to shift to colon from small intestine. The possible inference drawn from the study was that the female worms being more voracious blood suckers shifted in large number to colon as these were more exposed to anti Ac-APR-1 immunoglobulin [87].

Experimentally, immunization in rats with *Clonorchis sinensis* cysteine protease (CsCp) produced 31.5% protection [88]. Cathepsin B-like cysteine proteases were also found immunogenic in *H. contortus* in sheep [43]. In another study on goats, these cysteine proreases of *Haemonchus* also provided protection [89]. Conclusively, proteases are of utmost importance in parasitic vaccine development strategies.

7 Conclusion and Future Perspective

Cysteine proteases are present in almost all life-forms including viruses. Parasitic cysteine protease enzymes are diverse, and being immunogenic in nature, play important role in the in situ survival of the parasites. These enzymes are being extensively exploited for developing efficient diagnostics and vaccines against the parasitic diseases of economic significance. The cysteine proteases modulate either Th1 or Th2 responses to protect the parasites inside the host. At the same time, being potent immunogens, induce acquired immunity in the host and confer effective protection against future infections. Undoubtedly, cysteine proteinases as vaccines are claimed to be a preferred choice over chemotherapeutic control of the parasitic disease in the field.

Due to the ubiquitous presence of the cysteine proteases in helminth parasites, these molecules represent intriguing targets for development of antiparasitic strategies for field application. Homologous family members occur in nearly every major parasitic organism, and in light of the fact that the catalytic mechanism is identical in each of these homologue, it is reasonable to infer that a relatively large inhibitor library would contain potential leads for a number of different parasitic diseases. This would provide a very cost-effective approach to new antiparasitic chemotherapy – a necessity while dealing with diseases that are endemic in economically poor regions of the world including the Indian subcontinent. Although cysteine proteinases proved as diagnostic markers in fluke infections especially fasciolosis, however further research is required to fully exploit these molecules as potential vaccine, as an integrated strategy for ongoing antiparasitic drug development programme worldover.

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Erratum to: Matrix Metalloproteinases in Parasitic Infections

Fabrizio Bruschi and Barbara Pinto

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The original version of the book was inadvertently published without updating the order of the reference list provided by author, which has been now corrected in chapter "Matrix Metalloproteinases in Parasitic Infections". The erratum chapter and the book have been updated with the change.

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