Sajal Chakraborti · Tapati Chakraborti
Naranjan S. Dhalla *Editors*

Proteases in Human Diseases

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This book ***Proteases in Human Disease*** *is dedicated to Prof. Asis Datta, who was born in 1944 at Taki, West Bengal, India. His individual and group achievements in the field of molecular biology and genetic engineering have been spectacular and have secured him unique position in several areas of frontier research. After getting Ph.D. (in 1968) and D.Sc. in Biochemistry from Calcutta University and spending several years in USA (1968–1973) as a postdoctoral research fellow and Assistant Virologist, he joined School of Life Sciences, Jawaharlal Nehru University (JNU) in 1973 and became Professor of Biochemistry and Molecular*

Biology (1978) and moved on to become its Rector (1993–1996) and later its Vice-Chancellor (1996–2002). In two spells (1976–1977 and 1980–1981), he worked with the Nobel Laureate Prof. Severo Ochoa at Roche Institute of Molecular Biology, USA. At JNU, Prof. Datta initiated research on gene expression and its regulation in eukaryotic cells. On completion of his tenure as Vice-Chancellor, Jawaharlal Nehru University, Prof. Datta assumed charge as Founder Director, National Institute for Plant Genome Research. In addition to professional assignments, Prof. Datta provided leadership in development programs of enormous importance to India in education, science, and technology.

Professor Datta had abandoned a promising career in the United States and returned to India to promote scientific research and to build institutions, which could take their place on par with any in the world. With his in-depth commitment to science and technology, Prof. Datta believes that scholarly endeavor does not exclude a continuing commitment to build a "better, safer and healthier world". Participating in the process of scientific discovery does not preclude a full engagement with courage and conviction in the pursuit of social goals and ideals.

Professor Asis Datta has researched and taught at many internationally acclaimed institutions. He has been making significant contributions in basic and applied Biochemistry and Molecular Biology for more than three decades with numerous scientific papers in reputed international journals and several patents. Indeed, he is the

first person who has patented genes from India. Professor Datta and his team linked New Biology with agriculture, medicine, science, industry, and society to make a global partner in biotechnology. Professor Asis Datta is known for his work on pathogenic yeast, Candida albicans *as a model system, which opened up the possibility of designing a therapy to combat candidiasis. In addition, the scientific/research contributions have been vital in areas of food/nutritional security and use of genetically modified food. His group is known for pioneering contributions in molecular biology and identification and manipulation of novel genes leading to the production of transgenics of high societal value. His unique achievement that ranges from isolation of novel genes to production of transgenics and their field release have all been carried out successfully by his group.Professor Datta received several prestigious awards such as Shanti Swaroop Bhatnagar Award (1980), Guha Memorial Award (1988); Sir Amulya Rattan Oration Award (1988); First G.D. Birla Award for Science and Technology (1991); Dr. Nitya Anand Endowment Award, INSA (1993), The Federation of Indian Chamber of Commerce and Industry Award for R&D in Life Sciences (1994); Om Bhasin Award for Science and Technology (1995), Third World Academy of Sciences Awards (TWAS) in Biology (1996), Goyal Prize in Life Sciences (1996); Ranbaxy Award in Medical Sciences (1996); Indira Gandhi Priyadarshini Award (2000); R.D. Birla Award for Bio-Medical (2001), Dr. B.R. Ambedkar Centenary Award for Excellence in*

Biomedical Research, ICMR, Govt. of India (2003), Bashambar Nath Chopra Lecture Award (2004). Government of India honored Prof. Asis Datta with Padma Shri (1999) and Padma Bhushan (2008). Professor Datta was the president of the National Academy of Sciences (India) (2009–2011). He received life time achievement award by Biotech Research Society (2011), Priyadarshini Gold Medal Award for Outstanding Achievements (2011), G.M. Modi Award for Innovative Science and Technology (2011), Bharat Ratna Mother Teresa Gold Medal Award (2014), Pandit Jawaharlal Nehru Gold Medal Award (2014). He is a Fellow of Third World Academy of Sciences, Indian National Science Academy, Indian Academy of Science and National Science Academy. Several Universities awarded Doctor of Science (Honoris Causa) to Prof. Datta for his wide-ranging contributions. Professor Datta was the General President of Indian Science Congress Association for 2003–2004. He was awarded Asutosh Mookerjee Medal Award at the Ninety-two Session of Indian Science Congress, January 2005 and honored Life Time Achievement Award for the year 2005–2006 in the Ninety-Three Session of Indian Science Congress, January 2006. His relentless effort throughout has established a vibrant school of research on "structure-function-application" of eukaryotic genes, which led to the establishment of the National Institute Plant Genome Research, India's first and only one research center of its kind.

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

Preface

From dawn till dusk I sit here before my door and I know the happy moment will arrive of a sudden.
When I will surely see… In the meanwhile the air is filling with the perfume of promise.

Rabindranath Tagore (Gitanjali: Song of offerings)

Proteases, for a long time have received the attention of biochemists as degrading enzymes. The first scientific documented research was published by P.A. Levine entitled "The Cleavage Products of Proteoses" in the first issue of the *Journal of Biological Chemistry* (October 1, 1905). Now, after about 110 years and with more than 468,000 entries in MEDLINE database, proteases remain one of the focused topics of current research. Approximately 2% of the genes in most organisms are transcribed for proteases, which is second in number only to transcription factors. Proteases are involved in virtually every physiological and pathological process. Thus, proteases are considered as one of the important targets in drug development.

Proteases have likely arisen at the earliest stage of protein evolution as simple destructive enzymes necessary for protein catabolism and generation of amino acids in primitive organisms. For many years, studies on proteases have been focused mainly on their roles associated with protein degradation. However, it has now become clear that their role is not limited to breaking down of proteins and have multiple functions: it modulates enzymes activity, regulates membrane function, alters receptor channel properties, affects transcription, cell cycle, and reproduction, forms active peptides and many more than we had assumed. The hydrolysis of peptide bonds often involves multiple steps, for example, ubiquitination often requires the activity of enzyme complexes. Their activation, modification, and inactivation, therefore, play important roles in biological functions. Inhibitors of many of the proteases have now been well characterized and they attract much importance because of their great therapeutic potential in a number of diseases and some of which are already in clinical use.

Proteolytic enzymes, which are known to play important roles in numerous cellular and extracellular processes in health and diseases, are characterized. In addition to new discoveries of proteolytic principles in a variety of biological systems, proteases have gained importance as laboratory tools in the experimental

investigation of peptides and proteins. An outstandingly large number of publications on recent developments led to the recognition of the serine, cysteine (especially calpains and caspases), metallo-, threonine, glutamic, and aspartic proteases in different diseases such as allergy, asthma, atherosclerosis, cancer, AIDS, Alzheimer's and Parkinson's diseases, and also parasite-mediated diseases such as malaria and leishmaniasis. Substantial data have been accumulated implicating the role of proteases in numerous types of cancers including cancer of the breast, the gastrointestinal and urological tract, the lungs, and the brain. A strong and substantial clinical relation exists between matrix metalloproteases (MMPs) and cancer, and they are widely studied to predict disease progression among cancer patients. There is substantial hope that substances designed to affect MMPs or turn off the MMPs activation may prove useful for treatment against a variety of cancers, which could open new avenues in cancer therapy.

In this book, we have presented 24 state-of-the-art articles. Several contributors in this book illuminate our understanding on the role of different proteases with their findings, interests, and problems. We are confident that researchers' especially young scholars will benefit out of that. Some of these articles emphasize existing concepts while some shed light on newer ideas. In fact, these are the flowers of the spring. So, "let a hundred flowers blossom and a hundred schools of thought contend."

We like to extend our sincere thanks to all the authors for their excellent writing skills covered with current information that make every article a pleasure to read. We are thankful to Dr. Naren Agarwal, Dr. Madhurima Kahali, Dr. Nidhi Chandhoke, and Dr. Praveen Kumar (Biomedical Division, Springer, New Delhi) for the time and energy they spent for achieving our goal. We are also thankful to Prof. Sankar Kumar Ghosh, Vice Chancellor, University of Kalyani for his encouragement.

Kalyani, India — Sajal Chakraborti
Kalyani, India — Tapati Chakraborti
Winnipeg, Canada — Naranjan S. Dhalla

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About the Editors

Dr. Sajal Chakraborti is Professor of Biochemistry at the University of Kalyani, West Bengal, India. His research covers the role of proteases in regulating pulmonary vascular tone under oxidant and calcium signaling phenomena. He has been engaged in teaching and research in biochemistry for the past 40 years.

Dr. Tapati Chakraborti is Professor of Biochemistry at the University of Kalyani, West Bengal, India. Her research covers role of proteases in the pathophysiology of leishmaniasis. She is also doing research on the role of proteases in regulating pulmonary vascular tone. She has been engaged in teaching and research in biochemistry for the past 30 years.

Dr. Naranjan S. Dhalla is Distinguished Professor at the University of Manitoba, Winnnipeg, 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
Involvement of Proteases in Various Diseases

Matrix Metalloproteinases in Breast Carcinoma

Immunohistology and Prognosis

1

Irena Ranogajec

Abstract

Since breast carcinoma is a group of heterogeneous tumors, the prognosis for each of this is determined in terms of a whole range of clinicopathological factors which can be divided into traditional (tumor size, lymph node status, histological grade, hormone receptors, proliferation index) and new factors, most of which are still being researched. This chapter shows the immunohistology and the prognostic value of the analysed gelatinases (MMP-2 and MMP-9) in breast carcinoma patients. Their expression in breast carcinoma patients is an unfavorable prognostic indicator of the disease, and an indicator of the need for more aggressive treatment in patients with negative lymph nodes. In the future the inhibition of these proteins could play a role in preventing breast carcinoma and in stopping the development of metastases in already existing breast carcinoma. Therefore, there is need for the incorporation of new prognostic factors into future studies and clinical trials that will provide new approach for the breast carcinoma patients.

Keywords

Matrix metalloproteinases · Breast carcinoma · Prognosis · Immunohistology · MMP-2 · MMP-9

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1 Introduction

Breast carcinoma is the most frequent malignant tumor in women and it accounts for 27% of all malignant diseases affecting women, whereas it is rare in men. The highest incidence of breast carcinoma has been reported in developed countries, Northern America and Western Europe, and the lowest in Africa and Asia. However, growth of the number of newly detected breast carcinoma patients has recently been observed insofar lower risk countries (China, India, Japan) [1–3]. According to the WHO International Agency for Research on Carcinoma, breast carcinoma is the most frequent carcinoma in women in developed and less developed countries, and its incidence increases at the rate of 10%. In Europe the breast is the most frequent carcinoma site in women; breast carcinoma accounts for 25.5% of all deaths due to newly detected carcinoma sites in women, and for 17.5% of all deaths due to carcinoma in women. The carcinoma risk is 60% higher in Western than in Eastern Europe [4].

Breast carcinoma is also an important health problem for Croatia's female population. It accounts for 22% of new cancer cases, and in terms of incidence and mortality it is the most frequent form of carcinoma in women. According to the records of the National Carcinoma Register, breast carcinoma is diagnosed in about 2,300 women a year, and about 800–900 women die. The highest percentage of deaths due to breast carcinoma and the cancer in general has been reported in the 40–59-year group. Both the incidence and the mortality due to breast carcinoma in Croatia are on the increase, breast carcinoma being the most frequent single cause of death in women aged 35–59 years [5, 6].

In most cases surgical treatment (tumorectomy, mastectomy, dissection of axillary lymph nodes) is just the beginning of therapy, followed by adjuvant chemotherapy, postoperative radiation and, in some cases, adjuvant hormone therapy. Patients with an expression of the receptor of epidermal growth factor 2 (HER-2/neu) in tumor cells are also treated by an antibody blocking the receptor for this factor. According to the currently generally accepted opinion, the majority of women with negative lymph nodes and tumors up to 1–2 cm in size are healed by local therapy only in more than 70% of cases and do not benefit from chemotherapy. Metastases will develop in 20–30% of women in this group, and recent research is focused on detecting patient subgroups that would benefit most from adjuvant therapy [7]. Since breast carcinoma is a group of heterogeneous tumors, the prognosis for each is determined in terms of a whole range of clinical and pathological factors which can be divided into traditional and new factors, most of which are still being researched. The probability of recurrence and the length of survival depend on the stage of the disease (tumor size and histological grade, lymph node involvement, total positive node count), hormone receptor status, proliferation of malignant cell activity, oncogene expression or amplification (HER-2) and general condition of the patient. Since recurrence and repeated surgery can be expected in 25–30% of patients with breast carcinoma and negative lymph nodes, increasing attention is being focused on the discovery of new prognostic

markers which might help this group of patients by providing an additional future therapeutic approach [1, 2]. Matrix metalloproteinases have recently been studied intensively as possible prognostic factors in breast cancer patients. The numerous recent studies suggest their role in carcinogenesis and especially in the dissemination of breast carcinoma [8–15].

Matrix metalloproteinases (matrixins) belong to the family of zinc-dependent structural endoproteinases. In physiological conditions their role is essential in the degradation of the extracellular matrix during development, in the angiogenesis process, ovulation, involution of glandular breast tissue, and wound healing [16]. In 1962 Gross and Lapiere showed that skin cells from metamorphosing tadpole tails, when cultivated on collagen gel, released an enzyme which can degrade the triple collagen helix at neutral pH and 27 °C. The same study described a similar activity in cultures of embryonic skin, post-partial uterus and bone cells [17]. After that first discovery, collagenases were demonstrated in human tissue (MMP-1) and neutrophils (MMP-8). Over time other MMPs were discovered, starting with gelatinase A (MMP-2) and stromelysin (MMP-3), all the way to the identification of enamalysin (MMP-20) [16]. So far more than 20 different human metalloproteinases are known, and they are divided into four basic groups. The first group are collagenases which are involved in the remodeling and degradation of collagen; the second group are gelatinases (or type IV collagenases) which degrade gel and collagen type IV. The third group are stromelysins and matrilisins which degrade various extracellular matrix substances, including proteoglycanes and the non-collagen protein substance. The fourth group are membrane-type metalloproteinases which catalyze various ECM substrates and act as fibrinolytic enzymoplasmin. Proteinase inhibitors stimulating cellular proliferation and inhibiting cellular apoptosis have also been reported [8, 9]. All the metalloproteinases have some common features: each degrades at least one component of the basement membrane; they are active at physiological pH status; they need two zinc molecules for their activity; they are inhibited by metal chelases or tissue metalloproteinase inhibitors. Different physiological substances can inhibit MMPs; such as α2-macroglobulin molecules are larger molecules serum proteins which have an inhibitory effect but cannot cross into tissues. TIMP are smaller molecules displaying expression in different tissues and fluids; TIMP-1, TIMP-2, TIMP-3, and TIMP-4 are known so far. The amino-terminal domain present in all TIMPs is responsible for inhibitory activity. TIMPs form non-covalent complexes with all active MMPs in a 1:1 ratio. The balance between proteases and inhibitors is the key factor determining proteolytic activity. Moreover, MMPs are synthesized in the inactive zymogen zone and require additional extracellular activation. These proenzymes remain inactive owing to interaction between cysteine in the proregion and zinc ions in the active site; the interaction blocks transition into the active form, and this interruption leads to enzyme activation. Trypsin 2, cathepsin, elastases, and plasmin/the plasminogenic system are factors which can lead to transition into the active form and once activated, MMPs can activate the others; MMP-3 can activate MMP-2, MMP-9, and MMP-7. Gelatinases, MMP-2 and MMP-9 can activate one another. The recently discovered MT-MMP subgroup can activate proMMP-2

through the transmembrane domain which is the most important in the process of MMP-2 activation [8, 10]. All latent MMPs comprise at least three domains: 1. the hydrophobic prepeptide domain required for signal secretion; 2. the amino-terminal propeptide domain removed by activation; 3. the catalytic domain containing zinc. This basic structure is present in all matrix metalloproteinases [8, 10].

2 Matrix Metalloproteinases in Breast Carcinoma

The role of metalloproteinases in the carcinogenesis of breast cancer cells is related to tumor initiation and growth, primarily by promotion of angiogenesis in the tumor, and activation of stimulating growth factors. The activation of growth factors and their receptors, and degradation of their inhibitors, is another significant mechanism of their activity [9, 10, 12]. Tumor cell metalloproteinases are probably responsible for invasive tumor growth, while stromal elements mainly influence the remodeling process and the desmoplastic reaction of the tumor [18]. Angiogenesis is stimulated by degradation of the barrier which permits endothelial invasion, and by the release of factors promoting the angiogenic phenotype. Neoangiogenesis is a key moment in the stimulation of tumor growth and development of metastases. Angiogenesis develops in several steps, including the release of angiogenic factors, release of proteolytic enzymes, migration within the extracellular matrix, EC proliferation, and formation and differentiation of microvascular spaces. Proteases are required for the invasion of extracellular space of malignant cells; metastatic cells use proteases to cross the basement membranes, connective tissue and, after that, the basement membranes of small blood vessels and lymph vessels. Type IV collagen is the main structural protein of the basement membrane and extracellular matrix. Studies have shown a correlation between the enhanced expression of MMP-2 and MMP-9 and the occurrence of metastases. It has also been demonstrated that the enhanced expression of MMP in the tumor is correlated with its higher aggressiveness and metastasising capacity [10]. MMP-2, but not MMP-9, releases the ectodomain of the FCG receptor. Since the hydrolysed ectodomain retains the capacity to bind FCG, it can modulate FCG mitogenic and angiogenic activities. Stromelsyn-3 can affect the promotion of MCF-7 cell growth by releasing the extracellular matrix growth factor [13]. The field of carcinoma cell invasion and metastasizing and inhibition by MMP inhibitors has also been studied so far in carcinomas of the pancreas, head and neck, glioma and other CNS tumors, and gastric carcinoma and melanoma. Pathological processes related to MMP activity, the subject of continuous research, include tissue destruction in fibrotic diseases and the weakening of the extracellular matrix in arthritis, oral pathology and periodontal diseases, liver and kidney fibrosis, endometriosis, and aortal aneurysm and heart failure due to the weakening of the extracellular matrix [16]. In situ hybridization techniques have shown metalloproteinases to display expression in tumor cells, and in stromal and inflammatory cells stimulated by factors released by tumor cells [18].

2.1 Matrix metalloproteinase-2 (MMP-2) belongs to the gelatinase subgroup (type IV collagenases) and is also called gelatinase A, 72-kD gelatinase; type IV collagenase is involved in the degradation of the basement membrane, elastase, and denatured type I, II, and III collagen [8, 16, 19]. The MMP-2 gene is located in chromosome 16q13, it is 17 kb long with 13 exons 110–901 bp in size and 12 introns 175–4.350 bp in size. 1–4 and 8–12 introns of type IV collagenase coincide with intron localizations in genes for MMP-1 and MMP-3, indicating also the close structural correlation of these genes with metalloproteinases. Unlike the stromelysin genes, there is no TATA box in the promotor region, but there are two GC boxes. There is no CAAT box, but the potential binding site to the transcription factor AP-2 is on the first exon [20]. In 1972 Harris and Kane studied gelatinase activity in rheumatoid sinovial tissue, probably related to MMP-2 activity. In 1978 Sellers et al. separated gelatinase activity from the action of collagenase 1 and stromelysin 1 in a hare cell culture. Collier described the chemical structure of gelatinase which consists of a triple repeat domain of type II fibronectin type introduced into the catalytic domain [16]. MMP-2 is secerned in the latent, proenzyme form, and activated through proteolytic modification by the activator MMP-2, i.e., the membrane type 1 (MT1-MMP), the enhanced activity of which in malignant breast tumors has so far been discussed in several studies [21–23]. ProMMP-2 activation does not depend on uPA/plasmin activity, but it can be activated by thrombin, plasmin, MMP-1 and MMP-7. Nevertheless, activation under the influence of MT-MMP on the surface of the cell appears to be physiologically the most acceptable [16]. ProMMP-2 forms a complex with TIMP-2, and this complex binds on the surface of the cell with MT1-MMP and, by releasing TIMP-2, MT1-MMP then activates proMMP-2. The activation of proMMP-2 through MT2-MMPa is direct and does not depend in TIMP-2. MMP-2 is suppressed by inhibitor TIMP-2 [24]. This inhibitor is related to a reduced metastatic potential and invasion; it is a nonglycosyling 21 kDa protein [10]. Although MMP-2 is referred to as gelatinase, this enzyme can attack the native form of collagen type I, and elastin and other proteins [16].

2.2 Matrix metalloproteinase-9 (MMP-9) is also known as gelatinase B, and is part of the gelatinase group. Like MMP-2 it degrades gel (denatured collagen), elastin, laminin, collagen type IV, V, XI and XVI, but not interstitial collagen. It also activates growth factors such as TGFb and pro TNF [8, 25–27]. Sopata et al. isolated it from human neutrophil granulocytes. Gelatinase B is the largest metalloproteinase and weighs 92 kDa. It is made up of three fibronectin domains and a similar collagen V domain. TIMP-1 binds to proMMP-9 creating a complex which regulates activation into MMP-9. Latent MMP-9 activators can include tripsin, as the most efficient, plasmin, cathepsin G, chimotripsin, tissue kallikrein, elastase and proteinase. ProMMP-9 can be activated by a cascade reaction through an intermediate link by the influence of other metalloproteinases: MMP-1, MMP-2, MMP-3, MMP-7, MMP-10 and MMP-13 [8]. So far MMP-9 expression in tumor cells and in the stroma of breast carcinoma has been studied by immunohistochemical methods or in situ hybridisation techniques [28–31]. It has also been

demonstrated that an MMP-9 promoter with a T allele displays a significantly high transcription activity as compared with the C allele. Accordingly, genetic polymorphism is also important for gene expression and, thereby, MMP-9 activity, ultimately enhancing the risk of tumor invasion and disease progression in patients with such a CT/TT genotype of the MMP-9 gene [32]. It has been demonstrated that fibroblasts, i.e., the stromal cells of breast carcinoma, produce MMP-9, with the involvement of TNF-alpha and TGF-beta inductors produced by breast carcinoma cells [33]. Fibroblasts can also be active in regulating the invasion of breast carcinoma cells through the production of thrombospondin-1 in fibroblasts which in turn induces MMP-9 production [34]. FGF also induces MMP-9 expression, as demonstrated by cell lines in vitro; this is related to the enhanced activity of NF-kappa B and AP-1 [35]. Because of the degradation of type IV collagen, which is particularly abundant in the basement membrane, MMP-2 and MMP-9 are most frequently and most strongly involved in tumor initiation, tumor growth and metastasizing, especially in breast carcinoma. In this regard another important is the promotion of angiogenesis by MMP-2 and MMP-9 which includes the degradation of the basement membrane of vascular, interstitial spaces, and the release of VEGF which is an angiogenic factor [22].

3 Immunohistology and Prognosis

Gelatinases and their tissue inhibitors can be valuable diagnostic and prognostic markers in breast carcinoma patients. On the basis of current and future studies by various authors they could eventually be incorporated into a standard prognostic group for breast carcinoma patients. In view of the foregoing, quite a few studies focused on the influence and application of metalloproteinase inhibitors and the future therapeutic potential of these substances. Numerous studies have shown a correlation of metalloproteinases with the initiation and progression of tumor growth [10, 12, 24].

The expression of MMP-2 in different tissues has been analysed immunohistochemically and by in situ hybridisation. MMP-2 is an enzyme present in normal tissue, most strongly in stromal elements. In breast carcinoma the very tumor cells modulate the level of MMP-2 by MT1-MMPa and TIMPa action. Since both tumor cells and the surrounding stroma in tumorous tissue display MMP-2 expression, a possible explanation is that tumor cell MMP influences invasive growth, while stromal elements influence the remodeling process and the desmoplastic reactions around the tumor cells [10]. In vivo studies have also confirmed the claim that stromal cells, i.e., fibroblasts, in most carcinomas play an important role in MMP-2 production. Along with carcinoma cells, fibroblasts are also probably stimulated to produce the higher MMP-2 levels observed in malignant tumors [12]. So far numerous studies have demonstrated the positive correlation between enhanced MMP-2 activity and tumor invasiveness and metastatic capacity [10, 18, 24, 25, 28, 29, 36–38]. In breast carcinoma cells enhanced MMP-2 expression was observed

compared with expression in benign breast lesions, and in vitro studies have also shown that active MMP-2 expression is linked with the more aggressive malignant cell potential in breast carcinoma cell lines [24]. The level of pro-MMP2/MMP-2 affects the determination of cell invasive and metastatic capacity, and the higher MT-MMPa level influences MMP-2 activation in carcinoma of the breast, cervix, and lungs by promoting invasiveness and metastatic capacity [10].

The expression of MMP-9 is significantly higher in malignant breast carcinoma cells than in benign lesions. As observed, MMP-9 expression is the highest in the most aggressive invasive ductal carcinoma (NOS) of the breast, while the value of the TIMP-1 inhibitor is the lowest. These results show that the MMP-9/TIMP-1 imbalance can influence the development and growth of ductal carcinoma (NOS), and indicated the correlation between MMP-9/TIMP-1 and tumor size [22]. It has been demonstrated that the high expression of MMP-9, especially in stromal breast carcinoma cells, is related to the less differentiated ductal type tumors with a poorer prognosis of survival and with the shorter recurrence time in breast carcinoma patients [25]. Recent studies have also demonstrated the presence of higher MMP-9 expression in cells of lobular carcinoma in situ, both immunohistochemically and by the RT-PCR method; this makes MMP-9 an interesting therapeutic and chemoprotective agent in the future treatment of lobular neoplasias [39].

In view of the foregoing considerations quite a few studies have considered the experimental models of cell lines in examining the influence and application of metalloproteinase inhibitors and the future therapeutic potential of these substances. Numerous studies have demonstrated the correlation between metalloproteinases and tumor growth initiation and progression [10, 12, 24]. Thus, the integration of their inhibitors is linked with the prevention of carcinogenic growth and the inhibition of invasion and metastasizing [10].

3.1 Our study of matrix metalloproteinases in breast carcinoma hypothesized that the enhanced expression of gelatinases (MMP-2, MMP-9) in tumor and stromal cells in invasive breast carcinoma patients ought to be an unfavorable prognostic factor, and suggested the need for more aggressive treatment in patients with negative lymph nodes and HER-2 protein expression [40, 41]. The median age of breast carcinoma patients in this study was 56 years and the majority of them was postmenopausal, had a tumor less than 2 cm, ductal histological type, estrogen positive, intermediate histological grade, with a low proliferative index. Lymph node metastases were found in 36.9% of patients and HER-2 expression in 15.9% of our study of matrix metalloproteinases. In 48.1% of patients more than 100 tumor newly formed vessels/mm^2 were found (Table 1) [40, 41].

3.2 The expression of MMP-2 and MMP-9 in tumor cells, in our study, was evaluated by the semiquantitative method as cytoplasmic and membrane staining. The expression of MMP-2 and MMP-9 in tumor and stromal cells was compared with standard clinicopathological prognostic factors and patient survival. We showed MMP-2 and MMP-9 expression in cytoplasm and membrane of the tumor cells and also positivity of stromal cells of the tumor in breast carcinoma patients (Fig. 1 and 2) [40, 41].

Table 1 Overview of studied clinicopathological factors in 138 breast carcinoma patients [41]

Factor	Number of patients	%
Age		
<50 years	49	35.5
>50 years	89	64.5
Tumor size		
<2 cm	74	53.6
>2 cm <5 cm	57	41.3
>5 cm	7	5.1
Histological tumor type		
Ductal	81	58.7
Lobular	30	21.7
Other	27	19.6
Tumor grade		
1	21	15.2
2	73	52.8
3	44	32.0
Vascular invasion		
Negative	134	97.1
Positive	4	2.9
New blood vessels/square mm		
<50	19	14.5
>50 < 100	49	37.4
>100	63	48.1
Ki-67		
<10%	81	60.9
>10%	52	39.1
Lymph nodes		
Negative	60	43.5
Positive	51	37.0
Unknown	27	19.5
Estrogen receptors		
Negative	43	42.6
Positive	58	57.4
Progesteron receptors		
Negative	56	55.4
Positive	45	44.6
HER-2		
Negative	116	84.1
Positive	22	15.9

In 2004 Pellikainen et al. also demonstrated MMP-2 and MMP-9 expression in the cytoplasm of tumor and stromal cells. Positive MMP-2 stromal cells (46%) were associated with strong HER-2 expression in the group of patients with negative

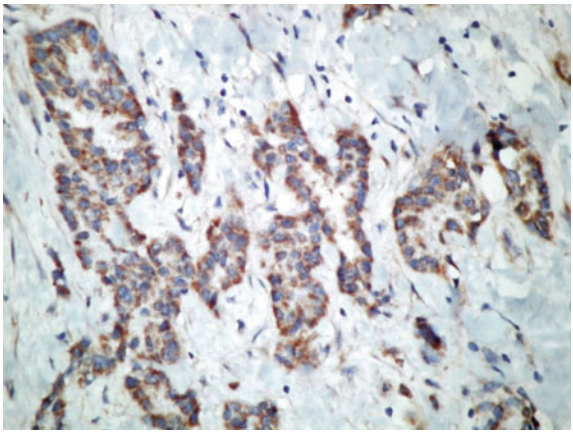

Fig. 1 Membrane and cytoplasmic MMP-2 positivity of tumor cells. (MMP-2 × 40) [41]

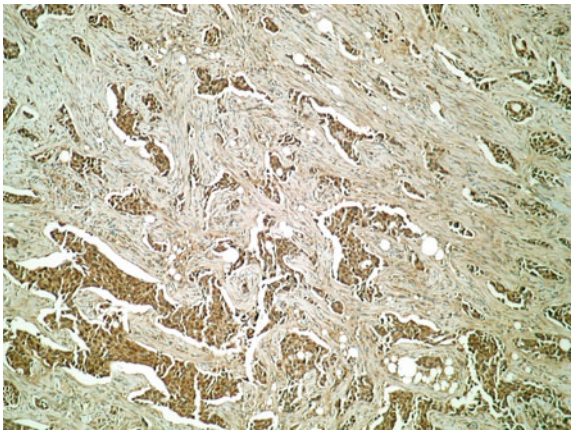

Fig. 2 MMP-9 positivity of tumor and stromal cells. (MMP-9 × 10)

lymph nodes, and this expression is correlated with more aggressive factors. The strong MMP-2 expression in tumor cells was associated with strong stromal expression to a statistically significant degree ($p = 0.009$). Stromal MMP-9 expression was observed in 38% of cases and was correlated, to a statistically significant degree, with HER-2 expression in hormone positive tumors. MMP-2 expression in tumor cells was not correlated with HER-2 expression [25]. In 1999 Jones et al. demonstrated MMP-2 expression in more than 90% of studied breast carcinoma patients, mainly in terms of cytoplasmatic staining; membrane expression was noted in 34% of patients. MMP-9 expression was seen in 68 of patients as tumor cell or stroma staining [29]. In 2002 Singer et al. confirmed the importance of cell–cell interaction and demonstrated, on an in vitro fibroblast and tumor cell culture, enhanced expression and activity of both gelatinases, MMP-2 and MMP-9. They thereby substantiated the claims regarding the importance of stroma in tumor progression through the release of angiogenic substances, cytokines affecting cellular growth, and protein degrading enzymes such as PDGF, EFG, FGF, IL-1,

TNFα, and EMMPRIN [42]. In the opinion of some authors—since both the tumor cells and the surrounding stroma in tumor tissue display MMP-2 expression—the possible explanation might be that tumor cell MMP affects invasive growth, while stromal elements influence the remodeling process and desmoplastic reaction round the tumor cells. In vivo studies also corroborate the claim that, in most carcinoma cases, stromal cells, i.e., fibroblasts, play an important role in MMP-2 production. Along with carcinoma cells, fibroblasts are also probably stimulated to produce the higher MMP-2 levels noted in malignant tumors [12]. In 2006 Tetu et al. demonstrated stromal MMP-2 expression in about 50% of breast carcinoma patients, whereas tumor cells were not positive as shown by the results obtained by in situ hybridisation. The same author has demonstrated that breast cancer cells produce factors (TGF, PDGF, EMMPRIN) inducing stromal cells to produce proteases which in their turn stimulate tumor cells by binding to receptors. These results support the hypothesis that biological behavior of the tumor does not depend only on its characteristics but also on its stroma. Moreover, this finding conceals possible therapeutic potential since reactive stromal cells display greater genetic stability than carcinoma cells and are hence theoretically less subject to mutation and resistance to therapy [24].

3.3 Our study presented the correlation of tumor and stromal MMP-2 and MMP-9 expression with other prognostic factors in breast carcinoma patients (Table 2). The results have shown a correlation between the studied factors and MMP-2 expression in tumor cells, but the correlation is not statistically significant ($p > 0.05$). However, stromal MMP-2 expression showed a statistically significant difference with regard to neoangiogenesis and tumor size, meaning that a stronger MMP-2 stromal expression also denotes stronger neoangiogenesis and greater probability of >5 cm tumors [40, 41].

In 2006 Liu et al. demonstrated the statistically significant correlation between MMP-2 expression and tumor size, tumor grade and metastasis development, concluded that MMP-2 expression can reflect the possible invasiveness of breast cancer and that, therefore, different selective MMP inhibitors could eventually be used as potential anti-metastatic drugs taking tumor size into account [43]. A group of Chinese authors, Peihong et al., showed in 2007 a strong MMP-2 expression in DCIS, and thereby demonstrated MMP-2 correlation with tumor invasion, i.e., more aggressive biological tumor potential, and suggested the possible use of MMP-2 expression as an early prognostic factor of invasiveness [44]. On the other hand, another group of authors, Kim et al., failed in 2006 to demonstrate a statistically significant difference in MMP-2 between DCIS and DIC [30]. In 2007 Ogura et al. demonstrated the statistical significance of MMP-2 expression in patients with T1 N0 grade breast carcinoma and recurrence within 10 years, and thereby suggested the possible use of MMP-2 as a promising predictor of recurrence risk in patients with earlier breast cancer stages [45].

The tumor cell MMP-9 expression in our study demonstrated a statistically significant correlation with the histological tumor type (ductal and other tumor types) ($p < 0.05$, data not shown), hormone status ($p = 0.02$), and a marginal

Table 2 Correlation of MMP-2 and MMP-9 expression with clinicopathological factors [40]

Prognostic factors	MMP-2 tumor cells *p*	MMP-2 stromal cells *p*	MMP-9 tumor cells *p*	MMP-9 stromal cells *p*	MMP-2/MMP-9 tumor cells *p*	MMP-2/MMP-9 stromal cells *p*
Age	0.28	0.32	0.06	0.53	0.55	0.35
Tumor type	0.63	0.52	0.06	0.08	0.50	0.51
Tumor grade	0.86	0.97	0.25	0.67	0.87	0.73
Vascular invasion	0.64	0.26	0.51	0.45	0.64	0.27
Estrogen receptors	0.25	0.47	**0.02***	0.44	0.46	0.53
Progesteron receptors	0.42	0.57	**0.02***	0.43	0.31	0.37
Lymph node	0.99	0.24	0.99	0.96	0.83	0.41
HER-2	0.47	0.28	0.08	0.41	0.52	0.49
Ki-67	0.40	0.14	0.12	0.12	0.39	0.06
Neoangiogenesis	0.28	**0.04***	0.78	0.74	0.13	0.67
Tumor size	0.31	**0.01***	0.94	0.46	0.39	**0.01***

*Significantly different

significance for HER-2 expression ($p = 0.08$) and patient age ($p = 0.06$). This means that there is a statistically significant correlation between higher MMP-9 expression in tumor cells and positive estrogen and progesterone receptors, ductal and other breast carcinoma types, HER-2 expression, and postmenopausal patient status [40, 41].

In 2006 Jinga et al. compared gelatinase activity and expression with prognostic factors in breast carcinoma patients (tumor stage, histological type and grade, tumor size, nodal status, and NPI). Because of the small number of patients in the studied groups they failed to demonstrate statistically the correlation between MMP and the tumor stage and histological grade: a weak positive correlation was found between tumor size and MMP-9, while correlation between MMP-9 expression and histological tumor type was statistically significant. Thus, in invasive breast carcinoma NOS MMP-9 expression was the strongest, and TIMP-1 value the lowest. Positive correlation was found between MMP-2 expression and lymph node status; enhanced MMP-2 expression and reduced TIMP-2 expression were found in patients with tumor cell invasion into lymph nodes. A positive correlation was found between MMP-2 expression and estrogen receptor status in breast tumors; in tumors with a stronger ER expression a stronger MMP-2 activity was observed. Estradiol is deemed to stimulate, through estrogen receptors, the signal transduction cascade leading to gelatinase (MMP-2 and MMP-9) activation. Gelatinases can be considered to be valuable diagnostic and prognostic markers in breast carcinoma patients, and in the future they can be incorporated into the prognostic factor group [22]. In 2007 Nilsson et al. demonstrated that estradiol and tamoxifen regulate

MMP-2 and MMP-9 in hormone positive breast carcinoma. During tamoxifen therapy MMP-2 and MMP-9 activity is enhanced by MMP modulation through anti-angiogenic fragments [46]. In 2005 Di et al. demonstrated in their in vitro study that gelatinase expression can be stimulated by estrogen in hormone dependent breast carcinomas which have positive estrogen as well as progesterone receptors. Thus, during therapy the blocking of estrogen or aromatase by aromatase inhibitors can reduce tumor growth and the tumor metastatic potential [31].

In our results the stromal expression of MMP-9 was correlated, to a statistically significant extent, with lobular breast carcinoma ($p = 0.02$, data not shown), whereas for ductal carcinoma the statistical significance was marginal ($p = 0.08$) [40, 41]. A similar result was confirmed by Jones et al. by demonstrating a statistically significant correlation between MMP-9 and the histological tumor type; in invasive lobular carcinoma, unlike the invasive ductal type, a more homogeneous cytoplasmatic MMP-9 expression was observed [29]. In 2008 Dengfeng et al. correlated MMP-9 expression with lobular carcinoma in situ. Accordingly, activated MMP-9 is believed to be involved in the formation of the typical *indian file* histological picture of lobular carcinoma; similarly, MMP-9 RNA and protein are believed to act as precursors of (LCIS) stage invasive lobular carcinoma, and factors activating it can trigger the development of the invasive disease. This makes MMP-9 an interesting therapeutic and chemopreventive target for patients with lobular invasion of the infiltrating or non-infiltrating type [39]. Similarly, Pellikainen correlates stromal MMP-9 expression with poor tumor differentiation, hormone-negative tumors, and ductal carcinoma [25]. In 2004 Rahko et al. demonstrated MMP-9 expression in 61.3% of patients with breast cancer. With MMP-9 expression, 5-year DFS amounted to 37%, as compared with 63% of patients with negative MMP-9 in the subgroup of hormone negative tumors. The study did not confirm the correlation of MMP-9 expression with clinical stage, histological prognostic factors, and hormone status [26].

Our study also analysed the tumoral and stromal MMP-2 and MMP-9 coexpression, and the correlation between this coexpression and other prognostic factors (Table 2). The results show a correlation with all factors, but it is statistically significant in stromal MMP-2/MMP-9 coexpression regarding tumor size ($p = 0.01$), i.e., positive tumors were more often larger [40, 41].

3.4 Our study analysed the correlation between the number of deaths and recurrences, and the MMP-2 and MMP-9 expression in tumor cells and stroma; correlation was demonstrated between tumor cell MMP-2 and the number of deaths ($p = 0.08$, data not shown), but it was marginal in terms of statistical significance; accordingly, the stronger the tumor cell MMP-2 expression the higher is the probable number of deaths for breast carcinoma patients. Survival and recurrence probability curves (Kaplan–Meier) over months showed no statistically significant difference between positive and negative MMP-2 and MMP-9 expression in 138 analysed patients ($p > 0.05$) with the exception of tumor cell MMP-2 expression as related to overall survival, where a statistically significant difference was observed between the curves for the positive and negative group ($p = 0.025$) (Fig. 3). This

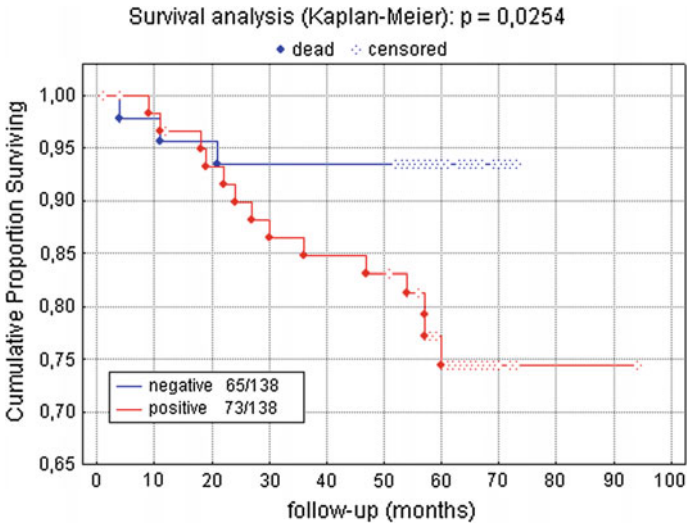

Fig. 3 Overall survival in relation to MMP-2 expression in breast tumor cells [40]

means that breast cancer patients with tumor cell MMP-2 expression die faster during the period of observation as compared with the group with negative MMP-2, and this difference is statistically significant [40, 41].

In 2003 Talvensaari-Matilla et al. demonstrated a statistically significant correlation between MMP-2 expression and survival. In the group of patients with negative progesterone receptors and MMP-2 positive tumors the rate of survival was 58%, and in the group of MMP-2 negative tumors survival observed over 10 years was 95% (p = 0.005). These results showed for the first time that negative MMP-2 in breast carcinoma patients with negative hormone receptors can serve as a marker indicating a much better prognosis [47]. Even earlier, in 1999, the same authors demonstrated a higher recurrence risk in patients with positive lymph nodes and MMP-2 expression in younger than 40 years, but the difference was not statistically significant [48]. In 2006 Tetu et al. showed, when MMP-2, TIMP-2, and MMP-14 were analysed together, that survival was the poorest for patients with strong stromal MMP-2 and MMP-14 and weak stromal TIMP-2 expression (five-year survival rate: 50%); it was the best with weak stromal MMP-2 and MMP-14 expression, and strong stromal TIMP-2 expression (5-year survival = 74%); however, the difference was not statistically significant [24]. In 2003 Wang et al. demonstrated the correlation between MMP-9 and metastases, i.e., positive lymph nodes. The correlation between MMP-9 expression and overall survival was also demonstrated, and the group of patients with survival longer than 3 years had a lower MMP-2 expression unlike the group with survival of less than 3 years [49]. Pellikainen also demonstrated that positive stromal MMP-9 expression is a predictor of a shorter DFS and shorter overall survival in estrogen positive tumors; thus, the rate of 5-year survival in negative stromal MMP-9 tumors was 89% as compared with 70% in positive stromal MMP-9 tumors [25].

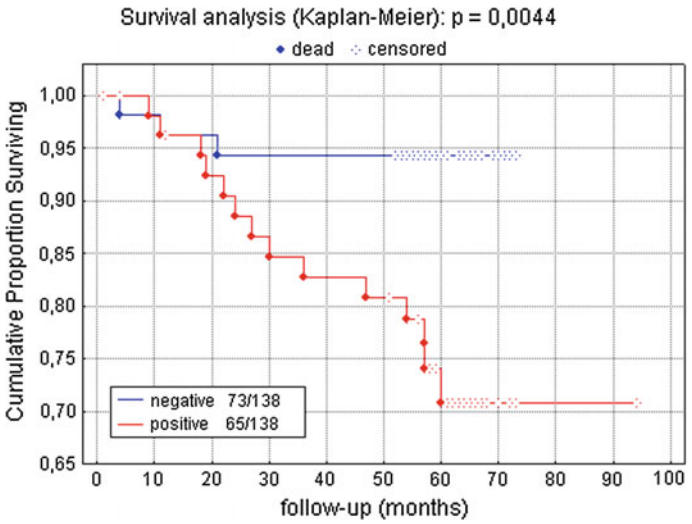

Fig. 4 Overall survival in relation to MMP-2/MMP-9 coexpression in breast tumor cells [40]

Considering the course of the disease, the correlation between tumoral MMP-2/MMP-9 coexpression, in our study, and the number of deaths and recurrences demonstrated a statistically significant correlation between tumoral MMP-2/MMP-9 coexpression and the number of deaths ($p = 0.001$, data not shown). The classic survival probability curve (Kaplan–Meier) showed a statistically significant difference between positive and negative tumoral MMP-2/MMP-9 coexpression over the followed-up months ($p = 0.004$) (Fig. 4). In other words, after a 60-month follow-up 30% of breast cancer patients with a positive tumoral MMP-2/MMP-9 coexpression died, as compared with only 5% deaths in the group with negative tumoral MMP-2/MMP-9 staining. This confirmed the value of MMP-2/MMP-9 coexpression for the clinical course of breast cancer patients [40, 41]. In 2004 Li et al. studied the prognostic value of immunohistochemical MMP-2/MMP-9 coexpression in breast cancer patients with negative lymph nodes. Positive MMP-2 expression was found in 56.7% of tumors and positive MMP-9 in 59.6%. In this patients group a statistically significant correlation was established between positive MMP-2 and tumor size and histological grade, whereas MMP-2/MMP-9 coexpression was statistically significant with respect to DFS ($p = 0.013$) but not with respect to overall survival ($p = 0.122$). This showed that gelatinases are poor prognostic factors with regard to the shorter survival and demonstrated the statistically significant value of MMP-2/MMP-9 coexpression in patients with negative lymph nodes [28].

In 2003 Fan et al. demonstrated a stronger MMP-2 and MMP-9 expression in breast cancer with positive lymph nodes. Moreover, there was a statistically significant correlation between gelatinases and tumor size and shorter survival. Univariate analysis confirmed MMP-2 and MMP-9 as predictors of an unfavorable

prognosis regarding overall survival ($p < 0.05$) [50]. In 2009 Shah et al. confirmed, in their recent study, that MMP-2 and MMP-9 expression in breast cancer patients and negative lymph nodes can predict the risk of metastasis development in lymph nodes, and that such patients need to be followed-up more closely, with targeted gelatinase inhibitor therapy added in the future [51].

3.5 Multivariate analysis of specific survival, in our study, showed that patients with tumor size larger than 5 cm with positive lymph nodes and coexpression of MMP-2/MMP-9 in tumor cells had an independent prognostic significance (Table 3). Coexpression of MMP-2/MMP-9 in tumor cells was an independent poor prognostic factor in our breast carcinoma patients [40, 41].

Talvensaari-Matilla et al. showed that after a 10-year follow-up 56% of patients with a strongly positive MMP-2 were alive, as compared with 88% of patients with negative MMP-2 regardless of other prognostic factors. In this study MMP-2 was confirmed for the first time, by multivariate analysis, as an independent prognostic factor for survival in breast cancer patients, increasing the death risk 3.6 times over a 10-year follow-up [47].

Our study has demonstrated the value of MMP-2 and MMP-9 determination in breast cancer patients since the expression of these proteins in breast cancer, along with the already existing traditional prognostic factors, represents an additional piece of information on poor prognosis in breast cancer patients. The expression of MMP-2 and MMP-9 (tumor cells and stroma) and their coexpression in breast cancer patients is an unfavorable prognostic indicator of the disease, and an indicator of the need for more aggressive treatment in patients with negative lymph nodes [40, 41].

The latest studies and their results provide information on so far statistically insignificant results in terms of the effect of MMP-2 and MMP-9 expression as independent poor prognostic factors of survival in breast cancer patients, but in combination with other prognostic factors they can provide valuable information, especially in the group with negative lymph nodes and negative hormone receptors. However, considering the still inconsistent results and the still unexplained true nature of behavior of these markers, many authors refer to the necessary additional study of these markers and of the entire metalloproteinase group since this field continues to yield more and more information on the importance of cell–cell interaction and on the influence of the extracellular matrix in carcinogenesis and in the evaluation of the biological tumor behavior.

Table 3 Multivariate model of breast carcinoma specific survival [40]

Risk factors	P	SE	OD	95% CI
Tumor size >5 cm	**0.0044**	1.0241	19.4076	2.560–147.09
Lymph node positivity	**0.0068**	0.6105	5.3544	1.600–17.910
Tumor cell MMP-2/MMP-9 coexpression	**0.0022**	0.8460	13.961	2.619–74.409

OD Odds ratio *CI* Confidence intervals

Where breast carcinoma is involved, in the future the implementation of additional methods will slowly shift from classis prognostic parameters to new markers which can help in selecting therapy and be factors predicting its use. In molecular diagnostics, the basis of additional research in breast cancer continues to be the determination of hormone receptors and the HER-2 status. However, the incorporation of new prognostic factors into the increasing number of studies and clinical trials will provide new contributions to the coming era of personalized medicine and help in the choice of the best individual therapeutic option for each patient.

Theoretically speaking, in the future we can expect that the main contribution of the study of all these compounds will provide for the appearance of a new approach in adjuvant or neoadjuvant therapy of breast cancer patients. Analogously, since metalloproteinases, as demonstrated by recent research, are involved in the initiation of carcinogenesis, in the future their inhibitors can be taken into account in the evaluation of the quest of appropriate chemopreventive substances.

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References

1. Harris JR, Lippman ME, Morrow M, Hellman S (1996) Diseases of the breast. Lippincott, Philadelphia
2. Lester SC, Cotran RS (1999) The Breast. In: Robbins SLG, Cotran RS, Kumar V (eds) Pathologic basis of disease, 6th edn. WB Sounders, Philadelphia, pp p1093–p1120
3. Harris JP, Hellman S, Henderson IC, Kinne DW (1987) Breast diseases. Lippincott, Philadelphia
4. Parkin DM, Bray F, Ferlay J, Pisani P (2002) Global cancerstatistics. CA Cancer J Clin 55 (2):74–108
5. Hrvatski zavod za javno zdravstvo (2004). Incidencija raka u Hrvatskoj 2002. Bilten; 25
6. Šamija M, Juzbašić S, Šeparović V, Vrdoljak VD (2007) Tumori dojke. Medicinska naklada, Zagreb
7. Vukas D (2004) Tumori dojke. In: Mršić-Krmpotić Z, Roth A et al (eds) Internistička onkologija, 1st edn. Medicinska naklada, Zagreb, pp 313–345
8. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function and biochemistry. Circ Res 92:827–839
9. Duffy MJ, Maguire TM, Hill A et al (2000) Metalloproteinases: role in breast cancerogenesis, invasion and metastasis. Breast Cancer Res 4:252–257
10. John A, Tuszynski G (2001) The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. Pathol Oncol Res 7:14–23
11. Jiang WG, Davies G, Martin TA et al (2006) Expression of membrane type-1 matrix metalloproteinase, MT1-MMP in human breast cancer and its impact on invasiveness of breast cancer cells. Int J Mol Med 4:583–590
12. Caudroy S, Polette M, Tournier JM et al (1999) Expression of the extracellular matrix metallo-proteinase inducer (EMMPERIN) and the matrix metalloproteinase-2 in bronchopulmonary and breast lesions. J Histochem Cytochem 47:1575–1580
13. Nakopolou L, Panayotopoulou EG, Giannopolou I et al (2002) Stromelysin-3 protein expression in invasive breast cancer: relation to proliferation, cell survival and patients outcome. Mod Pathol 11:1154–1161

14. Wei L, Shi YB (2005) Matrix metalloproteinase stromelysin-3 in development and pathogenesis. Histopathol 1:177–185
15. Ross JS, Linette GP, Stec J et al (2004) Breast cancer biomarkers and molecular medicine: part II. Expert Rev Mol Diagn 2:169–188
16. Woessner JF, Nagase H (2000) Matrix metalloproteinases and TIMPs (protein profile). Oxford University Press, New York
17. Gross J, Lapiere CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci 48:1014–1022
18. Szabo KA, Singh G (2005) Modulation of monocyte matrix metalloproteinase-2 by breast adenocarcinoma cells. Breast Cancer Res 7:661–668
19. Sellers A, Reynolds JJ, Meikle MC (1978) Neutral metallo-proteinases of rabbit bone. Separation in latent forms of distinct enzymes that when activated degrade collagen, gelatin and proteoglycans. Biochem J 171:493–496
20. Huhtala P et al (1990) Structure of the human type IV collagenase gene. J Biol Chem 265:11077–11082
21. Jayaram G, Elsayed EM (2005) Cytologic evaluation of prognostic markers in breast carcinoma. Acta Cytol 49:605–610
22. Jinga DC, Blidaru A, Condrea I et al (2006) MMP-9 and MMP-2 gelatinases and TIMP-1 and TIMP-2 inhibitors in breast cancer: correlations with prognostic factors. J Cell Mol Med 10 (2):499–510
23. Chabottaux V, Sounni NE, Pennington CJ et al (2006) Membrane-type 14 matrix metalloproteinase promotes breast cancer growth and metastases. Cancer Res 66:5165–5172
24. Tetu B, Brisson J, Wang CS et al (2006) The influence of MMP-14, TIMP-2 and MMP-2 expression on breast cancer prognosis. Breast Cancer Res 8(3):R28
25. Pellikainen JM, Ropponen KM, Kataja VV et al (2004) Expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 in breast cancer with a special reference to activator protein-2, Her-2, and prognosis. Clin Cancer Res 10:7621–7628
26. Rahko E, Jukkola A, Melkko J et al (2004) Matrix metalloproteinase-9 (MMP-9) immunoreactive protein has modest prognostic value in locally advanced breast carcinoma patients treated with an adjuvant antiestrogen therapy. Anticancer Res 24:4247–4253
27. Scorilas A, Karameris A, Arnogiannaki N et al (2001) Overexpression of matrix-metalloproteinase-9 in human breast cancer: a potential favourable indicator in node-negative patients. Br J Cancer 84:1488–1496
28. Li HC, Cao DC, Liu Y et al (2004) Prognostic value of matrix metalloproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma. Breast Cancer Res Treat 88:75–85
29. Jones JL, Glynn P, Walker RA (1999) Expression of MMP-2 and MMP-9, their inhibitors, and the activator MT1-MMP in primary breast carcinomas. The J Pathol 189:161–168
30. Kim HJ, Park CI, Park BW et al (2006) Expression of MT1-MMP, MMP-2, MMP-9 and TIMP-2 m RNAs in ductal carcinoma in situ and invasive ductal carcinoma of the breast. Yonsei Med J 47(3):333–342
31. Di GH, Lu JS, Song CG et al (2005) Over expression of aromatase protein is highly related to MMPs levels in human breast carcinomas. J Exp Clin Cancer Res 24(4):601–607
32. Sadeghi M, Motovali-Bashi M, Hojati Z (2009) MMP-9 promoter polymorphism associated with tumor progression of breast cancer in Iranian population. J Integr Biol 6:33–37
33. Stuelten CH, DaCosta BS, Arany PR et al (2005) Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-alpha and TGF-beta. J Cell Sci 118: 2143–2153
34. Wang TN, Albo D, Tuszynski GP (2002) Fibroblasts promote breast cancer cell invasion by upregulating tumor matrix metalloproteinase-9 production. Surgery 132:220–225
35. Lungu G, Covaleda L, Mendes O et al (2008) FGF-1-induced matrix metalloproteinase-9 expression in breast cancer cells is mediated by increased activities of NF-kappaB and activating protein-1. Mol Carcinog 47(6):424–435

36. Yokohama K, Kamata N, Fujimoto R et al (2003) Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas. Int J Oncol 22:891–898
37. Talvensaari-Mattila A, Paakko P, Hoyhtya M et al (1998) Matrix metalloproteinase-2 immunoreactive protein a marker of aggressiveness in breast carcinoma. Cancer 83(6): 1153–1162
38. Leppa S, Saarto T, Vehmanen L et al (2004) A high serum matrix metalloproteinase-2 level is associated with an adverse prognosis in node-positive breast carcinoma. Clin Cancer Res 3:1057–1063
39. Dengfeng C, Polyak K, Halushka M et al (2008) Serial analysis of gene expression of lobular carcinoma in situ identifies downregulation of claudin 4 and over expression of matrix metalloproteinase 9. Breast Caner Res 10:R91
40. Ranogajec I, Jakić-Razumović J, Puzović V, Gabrilovac J (2012) Prognostic value of matrix metalloproteinase-2 (MMP-2), matrixmetalloproteinase-9 (MMP-9) and aminopeptidase N/CD13 in breast cancer patients. Med Oncol 29(2):561–569
41. Ranogajec I (2014) Gelatinase (MMP-2, MMP-9) and Aminopeptidase N/CD 13 in Breast Carcinoma. Immunohistology and Prognosis, Lap Lambert, Berlin
42. Singer CF, Kronsteiner N, Marton E et al (2002) MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal-epithelial interactions. Breast Cancer Res 72:69–77
43. Liu SC, Yang SF, Yeh KT et al (2006) Relationships between the level of matrix metalloproteinase-2 and tumor size of breast cancer. Clin Chim Acta 371:92–96
44. Peihong S, Perry F (2007) Expression of nm23, MMP-2, TIMP-2 in breast neoplasmin Zhen-Zhou Center Hospital, China. Ethio Med J 45:79–83
45. Ogura S, Ohdaira T, Hozumi Y et al (2007) Metastasis-related factors expressed in pT1, pN0 breast cancer: assessment of recurrence risk. J Surg Oncol 96:46–53
46. Nilsson UW, Garvin S, Dabrosin C (2007) MMP-2 and MMP-9 activity is regulated by estradiol and tamoxifen in cultured human breast cancer cells. Breast Cancer Res 102(3):253–261
47. Talvensaari-Mattila A, Pääkkö P, Turpeenniemi-Hujanen T (2003) Matrix metalloproteinase-2 (MMP-2) is associated with survival in breast carcinoma. Br J Cancer 6:1270–1275
48. Talvensaari-Mattila A, Pääkkö P, Turpeenniemi-Hujanen T (1999) MMP-2 positivityand age less than 40 years increases the risk for recurrence in premenopausal patients with node-positive breast carcinoma. Breast Cancer Res Treat 58:287–293
49. Wang HY, Zhang XB, Wang M (2003) Expression of matrix metalloproteinase-9 (MMP-9) and laminin-receptor in breast carcinoma and their correlation with tumor metastasis and prognosis. Ai Zheng 22(5):529–532
50. Fan SQ, Wei QY, Li MR et al (2003) Expression and clinical significance of MMP-2, MMP-9, TIMP-1 and TIMP-2 in breast carcinoma. Ai Zheng 22(9):968–973
51. Shah FD, Shukla SN, Shah PM et al (2009) Clinical significance of matrix metalloproteinase 2 and 9 in breast cancer. Indian J Cancer 46(3):194–202

Multifaceted Role of Matrix Metalloproteases on Human Diseases

2

Soumitra Roy, Asmita Pramanik, Tapati Chakraborti and Sajal Chakraborti

Abstract

Matrix metalloproteases (MMPs) are important enzymes required in extracellular matrix (ECM) degradation for creating the cellular environments to maintain numerous physiological processes ranging from development to wound repair. However, MMP activity is strictly controlled and imbalance in the levels of MMP family members and its inhibitors has been implicated as an etiological factor in several diseases. Herein, involvement of MMPs and their natural inhibitors, tissue inhibitors of metalloproteases (TIMPs), in several disease processes have been considered for discussion.

Keywords

MMP · TIMP · AD · PD · ALS

1 Introduction

Matrix metalloproteases (MMPs) are zinc-containing endopeptidases capable of degrading various components of extracellular matrix (ECM) [1]. They are produced as latent zymogens (pro-MMPs). Once activated, they participate in the regulation of

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diverse physiological and pathological processes. Since its discovery about a few decades ago, MMPs have emerged as crucial mediators in defining interaction of cells with their surrounding microenvironment [2]. MMPs can be categorized into five major groups according to their substrate specificity: collagenases, gelatinases, membrane-type metalloproteases, stromelysins, and matrilysins [3].

MMP activity is tightly regulated at the transcriptional as well as at the post-translational level. MMPs enzymatic activities are controlled by tissue inhibitors of metalloproteases (TIMPs) after secretion in the extracellular milieu [4]. Among the four distinct TIMP molecules (TIMP-1, TIMP-2, TIMP-3, and TIMP-4), TIMP-1 is the major endogenous inhibitor of pro and active MMP-9 and inhibits their activity by forming a noncovalent complex [5].

The ECM is a three-dimensional, extracellular scaffold required for maintenance of life. Every tissue and organ is composed of ECM generated in early embryonic stages. The ECM provides structural support for organs, tissues, cell layers and for individual cells as substrates for cell motility. The function of ECM is much more than to provide physical support for tissues and organs; the ECM is constantly remodeled to provide a dynamic structure during tissue homeostasis [6]. MMPs can impact the development of several diseases through different pathophysiological mechanisms, mainly via tissue destruction and ECM degradation. Imbalance in MMP/TIMP ratio can cause a threat to mortality and severity of diseases. This chapter examines the detrimental role of MMPs and their endogenous inhibitors in the development of variety of pathological conditions, including neurodegenerative and lung diseases.

2 Structure and Function of MMPs

Members of the MMP family (Table 1) were initially grouped according to their preferred substrates, e.g., gelatinases, collagenases, matrilysins, and stromelysins. A sequential numbering system for MMPs was employed according to the chronology of their discovery when it became evident that more MMPs exist than was initially assumed and that the names based on substrate specificity were not sufficient. Some MMPs, like MMP-4, MMP-5 and MMP-6 are missing in the nomenclature as further studies showed that either the gene products did not exist or were identical to previously predicted MMP members [5]. MMPs are synthesized as pre proenzymes and secreted as latent pro-MMPs. The primary structures of vertebrate MMPs contain several domains. The propeptide domain consists of conserved PRCG(V/N)PD sequence. The conserved Cys within this sequence, called cysteine switch, coordinates with the catalytic zinc to prevent latent pro-MMPs from becoming inappropriately activated [7]. This sequence is missing in MMP-21 [8]. Stromelysin 3 (MMP-11) and *Xenopus* MMP possess a proprotein converting sequence R*X*(K/R)R at their C-terminal end. The catalytic domain possesses a zinc binding segment HE*XX*H*XX*G*XX*H and a unique methionine containing conserved stretch, called 'Met-turn' [9]. This domain is made up of three α-helices,

Table 1 Secreted MMPs and their substrates

MMP	Alternate names	Selected substrates
MMP-1	Collagenase-1	Collagen I, II, III, entactin, perlectan, IGFBP-2 and -3, proIL-1β, IL-1β
MMP-2	Gelatinase A	Gelatin, collagen IV, V, XI, laminin, aggrecan, proTGF-β, proTNF-α, IGFBP-3 and -5
MMP-3	Stromelysin-1	Aggrecan, laminin, fibronectin, fibrinogen, MCP-1 to -4, proMMP-1, -3, -7,-8, -9, -13
MMP-7	Matrilysin	Plasminogen, pro-α-defensin, FasL, proTNF-α, E-cadherin, syndecan, proMMPs
MMP-8	Collagenase-2	Collagen I-III, VII, X, aggrecan, fibronectin, proTNF-α, IGF-BP, MCP-1, angiotensin
MMP-9	Gelatinase B	Gelatin, collagen IV, V, XI, pro-IL-8, ProTNF-α, proTGF-β, proMMP-2, -9, -13
MMP-10	Stromelysin-2	Gelatins, fibronectin, proteoglycan, pro-MMP-1, -8, -10
MMP-11	Stromelysin-3	Fibronectin, laminin, aggrecan, IGFBP-1
MMP-12	Metalloelastase	Elastin, fibronectin, laminin, plasminogen, proTNF-α
MMP-13	Collagenase-3	Collagen I, II, III, entactin, aggrecan, tenascin, proTNF-α, proMMP-9, -13
MMP-14	MT1-MMP	Collagen I, II, III, laminin, fibronectin, proMMP-2, -13, CD44, tissue transglutaminase
MMP-15	MT2-MMP	Pro-MMP-2, pro-TNF-α, tissue transglutaminase 1
MMP-16	MT3-MMP	Collagen III, proMMP-2, proTNF-α, tissue transglutaminase
MMP-17	MT4-MMP	Gelatin, fibronectin, fibrin, proMMP-2, ADAMTS-4, TIMPs, proTNF-α
MMP-18	Collagenase-4	Collagen I, II, III
MMP-19	Stromelysin-4	Collagen IV, gelatin, laminin
MMP-20	Enamelysin	Amelogenin, aggrecan, cartilage oligomeric matrix protein (COMP)
MMP-21		Gelatin, α1-antitrypsin
MMP-24	MT5-MMP	ProMMP-2
MMP-25	MT6-MMP	Collagen IV, gelatin, fibrin, fironectin, proMMP-2 and -9, TIMPs, uPAR
MMP-26	Matrilysin-2	Collagen IV, fibronectin, fibrin, fibrinogen, proMMP-9 MMP-27 Gelatin, casein
MMP-28	Epilysin	Neural cell adhesion molecule (NCAM), casein

uPAR urokinase-type plasminogen activator receptor; *IGFBP-1* insulin-like growth factor binding protein

one five-stranded β-sheet and bridging loops [10]. This basic topology including the strictly conserved methionine containing 'Met-turn' is part of a larger metalloprotease family called metzincins, which include matrixins, adamalysin, astacins, and serralysins [9]. MMP catalytic domains are coordinated with zinc and calcium ions which are needed for their stability and enzymatic activity. Gelatinases (MMP-2 and MMP-9) contain three fibronectin-type II repeats which are located in the

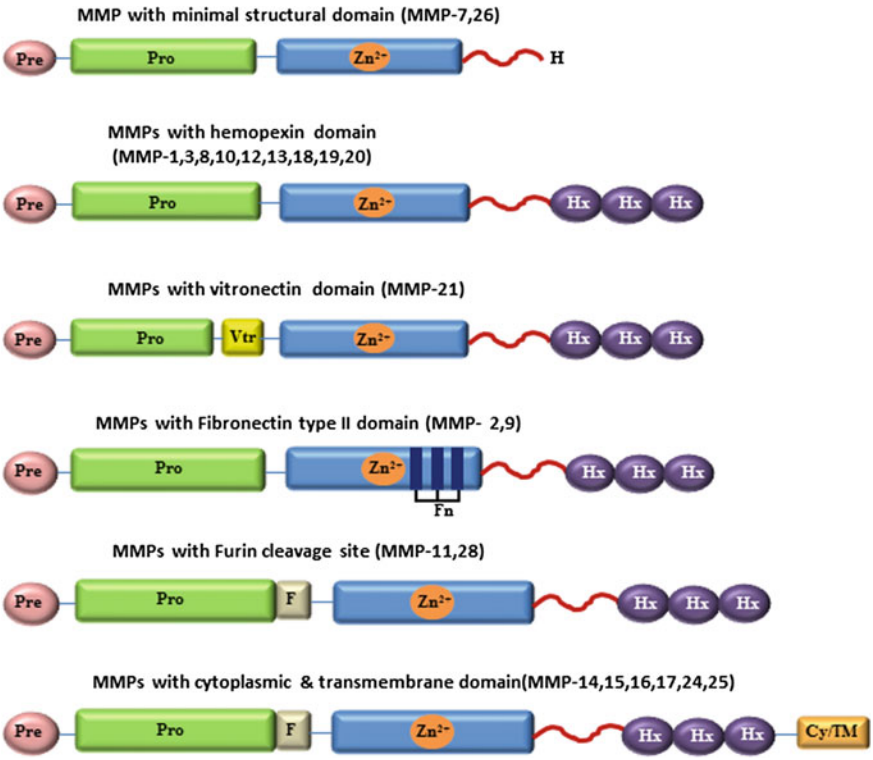

Fig. 1 Schematic representation of secreted MMP domain structure. The domain organization of MMPs is as indicated: *Pre* prepeptide; *Pro* propeptide; *Cat* catalytic domain; Zn^{2+} active-site zinc; *H* hinge region; *Hx* hemopexin domain; *Fn* fibronectin domain; *Vtr* vitronectin insert; *Cy/TM*, cytoplasmic/transmembrane domain

catalytic domain (Fig. 1). These repeats interact with collagens and gelatins [11, 12]. The C-terminal hemopexin-like domain has an ellipsoidal disk shape with a four bladed β-propeller structure; each blade comprising an α-helix and four antiparallel β-strands [13]. The hemopexin domain of collagenases is necessary for cleavage of triple helical interstitial collagens [14], although the proteolytic activity can be retained by the catalytic domains alone [15].

3 Role of MMPs in Neurodegenerative Diseases

3.1 Aging

Neurodegenerative diseases may be caused by multiple factors which include complex interactions of genetic elements and environmental factors. One of the

main features of aging is accumulation of advanced glycation end products (AGE). Activation of the receptor for AGE (RAGE) causes release of proinflammatory cytokines and free radicals which contributes to inflammatory processes [16]. A few reports suggest that vascular-derived insults could be a causal factor in propagating aging and to some of the age-related ailments, for instance, Alzheimer's disease (AD) [17, 18]. Age-related vascular diseases can indeed be triggered by dysregulation in the physiological balance between MMPs and TIMPs [19].

RNA profiling and DNA microarray analysis have shown TIMP-2, a potent inhibitor of MMP-2, to be a common biomarker of aging in heart and cerebellum of multiple mice strains, suggesting that an increase in the level of TIMP-2 may modulate impaired angiogenesis and fibrosis in aged tissues [20]. This study is corollary of the role of TIMPs in aging as transcription profiling data by Zahn et al. [21] indicates that TIMP-1 is the key gene product that exhibits maximum change in gene expression in several age-related human tissues. Elevated level of MMP-9 has been observed to be associated with aging and circulating MMP-9 and TIMP-1 levels in patients with brain ischemia and aging [22]. Moreover, Safciuc et al. [23] showed that microvessels in the brain of aged rats exhibit diminished MMP-2 activity and specific occurrence of MMP-9. Liu et al. [24] have suggested that an increase in MMP-12 could contribute to aging-associated neuroinflammation as they showed high level of MMP-12 in microglia in the brain of aging mice.

3.2 Alzheimer's Disease

Alzheimer's Disease (AD) is the commonest neurodegenerative disorder. Major characteristics of AD include neuronal cell death mediated brain atrophy, decreased dendritic arborization in the cerebral cortex and other subcortical areas. The hallmarks of AD include abundance of amyloid plaques in the nerve cells of the brain and neurofibrillary tangles, made up of misfolded proteins [25]. The relationship between MMPs and AD has been studied intensely. Using immunochemical methods Bjerke et al. [26] suggested that MMP-9 and TIMP-1 could be used as biomarkers of AD in addition to T-tau, P-tau, Aβ1–42, and white matter lesions. Another group proposed that high abundance of MMP-9 in a protease cascade, responsible for degradation of pronerve growth factor (proNGF) mature NGF (mNGF), resulting in degradation of mNGF which may cause the pathogenesis of cognitive deficits in AD [27] (Fig. 2). Lorenzl et al. [28] also documented higher levels of MMP-9 in serum of AD patients. MMP-9 expression was also demonstrated to be induced in neuronal cytoplasm, neurofibrillary tangles, amyloid plaques, and vascular tissue in AD patients [29], as well as in cultured rat astrocytes upon stimulation with Aβ [30]. Latent MMP-9 detected in pyramidal neurons of AD patients and near amyloid plaques by Backstrom et al. [31]. They also proposed that the lack of active MMP-9 contributes to the accumulation of insoluble beta-amyloid peptides in plaques. Yan et al. [32] demonstrated that MMP-9 can degrade Aβ fibrils in vitro, as well as amyloid plaques in brain of aged APP/PS1 (double-transgenic AD mouse model expressing variants of amyloid precursor

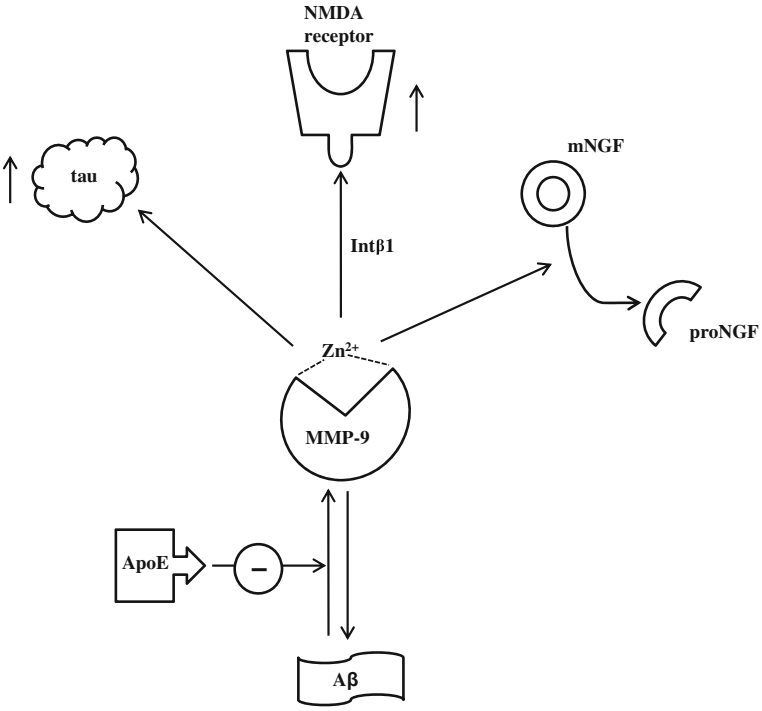

Fig. 2 MMP-9 in AD pathology: MMP-9 is responsible for degradation of pro nerve growth factor (proNGF) to mature NGF (mNGF) as well as Aβ fibrills. ApoE inbits Aβ induced MMP-9 synthesis. Activation of NMDA receptor is mediated by MMP-9 in integrin β1 (Intβ1) dependent manner which may facilitate AD. MMP-9 also mediates oligomeric deposition of tau protein in brain regions

protein (APP) and presenelin-1 (PS1) genes) and APPsw (APP gene bearing the Swedish mutation) mice, and increased MMP activity was selectively observed in compact Thioflavin S-positive plaques (a staining method to detect amyloid plaques). In rat primary astrocytes, treatment with oligomeric Aβ decreases MMP-2 expression and extracellular activity, whereas, in the brain of APP/PS1 AD mice, they found an increase in MMP-2 activity as well as mRNA level using immunohistochemistry and real time PCR, respectively. Furthermore, they suggested that Aβ can directly decrease the expression and activation of MMP-2 in astrocytes, while stimulates microglia to produce proinflammatory cytokines such as IL-1β and TGFβ, which in turn could induce MMP-2 expression and contribute to disease condition [33]. Liao et al. [34] using a transgenic mouse model of AD demonstrated expression of MMP-2 and membrane-type matrix metalloprotease (MT1-MMP), a potent activator of MMP-2, in reactive astrocytes around amyloid plaques and suggested their involvement in degradation of Aβ. In another finding, MMP-12 has been shown to exacerbate the proteolytic cascade by subsequent activation of other MMPs such as MMP-2 and MMP-3 [35]. By zymographic

study, Horstmann et al. [36] showed that MMP-3 levels were significantly elevated in plasma as well as in cerebrospinal fluid (CSF) of AD patients, whereas MMP-2 levels were significantly decreased. Interestingly in another study the levels of MMP-2 and MMP-3 were significantly down regulated in CSF of AD patients [37]. In a study, MMP-3 was demonstrated to express in astrocytes, microglia, and in endothelial cells in the brain as well as near-neuritic plaques in AD [38]. MMP-3 has also been shown to be induced in response to Aβ peptides in cultured astrocytes and neuronal cells [39]. A connection was found between MMP-3 and APOE 4 (apolipoprotein E) alleles and the presence of both is a risk factor for developing AD [40]. Furthermore, Aβ1–42 oligomers induce loss of barrier integrity at the blood–CSF barrier and were linked to increased MMP-3 expression and MMP activity [41]. Other MMPs, like MMP-1 has also been reported to be at increased level in brain of AD patients [42].

3.3 Parkinson's Disease

Parkinson's disease (PD) is a progressive movement disorder of the central nervous system that deteriorates over time mostly affecting middle- and old-aged people. Area of the brain called substantia nigra is mainly affected in PD. The exacerbated neurons emanate a chemical called dopamine that transmits signal to the brain to control movement and coordination of the body. With progression of PD, the amount of dopamine is downregulated in the brain, causing inability in controlling movement by the affected individual. Another hallmark of PD is the formation of Lewy bodies, an abnormal aggregate of proteins inside the nerve cells. These protein aggregates form fibrils and composed mainly of α-synuclein. The biological role of these protein aggregates is unclear [43]. Other symptoms of PD include mood and sleep disorders, dementia, and partial autonomic nervous system impairment [44]. It has been postulated that prolonged overactivation of microglia and production of proinflammatory cytokines could lead to neuronal degeneration in PD [45]. The hypothesis is supported by other studies which also predict involvement of active microglia in triggering neurodegeneration of dopaminergic neurons in the substantia nigra by lipopolysaccharide (LPS) [46, 47]. Initial neurotoxic insult to dopaminergic neurons could result in the release of certain factors that activate microglia to be damaging [48], however, the mechanism of this activation is largely unknown. MMP-3, produced by stress related dopaminergic neurons, could participate in microglial activation in the absence of any other inflammatory molecule as report suggests it could activate microglia, leading to the release of cytokines and receptors for phagocytosis of apoptotic cells [49]. In an in vitro study, Kim et al. [49] showed that MMP-3 could trigger microglia to produce proinflammatory cytokines, which in turn causes apoptosis of neuron, propagating further induction of apoptosis in neighboring dopaminergic neuronal cells. The presence of active microglia and extensive loss of dopaminergic neurons has been shown in a postmortem study by McGeer et al. [50] when they administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to monkeys for

5–14 years. In MMP-3 deficient mice using a broad spectrum MMP inhibitor, it has been shown that depletion of MMP-3 can significantly reduce MPTP induced degeneration of nigrostriatal dopaminergic neurons in the brain [51]. Involvement of MMP-9 was found in both striatum and substantia nigra after MPTP treatment and pharmacological MMP inhibitors protected against MPTP neurotoxicity [52]. The same group also demonstrated that post mortem analysis of brain tissue from PD patients did not display any change in the activities of MMP-9 and MMP-1 in substantia nigra, cortex or hippocampus, whereas MMP-2 was markedly down-regulated in the substantia nigra [53]. Additionally, they showed that MMP-9 was restricted predominantly in neurons and MMP-2 in astrocytes and microglia. In the same study, TIMP-2 levels were unaltered, whereas TIMP-1 was increased in substantia nigra but not in the hippocampus and cortex [53]. The increase in MMP-9 expression in substantia nigra and striatum in mice acutely injected with MPTP has been reported in reactive microglia and astrocytes, which indicates plausible role of MMP-9 in the onset of neuroinflammation in PD [54].

3.4 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is characterized by degeneration of motor neurons in the brain, brainstem, and spinal cord. In ALS all voluntary muscles are affected and manifested by muscular atrophy and weakness followed by paralysis and eventually respiratory failure and death [55]. Involvement of MMPs in this scenario was indicated by Lim et al. [56] when they studied brain and spinal cord specimens of ALS patients. They found MMP-2 in astrocytes and MMP-9 in pyramidal neurons in the spinal cord of ALS patient. In addition to that, MMP-2 activity was decreased in motor cortex, whereas MMP-9 activity was increased in spinal cord. Another group showed reduced MMP-9 activity during disease progression, with the peak at the onset of ALS and described a similar profile for MMP-2 [57]. Two separate groups have found elevated levels of both pro and active form of MMP-9 in the serum of ALS patients relative to healthy controls [58, 59]. In mild cases of ALS, expressions of MMP-2, MMP-9, TIMP-1 and MT-MMP-1 are elevated in serum compared to CSF, whereas MMP-2, MT-MMP-1 and TIMP-1 were either increased or remain unchanged with concomitant decrease in MMP-9 level [60]. Moreover, Fang et al. [61] found an increase in the levels of MMP-9 in CSF and skin in patients suffering from rapidly progressing ALS and suggested possible involvement of MMP-9 in progression of the disease, poor survival of the patients and neurodegeneration. Nevertheless, MMP-2 showed a slow but progressive decrease with the development of the disease [61]. In an ALS model with mice expressing mutant superoxide dismutase (SOD1), a reduction of MMP-9 function using gene ablation, viral gene therapy, or pharmacological inhibition has been shown to significantly delay muscle denervation, thereby assigning MMP-9 as a candidate therapeutic target for ALS [62]. Kaplan et al. [62] focused on the early stage of the disease and expression of MMP-9 by neurons, whereas another study by Kiaei et al. [63] at the later stages of

the disease revealed expression of MMP-9 by activated microglia, however, since the abrogation of MMP-9 gene does not rescue transgenic SOD1 mice from death, ALS indeed has complex background.

3.5 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic, autoimmune disease of the brain and spinal cord (central nervous system). In MS, the body's immune system causes inflammation in the protective myelin sheath that surrounds nerve fibers and blocks messages between brain and the rest of the body. As the disease progresses the nerves start to deteriorate or become permanently damaged. MS can be categorized into four major groups. (1) Relapsing–remitting MS (RRMS) happens in majority of patients suffering from MS. Alternate periods of remission (improvement of symptom) and relapses (deterioration) are seen to occur in a patient the disease. (2) Secondary progressive MS (SPMS), which is characterized by progressive deterioration of the symptoms, affects some individuals affected by RRMS. (3) Primary progressive MS (PPMS). Individuals in this category display progressive worsening of the disease with no remissions or relapses. (4) Progressive relapsing MS (PRMS) is the rarest type. The condition is characterized by a progressive worsening of the situation from the beginning. However, there are occasional relapse periods.

Cuzner et al. [64] suggested presence of MMP-9 and its inhibitor in reactive astrocytes in the CNS of MS patients. Increased levels of MMP-9 were found in CSF of acute phase and primary progressive MS patients [65]. Maeda et al. [65] showed expression of MMP-1, MMP-2, MMP-3 and MMP-9 in macrophages in active MS and necrotic lesions and in small numbers in astrocytes in acute and chronic MS lesions. CSF samples from patients suffering from both RRMS and PPMS and demonstrated up regulation of MMP-9 in all the RRMS cases throughout both stages of the disease [66]. However, in PPMS patients, elevated MMP-9 was found only in about half of the samples and in smaller quantity than in the relapsing–remitting form and suggesting T-cells and macrophages are responsible for the secretion of MMP-9 in MS. Moreover, Lepert et al. [66] suggested that elevation in MMP-9 level throughout the progress of the disease may cause damage of neighboring tissue and neuronal cell loss. Employing MRI study Lee et al. [67] showed elevated levels of MMP-9 in serum of MS patients, along with an increase in TIMP-1 and TIMP-2 levels and proposed that an abnormality in the inhibitory response to metalloproteases might play an aetiological role in the chronicity of multiple sclerosis. However, in another MRI study, elevated levels of MMP-9, but not TIMP-1, in serum were observed in RRMS patients and this imbalance of MMP-9 and TIMP-1 was postulated to take part in new lesions in MS [68]. Analysis of mRNA also showed an increase in the levels of MMP-1, MMP-3, MMP-7, MMP-9 and TIMP-1 in blood monocytes of MS patients [69]. In an interesting study by Althoff et al. [70] using the encephalomyelitis model, mice that genetically modified to constitutively express TIMP-1 in the CNS had a

normal phenotype but had reduced symptoms of experimental autoimmune encephalomyelitis. MMP-9 deficient mice have been observed to be less susceptible to the development of experimental autoimmune encephalomyelitis [71].

3.6 Japanese Encephalitis

Japanese encephalitis (JE) that normally affects children is mediated by a single-stranded RNA virus, called JE virus (JEV) which results in severe neurological disorders [72, 73]. JE virus is from the genus *Flavivirus* and is closely related to West Nile and Saint Louis encephalitis viruses. Humans are infected by JE virus through the bite of infected mosquitos. It infects the central nervous system leading to acute encephalitis. JEV infection causes severe damage to neurons and in various parts of the brain such as thalamus, brainstem and striatum [74].

The exact mechanism of neuronal cell death in JE is not fully clear yet, however some studies indicate the role of MMPs in neuronal cell death. Recent study suggests that JEV infection causes upregulation in the expression of MMPs (MMP-2, -7, -9) and TIMPs (TIMP-1 and -3) and can contribute to the severity of the disease [75]. Pieces of evidence also suggests expression of MMP-9 is induced during JEV infection in rat brain astrocytes through generation of reactive oxygen species (ROS) during JEV infection [76, 77]. Higher concentrations of MMP-2, TIMP-2 and TIMP-3 have been demonstrated in cerebrospinal fluid (CSF) and in serum of JEV infected children compared with control [78]. Additionally, higher serum concentrations of MMP-9 and MMP-7 have also been detected in JEV patients compared with healthy control.

3.7 MMPs in Glaucoma

Glaucoma, a neurodegenerative disease, is related to a group of eye disorders that cause impairment to the optic nerve that transmits signal from the eye to the brain. Glaucoma normally has few or no early symptoms. Glaucoma can be categorized into two major divisions: open angle glaucoma and closed angle glaucoma. In open angle glaucoma the retinal ganglion cells (RGCs) degenerate slowly without any display of symptoms at the early stage while vision loss occurs quickly at later stage. The most predominant and major risk factor for glaucoma is amplification in intraocular pressure (IOP) mainly used to diagnose the disease. Glaucoma is associated with apoptosis of RGCs and optic nerve degeneration which may lead to vision loss [79].

The mechanism of RGC and optic nerve degeneration is unclear till date. However, role of MMPs in the pathophysiology of glaucoma has been reported by some authors. MMPs are involved in modulating the trabecular ECM to maintain stable aqueous humor outflow resistance and IOP [80]. MMP-9 was shown to have a role in glaucoma by promoting loss of laminin, RGC apoptosis, and elevated IOP [81]. In steroid-induced glaucoma (SIG), role of MMPs in the regulation of outflow

resistance has also been studied. SIG, a form of open angle glaucoma, results from continuous use of corticosteroid usage causes a decreased trabecular outflow leading to increased IOP [82]. Several MMPs (MMP-2, MMP-9, and MMP-13) have shown to be elevated in steroid induced mice model of glaucoma [83]. Gerometta et al. [84] has shown that mRNA expression of MMP-1 was elevated in steroid induced sheep model of glaucoma.

4 Role of MMPs in Lung Diseases

4.1 MMP in Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a lung disease that causes difficulty in breathing. It is caused by injury to the lungs, usually from smoking. COPD is mainly referred to a mix of two diseases: Chronic bronchitis in which the bronchial tubes that carry air to the lungs are inflamed and increases mucus production. This results in narrowing and blockage in the airways, making it harder in the respiration process. Emphysema in which the tiny air sacs in the lungs are damaged and lose their flexibility, less air gets in and out of the lungs, which makes someone feel short of breath. COPD gets worse over time. The damage to the lung can't be undone. But preventive measures could be taken to check more damage and to feel better. Hallmark of COPD include elevated level of alveolar macrophages, neutrophils and cytotoxic T-lymphocytes and the release of multiple inflammatory mediators (lipids, chemokines, cytokines, growth factors). Worsening of inflammation may be caused by a high level of oxidative stress. There is also increased evidence for involvement of MMPs in this scenario [85].

Role of MMPs in COPD has been established by several studies on human subjects. Sputum of COPD patients demonstrated to have an increased MMP-2 and MMP-9 activity and higher levels of TIMP-1 [86]. Active MMP-9 was found in the sputum from 85% of COPD patients, whereas pro-MMP-2 (72 kDa) was found in 50% of COPD patients and in only 5% of controls [86]. COPD patients also showed higher levels of TIMP-1. This finding was substantiated by similar study done by Beeh et al. [87], who demonstrated an increase in MMP-9 and TIMP-1 levels in sputum from COPD patients, as well as an increased ratio of MMP-9 to TIMP-1. In bronchoalveolar lavage fluid (BAL) from COPD patients, there is an increase in collagenase activity (possibly due to elevated MMP-8 level) when compared with healthy controls. Additionally, MMP-9 has been observed to be present in the majority of COPD patients, whereas it was absent in control subjects matched for sex and smoking status [88]. In smokers with emphysema, MMP-8 and MMP-9 levels in BAL fluid were significantly upregulated compared to smokers without emphysema [89]. Russel et al. [90] found that cultured alveolar macrophages from COPD patients release higher amounts of MMP-9 than the alveolar macrophages from healthy smokers and nonsmokers.

Activity and expression of MMPs are up regulated in alveolar macrophages from COPD patients exposed with either tobacco smoke or wood smoke [91]. The study demonstrated increased macrophage elastolytic activity in COPD patients and suggested that the enzymatic activity is imparted by MMP-12. MMP-9 activity was increased in both COPD groups, whereas MMP-2 activity was higher in samples from wood smoke induced COPD than in those from COPD patients afflicted by tobacco smoking and in healthy controls. RT-PCR study revealed increased MMP-2 and MMP-12 expression in COPD patients. No significant difference has, however, been observed in alveolar macrophages from COPD patients when compared with healthy controls. However, earlier studies have shown up regulation in MMP-9 and MMP-1 mRNA levels in alveolar macrophages from emphysematous lung [92]. Immunohistochemical analysis on human lung tissue demonstrated elevated expression of MMP-1, -2, -8 and -9 in COPD patients [93]. MMP-1 and -2 were localized mainly in alveolar and interstitial macrophages and in epithelial cells, whereas MMP-8 and MMP-9 were primarily detected in neutrophils [93]. RT-PCR and ELISA studies revealed that mRNA and protein expression as well as MMP-1 activity were increased in the lung parenchyma of emphysema patients compared to control patients [94]. Studies of a group of researchers revealed an imbalance between MMP-9 and TIMP-1 levels in human lung tissue, which is associated with cigarette smoking [95]. They found positive correlation of MMP-9 level and the MMP-9/TIMP-1 ratio in the lung tissue extracts from cigarette smoking individual. However, MMP-9 levels were negatively correlated with FEV1 (forced expiratory volume; how much air a person can exhale during a forced breath), suggesting role of MMP-9 in the progression of airway obstruction in smokers. Genetic studies revealed that MMP-1 and MMP-12 polymorphisms are causal factors for worsening of lung function in smokers [96]. Polymorphism in the MMP-9 promoter region (-1562C/T) is responsible for the development of smoking-induced emphysema [97]. The allele frequency was greater in smokers with distinct emphysema compared with those without emphysema.

4.2 MMPs and Asthma

Asthma is a chronic disease of the airways that is manifested by inflammation resulting in narrowing of the air passage. Asthma causes repeated phases of wheezing (a high-pitched whistling sound made during breathing), shortness of breath, chest pain, and cough. The frequency of cough increases during night or early in the morning and it can be problematic as it hampers daily activities which may cause lethal asthma attack. Asthma cannot be cured completely, but its dangerous symptoms can be lessened by medication. Several reports indicate role of MMPs associated with asthma.

Zymographic analysis of sputum samples from patients with asthma revealed an increase in the activity of MMP-2 and MMP-9 and higher levels of TIMP-1 in their sputum [86]. Patients with severe asthma had increased MMP-9 levels and activity in their sputum than patients with mild asthma and normal individual.

Allergen challenge increases the MMP-9/TIMP-1 ratio and consequently an increase in MMP-9 activity. Moreover, Inhalation of budesonide (which reduces inflammation) had no effect on MMP-9 or TIMP-1 in patients with mild asthma [98]. Report suggests an increase in the MMP-9 levels, but not TIMP-1 level in allergen induced sputum of asthmatic patients [99]. Additionally, this study also indicates a significant correlation between MMP-9 levels at 6 h and the maximum percent fall in FEV1 (forced expiratory volume in 1 s; a parameter for the degree of airway obstruction in obstructive lung diseases) during the late response [99]. MMP-1 and MMP-2 are involved in IL-13 induced elastin, a protease known for its role in airway remodeling, expression in airway fibroblast in mild asthmatic subjects [100]. Inhibition of MMP expression reverses the effect of IL-13 induced suppression of elastin. Gelatin zymography and enzyme immunoassay of sputum samples revealed significantly increased activities of pro-MMP-9 in acute asthmatic patients than in stable asthmatic patients. [101]. Elevated MMP-9 activity significantly decreased after 7 and 28 days of therapy. Tenascin-C, an extracellular matrix protein increases MMP-1 expression in asthma derived airway smooth muscle cells and bronchial biopsies of asthmatic patients when compared with control [102]. Levels of MMP-9 in the sputum were significantly increased in patients with toluene diisocyanate (TDI)-induced asthma [103]. In a study in patients ventilated because of acute severe asthma, a 10- to 160-fold increase of MMP-9 and activated forms (46 and 26 kDa) of stromelysin-1 (MMP-3) in epithelial lining fluid was found [104]. About fourfold circadian increase in MMP-9 was found in bronchoalveolar lavage (BAL) fluid, and a twofold increase in MMP-9/TIMP-1 ratio in subjects with nocturnal asthma [105]. High molecular weight form of MMP-9 has been observed to be significantly higher in BAL fluid in patients with severe asthma, and MMP-9 level was correlated with BAL neutrophils [106]. A marked increase in MMP-9 production and activity has been observed in the plasma of patients with severe acute asthma following stimulation with Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) and phorbol-12-myristate-13-acetate (PMA) [107]. MMP-9 immunoreactivity was identified in endobronchial biopsy specimens from all the asthmatic subjects, but could not be identified in healthy controls. Immunoreactivity of MMP-9 was found in bronchial epithelium and extracellular matrix in submucosa [108]. Immunohistochemical analysis revealed that in a higher percentage of severe asthmatic patients MMP-9 staining of the subepithelial basement membrane is prominent compared to control subjects. MMP-9 levels in the BAL fluid were also increased in patients with severe asthma [109]. RT-PCR analysis demonstrated up regulation of mRNA transcripts for MMP-1 and TIMP-1 in cell pellets of induced sputum from asthmatic patients compared with control subjects [110]. The intensity of MMP-1 mRNA expression was inversely correlated with the FEV(1) in asthmatic patients. Vermaelen et al. [111] have shown that MMP-9 deficiency inhibits the development of allergic airway inflammation by impairing the recruitment of dendritic cells into the airways and the local production of dendritic cell-derived proallergic chemokines.

5 Conclusion and Future Perspective

An impaired pattern of MMPs and TIMPs is associated with an elevated risk in several human diseases. Currently there is no available clinical therapy to completely cure or postpone neurodegenerative diseases. Therefore, novel therapeutic approaches are needed to prevent the progression of the disease for a better and healthy lifespan of patients. More basic research is also required to fully understand the diverse roles of MMPs in the pathophysiology of neurodegenerative diseases in order to design plausible MMP inhibitors to set strategies for prevention or cure of chronic neurodegenerative diseases. The development of new inhibitors is also necessary and that may also alleviate the pain inflicted in patients with asthma and COPD. Targeted delivery of MMP inhibitors directly to the lung might result in fewer side effects, which needs to be explored.

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References

1. Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52–67
2. Gross J, Lapiere CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci U S A 48:1014–1022
3. Bourboulia D, Stetler-Stevenson WG (2010) Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): positive and negative regulators in tumor cell adhesion. Semin Cancer Biol 20:161–168
4. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T (2003) Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253:269–285
5. Woessner JF Jr, Nagase H (2000) Functions of the TIMPs. Matrix metalloproteinases and TIMPs. Oxford University Press; Oxford, New York, NY, pp 130–135
6. Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science 326:1216–1219
7. Becker JW, Marcy AI, Rokosz LL, Axel MG, Burbaum JJ, Fitzgerald PM, Cameron PM, Esser CK, Hagmann WK, Hermes JD, Springer JP (1995) Stromelysin-1: three-dimensional structure of the inhibited catalytic domain and of the C-truncated proenzyme. Protein Sci 4:1966–1976
8. Gururajan R, Grenet J, Lahti JM, Kidd VJ (1998) Isolation and characterization of two novel metalloproteinase genes linked to the Cdc2L locus on human chromosome 1p36.3. Genomics 52:101–106
9. Bode W, Gomis-Rüth FX, Stöckler W (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett 331:134–140
10. Dhanaraj V, Ye QZ, Johnson LL, Hupe DJ, Ortwine DF, Dunbar JB Jr, Rubin JR, Pavlovsky A, Humblet C, Blundell TL (1996) X-ray structure of a hydroxamate inhibitor complex of stromelysin catalytic domain and its comparison with members of the zinc metalloproteinase superfamily. Structure 4:375–386

11. Allan JA, Docherty AJ, Barker PJ, Huskisson NS, Reynolds JJ, Murphy G (1995) Binding of gelatinases A and B to type-I collagen and other matrix components. Biochem J 309:299–306
12. Steffensen B, Wallon UM, Overall CM (1995) Extracellular matrix binding properties of recombinant fibronectin type II-like modules of human 72-kDa gelatinase/type IV collagenase. High affinity binding to native type I collagen but not native type IV collagen. J Biol Chem 270:11555–11566
13. Gomis-Rüth FX, Gohlke U, Betz M, Knäuper V, Murphy G, López-Otín C, Bode W (1996) The helping hand of collagenase-3 (MMP-13): 2.7 A crystal structure of its C-terminal haemopexin-like domain. J Mol Biol 264:556–566
14. Bode W (1995) A helping hand for collagenases: the haemopexin-like domain. Structure 3:527–530
15. Clark IM, Cawston TE (1989) Fragments of human fibroblast collagenase. Purification and characterization. Biochem J 263:201–206
16. Srikanth V, Maczurek A, Phan T, Steele M, Westcott B, Juskiw D, Münch G (2011) Advanced glycation endproducts and their receptor RAGE in Alzheimer's disease. Neurobiol Aging 32:763–777
17. Salat DH (2011) The declining infrastructure of the aging brain. Brain Connect 1:279–293
18. Zlokovic BV (2011) Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. Nat Rev Neurosci 12:723–738
19. Dollery CM, McEwan JR, Henney AM (1995) Matrix metalloproteinases and cardiovascular disease. Circ Res 77:863–868
20. Park SK, Kim K, Page GP, Allison DB, Weindruch R, Prolla TA (2009) Gene expression profiling of aging in multiple mouse strains: identification of aging biomarkers and impact of dietary antioxidants. Aging Cell 8:484–495
21. Zahn JM, Sonu R, Vogel H, Crane E, Mazan-Mamczarz K, Rabkin R, Davis RW, Becker KG, Owen AB, Kim SK (2006) Transcriptional profiling of aging in human muscle reveals a common aging signature. PLoS Genet 2:e115
22. Romero JR, Vasan RS, Beiser AS, Au R, Benjamin EJ, DeCarli C, Wolf PA, Seshadri S (2010) Association of matrix metalloproteinases with MRI indices of brain ischemia and aging. Neurobiol Aging 31:2128–2135
23. Safciuc F, Constantin A, Manea A, Nicolae M, Popov D, Raicu M, Alexandru D, Constantinescu E (2007) Advanced glycation end products, oxidative stress and metalloproteinases are altered in the cerebral microvasculature during aging. Curr Neurovasc Res 4:228–234
24. Liu Y, Zhang M, Hao W, Mihaljevic I, Liu X, Xie K, Walter S, Fassbender K (2013) Matrix metalloproteinase-12 contributes to neuroinflammation in the aged brain. Neurobiol Aging 34:1231–1239
25. Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E (2011) Alzheimer's disease. Lancet 377(9770):1019–1031
26. Bjerke M, Zetterberg H, Edman Å, Blennow K, Wallin A, Andreasson U (2011) Cerebrospinal fluid matrix metalloproteinases and tissue inhibitor of metalloproteinases in combination with subcortical and cortical biomarkers in vascular dementia and Alzheimer's disease. J Alzheimers Dis 27:665–676
27. Bruno MA, Mufson EJ, Wuu J, Cuello AC (2009) Increased matrix metalloproteinase 9 activity in mild cognitive impairment. J Neuropathol Exp Neurol 68:1309–1318
28. Lorenzl S, Buerger K, Hampel H, Beal MF (2008) Profiles of matrix metalloproteinases and their inhibitors in plasma of patients with dementia. Int Psychogeriatr 20:67–76
29. Asahina M, Yoshiyama Y, Hattori T (2001) Expression of matrix metalloproteinase-9 and urinary-type plasminogen activator in Alzheimer's disease brain. Clin Neuropathol 20:60–63
30. Deb S, Wenjun Zhang J, Gottschall PE (2003) Beta-amyloid induces the production of active, matrix-degrading proteases in cultured rat astrocytes. Brain Res 970:205–213

31. Backstrom JR, Lim GP, Cullen MJ, Tökés ZA (1996) Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1-40). J Neurosci 16:7910–7919
32. Yan P, Hu X, Song H, Yin K, Bateman RJ, Cirrito JR, Xiao Q, Hsu FF, Turk JW, Xu J, Hsu CY, Holtzman DM, Lee JM (2006) Matrix metalloproteinase-9 degrades amyloid-beta fibrils in vitro and compact plaques in situ. J Biol Chem 281:24566–24574
33. Li W, Poteet E, Xie L, Liu R, Wen Y, Yang SH (2011) Regulation of matrix metalloproteinase 2 by oligomeric amyloid β protein. Brain Res 1387:141–148
34. Liao MC, Van Nostrand WE (2010) Degradation of soluble and fibrillar amyloid beta-protein by matrix metalloproteinase (MT1-MMP) in vitro. Biochemistry 49:1127–1136
35. Ito S, Kimura K, Haneda M, Ishida Y, Sawada M, Isobe K (2007) Induction of matrix metalloproteinases (MMP3, MMP12 and MMP13) expression in the microglia by amyloid-beta stimulation via the PI3 K/Akt pathway. Exp Gerontol 42:532–537
36. Horstmann S, Budig L, Gardner H, Koziol J, Deuschle M, Schilling C, Wagner S (2010) Matrix metalloproteinases in peripheral blood and cerebrospinal fluid in patients with Alzheimer's disease. Int Psychogeriatr 22:966–972
37. Mlekusch R, Humpel C (2009) Matrix metalloproteinases-2 and -3 are reduced in cerebrospinal fluid with low beta-amyloid1-42 levels. Neurosci Lett 466:135–138
38. Wang XX, Tan MS, Yu JT, Tan L (2014) Matrix metalloproteinases and their multiple roles in Alzheimer's disease. Biomed Res Int 2014:908636
39. Deb S, Gottschall PE (1996) Increased production of matrix metalloproteinases in enriched astrocyte and mixed hippocampal cultures treated with beta-amyloid peptides. J Neurochem 66:1641–1647
40. Saarela MS, Lehtimäki T, Rinne JO, Hervonen A, Jylhä M, Röyttä M, Ahonen JP, Mattila KM (2004) Interaction between matrix metalloproteinase 3 and the epsilon4 allele of apolipoprotein E increases the risk of Alzheimer's disease in Finns. Neurosci Lett 367:336–339
41. Brkic M, Balusu S, Van Wonterghem E, Gorlé N, Benilova I, Kremer A, Van Hove I, Moons L, De Strooper B, Kanazir S, Libert C, Vandenbroucke RE (2015) Amyloid β oligomers disrupt blood-CSF barrier integrity by activating matrix metalloproteinases. J Neurosci 35:12766–12778
42. Leake A, Morris CM, Whateley J (2000) Brain matrix metalloproteinase 1 levels are elevated in Alzheimer's disease. Neurosci Lett 291:201–203
43. Trojanowski JQ, Lee VM (1998) Aggregation of neurofilament and alpha-synuclein proteins in lewy bodies: implications for the pathogenesis of parkinson disease and lewy body dementia. Arch Neurol 55:151–152
44. Marsden CD, Obeso JA (1994) The functions of the basal ganglia and the paradox of stereotaxic surgery in Parkinson's disease. Brain 117:877–897
45. Barcia C, Ros CM, Annese V, Gómez A, Ros-Bernal F, Aguado-Yera D, Martínez-Pagán ME, de Pablos V, Fernandez-Villalba E, Herrero MT (2011) IFN-γ signaling, with the synergistic contribution of TNF-α, mediates cell specific microglial and astroglial activation in experimental models of Parkinson's disease. Cell Death Dis 2:e142
46. Gao HM, Jiang J, Wilson B, Zhang W, Hong JS, Liu B (2002) Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J Neurochem 81:1285–1297
47. Castaño A, Herrera AJ, Cano J, Machado A (1998) Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. J Neurochem 70:1584–1592
48. Lukens JR, Barr MJ, Chaplin DD, Chi H, Kanneganti TD (2012) Inflammasome-derived IL-1β regulates the production of GM-CSF by CD4(+) T cells and γδ T cells. J Immunol 188:3107–3115

49. Kim YS, Kim SS, Cho JJ, Choi DH, Hwang O, Shin DH, Chun HS, Beal MF, Joh TH (2005) Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. J Neurosci 25:3701–3711
50. McGeer PL, Schwab C, Parent A, Doudet D (2003) Presence of reactive microglia in monkey substantia nigra years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration. Ann Neurol 54:599–604
51. Kim YS, Choi DH, Block ML, Lorenzl S, Yang L, Kim YJ, Sugama S, Cho BP, Hwang O, Browne SE, Kim SY, Hong JS, Beal MF, Joh TH (2007) A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. FASEB J 21:179–187
52. Lorenzl S, Calingasan N, Yang L, Albers DS, Shugama S, Gregorio J, Krell HW, Chirichigno J, Joh T, Beal MF (2004) Matrix metalloproteinase-9 is elevated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in mice. Neuromol Med 5:119–132
53. Lorenzl S, Albers DS, Narr S, Chirichigno J, Beal MF (2002) Expression of MMP-2, MMP-9, and MMP-1 and their endogenous counterregulators TIMP-1 and TIMP-2 in postmortem brain tissue of Parkinson's disease. Exp Neurol 178:13–20
54. Annese V, Herrero MT, Di Pentima M, Gomez A, Lombardi L, Ros CM, De Pablos V, Fernandez-Villalba E, De Stefano ME (2015) Metalloproteinase-9 contributes to inflammatory glia activation and nigro-striatal pathway degeneration in both mouse and monkey models of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism. Brain Struct Funct 220:703–727
55. Garbuzova-Davis S, Rodrigues MC, Hernandez-Ontiveros DG, Louis MK, Willing AE, Borlongan CV, Sanberg PR (2011) Amyotrophic lateral sclerosis: a neurovascular disease. Brain Res 1398:113–125
56. Lim GP, Backstrom JR, Cullen MJ, Miller CA, Atkinson RD, Tökés ZA (1996) Matrix metalloproteinases in the neocortex and spinal cord of amyotrophic lateral sclerosis patients. J Neurochem 67:251–259
57. Soon CP, Crouch PJ, Turner BJ, McLean CA, Laughton KM, Atkin JD, Masters CL, White AR, Li QX (2010) Serum matrix metalloproteinase-9 activity is dysregulated with disease progression in the mutant SOD1 transgenic mice. Neuromuscul Disord 20:260–266
58. Beuche W, Yushchenko M, Mäder M, Maliszewska M, Felgenhauer K, Weber F (2000) Matrix metalloproteinase-9 is elevated in serum of patients with amyotrophic lateral sclerosis. NeuroReport 11:3419–3422
59. Demestre M, Parkin-Smith G, Petzold A, Pullen AH (2005) The pro and the active form of matrix metalloproteinase-9 is increased in serum of patients with amyotrophic lateral sclerosis. J Neuroimmunol 159:146–154
60. Niebroj-Dobosz I, Janik P, Sokołowska B, Kwiecinski H (2010) Matrix metalloproteinases and their tissue inhibitors in serum and cerebrospinal fluid of patients with amyotrophic lateral sclerosis. Eur J Neurol 17:226–231
61. Fang L, Huber-Abel F, Teuchert M, Hendrich C, Dorst J, Schattauer D, Zettlmeissel H, Wlaschek M, Scharffetter-Kochanek K, Tumani H, Ludolph AC, Brettschneider J (2009) Linking neuron and skin: matrix metalloproteinases in amyotrophic lateral sclerosis (ALS). J Neurol Sci 285:62–66
62. Kaplan A, Spiller KJ, Towne C, Kanning KC, Choe GT, Geber A, Akay T, Aebischer P, Henderson CE (2014) Neuronal matrix metalloproteinase-9 is a determinant of selective neurodegeneration. Neuron 81:333–348
63. Kiaei M, Kipiani K, Calingasan NY, Wille E, Chen J, Heissig B, Rafii S, Lorenzl S, Beal MF (2007) Matrix metalloproteinase-9 regulates TNF-alpha and FasL expression in neuronal, glial cells and its absence extends life in a transgenic mouse model of amyotrophic lateral sclerosis. Exp Neurol 205:74–81
64. Cuzner ML, Gveric D, Strand C, Loughlin AJ, Paemen L, Opdenakker G, Newcombe J (1996) The expression of tissue-type plasminogen activator, matrix metalloproteases and

endogenous inhibitors in the central nervous system in multiple sclerosis: comparison of stages in lesion evolution. J Neuropathol Exp Neurol 55:1194–1204
65. Maeda A, Sobel RA (1996) Matrix metalloproteinases in the normal human central nervous system, microglial nodules, and multiple sclerosis lesions. J Neuropathol Exp Neurol 55:300–309
66. Leppert D, Ford J, Stabler G, Grygar C, Lienert C, Huber S, Miller KM, Hauser SL, Kappos L (1998) Matrix metalloproteinase-9 (gelatinase B) is selectively elevated in CSF during relapses and stable phases of multiple sclerosis. Brain 121:2327–2334
67. Lee MA, Palace J, Stabler G, Ford J, Gearing A, Miller K (1999) Serum gelatinase B, TIMP-1 and TIMP-2 levels in multiple sclerosis. A longitudinal clinical and MRI study. Brain 122:191–197
68. Waubant E, Goodkin DE, Gee L, Bacchetti P, Sloan R, Stewart T, Andersson PB, Stabler G, Miller K (1999) Serum MMP-9 and TIMP-1 levels are related to MRI activity in relapsing multiple sclerosis. Neurology 53:1397–1401
69. Kouwenhoven M, Ozenci V, Gomes A, Yarilin D, Giedraitis V, Press R, Link H (2001) Multiple sclerosis: elevated expression of matrix metalloproteinases in blood monocytes. J Autoimmun 16:463–470
70. Althoff GE, Wolfer DP, Timmesfeld N, Kanzler B, Schrewe H, Pagenstecher A (2010) Long-term expression of tissue-inhibitor of matrix metalloproteinase-1 in the murine central nervous system does not alter the morphological and behavioral phenotype but alleviates the course of experimental allergic encephalomyelitis. Am J Pathol 177:840–853
71. Dubois B, Masure S, Hurtenbach U, Paemen L, Heremans H, van den Oord J, Sciot R, Meinhardt T, Hämmerling G, Opdenakker G, Arnold B (1999) Resistance of young gelatinase B-deficient mice to experimental autoimmune encephalomyelitis and necrotizing tail lesions. J Clin Invest 104:1507–1515
72. Kalita J, Kumar S, Vijaykumar K, Palit G, Misra UK (2007) A study of CSF catecholamine and its metabolites in acute and convalescent period of encephalitis. J Neurol Sci 252:62–66
73. Misra UK, Kumar S, Kalita J, Ahmad A, Khanna VK, Khan MY, Palit G (2009) A study of motor activity and catecholamine levels in different brain regions following Japanese encephalitis virus infection in rats. Brain Res 1292:136–147
74. Kumar S, Kalita J, Saxena V, Khan MY, Khanna VK, Sharma S, Dhole TN, Misra UK (2009) Some observations on the tropism of Japanese encephalitis virus in rat brain. Brain Res 1268:135–141
75. Shukla V, Kumar Shakya A, Dhole TN, Misra UK (2012) Upregulated expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in BALB/c mouse brain challenged with Japanese encephalitis virus. NeuroImmunoModulation 19:241–254
76. Tung WH, Tsai HW, Lee IT, Hsieh HL, Chen WJ, Chen YL, Yang CM (2010) Japanese encephalitis virus induces matrix metalloproteinase-9 in rat brain astrocytes via NF-κB signalling dependent on MAPKs and reactive oxygen species. Br J Pharmacol 161:1566–1583
77. Yang CM, Lin CC, Lee IT, Lin YH, Yang CM, Chen WJ, Jou MJ, Hsiao LD (2012) Japanese encephalitis virus induces matrix metalloproteinase-9 expression via a ROS/c-Src/PDGFR/PI3 K/Akt/MAPKs-dependent AP-1 pathway in rat brain astrocytes. J Neuroinflammation 9:12
78. Shukla V, Shakya AK, Dhole TN, Misra UK (2013) Matrix metalloproteinases and their tissue inhibitors in serum and cerebrospinal fluid of children with Japanese encephalitis virus infection. Arch Virol 158:2561–2575
79. Quigley HA (1999) Neuronal death in glaucoma. Prog Retin Eye Res 18:39–57
80. De Groef L, Van Hove I, Dekeyster E, Stalmans I, Moons L (2013) MMPs in the trabecular meshwork: promising targets for future glaucoma therapies? Invest Ophthalmol Vis Sci 54:7756–7763

81. Guo L, Moss SE, Alexander RA, Ali RR, Fitzke FW, Cordeiro MF (2005) Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and IOP-induced effects on extracellular matrix. Invest Ophthalmol Vis Sci 46:175–182
82. Kumar S, Shah S, Tang HM, Smith M, Borrás T, Danias J (2013) Tissue plasminogen activator in trabecular meshwork attenuates steroid induced outflow resistance in mice. PLoS ONE 8:e72447
83. Kumar S, Shah S, Deutsch ER, Tang HM, Danias J (2013) Triamcinolone acetonide decreases outflow facility in C57BL/6 mouse eyes. Invest Ophthalmol Vis Sci 54:1280–1287
84. Gerometta R, Kumar S, Shah S, Alvarez L, Candia O, Danias J (2013) Reduction of steroid-induced intraocular pressure elevation in sheep by tissue plasminogen activator. Invest Ophthalmol Vis Sci 54:7903–7909
85. Elkington PT, Friedland JS (2006) Matrix metalloproteinases in destructive pulmonary pathology. Thorax 61:259–266
86. Cataldo D, Munaut C, Noël A, Frankenne F, Bartsch P, Foidart JM, Louis R (2000) MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. Int Arch Allergy Immunol 123:259–267
87. Beeh KM, Beier J, Kornmann O, Buhl R (2003) Sputum matrix metalloproteinase-9, tissue inhibitor of metalloprotinease-1, and their molar ratio in patients with chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and healthy subjects. Respir Med 97:634–639
88. Finlay GA, Russell KJ, McMahon KJ, D'Arcy EM, Masterson JB, Fitzgerald MX, O'Connor CM (1997) Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid of emphysematous patients. Thorax 52:502–506
89. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, Kawakami Y (1999) Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. Am J Respir Crit Care Med 159:1985–1991
90. Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, Barnes PJ (2002) Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol 26:602–609
91. Montano M, Beccerril C, Ruiz V, Ramos C, Sansores RH, Gonzalez-Avila G (2004) Matrix metalloproteinases activity in COPD associated with wood smoke. Chest 125:466–472
92. Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, Fitzgerald MX, O'Connor CM (1997) Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. Am J Respir Crit Care Med 156:240–247
93. Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C, Selman M (2000) Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. Chest 117:684–694
94. Imai K, Dalal SS, Chen ES, Downey R, Schulman LL, Ginsburg M, D'Armiento J (2001) Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema. Am J Respir Crit Care Med 163:786–791
95. Kang MJ, Oh YM, Lee JC, Kim DG, Park MJ, Lee MG, Hyun IG, Han SK, Shim YS, Jung KS (2003) Lung matrix metalloproteinase-9 correlates with cigarette smoking and obstruction of airflow. J Korean Med Sci 18:821–827
96. Joos L, He JQ, Shepherdson MB, Connett JE, Anthonisen NR, Pare PD, Sandford AJ (2002) The role of matrix metalloproteinase polymorphisms in the rate of decline in lung function. Hum Mol Genet 11:569–576
97. Minematsu N, Nakamura H, Tateno H, Nakajima T, Yamaguchi K (2001) Genetic polymorphism in matrix metalloproteinase-9 and pulmonary emphysema. Biochem Biophys Res Commun 289:116–119

98. Mattos W, Lim S, Russell R, Jatakanon A, Chung KF, Barnes PJ (2002) Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids. Chest 122:1543–1552
99. Boulay ME, Prince P, Deschesnes F, Chakir J, Boulet LP (2004) Metalloproteinase-9 in induced sputum correlates with the severity of the late allergen-induced asthmatic response. Respiration 71:216–224
100. Ingram JL, Slade D, Church TD, Francisco D, Heck K, Sigmon RW, Ghio M, Murillo A, Firszt R, Lugogo NL, Que L, Sunday ME, Kraft M (2016) Role of matrix metalloproteinases-1 and -2 in interleukin-13-suppressed elastin in airway fibroblasts in asthma. Am J Respir Cell Mol Biol 54:41–50
101. Lee YC, Lee HB, Rhee YK, Song CH (2001) The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. Clin Exp Allergy 31:1623–1630
102. Rogers NK, Clements D, Dongre A, Harrison TW, Shaw D, Johnson SR (2014) Extra-cellular matrix proteins induce matrix metalloproteinase-1 (MMP-1) activity and increase airway smooth muscle contraction in asthma. PLoS ONE 9:e90565
103. Park HS, Kim HA, Jung JW, Kim YK, Lee SK, Kim SS, Nahm DH (2003) Metalloproteinase-9 is increased after toluene diisocyanate exposure in the induced sputum from patients with toluene diisocyanate-induced asthma. Clin Exp Allergy 33:113–118
104. Lemjabbar H, Gosset P, Lamblin C, Tillie I, Hartmann D, Wallaert B, Tonnel AB, Lafuma C (1999) Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. Am J Respir Crit Care Med 159:1298–1307
105. Pham DN, Chu HW, Martin RJ, Kraft M (2003) Increased matrix metalloproteinase-9 with elastolysis in nocturnal asthma. Ann Allergy Asthma Immunol 90:72–78
106. Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE (2003) Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. J Allergy Clin Immunol 112:1064–1071
107. Belleguic C, Corbel M, Germain N, Lena H, Boichot E, Delaval PH, Lagente V (2002) Increased release of matrix metalloproteinase-9 in the plasma of acute severe asthmatic patients. Clin Exp Allergy 32:217–223
108. Han Z, Junxu Zhong N (2003) Expression of matrix metalloproteinases MMP-9 within the airways in asthma. Respir Med 97:563–567
109. Wenzel SE, Balzar S, Cundall M, Chu HW (2003) Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. J Allergy Clin Immunol 111:1345–1352
110. Cataldo DD, Gueders M, Munaut C, Rocks N, Bartsch P, Foidart JM, Noel A, Louis R (2004) Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases mRNA transcripts in the bronchial secretions of asthmatics. Lab Invest 84:418–424
111. Vermaelen KY, Cataldo D, Tournoy K, Maes T, Dhulst A, Louis R, Foidart JM, Noel A, Pauwels R (2003) Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma. J Immunol 171:1016–1022

Complex Mechanisms of Matrix Metalloproteinases Involvement in Endometrial Physiology and Pathology—An Update

3

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Abstract

Matrix metalloproteinases (MMPs) belong to a multigenic family of proteolytic enzymes with great structural variability which provide a complex intervention in pathophysiological conditions. Our review is focused on both MMPs key role in physiological reproductive events, such as embryo implantation, uterine involution, normal endometrial cycle, and on their role in the main endometrial pathologies. MMPs activity is closely regulated by tissue inhibitors of MMPs (TIMPs). MMP: TIMP imbalance has been incriminated in various pathological conditions, including endometrial cancer and endometriosis. Accumulated data support the involvement of a large spectrum of MMPs and TIMPs in endometrial carcinogenesis. Strong MMP-2 and weak TIMP-2 tissue immunoexpressions have a powerful prognosis value, while MMP-9 high expression suggests its important involvement in endometrial tumor invasiveness. Endometriosis development implies an accumulation of events showing partial overlap with endometrial carcinogenesis and invasion, requiring MMPs involvement. Therefore, increased levels of several MMPs have been detected in peritoneal fluid and/or endometrial tissue of patients diagnosed with endometriosis. Endometriotic mesenchymal stem cells (MSCs) may be involved in the pathogenesis of endometriosis due to their upregulated expression for markers of migration and angiogenesis, such as MMP-2, MMP-3, MMP-9, and VEGF. The hypothesis of therapeutic benefits of synthetic MMPs inhibitors, added to the progesterone or progestins action, has been based on the complex MMPs involvement in

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endometrial pathology. Future research is necessary to elucidate the complex interactions between molecules involved in proliferation, angiogenesis and apoptosis, opening new perspectives in the early diagnosis and treatment of endometrial neoplasia and endometriosis.

Keywords
Matrix metalloproteinases (MMPs) · Tissue inhibitors of MMPs (TIMPs) · Endometrial cycle · Endometrial carcinoma · Endometriosis

1 Introduction

Matrix degrading metalloenzymes, matrix metalloproteinases, matrixins, or metalloproteinases (MMPs) [1] belong to a multigenic family of proteolytic, zinc-dependent enzymes, functioning at neutral pH [2]. MMPs have latent forms, proenzymes, inactive zymogens, or pro-MMPs that require proteolytic activation [3].

Matrix metalloproteinases (MMPs) family consists of endopeptidases that share homologous protein sequences, with conserved domain structures and specific domains related to substrate specificity and recognition of other proteins [2]. MMPs structure consists of a *signal peptide* which directs their secretion from the cell, a *propeptide* which is essential for pro-MMP latent form preservation, a *catalytic domain* which contains the highly conserved Zn^{2+}-binding site, a proline-rich *hinge region* which links the catalytic domain to the C-terminal *hemopexin-like* domain which determines MMPs' substrate specificity and mediates the interactions with endogenous inhibitors [4]. Their possibility of great structural variability provides a complex MMPs intervention in pathophysiological conditions.

MMPs, together with cysteine proteinases, aspartic proteinases, and serine proteinases are mainly involved in extracellular matrix (ECM) and basement membranes (BMs) degradation [5].

MMPs possess a key role in embryogenesis and in physiological activities, such as proliferation, cell motility, remodeling, wound healing, angiogenesis, and reproductive events, such as ovulation, embryo implantation, uterine, breast, and prostate involution, menstruation, and endometrial proliferation [6–8].

MMPs have distinct substrate spectra [3] and their activity is closely regulated by their endogenous inhibitors, TIMPs.

MMP: TIMP imbalance has been incriminated in various pathologic conditions, as tumor invasion, rheumatoid arthritis inflammation, atherosclerosis, aneurysms, nephritis, tissue ulcers, fibrosis, and endometriosis [1, 9].

2 Types of MMPs

Currently, 23 different human MMPs and their codifying genes have been identified [1, 2, 10]. Based on substrate specificity, sequence similarity, and domain organization, vertebrate MMPs are classified into seven groups: collagenases (collagenase-1 or MMP-1, collagenase-2 or MMP-8, and collagenase-3 or MMP-13), gelatinases (gelatinase A or MMP-2 and gelatinase B or MMP-9), stromelysines (stromelysin-1 or MMP-3 and stromelysin-2 or MMP-10), stromelysin-like MMPs (stromelysin-3 or MMP-11 and metalloelastase or MMP-12), matrilysins (matrylisin or MMP-7 and matrylisin-2 or MMP-26), membrane type MMPs (MT1-MMP or MMP-14, MT2-MMP or MMP-15, MT3-MMP or MMP-16, MT4-MMP or MMP-17, MT5-MMP or MMP-24, and MT6-MMP or MMP-25), and other MMPs (MMP-19, MMP-20 or enamelysin, MMP-22, MMP-23, MMP-27, and MMP-28 or epilysin) [1].

3 Mechanisms of MMPs Regulation

MMPs need activation for their in vivo action, thus zymogen forms codified by MMPs genes have to be transformed into active proteinases [11]. MMPs regulation may take place during transcription or secretion by activation or by inhibition of the activated forms.

MMPs can be activated in vitro by proteases, including plasmin, MT-MMPs, and MMP-3, or by treatment with organomercurial compounds. While these enzymes are weakly expressed in normal adult tissues, they become upregulated during normal or pathological remodeling process [7].

MMPs regulation during secretion may be achieved by MMP-7 storage in the secretory component of the exocrine glands and by MMP-8 and MMP-9 storage in secretory granules of eosinophils and neutrophils and their release during active secretion. In vivo expression of MMPs may be induced by exogenous signals, e.g., growth factors, cytokines, which modulate MMP mRNA half-life [12], or altered cell–cell or cell–matrix interactions.

The inhibition of MMPs is mainly attributed to a stoichiometric binding of TIMPs which are able to regulate both the zymogens and the active forms [3].

4 MMPs Physiological Functions

MMPs are involved in embryogenesis and tissue remodeling, due to their capacity of regulation of ECM proteins and variable soluble factors. MMP-1 contributes to keratinocyte migration [7], while MMP-2 and MMP-3 are important in mammary gland branching during morphogenesis [13]. MMP-2 and MMP-9 are involved in lipogenesis [14] and angiogenesis [15], in association with MMP-13 and

MT1-MMP, as demonstrated by inhibition of this process with endostatin (endogenous angiogenic inhibitor) [7].

MMP-9 is involved in endochondral bone formation, being associated to MT1-MMP which regulates skeletal muscle and connective tissue growth [7].

As the reproductive system needs remodelation, MMPs are expected to be involved in the related processes. During postpartum uterus involution, gelatinase A, collagenase-2 and -3, stromelysins, and matrilysin are upregulated [7]. Experimental studies also demonstrate the significant involvement of gelatinase A, stromelysins, and matrilysin in estrous cycle [7]. Moreover, a MMP-9 role has been demonstrated in the implantation process [7].

Experiments in MMPs deficient mutant mice support a functional redundancy between deficient enzymes and the components of the plasminogen system or between different MMPs [7, 16].

5 Endogenous MMP Inhibitors

The extracellular activity of MMPs is strictly controlled by their specific inhibitors and by nonspecific inhibitors, e.g., α2-macroglobulin [12].

5.1 TIMPs

Natural inhibitors of MMPs or TIMPs [17] have N- and C-terminal domains, each containing three conserved disulfide bonds [18]. The N-terminal domain acts as a MMP inhibitor [7].

TIMPs gene family comprises only four members: TIMP-1, TIMP-2, TIMP-3, and TIMP-4.

TIMP-1, expressed by fibroblasts nuclei [19], acts as a broad spectrum MMP inhibitor, except for MT1-MMP and MMP-2 [12]. TIMP-1 has the ability to bind to the hemopexin domain of latent MMP-9 and forms a TIMP-1-pro-MMP-9 complex which blocks the enzyme [20]. Moreover, TIMP-1 binds and consequently inactivates MMP-1, MMP-2, and MMP-3. Several growth factors, cytokines, and phorbol esters may act as TIMP-1 activators by enhancing its expression in cell cultures [20].

TIMP-2 has a constitutive expression, exhibiting the capacity to bind to the hemopexin domain of pro-MMP-2 and to inhibit most MMPs, except MMP-9 [7]. TIMP-2 role is dual, depending on the TIMP-2-MT1-MMP-pro-MMP-2 complex formation. A threshold level of TIMP-2 is required for the trimolecular complex construction, which leaves sufficient MT1-MMP uninhibited to cleave pro-MMP-2. Higher TIMP-2 concentrations are preventing MMP-2 processing by the inhibitory action on free MT1-MMP [21].

TIMP-3 expression is noticed during normal cell cycle progression or as a response to mitogenic stimulation. TIMP-3 may be inhibited by TNF-α in fibroblasts [12]. TIMP-3 may inhibit several MMPs, such as: MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, and MT1-MMP. TIMP-3 has a unique characteristic among the TIMP family as it is bound to the ECM sulfated glycosaminoglycans [20, 21] rather than as a free soluble protein. TIMP-3 has a broader inhibitory spectrum, including members of ADAM (a disintegrin and metalloproteinase domain) and ADAM-TS (aggrecanases—ADAMs with thrombospondin domains) families, as proteases involved in bioactivity of cytokines and growth factors regulation [7, 17]. TIMP-3 shows a better inhibitory effect for ADAM-17 and aggrecanases than that for MMPs, as has been proved by kinetic studies [22]. Supplementary, TIMP-3 shows a proapoptotic activity, either by TNF-α cell receptor 1 stabilization, either by Fas or by TNF-related apoptosis, as has been demonstrated in tumoral cells [22].

TIMP-4 is the most recently identified member of the TIMP family, being expressed in heart but also at the sites of tissue injury, i.e., vascular lesions and dermal wounds. TIMP-4 is able to inhibit MMP-2, MMP-7, MMP-9, and MT1-MMP [17].

Although TIMPs are considered as endogenous inhibitors, wild-type TIMPs could have drawbacks because of multiple MMPs inhibition and TIMP-3 supplementary inhibition of ADAMs and ADAM-TS [12]. As a consequence, a successful therapeutic application would be targeting specific proteinases by development of engineered TIMPs exhibiting altered specificity [12].

In addition to MMPs inhibiting activities, TIMPs have other biological functions. Moreover, TIMP-1 and TIMP-2 exhibit cell growth-promoting activities, antiapoptotic activity, and erythroid-potentiating activity [12]. TIMP-2 is able to inhibit FGF-b induced endothelial growth, in vivo angiogenesis, and in vitro endothelial cell proliferation by means of a MMP-independent mechanism [20, 23]. Likewise, TIMP-3 can also prevent VEGF to VEGF receptor-2 binding, thereby inhibiting downstream signaling and angiogenesis [24].

5.2 Miscellaneous Inhibitors of MMPs

MMPs may be inhibited by several other proteins, as tissue factor pathway inhibitor-2 (a serine protease inhibitor), C-terminal fragment of the procollagen C-terminal proteinase enhancer protein, membrane-bound β-amyloid precursor protein, RECK (reversion-inducing cysteine-rich protein with kazal motifs), a GPI (Glycosylphosphatidylinositol)-anchored glycoprotein [25, 26], chlorotoxin, a scorpion toxin [27], and plasma-macroglobulins (general endopeptidase inhibitors by enzymes trapping within the macroglobulin after proteolysis of the bait region of the inhibitor), as the most active MMP-1 inhibitors [28].

6 MMPs Involvement in Endometrial Physiology

Female genital tract can be interpreted as a highly dynamic environment, coordinated by ovarian steroid hormones, in combination with cytokines and local growth factors. Uterine mucosa is made up of a highly vascularized tissue adapted to create conditions for pregnancy, fetal support, and allows a complex invasive mechanism during implantation and placentation [6].

Although initially only a proteolytic function has been attributed to MMPs, relatively recent studies have shown their interference in the processes of differentiation, proliferation, angiogenesis, and apoptosis, due to their ability to catalyze the hydrolysis of cytokines precursors, growth factors, hormone receptors, serum amyloid A, IGFBP (Insulin-like growth factor-binding protein), proteinase inhibitors, and IL-1β [29]. Thus, complex activities of MMPs and TIMPs are responsible for uterine reshufflings.

Some MMPs, such as MMP-9, are involved in implantation [7], while other MMPs, such as matrilysin, stromelysin, gelatinase A, collagenase-2 and collagenase-3, are responsible for postpartum uterine involution [7].

6.1 MMPs and TIMPs Dynamic Expression in Endometrial Physiology

A dynamic MMPs and TIMPs expression, under hormonal regulation, has been associated to the endometrium physiology, both in common endometrial cycle physiology and in pregnancy, their abnormal levels being responsible of several pregnancy-related conditions.

6.1.1 MMPs Expression in Endometrial Cycle

MMPs expression during various phases of endometrial cycle registers quantifiable variations [6, 30] (Table 1).

Moreover, MMPs may be epithelial-specific, such as MMP-7, stromal or vascular- specific, such as MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, MMP-11, MMP-23, and MT1-MMP, or specifically expressed by resident immune cells (neutrophils, eosinophils, and macrophages), such as MMP-8, MMP-9, and MT1-MMP [30].

Supplementary, a possible compensatory role may be expressed by some MMPs. Thus, although MMP-7 has a key role in achieving endometrial cyclic sequences, as a response to estrogen stimulation, experimental models show that its absence does not result in cycle or fertility abnormalities, due to compensatory role of MMP-3 and MMP-10 [30].

Regarding MMPs expression along the endometrial cycle (Table 1), an increased stromal expression of MMP-1, MMP-2, MMP-3, MMP-10, MMP-11, and MT1-MMP, along with an enhanced MMP-9 expression in developing

Table 1 MMPs expression in endometrial cycle and pregnancy-related conditions

MMP	Proliferative phase	Secretory phase		Menstrual phase	Pregnancy		Childbirth		Uterine involution
		Early	Advanced		Decidua	Trophoblast	Decidua	Trophoblast	
MMP-1	+	–	–	+	+	+	+	–	?
MMP-2	++ (mainly S)	++ (mainly S, V)	++ (S, V)	++ (S, V)	++/?	+/++	?	+	?
MMP-3	+/−	–	–	++ (basal E, S, V)	+	+	+	–	?
MMP-7	++ (only E)	–	+ (only E)	+++ (only E)	–	++/+	–	++	+++
MMP-9	−/+	−/+/++	−/+/++	++ (S and V)	+	++	++	++	?
MMP-10	–	–	+	++	?	−/?	?	?	?
MMP-11	++	–	+	+++	+/−	+	?	?	?
MMP-13	?	?	?	?	?	++/?	?	?	++
MMP-14	?	?	?	?	+/?	+/?	?	?	?
MT1-MMP	?	?	?	?	+/?	++/?	?	?	?
MT2-MMP	?	?	?	?	−/?	+/?	?	?	?

− Absent; + focal; ++ moderate; +++ intense; ? unknown; E (epithelium); S (stroma); V (vessels)

arterioles, and strong MMP-7 epithelial expression are characteristic for the proliferative phase [30].

The secretory phase is characterized by increased stromal and vascular MMP-2 expression and, therefore, this pattern of expression is suggestive for MMP-2 involvement in angiogenesis [30]. The advanced secretory phase is supplemented by epithelial MMP-7 expression, along with MMP-10 and MMP-11 (Table 1).

During menstruation, there is an increased epithelial MMP-7 expression, along with MMP-11 and MMP-2 stromal and vascular expression (Table 1). Moreover, MMP-3 expression in basal epithelium, stroma, and vessels, associated with stromal and vessels MMP-9, MMP-10, MMP-1, and MT1-MMP expression have been demonstrated [30].

In pregnancy, there is an increased expression of MMP-2, MMP-7, MMP-9, MMP-13, MT1-MMP, MMP-11, and MT2-MMP (Table 1).

Enhanced MMP-9 levels, along with MMP-7, MMP-1, MMP-2, and MMP-3 expression, during childbirth has been reported, while MMP-9 exhibits variable involvement in endometrial cycle, without consensus between different reports (Table 1).

6.1.2 Mechanisms of MMP Regulation During Endometrial Cycle

VEGF is intimately associated to MMPs expression in endometrium. A strong interaction between VEGF stromal cell expression, induced by estradiol, in possible association with hypoxia, and MMP-2 increased expression has been demonstrated [31].

Conversely, progesterone increases the expression of stromal cells Angiopoietin-1 (Ang-1), stabilizing vessels and blocking further unlimited angiogenesis [32].

Progesterone also induces tissue factor expression in decidualized stromal cells [31]. Tissue factor has a receptor for coagulation factor VII and its active form, and therefore has the ability to initiate the clotting cascade which promotes hemostasis.

Moreover, EGFR expression is induced by progesterone and ligand binding to EGFR is required for tissue factor expression by stromal cells [31].

Progesterone also induces stromal cells plasminogen activator inhibitor-1 (PAI-1) expression [32] which displays anti-fibrinolytic properties and restrains trophoblast invasion mediated by urokinase-type plasminogen activator [32].

Consequently, the progesterone-dominated midluteal phase has maximal hemostatic, anti-fibrinolytic, and antiproteolytic properties.

Progesterone withdrawal in the perimenstrual period, or progestin withdrawal and/or in vitro treatment with the antiprogestin RU486 result in tissue factor decrease and PAI-1 expression [32], creating a prohemorrhagic environment around endometrial blood vessels and promoting menstrual bleeding, a mechanism incriminated in the failure of conception.

The hemostatic factor expression represents a parallel progestational inhibition of stromal MMP-1, MMP-3, and MMP-9 expression in luteal phase, while progesterone withdrawal or treatment with RU486 enhances MMPs expression in stromal cells [32]. In contrast, neither progestins nor progestational withdrawal is

reflected in MMP-2 or TIMPs expression [32]. Progesterone withdrawal is also associated with upregulation of the neutrophil and macrophage chemoattractants, such as interleukin-8 (CXCL8), and macrophage chemoattractant protein-1 (CCL2) [32].

Thus, luteal phase and gestational endometrium are associated with reduced MMPs activity, resulting in stromal and underlying vascular stabilization in order to impede endometrial hemorrhage during pregnancy.

Conversely, perimenstrual progesterone withdrawal in non-fertile cycles is associated with increased MMPs expression as well as chemokines expression which stimulate leukocyte infiltration, resulting in a proteolytic environment, promoting menstrual bleeding and tissue sloughing [32].

6.1.3 TIMPs Expression in Endometrial Cycle

Co-expression of MMPs and TIMPs demonstrates the importance of their compensatory intervention in the endometrial turnover. The expression of various TIMPs during endometrial cycle phases shows quantifiable variations, with opposed levels of expression registered in pregnancy (MMPs are reduced and TIMPs are amplified) [6, 30] (Table 2).

TIMP-1 and TIMP-3 are expressed in both epithelial and stromal cells and exhibit a strong expression particularly in the luminal compartment of the endometrial epithelium [6], with marked TIMP-1 stromal expression variations, during endometrial cycle.

TIMP-1 and TIMP-2 expressions in endometrial small arterioles and capillaries, in the secretory phase, suggest their intervention in the vascular network stabilization during the endometrial cycle phases and pregnancy [6]. Moreover, TIMP-1 and TIMP-2 also exhibit an antiangiogenic function due to inhibition of VEGF expression [6].

Although TIMP-4 intervention during the endometrial cycle has not been yet demonstrated, it shows a typical expression in term deciduas [6].

6.1.4 MMPs and TIMPs Expression in Menstrual Phase and Regulation Mechanisms

While the endometrial cycle is associated with maximum hemostasis and vascular stability in the midluteal phase, being controlled by progestational induction of tissue factor and PAI-1 expression, inhibition of MMP activity, along with angiogenesis regulation [32], the menstruation is considered a controlled hemorrhage and tissue sloughing in non-fertile cycles. The mechanism involved in menstrual phase is initiated by progesterone withdrawal which diminishes the hemostatic effect and enhances MMP activity, resulting in controlled hemorrhage [32].

Although menstruation has been traditionally attributed to ischemic necrosis, as a consequence of vasospasm of the spiral arterioles, an increasing importance has recently been attributed to proteolysis performed by locally synthesized MMPs, as products of various types of endometrial cells [6, 30]. The loss of progesterone support during menstruation causes focal lysis of reticular fibers and collagen type I

Table 2 TIMPs expression in endometrial cycle and pregnancy-related conditions

TIMP	Proliferative phase	Early secretory phase	Advanced secretory phase	Menstrual phase	Pregnancy		Childbirth		Uterine involution
					Decidua	Trophoblast	Decidua	Trophoblast	
TIMP-1	+ (mainly S, V)	+ (mainly S, V)	++	+++	+/++	++	++	+	++
TIMP-2	+	+	+	++	+/++	−/++	++	+	++
TIMP-3	+/−	+	++	+	++	++	++	+	++
TIMP-4	?	?	?	?	+/++	+/++	++	+	?

− Absent; + focal; ++moderate; +++ intense; ? unknown; S (stroma); V (vessels)

and II, along with an increase in the local expression of different types of MMPs. Thus, MMP-1, MMP-2, and MMP-3 are increased, as a result of autoactivation. Supplementary, pro-MMP-9 is activated by MMP-3, in endothelium. MMP-1, MMP-10, and MMP-11 show an increased expression in stromal fibroblasts, while MMP-7 is enhanced in epithelium, preceding MMPs stromal expression.

MMP-2, MMP-3, and MMP-9 expressions in functional endometrial vessels suggest their involvement in the mechanism of vascular wall damage during menses [30].

A greater hypoxia in the superior regions of the endometrium during menstruation due to spiral arterioles constriction, followed by endometrial tissue shedding are hypothetically induced by an increased expression of MMP-1, MMP-2, and MMP-3. TNF-α seems to play a key role in the induction of apoptosis in the endometrial epithelium and a possible similar action is attributed to IL-1α and IL-1β. Moreover, "LEFTY (left-right determination factor)-A" protein is transitory expressed prior to menstruation [33] and shows an abnormal expression in patients with dysfunctional bleeding.

MT1-MMP and MT2-MMP have been also identified in stromal endometrial cells during the menstrual phase, their mechanism of action being incompletely deciphered.

TIMP-1 reaches its maximum level during menstruation and its vascular co-expression with TIMP-2 in the secretory endometrium and in areas of demarcation between necrotic and viable areas during menses demonstrates their role in vascular stability and in bleeding limitation during menses [30].

Although menstruation is considered as a process of tissue destruction, endometrial repair begins during the first 24 h after initiation of tissue fragmentation, as a mechanism of tissue damage minimization. The transition to the growth phase associated with estrogen secretion is achieved through a complex mechanism, in which VEGF, EGF, IGF, and FGF-b are also involved [33].

VEGF is co-localized with MMPs in newly formed endometrial capillaries, stimulating MMPs expression in vascular smooth muscle. The proinflammatory cytokines produced by epithelial and stromal cells regulate MMPs expression, being supplemented by an influx of lymphomyeloid cells prior to menstruation, co-mediating MMPs activation [33].

6.1.5 MMPs and TIMPs Expression in Pregnancy and Their Involvement in Related Pathology

Receptive endometrium, regulated by ovarian steroids exhibits complex events during pregnancy, such as: blastocyst attachment, implantation, and subsequent development of the placenta. During the last years, researches on MMPs inhibitors were focused mainly on endometrial tissue during implantation and pregnancy. The invasion of trophoblastic cells into the maternal endometrium requires a precisely regulated secretion of specific proteolytic enzymes for the degradation of the endometrial BM and ECM and plays a substantial role during human embryo implantation and placentation. This process is facilitated by MMPs activity regulated by TIMPs.

Thus, the expression of various MMPs during pregnancy registers variable expressions, most of them being quantifiable [6] (Table 1).

The expression of TIMPs in the endometrium also registers quantifiable variations during pregnancy [6] (Table 2). TIMPs decidual levels are increased, mainly that of TIMP-3 [8], while trophoblast levels of both TIMP-3 and TIMP-1 are constantly increased (Table 2). Supplementary, high serum levels of TIMP-1 and TIMP-2 have been detected in ongoing pregnancies [34].

All TIMPs levels are more increased in decidua at childbirth compared to that of trophoblast (Table 2).

Moreover, TIMP-1, TIMP-2, and TIMP-3 are also involved in uterine involution after childbirth (Table 2).

The proinvasive effects of preimplantation factor (PIF), an embryo derived peptide secreted by viable mammalian embryos, are associated with activation of the gelatinase activity of MMP-9 and inhibition of TIMP-1 expression [35].

MMP-9 deficiency results in placental abnormalities in experimental studies, similar to preeclampsia, as MMP-9 activity plays a major role in trophoblast invasion [35]. Thus, MMP: TIMP balance is a component of the mechanism required for PIF induction of trophoblast invasion.

PIF modulates trophoblast invasion via multiple signaling pathways, such as PI3K, MAPK, and JAK-STAT transduction pathways, as have been also identified for other proinvasive factors [35].

PIF upregulates variable proinflammatory cytokines, like IL-6 [35], in human endometrium. Consequently, PIF could promote the extravillous trophoblast invasive activity by modulation of IL-6 placental secretion [35].

It is now considered that MMP: TIMP imbalance and the expression pattern of integrins associated to PIF anomalies could be responsible of pregnancy pathology [35].

The family of IL-1 proinflammatory cytokines modulates MMP expression, facilitating cytotrophoblast endometrial invasion. IL-1α production at the maternal–fetal interface is critical for successful implantation, the exogenous antagonists of IL-1 receptor being able to prevent pregnancy. Thus, IL-1 receptor polymorphism may be associated to a high risk of recurrent miscarriage [33].

Progesterone inhibits MMP-3 and MMP-7 and gradually decreases MMP-9 expression. Thus, progesterone antagonists, such as onapristone, produce an increased MMP-9 expression [9].

Production of IL-1 regulates the expression of leptin (a circulating hormone that regulates food intake) secreted by cytotrophoblast. Leptin exerts a stimulatory effect on MMP-2 and MMP-9 released at the laterobasal domains of the syncytiotrophoblast and is responsible for trophoblast conversion toward an invasive phenotype [33].

The parturition requires inhibition of progesterone, performed also by mifepristone, resulting in cervical infiltration with immune cells and increased expression of MMP-1, MMP-3, MMP-8, MMP-9, TIMP-1, IL-1, IL-8, and TNF-α [6].

Thus, MMP-9 and TIMP-2 or MMP-2-TIMP-2 complex levels are markedly elevated in missed abortions or in mifepristone–misoprostol (steroidian antiprogestative and synthetic prostaglandin E1 combination) medical abortion [36].

Recent researches have shown that aberrant TNF-α increases the expression of MMP-1, MMP-3, and MMP-9 by decidual cells, resulting in abnormal integrin-mediated extravillous trophoblast invasion of the decidua and later onset of preeclampsia [37]. This could be reversed by IFN-γ with restoration of normal stepwise extravillous trophoblast invasion of the deciduas [37]. These findings suggest a mechanism by which IFN-γ derived from decidual NK cells counteracts the shallow trophoblast invasion, a mechanism strongly implicated in impaired decidual vascular remodeling, leading to the later development of preeclampsia [37].

6.2 MMPs and TIMPs Complex Mechanism of Endometrium Regulation

MMPs and TIMPs are regulated by local variations of cytokines and by modulation of gene transcription, by the means of steroid receptors related mechanisms.

As a consequence of exposure to high levels of estrogen, formation of ligand–ER complex results in expression of transcription factors *fos* and *jun* that bind activation element AP-1. Since the most MMPs gene promoters contain AP-1 elements, the mechanism of estrogenic MMPs regulation may be initiated [33].

AP-1 activation also occurs in transactivation of MMPs promoters by phorbol esters and by proinflammatory cytokines, such as IL-1 and TNF-α [33].

Most MMPs promoters contain an A3 amplifier of the polyoma virus, PEA-3, which is connected to the Ets (E-twenty six) transcription factors family. PEA-3 is involved in MMPs regulation by growth factors and cytokines, acting synergistically with AP-1 proteins in activation of MMPs genes transcription [33].

Progesterone stimulates the mechanisms which limit the expression of MMPs, as a consequence of progesterone inhibition of estrogen-induced *c-fos* expression. Thus, *c-jun* and *c-fos* are inhibited during pregnancy. During stromal decidualization, retinoic acid is synthesized, resulting in MMPs inhibitory effects, by sequestration of *fos* and *jun* proteins by retinoic acid receptor binding. Progesterone inhibits the expression of MMP-1 by inhibition of IL-1α release and suppression of this cytokine endometrial action [33].

Androgens inhibit MMPs expression by interacting with Ets proteins. As MMP-2 has no AP-1 site or PEA-3, it is constitutively expressed in the endometrium, being activated by its association with MT-MMPs and TIMP-2 [38].

TGF-β mediates the suppression of MMP-7 in the endometrial epithelium, as a response to progesterone but concomitantly induces the expression of TIMP-1 and TIMP-3 in stromal cells [33].

Among the cytokines that regulate MMPs endometrial expression, there is also a cytokine, LEFTY-A, originally designated as endometrial bleeding associated

factor (EBAF). LEFTY-A is a TGF-β family member secreted as a precursor of 42 kDa. The active form induces MAPK activity, inhibits TGF-β signaling, and induces the expression of pro-MMP-3, pro-MMP-7, and MMP-9 [33].

7 MMPs Involvement in Abnormal Endometrial Bleeding

Currently, the abnormal endometrial bleeding is considered as a disordered process of physiological menstrual cycle, due to impaired endometrial hemostasis and unrestrained aberrant angiogenesis. This abnormal bleeding can be related to bleeding diatheses, long-term progestin-only contraception, uterine leiomyomas, and endometrial polyps [32].

Bleeding diatheses are characterized by menorrhagia onset at menarche and the most common defect associated with menorrhagia is Von Willebrand's disease [32]. By combined hormonal contraceptives treatment applied in this disease, stromal cells are induced to secrete tissue factor and PAI-1 with consequent hemostatic effect [32].

Long-term progestin-only contraceptives induce reductions in endometrial blood flow resulting in hypoxia–reperfusion injury and free radical production. These induce aberrant angiogenesis by VEGF and Ang-2 increased expression and Ang-1 suppression as well as direct damage of blood vessels [32].

Endometrial biopsies demonstrate the presence of enlarged, thin walled blood vessels at bleeding sites despite increased tissue factor expression [32] and a general dramatic increase in immature and partially mature vessel number, area, and density [32], and perivascular extracellular matrix degeneration due to excess MMP-2 activity, culminating in bleeding and collapsed stroma [32].

The most common presentation in leiomyomas is menorrhagia, due to venule ectasia. This is the result of tumor and/or hypoxia-derived angiogenic factors, such as VEGF, FGF-b, PDGF, HBEGF, TGF-β, parathyroid hormone related protein, and prolactin [32]. There is also spiral artery vasodilation due to increased estrogen receptor expression [32]. The most common presentation in endometrial polyps is metrorrhagia, due to increased cyclo-oxygenases and MMPs production [32] and due to enhanced microvascular density by aberrant angiogenesis [32].

In contrast to physiological regulated cycle, anovulatory bleeding is associated with both impaired hemostasis due to an absence of progestational induction of tissue factor and PAI-1 and increased MMP and angiogenesis due to unrestrained estrogenic effects. On the other hand, long-term progestin-only contraceptives-associated bleeding reflects sustained hemostasis with persistently elevated tissue factor expression and vessel damage due to impaired endometrial blood flow with hypoxia-induced unrestrained angiogenesis [32]. Similar defects appear to account

for abnormal bleeding with myomas and endometrial polyps, though in the former condition macrovascular changes lead to menorrhagia while microvascular changes promote metrorrhagia in the latter [32].

8 MMPs Involvement in Endometrial Hyperplasia and Carcinoma

Endometrial carcinoma has the highest incidence among the malignancies of the feminine genital tract [39] and morphologic expressions of precursor lesions (atypical hyperplasia) and carcinoma are correlated to progressive accumulation of genes mutations. The imbalance between MMPs and TIMPs is incriminated in endometrial carcinogenesis, added to hormonal influences, adhesion molecules alteration, and apoptosis deregulation. Endometrial multistep carcinogenesis shows a partial correlation between molecular markers expression and progression of precursors or aggressiveness of carcinomas [39]. Accumulated data support the involvement of a large spectrum of MMPs and TIMPs in endometrial carcinogenesis, such as MMP-1, MMP-2, MMP-3, MMP-9, MT1-MMP, although MMPs real involvement in endometrial pathology is difficult to evaluate considering stromal remodeling during normal menstrual turnover [33, 40].

The progesterone decreased expression seems to be both responsible for an increased production of pro-MMPs [9, 41] and for MMPs activation [9]. Moreover, MMPs genetic polymorphism is considered as a risk factor for endometrial cancer susceptibility [42].

The effects of MMPs and their inhibitors depend on their ratio in local tumor microenvironment, thus individual markers analysis may not reveal the whole picture of the disease. The imbalance in favor of the proteinases is supported by the molar ratio of MMPs to TIMPs which is higher in carcinoma compared to non-neoplastic control tissues [43].

Numerous studies have demonstrated a high MMP-2, MMP-9, and TIMP-2 expression in both endometrial and cervical carcinoma [15, 44–50].

MMP-2 seems to be the main MMP involved in endometrial carcinomas aggressiveness [50], being activated by MT1-MMP and TIMP-2. MMP-2 stimulates cell proliferation and migration of epithelial cells, via fibronectin degradation. Due to pro-MMP-2 stimulation by MT1-MMP, MMP-2-MT1-MMP co-expression in the tumor invasive front has been reported [49]. MMP-2 shows a strong tumor expression, in correlation with histological grade and type, patients' age, and depth of myometrial invasion [39].

High MMP-2 expression is also correlated to a poor survival in endometrial carcinoma [51].

According to the literature reports, MMP-9 expression shows an increasing expression in endometrial hyperplasia and furthermore in carcinoma, in both stromal and epithelial components [49]. MMP-9 effects are attributed to its high affinity to collagen substrates, the capacity to generate fragments of angiostatin

type, the participation in the protection of tumor cells, and the ability to suppress the proliferation of T cells [52]. MMP-9 expression in smooth myocytes may be correlated to myometrial invasion mechanisms of endometrial neoplasia [49].

Although TIMP-2 expression is increased in endometrial carcinoma, it shows a weaker expression in grade 3 endometrial carcinoma when compared to grade 1 or 2, exhibiting an analogous pattern to that previously reported in breast, colorectal, hepatocellular, and gallbladder carcinomas [44].

Due to their synthesis and translocation from stromal to epithelial cells, MMP-2 and MMP-9 mRNAs have been located in endometrial stroma, including in endothelial cells, macrophages and fibroblasts, mainly in areas within or surrounding tumor aggregates, and show variable expression in epithelial tumor cells [39].

Increased co-expression of MMP-2 and MMP-9 is also correlated to the stage transition and the depth of myometrial invasion [53].

The significance of MMP-2 and TIMP-2 expression is different in terms of prognosis significance. Thus, TIMP-2 overexpression is associated to a favorable prognosis and overall survival in endometrial carcinoma patients but it is still debatable which of the two, MMP-2 or TIMP-2, has superior effect on the prognosis [54]. Moreover, MMP-2 seems to be superior to TIMP-2 in determining the prognosis in endometrial cancer if used separately [55]. Associated strong MMP-2 and weak TIMP-2 expressions have a powerful value of poor prognosis when compared to MMP-2 or TIMP-2 alone.

MMP-2 and TIMP-2 expression patterns are different in the two main types of endometrial cancer [55]. Type II endometrial tumors which consist mainly of serous and clear cell carcinomas show a positive immunoreaction for MMP-2 and negative for TIMP-2 and carry a higher mortality than type I tumors (endometrioid adenocarcinomas) in which MMP-2 immunostaining negativity is associated to a favorable prognosis [56]. Negative MMP-2 and TIMP-2 immunostainings in adenoacanthomas render a prognosis which resembles the prognosis of endometrioid adenocarcinoma [55]. In contrast, histologic types correlated with MMP-2 positivity and TIMP-2 negativity are associated to a poor prognosis.

MMP-9 and Bcl-2 are overexpressed, unlike steroid receptors and CD44-v6 variant, in carcinoma compared to atypical hyperplasia [57]. KAI1 (a metastasis suppressor protein) as well as ER and PR might modulate MMP-2 and MMP-9 expressions in endometrial cancer. The overlapping expression of these biomarkers suggests their possible cooperation, even at early stages of endometrial cancer growth, modulating the speed of tumor cell dissemination [58].

MMP-9 immunoexpression in the areas containing tumor-infiltrating CD3 lymphocytes suggests a possible role of these lymphocytes in mediating the endometrial cancer microenvironment [59].

MMP-9 overexpression is associated to LCN-2 (lipocalin-2) increased expression in several cancers, including high-grade endometrial cancer [60]. Accordingly, the immunopositivity of LCN-2 and MMP-9 are correlated with shorter survival in patients with high-grade endometrial carcinoma, LCN-2 overexpression being

associated with shorter overall and disease-free survival. Moreover, LCN2 immunopositivity has been associated with expression of the angiogenesis marker, VEGF-A, but not with several EMT (epithelial–mesenchymal transition)-related markers, like E-cadherin, N-cadherin, P-cadherin, β-catenin, nor with vascular invasion [61]. Furthermore, LCN2 is significantly associated with distant tumor recurrences, as well as with the S100A family of metastasis related genes, thereby this marker being associated with aggressive features and poor prognosis in endometrial cancer [61].

MMPs immunolocation in endometrial carcinoma reveals correlations between MMP-1 and TIMP-1 expressions and histologic stage, depth of infiltration, histologic type, and patients' age [39].

TIMP-1 slightly decreases in expression in hyperplasia and endometrial carcinoma, in both endometrial components, suggesting a MMP-9: TIMP-1 imbalance in endometrial carcinogenesis [49].

MMP-26 (matrilysin-2 or endometase) promotes matrix destruction in estrogen-dependent tumors, like type I endometrial carcinomas, contributing to malignant progression, by inactivating α1-antitrypsin serpin. Moreover, the transactivation of MMP-26 promoter activity and the enhancing of the endogenous MMP-26 expression by estrogen may represent one of the mechanisms involved in endometrial carcinogenesis [62].

A low constitutive expression of the ECM metalloproteinase inducer (Emmprin) or CD147, a member of the immunoglobulin superfamily [63], has been identified in most cells, being involved in different physiologic processes. High Emmprin expression is observed during remodeling processes associated to embryonic development, wound healing, inflammatory processes [63], and in malignancies. Emmprin has been identified as a modulator of tumor–stromal interaction and of a wide spectrum of molecular events, such as acquisition of anchorage-independent growth capacities, invasive abilities, and tumor angiogenesis [64]. Consequently, Emmprin overexpression in human cancers is correlated with aggressive behavior and poor prognosis. Emmprin modality of action implies stimulation of peritumoral fibroblasts or even tumor cells to produce large amounts of MMPs which facilitate tumor invasion and metastasis processes [65]. Emmprin promotes invasion via activation of urokinase-type plasminogen activator, nuclear factor kappa B (NF-κB), and c-Jun N-terminal kinase (JNK). Moreover, Emmprin stimulates tumor angiogenesis via VEGF [66].

High levels of Emmprin expression have a significant correlation with endometrial carcinoma recurrence [67]. Moreover, increased Emmprin expression is correlated to clinicopathological parameters (histology, depth of myometrial invasion, FIGO stage, cervical involvement, lymphatic vessels involvement, lymph node metastasis, and peritoneal cytology) [68]. Supplementary, strong Emmprin expression is significantly associated with disease-free and overall survival [68]. These findings suggest that low Emmprin expression might have the predictor value of a favorable prognosis in endometrial cancer [68].

EMT occurs during cancer progression, being responsible of tumor cells acquirement of migration, invasion, and metastasizing abilities [69] by induction of E-cadherin transcription repressors, such as Slug, Snail, Smad-interacting protein 1 (SIP1), and Twist [70].

An important role in mesenchymal phenotype promotion is attributed to NF-κB involvement in the transcription of several mesenchymal genes encoding MMP-2, MMP-9, VEGF, and Vimentin [68].

The inhibitory effect of Emmprin on cell proliferation, migration, and invasion in endometrial carcinoma is achieved by NF-κB, TGF-β, EGF, VEGF, MMP-2, and MMP-9 expression and results in increased expression of E-cadherin and reduced levels of Vimentin and Snail, suggesting Emmprin identification as a potential therapeutic target [68].

9 MMPs Involvement in Endometriosis and Updated Therapeutic Approach

Endometriosis development requires accumulation of events represented by the increased capacity of the migrated endometrial tissue to prevent apoptosis, to adhere to and to invade the host tissue, to proliferate, and to stimulate the host angiogenic activity [71]. These may be achieved by alterations in the apoptotic pathways, with upregulation of Bcl-2 and downregulation of Bax, and an increased expression of αvβ3 integrin receptors, as markers of neovascularization and cell adhesion to the extracellular matrix [71]. A recent study using tissue microarrays in endometriotic and normal endometrial tissues has found higher expression of survivin in endometriotic tissues compared to normal endometrium, with a higher glandular expression in non-ovarian than in ovarian endometriotic tissues and lower expression in stromal components [72].

Showing a similar pattern to that previously detected in cancer [73], an increased MMP activity [74] correlated to plasminogen and cathepsin D expression is detected in endometriosis. Moreover, β-catenin mutations result in intercellular adhesion anomalies, associated with E-cadherin mutations, both in endometriosis [75–77] and in female genital malignancies [78]. Consequently, endometriosis management could be oriented to catenin and cadherin signalization, along with MMP selective inhibitors.

In order to perform extracellular matrix degradation and to stimulate the sprouting of new vessels from preexisting capillaries, MMPs, VEGF, IL-8, and COX2 are required [71]. Therefore, increased levels of several MMPs have been detected in peritoneal fluid of patients diagnosed with endometriosis, such as: MMP-1, MMP-2, MMP-7, and MMP-9 or in endometrial tissue, such as MMP-2 and MMP-9 [79]. Moreover, the assessment of MMP-2 and MMP-9 levels in the follicular fluid of infertile patients demonstrates the direct correlation between pelvic endometriosis severity and MMP-2 higher serum levels [80]. Furthermore, MMPs involvement in endometriosis invasiveness is attributed to MMP-2 and

MT1-MMP, in correlation with TIMP-2, feature that underscores its possible key regulatory role [80].

MMPs seem to be activated by IL-1 and TNF-α, in endometriosis. TNF-α exhibits an inhibitory action on TIMP-2 expression in vitro, increasing the MMP-2/MMP-9/TIMP-1: TIMP-2 ratio imbalance [81]. Taking into account the regulatory role of MMP-1, MMP-3, pro-MMP-9, or MMP-9, low TIMP-1 levels may be considered as a result of characteristic MMP-TIMP-1 complexes detected in the endometriotic peritoneal fluid [20]. Supplementary, MMP hemopexin domain is probably recognized by T-like autoantibodies, except MMP-7 [82], as an important pathway of the characteristic immunologic pattern of endometriosis.

A specific MMP, MMP-27, is expressed in a subset of endometrial macrophages (CD45+, CD163+, and CD206+) related to both menstruation and endometriotic lesions, with different patterns of expression in ovarian and peritoneal locations [83].

New data have demonstrated that selected MMPs confer a degree of genetic susceptibility for endometriosis, as haplotypes of angiotensin I-converting enzyme (ACE) and MMP-2 genes are not associated with endometriosis, while those of MMP-1, MMP-3, and MMP-9 genes are related to a high risk for the disease [84]. Moreover, a comparative analysis of the allelic polymorphism of MMPs gene family, which included MMP-3 (rs3025058), MMP-7 (rs11568818), MMP-9 (rs17576, rs2250889), MMP-12 (rs2276109), and MMP-13 (rs2252070), has found important differences in the incidence of particular MMP-3 and MMP-9 allelic combinations in patients with endometriosis [85]. Supplementary, a recent study points out that DNA methylation at the promoter region of MMP-9 gene can enhance the expression of MMP-9 in ectopic endometrial stromal cells [86].

A comparative analysis of MSCs phenotypes, differentiation potential, gene expression for pattern recognition receptors (PRRs) and proinflammatory cytokine release, along with markers of migration and angiogenesis, between eutopic and ectopic locations, showed that TGF-β exhibits significant downregulation, while IL-10 exhibits a significant increase in endometriotic MSCs. Moreover, these cells show an upregulated expression for markers of migration and angiogenesis such as MMP-2, MMP-3, MMP-9, and VEGF, respectively. By exhibiting this distinct immune phenotype, the endometriotic MSCs may be responsible for the reduced immunosuppressive host reaction [87].

Relatively recent data show that Lipoxin A4 (LXA4), a member of the lipid-derived mediators generated at sites of vascular and mucosal inflammation [88], suppresses the development of endometriosis, by anti-inflammatory, anti-proliferative and anti-invasive effects on ectopic endometrial tissue, by p38 MAPK (mitogen-activated protein kinase) downregulation mediated by ALX receptors (LXA4 receptors). LXA4 also decreases the invasive activity of endometriotic stromal cells by suppressing the expression and activity of MMP-9 [89, 90].

The results of a prospective experimental study suggested that resveratrol is a potential agent for the treatment of endometriosis and may be an alternative to leuprolide acetate (LA). This possible therapy has been supported by significantly

reduced immunoreactivity to MMP-2, MMP-9, and VEGF of surgically induced endometriotic implants by administration of resveratrol, LA or both, correlated with decreased plasmatic and peritoneal levels of IL-6, IL-8, and TNF-α, in experimental models [91].

10 MMPs Inhibitors Spectrum and Potential Utility in Endometrium

The hypothesis of therapeutic benefit of MMP inhibition has been based on the complex MMPs involvement in endometrial pathology.

Added to TIMPs, as previously mentioned, several natural inhibitors or compounds obtained from natural sources have been identified, such as tissue factor pathway inhibitor-2, membrane-bound β-amyloid precursor protein (MMP-2 inhibitor), RECK (inhibition of MMP-9, MMP-2, and MT1-MMP), chlorotoxin (MMP-2 inhibitor), α-macroglobulins (inhibitors of the most MMPs), added to hydroxamates (BE16627B and matlystatin B), and BE16627B (broad spectrum MMPs inhibitor) [48, 92].

In the early 80s, the first synthetic MMP inhibitor was produced. Consequently, several MMPs inhibitors have been tested in experimental models, such as: batimastat, CDP-845 (inhibitor of stromelysin-1), RO32-3555 (hydroxamate-based selective gelatinase inhibitor), hydroxamate inhibitors (GI168 and GI173) [93], GI173 (gelatinases inhibitor), GI168 (stromelysin-1 inhibitor), ilomastat or GM6001 (related batimastat compound) [93], marimastat [94], chelating agents (EDTA, 1,10-phenanthroline, or EGTA—inhibitory action on matrixins), cysteine and dithiothreitol (MMP-3 inhibition), phosphonamidate compounds (MMP-3 inhibitors), nonpeptide hydroxamate inhibitors (MMP-3 inhibitors), pseudopeptide-hydroxamate compounds (MMP-3 inhibitors) [95], and SC-44463 (broad range MMP inhibitor and of pro-MMP-3 activation in cultures) [96].

The mechanisms of action had been thought to be: fibrotic changes, angiogenesis inhibition, constriction of invasive growth resulting in an increased interstitial pressure, compression of blood vessels, followed by ischemia and subsequent necrosis, inhibition of collagen degradation, and collagen biosynthesis stimulation ("encapsulation" of primary or secondary tumors) [93, 94].

However, in cancer models, synthetic MMP inhibitors are applied when the tumor diameter is small and they lack stromal tissue, the source of most MMP activity. Therefore, the clinical effects may be amplified.

Considering the local environment, Progesterone may be considered an endometrial MMP inhibitor, acting by negative control of the production of MMP-1 (interstitial collagenase) and MMP-3 (stromelysin-1), in experimental models [97]. Withdrawal of progesterone results in increased stromal cell MMP-1, MMP-2, and MMP-3 production [97], in downregulation of TIMP-2 [98], without any change in expression or secretion of TIMP-1 and TIMP-3 [97].

Progestins are able to continuously inhibit the production of MMP-1 and MMP-3 in cultures that have been designed to mimic the control of long-acting progestogenic contraceptives on endometrium. Although progesterone has the potential to inhibit MMP production by stromal cells within the short term, it may lose the capacity to maintain this inhibition in the longer term administration.

Endometrial stromal cells show variable immunopositivity for endothelin-1 and TIMPs in cultures [99], corresponding to focal endometrial tissue breakdown at menstruation due to local, rather than endocrine regulation. Endothelin-1, TNF-α and IL-1 stimulate MMP-1 and MMP-3 production in short-term culture [100]. Endometrial paracrine actions of both IL-1 [101] and TGF-β [102] may modify the production of certain endometrial MMPs, while mast cells mediators modulate both MMPs production and activation by stromal cells [103]. In slow release progestins users, the endometrium shows an increased number of macrophages, neutrophils, and eosinophils and their products are stimuli for local MMP expression, overriding the inhibitory effect of the progestins on MMP production.

Progestins responses variability could be accounted to endometrial PR subtypes in normal cycling women, compared with those using progestogenic steroids. Surprisingly, women receiving slow release progestins show increased total immunoreactive endometrial PR and PR mRNA [104]. PR B is a stronger activator of target genes while PR A can act as a dominant repressor of PR B and other hormone receptors.

ARs have been demonstrated as cycle dependent in normal endometrium [105], showing a B subtype dominance [106]. Some of the synthetic progestins may also exhibit androgenic activity and regulate MMPs expression by ARs activation [107]. As a consequence, the identification of endometrial ARs and their subtypes could be relevant in administration of synthetic progestins and may offer important information related to their mechanism of endometrial MMPs regulation. In this regard, it is assumed that androgens have a significant role in MMP-1 regulation. The secretion and production of MMP-1 is inhibited by testosterone by specific ARs binding, in the same manner to that noticed for progesterone, in human endometrial stromal cells cultures [37].

11 Conclusions

MMPs are involved in many biological processes, mainly due to their ability of ECM proteolysis and/or capacity to initiate unrevealed functions. Recent progresses were made in understanding biochemical and structural aspects of MMPs, and their molecular complexes with TIMPs allow them to play a complex role in endometrial physiology and pathology by facilitating both turnover and invasion mechanisms.

MMPs: TIMPs ratio imbalances are involved in hormonal disorders and furthermore in hyperplasia, as steps along endometrial malignant transformation. Partially using the mechanisms already identified in carcinogenesis, an increased MMP activity is also detected in endometriosis.

Future studies are necessary to elucidate the complex interactions between molecules involved in proliferation, angiogenesis, and apoptosis, as part of EMT mechanism, opening new perspectives in the early diagnosis and treatment of endometrial neoplasia.

The design of potent specific inhibitors for MMPs represents a challenge for scientists, not only for gaining insights into the biological roles of MMPs but also for the development of new targeted therapies in endometrial neoplasia and endometriosis.

References

1. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function and biochemistry. Circ Res 92:827–839
2. Ii M, Yamamoto H, Adachi Y et al (2006) Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp Biol Med 231:20–27
3. Brummer O, Bohmer G, Hollwitz B et al (2002) MMP-1 and MMP-2 in the cervix uteri in different steps of malignant transformation-an immunohistochemical study. Gynecol Oncol 84:222–227
4. Overall CM (2002) Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. Mol Biotechnol 22:51–86
5. Johansson N, Ala-Aho R, Uitto V et al (2000) Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase. J Cell Sci 113:227–235
6. Curry TE Jr, Osteen KG (2003) The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endocr Rev 24:428–465
7. Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C (2004) Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. Int J Dev Biol 48:411–424
8. Hashizume K (2007) Analysis of utero-placental-specific molecules and their functions during implantation and placentation in the bovine. J Reprod Dev 53:1–11
9. Osteen KG, Igarashi TM, Bruner-Tran KL (2003) Progesterone action in the human endometrium: induction of a unique tissue environment which limits matrix metalloproteinase (MMP) expression. Front Biosci 8:78–86
10. Yadav L, Puri N, Rastogi V et al (2014) Matrix metalloproteinases and cancer—roles in threat and therapy. Asian Pac J Cancer Prev 15:1085–1091
11. Shiomi T, Okada Y (2003) MT1-MMP and MMP-7 in invasion and metastasis of human cancers. Cancer Metastasis Rev 22:145–152
12. Amalinei C, Caruntu ID, Balan R (2007) Biology of metalloproteinases. Rom J Morphol Embryol 48:316–320
13. Wiseman BS, Sternlicht MD, Lund LR et al (2003) Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. J Cell Biol 162:1123–1133
14. Bouloumie A, Sengenes C, Portolan G et al (2001) Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. Diabetes 50:2080–2086
15. Lambert V, Wielockx B, Munaut C et al (2003) MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. FASEB J 17:2290–2292
16. Solberg H, Rinkenberger J, Danø K et al (2003) A functional overlap of plasminogen and MMPs regulates vascularization during placental development. Development 130:4439–4450

17. English JL, Kassiri Z, Koskivirta I et al (2006) Individual TIMP deficiencies differentially impact pro-MMP-2 activation. J Biol Chem 281:10337–10346
18. Lee MH, Murphy G (2004) Matrix metalloproteinases at a glance. J Cell Science 117:4015–4016
19. Mannello F, Gazzanelli G (2001) Tissue inhibitors of metalloproteinases and programmed cell death: Conundrums, controversies and potential implications. Apoptosis 6:479–482
20. Collette T, Bellehumeur C, Kats R et al (2004) Evidence for an increased release of proteolytic activity by the eutopic endometrial tissue in women with endometriosis and for involvement of matrix metalloproteinase-9. Hum Reprod 19:1257–1264
21. Seo DW, Li H, Guedez L et al (2003) TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. Cell 114:171–180
22. Wang WM, Ge G, Lim NH et al (2006) TIMP-3 inhibits the procollagen N-proteinase ADAMTS-2. Biochem J 398:515–519
23. Yu Q, Stamenkovic I (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. Genes Dev 14:163–176
24. Qi JH, Ebrahem Q, Moore N et al (2003) A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. Nat Med 9:407–415
25. Liu K, Wahlberg P, Hagglund AC, Ny T (2003) Expression pattern and functional studies of matrix degrading proteases and their inhibitors in the mouse corpus luteum. Mol Cell Endocrinol 31:131–140
26. Oh J, Seo DW, Diaz T et al (2004) Tissue inhibitors of metalloproteinase 2 inhibits endothelial cell migration through increased expression of RECK. Cancer Res 64:9062–9069
27. Baker AH, Edwards DR, Murphy G (2002) Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 115:3719–3727
28. Jiang Y, Goldberg ID, Shi YE (2002) Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene 28:2245–2252
29. Velasco G, Pendás AM, Fueyo A et al (1999) Cloning and characterization of human MMP-23, a new matrix metalloproteinase predominantly expressed in reproductive tissues and lacking conserved domains in other family members. J Biol Chem 274:4570–4576
30. Freitas S, Meduri G, Le Nestour E et al (1999) Expression of metalloproteinases and their inhibitors in blood vessels in human endometrium. Biol Reprod 61:1070–1082
31. D'Ippolito S, Marana R, Di Nicuolo F et al (2012) Effect of low molecular weight heparins (LMWHs) on antiphospholipid antibodies (aPL)-mediated inhibition of endometrial angiogenesis. PLoS ONE 7(1):e29660. doi:10.1371/journal.pone.0029660
32. Lockwood CJ (2011) Mechanisms of normal and abnormal endometrial bleeding. Menopause 18:408–411
33. Cornet PB, Galant C, Eeckhout Y et al (2005) Regulation of matrix metalloproteinase-9/gelatinase B expression and activation by ovarian steroids and LEFTY-A/endometrial bleeding-associated factor in the human endometrium. J Clin Endocrinol Metab 90:1001–1011
34. Nissi R, Talvensaari-Mattila A, Kotila V et al (2013) Circulating matrix metalloproteinase MMP-9 and MMP-2/TIMP-2 complex are associated with spontaneous early pregnancy failure. Reprod Biol Endocrinol 11:2. doi:10.1186/1477-7827-11-2
35. Moindjie H, Santos ED, Loeuillet L et al (2014) Preimplantation factor (PIF) promotes human trophoblast invasion. Biol Reprod 91:118. doi:10.1095/biolreprod.114.119156
36. Zhuang Y, Qian Z, Huang L (2014) Elevated expression levels of matrix metalloproteinase-9 in placental villi and tissue inhibitor of metalloproteinase-2 in decidua are associated with prolonged bleeding after mifepristone-misoprostol medical abortion. Fertil Steril 101:166–171

37. Lockwood CJ, Basar M, Kayisli UA et al (2014) Interferon-γ protects first-trimester decidual cells against aberrant matrix metalloproteinases 1, 3, and 9 expression in preeclampsia. Am J Pathol 184:2549–2559
38. Ishikawa T, Harada T, Kubota T, Aso T (2007) Testosterone inhibits matrix metalloproteinase-1 production in human endometrial stromal cells in vitro. Reproduction 133:1233–1239
39. Graesslin O, Cortez A, Fauvet M et al (2006) Metalloproteinase-2, -7 and -9 and tissue inhibitor of metalloproteinase-1 and -2 expression in normal, hyperplastic and neoplastic endometrium: a clinical-pathological correlation study. Ann Oncol 17:637–645
40. Galant C, Berliere M, Dubois D et al (2004) Focal expression and final activity of matrix metalloproteinases may explain irregular dysfunctional endometrial bleeding. Am J Pathol 165:83–94
41. Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69:562–573
42. Yi YC, Chou PT, Chen LY et al (2010) Matrix metalloproteinase-7 (MMP-7) polymorphism is a risk factor for endometrial cancer susceptibility. Clin Chem Lab Med 48:337–344
43. Ueno H, Yamashita K, Azumano I et al (1999) Enhanced production and activation of matrix metalloproteinase-7 (matrilysin) in human endometrial carcinomas. Int J Cancer 84:470–477
44. Määtä M, Soini Y, Liakka A, Autio-Harmainen H (2000) Localisation of MT1-MMP, TIMP-1, TIMP-2, and TIMP-3 messenger RNA in normal, hyperplastic, and neoplastic endometrium. Enhanced expression by endometrial adenocarcinomas is associated with low differentiation. Am J Clin Pathol 114:402–411
45. Hojilla CV, Mohammed FF, Khokha R (2003) Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. Br J Cancer 10:1817–1821
46. Amalinei C, Caruntu ID, Balan R et al (2008) Contribution of immunohistochemistry and flow-cytometry in the study of endometrial pathology. Rev Med Chir Soc Med Nat Iasi 112:437–448
47. Amalinei C, Caruntu ID, Giusca SE, Balan RA (2010) Matrix metalloproteinases involvement in pathologic conditions. Rom J Morphol Embryol 51:215–228
48. Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174
49. Amalinei C, Cianga C, Balan R et al (2011) Immunohistochemical analysis of steroid receptors, proliferation markers, apoptosis related molecules, and gelatinases in non-neoplastic and neoplastic endometrium. Ann Anat 193:43–55
50. Park DW, Ryu HS, Choi DS et al (2001) Localization of matrix metalloproteinases on endometrial cancer cell invasion in vitro. Gynecol Oncol 82:442–449
51. Honkavuori M, Talvensaari-Mattila A, Soini Y et al (2007) MMP-2 expression associates with CA 125 and clinical course in endometrial carcinoma. Gynecol Oncol 104:217–221
52. McLaughlin B, Weiss JB (1996) Endothelial-cell-stimulating angiogenesis factor (ESAF) activates progelatinase A (72 kDa type IV collagenase), prostromelysin 1 and procollagenase and reactivates their complexes with tissue inhibitors of metalloproteinases: a role for ESAF in non-inflammatory angiogenesis. Biochem J 317:739–745
53. Aglund K, Rauvala M, Puistola U et al (2004) Gelatinases A and B (MMP-2 and MMP-9) in endometrial cancer—MMP-9 correlates to the grade and the stage. Gynecol Oncol 94:699–704
54. Honkavuori-Toivola M, Talvensaari-Mattila A, Soini Y et al (2012) Immunoreactivity for TIMP-2 is associated with a favorable prognosis in endometrial carcinoma. Tumor Biol 33:935–941
55. Honkavuori-Toivola M, Santala M, Soini Y et al (2013) Combination of strong MMP-2 and weak TIMP-2 immunostainings is a significant prognostic factor in endometrial carcinoma. Dis Markers 35:261–266

56. Talvensaari-Mattila A, Santala M, Soini Y, Turpeenniemi-Hujanen T (2005) Prognostic value of matrix metalloproteinase-2 (MMP-2) expression in endometrial endometrioid adenocarcinoma. Anticancer Res 25:4101–4105
57. Laas E, Ballester M, Cortez A et al (2014) Supervised clustering of immunohistochemical markers to distinguish atypical endometrial hyperplasia from grade 1 endometrial cancer. Gynecol Oncol 133:205–210
58. Grybos A, Bar J (2014) The relationships between the immunoexpression of KAI1, MMP-2, MMP-9 and steroid receptors expression in endometrial cancer. Folia Histochem Cytobiol 52:187–194
59. Jedryka M, Chrobak A, Chelmonska-Soyta A et al (2012) Matrix metalloproteinase (MMP)-2 and MMP-9 expression in tumor infiltrating CD3 lymphocytes from women with endometrial cancer. Int J Gynecol Cancer 22:1303–1309
60. Srdelić Mihalj S, Kuzmić-Prusac I, Zekić-Tomaš S et al (2014) Lipocalin-2 and matrix metalloproteinase-9 expression in high-grade endometrial cancer and their prognostic value. Histopathology 17. doi:10.1111/his.12633
61. Mannelqvist M, Stefansson IM, Wik E et al (2012) Lipocalin 2 expression is associated with aggressive features of endometrial cancer. BMC Cancer 12:169
62. Nishi H, Kuroda M, Isaka K (2013) Estrogen and estrogen receptor induce matrix metalloproteinase-26 expression in endometrial carcinoma cells. Oncol Rep 30:751–756
63. Nabeshima K, Iwasaki H, Nishio J et al (2006) Expression of emmprin and matrix metalloproteinases (MMPs) in peripheral nerve sheath tumors: emmprin and membrane-type (MT)1-MMP expressions are associated with malignant potential. Anticancer Res 26:1359–1367
64. Yan L, Zucker S, Toole BP (2005) Roles of the multifunctional glycoprotein, emmprin (basigin; CD147), in tumour progression. Thromb Haemost 93:199–204
65. Muramatsu T, Miyauchi T (2003) Basigin (CD147): a multifunctional transmembrane protein involved in reproduction, neural function, inflammation and tumor invasion. Histol Histopathol 18:981–987
66. Tang Y, Nakada MT, Kesavan P et al (2005) Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. Cancer Res 65:3193–3199
67. Ueda K, Yamada K, Urashima M et al (2007) Association of extracellular matrix metalloproteinase inducer in endometrial carcinoma with patient outcomes and clinico-pathogenesis using monoclonal antibody 12C3. Oncol Rep 17:731–735
68. Nakamura K, Kodama J, Hogo A, Hiramatsu Y (2012) Role of emmprin in endometrial cancer. BMC Cancer 12:191–201
69. Yang J, Weinberg RA (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 14:818–829
70. Yang J, Mani SA, Donaher JL et al (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117:927–939
71. Khoufache K, Bazin S, Girard K et al (2012) Macrophage migration inhibitory factor antagonist blocks the development of endometriosis in vivo. PLoS ONE 7:e37264
72. Londero AP, Calcagno A, Grassi T et al (2012) Survivin, MMP-2, MT1-MMP, and TIMP-2: their impact on survival, implantation, and proliferation of endometriotic tissues. Virchows Arch 461:589–599
73. Suzumori N, Ozaki Y, Ogasawara M, Suzumori K (2001) Increased concentrations of cathepsin D in peritoneal fluid from women with endometriosis. Mol Hum Reprod 7:459–462
74. Mizumoto H, Saito T, Ashihara K et al (2002) Expression of matrix metalloproteinases in ovarian endometriomas: immunohistochemical study and enzyme immunoassay. Life Sci 71:259–273
75. Scotti S, Regidor PA, Schindler AE, Winterhager E (2000) Reduced proliferation and cell adhesion in endometriosis. Mol Hum Reprod 6:610–617

76. Chen GT, Tai CT, Yeh LS et al (2002) Identification of the cadherin subtypes present in the human peritoneum and endometriotic lesions: potential role for P-cadherin in the development of endometriosis. Mol Reprod Dev 62:289–294
77. Ueda M, Yamashita Y, Takehara M et al (2002) Gene expression of adhesion molecules and matrix metalloproteinases in endometriosis. Gynecol Endocrinol 16:391–402
78. Moreno-Bueno G, Gamallo C, Pérez-Gallego L et al (2001) beta-Catenin expression pattern, beta-catenin gene mutations, and microsatellite instability in endometrioid ovarian carcinomas and synchronous endometrial carcinomas. Diagn Mol Pathol 10:116–122
79. Jana SK, Banerjee P, Mukherjee R et al (2013) HOXA-11 mediated dysregulation of matrix remodeling during implantation window in women with endometriosis. J Assist Reprod Genet 30:1505–1512
80. Malvezzi H, Aguiar VG, Paz CC et al (2013) Increased circulating MMP-2 levels in infertile patients with moderate and severe pelvic endometriosis. Reprod Sci 20:557–562
81. Antsiferova Y, Sotnikova N (2012) The local immune mechanisms involved in the formation of endometriotic lesions. In: Chaudhury K, Chakravarty B (eds) Endometriosis—basic concepts and current research trends. ISBN 978-953-51-0524-4, 11: 211-244. doi:10.5772/29965
82. Yeaman GR, Collins JE, Lang GA (2002) Autoantibody responses to carbohydrate epitopes in endometriosis. Ann N Y Acad Sci 955:174–182
83. Cominelli A, Gaide Chevronnay HP, Lemoine P et al (2014) Matrix metalloproteinase-27 is expressed in CD163+/CD206+ M2 macrophages in the cycling human endometrium and in superficial endometriotic lesions. Mol Hum Reprod 20:767–775
84. Trovó de Marqui AB (2012) Genetic polymorphisms and endometriosis: contribution of genes that regulate vascular function and tissue remodeling. Rev Assoc Med Bras 58:620–632
85. Iarmolinskaia MI, Molotkov AS, Bezhenar VF et al (2014) Association of matrix metalloproteinases' polymorphisms of MMP3 and MMP9 with development of genital endometriosis. Genetika 50:230–235
86. Yuan C, Zhang L, Gao Y et al (2014) DNA demethylation at the promoter region enhances the expression of MMP-9 in ectopic endometrial stromal cells of endometriosis. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 30:1258–1261
87. Koippallil Gopalakrishnan Nair AR, Pandit H, Warty N, Madan T (2015) Endometriotic mesenchymal stem cells exhibit a distinct immune phenotype. Int Immunol 27:195–204
88. Fierro IM, Colgan SP, Bernasconi G et al (2003) Lipoxin A4 and aspirin-triggered 15-epi-LipoxinA4 inhibit human neutrophil nigration: comparisons between synthetic 15 epimers in chemotaxis and transmigration with microvessel endothelial cells and epithelial cells. J Immunol 170:2688–2694
89. Kumar R, Clerc AC, Gori I et al (2014) Lipoxin A_4 prevents the progression of de novo and established endometriosis in a mouse model by attenuating prostaglandin E_2 production and estrogen signaling. PLoS ONE 24:e89742
90. Wu R, Zhou W, Chen S et al (2014) Lipoxin A4 suppresses the development of endometriosis in an ALX receptor-dependent manner via the p38 MAPK pathway. Br J Pharmacol 171:4927–4940
91. Bayoglu Tekin Y, Guven S, Kirbas A et al (2015) Is resveratrol a potential substitute for leuprolide acetate in experimental endometriosis? Eur J Obstet Gynecol Reprod Biol 184:1–6
92. Tamaki K, Tanzawa K, Kurihara S et al (1995) Synthesis and structure-activity relationships of gelatinase inhibitors derived from matlystatins. Chem Pharm Bull 43:1883–1893
93. Conway JG, Trexler SJ, Wakefield JA et al (1996) Effect of matrix metalloproteinase inhibitors on tumor growth and spontaneous metastatsis. Clin Exp Metastasis 14:115–124
94. Brown PD (1998) Matrix metalloproteinase inhibitors. Angiogenesis 1:142–154
95. Bottomley KM Borkakoti N, Bradshaw D et al (1997) Inhibition of bovine nasal cartilage degradation by selective matrix metalloproteinase inhibitors. Biochem J 323:483–488

96. Rigot V, Marbaix E, Lemoine P et al (2001) In vivo perimenstrual activation of progelatinase B (proMMP-9) in the human endometrium and its dependence on stromelysin 1 (MMP-3) ex vivo. Biochem J 358:275–280
97. Salamonsen LA, Butt AR, Hammond FR et al (1997) Production of endometrial matrix metalloproteinases, but not their tissue inhibitors, is modulated by progesterone withdrawal in an in vitro model for menstruation. J Clin Endocrinol Metab 82:1409–1415
98. Prifti S, Lelle I, Zhong G et al (2004) Matrix metalloproteinase 2 and tissue inhibitor of metalloproteinase 2 expression is not regulated by norgestimate or norelgestromin. Gynecol Endocrinol 18:23–27
99. Hampton AL, Nie G, Salamonsen LA (1999) Progesterone analogues similarly modulate endometrial matrix metalloproteinase-1 and matrixmetalloproteinase-3 and their inhibitor in a model for long-term contraceptive effects. Mol Hum Reprod 5:365–371
100. Rawdanowicz TJ, Hampton AL, Nagase H et al (1994) Matrix metalloproteinase production by cultured human endometrial stromal cells: identification of interstitial collagenase, gelatinase-A, gelatinase-B, and stromelysin-1 and their differential regulation by interleukin-1 alpha and tumor necrosis factor-alpha. J Clin Endocrinol Metab 79:530–536
101. Singer CF, Marbaix E, Lemoine P et al (1999) Local cytokines induce differential expression of matrix metalloproteinases but not their tissue inhibitors in human endometrial fibroblasts. Eur J Biochem 259:40–45
102. Bruner KL, Rodgers WH, Gold LI et al (1995) Transforming growth factor beta mediates the progesterone suppression of an epithelial metalloproteinase by adjacent stroma in the human endometrium. Proc Natl Acad Sci USA 92:7362–7366
103. Zhang Y, McCluskey K, Fujii K, Wahl LM (1998) Differential regulation of monocyte matrix metalloproteinase and TIMP-1 production by TNF-alpha, granulocyte-macrophage CSF, and IL-1 beta through prostaglandin-dependent and -independent mechanisms. J Immunol 161:3071–3076
104. Lau TM, Witjaksono J, Affandi B, Rogers PA (1996) Expression of progesterone receptor mRNA in the endometrium during the normal menstrual cycle and in Norplant users. Hum Reprod 11:2629–2634
105. Mertens HJ, Heineman MJ, Koudstaal J et al (1996) Androgen receptor content in human endometrium. Eur J Obstet Gynecol Reprod Biol 70:11–13
106. Wilson CM, McPhaul MJ (1996) A and B forms of the androgen receptor are expressed in a variety of human tissues. Mol Cell Endocrinol 120:51–57
107. Schneikert J, Peterziel H, Defossez PA et al (1996) Androgen receptor-Ets protein interaction is a novel mechanism for steroid hormone-mediated down-modulation of matrix metalloproteinase expression. J Biol Chem 271:23907–23913

Plasmodium Proteases as Therapeutic Targets Against Malaria

4

Asrar Alam

Abstract

Malaria is a major global parasitic disease responsible for tremendous health burden and mortality in tropical and subtropical regions of the world. *Plasmodium falciparum* is the causative agent of severe malaria, which accounts for most of the global malaria-related deaths, mainly in sub-Saharan Africa. Despite the enormous global efforts to curb the spread of the disease and significant decline in malaria-related deaths in the last decade, development of parasite resistance to currently used drugs is widespread, which necessitates the development of novel antimalarial targeting crucial parasite molecules. Parasite proteases are a group of molecules crucial for the development and propagation of the parasite inside the host cell. The major parasite-specific processes dependent on protease activity for their completion are hemoglobin degradation, merozoite egress from the host cell, and invasion of the host cells. A number of proteases of various classes are found in *P. falciparum*, many of which have the potential to be used as antimalarial drug targets. I this chapter, I have described the role of the proteases, which have the potential to be targeted for antimalarial drug development and the progresses made in the direction of drug development against these targets.

Keywords

Malaria · *Plasmodium falciparum* · Asexual blood stages · Proteases · Hemoglobin degradation · Merozoite egress · Merozoite invasion · Other cellular proteases

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1 Introduction

Malaria is an ancient disease caused by protozoan parasite of the genus *Plasmodium*. Till date malaria remains a major health burden for impoverished regions of the world with limited sanitation and healthcare facilities. In humans, malaria is caused by five *Plasmodium* species, namely, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae,* and *P. cynomolgi*. Of the five species, *P. falciparum* is the causative agent of severe malaria, responsible for most of the malaria-related deaths. Due to the combined global efforts to curb the menace of malaria, disease cases have come down by 37% globally and by 42% in Africa between 2000 and 2015. The more encouraging outcome of the efforts has been the drastic reduction in malaria-related deaths that have come down by 60% globally and 66% in the African regions in the same time span [1].

Despite these successes in malaria control, complete malaria eradication remains an ambitious goal for the global scientific community and healthcare providers. A completely protective malaria vaccine is still a dream for the scientists and the available arsenals of antimalarial drugs are limited. Till date major antimalarials are natural compounds or their derivatives and the knowledge about their mechanisms of action are limited. In addition, the ability of the parasite to develop resistance against antimalarials is common. The parasite quickly develops resistance against the extensively used antimalarials, especially when they are used as a monotherapy, although successes have been achieved to counter drug resistance using combination therapy [2–11]. Due to above-mentioned reasons, development of novel antimalarial drugs remains a high priority.

For development of new drugs, identification of crucial parasite-specific molecules is required, which could be used for specific targeting. One group of such molecules is parasite proteases. They constitute the major virulence factors in various parasitic diseases and are crucial for the pathogens for their survival and ability to cause the diseases [12]. Malarial proteases are crucial molecules for parasite development and virulence. Some of these proteases possess parasite-specific functions and structural features that could be specifically targeted by drugs. Potential of these molecules as antimalarial drug targets has been substantiated by the use of protease inhibitors as drugs against a number of diseases. Protease inhibitors are already in use as drugs against human immunodeficiency virus (HIV) [13], hepatitis C virus (HCV) [14], and in the treatment of hypertension [15] and coagulopathies [16].

These groups of molecules are widespread in organisms and carry out a variety of biological processes by regulating the fate, localization, and activity of the target proteins [17]. Through evolution they have adapted to a variety of physiological conditions found in complex organisms and employ different mechanisms for substrate hydrolysis. Depending on the mechanisms of action, proteases have been classified into different classes (serine, cysteine, metallo, aspartic, threonine, and glutamic proteases). Serine, threonine, and cysteine proteases rely upon the nucleophilic character of the side chains of serine, threonine, and cysteine, respectively, at

the active site. These side chains directly attack the peptide bond to form a transient, covalent enzyme–substrate intermediate, which breaks to give rise to the product and the enzyme. Aspartic and metalloproteases employ water molecule as nucleophile and do not form a covalent intermediates [18]. A number of proteases of various classes are found in *Plasmodium*. This chapter describes the available information on *P. falciparum* proteases crucial for parasite development in the human host and the studies related to drug development efforts to target these molecules.

2 Proteases as Potential Antimalarial Drug Targets

Malaria parasite completes its life cycle inside the vertebrate and invertebrate host cells. The host cell provides a rich source of nutrients for the parasite and a safe niche where the parasite is protected from the host immune responses. Asexual erythrocytic life cycle is responsible for the clinical manifestations of malaria, which starts when the merozoites released from liver infect the erythrocytes. The intracellular parasite feeds on erythrocyte hemoglobin and develops into more metabolically active trophozoite, which then undergoes nuclear division to transform into multinucleated schizont. After maturation, schizonts rupture to release the merozoites, which start a new wave of infection by invading new erythrocytes.

Completion of parasite life cycle in vertebrate and invertebrate hosts is dependent on a number of proteolytic activities. Inhibitor studies have shown that cysteine, aspartic, metallo, and serine proteases play critical roles in parasite-specific processes during the erythrocytic cycle such as hemoglobin digestion, merozoite release from infected erythrocytes, and invasion of fresh erythrocytes by the released merozoites. In addition a number of proteases are involved in metabolic processes necessary for parasite survival (Fig. 1). These processes have been the focus of drug development efforts [19]. In addition to erythrocytic stages, liver stages of the parasite are also attractive drug targets due to their low numbers and distinct metabolism [20, 21]. Liver stage parasites also employ proteolytic activities for their maturation and subsequent dispersal in the blood stream [22, 23]. Aspartate, cysteine, and metalloproteases are involved in hemoglobin degradation, a pathway necessary for parasite growth in the blood stages, whereas serine and cysteine proteases are crucial for the parasite egress and invasion at blood and liver stages. Table 1 summarizes the role of parasite proteases in *P. falciparum* development and propagation. Data mining revealed the presence of 92 putative proteases in *P. falciparum* genome [24]. Further analysis of *P. falciparum* genome database revealed the presence of 124 predicted proteases [25]. The roles of proteases in various parasite-specific processes and their potential as drug targets are described below.

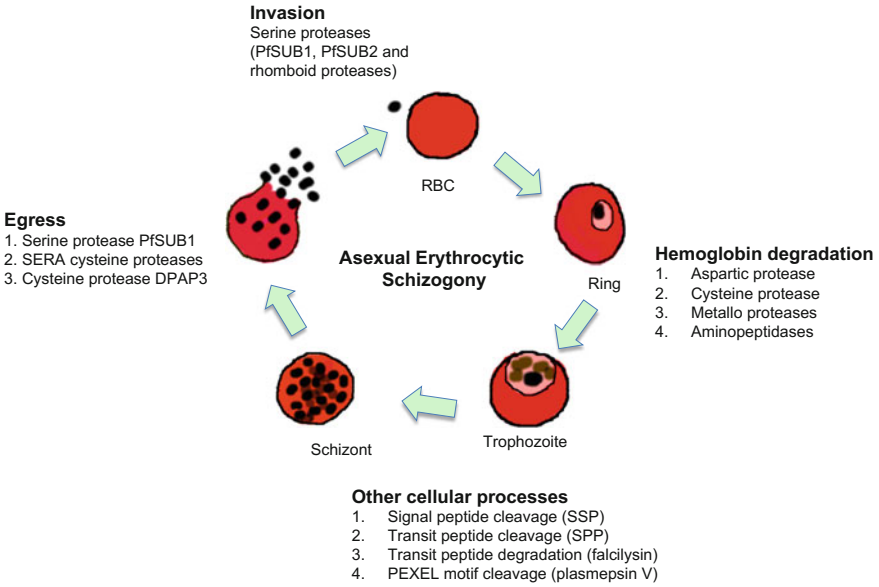

Fig. 1 Role of proteases in asexual erythrocytic life cycle of malaria parasite

3 Proteases Involved in Hemoglobin Digestion

P. falciparum has a limited capacity to synthesize amino acids [26]. In the blood stages, it relies on degradation of abundant host cell molecule hemoglobin for its utilization [27, 28]. During the trophozoite stage, parasite proteases degrade most of the hemoglobin [29] and supply the parasite with amino acids for protein synthesis and metabolism [30]. *Plasmodium* ingests hemoglobin by an invagination spanning from parasite plasma membrane to parasitophorous vascuolar membrane known as cytostome. The cytostome takes host cell cytoplasm and pinches off to form double-membrane transport vesicles loaded with hemoglobin, which fuse with food vacuoles to empty their contents there. Food vacuoles are acidic compartments with a pH between 5.0 and 5.4 [31, 32] and provide the site for hemoglobin degradation and heme detoxification [33].

Hydrolysis of hemoglobin is a semi-ordered process mediated by the action of a series of proteases. Aspartic [34–36] and metalloproteases [37] are involved in hemoglobin degradation in the food vacuole. Four proteases predominantly carry out hemoglobin degradation (aspartic proteases plasmepsin 1 and 2, cysteine protease falcipain 2, and metalloprotease falcilysin) [38]. Plasmepsin 1 and 2 possess the ability to cleave hemoglobin under non-denaturing conditions between Phe33 and Leu34 in the hinge region of the alpha globin chain, which probably allows the globin subunits to unwind that facilitates further proteolysis [33, 39]. After the initial cleavage by plasmepsins 1 and 2, falcipain 2 cleaves the globin fragments

Table 1 Role of proteases in development of *P. falciparum* parasitic stages

Parasitic-specific process	Class of protease involved	Name of the protease	Putative function	References
Hemoglobin degradation	Aspartic	Plasmepsin 1	Food vacuole hemoglobinase (initial cleavage of hemoglobin)	[41, 44]
		Plasmepsin 2	Food vacuole hemoglobinase (initial cleavage of hemoglobin)	[41, 44]
		Plasmepsin 4	Food vacuole hemoglobinase	[41, 44]
		Histo-aspartic protease	Food vacuole hemoglobinase	[41, 44]
	Cysteine	Falcipain 2	Food vacuole hemoglobinase (active at early trophozoite stage)	[57–59]
		Falcipain 3	Food vacuole hemoglobinase (active at late trophozoite stage)	[57–59]
	Metallo	Falcilysin	Food vacuole hemoglobinase (hydrolysis of peptide fragments generated by aspartic and cysteine proteases)	[37]
	Amino	M1 alanyl aminopeptidase	Hydrolysis of peptides generated by hemoglobin degradation; essential for parasite growth	[70, 71]
		M17 leucine aminopeptidase	Hydrolysis of peptides generated by hemoglobin degradation, essential for parasite growth	[70, 71]
Egress	Aspartic	Plasmepsin 2	In vitro digestion of spectrin, protein 4.1 and actin (probable role in RBC cytoskeleton degradation)	[84]
	Cysteine	Falcipain 2	In vitro digestion of ankyrin and protein 4.1 (probable role in RBC cytoskeleton degradation)	[85, 86]
		SERA5	Merozoite egress from RBCs (exact mechanism unknown)	[100–103]
		SERA6	Active site Cys mutant and blockade of processing by PfSUB1 lethal for parasite (probable role in egress)	[104]
		DPAP3	Probable role in PfSUB1 maturation	[101]
	Serine	PfSUB1	Mediates egress by causing maturation of SERA5 and SERA6	[100]
Invasion	Serine	PfSUB1	Primary processing of MSP1 complex	[123]
		PfSUB2	Secondary processing of MSP1 complex (sheddase activity) and processing of AMA1	[115, 124, 125]
		PfROM1	Intramembrane cleavage of AMA1	[125, 130]
		PfROM4	Intramembrane cleavage of EBA-175	[131]
Other cellular processes	Threonine	ClpQ	Exact substrate not known	[143]
		Stromal processing protease (SPP)	Cleavage of transit peptide	[146]
	Metallo	Falcilysin	Degradation of transit peptide	[69]

(continued)

Table 1 (continued)

Parasitic-specific process	Class of protease involved	Name of the protease	Putative function	References
	Serine	ClpP	Exact substrate not known	[147]
	Signal peptidase	SSP	Cleavage of signal peptide	[24, 150]
	Aspartic	Plasmepsin V	Cleavage of PEXEL motif	[155]

[38]. It has been demonstrated that plasmepsin 1 can degrade native hemoglobin efficiently under nonreducing conditions while plasmepsin 2 has a preference for denatured hemoglobin. Although falcipains cleave denatured globin but not native hemoglobin [39], under mild reducing conditions falcipain 2 cleaves both native and denatured globin [40]. Falcilysin further cleaves the globin fragments into smaller peptides [41]. The digested peptides are then transported to parasite cytosol, where cytosolic aminopeptidases hydrolyse these peptides to free amino acids [42].

Human hemoglobin lacks a single amino acid isoleucine. Liu et al. [43] demonstrated that *P. falciparum* could grow in a medium containing only a single amino acid isoleucine. This finding indicated that host hemoglobin is sufficient to provide the rest of the amino acid requirements of the parasite. Knockout parasite lines of the hemoglobin-degrading enzymes (falcipain-2, plasmepsins, alone or in combination) grown in this medium showed reduced growth. A potent inhibitor of plasmepsin pepstatin A did not significantly affect the growth of wild-type parasites but was able to kill the parasites lacking falcipain-2 at low concentrations. It was even more effective in killing the parasites triple knockout parasites lacking falcipain-2, plasmepsin 1, and 4. This study provided evidence that both plasmepsins and falcipain-2 are hemoglobinases with overlapping functions. While falcipain-2 was essential for parasite survival plasmepsins provided subsidiary roles and were dispensable for the parasite [43], different classes of hemoglobin-degrading proteases and their role in the parasite are summarized below.

3.1 Aspartic Proteases (Plasmepsins)

P. falciparum genome data predicted the presence of 10 plasmepsin genes. Expression, localization, and enzymatic data suggested that four of the plasmepsins (plasmepsin 1, plasmepsin 2, plasmepsin 4, and a histo-aspartic protease (HAP)) were found in the food vacuole and were capable to degrade hemoglobin [41, 44]. Gene knockout of *P. falciparum* food vacuole plasmepsins revealed that growth rates were reduced by 30–35% upon plasmepsin 1 and plasmepsin 4 disruption, whereas disruption of plasmepsin 2 did not cause any growth defect. Disruption of HAP caused a slight drop in growth rates that was not statistically significant [45]. In another similar study, targeted gene disruption by double crossover homologous recombination was carried out for plasmepsin 1, plasmepsin 2, HAP, and

plasmepsin 4/plasmepsin 1. All the four knockout parasites were morphologically normal. In amino acid-limited medium, they exhibited slower growth rate as compared to the parental strain. Sensitivity of the knockout parasites to aspartic and cysteine protease inhibitors changed minimally, compared to wild-type 3D7 parasite, suggesting the functional redundancy of these proteases [46].

Among the two major hemoglobin-degrading plasmepsins, 1 and 2, plasmepsin 2 was found to be 5–10-fold more efficient on peptide substrates and also had 80-fold higher inhibition constant compared to plasmepsin 1 [47]. Because of their higher hemoglobin-degrading activities, a number of studies on drug screening have been focused on plasmepsins 1 and 2 [48–54]. Due to their redundant function, it is suggested that in order to achieve higher antiparasite activity, as many as of these enzymes have to been targeted by the inhibitors [55] and inhibitors targeting multiple plasmepsins could be used as antimalarials [56].

3.2 Cysteine Proteases (Falcipains)

P. falciparum genome encodes four clan CA cysteine proteases known as falcipains (falcipain 1, falcipain 2, falcipain 2′, and falcipain 3). These proteases have been biochemical and genetically characterized [57–59]. Genetic disruption of falcipain 2 caused block in hemoglobin degradation resulting in accumulation of undegraded hemoglobin in the food vacuole, similar to that caused by cysteine protease inhibitors [58]. Falcipain 3 was refractory to genetic deletion, although a tagged copy of the gene could readily be produced. Falcipain 1 and falcipain 2′ were dispensable for the parasite as their disruption did not cause any defect in parasite phenotype [59]. Based on these studies, falcipains 2 and 3 appear to be crucial hemoglobinases with redundant functions [57–59].

Activities of falcipain 2 and 3 have been assessed on peptide substrates in vitro. Falcipain 2 accounted for 90% of the cysteine protease activity on peptide substrates in trophozoite lysates [40], while falcipain 3 showed relatively lesser activity on peptide substrates [57]. Differential activities of these enzymes correlated with their timings of expression. Falcipain 2 was predominantly expressed in early trophozoites when parasite growth is maximum and hence maximum hemoglobin degradation is required while falcipain 3 showed maximum expressions in late trophozoites when the nutrient requirement is minimal and parasite prepares for division [58]. Falcipain 2′ did not exhibit any biochemical differences with falcipain 2. Gene knockout of falcipain 2′ did not cause any phenotypic defect in the parasite and the biological role of this enzyme remains unknown [60–62].

Due to the crucial role of falcipains 2 and 3, numerous studies have been focused on designing of their inhibitors [38, 63]. These inhibitors caused accumulation of undegraded hemoglobin in the food vacuole resulting in swollen morphology. They have also inhibited parasite development and have been effective in cure of experimental malaria [28, 40, 64–67].

3.3 Metalloproteases

Following degradation by aspartic and cysteine proteases, peptide fragments generated from hemoglobin are further hydrolyzed by metalloprotease falcilysin [37]. Specificity analysis using hydrolysis of a series of random peptide substrates revealed that the enzyme was highly active at acidic pH, consistent with its role in hemoglobin degradation. Surprisingly, the enzyme also showed robust activity at neutral pH and with slightly different substrate specificities [68]. Further studies revealed that in addition to food vacuole falcilysin was also translocated to the apicoplast. Based on in vitro studies, it was suggested that this enzyme might have a role in transit peptide hydrolysis [69].

3.4 Aminopeptidases

Nine aminopeptidases are annotated in *P. falciparum* genome. These enzymes catalyze the hydrolysis of amino acids from the N-terminus. Five aminopeptidases are involved in hydrolysis of peptides generated by hemoglobin digestion [70]. Two of these enzymes M1 alanyl aminopeptidase (PfM1AAP) and M17 leucine aminopeptidase (PfM17LAP) are essential for *P. falciparum* growth and development as inhibitors of these peptidases were found to be lethal to *P. falciparum* culture in vitro [71].

4 Proteases Involved in Parasite Egress from Erythrocytes

Erythrocyte rupture by malaria parasites is a temporally regulated process. Different models of erythrocyte rupture have been proposed based on live video microscopic and electron microscopic studies [72–76]. Although the exact mechanism of egress is obscure, the most widely experimentally supported model of erythrocyte rupture suggests that egress is a two-step process, in which the degradation of parasitophorous vacuole membrane takes place prior to RBC membrane rupture [74]. This model was supported by electron microscopy (EM)-based evidences demonstrating that highly mature schizonts occasionally lack a discrete parasitophorous vacuole membrane (PVM) [77, 78]. Similar results were obtained by immune electron microscopy of mature schizonts with antibody against parasitophorous vacuole (PV) resident protein S-antigen [74].

Mechanistically egress consists of destabilization of erythrocyte cytoskeleton, PVM, and the erythrocyte membrane. Studies based on serine, cysteine, and aspartic protease inhibitors suggest that egress is dependent on proteolytic activities [79–83]. Analysis on unruptured schizonts in the protease inhibitor-treated parasites revealed the defects in processing of merozoite surface protein (MSP1) [82] and PV protein p126 that is now known as SERA5 [83].

In vitro studies have suggested that hemoglobin-degrading proteases plasmepsin 2 and falcipain 2 could also have role in egress. Plasmepsin 2 digested erythrocyte cytoskeleton proteins spectrin, protein 4.1, and actin at neutral pH in vitro. Detection of this protease in the cytoplasm of schizont-infected erythrocytes was also suggestive of its role in destabilization of host cell membrane skeleton [84]. Falcipain 2 digested cytoskeleton proteins ankyrin and protein 4.1 at neutral pH in vitro [85, 86]. A peptide based on ankyrin cleavage site was found to be inhibitory to falcipain 2 activity and merozoite egress when added to schizont-stage parasites. Localization of falcipain 2 in PV and subcellular structures extending into the erythrocyte cytosol also provide strength to its possible involvement in erythrocyte cytoskeleton destabilization [87]. Surprisingly, plasmepsin 2 and falcipain 2 were dispensable for the parasite as shown by gene disruption studies and these knockout lines showed no defect in egress [43, 45, 46, 57, 58]. Although the role of these proteases in egress has not been conclusively ascertained, they seem to be redundant in function [88].

The role of a protease in egress was demonstrated in an exquisite study by Aly and Matuschewski [89] using reverse genetics approach. They showed that disruption of a *P. berghei* gene termed as "egress cysteine protease 1" (*ecp1*) inhibited the release of sporozoites from mosquito oocyst [89]. *ecp1* belongs a family of cysteine proteases known as serine repeat antigens (SERAs). There are nine members of SERA proteases found in *P. falciparum* genome. Eight of them are arranged on chromosome 2 in a head-to-tail tandem orientation, whereas the ninth member SERA9 is located on chromosome 9 [90]. *P. berghei ecp1* was found to be ortholog of *P.falciparum* SERA8.

The central regions of SERA proteins show homology to papain family cysteine proteases. However, in some SERAs active site cysteine is substituted with a serine residue [91–93]. In *P. falciparum*, SERAs 1–5 and SERA9 possess active site serine and SERAs 6–8 possess active site cysteine [93]. Proteolytic activity of *P. falciparum* SERA5, the most highly expressed member, has been demonstrated in vitro on peptide substrates [94]. To study the essentiality of *P. falciparum* SERAs, gene knockout studies were conducted. All but SERA5 and SERA6 could not be disrupted suggesting the essential function of these proteins for the parasite [90, 95].

SERA5, also known as SERA, is the most highly expressed member and a number of studies have suggested its involvement in merozoite egress from erythrocytes. It is synthesized as a 126 kDa precursor (p126) [96] and secreted into the lumen of the parasitophorous vacuole. Around the time of schizont rupture, it undergoes proteolytic processing into multiple fragments. These processing seem to be essential for its role in egress. The full-length p126 is processed into a 47-kDa N-terminal and a 73-kDa fragment (P73) C-terminal fragments. P73 is processed into 56-kDa and 18-kDa fragments and the 56-kDa fragment is further processed to form a 50-kDa central domain and a 6-kDa fragment [97]. The complex of N-terminal 47-kDa and C-terminal 18-kDa fragments is found associated with the merozoite surface and the central 50-kDa fragment is detected in the

culture medium after schizont rupture [98, 99]. The cleavage that produces p47 and p73 from p126 and cleavage of p73 to p56 and p18 is carried out by PfSUB1. p56 is cleaved by an unknown protease to produce p50 and a 6-kDa fragment [88].

Evidence for involvement of SERA5 and other SERA family members came from inhibitor studies. A specific inhibitor of PfSUB1 was found to block the rupture of schizonts. Further biochemical analysis revealed that PfSUB1 mediated rupture of schizonts through the processing of SERA5 and other SERA family members including SERA4 and SERA6 [100]. In a similar but reciprocal approach to identify the mediators of merozoite egress, using a chemical screen of 1200 covalent serine and cysteine protease inhibitors demonstrated that PfSUB1 and a cysteine protease DPAP3 as the regulators of this process. This study demonstrated that chemical blocking of PfSUB1 and DPAP3 resulted in blockade of SERA5 processing, correlating with the blockade in schizont rupture. Inhibition of DPAP3 resulted in reduced level of mature PfSUB1 suggesting that both the proteases regulate the efficient release of merozoites from the infected red blood cells by SERA5 processing [101]. Attempts to target the enzyme domain of SERA5 using peptides resulted in the block of merozoite egress [102]. In another study, incubation of *P. falciparum* culture with the SERA5 prodomain and a peptide derived from it resulted in block in schizont rupture [103]. These studies also suggested the role of SERA5 in merozoite egress and the possibility of targeting the protein for parasite growth inhibition. In addition to SERA5, SERA6 is also supposed to have role in merozoite egress. It is cleaved in the PV by PfSUB1 just before egress. Mutation of active site cysteine or the blockade in processing by PfSUB1 was lethal for the parasite. Processing of *P. berghei* ortholog of SERA6 (PbSERA3) by PfSUB1 converted it into an active cysteine protease. Taken together, these findings suggest the role of SERA6 in merozoite egress [104].

Based on these studies, PfSUB1 appears to be involved in maturation of multiple SERA proteases and hence is an important regulator of merozoite egress. Its role in egress at blood stages and expression in liver stages raised the possibility of similar role in liver stages. A conditional mutagenesis-based invalidation of *P. berghei* SUB1 in liver stages revealed that SUB1-deficient parasites failed to egress from hepatocytes [23]. Based on its role in both blood and liver stages, PfSUB1 qualifies an attractive multistage target against malaria. Molecular modeling, substrate specificity, and characterization of PfSUB1 and its homologs from *Plasmodium vivax*, *Plasmodium knowlesi,* and *P. berghei* revealed many unusual features in SUB1 substrate-binding cleft, although SUB1 from all the species cleaved the conserved parasite substrates. Two peptidyl alpha-ketoamides based on an authentic PfSUB1 substrate inhibited SUB1 from all the species [105]. In another study, high throughput screening of a proprietary library of compounds against PfSUB1 identified hydrazine 2 as an inhibitor of PfSUB1 [106]. Due to its potential as an attractive multistage druggable target, identification of potent inhibitors against PfSUB1 could pave the way for the development antimalarials acting at schizont rupture.

5 Proteases Involved in Erythrocyte Invasion by Merozoites

Malaria parasites efficiently invade their host cells to survive inside the host and protect themselves from the host immune responses. Apicomplexan parasites utilize their specialized invasion apparatuses to identify, penetrate, and establish themselves within the host cells. Host cell entry is a crucial checkpoint where parasite development can be blocked, and hence an important target for antimalarial drug development [107]. Evidences for the involvement of parasite proteases in invasion came from inhibitor studies. Protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and chymostatin blocked merozoite invasion in P. *falciparum* and a number of other *Plasmodium* species [108, 109]. The chymostatin-sensitive effect was reversed by pretreatment of RBCs with chymotrypsin, suggesting that parasite-induced proteolytic modification of RBCs was involved in chymostatin-sensitive step [108, 110, 111]. Inhibitor studies on simian malaria parasite *P. knowlesi* revealed that treatment of isolated, invasive merozoites with *N*-tosyl phenylalanyl chloromethyl ketone, or *N*-tosyl lysyl chloromethyl ketone prevented primary attachment of parasites to host cells, whereas chymostatin blocked invasion at a later stage suggesting the involvement of multiple proteolytic activities in the pathway [81]. Consistent with this, a *P. falciparum* serine protease activity that mediated an essential processing and shedding of a major merozoite surface protein (merozoite surface protein 1; MSP1) at invasion was identified that was highly sensitive to inhibition by PMSF but not by chymostatin [112].

Invasion is a rapid process, taking about 60 s for the egressed merozoites to invade new erythrocytes. These merozoites distinguish between erythrocytes and other cells by initial low-affinity reversible contacts between merozoite and erythrocyte surface molecules [113]. The initial attachment occurs anywhere between the merozoite surface and erythrocyte; hence, the apical end of the merozoite orientates toward the erythrocyte surface in the subsequent steps. This orientation results in the formation of a tight junction between the merozoite apical end and the erythrocyte surface. This junction moves from anterior to the posterior end of the merozoite with the help of actin-myosin motor as it invades the erythrocytes [114]. The interaction between the merozoite and the erythrocyte molecules during the movement of the tight junction involves activity of parasite proteases [116–118]. Studies in *Toxoplasma gondii* and *P. falciparum* have revealed that micronemal secretion is induced upon initial contact. These secretory proteins are exposed at the parasite surface and mediate parasite orientation and formation of tight junction. Soon after microneme secretion, another apical organelles known as rhoptries discharge their contents, which are also supposed to have role in tight junction formation, although their precise role is not known [119, 120].

The initial interaction between the merozoite and the host cell is mediated by a protein complex on the merozoite surface known as merozoite surface protein (MSP1) complex MSP1 complex is composed of MSP1 and its associated proteins MSP6 and MSP7 [121–123]. This complex is processed in the parasitophorous

vacuole by the serine protease PfSUB1. This processing termed as "primary processing" makes the merozoite competent for the initial interaction [124]. After initial interaction, micronemal secretions mediate the high affinity interaction resulting in the formation of tight junction. This tight junction moves from anterior to posterior pole of the parasite in actomyosin motor-dependent manner. During this movement, shedding of MSP1 complexes and another micronemal protein apical membrane antigen (PfAMA1) takes place that is essential for invasion. Cleavage of both these proteins takes place at the juxtamembrane site. MSP1 is cleaved at the site distal to the epidermal growth factor-like domain at its C-terminal, shedding the MSP1 complex except the C-terminal region known as $MSP1_{19}$ that enters into the host cell following invasion [125]. Similarly, cleavage of AMA1 29 residues away from the transmembrane domain releases the bulk of the ectodomain. In this way the juxtamembrane "stub" along with the transmembrane and cytoplasmic domains enters into the host cell after invasion [126]. In vitro studies on the shedding of these parasite proteins strongly suggest that micronemal subtilisin-like serine protease PfSUB2 is the most likely candidate for these processing events, and hence termed as "merozoite surface sheddase" (MESH) [116].

By reverse genetics approaches both PfSUB1 and PfSUB2 appear to be essential for the parasite [100, 105]. Based on the numerous studies on their role in merozoite egress and invasion, these two proteases appear to be promising drug targets against malaria. Recently, efforts have been made to identify PfSUB1 and PfSUB2 inhibitors. Characterization of PfSUB1 and its orthologs in *P. knowlesi* and *P. berghei* revealed that cleavage sites in the parasite substrates of these proteases in conserved despite the differences in the enzyme substrate-binding sites. Consistent with these features, two peptidyl alpha-ketoamide inhibitors of PfSUB1 also inhibited its orthologs [127]. A low toxic natural pentacyclic triterpene maslinic acid (MA) was inhibitory to *P. falciparum* transition from ring to schizont stage. MA also inhibited the processing of MSP1 complex and hence supposed to target PfSUB1 [128]. In an in vitro study, PfSUB2 prodomain selectively inhibited the shedding of MSP1 and AMA1, it could be used an attractive design for PfSUB2 inhibitors. Structural study of PfSUB2 prodomain using nuclear magnetic resonance (NMR) identified a likely catalytic domain-binding interface region in it, which could act as a design for peptidomimetic inhibitor against the enzyme [129].

In addition to subtilisin-like proteases, intramembrane rhomboid proteases also have role during parasite invasion by cleaving the adhesins inside the parasite membrane. Rhomboid proteases carry out intramembrane proteolysis, and hence have their catalytic triad embedded within the membrane bilayer that is surrounded by a hydrophilic cavity [130]. *P. falciparum* rhomboid proteases are largely uncharacterized till date with two members PfROM1 and PfPROM4 partially characterized. PfROM1 andPfROM4 carried out the intramembrane cleavage of the adhesins AMA1 [126, 131] and erythrocyte-binding antigen 175 (EBA-175), respectively [132]. In addition, they were able to cleave a variety of adhesins involved in host–parasite interaction within the transmembrane domains [131].

Although this group of proteases seems to be crucial for the invasion process, they need to be extensively characterized and evaluated for druggability.

Overall, proteolytic enzymes involved in invasion are considered to be attractive targets of antimalarial drug development. Invasion is a very rapid process, taking place within a minute of merozoite release; the access of parasite molecules involved in invasion is debatable. Dowse et al. [132] suggested that proteases involved in invasion have the potential to serve as drug targets as their biosynthesis and maturation start much earlier in the life cycle, making them available for targeting by drugs [132].

6 Other Cellular Proteases

In addition to the above-described proteases, a number of parasitic proteases are involved in regular metabolic processes and cell cycle regulation in the parasite, with unique parasite-specific features. The parasite possesses a single mitochondrion and a relict plant plastid-like organelle called apicoplast [133–137]. The mitochondrion possesses some unique biochemical features. It is defective in tricarboxylic acid cycle and does not appear to oxidize glucose to produce ATP in the blood stages of the parasite [138], although it seems to be involved in pyrimidine biosynthesis [139]. An intriguing *P. falciparum* protease is ClpQ threonine protease (heat shock loci V or HslV) is localized in the mitochondrion [140, 141]. Its ATPase partner is ClpY (heat shock loci U or HslU) [142]. These two proteins interact to form ClpQY machinery, a prokaryotic predecessor of the eukaryotic proteosomal machinery [142]. ClpQ appears to be critical for parasite survival as the disruption of the interaction between ClpQ and ClpY using peptide inhibitors resulted in the death of asexual blood stages in a phenotype resembling apoptosis [143].

The apicoplast is crucial for the biosynthesis of parasite heme, isopentenyl diphosphate, fatty acids, and isoprenoid precursors [144, 145]. The apicoplast possesses its own genome but 95% of its proteins are nuclear-encoded and transported to the organelle. Targeting of proteins to apicoplast through the secretory pathway is mediated by bipartite signal and transit peptide sequences. Two parasite proteases are involved in the targeting process; the stromal processing protease (SPP) cleaves the transit peptide to release the mature protein [146] and the metalloprotease falcilysin carries out the degradation of the transit peptide [69]. Another serine protease ClpP dependent on ATPase for functioning has been localized in the apicoplast and its inhibitor was found to significantly inhibit parasite growth in vitro [147].

Parasite signal peptide peptidases mediate protein trafficking to their destinations within the parasite and host cytosol [24, 147–149]. The *P. falciparum* signal peptide peptidase (PfSPP) is essential for parasite growth inside the erythrocytes and could be targeted by small molecule inhibitors [150]. Gamma secretase and

signal peptide peptidase inhibitor LY411,575 was evaluated for the targeting *Plasmodium berghei* liver stages in human hepatoma cell lines and in mouse primary hepatocytes. LY411,575 was found to be inhibitory for normal liver stage development at nanomolar concentration (IC_{50} value of 80 nM). This inhibitor also decreased the liver stage parasite load in vivo and also conferred 55% resistance to cerebral malaria in mice [151].

The malaria parasite contains a minimal endoplasmic reticulum-associated degradation (ERAD) network relative to higher eukaryotes. *P. falciparum* is highly sensitive to the inhibition of protease component of this system (PfSSP). The compounds inhibiting PfSSP also showed low nanomolar activity against liver stage malaria parasites [152]. Many nuclear-encoded proteins are targeted to subcellular organelles like apicoplast and mitochondria using the signal and transit peptides. Upon reaching the target, the transit peptides are cleaved to release the proteins. Metalloprotease falcilysin is known to cleave the transit peptide in malaria parasite [68, 69]. Parasite exports a number of proteins to modify the host erythrocytes. These proteins contain a pentameric (RxLxE/Q/D) motif required for export into the erythrocytes known as PEXEL (Plasmodium EXport ELement) motif, which is responsible for trafficking of these proteins into the host cytosol [153, 154]. An ER-resident aspartic protease plasmepsin V is responsible for cleavage of PEXEL and facilitating trafficking of the proteins [155].

7 Conclusion

Despite the availability of drugs and significant successes in reducing malaria-related illnesses and deaths globally, malaria still poses a serious threat to human health. Due to the ability of the parasite to quickly acquire resistance against the drugs, development of novel drugs against parasite-specific molecules remains a priority. The parasite genome encodes proteases of many different classes, many of which carry out processes crucial for parasite survival and propagation. These molecules have been extensively studied and many of these have been shown promises as potential drug targets because they posses unique parasite-specific features that could be specifically targeted. The success of protease inhibitors as drugs has already been shown against other infectious agents like HIV and HCV, invoking interest in the use of malarial protease inhibitors as drugs. Overall, malarial proteases represent an intriguing group of molecules that could be utilized for specific targeting by the novel drugs.

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References

1. World Malaria Report (2015) World Health Organization
2. Trape JF, Legros F, Ndiaye P, Konate L et al (1989) Chloroquine-resistant *Plasmodium falciparum* malaria in Senegal. Trans R Soc Trop Med Hyg 83:761
3. Zucker JR, Ruebush TK, Obonyo C et al (2003) The mortality consequences of the continued use of chloroquine in Africa: experience in Siaya, western Kenya. Am J Trop Med Hyg 68:386–390
4. Sibley CH, Hyde JE, Sims PF et al (2001) Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? Trends Parasitol 17:582–588
5. Wongsrichanalai C, Pickard AL, Wernsdorfer WH et al (2002) Epidemiology of drug-resistant malaria. Lancet Infect Dis 2:209–218
6. TerKuile F, White NJ, Holloway P et al (1993) *Plasmodium falciparum*: in vitro studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria. Exp Parasitol 76:85–95
7. White NJ (2008) Qinghaosu (artemisinin): the price of success. Science 320:330–334
8. Ittarat W, Pickard AL, Rattanasinganchan P et al (2003) Recrudescence in artesunate treated patients with falciparum malaria is dependent on parasite burden not on parasite factors. Am J Trop Med Hyg 68:147–152
9. Travassos MA, Laufer MK (2009) Resistance to antimalarial drugs: molecular, pharmacologic, and clinical considerations. Pediatr Res 65:64R–70R
10. Dondorp AM, Nosten F, Yi P et al (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–467
11. Anderson TJ, Nair S, Nkhoma S et al (2010) High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in Western Cambodia. J Infect Dis 201:1326–1330
12. McKerrow JH, Caffrey C, Kelly B et al (2006) Proteases in parasitic diseases. Annu Rev Pathol 1:497–536
13. Flexner C, Bate G, Kirkpatrick P (2005) Tipranavir. Nat Rev Drug Discov 4:955–956
14. Melnikova I (2008) Hepatitis C therapies. Nat Rev Drug Discov 7:799–800
15. Smith CG, Vane JR (2003) The discovery of captopril. FASEB J 17:788–789
16. Melnikova I (2009) The anticoagulants market. Nat Rev Drug Discov 8:353–354
17. Turk B (2006) Targeting proteases: successes, failures and future prospects. Nat Rev Drug Discov 5:785–799
18. Neurath H (1989) in The diversity of proteolytic enzymes.In: Beynon RJ, Bond JS (eds) Proteolytic enzymes: a practical approach. IRL Press, Oxford, pp 1–13
19. Rosenthal PJ (2002) Hydrolysis of erythrocyte proteins by proteases of malaria parasites. Curr Opin Hematol 9:140–145
20. Gardner MJ, Hall N, Fung E et al (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:498–511
21. Mazier D, Rénia L, Snounou G (2013) A pre-emptive strike against malaria's stealthy hepatic forms. Nat Rev Drug Discovery 8:854–864
22. Suarez C, Volkmann K, Gomes AR et al (2013) The malarial serine protease SUB1 plays an essential role in parasite liver stage development. PLoS Pathog 9:e1003811
23. Tawk L, Lacroix C, Gueirard P et al (2013) A key role for Plasmodium subtilisin-like SUB1 protease in egress of malaria parasites from host hepatocytes. J Biol Chem 288:33336–33346
24. Wu Y, Wang X, Liu X et al (2003) Data-mining approaches reveal hidden families of proteases in the genome of malaria parasite. Genome Res 13:601–616
25. Lilburn TG, Hong C, Zhan Zhou Z et al (2011) Protease-associated cellular networks in malaria parasite *Plasmodium falciparum*. BMC Genom 12(Suppl 5):S9
26. Sherman IW (1977) Amino acid metabolism and protein synthesis in malarial parasites. Bull World Health Organ 55:265–276

27. McKerrow JH, Sun E, Rosenthal PJ et al (1993) The proteases and pathogenicity of parasitic protozoa. Annu Rev Microbiol 47:821–853
28. Rosenthal PJ, Meshnick SR (1996) Hemoglobin catabolism and iron utilization by malaria parasites. Mol Biochem Parasitol 83:131–139
29. Wood PA, Eaton JW (1993) Hemoglobin catabolism and host-parasite heme balance in chloroquine-sensitive and chloroquine-resistant *Plasmodium berghei* infections. Am J Trop Med Hyg 48:465–472
30. Scheibel LW, Sherman IW (1988). In: Wernsdorfer WH, McGregor I (eds) Malaria. Churchill Livingstone, Edinburgh, vol 1, pp 219–252
31. Yayon A, Cabantchik ZI, Ginsburg H (1984) Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine. EMBO J 3:2695–2700
32. Krogstad DJ, Schlesinger PH, Gluzman IY (1985) Antimalarials increase vesicle pH in *Plasmodium falciparum*. J Cell Biol 101:2302–2309
33. Goldberg DE, Slater AF, Cerami A et al (1990) Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle. Proc Natl Acad Sci USA 87:2931–2935
34. Francis SE, Gluzman IY, Oksman A et al (1994) Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. EMBO J 13:306–317
35. Hill J, Tyas L, Phylip LH et al (1994) High level expression and characterisation of Plasmepsin II, an aspartic proteinase from *Plasmodium falciparum*. FEBS Lett 352:155–158
36. Rosenthal PJ, McKerrow JH, Aikawa M et al (1988) A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium falciparum*. J Clin Invest 82:1560–1566
37. Banerjee R, Liu J, Beatty W et al (2002) Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. Proc Natl Acad Sci USA 99:990–995
38. Goldberg DE (2005) Hemoglobin degradation. Curr Top Microbiol Immunol 295:275–291
39. Gluzman IY, Francis SE, Oksman A et al (1994) Order and specificity of the *Plasmodium falciparum* hemoglobin degradation pathway. J Clin Invest 93:1602–1608
40. Shenai BR, Sijwali PS, Singh A (2000) Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. J Biol Chem 275:29000–29010
41. Eggleson KK, Duffin KL, Goldberg DE (1999) Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. J Biol Chem 274:32411–32417
42. Gavigan CS, Dalton JP, Bell A (2001) The role of aminopeptidases in haemoglobin degradation in *Plasmodium falciparum*-infected erythrocytes. Mol Biochem Parasitol 117:37–48
43. Liu J, Istvan ES, Gluzman IY et al (2006) *Plasmodium falciparum* ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. Proc Natl Acad Sci USA 103:8840–8845
44. Liu K, Shi H, Xiao H et al (2009) Functional profiling, identification, and inhibition of Plasmepsins in intraerythrocytic malaria parasites. Angew Chem Int Ed 48:8293–8297
45. Omara-Opyene AL, Moura PA, Sulsona CR (2004) Genetic disruption of the *Plasmodium falciparum* digestive vacuole plasmepsins demonstrates their functional redundancy. J Biol Chem 279:54088–54096
46. Liu J, Gluzman IY, Drew ME (2005) The role of *Plasmodium falciparum* food vacuole plasmepsins. J Biol Chem 280:1432–1437
47. Moon RP, Bur D, Loetscher H et al (1998) Studies on plasmepsins I and II from the malarial parasite *Plasmodium falciparum* and their exploitation as drug targets. Adv Exp Med Biol 436:397–406

48. Ersmark K, Feierberg I, Bjelic S et al (2004) Potent inhibitors of the *Plasmodium falciparum* enzymes plasmepsin I and II devoid of cathepsin D inhibitory activity. J Med Chem 47:110–122
49. Johansson PO, Lindberg J, Blackman MJ et al (2005) Design and synthesis of potent inhibitors of plasmepsin I and II: X-ray crystal of inhibitor in complex with plasmepsin II. J Med Chem 48:4400–4409
50. Muthas D, Nöteberg D, Sabnis YA et al (2005) Synthesis, biological evaluation, and modeling studies of inhibitors aimed at the malarial proteases plasmepsins I and II. Bioorg Med Chem 13:5371–5390
51. Mohapatra SC, Tiwari HK, Single M (2010) Antimalarial evaluation of copper(II) nanohybrid solids: inhibition of plasmepsin II, a hemoglobin-degrading malarial aspartic protease from *Plasmodium falciparum*. J Biol Inorg Chem 15:373–385
52. Gupta D, Yedidi RS, Varghese S et al (2010) Mechanism-based inhibitors of the aspartyl protease plasmepsin II as potential antimalarial agents. J Med Chem 53:4234–4247
53. Dali B, Keita M, Megnassan E et al (2012) Insight into selectivity of peptidomimetic inhibitors with modified statine core for plasmepsin II of *Plasmodium falciparum* over human cathepsin D. Chem Biol Drug Des 79:411–430
54. Rasina D, Otikovs M, Leitans J et al (2016) Fragment-based discovery of 2-aminoquinazolin-4(3H)-ones as novel class nopeptidomimetic inhibitors of the plasmepsins I, II, and IV. J Med Chem 59:374–387
55. Ersmark K, Samuelsson B, Hallberg A (2006) Plasmepsins as potential targets for new antimalarial therapy. Med Res Rev 26:626–666
56. Nezami A, Kimura T, Hidaka K et al (2003) High-affinity inhibition of a family of *Plasmodium falciparum* proteases by a designed adaptive inhibitor. Biochemistry 42:8459–8464
57. Sijwali PS, Shenai BR, Gut J et al (2001) Expression and characterization of the *Plasmodium falciparum* haemoglobinase falcipain-3. Biochem J 360:481–489
58. Sijwali PS, Rosenthal PJ (2004) Gene disruption confirms a critical role for the cysteine protease falcipain-2in hemoglobin hydrolysis by *Plasmodium falciparum*. Proc Natl Acad Sci USA 101:4384–4389
59. Sijwali PS, Koo J, Singh N et al (2006) Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*. Mol Biochem Parasitol 150:96–106
60. Singh N, Sijwali PS, Pandey KC et al (2006) Plasmodium falciparum: biochemical characterization of the cysteineprotease falcipain-2. Exp Parasitol 112:187–192
61. Goh LL, Sim TS (2005) Characterization of amino acid variation at strategic positions in parasite and human proteases for selective inhibition of falcipains in *Plasmodium falciparum*. Biochem Biophys Res Commun 335:762–770
62. Jeong JJ, Kumar A, Hanada T et al (2006) Cloning and characterization of *Plasmodium falciparum* cysteine protease, falcipain-2B. Blood Cells Mol Dis 36:429–435
63. Rosenthal PJ (2004) Cysteine proteases of malaria parasites. Int J Parasitol l34:1489–1499
64. Rosenthal PJ, Wollish WS, Palmer JT (1991) Antimalarial effects of peptide inhibitors of a *Plasmodium falciparum* cysteine proteinase. J Clin Invest 88:1467–1472
65. Singh A, Rosenthal PJ (2001) Comparison of efficacies of cysteine protease inhibitors against five strains of *Plasmodium falciparum*. Antimicrob Agents Chemother 45:949–951
66. Batra S, Sabnis YA, Rosenthal PJ et al (2003) Structure-based approach to falcipain-2 inhibitors: synthesis and biological evaluation of 1,6,7-trisubstituted dihydroisoquinolines and isoquinolines. Bioorg Med Chem 11:2293–2299
67. Rosenthal PJ, Lee GK, Smith RE (1993) Inhibition of a Plasmodium vinckei cysteine proteinase cures murine malaria. J Clin Invest 91:1052–1056
68. Murata CE, Goldberg DE (2003) *Plasmodium falciparum* falcilysin: a metalloprotease with dual specificity. J Biol Chem 278:38022–38028

69. Ponpuak M, Klemba M, Park M et al (2007) A role for falcilysin in transit peptide degradation in the *Plasmodium falciparum* apicoplast. Mol Microbiol 63:314–334
70. Curley GP, O'Donovan SM, McNally J et al (1994) Aminopeptidases from *Plasmodium falciparum*, *Plasmodium chabaudi chabaudi* and *Plasmodium berghei*. J Eukaryot Microbiol 41:119–123
71. Mistry SN, Drinkwater N, Ruggeri C et al (2014) Two-pronged attack: dual inhibition of *Plasmodium falciparum* M1 and M17 metalloaminopeptidases by a novel series of hydroxamic acid-based inhibitors. J Med Chem 57:9168–9183
72. Winograd E, Clavijo CA, Bustamante LY et al (1999) Release of merozoites from *Plasmodium falciparum*-infected erythrocytes could be mediated by a non-explosive event. Parasitol Res 85:621–624
73. Glushakova S, Yin D, Li T et al (2005) Membrane transformation during malaria parasite release from human red blood cells. Curr Biol 15:1645–1650
74. Wickham ME, Culvenor JG, Cowman AF (2003) Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte. J Biol Chem 278:37658–37663
75. Salmon BL, Oksman A, Goldberg DE (2001) Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. Proc Natl Acad Sci USA 98:271–276
76. Soni S, Dhawan S, Rosen KM et al (2005) Characterization of events preceding the release of malaria parasite from the host red blood cell. Blood Cells Mol Dis 35:201–211
77. Langreth SG, Jensen JB, Reese RT et al (1978) Fine structure of human malaria in vitro. J Protozool 25:443–452
78. Aikawa M (1971) Parasitological review. Plasmodium: the fine structure of malarial parasites. Exp Parasitol 30:284–320
79. Banyal HS, Misra GC, Gupta CM et al (1981) Involvement of malarial proteases in the interaction between the parasite and host erythrocyte in *Plasmodium knowlesi* infections. J Parasitol 67:623–626
80. Dutta GP, Banyal HS (1981) In vitro susceptibility of erythrocytes of Presbytis entellus (Indian Langur) to *Plasmodium knowlesi* & blocking of merozoite invasion process by certain protease inhibitors. Ind J Exp Biol 19:9–11
81. Hadley T, Aikawa M, Miller LH (1983) Plasmodium knowlesi: studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. Exp Parasitol 55:306–311
82. Lyon JA, Haynes JD (1986) *Plasmodium falciparum* antigens synthesized by schizonts and stabilized at the merozoite surface when schizonts mature in the presence of protease inhibitors. J Immunol 136:2245–2251
83. Delplace P, Bhatia A, Cagnard M et al (1988) Protein p126: a parasitophorous vacuole antigen associated with the release of *Plasmodium falciparum* merozoites. Biol Cell 64:215–221
84. Le Bonniec S, Deregnaucourt C, Redeker V et al (1999) Plasmepsin II, an acidic hemoglobinase from the *Plasmodium falciparum* food vacuole, is active at neutral pH on the host erythrocyte membrane skeleton. J Biol Chem 274:14218–14223
85. Dua M, Raphael P, Sijwali PS et al (2001) Recombinant falcipain-2 cleaves erythrocyte membrane ankyrin and protein 4.1. Mol Biochem Parasitol 116:95–99
86. Hanspal M, Dua M, Takakuwa Y et al (2002) *Plasmodium falciparum* cysteine protease falcipain-2 cleaves erythrocyte membrane skeletal proteins at late stages of parasite development. Blood 100:1048–1054
87. Dhawan S, Dua M, Chishti AH et al (2003) Ankyrin peptide blocks falcipain-2-mediated malaria parasite release from red blood cells. J Biol Chem 278:30180–30186
88. Blackman MJ (2008) Malarial proteases and host cell egress: an 'emerging' cascade. Cell Microbiol 10:1925–1934
89. Aly AS, Matuschewski K (2005) A malarial cysteine protease is necessary for Plasmodium sporozoite egress from oocysts. J Exp Med 202:225–230

90. Miller SK, Good RT, Drew DR et al (2002) A subset of *Plasmodium falciparum* SERA genes are expressed and appear to play an important role in the erythrocytic cycle. J Biol Chem 277:47524–47532
91. Bzik DJ, Li WB, Horii T et al (1988) Amino acid sequence of the serine-repeat antigen (SERA) of *Plasmodium falciparum* determined from cloned cDNA. Mol Biochem Parasitol 30:279–288
92. Gor DO, Li AC, Rosenthal PJ (1998) Protective immune responses against protease-like antigens of the murine malaria parasite *Plasmodium vinckei*. Vaccine 16:1193–1202
93. Kiefer MC, Crawford KA, Boley LJ et al (1996) Identification and cloning of a locus of serine repeat antigen (sera)-related genes from *Plasmodium vivax*. Mol Biochem Parasitol 78:55–65
94. Hodder AN, Drew DR, Epa VC et al (2003) Enzymic, phylogenetic, and structural characterization of the unusual papain-like protease domain of *Plasmodium falciparum* SERA5. J Biol Chem 278:48169–48177
95. McCoubrie JE, Miller SK, Sargeant T et al (2007) Evidence for a common role for the serine-type *Plasmodium falciparum* serine repeat antigen proteases: implications for vaccine and drug design. Infect Immun 75:5565–5574
96. Delplace P, Dubremetz JF, Fortier B et al (1985) A 50 kilodalton exoantigen specific to the merozoite release-reinvasion stage of *Plasmodium falciparum*. Mol Biochem Parasitol 17:239–251
97. Debrabant A, Maes P, Delplace P, Dubremetz JF, Tartar A, Camus D (1992) Intramolecular mapping of *Plasmodium falciparum* P126 proteolytic fragments by N-terminal amino acid sequencing. Mol Biochem Parasitol 53:89–95
98. Pang XL, Mitamura T, Horii T (1999) Antibodies reactive with the N-terminal domain of *Plasmodium falciparum* serine repeat antigen inhibit cell proliferation by agglutinating merozoites and schizonts. Infect Immun 67:1821–1827
99. Li J, Matsuoka H, Mitamura T et al (2002) Characterization of proteases involved in the processing of *Plasmodium falciparum* serine repeat antigen (SERA). Mol Biochem Parasitol 120:177–186
100. Yeoh S, O'Donnell RA, Koussis A et al (2007) Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. Cell 131:1072–1083
101. Arastu-Kapur S, Ponder EL, Fonovic UP et al (2008) Identification of proteases that regulate erythrocyte rupture by the malaria parasite *Plasmodium falciparum*. Nat Chem Biol 4:203–213
102. Fairlie WD, Spurck TP, McCoubrie JE et al (2008) Inhibition of malaria parasite development by a cyclic peptide that targets the vital parasite protein SERA5. Infect Immun 76:4332–4344
103. Alam A, Chauhan VS (2012) Inhibitory potential of prodomain of *Plasmodium falciparum* protease serine repeat antigen 5 for asexual blood stages of parasite. PLoS ONE, e30452
104. Ruecker A, Shea M, Hackett F et al (2012) Proteolytic activation of the essential parasitophorous vacuole cysteine protease SERA6 accompanies malaria parasite egress from its host erythrocyte. J Biol Chem 287:37949–37963
105. Withers-Martinez C, Suarez C, Fulle S et al (2012) Plasmodium subtilisin-like protease 1 (SUB1): insights into the active-site structure, specificity and function of a pan-malaria drug target. Int J Parasitol 42:597–612
106. Gemma S, Giovani S, Brindisi M et al (2012) Quinolylhydrazones as novel inhibitors of *Plasmodium falciparum* serine protease PfSUB1. Bioorg Med Chem Lett 22:5317–5321
107. Cowman AF, Crabb BS (2006) Invasion of red blood cells by malaria parasites. Cell 124:755–766
108. Dejkriengkraikhul P, Wilairat P (1983) Requirement of malarial protease in the invasion of human red cells by merozoites of *Plasmodium falciparum*. Z Parasitenkd 69:313–317

109. Breton CB, Blisnick T, Jouin H et al (1992) *Plasmodium chabaudi* p68 serine protease activity required for merozoite entry into mouse erythrocytes. Proc Natl Acad Sci USA 89:9647–9651
110. Dluzewski AR, Rangachari K, Wilson RJ et al (1986) Protease inhibitors and inhibition of erythrocyte invasion. Exp Parasitol 62:416–422
111. McPherson RA, Donald DR, Sawyer WH et al (1993) Proteolytic digestion of band 3 at an external site alters the erythrocyte membrane organisation and may facilitate malarial invasion. Mol Biochem Parasitol 62:233–242
112. Blackman MJ, Holder AA (1992) Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1. Mol Biochem Parasitol 50:307–315
113. Bannister LH, Dluzewski AR (1990) The ultrastructure of red cell invasion in malaria infections: a review. Blood Cells 16:257–292
114. Keeley A, Soldati D (2004) The glideosome: a molecular machinepowering motility and host-cell invasion by Apicomplexa. TrendsCell Biol 14:528–532
115. Harris PK, Yeoh S, Dluzewski AR et al (2005) Molecular identification of a malaria merozoite surface sheddase. PLoS Pathog 1(e29):0241–0251
116. Brossier F, Jewett TJ, Sibley LD et al (2005) A spatiallylocalized rhomboid protease cleaves cell surface adhesins essentialfor invasion by Toxoplasma. Proc Natl Acad Sci USA 102:4146–4151
117. Dowse TJ, Pascall JC, Brown KD et al (2005) Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion. Int J Parasitol 35:747–756
118. Alexander DL, Mital J, Ward GE et al (2005) Identification of the moving junction complex of Toxoplasma gondii: A collaboration between distinct secretory organelles. PLoS Pathog 1:e17
119. Singh S, Alam MM, Pal-Bhowick I et al (2010) Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. PLoS Pathog 6: e1000746
120. Blackman MJ, Heidrich H-G, Donachie S et al (1990) A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J Exp Med 172:379–382
121. Goel VK, Li X, Chen H et al (2003) Band 3 is a host receptor bindingmerozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. Proc Natl Acad Sc USA 100:5164–5169
122. Li X, Chen H, Oo TH et al (2004) A co-ligand complex anchors *Plasmodium falciparum* merozoites to the erythrocyte invasion receptor band 3. J Biol Chem 279:5765–5771
123. Child MA, Epp C, Bujard H et al (2010) Regulated maturation of malaria merozoite surface protein-1 is essential for parasite growth. Mol Microbiol 78:187–202
124. Barale J-C, Blisnick T, Fujioka H et al (1999) *Plasmodium falciparum* subtilisin-like protease 2, a merozoite candidate for the merozoite surface protein 1-42 maturase. Proc Natl Acad Sc USA 96:6445–6450
125. Howell SA, Hackett F, Jongco AM et al (2005) Distinct mechanisms govern proteolytic shedding of a key invasion protein in apicomplexan pathogens. Mol Microbiol 57:1342–1356
126. Uzureau P, Barale JC, Janse CJ et al (2004) Gene targeting demonstrates that the *Plasmodium berghei* subtilisin PbSUB2 is essential for red cell invasion and reveals spontaneous genetic recombination events. Cell Microbiol 6:65–78
127. Moneriz C, Mestres J, Bautista JM et al (2011) Multi-targeted activity of maslinic acid as an antimalarial natural compound. FEBS J 278:2951–2961
128. He Y, Chen Y, Oganesyan N et al (2012) Solution NMR structure of a sheddase inhibitor prodomain from the malarial parasite *Plasmodium falciparum*. Proteins 80:2810–2817

129. Urban S (2006) Rhomboid proteins: conserved membrane proteases with divergent biological functions. Genes Dev 20:3054–3068
130. Baker RP, Wijetilaka R, Urban S (2006) Two Plasmodium rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. PLoS Pathog 2:e113
131. O'Donnell RA, Hackett S, Howell SA et al (2006) Intramembrane proteolysis mediates shedding of a key adhesin during erythrocyte invasion by the malaria parasite. J Cell Biol 174:1023–1033
132. Dowse TJ, Koussis K, Blackman MJ, Soldati-Favre D (2008) Roles of proteases during invasion and egress by Plasmodium and Toxoplasma. In: Burleigh BA, Soldati-Favre D (eds) Molecular mechanism of parasite invasion. Landes Biosciences and Springer Science +Business Media
133. Waller RF, McFadden GI (2005) The apicoplast: a review of the derived plastid of apicomplexan parasites. Curr Issues Mol Biol 7:57–79
134. Goodman CD, Su V, McFAdden GI (2007) The effects of anti-bacterials on the malaria parasite *Plasmodium falciparum*. Mol Biochem Parasitol 152:181–191
135. Schlitzer M (2007) Malaria chemotherapeutics part I: History of antimalarial drug development, currently used therapeutics, and drugs in clinical development. ChemMedChem 2:944–986
136. Schlitzer M (2006) Selective enzyme inhibitor instead of an "iron-triggered cluster bomb". Pharm Unserer Zeit 35:8–9
137. Dahl EL, Rosenthal PJ (2008) Apicoplast translation, transcription and genome replication: targets for antimalarial antibiotics. Trends Parasitol 24:279–284
138. Vaidya AB, Mather MW (2009) Mitochondrial evolution and functions in malaria parasites. Annu Rev Microbiol 63:249–267
139. Painter HJ, Morrisey JM, Mather MW, Vaidya AB (2007) Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. Nature 446:88–91
140. Tschan S, Kreidenweiss A, Stierhof YD et al (2010) Mitochondrial localization of the threonine peptidase PfHslV, a ClpQ ortholog in *Plasmodium falciparum*. Int J Parasitol 40:1517–1523
141. Ramasamy G, Gupta D, Mohmmed A et al (2007) Characterization and localization of *Plasmodium falciparum* homolog of prokaryotic ClpQ/HslV protease. Mol Biochem Parasitol 152:139–148
142. Sousa MC, Trame CB, Tsuruta H et al (2000) Crystal and solution structures of an HslUV protease-chaperone complex. Cell 103:633–643
143. Rathore S, Jain S, Sinha D et al (2011) Disruption of a mitochondrial protease machinery in *Plasmodium falciparum* is an intrinsic signal for parasite cell death. Cell Death Dis 2:e231
144. Ralph SA, van Dooren GG, Waller RF et al (2004) Tropical infectious diseases: metabolic maps and functions of the *Plasmodium falciparum* apicoplast. Nat Rev Microbiol 2:203–216
145. Yeh E, DeRisi JL (2011) Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. PLoS Biol 9:e1001138
146. van Dooren GG, Su V, D'Ombrain MC et al (2002) Processing of an apicoplast leader sequence in *Plasmodium falciparum* and the identification of a putative leader cleavage enzyme. J Biol Chem 277:23612–23619
147. Rathore S, Sinha D, Asad M et al (2010) A cyanobacterial serine protease of *Plasmodium falciparum* is targeted to the apicoplast and plays an important role in its growth and development. Mol Microbiol 77:873–890
148. Sharma S, Pradhan A, Chauhan VS et al (2005) Isolation and characterization of type I signal peptidase of different malaria parasites. J Biomed Biotechnol 2005:301–309
149. Tuteja R, Pradhan A, Sharma S (2008) *Plasmodium falciparum* signal peptidase is regulated by phosphorylation and required for intra-erythrocytic growth. Mol Biochem Parasitol 157:137–147

150. Li X, Chen H, Bahamontes-Rosa N et al (2009) *Plasmodium falciparum* signal peptide peptidase is a promising drug target against blood stage malaria. Biochem Biophys Res Commun 380:454–459
151. Parvanova I, Epiphanio S, Fauq A et al (2009) A small molecule inhibitor of signal peptide peptidase inhibits Plasmodium development in the liver and decreases malaria severity. PLoS ONE 4:e5078
152. Harbut MB, Velmourougane G, Dalal S et al (2011) Bestatin-based chemical biology strategy reveals distinct roles for malaria M1- and M17-family aminopeptidases. Proc Natl Acad Sci USA 108:E526–E534
153. Chang HH, Falick AM, Carlton PM et al (2008) N-terminal processing of proteins exportedby malaria parasites. Mol Biochem Parasitol 160:107–115
154. Boddey JA, Moritz RL, Simpson RJ et al (2009) Role of the Plasmodium export element intraffi cking parasite proteins to the infected erythrocyte. Traffi c 10:285–299
155. Boddey JA, Hodder AN, Gunther S et al (2010) An aspartyl protease directs malaria effectorproteins to the host cell. Nature 463:627–631

Role of Metalloproteinases in Melanoma Growth and Progression

5

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Abstract

Matrix metalloproteinases (MMPs) are zinc-containing endopeptidases with an extensive range of substrate specificities. Together, these enzymes are able to degrade various components of extracellular matrix (ECM) proteins and driven important physiological and pathological process, such as reproduction, wound healing, inflammation, and cancer development. In melanoma, MMPs are involved in growth, invasion, and metastasis process. In this disease, as in other tumors, the degradation of ECM permits angiogenesis and the migration of melanoma cells to distant sites. In this chapter, we focus on the functional characteristic of the main MMPs and their contributions to melanoma development.

Keywords

Melanoma · Metalloproteinases · Progression · Metastasis

1 Introduction

Matrix metalloproteinases (MMPs), also known as matrixins, form an important group of endopeptidases, active at neutral pH, which can cleave almost every component of extracellular matrix (ECM) proteins and other non-ECM proteins.

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There are about 27 different MMPs discovered so far (24 in mammals), subdivided in groups according to substrate specificity and structural integrity. Structurally, MMPs present high similarity, all have zinc in their catalytic site and their activity is dependent of calcium [1, 2]. These endopeptidases play a central role in normal physiological processes such as proliferation, cell motility, remodeling, wound healing, angiogenesis, and control key events of reproduction as menstruation, endometrial proliferation, embryogenesis, ovulation, and implantation [3].

MMPs are secreted by several cell types including inflammatory and tumor cells, fibroblast, and keratinocytes as inactive proenzymes, referred to as zymogens or pro-MMPs, requiring proteolytic activation. The activity of MMPs is regulated by their endogenous inhibitors, tissue inhibitors of MMPs (TIMPs). The imbalances in the expression of MMPs and TIMPs have been linked to the pathogenesis of various conditions, such as rheumatoid arthritis, atherosclerosis, aneurysm, ulcers, fibrosis, endometriosis, and tumor progression [3, 4].

The role of MMPs in cancer progression is well established and has been extensively studied in recent years. MMPs not only degrade the ECM, but their activity directs cell growth, expression of growth factors and their receptors, and many other important molecules in tumor pathogenesis. In melanomas, MMPs play a crucial role in tumor growth and establishment of metastasis now becoming important therapeutic targets in this condition [5]. In this chapter we discuss about the role of the major MMPs involved in the pathogenesis and progression of cancer, in special in melanoma.

2 General Features

MMPs were discovered in 1962, and the interstitial collagenase (MMP-1) was first isolated in 1968 [6, 7]. Originally, MMPs were classified according to the specificity of components of the ECM and structural features, in five groups: collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs), and other [2]. Currently, desintegrin and metalloproteinase (ADAMs) and ADAM with motifs thrombospondin (ADAMTS) are also classified as MMPs. These enzymes have a methionine residue conserved in the active site and depend on the zinc ion to enzymatic reactions [8]. Table 1 reports the main matrix metalloproteinases already described with their respective substrates.

All members of the MMP family share a common structural organization including (1) a pro-domain that maintains the enzyme in its latent form; (2) a catalytic domain containing the consensus sequence HEXXHXXGXXH, which binds a Zn^{+2} atom and is essential for catalysis; and (3) a carboxy-terminal hemopexin-like domain, which depending on the MMP is involved in substrate recognition and/or interactions with the TIMPs. A hinge region links the catalytic domain with the hemopexin-like domain. Figure 1 shows the basic structure of

Table 1 Classification of some human metalloproteinases (MMPs) and their substrates

MMP subgroup	MMP number	Alternative name	Substrate
Collagenases	MMP-1	Interstitial collagenase; collagenase-1	Collagen type I, II, and III
	MMP-8	Neutrophil collagenase; collagenase-2	Collagen type I and II
	MMP-13	Collagenase-3	Collagen type II (more effectively), I, and III collagens; gelatin
Gelatinases	MMP-2	Gelatinase A	Pro-MMP-9, fibronectin, collagen type I and IV, denaturated collagen
	MMP-9	Gelatinase B	Fibronectin, gelatin, elastin, collagen IV, V, VII, X, and type I denaturated collagen
Stromelysins	MMP-3	Stromelysin-1	Fibronectin, laminin, elastin, proteoglycan, collagen IV, V, IX, and X., Pro-MMP-1
	MMP-10	Stromelysin-2	Fibronectin, laminin, elastin, proteoglycan and collagen IV, V, IX, and X
Matrilysins	MMP-7	Matrilysin-1	Fibronectin, elastin, collagen IV, β-4 integrin
Membrane-type	MMP-14	MT1-MMP	Pro-MMP-2, pro-MMP-13, collagen type I, II and III, fibronectin, laminin-1, gelatin
	MMP-15	MT2-MMP	Pro-MMP-2, pro-MMP-13, laminin, fibronectin, and tenascin
	MMP-16	MT3-MMP	Pro-MMP-2, gelatin, casein, type III collagen, and fibronectin
Other types	MMP-12	Metalloelastase	Elastin, collagen type IV, gelatin type I, fibronectin α1-antitrypsin
	MMP-20	Enamelysin	Enamel matrix

MMP-2 and MMP-9, which have an additional fibronectin type II (FN) domain, located between the catalytic domain and the active site [2].

Similar to all secreted proteinases, the catalytic activity of MMPs is regulated at four points—gene expression, compartmentalization (that is, the pericellular accumulation of enzymes), pro-enzyme (or zymogen) activation, and enzyme inactivation—and is further controlled by substrate availability and affinity [9]. The activity of MMPs in tissue is highly regulated to ensure they are in inactive form in the most steady-state conditions. By contrast, MMP expression can be increased during repair or remodeling processes, in inflamed tissues and tumors, and its

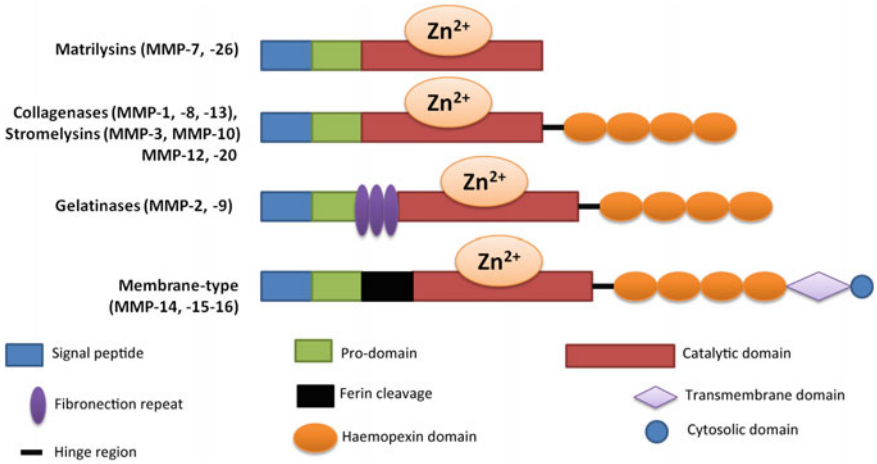

Fig. 1 Basic domain structural of MMP groups. At least, all human MMPs share signal peptide, pro-domain, and catalytic domain. Gelatinases MMP-2 and MMP-9 have an additional fibronectin domain in their catalytic domain. Membrane-type MMP as MMP-14 has a furin site, which allows its intracellular activation by this protein and transmembrane and cytosolic domains. With exception of matrilysins, all groups present the hinge region and a hemopexin-like domain in C-terminal, which displays functions in substrate recognition

production is regulated by specific signal that are temporally limited and spatially confined [2, 9]. MMPs are synthesized as pro-MMPs that require the enzymatic or chemical removal of pro-domain [10]. Various proteinases or ROS disrupt the interaction between the active site zinc atom in the catalytic domain and a conserved cysteine within the pro-domain. Exposure of the zinc atom results in the autolytic cleavage of the pro-domain, a process known as the "cysteine switch" [11].

Once activated, MMP catalytic activity is controlled, in part, by TIMPs, which act as specific proteinase inhibitors. The TIMP family comprises four homologous proteins: TIMP-1, TIMP-2, TIMP-3, and TIMP-4, weighing 21–29 kDa and domains N and C-terminal of 125 and 65 amino acids, respectively, each containing three disulfide bonds conserved. The TIMPs bind to the catalytic domain similar to the substrate and inhibit the enzymatic activity of both MMPs, active and latent form [12]. Concerning about TIMPs location, TIMP-1, TIMP-2, and TIMP-4 inhibitors were thought to be soluble, whereas TIMP-3 was in the ECM, bound proteoglycans [13, 14]. More recently, TIMPs have been shown to localize with cell surface proteins; however, this interaction has primarily been associated with metalloproteinase-independent functions for the various TIMPs and, as yet, has not been found to impact ECM turnover [15, 16]. The balance between MMPs and TIMPs is important to keep tissues health. A shift in the balance in favor of MMPs resulted in increased ECM proteolysis, as it occurs in inflammation or tumor progression, whereas a shift in balance in favor of TIMPs decreased ECM proteolysis leading to an accumulation of matrix, as seen in lung fibrosis [17, 18]. Importantly,

examination of the phenotypes of mice lacking different TIMPs demonstrated that TIMPs have many different roles, one of which is directly regulating ECM proteolysis. For example, the modulation of cell surface cytokines and cytokine receptors by MMPs can lead to increased inflammation (i.e., neutrophil chemotaxis). TIMP activity can inhibit shedding of these cell surface proteins, and as a result, indirectly regulate ECM turnover through control of inflammation [9, 18].

3 MMPs and Cancer

Cancer originates from mutations in genes that regulate cell proliferation and death, leading to uncontrolled outgrowth of tissue cells. Tumors are complex structures of mutated cells, presenting vascular growing and a dynamic stroma consisting of various nonmalignant cells, such as fibroblasts and myeloid cells. The tumor microenvironment is similar to an inflammatory response in a healing wound, which promotes angiogenesis, turnover of the ECM, and tumor cell motility [14, 19]. The knowledge about the involvement of MMPs in these processes lead to an association between MMPs and cancer and its progression. However, further studies showed that the cancer progression is much more complex than that derived from their contribution to the last invasive stages of the tumorigenic process. MMPs may be critically involved in disrupting the balance between growth and antigrowth signals in the tumor microenvironment, modulating its growth [14].

MMPs may target growth factor, cytokines, chemokines, cell adhesion molecules, angiogenic factors, and growth factor receptors, and contribute to adhesion, proliferation, migration, apoptosis, angiogenesis, and evasion of the immune system [8, 20–22]. Cell proliferation is driven by ligands for the epidermal growth factor receptor (EGFR). Genetic mutations in molecules involved in this system are observed in breast cancer and other cancers as ADAM proteinases. ADAM-10 and ADAM-17 are thought to promote the release of soluble EGF and the activation of TNF-α, which activate EGFR. This results in the up regulation of MMP-9, which in turn degrades E-cadherin, a potent control element of cell–cell adhesion and differentiation. This association between EGFR, MMP-9, and E-cadherin was thought to have an important role in ovarian cancer and metastasis, since activated EGFR and MMP-9 in these specimens colocalize with a region of reduced E-cadherin [23]. In mammary epithelium, over-expressed MMP-3 promotes the cleavage of E-cadherin resulting in epithelial–mesenchymal transition [24], which is related to the cancer progression and a poor prognostic.

Apoptosis in malignant cells can be inhibited through cleavage by MMPs of ligands or receptors that transduce proapoptotic signals. Fas ligand cleavage is mediated by MMP-7 in doxorubicin-treated cancer cells, reducing the effect of the chemotherapy by apoptosis. Indeed, MMP-7 expression may serve as a predictive marker for the resistance to chemotherapy in patients with non-small cell lung cancer [14, 25, 26].

One fundamental signaling pathway with essential roles in tissue homeostasis is the transforming growth factor-β (TGF-β) pathway. TGF-β normally exerts tumor-suppressive effects by enforcing cytostasis and differentiation. However, during tumor progression, accumulated mutations in TGF-β receptor become cells unresponsive to this factor. Furthermore, TGF-β can be activated to a tumor-promoting factor pro-form by proteolytic conversion by MMP-9 and other proteases, which is usually expressed by inflammatory cells [27]. Similarly, MMP-14 and MMP-2 proteolytically activate TGF-β1 [28]. These actions can selectively drive stroma-mediated invasion and metastasis of the tumor.

MMPs display important function in tumor angiogenesis. When components of the ECM degrade, MMPs facilitate migration of endothelial cells and blood vessels murals, stimulating the growth of tumor blood network [29, 30]. The degradation of ECM and plasmatic membrane from cells in the neighborhood activates the release of vascular endothelial growth factor (VEGF), basic fibroblast-derived growth factor (bFGF), and other factors related to angiogenesis. Gelatinases MMP-2 and MMP-9 degrade Type IV collagen, the major component of the ECM, and basal membrane of blood vessels. This process facilitates invasion, enabling the spread of tumor cells in circulation and the occurrence of metastases [31, 32]. In studies with experimental animals deficient in MMP-9 enzyme, metastasis was suppressed [33].

To produce metastases, tumor cells are required to cross several physical barriers, such as the endothelial basement membrane, and interact with inflammatory and local cells of the pre-metastatic niches [34]. In these processes, MMPs have important participation. In breast, colon, and lung tumor, MMP-1 cleaves and activates proteinase-activated receptors 1 (PAR-1), resulting in acute endothelial activation that generates a pro-inflammatory environment associated with tumor progression [35]. MMPs are able to increase osteolysis and dissemination of tumor into bone tissue. In rodent model of prostate cancer, MMP-7 promotes activation of osteoclast by releasing an active form of RANKL (Receptor Activator for Nuclear Factor κB Ligand) and promotes osteolysis and subsequent bone metastasis [36]. Taken together, these proteinases could serve as therapeutic targets to prevent metastasis to the bone in breast or prostate cancer [14].

4 MMPs in Melanoma Progression

Melanoma is a neoplasm derived from melanocytes, melanin specialized cells producers, which occurs in many anatomical sites including the skin, mucosal surfaces, conjunctiva, and uveal structures. Although only 2% of skin cancers are melanoma, this type is the most aggressive, with a high incidence of metastases and death [22].

MMPs are strongly involved in maintenance of skin integrity, but during the aging, changes occur in ECM deposition and degradation by these enzymes, which frequently lead to tumor development [5, 37]. In addition, exposure to UV radiation increases the expression of matrix metalloproteinases (MMPs) in human,

contributing to photoaging and photocarcinogenesis [38–40]. Photoaging is caused by an imbalance in equilibrium between the accumulation and degradation of ECM components that provide structural and functional supports to the skin tissue. Cumulative exposure to the sun results in continuous degradation of ECM proteins such as collagen and elastin and a decrease rate of renewal/syntheses of collage and also triggered important mutations pathways such as serine–threonine kinase *BRAF* and MAPK pathways, determining uncontrolled proliferation and migration of mutated cells [21, 40]. In addition to these effects, MMPs can modify signalization through growth factors receptors, as EGFR, and stimulate proliferation of tumor cells, contributing melanoma [5, 23].

Malignant melanoma cells express several MMPs including MMP-1, MMP-2, MMP-9, MMP-13, and MMP-14 as well as inhibitors of MMPs, TIMP-1, TIMP-2, and TIMP-3 [41]. The balance between MMPs and TIMPs determines the progression of melanoma [42], with higher expressions of MMPs associated to lower survival rates in patients [43].

The most studied MMPs for melanomas are MMP-2 and MMP-9, also known as gelatinase A and B, respectively. They are related to the most metastatic and invasive phenotypes of melanoma and its expression directly related to atypia and dedifferentiation of tumor cells [44, 45]. These enzymes degrade basement membrane proteins such as collagen in types IV, V, VII, X, XI and XIV, gelatin, elastin, fibronectin, TNF-α precursor, and IL-1β [46]. Studies using immunohistochemistry, tissue microarray, and tissue biopsies show that there is increased MMP-2 expression in primary and metastatic melanoma in common nevus in relation to the dysplastic tissue [47].

Malignant melanoma lesions in different stages show that MMPs are usually located in the tumor stroma interface and the invasive portion of the tumor [41]. In in vivo studies, MMP-2 activity was located in peripheral areas of the tumors, suggesting that the processing of type I collagen and fibronectin cleavage are needed to promote adhesion and cell migration [48]. MMP-2 also have a role in angiogenesis as demonstrated by in vitro studies, where the highest concentration of MMP-2 enhanced growth of tumor cells in matrigel enhances the migration of these cells in laminin and colony formation in agarose [49]. MMP-2 expression has been associated with malignant progression and indicated as a prognostic marker in melanoma [47].

MMP-9 expression in melanoma tumor cells was found exclusively during the horizontal growth phase but not during the vertical growth phase, suggesting that expression of MMP-9 is an early event in melanoma progression [50]. Further, melanomas expressing constitutively high levels of MMP-9 exhibited increased lung colonization in experimental lung metastasis models and are associated to more invasive forms [51, 52]. In melanoma angiogenesis, MMP-9 enhances the availability of VEGF that up-regulate MMP-2 expression in melanoma cells. Taken together, interaction between gelatinases and VEGF promotes tumor progression by regulating tumor angiogenesis and metastasis [40].

The collagenases, MMP-1, MMP-8, and MMP-13, are particularly relevant in melanoma for their ability to cleave native fibrillar collagen types I, II, III, and V. MMP-1, secreted by melanoma and activated stromal cells, contributes to the progression of melanoma by stimulate invasion and metastasis by breaking down interstitial collagen and by activating PAR-1. The expression of PAR-1 correlates with the depth of melanoma invasion, as described previously [35].

MMP-13 has also been detected in human melanoma, and evidence indicated that it is released by peritumoral fibroblasts as well as MMP-1 [53, 54]. Its expression in stromal cells immediately adjacent to the tumor is higher upon tumor cell invasion, indicating that MMP-13 is important to melanoma growth and invasion. Some studies showed that MMP-13 promotes vascularization through the proteolytic release of VEGF and in mice lacking MMP-13, the melanoma growth was decreased [55, 56].

In the opposite way, MMP-8 (collagenase-2) displays antimetastatic activities. Previous in vivo studies had shown that over-expression of wild-type MMP-8 in melanoma cells reduces lung metastasis formation. The mechanism involved seems to be through increased adhesion of cells expressing wild-type MMP-8 to type I collagen and laminin-1 when compared with control cells. In addition, tumor cells over-expressing wild-type MMP-8 have a reduced invasive capability through matrigel. Mutations in MMP-8 are found in 6.3% of melanoma and the most of its mutations were accompanied by a loss of heterozygosity and because it has been previously shown to protect against skin tumor development [57]. These findings suggest MMP-8 to be a tumor suppressor gene [8, 58].

Stromelysins and matrilysins are also associated to melanoma growth and metastasis. In vitro, MMP-3 (stromelysin) is expressed in more aggressive metastatic cells lines and is associated to melanoma metastasis formation. This MMP was just found in metastatic sites and its presence is correlated with shorter disease-free survival. The pro-invasive function in melanoma was attributed to its capability to activated MMP-1 and pro-MMP-13. It is expressed in metastatic melanoma and is correlated with shorter disease-free survival [5, 59].

MMP-7 or matrilysin is expressed and produced by primary cutaneous and metastatic melanomas [5]. Its expression in melanomas enhances tumor cell growth and metastasis, thereby reducing the survival rate. MMP-7 (called matrilysin) can efficiently degrade elastin. Upon UV irradiation, MMP-7 can cleave not only elastin but also many other substrates of the ECM, such as collagen type IV, entactin, fibronectin, laminin, and cartilage proteoglycan aggregates [40].

MT1-MMP (MMP-14) is localized at the invasive front of tumor invading cells and acts as a membrane activator of other soluble MMPs, such as MMP-2. Thus, expression of MT1-MMP is strongly involved in tumor invasion [60]. In addition to its ability to degrade multiple components of the ECM, MT1-MMP can degrade cell adhesion molecules and signaling receptors such as CD44 and E-cadherin and promoting the expression of VEGF, mediating endothelial cell migration and vascular formation processes [5, 61]. Regarding to MT2-MMP (MMP-15) and MT3-MMP (MMP-16), their expression is increased in primary and metastatic

melanoma, and also activates pro-MMP-2, contributing for tumor growth and metastasis [62].

MMP-12 is the most effective MMP against elastin and secreted by macrophages and fibroblasts in response to acute UV radiation. Because of that, MMP is associated to elastosis in photodamaged skin. In addition to elastin, MMP-12 can cleave other ECM components and pro-MMPs, such as pro-MMP-1, MMP-2, MMP-3, and MMP-9, all involved in melanoma growth and metastasis [40, 63, 64]. A recent study showed the association of the expression of MMP-12, a metalloelastase, with clinical features and prognosis melanoma. High MMP-12 expression was increased in cutaneous melanoma compared with that in normal skin, and significantly associated with tumor invasion, metastasis, and an unfavorable overall survival. From the results, MMP-12 expression was pointed as an independent prognostic marker of overall survival for patients with cutaneous melanoma [65].

5 Final Considerations

MMPs are important actors in melanoma development, growth, angiogenesis, and metastasis. Through specific roles organized in locally and temporally manner, MMPs determine the invasive capacity of the tumor and the occurrence of metastasis. In despite of this knowledge, clinical trials with certain MMP inhibitors have failure. It is possible that the multi-role of MMPs in the tumor environment, including protective actions in the development of melanoma, did not permit a driven antitumor action [8, 14]. Thus, recognizing some particular effects, polymorphism and mutations on MMPs involved in melanoma can change the treatment of this malignance. Efforts have been applied to discover new agents that restoring the protective nature of certain MMPs or increasing their proteolytic activity in tumors with a particular MMP mutation or deficiency can hinder the progression of the cancer.

References

1. Singh D, Srivastava SK, Chaudhuri TK et al (2015) Multifaceted role of matrix metalloproteinases (MMPs). Front Mol Biosci. doi:10.3389/fmolb.2015.00019
2. Marco M, Fortin C, Fulop T (2013) Membrane-type matrix metalloproteinases: key mediators of leukocyte function. J Leukoc Biol 94:237–246
3. Amalinei C, Caruntu ID, Balan RA (2007) Biology of metalloproteinases. Rom J Morphol Embryol 48(4):323–334
4. Pulkoski-Gross AE (2015) Historical perspective of matrix metalloproteinases. Front Biosci (Schol Ed) 7:125–149
5. Moro N, Mauch C, Zigrino P (2015) Metalloproteinases in melanoma. Eur J Cell Biol 93:23–29
6. Gross J, Lapiere CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci USA 48:1014–1022

7. Eisen AZ, Jeffrey JJ, Gross J (1968) Human skin collagenase. Isolation and mechanism of attack on the collagen molecule. Biochim Biophys Acta 151:637–645
8. Lopez-Otin C, Palavalli LH, Samuels Y (2009) Protective roles of matrix metalloproteinases: from mouse models to human cancer. Cell Cycle 8:3657–3662
9. Parks MC, Wilson CL, López-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4:617–629
10. Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodeling. Nat Rev Mol Cell Biol 8:221–233
11. Van Wart HE, Birkedal-Hansen H (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci USA 87:5578–5582
12. Moore CS, Crocker SJ (2012) An alternative perspective on the roles of TIMPs and MMPs in pathology. Am J Pathol 180:12–16
13. Arpino V, Brock M, Gill SE (2015) The role of TIMPs in regulation of extracellular matrix proteolysis. Matrix Biol 44–46:247–254
14. Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52–67
15. Jung KK, Liu XW, Chirco R, Fridman R, Kim HRC (2006) Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. EMBO J 25:3934–3942
16. Toricelli M, Melo F, Peres G (2013) Timp1 interacts with beta-1 integrin and CD63 along melanoma genesis and confers anoikis resistance by activating PI3-K signaling pathway independently of Akt phosphorylation. Mol Cancer 12:1–15
17. McCawley LJ, Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol 13:534–540
18. Selman M, Ruiz V, Cabrera S et al (2000) TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? Am J Physiol Lung Cell Mol Physiol 279:562–574
19. Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420(6917):860–867
20. Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174
21. Dye D, Medic S, Ziman M et al (2013) Melanoma biomolecules: independently identified but functionally interwined. Front Oncol. doi:10.3389/fonc.2013.00252
22. Sullivan R, LoRusso P, Boerner S et al (2015) Achievements and challenges of molecular targeted therapy in melanoma. Am Soc Clin Oncol Educ Book 2015:177–186. doi:10.14694/EdBook_AM.2015.35.177
23. Cowden Dahl KD, Symowicz J, Ning Y et al (2008) Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells. Cancer Res 68:4606–4613
24. Radisky DC, Levy DD, Littlepage LE et al (2005) Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature 436:123–127
25. Mitsiades N, Yu WH, Poulaki V et al (2001) Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity. Cancer Res 61:577–581
26. Liu H, Zhang T, Li X et al (2008) Predictive value of MMP-7 expression for response to chemotherapy and survival in patients with non-small cell lung cancer. Cancer Sci 99:2185–2192
27. Yu Q, Stamenkovic I (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 14:163–176
28. Mu D, Cambier S, Fjellbirkeland L et al (2002) The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. J Cell Biol 157:493–507

29. Van Wart H, Birkedal-Hansen HH (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci 87:5578–5582
30. Rodriguez D, Morrison C (1803) Overall C matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta 39–54:2010
31. Ribeiro R, Borges Junior P, Cardoso S et al (2008) Expression of matrix metalloproteinasis and their tissue inhibitors in basal cell carcinoma. J Bras Patol Med Lab 44:115–121
32. Lugowska I, Kowalska M, Fuksiewicz M et al (2015) Serum markers in early-stage and locally advanced melanoma. Tumour Biol 36:8277–8285
33. Itoh T, Tanioka M, Matsuda H et al (1999) Experimental metastasis is suppressed in MMP-9-deficient mice. Clin Exp Met 17:177–181
34. Kaplan RN, Riba RD, Zacharoulis S et al (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438:820–827
35. Boire A, Covic L, Agarwal A et al (2005) PAR-1 is a matrix metalloprotease-I receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell 120:303–313
36. Lynch CC, Hikosaka A, Acuff HB et al (2005) MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. Cancer Cell 7:485–496
37. Reed MJ, Corsa AC, Kudravi SA et al (2000) A deficit in collagenase activity contributes to impaired migration of aged microvascular endothelial cells. J Cell Biochem 77:116–126
38. O'Grady A, Dunne C, O'Kelly P et al (2007) Differential expression of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 in non-melanoma skin cancer: implications for tumour progression. Histopathology 51:793–798
39. Quan T, Qin Z, Xia W et al (2009) Matrix-degrading metalloproteinases in photoaging. J Investig Dermatol Symp Proc 14:20–24
40. Pittayapruek P, Meephansan J, Prapagan O et al (2016) Role of matrix metalloproteinases in photoaging and photocarcinogenesis. Int J Mol Sci 17(6):868
41. Mahabeleshwar G, Byzova T (2007) Angiogenesis in melanoma. Semin Oncol 34:555–565
42. Rudek M, Figg W, Dyer V et al (2001) Phase I clinical trial of oral COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic cancer. J Clin Oncol 19:584–592
43. Nikkola J, Vihinen P, Vuoristo M et al (2005) High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. Clin Cancer Res 11:5158–5166
44. Väisänen AH, Kallioinen M, Turpeenniemi-Hujanen T (2008) Comparison of the prognostic value of matrix metalloproteinases 2 and 9 in cutaneous melanoma. Hum Pathol 39:377–385
45. Ria R, Reale A, Castrovilli A et al (2010) Angiogenesis and progression in human melanoma. Dermatol Res Pract 2010. ID 185687 doi:10.1155/2010/185687
46. Hashizume K (2007) Analysis of utero-placental-specific molecules and their functions during implantation and placentation in the bovine. J Reprod Dev 53:1–11
47. Rotte A, Martinka M, Li G (2012) MMP-2 expression is a prognostic marker for primary melanoma patients. Cell Oncol 35:207–216
48. JiaoY Feng X, Zhan Y et al (2012) Matrix metalloproteinase-2 promotes alphavbeta3 integrin-mediated adhesion and migration of human melanoma cells by cleaving fibronectin. PLoS ONE 7:e41591
49. Iida J, Wilhelmson K, Price M et al (2004) Membrane type-1 matrix metalloproteinase promotes human melanoma invasion and growth. J Invest Dermatol 122:167–176
50. Schnaeker EM, Ossig R, Ludwig T et al (2004) Microtubule-dependent matrix metalloproteinase-2/matrix metalloproteinase-9 exocytosis: prerequisite in human melanoma cell invasion. Cancer 64:8924–8931
51. Cotignola J, Reva B, Mitra N et al (2007) Matrix metalloproteinase-9 (MMP-9) polymorphisms in patients with cutaneous malignant melanoma. BMC Med Genet 8:10. doi:10.1186/1471-2350-8-10

52. Farina A, Mackay A (2014) Gelatinase B/MMP-9 in tumour pathogenesis and progression. Cancers (Basel) 6(1):240–296
53. Airola K, Karonen T, Vaalamo M et al (1999) Expression of collagenases-1 and -3 and their inhibitors TIMP-1 and -3 correlates with the level of invasion in malignant melanomas. Br J Cancer 80:733–743
54. Wandel E, Grasshoff A, Mittag M et al (2000) Fibroblasts surrounding melanoma express elevated levels of matrix metalloproteinase-1 (MMP-1) and intercellular adhesion molecule-1 (ICAM-1) in vitro. Exp Dermatol 9:34–41
55. Zigrino P, Kuhn I, Bauerle T et al (2009) Stromal expression of MMP-13 is required for melanoma invasion and metastasis. J Invest Dermatol 129:2686–2693
56. Lederle W, Hartenstein B, Meides A et al (2010) MMP-13 as a stromal mediator in controlling persistent angiogenesis in skin carcinoma. Carcinogenesis 31(7):1175–1184
57. Balbin M, Fueyo A, Tester AM et al (2003) Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet 35:252–257
58. Gutierrez-Fernandez A, Fueyo A, Folgueras AR et al (2008) Matrix metalloproteinase-8 functions as a metastasis suppressor through modulation of tumor cell adhesion and invasion. Cancer Res 68:2755–2763
59. Vlaykova T, Kurzawski M, Tacheva T et al (2014) Investigation of the role of MMP3-1171insA polymorphism in cutaneous malignant melanoma a preliminary study. Biotechnol Biotechnl Equip 28:904–910
60. De Oliveira Poswar F, de Carvalho Fraga CA, Gomes ESB et al (2015) Protein expression of MMP-2 and MT1-MMP in actinic keratosis, squamous cell carcinoma of the skin, and basal cell carcinoma. Int J Surg Pathol 23:20–25
61. Oh ST, Kim HS, Yoo NJ et al (2011) Increased immunoreactivity of membrane type-1 matrix metalloproteinase (MT1-MMP) and b-catenin in high-risk basal cell carcinoma. Br Assoc Dermatol 165:1197–1204
62. Tatti O, Arjama M, Ranki A et al (2011) Membrane-type-3 matrix metalloproteinase (MT3-MMP) functions as a matrix composition-dependent effector of melanoma cell invasion. PLoS ONE 6:1–13
63. Tewari A, Grys K, Kollet J et al (2014) Upregulation of MMP-12 and its activity by UVA1 in human skin: potential implications for photoaging. J Investig Dermatol 134:2598–2609
64. Taddese S, Weiss AS, Jahreis G et al (2009) In vitro degradation of human tropoelastin by MMP-12 and the generation of matrikines from domain 24. Matrix Biol 28:84–91
65. Zhang Z, Zhu S, Yang Y et al (2015) Matrix metalloproteinase-12 expression is increased in cutaneous melanoma and associated with tumor aggressiveness. Tumor Biol 36:8593–8600

Cysteine Cathepsins: In Health and Rheumatoid Arthritis

6

Nalini Ganesan

Abstract

Proteases are enzymes which catalyze the irreversible hydrolysis of peptide bonds in proteins. Cysteine cathepsins belonging to proteases have also been termed as papain-like proteases because they resemble the overall fold of papain. The present chapter aims to focus on the historical aspects, structure, cellular distribution, biosynthesis, mechanism of catalysis, its regulation, physiological functions, and its association with rheumatoid arthritis. As these enzymes are also new therapeutic drug targets, information on available assays of cysteine cathepsins and their inhibitors are also highlighted which will help in the development of therapies in various diseases.

Keywords

Cysteine cathepsin · Rheumatoid arthritis · Cystatin · Lysosomal proteases · Activity regulation · Activity-based probes · Substrates-based probes

1 Introduction

The health of an organism is maintained by number of factors, of which the major contribution is governed by controlled biosynthesis, maturation, function, and terminal catabolism of proteins. The selective and well-controlled proteolytic events are accomplished by a group of proteases. These simple destructive enzymes likely

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arose at the earliest stages of protein evolution in primitive organisms [1]. The proteases constitute 1–4% of the genes per genomes sequenced to date [2] and are found in all known life forms from viruses to mammalian. In mammal, two main groups of cysteine proteases (CP) are present: cytosolic proteases (calpains type I and calpains type II) and lysosomal cathepsins [3]. More than 670 proteases and 200 endogenous proteases were encoded by the human genome [2, 4]. The cysteine proteases also known as cysteine cathepsin (CC) account for about 25% of the proteases and traditionally were believed to perform nonspecific bulk proteolysis within lysosomes. Cathepsin, derived from the Greek word "KATHEPSIN" meaning "to digest", was first proposed by Willstatter and Bamann [5] for the acid proteinase activity found in the aqueous extracts of several mammalian tissues.

The lysosomes discovered more than 50 years ago are membrane-bound organelles representing the important degradative compartments in eukaryotic cells. Some cell types, from the hematopoietic lineage, contain a specialized lysosomal compartment which secretes its contents into the extracellular environment in response to external stimuli [6]. These are termed as "secretory lysosomes" or lysosomal-related organelles sharing properties which are similar and differing from the conventional lysosomes. The acidic pH and presence of degradative proteins are found in both types of lysosomes but the secretory lysosomes have the ability to undergo regulated secretion [7].

In this chapter, the main aim is to review the various aspects of CC pertaining to its historical heritage, cellular localization, structure, biosynthesis, mechanism of catalysis, biological functions, structure-function relationship, activity regulation, available assays, therapeutic strategies, and its association with diseased state such as rheumatoid arthritis (RA) to give a current knowledge on CC.

2 Historical Heritage

The discovery of lysosomal cysteine proteases also known as cathepsins was in the first half of the twentieth century [8]. Since its discovery, it has been known that these enzymes nonselectively degrade proteins in lysosomes and are also responsible for a number of important cellular processes. The term cathepsin comprises proteases from three different mechanistic classes—the cysteine, serine, and aspartic proteases. In humans, 11 CC are known: cathepsin B, C (J, Dipeptidyl peptidase I or DPPI), F, H, K (O2), L, O, S, V (L2), X (P, Y, Z), and W (lymphopain) [9]. Being members of papain family, they belong to clan CA (cathepsin) of cysteine proteases. The papain-like thiol protease forms the largest subfamily (C1) among the cysteine protease clan CA.

Cathepsin C, the first pure enzyme, was found in the 1940s [10]. The enzymes cathepsin B and H were identified in the early 1970s followed soon after by cathepsin L [11]. In early 1980s, the amino acid sequences of rat cathepsins B and H were published [12]. The crystal structure of human cathepsin B was determined

in 1990 [13] indicating a rapid progress in this field. It is also known as the golden era of lysosomal cysteine protease research wherein 6 out of 11 known human enzymes were identified.

3 Cellular Localization

The physiological function of cathepsins can be partly attributed to the differences in localization of them in specific cell types. The expression profile of cathepsins B, H, L, C, X, F, O, and V in human tissues is ubiquitous indicating that these enzymes are involved in a normal cellular protein degradation and turnover [14]. On the other hand, cathepsins K, W, and S show a restricted cell or tissue specific distribution revealing their more specific role. Cathepsin K is highly expressed in osteoclasts, epithelial cells and in synovial fibroblast in RA joints [15], therefore reflecting the major role for cathepsin K in bone resorption. Cathepsin W is predominantly expressed in $CD8^+$ lymphocytes and natural killer cells [16] but its biological function still remains elusive. The antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells express cathepsin S [17]. There is high homology between cathepsin L and V but the former cellular expression is ubiquitous in contrast to restricted distribution of the latter to thymus and testis [18]. There is huge body of evidence that active cathepsins are also localized in other cellular compartments such as nucleus, cytoplasm, and plasma membrane. Nuclear cathepsin L plays a role in the regulation of cell cycle progression through proteolytic processing of the CDP/Cux transcription factor [19]. The interaction between nucleosomes and stefin B inhibits nuclear cathepsin L thereby delaying cell cycle progression [20]. Cathepsin L mediates the proteolysis of histone H3 which modulates cell cycle progression [21].

4 Structure

The crystal structure of papain [22] together with actinidin [23] has provided the first insights into the three-dimensional (3D) structure of cysteine cathepsins. They are small monomeric proteins with molecular weight around 30 kDa with the exception of cathepsin C which is tetrameric with 200 kDa. The CC structurally similar to papain [24] consists of two domains the L and R which specify the left and right domain of the catalytic unit and is in accordance with standard view as shown in (Fig. 1). The L-domain has three α-helices, one of which is vertical and also known as central helix having around 30 residues. The R-domain is a kind of β-barrel with the front strand forming a coiled structure. The bottom the barrel is enclosed by α-helix. The two domains separate at the top forming a V-shaped active

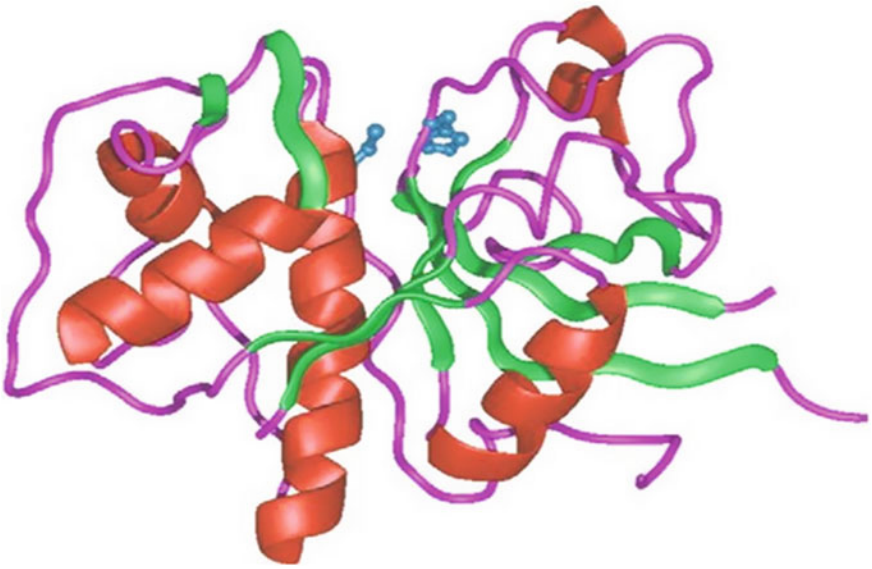

Fig. 1 General structure of cysteine cathepsin. The fold of the two chains form of native cathepsin is shown as purple ribbon with secondary structures α-helices and β-sheets shown in *red* and *green* respectively. The catalytic residues Cys 25 and His 159 are indicated in *ball–stick* representation

cleft. In the middle of the cleft resides the catalytic residues Cys 25 in the L-domain and His 159 in the R-domain. The proteolytic activity of the enzymes is mediated through these catalytic residues forming thiolate-imidazolium ion pair and has a low pka around 2.5–3.5 [25].

4.1 Substrate Binding Sites

Studies with known number of amino acid residues in peptides and substrates-mimicking inhibitor molecules have shown that the substrate binding sites are well defined in all CP. Schechter and Berger [26] have revealed that the substrate pocket binds at least seven amino acid residues in appropriate subsites. They proposed the nomenclature for the substrate residues (P) and the subsites (S) where they bind to the surface of the protease as shown in (Fig. 2). The non-primed side refers to the N-terminal and the primed refers to the C-terminal of the substrate.

The surface of the substrate binding sites is formed by four chain segments with two shorter loops on the L-domain and two longer loops on the right domain as shown in (Fig. 3). The additional loop with a disulphide linkage connects the two L-domains loops at the top. The substrate binding sites S1, S3, and S2′ are seen in the left and S2, S1′ on the right side of the active site of the enzyme.

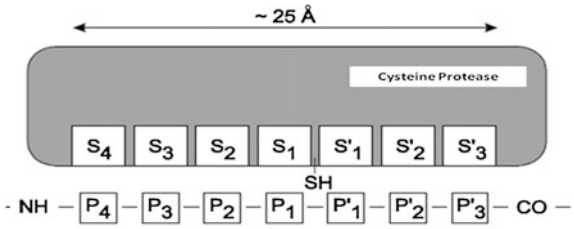

Fig. 2 Substrate residues P and substrate subsites S in a cysteine protease

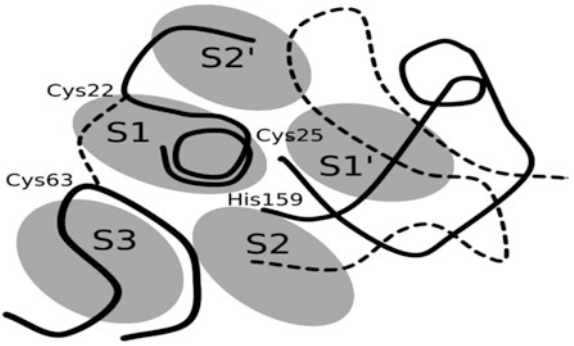

Fig. 3 Indicates the substrate binding sites on cysteine protease along the active side cleft

The substrates bind along the active site in an extended conformation [27]. The kinetic and structural studies by Turk et al. [28] have demonstrated that only five subsites are important for substrate binding. The S2, S1, and S1′ pockets are important for both backbone and side chain binding whereas S3 and S2 are essential only for amino acid side chain binding. Among the substrate binding sites, S2 and S1′ represent major specificity determinants. Most of the cysteine cathepsins are endopeptidases. In endopeptidases, the active cleft extends along the entire length of the two domains interface and in exopeptidases (Cathepsin B, C, H, and X) possesses additional features [29] of dual nature that reduce the number of substrate binding sites. They prevent the binding of longer peptidyl substrates and dock with charged N or C chain termini of substrates with electrostatic interactions.

4.2 Understanding Substrate Binding Sites

The substrate specificity of a protease depends upon the interaction between the surface of the protease and its substrates. There exist two complementary approaches to understand the interaction between protease and substrates [14]. One of them is the studies of the structure-function relationship and the other is the studies of substrate specificity and the combination of both has been used for more than two decades to know the specificity of CC for target proteins. In the first approach, certain residues in the enzyme are mutated to reveal their relevance and role in

certain interactions. In the second approach, the enzymes are left intact but the substrates are manipulated in size and composition.

It was observed that mutation of Glu245 in the S2 pocket of cathepsin B to Gln showed the importance of processing the substrate with an arginine in the P2 position, but had no effect on the phenylalanine substrate [30]. Another example is the mutation at the S1 site of cathepsin B which made the site more favorable for glycine [31]. The mutation of two histidine residues or the deletion of the additional loop of L-domain in the enzyme has shown its importance to the endopeptidase activity of cathepsin B [32]. Similarly attempts were made to understand the differences between the S2 sites of cathepsin L and K [33]. These studies demonstrated that we have a basic understanding of how specificity is encoded in structure. However, this knowledge is still not adequate to predict the selectivity for the substrates based on the sequence and atomic structure of the CC.

The prediction of highly selective substrates was found to be crucial for monitoring the activities of individual CC in biological samples. The focus of substrate studies therefore shifted to the discovery of substrate specific for individual cathepsins. These studies utilized peptidyl libraries in combination with scanning of individual position [34]. The screening of the P3 to P1′ sites of cathepsin B has revealed that Ab2-GIVRAK (Dnp)-OH peptide is the most favorable residue for cathepsin B. Among the mammalian CC, cathepsin K was found to have a unique preference for a proline residue in the P2 position, the primary determinant of its substrate specificity [35]. The processing of MHC class-II-associated invariant chain (Ii), a natural protein substrate of cathepsin S, resulted in peptides whose amino acid residues in position P3, P1, and P1′ were similar to a number of synthetic peptides [36]. It was also found that S1 and S3 sites of cathepsin V exhibited a broad specificity, while cathepsin L has preference for positively charged residues in the same positions. The S2 sites of both enzymes were found to require hydrophobic residues with a preference for phenyl alanine and leucine [37].

5 Biosynthesis, Processing and Trafficking of Cysteine Cathepsin

CC like any other protein is synthesized with a N-terminal signal peptide which targets the lumen of endoplasmic reticulum (ER). There is a co-translational cleavage of the signal peptide and initial N-linked glycosylation occurs within ER which generates a high mannose glycans content in the enzyme. Figure 4 illustrates the formation of cathepsin [38]. The next step involves the modification of mannose residues to mannose-6-phosphate in the Golgi apparatus. The recognition of cathepsin D by the initial enzyme in mannose-6-phosphate requires interaction with the surface loops in the structure [39]. The modified cathepsins bind to mannose-6-receptors for targeting to lysosomes. Upon initial acidification in the endosomes, the cathepsins are activated which leads to cleavage of the proregion and activation of cathepsin, resulting in further proteolytic processing in the lysosome

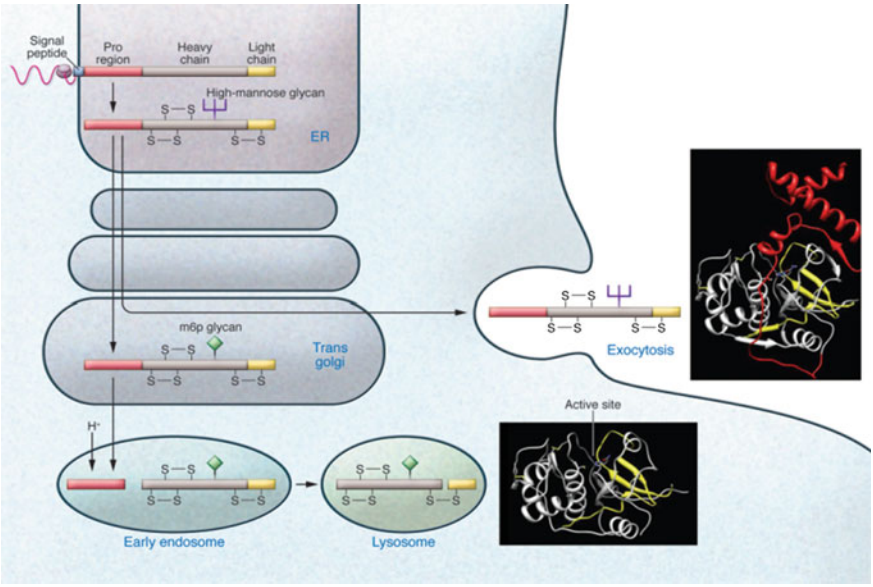

Fig. 4 Illustrates the steps involved in the biosynthesis, processing, and trafficking of cathepsin. *Ribbon diagrams* represent the structure of mature cathepsin L in the lysosome and extracellular matrix. The signal peptide directs the inactive form of the enzyme into the ER followed by glycosylation to produce a high content of mannose in cathepsin. *1* Transfer of proenzyme into Golgi apparatus for the phosphorylation of mannose to mannose-6-phosphate. This is the rate limiting step. *2* Upon acidification in the endosome, cleavage of the proregion of the enzyme. *3* Sorting of the enzyme into the lysosome by mannose-6-phosphate residues. Lysosomal proteolytic processing of the enzyme into light and heavy chains. *4* A small percentage of proenzyme secreted into the extracellular matrix (ECM) by exocytosis. *5* Structure of active cathepsin L. *6* Structure of cathepsin L without proregion

into heavy and light chains. The cleavage of the proregion occurs autocatalytically for both cathepsin B [40] and L [41] and the process is believed to involve intermolecular rather than intramolecular due to the distance between the active site and identified cleavage site. The unmodified mannose residues in portions of cathepsin results in shunting the enzyme into extracellular compartment by exocytosis.

6 Biological Functions

CC are well known for a long period of time that they are responsible for intracellular protein degradation [42]. Studies from gene knock out revealed that the intracellular protein degradation is not dependent on any single cathepsin. The majority of cathepsins (B, C, H, K, L, O, S, V, X, and W) are ubiquitously expressed in human tissues and are involved in bulk protein degradation which is considered as the main function of CC. Some of the CC are located in diverse

tissues which suggests that their action is not solely attributed to protein degradation. These functions include thyroglobulin processing and thyroid hormone release [43] and bone resorption action by cathepsins K [15], histone H3 processing by mouse cathepsin L [44] and the role of cathepsin X in integrin processing and T-cell signaling [45]. Yet another function of cathepsin L in epidermal homeostasis, regular hair follicle morphogenesis and cycling has been indicated [46] by gene knock out analysis in mice. All of these functions such as protein degradation, extracellular breakdown, apoptosis, and inflammation indicate the physiological importance of CC and a disturbance in these functions may lead to pathological conditions.

6.1 Cysteine Cathepsins and Extracellular Matrix Turnover

Extracellular matrix (ECM) is a complex macromolecular structure which is important for tissue homeostasis [47]. A number of enzymatic or nonenzymatic processes are involved in posttranslational modifications where this dynamic structure is remodeled. Among the enzymatic modifications, the proteolytic processing and degradation are most important. The main agents of ECM degradation are metalloproteases and serine proteinases because of their extracellular localization and are active at neutral pH [48]. In contrast to this, CC are effective at acidic pH and are mainly regarded as intracellular proteases. This view was shifted by the discovery that under specific physiological and pathological conditions, the cathepsins can be secreted into the extracellular space and can degrade the components of the ECM.

The ECM consists of two main classes of molecules: the proteoglycans and fibrous proteins which are substrates for CC [38]. The proteoglycans are glycosylated proteins which are covalently attached to glycosaminoglycans (GAGs). The GAGs such as chondroitin sulfate or keratin sulfate, heparan sulfate have a major effect on the activation and stability of CC by forming complex with them. Different GAGs have opposing effects on cathepsin activity. Chondroitin sulfate inhibits the degradation of elastin by cathepsin V and K [49] and collagenolytic activity of cathepsin S [50]. In contrast, chondroitin sulfate strongly increases the collagenolytic activity of cathepsin K [38, 51]. Among the CC, capable of cleaving aggrecan are cathepsin B, K, L, and S. Cathepsin B cleaves aggrecan as an endoprotease within the E1 region (G^{344}) in close proximity to the matrix metalloproteinase (MMP) cleavage site [52]. Cathepsin K was reported to cleave the G1, E1, and E2 domains of aggrecan [53]. Besides this, CC are also involved in cleaving the proteoglycans of the basement membrane [54] thus making them important in exerting pathological roles.

Collagen is the most abundant fibrous proteins which are degraded by MMPs, serine proteases, elastase, and CP. Among the CC, cathepsin K is the only physiologically relevant collagenase [55]. Its collagenolytic activity strongly depends on its complex formation with GAGs [56]. This interaction helps in unfolding of the triple helix of collagen and makes it more susceptible to degradation. In contrast to

other collagenases like the MMPs and neutrophil elastase, cathepsin K has the ability to attack at multiple sites of the collagen molecule [57]. Besides the triple helix, cathepsin K can also cleave the telopeptides which are the common feature of other cathepsins (cathepsins B, L, S).

Another important feature of cathepsin K which distinguishes it from other CC is its ability to accept proline residue in P1 and P2 positions [58]. This is essential for degradation of collagens because of the presence of highly repetitive motifs Gly-Pro-X and Gly-X-Hyp in this fibrous protein. The combined effect of GAG complex and active site specificity for proline residue [59] is required for effective cleavage of collagen. Cathepsin V was found to lack collagenolytic activity [55] because it cannot accept proline in P2 position [34]. Cathepsin L was suggested to be in addition to metalloproteases, an important enzyme in generation of endostatin, an angiogenesis inhibitor, by processing the C-terminus of collagen XVIII in both mouse and human system [60]. Elastin is another major ECM protein. Among CC, cathepsin K, S, and V are potent elastases while cathepsin L has only weak elastinolytic activity [61]. This property is due to the discovery of two hydrophobic exosites which stabilize the complex between elastin and the enzyme. The studies have revealed that cathepsin V variant is generated because the exosites are distant from the active site [62]. Fibronectin, another ECM protein, was found to be cleaved by cathepsin S, L, and B [63]. Other ECM-related cathepsin substrates are osteocalcin, osteonectin, and heparanase.

6.2 Cysteine Cathepsins and Antigen Processing and Presentation

One of the main biological functions of CC cathepsins is in antigen presentation and induction of immunity [64]. They are constitutively expressed in most cell types, especially in macrophages and dendritic cells. Among the CC, cathepsin S and L were found to be expressed in professional and nonprofessional antigen-presenting cells such as B cells, macrophages, specialized thymic epithelial cells, intestinal epithelium, and dendritic cells.

CC are involved in two critical stages of MHC class-II-mediated antigen presentation that is the degradation of invariant chain, a chaperone molecule critical for MHC class II assembly, transport, and generation of class II binding peptides in the endocytic compartment [65]. MHC class II α/β heterodimers assemble in the endoplasmic reticulum (ER) with the assistance of invariant chain (Ii) and this glycoprotein promotes the proper folding and assembly of MHC molecule [66]. A motif in the cytoplasmic tail of Ii directs the MHC class II: li complex to the endosomal/lysosomal pathway and CC mediates the stepwise degradation of the invariant chain leaving behind only class-II-associated Ii peptide (CLIP). This peptide associates with HLA-DM (H2 in mice) at the MHC class II peptide binding groove [67]. This interaction results in the removal of CLIP in exchange for a diverse array of peptide of self or foreign origin derived from the endosomes and lysosomes. The MHC class II complexes reach the plasma membrane by a poorly understood

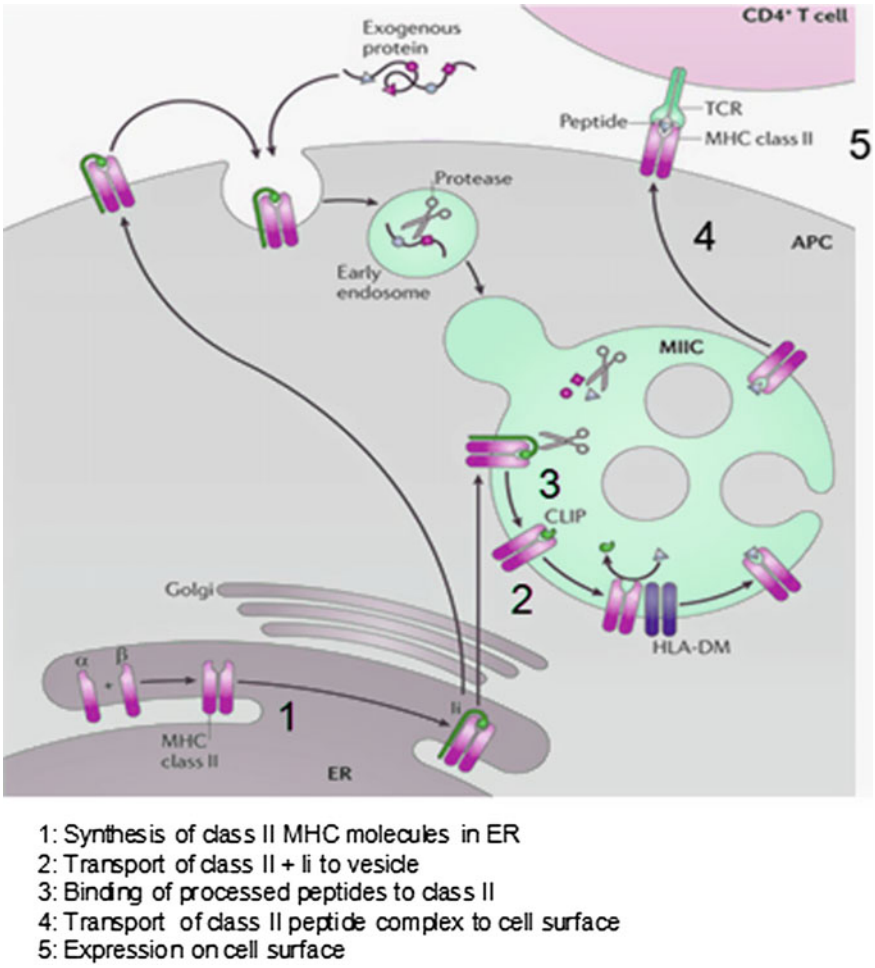

Fig. 5 The role of cathepsins in MHC class II antigen processing and presentation

mechanism on peptide binding [68]. Cathepsin S is regarded as the major protease involved in MHC II Ag processing and presentation and the steps involved are illustrated [69] in Fig. 5. Studies on antigen presentation with Cat S−/− bone marrow-derived APCs revealed an impaired Ii degradation and diminished presentation of exogenous antigens compared with the wild-type mice. In spite of reduced presentation of exogenous antigens by B cells, the number of CD+4 T cells remained normal in Cat S−/− mice [70]. The role of cathepsin L as an essential protease in MHC II antigen presentation in thymus was confirmed by the studies of Nakagawa et al. [71]. In humans, this proposed function of mouse cathepsin L is performed by its homologue cathepsin V [72]. These data reveal that both the cathepsins participate in the late stages but display a distinct function in different cell types. This differential

function displayed by these cathepsins helps in not only limiting immunogenicity but may also comprise tolerance to self-proteins [73]. The study by Chyi-Song Hsieh et al. has shown that cysteine proteases such as Cathepsin S and L participate in antigen processing and display qualitative and quantitative differences in the peptide repertoires displayed by MHC class II molecules [74]. The results by Beers et al. [75] suggest that in vivo, both professional and nonprofessional MHC class-II-expressing APC use Cat S, but not Cat L, for MHC class-II-mediated Ag presentation.

6.3 Cysteine Cathepsin and Apoptosis

The use of factors like TNFα and H_2O_2 in cell culture studies have shown that the lysosomes and lysosomal proteases are involved in apoptosis [76–79]. Evidences from in vitro [80] and in vivo [81] studies revealed that cathepsins take part in the process of programmed cell death. A number of direct or indirect stimuli cause lysosomal damage with the release of cathepsins (B, H, K, L, and S) into the cytosol [82–84]. In turn these enzymes cleave Bid, a cytoplasmtic factor which releases cytochrome C and forms a ternary complex with protein Apaf-1 and procaspase (pC9) leading to the activation of caspase-9 (C9) and caspase-3 (C3). Apoptosis is also triggered by the release of apoptosis inducing factor (AIF) by cathepsin-D-activated Bax. Lysosomal mediated apoptosis is shown in Fig. 6. Salt-induced hepatic apoptosis [85] and oxidative stress induced apoptosis [86] cause the translocation of cathepsins from lysosomes into the cytosol resulting in cell death. A diminished release of cathepsin B was observed in hepatocytes of B−/B− mice by TNF α induced apoptosis [87]. A reduction in the activity of lysosomal hydrolases and a resistance to TNF α induced apoptosis were observed in fibroblast isolated from patients with inclusion cell disease [83].

The cleavage at the residue Arg 65 in the flexible loop of the Bid molecule facilitates the release of cytochrome C by cathepsins B, H, K, L, and S. An impairment in this process was noted in hepatocytes isolated from Bid deficient (B−/B−) mouse cells highlighting the physiological importance of this pathway [88]. The mechanism by which the lysosomes mediate programmed cell death is still not clearly understood and warrants more studies.

6.4 Cathepsin and Bone Remodeling

Bone development and homeostasis are mediated by osteoblasts and osteocytes [89]. The involvement of CP in bone matrix degradation was evident from the studies using cysteine protease inhibitors such as leupeptin, antipain, and E-64 in isolated osteoclasts [90]. The presence of intact collagen fibrils in E-64 treated osteoclasts confirms the role of CC in the breakdown of phagocytosed collagen [91]. The main enzyme in the degradation of organic bone matrix was found to be cathepsin K [92, 93] as it was expressed widely in the osteoclasts. Previously, this function was attributed to cathepsin B and L. These cathepsins cleave only the

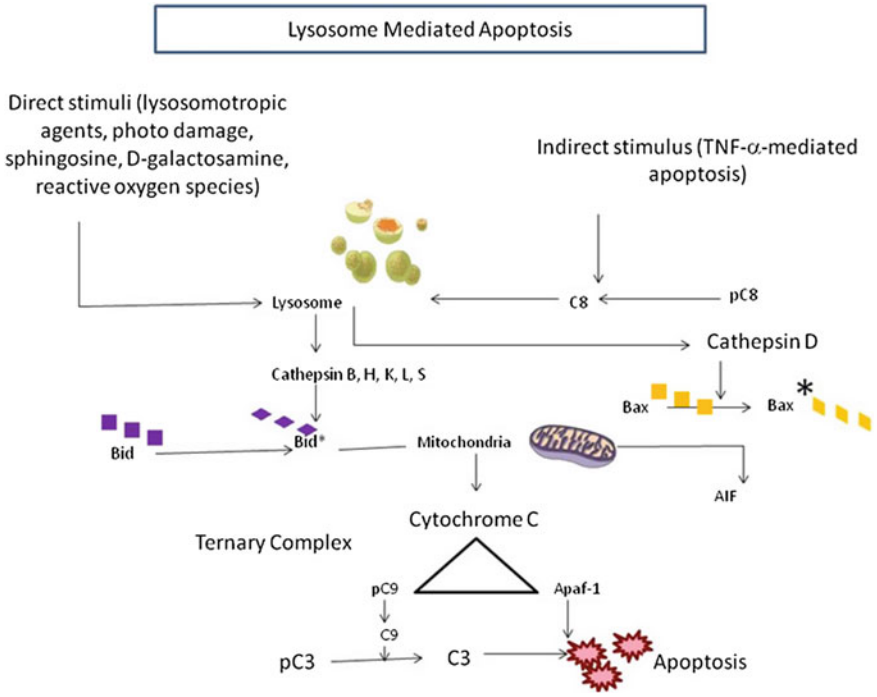

Fig. 6 Illustrates lysosomal mediated apoptosis

telopeptides and not the native triple helix resulting in monomers of type I collagen [93] whereas cathepsin K cleaves not only the telopeptides but at multiple sites within the native triple helix [57]. The degradation of collagen by cathepsin K involves a complex formation with glycosaminoglycans in bone and cartilage [51]. The use of cathepsin K inhibitors in vitro and in vivo experiments revealed a marked reduction in bone resorption [94].

6.5 Cathepsins, Lipid Metabolism and Inflammation

Lipids or modified lipoproteins affect CC cellular expression and localization. The cathepsins exhibit diverse functions in lipid metabolism and thus contribute to atherosclerosis. Sun et al. [95] demonstrated that free cholesterol accumulation stimulated cathepsin K expression via activation of toll-like receptors and p38 mitogen-activated protein kinase. Likewise macrophages also expressed higher level of cathepsin B and L when exposed to oxidized low-density lipoprotein or 7-hydroxycholesterol [96]. The lysosomal membrane damage and translocation of its content into the cytoplasm are because of the uptake of oxidized LDL. The released enzymes contribute to apoptosis of the macrophage [96]. The studies with HDL and/or vitamin E have found to reduce the harmful effects of oxidized LDL on lysosomes [97].

The cathepsin K-deficient apo E−/− bone marrow (BM) derived macrophages showed an increase in uptake of modified LDL and storage of cholesterol ester [98]. The increased uptake is mediated by CD36 and caveolins [99]. Cathepsins may also play a role in (modified) LDL degradation. Cathepsin F degrades LDL more extensively than cathepsin K and cathepsin S [100]. The deficiency of cathepsin K leads to foam cell formation. Cathepsin F mediated proteolytic modification of LDL leads to aggregation, fusion of LDL particles which in turn binds to proteoglycans subsequently leading to the accumulation of lipid droplets in arterial wall which is the key feature in atherosclerosis. The proteolysis of pre-HDL by cathepsin results in reduction of cholesterol efflux from macrophages [101]. The reverse cholesterol transport by HDL was found to be decreased in cathepsin K and cathepsin S deficient macrophages. Tertov and Orekhov [102] have reported that cathepsin D is involved in the initial degradation of LDL and the increased uptake and decreased lysosomal degradation of modified LDL lead to accumulation of foam cells in human aortic smooth muscle cells.

The relocation of cathepsins in the cytosol and its contribution to apoptosis point out toward its atherosclerotic stimulating role. The reduced lysosomal degradation of LDL by cathepsins reveals the atherosclerotic protection function. The cathepsins thus have both atherosclerosis-stimulating or protective role. The atherosclerotic-stimulating or protective role of cathepsins is under question.

7 Activity Regulation

The prime physiological function of CC is proteolytic breakdown of peptides and proteins. There are various ways by which this activity is kept under control to prevent harmful effects which include compartmentalization of cathepsins within lysosomes or organelles, zymogen activation, pH changes or the existence of small molecule inhibitors A combination of all these factors may also exist for effective enzyme function [8, 103].

Like most of the enzymes, CC are also synthesized as inactive forms. These zymogens need to be activated to perform its function. The human propeptide chain in CC positions on the active site cleft of the enzyme in a direction opposite to that of a substrate thereby preventing substrate binding. This was evident from crystal structures of human procathepsin B [104], procathepsin L [105], procathepsin K [106], and procathepsin X [107]. The interactions in the propeptide and between the propeptide and mature enzyme are hydrophobic, salt bridge formation or hydrogen bonding. The autocatalytic processing of cathepsin X can be prevented by disulphide bond formation between cysteines residues in propeptide and active site [107]. It has been suggested that an additional sorting motif other than mannose 6 phosphate is essential for activation of cathepsin S [108]. It is also postulated that cathepsin D, an aspartic protease, is responsible for intracellular processing of cathepsin L [109]. The activation of recombinant cathepsin B has been demonstrated by providing an acidic environment [110]. It is now evident that both

unimolecular and bimolecular mechanisms are involved in autoactivation of cathepsins [111]. The activation of endopeptidases such as the cathepsins B, H, L, S, and K is by removal of propeptide, but however the activation of exopeptidases such as cathepsin C and X requires the help of endopeptidases [112]. The presence of negatively charged molecules like GAGs and dextran sulfate is involved in acceleration of autocatalytic activation of CC [113]. The propeptides themselves act as potent inhibitors which was evident from studies using synthetic propeptides. The synthetic propeptide of cathepsin B inhibits the enzyme at pH 6 and with a weaker inhibition at pH 4 [114]. Carmona et al. [115] have indicated that the inhibitory effect resides in the first 20 residues of the N-terminal region of the propeptide of cathepsin L. A very little similarity exists in amino acid sequences and length of the propeptides [116] of CC which contrasts to strong homology between the mature forms of the enzymes. This structural difference in propeptides and the mature enzymes can be related to the requirement of selective inhibition and prevention of continued inhibition during processing and degradation of the enzyme in the lysosomal compartment, respectively [14].

The endogenous inhibitors which are the major regulators of CC can be classified into four types: cystatins, thyropins, serpins (propeptide like inhibitors) and the β-barrel type inhibitors [117].

The cystatins are the best characterized inhibitors and are further classified into three: stefins which are intracellular (Family I), extracellular and/or transcellular cystatins (Family II), and intracellular cystatins known as kininogens (Family III) [118]. Based on the physiological function, they can be classified as emergency and regulatory inhibitors [119]. The cystatins and thyropins are emergency inhibitors [120] primarily acting on the escaped proteases from invaded pathogens. The regulatory inhibitors co-localized with the target enzymes are classified into threshold, buffer, and delay type. These inhibitors block and modulate the protease activity. The propeptides of lysosomal cathepsins complexed with the substrates belong to buffer type of inhibitors. The levels of these endogenous inhibitors are balanced under physiological conditions [117].

An rapid increase in number of cystatins from various sources led to a new type of classification of cystatins. The basis for this classification depends on the presence of number of cystatin-like domains and disulphide linkages [121]. According to this classification, stefins, cystatins, and kininogens are inhibitory in nature [122] whereas fetuins, a histidine rich glycoprotein, are non-inhibitory homologs of cystatins [123–125]. The mutational change in the structurally important region has resulted in the loss of their inhibitory nature [126].

Type 1 cystatins are major eukaryotic intracellular protein having around 100 amino acid residues, lacking carbohydrates, disulphide bonds, and signal peptides [126, 127].

Type 2 cystatins are more widely distributed extracellular proteins with a single chain of $\sim$115 amino acid residues and signal peptide which facilitates its secretion into extracellular milieu [128].

Kininogens are multidomain glycoproteins belonging to Type 3 cystatins present predominantly in blood of mammals [128]. Thyrotropins have structural homology with thyroglobulin type I domain and also inhibits cathepsin L [129, 130].

7.1 Cysteine Protease–Cystatin Interaction

The mechanism of CP inhibition by cystatins is explained by various spectroscopic, kinetic, and crystallographic studies. This inhibition has been found to be a simple, reversible, and second-order type. These studies have also indicated that though the enzymes have a blocked active center, they bind with a lower affinity with cystatins [131]. The cysteine protease–cystatin interactions are hydrophobic in nature involving binding regions of the cystatin with residues in active site of the enzymes [118]. The structural studies by Stubbs et al. [132] have shown that a tripartite complex is formed between the amino terminal and two hairpin loops of the cystatin which involves hydrophobic interactions. This complex is complementary and strengthens the binding with the S2 subsite of papain [133]. These inhibitors are able to differentiate the interacting regions of exo- and endopeptidases.

8 Assay of Cysteine Cathepsins

The activity of cathepsin is quantitated by using substrates assays whereas immunological assays such as ELISA measure cathepsins as protein. Immunohistochemistry enables to detect these proteins in biopsy samples. Cox et al. [134] developed an immunoassay and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for measuring the activity and protein mass of cathepsin S, respectively. It was of interest to note from these studies that only 0.4–1% of the enzyme was active in circulation because majority of the enzyme was bound with its inhibitor. The disadvantage of these substrate-based assays and ELISA is the inability to discriminate between the active and inactive forms of proteases and hence cannot provide selective and sensitive readouts of cathepsin activity in disease.

A lot of progress was directed toward the quantitation of cathepsins by substrate-based probes and activity-based probes. The substrate-based probes consist of a protease recognition sequence and a signal moiety which permits its detection noninvasively. The recognition sequence renders the specificity toward the protease and the signal molecule gets activated and cleaved by the target protease. Mamhood et al. [135] has developed an in vivo method to image tumor-associated lysosomal protease activity in a xenograft mouse model using autoquenched near-infrared fluorescence probes.

Prosense 680, a broad-spectrum polymeric substrate containing a near-infrared fluorophore has been used to localize cathepsin protease activity in in vivo molecular imaging of lung cancer [136]. Cortez-Retamozo et al. [137] have utilized the combination of enzymes targeted sensor and noninvasive molecular imaging techniques to evaluate the severity of airway inflammation. Likewise, infrared (NIR) fluorescent probes were used to localize cathepsin activity in intestinal polyps of mice indicating inflammatory reactions that are etiologically linked with cancer progression and form a suitable approach for monitoring the response to therapy [138].

A chemical concept of "Reverse Design" involves redesigning the electrophilic group in the probe by another moiety which improves the selectivity of the cathepsins [139]. The newly developed activity-based probe AW-091 was demonstrated to be highly selective for cathepsin S in vitro and proved useful in monitoring cysteine cathepsin activity in vivo in zymosan-induced mouse model of inflammation [140].

Another new method for analyzing protease activity is the use of activity-based probes (ABPs). This method permits the selective covalent binding with the active protease and also visualization of the enzyme within the diseased site [141]. These probes contain a domain which binds to the protease of interest, a spacer which has recognition domain which confers specificity and a tagged signal element which quantifies the enzyme. Activity-based protein profiling has emerged as a powerful chemical proteomic strategy to characterize enzyme function directly in native biological systems on a global scale [142]. Blum et al. [143] have shown that an intravenous injection of NIRF-ABPs into mice bearing grafted tumors helps in visualizing the whole-body image and direct monitoring of cathepsin B and L activity. The synthesis and characterization of several ABPs that target active cathepsin X have also been developed [144].

The main limitation of substrate-based probes is that single protease can convert hundreds of substrate probes, therefore compromising accuracy in quantitation of the protease. The drawback in ABPs is that the covalent binding of the signal probe with the active site of the enzyme results in reduction of amplification. A comparative analysis of substrates and activity-based probes as tools for noninvasive optical imaging of cysteine protease activity has been demonstrated [145]. Edgington et al. [146] highlight the strengths and weaknesses of both substrate-based and activity-based probes and their applications for imaging cysteine proteases which are important biomarkers for multiple human diseases.

Nanocarrier systems are an interesting approach to providing in vivo localization of drugs and probes in tumors. Nanocarriers contain surface-conjugated probes targeting proteases providing a unique mechanism by which tumors may be visualized. One such study demonstrates the use of a cathepsin B inhibitor, LNC-NS-629, which has been tethered onto a liposomal nanocarrier [147] primarily for the delivery of adjuvant therapy to tumor sites.

These detection technologies will help in validating the proteases with diagnostic and/or prognostic value in various diseases such as cancer, rheumatoid arthritis, osteoarthritis, cardiovascular diseases, and asthma.

9 Cysteine Cathepsin, Therapeutic Strategies, and Rheumatoid Arthritis

An imbalance in enzymatic activity of CC is observed in a number of pathological conditions such as RA, osteoarthritis, cancer, neurological disorders, osteoporosis, and lysosomal storage disorders [148, 149]. CC have been considered as valuable

therapeutic targets because of their involvement in these numerous pathologies. They have gathered a lot of interest in various areas such as cancer, viral infections, cardiovascular disease, osteoarthritis, and RA. The selective expression of cathepsin K and S in immune cells has been exploited in development of inhibitors and has made them as potential targets in diseased states. The rationale for targeting cathepsin K inhibitor as anti-resorptive agent has been reviewed extensively [150]. The use of appropriate cell-based studies and animal model has made a good progress in development of cathepsin K inhibitors [151]. The neutralizing monoclonal antibody (Mab) 2A2 against cathepsin B has been found to impair the degradation of intra- and extracellular matrix of MCF-10A neo cell line [152]. The properties of cathepsin inhibitors such as non-covalent reversible binding, high selectivity, and low probability of an adverse immune response have helped its development. These inhibitors have sequences similar to the substrate and are attached to nitriles, aldehydes α-keto heterocycles or aliphatic ketones as war heads. Cathepsin K inhibitors are peptidic in nature and have entered clinical studies. Early human data support a promising future for this therapeutic class of osteoporosis drugs [153].

Autoimmune disease is characterized as the host's recognition of self-antigens as foreign entities, inducing a subsequent immune response against these self-antigens, resulting in an inappropriate inflammatory response. RA an autoimmune disease characterized by erosive joint synovitis leading to the destruction of bone and cartilage [154]. This chronic inflammatory state results due to the accumulation of monocytes/macrophages activated by interleukin 1β in rheumatoid synovium. The caspase-3 activity was increased 0.7-fold in llsAM114, a proteasome inhibitor (PI) IL-1-β treated peripheral blood macrophage cultured cells from RA patients in comparison with unstimulated PI-treated cells [155]. The destructive metalloproteinases in articular joints of RA are produced by stimulation of synoviocytes and chondrocytes by the cellular products such as cytokines and histamine [156].

The cathepsins B and L were found to be elevated in the synovial fluid and synovial lining tissues in RA patients [157, 158]. Immunohistochemistry studies by Hansen et al. [159] have shown that normal synovium exhibited limited expression of these enzymes when compared to RA synovium. Keyszer and co-authors [160] have shown that the expression of MMP mRNA only was more pronounced in RA synovium compared to OA. However, immunohistochemical evaluation of proteins like MMP and cysteine proteinases were more prominent in RA synovial lining compared with OA. The cytokines induce a selective secretion of cathepsins B and L by synovial fibroblast-like cells and that cell-mediated articular degradation is a highly regulated process [161]. A marked increase in the activity of collagenolytic lysosomal enzyme, cathepsin L, was observed during the initiation and development of antigen-induced arthritis [162]. The studies with selective inactivators of CP demonstrate that cathepsin B L, and/or S are involved in bone resorption [163].

The ribozymes cleaving cathepsin L mRNA were able to decrease the synthesis of this protease in RA synovial fibroblasts thereby reducing the invasiveness into cartilage in mouse model [164].

Hou et al. [165] have investigated the expression of cathepsin K in synovial fibroblast and found a correlation between cathepsin K expression and disease severity. These studies have also shown that cocultures of RA synovial fibroblasts on cartilage disk had the ability to degrade collagen fibrils. The presence of inhibitors was effective in reducing the activity of cathepsin K and not cathepsin L, B, and S. The collagenolytic activity of cathepsin K in bone and cartilage requires interaction with soluble glycosaminoglycans [53]. Proinflammatory cytokines such as −1β and tumor necrosis factor alpha induces expression of cathepsin K in the rheumatoid synovium indicating that it can be a specific marker for RA [166]. A significant increase in serum cathepsin K levels correlating with the radiological destruction was observed in RA patient compared with healthy individuals [167]. The activated macrophages in the inflammatory process secrete the pro and mature forms of cathepsins into the cartilage matrix [168].

CatS-deficient mice showed diminished susceptibility to collagen-induced arthritis, suggesting a potential therapeutic target for regulation of immune responsiveness [169]. The in vivo administration of vinyl sulfone, an inhibitor of cathepsin S, resulted in a decrease in inflammation of the joint in adjuvant-induced arthritis [170]. Cathepsin S has a weaker collagenolytic activity when compared to cathepsin K which reveals its lesser involvement in collagen degradation [171]. Pozgan et al. [172] have observed a significant elevation in cathepsin L and S in the synovial fluid of RA patients. Interleukin-6 was also increased indicating that it stimulates the proteases during inflammation. Synovial fluid levels of cathepsin S and L were higher than the paired serum samples in RA patients. These proteases were also found to correlate with DA28 and CRP levels in seropositive patients [173]. It was noticed that anti-collagen antibodies did not induce arthritis in mice which was deficient in cathepsin C thus ruling out its pathological role in acute inflammatory arthritis [174].

In spite of the ubiquitous expression of the CC in human tissues, the multitude of functions demonstrated by them in healthy individuals has made them attractive therapeutic targets in diseased states.

10 Perspectives

Over the past few decades, tremendous advances have been made in understanding the structure, properties, biological functions, and inhibitors of cysteine cathepsins. Their main function is the catabolism of intracellular and endocytosed proteins. There is increasing urge to understand the biology of these proteins under physiological and pathological states. In pathological states, the inhibitors of CC form a therapeutic target for drug design. The modern techniques such as protease microarrays, in vivo imaging of CC and computational approaches can lead to identifying more substrates and inhibitors of CC. The combination of the improving

knowledge and new creative strategies can lead a rapid progress in basic and applied research which provides starting points for the development of new therapeutic modalities for various human diseases.

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References

1. López-Otín C, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283:30433–30437
2. Brömme D, Wilson S (2100) Role of cysteine cathepsins in extracellular proteolysis. In: Extracellular matrix degradation. Springer, Berlin, Heidelberg, pp 22–51
3. McGrath ME (1999) The lysosomal cysteine proteases. Annu Rev Biophys Biomol Struct 28:181–204
4. Barrett AJ, Rawlings ND (2007) 'Species' of peptidases. Biol Chem 388:1151–1157
5. Willstätter R, Bamann E (1929) Über die proteasen der magenschleimhaut. Erste abhandlung über die enzyme der leukocyten, Hoppe-Seyler's Z. Physiol Chem 180:127–143
6. Holt OJ, Gallo F, Griffiths GM (2006) Regulating secretory lysosomes. J Biochem 140:7–12
7. Blott EJ, Griffiths GM (2002) Secretory lysosomes. Nat Rev Mol Cell Biol 3:122–131
8. Turk V, Turk B, Turk D (2001) Lysosomal cysteine proteases: facts and opportunities. EMBO J 20:4629–4633
9. Rossi A, Deveraux Q, Turk B, Sali A (2004) Comprehensive search for cysteine cathepsins in the human genome. Biol Chem 385:363–372
10. Gutman HR, Fruton JS (1948) On the proteolytic enzymes of animal tissues VIII. An intracellular enzyme related to chymotrypsin. J Biol Chem 174:851–858
11. Barrett AJ, Rawlings ND, Woessner JF Jr (eds) (1998) Handbook of proteolytic enzymes. Academic Press, London
12. Takio K, Towatari T, Katunuma N, Teller DC, Titani K (1983) Homology of amino acid sequences of liver cathepsins B and H with that of papain. Proc Natl Acad Sci USA 80:3660–3670
13. Musil D, Zucic D, Turk D, Engh RA, Mayr I, Huber R, Popovic T, Turk V, Towatari T, Katunuma N (1991) The refined 2.15A X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. EMBO J 10:2321–2330
14. Turk V, Stoka V, Vasiljeva O, Renkoa M, Sun T, Turk B, Turk D (2012) Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochem Biophys Acta 1824:68–88
15. Salminen-Mankonen HJ, Morko J, Vuorio E (2007) Role of cathepsin K in normal joints and in the development of arthritis. Curr Drug Targets 8:315–323
16. Wex T, Wex H, Hartig R, Wilhelmsen S, Malfertheiner P (2003) Functional involvement of cathepsin W in the cytotoxic activity of NK-92 cells. FEBS Lett 552:115–119
17. Hsing LC, Rudensky AY (2005) The lysosomal cysteine proteases in MHC class II antigen presentation. Immunol Rev 207:229–241
18. Bromme D, Li Z, Barnes M, Mehler E (1999) Human cathepsin V functional expression, tissue distribution, electrostatic surface potential, enzymatic characterization, and chromosomal localization. Biochemistry 38:2377–2385
19. Goulet B, Baruch A, Moon NS, Poirier M, Sansregret LL, Erickson A, Bogyo M, Nepveu A (2004) A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor. Mol Cell 14:207–219

20. Ceru S, Konjar S, Maher K, Repnik U, Krizaj I, Bencina M, Renko M, Nepveu A, Zerovnik E, Turk B, Kopitar-Jerala N (2010) Stefin B interacts with histones and cathepsin L in the nucleus. J Biol Chem 285:10078–10086
21. Adams-Cioaba MA, Krupa JC, Xu C, Mort JS, Min J (2011) Structural basis for the recognition and cleavage of histone H3 by cathepsin L. Nat Commun 2:197
22. Kamphuis IG, Kalk KH, Swarte MB, Drenth J (1984) Structure of papain refined at 1.65 A resolution. J Mol Biol 179:233–256
23. Baker EN, Dodson EJ (1980) Crystallographic refinement of the structure of actinidin at 1.7 Å resolution by fast fourier least-squares methods. Acta Crystallogr Sect A 36:559–572
24. Guncar G, Pungercic G, Klemencic I, Turk V, Turk D (1999) Crystal structure of MHC class II-associated p41 Ii fragment bound to cathepsin L reveals the structural basis for differentiation between cathepsins L and S. EMBO J 18:793–803
25. Pinitglang S, Watts AB, Patel M, Reid JD, Noble MA, Gul S et al (1997) A classical enzyme active center motif lacks catalytic competence until modulated electrostatically. Biochemistry 36:9968–9982
26. Schechter I, Berger A (1967) On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun 27:157–162
27. Turk D, Turk B, Turk V (2003) Papain-like lysosomal cysteine proteases and their inhibitors: drug discovery targets? Biochem Soc Symp 70:15–30
28. Turk D, Guncar G, Podobnik M, Turk B (1998) Revised definition of substrate sites of papain-like cysteine proteases. Biol Chem 379:137–147
29. Vasiljeva O, Reinheckel T, Peters C, Turk D, Turk V, Turk B (2007) Emerging roles of cysteine cathepsins in disease and their potential as drug targets. Curr Pharm Des 13: 387–403
30. Hasnain S, Hirama T, Huber CP, Mason P, Mort JS (1993) Characterization of cathepsin B specificity by site-directed mutagenesis—importance of Glu (245) in the S2-P2 specificity for arginine and its role in transition-state stabilization. J Biol Chem 268:235–240
31. Fox T, Mason P, Storer AC, Mort JS (1995) Modification of S1 subsite specificity in the cysteine protease cathepsin-B. Protein Eng 8:53–57
32. Illy C, Quraishi O, Wang J, Purisima E, Vernet T, Mort JS (1997) Role of the occluding loop in cathepsin B activity. J Biol Chem 272:1197–1202
33. Lecaille F, Chowdhury S, Purisima E, Bromme D, Lalmanach G (2007) The S2 subsites of cathepsins K and L and their contribution to collagen degradation. Protein Sci 16:662–670
34. Choe Y, Leonetti F, Greenbaum DC, Lecaille F, Bogyo M, Bromme D, Ellman JA, Craik CS (2006) Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. J Biol Chem 281:12824–12832
35. Alves MFM, Puzer L, Cotrin SS, Juliano MA, Juliano L, Bromme D, Carmona AK (2003) S3 to S3′ subsite specificity of recombinant human cathepsin K and development of selective internally quenched fluorescent substrates. Biochem J 373:981–986
36. Ruckrich T, Brandenburg J, Cansier A, Muller M, Stevanovic S, Schilling K, Wiederanders B, Beck A, Melms A, Reich M, Driessen C, Kalbacher H (2006) Specificity of human cathepsin S determined by processing of peptide substrates and MHC class II-associated invariant chain. Biol Chem 387:1503–1511
37. Puzer L, Cotrin SS, Alves MF, Egborge T, Araújo MS, Juliano MA, Juliano L, Brömme D, Carmona AK (2004) Comparative substrate specificity analysis of recombinant human cathepsin V and cathepsin L. Arch Biochem Biophys 430:274–283
38. Reiser J, Adair B, Reinheckel T (2010) Specialized roles for cysteine cathepsins in health and disease. J Clin Invest 120:3421–3431
39. Baranski TJ, Faust PL, Kornfeld S (1990) Generation of a lysosomal enzyme targeting signal in the secretory protein pepsinogen. Cell 63:281–291
40. Mach L, Mort JS, Glossi J (1994) Non covalent complexes between the lysosomal proteinase cathepsin B and its propeptide account for stable, extracellular, high molecular mass form of the enzyme. J Biol Chem 269:13036–13040

41. Ménard R, Carmona E, Takebe S, Dufour E, Plouffe C, Mason P, Mort JS (1998) Autocatalytic processing of recombinant human procathepsin L. Contribution of both intermolecular and unimolecular events in the processing of procathepsin L in vitro. J BiolChem 273:4478–4484
42. Stoka V, Turk B, Turk V (2005) Lysosomal cysteine proteases: structural features and their role in apoptosis. IUBMB Life 57:347–353
43. Jordans S, Jenko-Kokalj S, Kühl NM, Tedelind S, Sendt W, Brömme D, Turk D, Brix K (2009) Monitoring compartment-specific substrate cleavage by cathepsins B, K, L, and S at physiological pH and redox conditions. BMC Biochem 10:23
44. Duncan EM, Muratore-Schroeder TL, Cook RG, Garcia BA, Shabanowitz J, Hunt DF, Allis CD (2008) Cathepsin L proteolytically processes histone H3 during mouse embryonic stem cell differentiation. Cell 135:284–294
45. Obermajer N, Repnik U, Jevnikar Z, Turk B, Kreft M, Kos J (2008) Cysteine protease cathepsin X modulates immune response via activation of beta (2) integrins. Immunology 124:76–88
46. Roth W, Deussing J, Botchkarev VA, Pauly-Evers M, Saftig P, Hafner A, Schmidt P, Schmahl W, Scherer J, Anton-Lamprecht I, Von Figura K, Paus R, Peters C (2000) Cathepsin L deficiency as molecular defect of hyperproliferation of keratinocytes and perturbation of hair follicle cycling. FASEB J 14:2075–2086
47. Frantz C, Stewart KM, Weaver VM (2010) The extracellular matrix at a glance. J Cell Sci 123:4195–4200
48. Werb Z (1997) ECM and cell surface proteolysis: regulating cellular ecology. Cell 91: 439–442
49. Yasuda Y, Li Z, Greenbaum D, Bogyo M, Weber E, Brömme D (2004) Cathepsin V, a novel and potent elastolytic activity expressed in activated macrophages. J Biol Chem 279: 36761–36770
50. Sage J, Mallevre F, Barbarin-Costes F, Samsonov SA, Gehrcke JP, Pisabarro MT, Perrier E, Schnebert S, Roget A, Livache T, Nizard C, Lalmanach G, Lecaille F (2013) Binding of chondroitin 4-sulfate to cathepsin S regulates its enzymatic activity. Biochemistry 52: 6487–6498
51. Li Z, Yasuda Y, Li W, Bogyo M, Katz N, Gordon RE, Fields GB, Brömme D (2004) Regulation of collagenase activities of human cathepsins by glycosaminoglycans. J Biol Chem 279:5470–5479
52. Mort JS, Magny MC, Lee ER (1998) Cathepsin B: an alternative protease for the generation of an aggrecan 'metalloproteinase' cleavage neoepitope. Biochem J 335:491–494
53. Hou WS, Li Z, Buttner FH, Bartnik E, Brömme D (2003) Cleavage site specificity of cathepsin K toward cartilage proteoglycans and protease complex formation. Biol Chem 384:891–897
54. Fonović M, Turk B (2014) Cysteine cathepsins and extracellular matrix degradation. Biochim Biophys Acta 1840:2560–2570
55. Panwar P, Du X, Sharma V, Lamour G, Castro M, Li H, Brömme D (2013) Effects of cysteine proteases on the structural and mechanical properties of collagen fibers. J Biol Chem 288:5940–5950
56. Li Z, Hou WS, Brömme D (2000) Collagenolytic activity of cathepsin K is specifically modulated by cartilage-resident chondroitin sulfates. Biochemistry 39:529–536
57. Kafienah W, Brömme D, Buttle DJ, Croucher LJ, Hollander AP (1998) Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. Biochem J 331:727–732
58. Lecaille F, Weidauer E, Juliano MA, Brömme D, Lalmanach G (2003) Probing cathepsin K activity with a selective substrate spanning its active site. Biochem J 375:307–312
59. Li ZQ, Hou WS, Escalante-Torres CR, Gelb BD, Bromme D (2002) Collagenase activity of cathepsin K depends on complex formation with chondroitin sulphate. J Biol Chem 277:28669–28676

60. Veillard F, Saidi A, Burden RE, Scott CJ, Gillet L, Lecaille F, Lalmanach G (2011) Cysteine cathepsins S and L modulate anti-angiogenic activities of human endostatin. J Biol Chem 286:37158–37167
61. Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P (1998) Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. J Clin Invest 102:576–583
62. Du X, Chen NL, Wong A, Craik CS, Brömme D (2013) Elastin degradation by cathepsin V requires two exosites. J Biol Chem 288:34871–34881
63. Taleb S, Cancello R, Clement K, Lacasa D (2006) Cathepsin S promotes human preadipocyte differentiation: possible involvement of fibronectin degradation. Endocrinology 147:4950–4959
64. Blum JS, Cresswell P (1988) Role for intracellular proteases in the processing and transport of class II HLA antigens. Proc Natl Acad Sci USA 85:3975–3979
65. Rudensky A, Beers C (2006) Lysosomal cysteine proteases and antigen presentation. Ernst Schering Res Found Workshop 56:81–95
66. Cresswell P (1996) Invariant chain structure and MHC class II function. Cell 84:505–507
67. Riese RJ, Wolf PR, Bromme D, Natkin LR, Villadangos JA, Ploegh HL, Chapman HA (1996) Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. Immunity 4:357–366
68. Martin WD, Hicks GG, Mendiratta SK, Leva HI, Ruley HE, Van Kaer L (1996) H-2M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. Cell 84:543–550
69. Jacques N, Marlieke LMJ, Petra P, Oddmund B (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol 11:823–836
70. Nakagawa TY, Rudensky AY (1999) The role of lysosomal proteinases in MHC class II-mediated antigen processing and presentation. Immunol Rev 172:121–129
71. Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, Villadangos JA, Ploegh H, Peters C, Rudensky AY (1998) Cathepsin L: critical role in li degradation and CD4 Tcell selection in the thymus. Science 280:450–453
72. Tolosa E, Li W, Yasuda Y, Wienhold W, Denzin LK, Lautwein A, Driessen C, Schnorrer P, Weber E, Stevanovic S, Kurek R, Melms A, Bromme D (2003) Cathepsin V is involved in the degradation of invariant chain in human thymus and is overexpressed in myasthenia gravis. J Clin Invest 112:517–526
73. Zou J, Henderson L, Thomas V, Swan P, Turner AN, Phelps RG (2007) Presentation of the good pasture autoantigen requires proteolytic unlocking steps that destroy prominent T cell epitopes. J Am Soc Nephrol 18:771–779
74. Hsieh CS, deRoos P, Honey K, Beers C, Rudensky AY (2002) A role for cathepsin L and cathepsin S in peptide generation for MHC class II presentation. J Immunol 168:2618–2625
75. Beers C, Burich A, Kleijmeer MJ, Griffith JM, Wong P, Rudensky A (2005) cathepsin S controls MHC class II-mediated antigen presentation by epithelial cells in vivo. J Immunol 174:1205–1212
76. Foghsgaard L, Wissing D, Mauch D, Lademann U, Bastholm L, Boes M, Elling F, Leist M, Jäättelä M (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J Cell Biol 153:999–1010
77. Boya P, Andreau K, Poncet D, Zamzami N, Perfettini JL, Metivier D, Ojcius DM, Jäättelä M, Kroemer G (2003) Lysosomal membrane permeabilization induces cell death in amitochondrion-dependent fashion. J Exp Med 197:1323–1334
78. Cirman T, Oresić K, Mazovec GD, Turk V, Reed JC, Myers RM, Salvesen GS, Turk B (2004) Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. J Biol Chem 279:3578–3587
79. Yin L, Stearns R, Gonzalez-Flecha B (2005) Lysosomal and mitochondrial pathways in H_2O_2-induced apoptosis of alveolar type II cells. J Cell Biochem 94:433–445

80. Vancompernolle K, Van Herreweghe F, Pynaert G, Van de Craen M, De Vos K, Totty N, Sterling A, Fiers W, Vandenabeele P, Grooten J (1998) Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. FEBS Lett 438:150–158
81. Yamashima T, Kohda Y, Tsuchiya K, Ueno T, Yamashita J, Yoshioka T, Kominami E (1998) Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on 'calpain-cathepsin hypothesis'. Eur J Neurosci 10:1723–1733
82. Guicciardi ME, Leist M, Gores GJ (2004) Lysosomes in cell death. Oncogene 23:2881–2890
83. Tardy C, Autefage H, Garcia V, Levade T, Andrieu-Abadie N (2004) Mannose 6-phosphorylated proteins pre pequired for tumor necrosis factor-induced apoptosis: defective response in I-cell disease fibroblasts. J Biol Chem 279:52914–52923
84. Reiners JJ Jr, Caruso JA, Mathieu P, Chelladurai B, Yin XM, Kessel D (2002) Release of cytochrome C and activation of pro-caspase-9 following lysosomal photodamage involves Bid cleavage. Cell Death Differ 9:9344–9944
85. Roberts LR, Adjei PN, Gores GJ (1999) Cathepsins as effector proteases in hepatocyte apoptosis. Cell Biochem Biophys 30:71–88
86. Turk B, Turk V, Turk D (1997) Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. Biol Chem 378:141–150
87. Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, Kaufmann SH, Gores GJ (2000) Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome C. J Clin Invest 106:1127–1137
88. Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, Ellerby LM, Bredesen D, Freeze H, Abrahamson M, Bromme D, Krajewski S, Reed JC, Yin XM, Turk V, Salvesen GS (2001) Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J Biol Chem 276:3149–3157
89. Teitelbaum SL (2006) Osteoclasts; culprits in inflammatory osteolysis. Arthritis Res Ther 8:201
90. Delaisse JM, Boyde A, Maconnachie E, Ali NN, Sear CH, Eeckhout Y, Vaes G, Jones SJ (1987) The effects of inhibitors of cysteine-proteinases and collagenase on the resorptive activity of isolated osteoclasts. Bone 8:305–313
91. Everts V, Beertsen W, Tigchelaar-Gutter W (1985) The digestion of phagocytosed collagen is inhibited by the proteinase inhibitors leupeptin and E-64. Coll Relat Res 5:315–336
92. Littlewood-Evans A, Kokubo T, Ishibashi O, Inaoka T, Wlodarski B, Gallagher JA, Bilbe G (1997) Localization of cathepsin K in human osteoclasts by in situ hybridization and immunohistochemistry. Bone 20:81–86
93. Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD, Delaissé JM (1998) The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 273:32347–32352
94. Votta BJ, Levy MA, Badger A, Bradbeer J, Dodds RA, James IE, Thompson S, Bossard MJ, Carr T, Connor JR, Tomaszek TA, Szewczuk L, Drake FH, Veber DF, Gowen M (1997) Peptide aldehyde inhibitors of cathepsin K inhibit bone resorption both *in vitro* and *in vivo*. J Bone Miner Res 12:1396–1406
95. Sun Y, Ishibashi M, Seimon T, Lee M, Sharma SM, Fitzgerald KA, Samokhin AO, Wang Y, Sayers S, Aikawa M, Jerome WG, Ostrowski MC, Bromme D, Libby P, Tabas IA, Welch CL, Tall AR (2009) Free cholesterol accumulation in macrophage membranes activates Toll-like receptors and p38 mitogen-activated protein kinase and induces cathepsin K. Circ Res 104:455–465
96. Li W, Yuan XM (2004) Increased expression and translocation of lysosomal cathepsins contribute to macrophage apoptosis in atherogenesis. Ann N Y Acad Sci 1030:427–433
97. Li W, Yuan XM, Olsson AG, Brunk UT (1998) Uptake of oxidized LDL by macrophages results in partial lysosomal enzyme inactivation and relocation. Arterioscler Thromb Vasc Biol 18:177–184

98. Lutgens E, Lutgens SP, Faber BC, Heeneman S, Gijbels MM, de Winther MP, Frederik P, van der Made I, Daugherty A, Sijbers AM, Fisher A, Long CJ, Saftig P, Black D, Daemen MJ, Cleutjens KB (2006) Disruption of the cathepsin K gene reduces atherosclerosis progression and induces plaque fibrosis but accelerates macrophage foam cell formation. Circulation 11:98–107
99. Lutgens S, Kisters N, Lutgens E, van Haaften R, Evelo C, de Winther M, Saftig P, Daemen M, Heeneman S, Cleutjens K (2006) Gene profiling of cathepsin K deficiency in atherogenesis: profibrotic but lipogenic. J Pathol 210:334–343
100. Oorni K, Sneck M, Bromme D, Pentikainen MO, Lindstedt KA, Mayranpaa M, Aitio H, Kovanen PT (2004) Cysteine protease cathepsin F is expressed in human atherosclerotic lesions, is secreted by cultured macrophages, and modifies low density lipoprotein particles in vitro. J Biol Chem 279:34776–34784
101. Lindstedt L, Lee M, Oorni K, Bromme D, Kovanen PT (2003) Cathepsins F and S block HDL3-induced cholesterol efflux from macrophage foam cells. Biochem Biophys Res Commun 312:1019–1024
102. Tertov VV, Orekhov AN (1997) Metabolism of native and naturally occurring multiple modified low density lipoprotein in smooth muscle cells of human aortic intima. Exp Mol Pathol 64:127–145
103. Twining SS (1994) Regulation of proteolytic activity in tissues. Crit Rev Biochem Mol Biol 29:315–383
104. Cygler M, Sivaraman J, Grochulski P, Coulombe R, Storer AC, Mort JS (1996) Structure of rat procathepsin B: model for inhibition of cysteine protease activity by the proregion. Structure 4:405–416
105. Coulombe R, Grochulski P, Sivaraman J, Menard R, Mort JS, Cygler M (1996) Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. EMBO J 15:5492–5503
106. LaLonde JM, Zhao BG, Janson CA, D'Alessio KJ, McQueney MS, Orsini MJ, Debouck CM, Smith WW (1999) The crystal structure of human procathepsin K. Biochemistry 38:862–869
107. Sivaraman J, Nagler DK, Zhang RL, Menard R, Cygler M (2000) Crystal structure of human procathepsin X: a cysteine protease with the proregion covalently linked to the active site cysteine. J Mol Biol 295:939–951
108. Nissler K, Kreusch S, Rommerskirch W, Strubel W, Weber E, Wiederanders B (1998) Sorting of non-glycosylated human procathepsin S in mammalian cells. Biol Chem 379:219–224
109. Nishimura Y, Kawabata T, Furuno K, Kato K (1989) Evidence that aspartic proteinase is involved in the proteolytic processing event of procathepsin L in lysosomes. Arch Biochem Biophys 271:400–406
110. Mach L, Mort JS, Glossl J (1994) Maturation of human procathepsin B. Proenzyme activation and proteolytic processing of the precursor to the mature proteinase, in vitro, are primarily unimolecular processes. J Biol Chem 269:13030–13035
111. Rozman J, Stojan J, Kuhelj R, Turk V, Turk B (1999) Autocatalytic processing of recombinant human procathepsin B is a bimolecular process. FEBS Lett 459:358–362
112. Dahl SW, Halkier T, Lauritzen C, Dolenc I, Pedersen J, Turk V, Turk B (2001) Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing. Biochemistry 40:1671–1678
113. Caglic D, Pungercar JR, Pejler G, Turk V, Turk B (2007) Glycosaminoglycans facilitate procathepsin B activation through disruption of propeptide-mature enzyme interactions. J Biol Chem 282:33076–33085
114. Fox T, de Miguel E, Mort JS, Storer AC (1992) Potent slow-binding inhibition of cathepsin B by its propeptide. Biochemistry 31:12571–12576

115. Carmona E, Dufour E, Plouffe C, Takebe S, Mason P, Mort JS, Menard R (1996) Potency sand selectivity of the cathepsin L propeptide as an inhibitor of cysteine proteases. Biochemistry 35:8149–8157
116. Turk B, Turk D, Turk V (2000) Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta 1477:98–111
117. Turk V, Turk B, Guncar G, Turk D, Kos J (2002) Lysosomal cathepsins: structure, role in antigen processing and presentation and cancer. Adv Enzyme Regul 4:285–303
118. Grzonka Z, Jankowska E, Kasprzykowski F, Kasprzykowska R, Lankiewicz L, Wiczk W, Wieczerzak E, Ciarkowski J, Drabik P, Janowski R, Kozak M, Jaskólski M, Grubb A (2001) Structural studies of cysteine proteases and their inhibitor. Acta Biochimica Polonica 48: 1–20
119. Turk B, Turk D, Salvesen GS (2002) Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators. Curr Pharm Des 8:1623–1637
120. Ogrinc T, Dolenc I, Ritonja A, Turk V (1993) Purification of the complex of cathepsin L and the MHC class II-associated invariant chain fragment from human kidney. FEBS Lett 336:555–559
121. Rawlings ND, Barrett AJ (1990) Evolution of proteins of the cystatin superfamily. J Mol Evol 30:60–71
122. Abrahamson M, Alvarez-Fernandez M, Nathanson CM (2003) Cystatins. Biochem Soc Symp 70:179–199
123. Reynolds JL, Skepper JN, McNair R, Kasama T, Gupta K, Weissberg PL, Jahnen-Dechent W, Shanahan CM (2005) Multifunctional roles for serum protein fetuin-A in inhibition of human vascular smooth muscle cell calcification. J Am Soc Nephrol 16:2920–2930
124. Jones AL, Hulett MD, Parish CR (2005) Histidine-rich glycoprotein: a novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. Immunol Cell Biol 83:106–118
125. Brown WM, Dziegielewska KM (2009) Friends and relations of the cystatin superfamily—new members and their evolution. Protein Sci 6:5–12
126. Kordis D, Turk V (2009) Phylogenomic analysis of the cystatin superfamily in eukaryotes and prokaryotes. BMC Evol Biol 9:266
127. Turk V, Bode W (1991) The cystatins: protein inhibitors of cysteine proteinases. FEBS Lett 285:213–219
128. Abrahamson M, Barrett AJ, Salvessen G, Grubb A (1986) Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. J Biol Chem 261:11282–11289
129. Lenarcic B, Bevec T (1998) Thyropins—new structurally related proteinase inhibitors. Biol Chem 379:105–111
130. Bevec T, Stoka V, Pungercic G, Dolenc I, Turk V (1996) Major histocompatibility complex class II-associated p41 invariant chain fragment is a strong inhibitor of lysosomal cathepsin L. J Exp Med 183:1331–1338
131. Bode W, Engh R, Musil D, Laber B, Stubbs M, Huber R, Turk V (1990) Mechanism of interaction of cysteine proteinases and their proteininhibitors as compared to the serine proteinase–inhibitor interaction. Biol Chem Hoppe-Seyler 371:111–118
132. Stubbs MT, Laber B, Bode W, Huber R, Jerala R, Lenarcic B, Turk V (1990) The refined 2.4 A X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. EMBO J 9:1939–1947
133. Abrahamson M, Ritonja A, Brown MA, Grubb A, Machleidt W, Barrett AJ (1987) Identification of the probable inhibitory reactive sites of the cysteine proteinase-inhibitors human cystatin C and chicken cystatin. J Biol Chem 262:9688–9694
134. Cox JM, Troutt JS, Knierman MD, Siegel RW, Qian Y-W, Ackermann BL, Konrad RJ (2012) Determination of cathepsin S abundance and activity in human plasma and implications for clinical investigation. Anal Biochem 430:130–137

135. Mahmood U, Tung CH, Bogdanov A, Weissleder R (1999) Near-infrared optical imaging of protease activity for tumor detection. Radiology 213:866–870
136. Grimm J, Kirsch DG, Windsor SD, Kim CFB, Santiago PM, Ntziachristos V, Jacks T, Weissleder R (2005) Use of gene expression profiling to direct *in vivo* molecular imaging of lung cancer. Proc Natl Acad Sci USA 102:14404–14409
137. Cortez-Retamozo V, Swirski FK, Waterman P, Yuan H, Figueiredo JL, Newton AP, Upadhyay R, Vinegoni C, Kohler R, Blois J et al (2008) Real-time assessment of inflammation and treatment response in a mouse model of allergic airway inflammation. J Clin Invest 118:4058–4066
138. Gounaris E, Tung CH, Restaino C, Maehr R, Kohler R, Joyce JA, Plough HL, Barrett TA, Weissleder R, Khazaie K (2008) Live imaging of cysteine-cathepsin activity reveals dynamics of focal inflammation, angiogenesis, and polyp growth. PLoS One 3:e2916
139. Watzke A, Kosec G, Kindermann M, Jeske V, Nestler HP, Turk V, Turk B, Wendt KU (2008) Selective activity-based probes for cysteine cathepsins. Angew Chem Int Ed 47: 406–409
140. Caglič D, Globisch A, Kindermann M, Lim N-H, Jeske V, Juretschke H-P, Bartnik E, Weithmann KU, Nagase H, Turk B et al (2011) Functional *in vivo* imaging of cysteine cathepsin activity in murine model of inflammation. Bioorg Med Chem 19:1055–1061
141. Wilkinson RD, Williams R, Scott CJ, Burden RE (2015) Cathepsin S: therapeutic, diagnostic, and prognostic potential. Biol Chem 396:867–882
142. Cravatt BF, Wright AT, Kozarich JW (2008) Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. Annu Rev Biochem 77:383–414
143. Blum G, von Degenfeld G, Merchant MJ, Blau HM, Bogyo M (2007) Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity based probes. Nat Chem Biol 3:668–677
144. Paulick MG, Bogyo M (2011) Development of activity-based probes for cathepsin X. ACS Chem Biol 6:563–572
145. Blum G, Weimer RM, Edgington LE, Adams W, Bogyo M (2009) Comparative assessment of substrates and activity based probes as tools for non-invasive optical imaging of cysteine protease activity. PLoS One 4:e6374
146. Edgington LE, Verdoes M, Bogyo M (2011) Functional imaging of proteases: recent advances in the design and application of substrate-based and activity-based probes. Curr Opin Chem Biol 15:798–805
147. Mikhaylov G, Klimpel D, Schaschke N, Mikac U, Vizovisek M, Fonovic M, Turk V, Turk B, Vasiljeva O (2014) Selective targeting of tumor and stromal cells by a nanocarrier system displaying lipidated cathepsin B inhibitor. Angew Chem Int Ed Engl 53:10077–10081
148. Kirschke H, Barrett AJ, Rawlings ND (1995) Proteinase 1: lysosomal cysteine proteases. Protein profile 2:1582–1643
149. Chapman HA, Riese RJ, Shi GP (1997) Emerging roles for cysteine protease in human biology. Annu Rev Physiol 59:63–68
150. Yasuda Y, Kaleta J, Brömme D (2005) The role of cathepsins in osteoporosis and arthritis: rationale for the design of new therapeutics. Adv Drug Deliv Rev 57:973–993
151. Grabowska U, Chambers TJ, Shiroo M (2005) Recent developments in cathepsin K inhibitor design. Curr Opin Drug Discov Devel 8:619–630
152. Premzl A, Zavasnik-Bergant V, Turk V, Kos J (2003) Intracellular and extracellular cathepsin B facilitate invasion of MCF-10A neo T cells through reconstituted extracellular matrix *in vitro*. Exp Cell Res 283:206–214
153. Deaton DN, Kumar S (2004) Cathepsin K inhibitors: their potential as anti-osteoporosis agents. Prog Med Chem 42:245–375
154. Schurigt U (2013) Role of cysteine cathepsins in joint inflammation and destruction in human rheumatoid arthritis and associated animal models. Inflamm Res 61:1021–1029

155. Chitra S, Nalini G, Lokeswari TS, Rajasekhar G (2016) The effect of proteasome inhibitor (AM114) on apoptosis in IL-1b-treated peripheral blood macrophage cultured cells from rheumatoid arthritis patients. Ind J Rheum 11:7–13
156. Murphy G, Nagasse H (2008) Reappraising metalloproteinases in rheumatoid arthritis and osteoarthritis: destruction or repair? Nat Clin Pract Rheumatol 4:128–135
157. Lenarcic B, Gabrijelcic D, Rozman B, Drobnic -Kossorok M, Turk V (1988) Human cathepsin B and cysteine proteinase inhibitors (CPIs) in inflammatory and metabolic joint diseases. Biol Chem Hoppe Seyler 369:257–261
158. Esser RE, Angelo RA, Murphey MD, Watts LM, Thornburg LP, Palmer JT et al (1994) Cysteine proteinase inhibitors decrease articular cartilage and bone destruction in chronic inflammatory arthritis. Arthritis Rheum 37:236–247
159. Hansen T, Petrow PK, Gaumann A, Keyszer GM, Eysel P, Eckardt A et al (2000) Cathepsin B and its endogenous inhibitor cystatin C in rheumatoid arthritis synovium. J Rheumatol 27:859–865
160. Keyszer G, Redlich A, Haupl T, Zacher J, Sparmann M, Engethum U et al (1998) Differential expression of cathepsins B and L compared with matrix metalloproteinases and their respective inhibitors in rheumatoid arthritis and osteoarthritis: a parallel investigation by semiquantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry. Arthritis Rheum 41:1378–1387
161. Lemaire R, Huet G, Zerimech F, Grard G, Fontaine C, Duquesnoy B et al (1997) Selective induction of the secretion of cathepsins B and L by cytokines in synovial fibroblast-like cells. Br J Rheumatol 36:735–743
162. Etherington DJ, Taylor MA, Henderson B (1988) Elevation of cathepsin L levels in the synovial lining of rabbits with antigen-induced arthritis. Br J Exp Pathol 69:281–289
163. Hill PA, Buttle DJ, Jones SJ, Boyde A, Murata M, Reynolds JJ et al (1994) Inhibition of bone resorption by selective inactivators of cysteine proteinases. J Cell Biochem 56:118–130
164. Schedel J, Seemayer CA, Pap T, Neidhart M, Kuchen S, Michel BA et al (2004) Targeting cathepsin L (CL) by specific ribozymes decreases CL protein synthesis and cartilage destruction in rheumatoid arthritis. Gene Ther 11:1040–1047
165. Hou WS, Li ZQ, Gordon RE, Chan K, Klein MJ, Levy R, Keysser M, Keyszer G, Bromme D (2001) Cathepsin K is a critical protease in synovial fibroblast-mediated collagen degradation. Am J Pathol 159:2167–2177
166. Hou WS, Li Z, Keyszer G, Weber E, Levy R, Klein MJ, Gravallese EM, Goldring SR, Bromme D (2002) Comparison of cathepsin K and S expression within the rheumatoid and osteoarthritic synovium. Arthritis Rheum 46:663–674
167. Skoumal M, Haberhauer G, Kolarz G, Hawa G, Woloszczuk W, Klingler A (2004) Serum cathepsin K levels of patients with longstanding rheumatoid arthritis: correlation with radiological destruction. Arthritis Res Ther 7:R65–R70
168. Reddy VY, Zhang QY, Weiss SJ (1995) Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages. Proc Natl Acad Sci USA 92:3849–3853
169. Nakagawa TY, Brissette WH, Lira PD, Griffiths RJ, Petrushova N, Stock J et al (1999) Impaired invariant chain degradation and antigen presentation and diminished collagen-induced arthritis in cathepsin S null mice. Immunity 10:207–217
170. Biroc SL, Gay S, Hummel K, Magill C, Palmer JT, Spencer DR et al (2001) Cysteine protease activity is up-regulated in inflamed ankle joints of rats with adjuvant-induced arthritis and decreases with *in vivo* administration of a vinyl sulfone cysteine protease inhibitor. Arthritis Rheum 44:703–711
171. Brömme D, Okamoto K, Wang BB, Biroc S (1996) Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. Functional expression of human cathepsin O2 in Spodoptera frugiperda and characterization of the enzyme. J Biol Chem 271:2126–2132

172. Pozgan U, Caglic D, Rozman B, Nagase H, Turk V, Turk B (2010) Expression and activity profiling of selected cysteine cathepsins and matrix metalloproteinases in synovial fluids from patients with rheumatoid arthritis and osteoarthritis. Biol Chem 391:571–579
173. Weitoft T, Larsson A, Manivel VA, Jorgen L, Knight A, Ronnelid J (2015) Cathepsin S and cathepsin L in serum and synovial fluid in rheumatoid arthritis with and without autoantibodies. Rheumatology 53:1–6
174. Adkison AM, Raptis SZ, Kelley DG, Pham CT (2002) Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. J Clin Invest 109:363–371

Proteases in Neuropathophysiology

7

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Abstract

Proteases in normal cells are important in performing essential biological processes in living systems. A balance between proteases and their inhibitors occurs for normal physiological functions and any disturbance of this balance usually leads to many diseases. The neuronal diseases are one of them. In this chapter, we will focus on the role of proteases and some protease inhibitors in various neurological disorders. Here, we would like to discuss about the role of different proteases (serine protease, cysteine protease, aspartic protease, matrix metallo protease, etc.) in neuropathology, like neuropathy, neuroinflammation, and also some neurological diseases, namely, Alzheimer's disease and Parkinson's disease. At the end of this chapter, we will also discuss about the involvement of serine proteases and their inhibitors in overall neurological disorder.

Keywords

Proteases · Neuropathy · Neuroinflammation · Alzheimer's · Parkinson's · Glioma

1 Introduction

Proteases or proteolytic enzymes are a group of enzymes which belong to the class of hydrolases. They hydrolyse peptide bonds of proteins without consuming energy because peptide bond hydrolysis is exothermic. These enzymes are widely distributed nearly in all plant and animal groups, including microorganisms. Proteases

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have adapted to variations in pH, reductive environment, and much other wide range of conditions through evolution and have been reported to use different mechanisms for various substrate hydrolysis in complex organisms. They are classified as either serine, cysteine or threonine proteases (acting as amino-terminal nucleophile hydrolases), or as aspartic, metallo, and glutamic proteases (no report so far with glutamic proteases in mammals) depending on their mechanism of action. Proteases specifically cleave protein substrates either from the N terminals or from the C terminals. The former class of proteases is called aminopeptidases and the latter one is called carboxypeptidases, respectively. Besides, endopeptidases are another class that cleave in the middle of the molecule [1]. By cleaving, proteases generate small peptides and amino acids needed for the body. Mammalian digestive enzymes (trypsin, chymotrypsin, and pepsin) and the lysosomal enzymes (cathepsin B and D) have the ability to modify protein structures by limited cleavage via activation of zymogens, blood clotting factors, and also by induction of lysis of fibrin clots and processing and transport of secretory proteins across the membranes. These are also used in many important biological processes such as regulation of metabolism, enzyme modification, complement system, apoptosis pathways, etc. [2].

Proteolytic enzymes are important because of their use in various forms of medical therapies such as reagents in laboratory, clinical, and industrial processes. Proteases represent a large group of industrial enzymes and this is about 60% of the total worldwide sale of enzyme [3].

It was estimated previously that about 2% of the human genes encode proteolytic enzymes and proteases have become an important therapeutic target because of their importance in many biological processes [4].

Studies suggest that uncontrolled proteolysis is associated with several diseases [5, 6]. The neuronal diseases are no exception to this as proteases have emerging roles in, neurodegenerative disorders and neuropathy, ischemia, neurological inflammation, traumatic brain injury, inflammatory and infectious diseases. This chapter basically describes the involvement of proteases in neurological disorder.

2 Role of MMPs in Neuroinflammation

Matrix metalloproteases (MMPs) comprise a large family of endopeptidases which require Zn^{2+} for their enzymatic activity. They play a crucial role in inflammation [7]. The activity of MMPs is regulated by endogenous inhibitors, TIMPs (tissue inhibitors of metalloproteases). As a therapeutic strategy, MMP inhibitors have been developed to target different kinds of inflammation-related diseases [8]. The gelatinases, MMP-2 and MMP-9, are the best studied members of the MMP family. They are found in the cerebrospinal fluid, serum, and extracellular matrix. MMP-9 and MMP-2 are activated by other proteases. MMP-2 is constitutively expressed in many tissues unlike MMP-9 which needs to be induced to elicit its activity. MMP-2 is activated by MMP-14 and MMP-9 is activated by MMP-3. The activity of MMP-9 and MMP-2 is inhibited by TIMP-1 and TIMP-2, respectively [9]. MMPs

also play important role in neuroinflammation and are involved in a wide range of diseases including Alzheimer's, multiple sclerosis, epilepsy, etc. MMP-2 and MMP-9 are upregulated in the CNS in these diseased conditions [10]. MMPs damage the blood–brain barrier, interrupt cell-matrix homeostasis, and trigger anoikis in order to induce brain cell death. Tetracycline and minocycline have been recently proposed for treating different types of neurological diseases as they are inhibitors of MMP-9 [11].

3 Role of MMPS in Neuropathic Pain

Neuropathic pain is associated with painful responses to non-painful mechanical stimuli. Proteases play an important role in the genesis of neuropathic pain. Mice lacking MMP-24 experience reduced neuropathic pain [12]. The lysosomal cysteine protease, cathepsin S, facilitates neuropathic pain through cleavage of the chemokine, CX3CL1 [13]. MMP-9 is upregulated in the sciatic nerve after nerve crush, leading to nerve demyelination by the degradation of the myelin basic protein [14]. It has been observed that ligation of the spinal cord induces a rapid but transient upregulation of MMP-9 in the dorsal root ganglia. MMP-9 expression has been found to increase in these neurons within 1 day of ligation [15]. It has also been observed that inhibition of MMP-9 can attenuate the early phase of neuropathic pain. However, TIMP-1 has been found to be extremely potent in preventing both, early and late phases, of neuropathic pain. Literature suggests that MMP-9 can induce symptoms of neuropathic pain via IL-1β [16]. Both MMP-9 and IL-1β are co-expressed in the dorsal root ganglia [16]. The precursor form of IL-1β exhibits no biological activity. Though caspase-1 is known to exhibit cleavage-induced activation, the enzyme gets inactivated after nerve injury. In such cases, MMP-2 and MMP-9 induce IL-1β cleavage [17]. It is reported that nerve injury mediated spontaneous discharge in the sensory neurons leads to the release of MMP-9 into the extracellular matrix where it cleaves pro-IL-1β to produce active IL-1β, which then acts on adjacent neurons to generate hyper-excitation. MMP-9 in soma of the dorsal root ganglia is translocated to the nerve terminals to activate the microglia. It is also reported that MMP-9 is required for the development of epilepsy [18].

Neuropathic pain in the late phase is blocked by TIMP-2. MMP-2 induces IL-1β cleavage in the late phase. MMP-2 is reported to activate astrocytes of the spinal cord. Inhibition of MMP-2 can suppress the induction of pERK in astrocytes of the spinal cord in the late phase [16]. Thus, MMP-2 contributes to the late phase of development of neuropathic pain by activating IL-1β and astrocytes.

Transient receptor potential vanilloid 1 (TRPV1) and cannabinoid receptors (CBRs) are reported to link MMP network and mechanisms of nociception. The concerned pathways diminish thermal hyperalgesia and induce the activity of TIMPs [19]. Activation of TRPV1R occurs in animals suffering from spinal cord injury induced neuropathic pain [20].

In systemic lupus erythematosus (SLE), peripheral neuropathy is often observed. The pathogenic mechanisms involve ischemic nerve damage due to vasculopathy and vasculitis of the nutritional vessels. Abnormal MMP levels have been reported in the serum of SLE patients [21]. MMP expression in mononuclear cells may be related to leukocyte trafficking through the vessel walls. The upregulation of MMP-3 and MMP-9 within the vessel walls leads to vascular damage in SLE.

Cortical spreading depression (CSD) is associated with slow propagating waves of depolarization that increase the expression of many genes, most prominently MMPs which alter the permeability of the blood–brain barrier and allow nitric oxide, adenosine, potassium, and other factors to sensitize the perivascular trigeminal afferent endings of the dura mater [22].

Migraine is usually characterized by recurrent bouts of severe throbbing head pain. The pathophysiology of this disorder includes neuronal hyperexcitability, hypoperfusion, abnormalities of neurotransmitters as well as neurogenic inflammation [23]. Highly significant increase in MMP activity in migraine patients has been found to be associated with atherogenic lipid alterations and hyperinsulinemia [24].

4 Role of Cysteine Proteases in Neuroinflammation

Neuroinflammation is associated with selective loss and/or dysfunction of neurons, thereby leading to neurodegenerative disorders. Lysosomal cathepsins, derived from microglia, are important markers of neuroinflammation. Microglial expression, release, and activity of cathepsins Cat B, D, H, L, and S (cysteine proteases) in the brain are reported to induce neuroinflammation [25]. On the other hand, the expression of stefin B (cystatin B), an endogenous inhibitor of cysteine cathepsin is localized in the cytosol, nucleus, and mitochondria. It is upregulated by activation of macrophages and induction of cellular stress. Mutations of stefin B gene induce the Unverricht–Lundborg disease (EPM1), a neurodegenerative disorder. Activation of stefin B-deficient microglia exhibits a significantly higher proportion of both pro-inflammatory M1 and anti-inflammatory M2 microglia in the stefin B-deficient mice brains compared with the control mice. Such stefin B-deficient mice are highly sensitive to the lipopolysaccharide (LPS)-induced sepsis, owing to the increased expression of caspase-11 and some pro-inflammatory cytokines, like IL-1 β and IL-18 [26].

5 Role of PARs and Serine Proteases in Neuropathic Pain

Protease-activated receptors (PARs) initiate and maintain neuropathic pain. Following nerve injury, alterations in neurons and neuron functions induce abnormal increase of some neuromodulators and neurotransmitters, such as substance P (SP), kinins, prostaglandins, etc. Such factors induce sensations of pain and ultimately

lead to PAR-mediated activation of glial cells (astrocytes and microglia), which induce the progress of neuropathic pain. Inflammatory factors have profound effect on PARs (especially PAR1 and PAR2) in the surrounding neurons and aggravate the status of pain. With the progress of neuroinflammatory pain, microglia are activated, release inflammatory factors and complements which in turn activate astrocytes and making the pain worse. Thus, PARs may play an important role in the induction and maintenance of chronic neuropathic pain [27].

Serine proteases are well-known enzymes involved in digestion of dietary proteins, coagulation of blood and maintenance of homeostasis [28]. SerpinA3N, a serine protease inhibitor is upregulated in the dorsal root ganglia (DRG) in mice suffering from neuropathic pain. Mice lacking SerpinA3N develop excessive neuropathic allodynia. T lymphocytes, infiltrating the DRG after nerve injury, release the leukocyte elastase (LE), which is inhibited by SerpinA3N derived from DRG neurons [29].

6 Proteases in Glioma

A glioma is a common type of tumor originating in the brain. It is called a glioma because it arises from glial cells. There are three types of normal glial cells that can produce tumors. Astrocyte, oligodendrocyte, and ependymal cells produce astrocytomas (including glioblastomas), oligodendrogliomas, and ependymomas, respectively. Mixed gliomas are the mixture of these different cells tumors. The "optic nerve glioma" and "brain stem glioma" are not the tissue type from which they originate. They are named for their locations. The invasive nature of brain tumor cells makes it an important cause of mortality and underscores the need to understand in more detail the mechanisms of tumor invasiveness. There are several steps involved in tumor invasion such as interaction of tumor cells with extracellular matrix (ECM) ligands, hydrolytic destruction of the matrix by proteolytic enzymes and the migration of those cells through the destructed area. Among these, the best correlation with the tissue invasiveness is the ability of tumor cells to digest the ECM by secretion of proteolytic enzymes. To degrade extracellular matrix components selectively and maintain a microenvironment facilitating tumor cell survival, increased synthesis, and secretion of several proteases namely, cysteine, serine, and metalloproteinases are induced. Protease profiling studies indicate that the expression of the serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR), of the cysteine protease cathepsin B and of the matrix metalloproteinases MMP-2 and MMP-9 are increased in astrocytomas of the inflamed brain [30]. CNS tissue contains three major groups of proteases and their inhibitors: (1) serine proteases, including tissue plasminogen activator (tPA), urokinase, and plasminogen activator inhibitors (PAIs); (2) matrix metalloproteases (MMPs) and tissue inhibitors of MMPs (TIMPs); and (3) cysteine proteases. Of these groups, most is known about the role of MMPs in tumor invasion. Interestingly, however, the level of expression of cathepsins B (cysteine proteases) and its

natural inhibitor, cystatin C, have shown a role in invasiveness in gliomas [31]. MMP-2 is the member of the MMP family that emerges as the most central in glioma invasiveness. Inhibitors of MMP-2 have been reported to block glioma invasion in vitro whereas increasing MMP-2 activity increases glioma invasiveness in the same [32, 33]. A recent ex vivo studies also demonstrated increased MMP-2 activity in resected glioblastoma specimens compared to normal brain or low-grade glioma [34]. The progressions of tumors from the premalignant to malignant state have been implicated in the increased levels of human cysteine proteases. Interaction with specific cysteine protease inhibitors (CPI) regulates the physiological activities of these proteases and a decrease in CPI activity may contribute to the malignant properties of brain tumors [35].

7 Alzheimer's Disease and Proteases

In 1907, Alois Alzheimer published a short note about a 56-year-old woman Auguste D, who was suffering from severe memory impairment [36]. After the death of that woman, Alzheimer saw some of the characteristic structural alterations like compromised neurons, intraneuronal tangles, and extracellular plaques in her brain which now are commonly accepted as neuropathological hallmarks of the disease carrying his name [37, 38]. Alzheimer disease (AD) is not a single disorder; at least two different types exist, among which 5% of the cases is due to mutations of certain genes [39]. Studies suggest that proteases are prominently involved in the pathobiology of this disease. The present part of the chapter describes the involvement of proteases in the development and progression of AD. Alzheimer's disease affects every tenth human aged over 65. It is a progressive degenerative encephalopathy, characterized by behavioral disorders and personality changes. Neuropathological hallmarks of AD are accelerated atrophy and loss of neurons from specific areas of the brain. The exact cause of Alzheimer's disease is still unknown. Most gene mutations in Alzheimer's disease are associated with the amyloid precursor protein and ultimately amyloid β. The proteases α-, β-, and γ-secretase are the three executioners of amyloid precursor protein processing and disruption of their balance and are suspected to result in Alzheimer's disease [40]. Basically this 40–43 amino acid long fragment is generated from the beta-amyloid precursor protein (betaAPP) by two distinct enzymes, namely the beta- and the gamma-secretases. Besides these amyloid beta peptide (Abeta)-forming proteases, a third cleavage performed by the so-called alpha-secretase takes place in the middle of the Abeta sequence and not only prevents its formation but also generates the secreted product sAPPalpha that possesses neurotrophic and neuroprotective properties. This functional upregulation is mediated by protein kinase C (PKC) agonists and ADAM proteases [41]. Abnormally folded Abeta protein fragments and tau precipitate in amyloid plaques and neuronal tangles, respectively. The interaction between the proteolytic pathways responsible for the generation and clearance of these fragments and disturbances in these pathways is believed to

provide a background for a novel understanding of Alzheimer disease as a multifactorial disorder [42].

In AD brain, certain enzymes such as calpains and cathepsins become dramatically upregulated. It has been seen that changes in the lysosomal cathepsins are an early and reliable cellular marker for AD [43–45]. It has been repeatedly shown that cathepsin D contributes to the intracellular clearance of Abeta peptides and remains responsible for their degradation [46]. Thus, for the prevention of the amyloid formation resulting from the digestion of Abeta, cathepsin D appears to play some important roles [47].

8 Proteolytic Stress and Its Effect on Parkinson's Disease

The cause and subsequent development of an irregular condition in Parkinson's disease (PD) has been studied for many years and recent indication suggests that insufficiencies in the ubiquitin-proteasome system (UPS) and proteolytic stress underlying nigral pathology in both familial and sporadic forms of the illness are responsible [48]. Mutations in alpha-synuclein, that cause the protein to misfold and resist proteasomal degradation, cause familial PD [49, 50]. In the same way, for hereditary PD, mutations in two enzymes (parkin and ubiquitin C-terminal hydrolase L1), involved in the normal purpose of the ubiquitin-proteasome system, are found to be responsible [51]. Additionally, structural and functional deficiencies in 26/20S proteasomes with growth and collection of potentially cytotoxic unusual proteins have been documented in the substantia nigra pars compacta of patients with sporadic PD [52]. So, a deficiency in protein handling seems to be a mutual factor in sporadic and the various familial forms of PD. Deficiencies in the UPS can induce protein escalation, formation of Lewy body-like inclusions, and dopaminergic cell death in both in vitro and in vivo model systems. Reduced UPS action is responsible for substantia nigra pars compacta (SNc) dopaminergic neurons vulnerability to the neurodegenerative progression and poor UPS PD activity is also responsible for an age-associated disorder [51]. The evidences that prove proteolytic stress play an important role in the cause and/or mechanism responsible for PD, should assist the improvement of novel model systems for studying hypotheses associated with the mechanism of neurodegeneration and for the study of familiar neuroprotective therapies [48].

Abnormality in the UPS system responsible for the development of both familial and sporadic PD helps to clarify clinical and neuropathological disorders [53]. The accumulation of intracellular unwanted proteins due to failure of the UPS is common to both type of PD and could be responsible for the introduction and/or development of nigrostriatal deterioration in these conditions. Damage to the proteosomal system could incidentally affect mitochondrial dysfunction [54] and the increase of oxidative stress [55]. Protein gathering is also thought to bring apoptotic cell death by upregulating pro-apoptotic signal, like Jun kinase. So, it is striking that neuronal death in the SNc in PD has been related with an apoptotic process [56].

9 Cysteine Protease's Role in Parkinson's Disease

The knowledge on the cellular and molecular mechanisms of midbrain, dopamine neurons worsening in Parkinson's disease (PD) is limited. Contribution of the calpains (calcium-dependent proteases) in the impairment of dopamine neurons has been recently reported in an animal model (mouse) of PD [57]. *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration has been shown to amplify calpain-facilitated proteolysis in nigral dopamine neurons. On the other hand, a calpain inhibitor (MDL-28170) or adenovirus-facilitated overexpression of the internal calpain inhibitor protein, calpastatin, inhibited proteolysis of calpain resulting in a significant attenuation of MPTP-induced loss of nigral dopamine neurons. At the same time, MPTP-induced locomotor shortages were eliminated and striatal postsynaptic activity markers were normalized with this calpain inhibitor-induced neuroprotection in the experimental animals. Behavioral improvements, however, could not linearly establish a relationship with restored levels of striatal dopamine in MPTP-treated, calpain-inhibited mice. Based on these results, the researchers feel that defence against nigral neuron degeneration in PD may be sufficient without necessitating striatal reinnervation to facilitate normalized locomotor activity. Increased calpain-related proteolytic activity was also found in human suffering from PD (as verified by immunohistochemical analyses of postmortem midbrain tissues) although that was absent in age-matched control subjects. On combining, these data appear to provide a new potent association between the proteolytic action of calpain (in an MPTP animal model of PD) and the etymology of neuronal injury in PD in humans [57].

Tyrosine nitration of α-synuclein can potentially create fibril development of original α-synuclein. It also can decrease the rate of deprivation by the 20S proteasome and the cysteine protease calpain I [58]. Alternative pathways of α-synuclein deprivation including dispensation by the lysosomal autophagy pathway and by cytoplasmic proteases, like calpain I, have also been projected [59, 60]. Quantitative analysis of m-calpain staining in the substantia nigra and locus coeruleus indicated an increased density of fibers and neuronal perikarya in parkinsonian patients [61].

DJ-1 may contribute an important diversified role in apparently distinct neurodegenerative disorders. Additionally, indecipherable forms of DJ-1 are radically amplified in the brains of sporadic PD patients [62] and perhaps it is also reproving DJ-1 in sporadic forms of this disease. The crystal structure of human DJ-1 shows a flavodoxin-like fold structure similar with the bacterial protease PH1704 and the stress inducible molecular chaperone Hsp31 isolated from *E. coli* and yeast [63, 64]. The crystal structure of DJ-1 also indicated that it exists as a dimer in solution. This dimer form of DJ-1 has been established in cultured cells [65, 66]. An active site has been documented near the dimer interface with resemblances to the active site catalytic triad (Cys-His-Asp/Glu) of cysteine proteases, linking residues Cys106, His126, and perhaps Glu18 although these residues do not show an direction favorable for proton transfer that is characteristic of cysteine protease

catalysis. DJ-1 is a component of the UPS and may confer protection by functioning as a molecular chaperone or protease to refold or promote the possibility of dual enzymatic purpose, i.e., chaperone and protease activities [62].

10 Neurodisorders Related to Serine Proteases

Serine proteases are a diversified specialized group of enzymes which involve a serine residue for nucleophilic catalysis. Associates of the serine protease family, like thrombin, possess a catalytic site containing a serine, histidine, and an aspartic acid residue [67]. Equilibrium is sustained between serine proteases and their particular inhibitors in many tissues of the body where they are included in a variety of physiological processes related with inflammation, complement activation, connective tissue turnover and repair. After activation, proteases may play a threat to tissues and other circulating proteins. So, they are regulated by their respective inhibitors [68–70]. Here in this part of the chapter, we restrict our argument to a brief mentioning of serine proteases and their inhibitors that have been exactly related with neurological disorders.

The serine proteases and their inhibitors (serpins) have been recommended to play a crucial role in the nervous system. Cultured sympathetic and sensory neurons have been found to release serine proteases which help in growth cone elongation and target cells secrete serpins that assist to inhibit growth cone elongation and stimulate firm synapse formation [71].

Vigorously proliferating Schwann cells secrete serine proteases which are involved in helping these cells to enter and subdivide large bundles of axons which ensure separate myelin ensheathment [72].

Recent evidence suggests that association of the serine protease family (e.g., thrombin, chymotrypsin, urokinase plasminogen activator, and kallikrein) shows an important role in normal change and pathology of the nervous system. Serine proteases and their potent inhibitors have been found to be increased in the neural parenchyma and cerebrospinal fluid after injury to the blood–brain barrier (BBB). Thrombin-like proteases play detrimental effects on different neuronal and non-neuronal cell populations in vitro. These effects can be prevented with specific serine protease inhibitors like serpins. Furthermore, it has been found that treatment with protease nexin-1, a serpin, inhibits thrombin-like proteases and promotes the survival and growth of spinal motor neurons during the period of programmed cell death after injury. Taken together, these views suggest that thrombin-like proteases play a negative role, but serpins promote the development and maintenance of neuronal cells. Changes in the balance between serine proteases and their inhibitors may lead to neuropathological states similar to Alzheimer's disease [73].

Kallikrein 6 (K6) is a newly identified associate of the Kallikrein family of serine proteases. K6 preferentially expressed in the adult central nervous system (CNS). It was established that K6 is predominantly expressed by inflammatory cells at sites of CNS inflammation in animal and human models of multiple sclerosis. This enzyme

participates in CNS inflammatory diseases and may represent a novel therapeutic target for the treatment of progressive inflammatory disorders, like multiple sclerosis [74]. Alterations of the levels of this enzyme were found in sera and central nervous system (CNS) in different neurological disorders namely, multiple sclerosis, neurodegeneration, Alzheimer's disease. The alterations have also been reported within the central nervous system of ischemic and active multiple sclerosis lesions [75–79]. In multiple sclerosis, several kallikreins have been linked to immune cell activation [80]. Kallikrein 1 (K1) and K6 can be treated as serological markers for progressive multiple sclerosis and also responsible for the advancement of neurological disability due to axonal injury and neuronal cell death [76]. Neurosin, another kallikrein-like serine protease, is highly expressed in the human brain and is responsible for aging. Decreased cerebrospinal fluid concentration of neurosin increases risk factor for developing of Alzheimer's disease [81].

Urokinase plasminogen activator is another important serine protease which cleans neurotoxic amyloid peptides from CNS. Neurotoxic amyloid peptides are the characteristic pathophysiological feature of Alzheimer's disease [82]. Urokinase plasminogen activator (PLAU) gene rs2227564 polymorphism might raise the risk of Alzheimer's disease [83]. Urinary-type plasminogen activator (uPA), after binding with uPA receptor (uPAR), encourages the activation of matrix metalloproteinase-9 (MMP-9). Experiments were carried out to identify the expression level of MMP-9, uPA and uPAR in postmortem brains from patients with Alzheimer's disease (AD) and vascular dementia (VD) and found that the neurons in the AD brains show high level of MMP-9 and uPAR. MMP-9 was activated by uPA and helped in degradation of Abeta. Immunolocalization of uPA in the neurosin of the AD brains was well expressed [84].

Abraham and Potter [85] suggested that serine protease inhibitor, 1-antichymotrypsin, plays a major role in different neuropathological diseases, like Alzheimer's disease, hereditary cerebral hemorrhage and in normal aging. Immunocytochemical study suggests that pericytes and astrocytes (near areas of neuronal or tissue loss) express this serine protease inhibitor. However, it is not expressed in Creutzfeldt–Jakob disease, familial amyloidotic neuropathy, primary amyloidosis, or secondary amyloidosis [68, 85].

Activated protein C (APC) is a plasma serine protease which is capable of different immunological and cytological activities. Animal injury studies indicated that recombinant APC and mutants' apc are remarkably capable of therapeutic approaches for a wide range of injuries including neurological injuries. In case of neurological injuries, APC reduces damage caused by ischemia in the brain, by acute brain trauma, and by chronic neurodegenerative conditions. Endothelial cell protein C receptor helps APCs for these neuroprotective effects. APC activates cell signalling networks by activating protease-activated receptors 1 and 3 with alterations in gene expression profiles. To minimize APC-induced bleeding risk, APC variants were engineered to have <10% anticoagulant activity but retain normal cell signalling (Lys191-193 mutated to Ala191-193). These variants are safe and could set the stage for ongoing and future clinical trials for ischemic stroke or for other brain pathologies [86].

Protein C also plays unique roles in therapies of neuropathological conditions, like multiple sclerosis. Relative proteomic profiles recognized protein C inhibitor within chronic active plaque samples of multiple sclerosis, suggesting dysregulation of molecules related with coagulation. In vivo administration of recombinant activated protein C diminished autoimmune encephalomyelitis by its anticoagulant and signalling functions and suppressed Th1 and Th17 cytokines in astrocytes and immune cells [87].

Pigment epithelium-derived factor (PEDF), a secretory protein derived from cultured pigment epithelial cells of the fetal human retina, is capable of inducing a neuronal phenotype in cultured human retinoblastoma cells. The differentiated cells also display increased expression of neuron-specific enolase and the 200 kDa neurofilament subunit. Amino acid and DNA sequencing data indicated that PEDF belongs to the serine protease inhibitor (serpins) family. PEDF could exert a paracrine effect in the embryonic retina which influences neuronal differentiation by a mechanism other than classical serine protease inhibition activity [88].

Neuroserpin is a novel serine protease inhibitor and is secreted from the axons of both PNS and CNS neurons. It is predominantly neuronal in origin and expressed predominantly in the formation and reorganization of synapses during nervous system development. In the adult central nervous system, it is found in areas in which the synaptic changes are associated with learning and memory. Neuroserpin is maintaining the equilibrium between proteolytic and anti-proteolytic activities at the synapse needed for the formation or reorganization of synaptic connections, as well as for synaptic plasticity in the adult nervous system [89].

11 Conclusion

Proteases play an eminent role in the neuropathophysiological conditions. Proteases like membrane matrix proteases (MMP-2, MMP-9, TIMP-1, and TIMP-2), cysteine proteases, serine proteases, and their inhibitors are important in this regard. Parkinson's disease, Alzheimer's disease, Lupus erythematosus, neuropathic pain, neurological inflammation, ischemia, and traumatic brain injury are mostly affected by these proteases. The interplay between proteases and protease inhibitors is therapeutically significant and needs to be further investigated.

References

1. Turk B (2006) Targeting proteases: successes, failures and future prospects. Nat Rev Drug Discov 5:785–799
2. Rani K, Rana R, Datt S (2012) Review on latest overview of proteases. Int J Curr Life Sci 2 (1):12–18
3. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635
4. Craik CS, Page MJ, Madison EL (2011) Proteases as therapeutics. Biochem J 435:1–16

5. Ledesma MD, Da Silva JS, Crassaerts K, Delacourte A, De Strooper B et al (2000) Brain plasmin enhances APP α-cleavage and Aβ degradation and is reduced in Alzheimer's disease brains. EMBO Rep 1:530–535
6. López-Otín C, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283:30433–30437
7. Yong VW (2005) Metalloproteinases: mediators of pathology and regeneration in the CNS. Nat Rev Neurosci 6:931–944
8. Hu J, Van den Steen PE, Sang Q-XA, Opdenakker G (2007) Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. Nat Rev Drug Discov 6:480–498
9. Murphy G, Willenbrock F (1995) Tissue inhibitors of matrix metalloendopeptidases. Methods Enzymol 248:496
10. Rosenberg GA (2002) Matrix metalloproteinases in neuroinflammation. Glia 39:279–291
11. Murata Y, Rosell A, Scannevin RH, Rhodes KJ, Wang X et al (2008) Extension of the thrombolytic time window with minocycline in experimental stroke. Stroke 39:3372–3377
12. Komori K, Nonaka T, Okada A, Kinoh H, Hayashita-Kinoh H et al (2004) Absence of mechanical allodynia and Aβ-fiber sprouting after sciatic nerve injury in mice lacking membrane-type 5 matrix metalloproteinase. FEBS Lett 557:125–128
13. Clark AK, Yip PK, Grist J, Gentry C, Staniland AA et al (2007) Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proc Natl Acad Sci 104:10655–10660
14. Chattopadhyay S, Myers RR, Janes J, Shubayev V (2007) Cytokine regulation of MMP-9 in peripheral glia: implications for pathological processes and pain in injured nerve. Brain Behav Immun 21:561–568
15. Ji R-R, Xu Z-Z, Wang X, Lo EH (2009) Matrix metalloprotease regulation of neuropathic pain. Trends Pharmacol Sci 30:336–340
16. Kawasaki Y, Xu Z-Z, Wang X, Park JY, Zhuang Z-Y et al (2008) Distinct roles of matrix metalloproteases in the early-and late-phase development of neuropathic pain. Nat Med 14:331–336
17. Schönbeck U, Mach F, Libby P (1998) Generation of biologically active IL-1β by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1β processing. J Immunol 161:3340–3346
18. Wilczynski GM, Konopacki FA, Wilczek E, Lasiecka Z, Gorlewicz A et al (2008) Important role of matrix metalloproteinase 9 in epileptogenesis. J Cell Biol 180:1021–1035
19. Rice A, Farquhar-Smith W, Nagy I (2002) Endocannabinoids and pain: spinal and peripheral analgesia in inflammation and neuropathy. Prostaglandins. Leukot Essent Fatty Acids (PLEFA) 66:243–256
20. DomBourian MG, Turner NA, Gerovac TA, Vemuganti R, Miranpuri GS et al (2006) B1 and TRPV-1 receptor genes and their relationship to hyperalgesia following spinal cord injury. Spine 31:2778–2782
21. Robak E, Wierzbowska A, Chmiela M, Kulczycka L, Sysa-Jedrejowska A et al (2006) Circulating total and active metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 in patients with systemic lupus erythomatosus. Mediat Inflamm
22. Richter F, Lehmenkühler A (2008) Cortical spreading depression (CSD). Der Schmerz 22:544–550
23. Dalkara T, Nozari A, Moskowitz MA (2010) Migraine aura pathophysiology: the role of blood vessels and microembolisation. Lancet Neurol 9:309–317
24. Bernecker C, Pailer S, Kieslinger P, Horejsi R, Möller R et al (2011) Increased matrix metalloproteinase activity is associated with migraine and migraine-related metabolic dysfunctions. Eur J Neurol 18:571–576
25. Fan K, Li D, Zhang Y, Han C, Liang J et al (2015) The induction of neuronal death by up-regulated microglial cathepsin H in LPS-induced neuroinflammation. J neuroinflamm 12:1
26. Kopitar-Jerala N (2015) The role of stefin B in neuro-inflammation. Front Cell Neurosci 9

27. Jian C, Wenjuan H, Huaizhen R (2009) Protease-activated receptors in neuropathic pain: an important mediator between neuron and glia. J Med Coll PLA 24:244–249
28. Shpacovitch V, Feld M, Hollenberg M, Luger T, Steinhoff M (2008) Role of protease-activated receptors in inflammatory responses, innate and adaptive immunity. J Leukoc Biol 83:1309–1322
29. Vicuña L, Strochlic DE, Latremoliere A, Bali KK, Simonetti M et al (2015) The serine protease inhibitor SerpinA3N attenuates neuropathic pain by inhibiting T cell-derived leukocyte elastase. Nat Med 21:518–523
30. Rao JS (2003) Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer 3:489–501
31. Strojnik T, Kos J, Židanik B, Golouh R, Lah T (1999) Cathepsin B immunohistochemical staining in tumor and endothelial cells is a new prognostic factor for survival in patients with brain tumors. Clin Cancer Res 5:559–567
32. Abe T, Mori T, Kohno K, Sciki M, Hayakawa T et al (1994) Expression of 72 kDa type IV collagenase and invasion activity of human glioma cells. Clin Exp Metas 12:296–304
33. Uhm JH, Dooley NP, Villemure J-G, Yong VW (1996) Glioma invasionin vitro: regulation by matrix metalloprotease-2 and protein kinase C. Clin Exp Metas 14:421–433
34. Sawaya RE, Yamamoto M, Gokaslan ZL, Wang SW, Mohanam S et al (1996) Expression and localization of 72 kDa type IV collagenase (MMP-2) in human malignant gliomas in vivo. Clin Exp Metas 14:35–42
35. Sivaparvathi M, McCutcheon I, Sawaya R, Nicolson GL, Rao JS (1996) Expression of cysteine protease inhibitors in human gliomas and meningiomas. Clin Exp Metas 14:344–350
36. Alzheimer A (1907) About a peculiar disease of the cerebral cortex. Allgemeine Zeitschrift fur Psychiatrie und Psychish-Gerichtlich Medicin 64:146–148
37. Bernstein HG (2005) Proteases and Alzheimer's disease: present knowledge and emerging concepts of therapy. In: Proteases in the brain. Springer, Berlin, pp 1–23
38. Fiorelli TN (2013) Proteolytic processing of the amyloid precursor protein during apoptosis and cell cycle: implications for Alzheimer's disease
39. Hoyer S (2004) Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. Eur J Pharmacol 490:115–125
40. Schmidt B (2003) Aspartic proteases involved in Alzheimer's disease. ChemBioChem 4:366–378
41. Vincent B (2004) ADAM proteases: protective role in Alzheimer's and prion diseases? Curr Alzheimer Res 1:165–174
42. De Strooper B (2010) Proteases and proteolysis in Alzheimer disease: a multifactorial view on the disease process. Physiol Rev 90:465–494
43. Bernstein H, Bruszis S, Schmidt D, Wiederanders B, Dorn A (1988) Immunodetection of cathepsin D in neuritic plaques found in brains of patients with dementia of Alzheimer type. J Hirnforsch 30:613–618
44. Bernstein H-G, Kirschke H, Wiederanders B, Schmidt D, Rinne A (1990) Antigenic expression of cathepsin B in aged human brain. Brain Res Bull 24:543–549
45. Cataldo AM, Nixon RA (1990) Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer brain. Proc Natl Acad Sci 87:3861–3865
46. Nakanishi H (2003) Neuronal and microglial cathepsins in aging and age-related diseases. Ageing Res Rev 2:367–381
47. Bendiske J, Bahr BA (2003) Lysosomal activation is a compensatory response against protein accumulation and associated synaptopathogenesis—an approach for slowing Alzheimer disease? J Neuropathol Exp Neurol 62:451–463
48. McNaught KSP, Olanow CW (2003) Proteolytic stress: a unifying concept for the etiopathogenesis of Parkinson's disease. Ann Neurol 53:S73–S86
49. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A et al (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science 276:2045–2047

50. Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M et al (1998) AlaSOPro mutation in the gene encoding α-synuclein in Parkinson's disease. Nat Genet 18:106–108
51. Gasser T (2001) Genetics of Parkinson's disease. J Neurol 248:833–840
52. Voges D, Zwickl P, Baumeister W (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu Rev Biochem 68:1015–1068
53. McNaught KSP, Olanow CW, Halliwell B, Isacson O, Jenner P (2001) Failure of the ubiquitin–proteasome system in Parkinson's disease. Nat Rev Neurosci 2:589–594
54. Tanaka Y, Engelender S, Igarashi S, Rao RK, Wanner T et al (2001) Inducible expression of mutant α-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum Mol Genet 10:919–926
55. Lee M, Hyun DH, Jenner P, Halliwell B (2001) Effect of proteasome inhibition on cellular oxidative damage, antioxidant defences and nitric oxide production. J Neurochem 78:32–41
56. Tatton NA, Maclean-Fraser A, Tatton WG, Perl DP, Warren CO (1998) A fluorescent double-labeling method to detect and confirm apoptotic nuclei in parkinson's disease. Ann Neurol 44:S142–S148
57. Crocker SJ, Smith PD, Jackson-Lewis V, Lamba WR, Hayley SP et al (2003) Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease. J Neurosci 23:4081–4091
58. Hodara R, Norris EH, Giasson BI, Mishizen-Eberz AJ, Lynch DR, Lee VMY, Ischiropoulos H (2004) Functional Consequences of α-Synuclein Tyrosine Nitration, Diminished binding to lipid vesicles and increased fibril formation. J Biol Chem 279(46):47746–47753
59. Surmeier D, Guzman J, Sanchez-Padilla J, Goldberg J (2010) Recent advances in Parkinson's disease: basic research. Elsevier, Amsterdam, pp 59–77
60. Mishizen-Eberz AJ, Guttmann RP, Giasson BI, Day GA, Hodara R et al (2003) Distinct cleavage patterns of normal and pathologic forms of α-synuclein by calpain I in vitro. J Neurochem 86:836–847
61. Mouatt-Prigent A, Karlsson J, Agid Y, Hirsch E (1996) Increased M-calpain expression in the mesencephalon of patients with Parkinson's disease but not in other neurodegenerative disorders involving the mesencephalon: a role in nerve cell death? Neuroscience 73:979–987
62. Moore DJ, West AB, Dawson VL, Dawson TM (2005) Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci 28:57–87
63. Tao X, Tong L (2003) Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. J Biol Chem 278:31372–31379
64. Wilson MA, Collins JL, Hod Y, Ringe D, Petsko GA (2003) The 1.1-Å resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. Proc Natl Acad Sci 100:9256–9261
65. Miller DW, Ahmad R, Hague S, Baptista MJ, Canet-Aviles R et al (2003) L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the ubiquitin-proteasome system. J Biol Chem 278:36588–36595
66. Moore DJ, Zhang L, Dawson TM, Dawson VL (2003) A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. J Neurochem 87:1558–1567
67. Stone SR, Maraganore JM (1992) Hirudin interactions with thrombin. In: Thrombin, Springer, Berlin, pp 219–256
68. Abraham CR, Selkoe DJ, Potter H (1988) Immunochemical identification of the serine protease inhibitor α 1-antichymotrypsin in the brain amyloid deposits of Alzheimer's disease. Cell 52:487–501
69. Cunningham DD, Long GL (1987) Proteases in biological control and biotechnology
70. Cuccioloni M, Mozzicafreddo M, Bonfili L, Cecarini V, Eleuteri AM et al (2009) Natural occurring polyphenols as template for drug design. Focus on serine proteases. Chem Biol Drug Des 74:1–15
71. Pittman R (1984) Neuron-target cell interactions may involve protease-inhibitor interactions, p 662

72. Kalderon N (1984) Schwann cell proliferation and localized proteolysis: expression of plasminogen-activator activity predominates in the proliferating cell populations. Proc Natl Acad Sci 81:7216–7220
73. Turgeon VL, Lloyd ED, Wang S, Festoff BW, Houenou LJ (1998) Thrombin perturbs neurite outgrowth and induces apoptotic cell death in enriched chick spinal motoneuron cultures through caspase activation. J Neurosci 18:6882–6891
74. Blaber SI, Ciric B, Christophi GP, Bernett MJ, Blaber M et al (2004) Targeting kallikrein 6 proteolysis attenuates CNS inflammatory disease. FASEB J 18:920–922
75. Diamandis EP, Yousef GM, Petraki C, Soosaipillai AR (2000) Human kallikrein 6 as a biomarker of Alzheimer's disease. Clin Biochem 33:663–667
76. Scarisbrick IA, Linbo R, Vandell AG, Keegan M, Blaber SI et al (2008) Kallikreins are associated with secondary progressive multiple sclerosis and promote neurodegeneration. Biol Chem 389:739–745
77. Zarghooni M, Soosaipillai A, Grass L, Scorilas A, Mirazimi N et al (2002) Decreased concentration of human kallikrein 6 in brain extracts of Alzheimer's disease patients. Clin Biochem 35:225–231
78. Uchida A, Oka Y, Aoyama M, Suzuki S, Yokoi T et al (2004) Expression of myelencephalon-specific protease in transient middle cerebral artery occlusion model of rat brain. Mol Brain Res 126:129–136
79. Scarisbrick I, Blaber S, Lucchinetti CF, Genain C, Blaber M et al (2002) Activity of a newly identified serine protease in CNS demyelination. Brain 125:1283–1296
80. Scarisbrick I, Blaber S, Tingling J, Rodriguez M, Blaber M et al (2006) Potential scope of action of tissue kallikreins in CNS immune-mediated disease. J Neurosci 178:167–176
81. Mitsui S, Okui A, Uemura H, Mizuno T, Yamada T et al (2002) Decreased cerebrospinal fluid levels of neurosin (KLK6), an aging-related protease, as a possible new risk factor for Alzheimer's disease. Ann N Y Acad Sci 977:216–223
82. Selkoe DJ (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399:A23–A31
83. Wu W, Jiang H, Wang M, Zhang D (2013) Meta-analysis of the association between urokinase-plasminogen activator gene rs2227564 polymorphism and Alzheimer's disease. Am J Alzheimer Dis Other Dement 28:517–523
84. Asahina M, Yoshiyama Y, Hattori T (2000) Expression of matrix metalloproteinase-9 and urinary-type plasminogen activator in Alzheimer's disease brain. Clin Neuropathol 20:60–63
85. Abraham CR, Potter H (1989) Alpha 1-antichymotrypsin in brain aging and disease. Prog Clin Biol Res 317:1037–1048
86. Griffin JH, Fernández JA, Lyden PD, Zlokovic BV (2016) Activated protein C promotes neuroprotection: mechanisms and translation to the clinic. Thromb Res 141:S62–S64
87. Han MH, Hwang S-I, Roy DB, Lundgren DH, Price JV et al (2008) Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. Nature 451:1076–1081
88. Steele FR, Chader GJ, Johnson LV, Tombran-Tink J (1993) Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. Proc Natl Acad Sci 90:1526–1530
89. Osterwalder T, Contartese J, Stoeckli E, Kuhn T, Sonderegger P (1996) Neuroserpin, an axonally secreted serine protease inhibitor. EMBO J 15:2944

Proteases in Wound Healing and Immunity

8

Namrata Singh and Debasish Bhattacharyya

Abstract

Proteases play a pivotal role in wound management. They are present in acute and chronic wound in different proportions. Balance between protease and their inhibitors are crucial for healing of wound because irregularity can lead to excessive extracellular matrix (ECM) degradation and deposition leading to impaired healing. Recent advancements in wound care established several means to control the level of proteases, such as MMP modulators including protease-modulating dressing, signaling molecules, peptides, and microRNA. Besides wound healing, proteases also play a significant role in immunity. They activate immune cells by proteolysis. Function of proteases in the areas of wound healing and immunity can be targeted as an alternative therapeutic approach for treatment.

Keywords

MMP · MicroRNA · ECM · Protease · Immunity

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1 Introduction

Wound is an injury caused by any physical or chemical means or a result of any disease. In a wound, rupture of the protective function of the skin occurs leading to disruption of continuity of epithelium, connective tissue, or underlying tissues/organs [1–4]. There are several types of wounds that can damage the protective layers such as abrasions, lacerations, rupture injuries, punctures, and penetrating wounds. Various wounds are superficial that need only local first aid of cleansing and dressing. But, some wounds are deeper and cannot be handled by first aid. They require medical attention to prevent infection and loss of function, because of damage to underlying structures like bone, muscle, tendon, arteries, and nerves. Thus, an important aspect in minimizing these effects and initiating proper care is careful asepsis.

2 Classification of Wound

There are various criteria for classification of wounds. Clinically, wounds can be categorized into acute and chronic wounds according to their rate of healing [5–7]. Wounds which can repair themselves and follow normal healing cascades with restoration of both functional and anatomical integrity are called acute wounds [5]. In contrast, chronic wounds fail to repair through the normal stages of healing [8]. In this type of wound, the process of healing is partial due to several reasons including infection, tissue hypoxia, necrosis, exudate, and excess levels of inflammatory cytokines. These factors can extend the stages of healing, such as hemostasis, inflammation, proliferation, or remodeling [9]. Chronic wound can be subclassified into three categories; vascular ulcers (e.g., venous and arterial ulcers), diabetics ulcers, and pressure ulcers [10]. A third category now emerged as complicated wound. This type is a special entity and is defined as a combination of an infection and tissue reformation defect. Infection persistently poses a hazard to the healing status of a wound, while the cause of the defect evolves the traumatic or postinfectious etiology or a wide tissue resection, e.g., tumor management. They cannot be closed primarily without complex surgical manipulation. There are various aspects on which closure of wound depends, such as the causes, location, physical characteristics, and healing potential of the wound.

3 Wound Healing

Wound healing is a complex and dynamic process where cascade of cellular events occurs including replacing, resurfacing, reconstitution, and restoration of skin integrity [11–14]. Curing of wound is followed by primary, secondary, and tertiary intention depending on the extent of tissue loss [15] primary intention is also called

'primary union' or 'first intention healing'. In this case, wound is precise and there is minimal damage to the local tissue and the epithelial basement membrane. Restoration of continuity occurs directly by fibrous adhesion, without formation of granulation tissue resulting in a thin scar. Surgical incisions, well-repaired lacerations, well-reduced bone fractures and healing after flap surgeries are examples of primary intention. In secondary intention of healing, wound is left open and closes naturally. In this type, edges of the wound are far apart and cannot be brought together thus healing occurs by adhesion of granulating surfaces. Granulations form the base and sides of the wound toward the surface. In contrast, healing by tertiary intention is of delayed primary closure. Wound cavity is gradually filled by granulations and a cicatrix (Fig. 1) [16].

Commonly, wound healing is divided into three distinct but overlapping phases (i) Inflammatory phase, (ii) Proliferation phase, (iii) Maturation phase [17] (Fig. 2). Inflammatory phase includes coagulation and recruitment of inflammatory cell at the site of injury. Proliferative phase includes angiogenesis, granulation tissue formation, epithelialization, and wound contraction. Finally, in the maturation

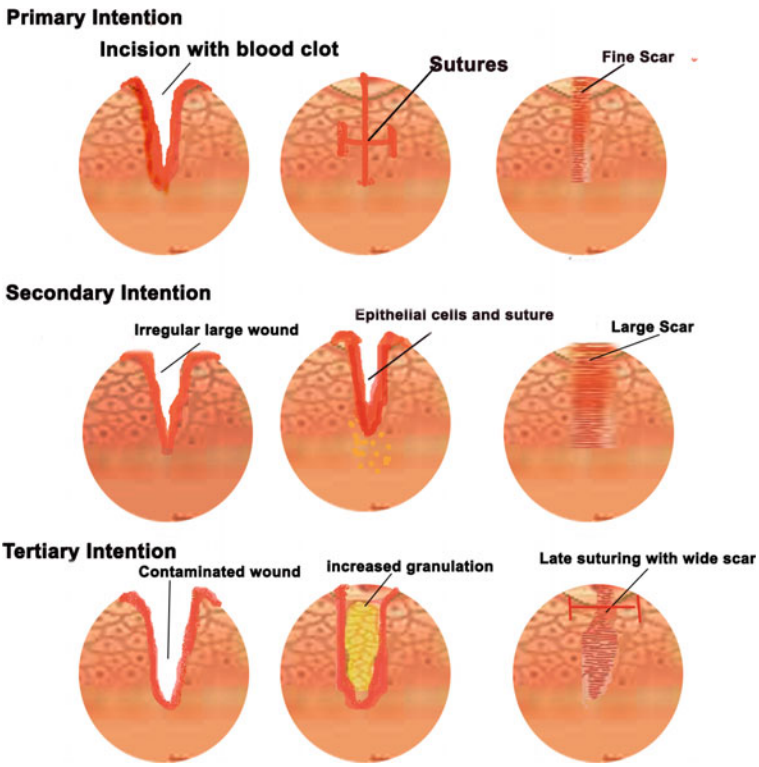

Fig. 1 Types of wound healing

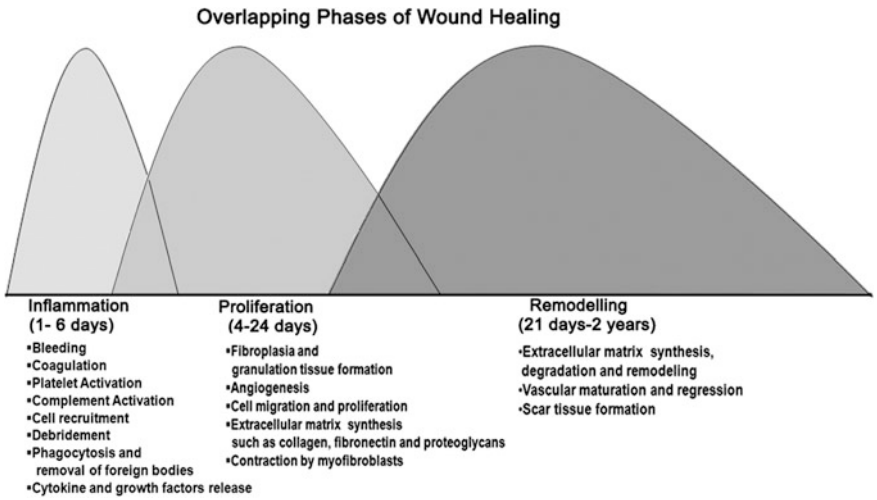

Fig. 2 Phases of acute wound healing

(remodeling) phase, type III collagen is mainly replaced by type I and cross-linked also aligned along tension lines, cells die out by programmed cell death, or apoptosis and scar formation occurs [18].

4 Cascades of Events in Wound Healing

4.1 Inflammation

The first stage of wound healing is inflammation. It begins immediately after tissue damage or injury. It arises in response to several factors such as physical trauma, intense heat, corrosive chemicals as well as infection by microbes. The inflammatory response prevents the spreading of damaging agents to nearby tissues, eradicates cell debris, pathogens, and establishes the platform for the repair process. Components of the coagulation cascade, inflammatory pathways and immune system get activated to prevent further loss of blood and body fluids, eliminate dead tissues, and prevent infection. Initially, hemostasis leads to contraction of injured blood vessels and initiation of blood clotting by activation and aggregation of platelets. Then formation of fibrin matrix takes place, which serves as scaffold for infiltrating cells. Therefore, complement system, the degranulation of platelets and the products of bacterial degradation give signals for activation of neutrophils for recruitment to the wounded area [19]. First, neutrophils predominate, subsequently these cells die out and monocytes become the dominant cell type in the wound.

Monocytes are recruited from the blood at the site of injury and differentiate into macrophages. Macrophages discharge additional proinflammatory cytokines, such as IL-1, TGF-β, and tumor necrosis factor-α (TNF-α). They also remove foreign debris and remain there for prolonged period of time depending on the level of injury and the amount of debris to be removed. Although neutrophils phagocytize bacteria, but monocytes are considered essential for coordinating advanced events in the response to wound. Though, recent studies have suggested that a deficiency in either of the cell types can be compensated by the redundancy in the inflammatory response [20]. Repair of small wounds can occur with less scar formation in absence of both cell types.

4.2 Proliferation

In general, proliferative phase follows and overlaps with inflammatory phase and begins after 2–3 days of injury which last for weeks. This phase consists of different sub phases are angiogenesis, granular formation tissues, epithelialization, and wound contraction. Formation of new blood vessels (angiogenesis) takes place thereby activating intracellular signaling cascades to provide nutrients and oxygen to the newly formed granulation tissue [18]. Reconstitution of dermis through formation of granulation tissue starts within 4 days after injury. Keratinocytes migrate over the injured dermis. During proliferation stage, mesenchymal cells convert into fibroblasts which form fibrin strands for cellular migration. These fibroblasts initially secrete several growth factors like interstitial growth factor-1, Transforming growth factor-β, platelet derived growth factor and epidermal growth factor. Meanwhile, components of basement membrane are secreted, such as collagen, glycosaminoglycans, and glycoproteins, e.g., fibronectin and tenascin resulting synthesis of a new collagen-rich matrix and this process is called fibroplasia. Initially, fibroblasts are secreted. Then, endothelial cells synthesize VEGF, bFGF, and PDGF, whereas keratinocytes synthesize TGF-α, TGF-β, and keratinocytes-derived autocrine factor [21]. These mediators act as stimulator and modulator for biosynthesis and epithelialization of extracellular matrix (ECM) and angiogenesis which eventually form mature scar [22]. Fibroblasts begin synthesizing collagen and proliferate to form granulation tissue. TGF-β induces fibroblasts to synthesize type I collagen and reduce matrix metallo proteases (MMP) production. Fibroblasts produce collagen and substances of extracellular matrix, like fibronectin, glycosaminoglycans, proteoglycans, and hyaluronic acid. Finally, in this proliferative stage, fibroblasts differentiated into myofibroblasts. Myofibroblasts are bringing the edges of a wound together and stimulate wound contraction. Cytokines play an important role in contraction, for example, TGF-β increases the rate and degree of contraction possibly through the induction of PDGF. At the end of this phase, these myofibroblasts die out by apoptosis [22, 23].

4.3 Remodeling (Maturation)

Remodeling is the last phase of wound healing. It begins by 2–3 weeks after injury and lasts for a year or more. This phase comprises remodeling of collagen from type III to type I. During this stage, all processes are activated, cellular activities are reduced and the many of newly formed capillaries in the wounded area is regressed and decreased. Maximum cells including endothelial cells, macrophages and myofibroblasts die away through apoptosis (i.e., programmed cell death) or leave the wound area keeping behind an agglomeration of cells mostly consisting collagen and other extracellular matrix proteins. This process is significant because its abnormality can lead to hypertrophic scarring and keloids formation. As the wound matures, the composition of the extracellular matrix is changed. Type III collagen that was deposited during the proliferative phase now get slowly degraded and replaced by a stronger variety of type I collagen which is secreted by fibroblasts, macrophages, and endothelial cells and it strengthens the repaired tissue [24, 25].

5 Difference Between Acute and Chronic Wound

Acute wounds generally heal without significant intrusions and exhibit accurate balance between production and degradation of molecules such as collagen. However in chronic wounds, there is physiological imbalance between two and thus displays obstruction in healing due to cellular and molecular abnormalities occurring within the wound bed [26, 27]. Earlier studies indicated that there is difference in the biochemical environment of the non-healing wound to healing wound. There are several reasons for delayed healing in chronic wounds, for example, obstinate infections, augmented inflammatory cytokines, formation of drug-resistant microbial biofilms, defective macrophage function, impaired neovascularization, abnormal matrix metalloproteinase regulation, poor oxygenation of the wound, cellular senescence, and the incapability of dermal and/or epidermal cells to respond to reparative stimuli [26, 28–31]. In chronic wound, infection, and abnormal secretion of vascular and interstitial cell adhesion molecule 1 by resident endothelial cells activate excessive inflammatory cells causing cell extravasation. Excessive inflammatory cells generate various ROS. These ROS along with proinflammatory cytokines induce MMPs and serine proteinases production that degrade structural elements of the ECM and cell membranes. This inactivates ECM components and growth factors necessary for normal cell function causing premature cell senescence [30, 32]. Inactivation of proteinase inhibitors also enhances this process. Moreover, there are other phenotypic irregularities commonly found in chronic wound are lower density of growth factor receptors and lowered mitogenic/motogenic potential [33, 34].

6 Proteases

Proteases are enzymes, which catalyze hydrolysis of proteins into smaller fragments, i.e., peptides. Proteases can be classified by several criteria such as specificity of the amino acid peptide bond hydrolysing, protein substrate, pH optima, amino acid involved at the catalytic site and involvement of metal ions. Proteases can also be divided into two major groups as exopeptidases and endopeptidases. Exopeptidases target the C- or N-terminal peptide linkages, whereas endopeptidases cleave peptide bonds distant from the termini of the protein substrate. Based on the functional group present at the active site, proteases are classified into four prominent groups such as serine proteases, aspartic proteases, cysteine proteases and metalloproteases [35]. There are also other few proteases which do not exactly suitable into these standard classifications, e.g., ATP-dependent proteases which require ATP for activity [36]. Likewise, based on their amino acid sequences, proteases are classified into different families and subdivided into groups that have diverged from a common ancestor [37].

6.1 Role of Proteases in Wound Healing

Over the decades, research in the area of pathophysiology of wound healing revealed a number of key factors essential for curing wounds. Proteases are one of those factors significantly involved in wound healing mechanism. They play an essential role in migration and activation of fibroblasts, remodeling of extracellular matrix and activation of growth factors [38]. During inflammatory stage of wound healing, proteases play a crucial role in debridement and removing of the necrotic tissues, foreign bodies, and bacteria load at the site of the wound [39–43]. Previous reports indicated that excessive proteolysis and degradation due to an increased level of proteases and an imbalance in protease/protease-inhibitor levels can impair healing of wound by increasing the period of inflammatory stage. Later in the proliferative phase, these proteases are expressed quickly at the budding tip of blood vessels to facilitate vascular invasion during angiogenesis. Finally, during the last phase of maturation and remodeling, proteases digest the extracellular matrix and assists in tissue remodeling. In fact, one of the major functions of proteases is to regulate the balance between tissue synthesis and tissue degradation. This requirement is maintained throughout the process of wound repair [44].

Background studies are inclined to the hypothesis that higher levels of proteases are always found in exudates from chronic wounds as compared to acute wounds. This observation contributes to the chronic character of the wound resulting in delay even failure of healing [41, 42, 45, 46]. Evidence showed that abnormal level of proteases can degrade growth factors and their receptors, granulation tissue, inhibits angiogenesis causing tissue damage leading to impaired healing of wound [47]. Growth factors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth

factor-beta (TGF-N) play significant roles in cell migration, proliferation, protein synthesis, and extracellular matrix formation during wound healing. Therefore, degradation of growth factors by proteases increases tissue destruction causing delayed wound healing [48]. It was estimated that more than 100 enzymes are involved in this complex process of tissue remodeling and repairing of wound [49]. The major proteases involved in wound healing are metalloproteases of the metzincin family [MMPs, ADAM (TS)s], serine proteases (e.g., plasmin), tolloids, meprins, pappalysins, whereas cysteine proteases play less prominent roles [50, 51].

Several regulatory proteins and hundreds of substrates have been identified for these proteases. Where its irregularities can delay the process of healing; for example, Neutrophil-derived elastase, plasmin, and matrix metalloproteases or MMP's are major proteases present in chronic wounds that delay the process of healing. Predominant protease activity in chronic wounds is offered by the neutrophil-derived elastase. Its presence supports the notion that healing is delayed in chronic wounds because the wound is trapped in the inflammatory phase of repair.

7 Matrix Metalloproteases and Their Role in Repairing of Wound

MMPs are a family of endoproteases, a group of calcium-dependent zinc-containing enzymes which consists 'classical' MMPs, membrane-bound MMPs, ADAMs (a disintegrin and metalloproteinases, also known as adamlysins) and ADAMTSs (ADAMs with thrombospondin motif) [52, 53]. They mostly involved in the degradation of ECM [54]. Based on the substrate preference and domain organization, MMPs are classified into seven groups: (1) collagenases, (2) gelatinases, (3) stromelysins, (4) matrilysins, (5) metalloelastases, (6) membrane-type MMPs (MT-MMPs), and (7) other MMPs [55]. Exudates from acute and chronic wounds contain matrix metalloproteases (MMPs). But the level of MMP is observed very low in acute wound as compared to chronic wound suggesting that expression of protease depends on the extent of injury [52, 53, 56, 57]. They play a crucial role in all phase of wound healing by modifying the wound matrix, allowing migration of cells and remodeling of tissues [57]. Different types of cell express several MMPs including MMP-1, 2, 3, 7, 9, 10, and 29 within the skin (keratinocytes, fibroblasts, endothelial cells, and inflammatory cells such as monocytes, lymphocytes, and macrophages) [Table 1] [58–65]. Range of signals, such as cytokines, hormones, and contact with other cell types or the ECM induces expression of MMPs. MMP-1, 3, and 9 plays a vital role among all MMPs in regulating chemokine signaling where they completely degrade chemokine or create receptor antagonists and increases chemokine activity [57, 66]. Previous reports also indicated that during wound repair, gene expression of MMP-2, and -3, -9, -11, -12, -13, and -14 increases. Additionally, investigators also pointed out the significant role of ADAM10, which can cleave CXCL16 from the cell surface and, therefore, allow it

Table 1 Different matrix metalloproteinases (MMPs) and their substrates

Subgroup	MMP	Trivial name	Substrates
Collagenases	MMP-1	Collagenases-1 (interstitial collagenase)	Collagens (I–III, Vll, Vlll, and X), gelatin, aggrecan, L-selectin, IL-Iß, proteoglycans, entactin, ovostatin, MMP-2, and MMP-9
	MMP-8	Collagenases-2 (neutrophil collagenase)	Collagens (I–III, V, VII, VIII, and X), gelatin, aggrecan, and fibronectin
	MMP-13	Collagenases-3	Collagens (I–IV, IX, X, and XIV), gelatin, plasminogen, (preliminary aggrecan, perlecan, fibronectin, osteonectin, and MMP-9
	MMP-18	Collagenases-4	Type I collagen
Gelatinases	MMP-2	Gelatinase A	Gelatin, collagen IV–VI and X, elastin, and fibronectin
	MMP-9	Gelatinase B	Collagens (IV, V, VI′, X, and XIV), gelatin, entactin, aggrecan, elastin, fibronectin, osteonectin, plasminogen, MBP, and IL-IB
Stromelysins	MMP-3	Stromelysin-1	Collagens (Ill–V and 1 > 0, gelatin, aggrecan, perlecan, decorin, laminin, elastin, casein, osteonectin, ovostatin, entactin, plasminogen, MBP, MMP-2/TlMP-2, MMP-7, MMP-8, MMP-9, and MMP-13
	MMP-10	Stromelysin-2	Collagens (III–V), gelatin, casein, aggrecan, elastin, MMP-I, and MMP-8
	MMP-11	Stromelysin-3	Unknown
Matrilysins	MMP-7	Matrilysin-1	Collagens (IV, X), gelatin, aggrecan, decorin, fibronectin, laminin, elastin, casein, transferrin, plasminogen, MBP, ß4-integrin, MMR-I, MMP-2, MMP-9, and MMP-9/TlMP-1
	MMP-26	Matrilysin-2	Collagen IV, fibronectin, fibrinogen, gelatin, α(1) proteinase inhibitor
MT-MMPs	MMP-14	MT1-MMP	Collagens (I–III), gelatin, casein, fibronectin, laminin, vitronectin, laminin, vitronectin, entactin, proteoglycans, MMP-2, and MMP-13
	MMP-15	MT2-MMP	Fibronectin, entactin, laminin, aggrecan, perlecan, and MMP-2
	MMP-16	MT3-MMP	Collagen III, gelatin, casein, fibronectin, and MMP-2
	MMP-17	MT4-MMP	Fibrin; fibrinogen; tumor necrosis factor precursor
	MMP-24	MT5-MMP	Fibronectin, but not collagen type I or laminin
	MMP-25	MT6-MMP	Progelatinase A

(continued)

Table 1 (continued)

Subgroup	MMP	Trivial name	Substrates
Other MMPs	MMP-12	Macrophage metalloelastase	Collagen IV, gelatin, elastin, casein, fibronectin, vitronectin, laminin, entactin, MBP, fibrinogen, fibrin, and plasminogen
	MMP-19	RASI-1	Type I collagen
	MMP-20	Enamelysin	Amelogenin; aggrecan; cartilage oligomeric matrix protein
	MMP-21	MMP identified on chromosome 1	Unknown
	MMP-23	CA-MMP	Unknown
	MMP-27	MMP identified on chromosome 1	Unknown
	MMP-28	Epilysin	Casein
	MMP-29		Unknown

to bind to its receptor and regulate T-cell activation in the wounded area [67]. All these data suggested that metalloproteinase, including both MMPs and ADAMs, are involved in regulation of chemokine activity.

7.1 MMP-1, MMP-8, and MMP-13 (Collagenases Group)

MMP-1, MMP-8, and MMP-13 are the main secreted endopeptidases capable of cleaving collagenous extracellular matrix [68]. An important aspect of these enzymes is their ability to cleave interstitial collagens I, II, and III at a specific site three-fourths from the N-terminus. These MMPs can also degrade a number of other ECM and non-ECM molecules. MMP-1 cleaves type II collagen whereas MMP-13 has intense activity than MMP-1 on type II collagen and MMP-8 has greatest activity against type I collagen. Fibroblasts and macrophages secrete MMP-1 during normal tissue remodeling. During wound healing, degradation of ECM triggers quick expression of MMP1 in basal keratinocytes at the migrating epithelial front in wounds. Expression of MMP-1 is induced when cells are in contact with type I collagen promoting migration. Its expression is controlled by the binding of type I collagen to $\alpha_2\beta_1$ integrin. At day 1, after wounding in migrating basal keratinocytes, MMP-1 expression rises at the wound edge followed by a steady decrease until re-epithelialization is over. Later in final stages of tissue remodeling in keratinocytes, laminin isoforms serve as a signal for the down-regulation of MMP-1. Collagenase-8 (MMP-8) is another most effective collagenase in initiating type I collagen degradation that is secreted by wound fibroblasts, neutrophils, and macrophages. An increased expression of MMP-8 in chronic wounds is detrimental to wound repair causing breakdown of type I collagen. Another collagenase, MMP-13 expressed by

fibroblasts plays a substantial role in the maturation of granulation tissue, comprising modulating myofibroblast function, inflammation, angiogenesis, and degradation of the matrix [38, 69, 70].

7.2 MMP-2 and MMP-9 (Gelatinases)

Gelatinases are matrix zinc-dependent metalloproteases capable of degrading denatured collagens as well as other extracellular matrix proteins [71]. MMP-2 and MMP-9 are also called gelatinases A and B, respectively that are expressed by chondrocytes, fibroblasts, endothelial cells, and macrophages. They can degrade type IV, V, VII, X, XI, and XII collagens, elastin, and fibronectin. Earlier researchers suggested their role in cell migration and re-epithelialization [72]. High level of these two gelatinases was observed in diabetic ulcers. After the initial cleavage of intact collagen by MMP-1, these two MMPs further degrade it into small fragments. Likewise, MMP-2 and MMP-9 can also degrade type IV collagen, which is the major type of collagen found in basement membranes. Expression of laminin-332 and increased keratinocyte migration directs expression of MMP-2 in acute wounds. Exogenous MMP2 stimulates cleave γ-2 chain of laminin-332 for epithelial migration [73, 74]. MMP-9 is expressed by epithelial injury, another gelatinase that play a significant role in wound healing and cell signaling [75]. It has also been implicated in re-epithelialization after injury. It has been reported that MMP-9 is significantly up-regulated during human respiratory epithelial healing [76]. Importance of MMP-9 in wound repair was studied using a MMP-9 deficient mouse model, where, MMP-9 coordinated epithelial wound repair and deficient showed wound healing with delayed re-epithelization and disordered collagen fibrillogenesis [77]. It also stimulates collagen contraction interacting with TGF-ß1 assisting in wound closure [78]. Hypoxia is an example of MMP-9 indicated increase in keratinocyte migration and MMP-9 activity [79]. Alternatively, there are reports that MMP-9 activates the movement of lung epithelial cell in culture by modifying adhesion to type IV collagen, whereas when injury of lung was severe, re-epithelialization was unaffected in MMP-9 deficient mice [80]. In the eye, MMP-9 slows cornea wound closure by inhibiting cell proliferation through Smad2 signaling [76]. Both MMP-2 and MMP-9 play a significant role in angiogenesis and remodeling phase of wound healing. They activate cytokines, including TNF-α and VEGF, and generate antiangiogenic peptides such as endostatin from type XVII collagen which is expressed in the basement membrane [81].

7.3 MMP-3 and MMP-10, MMP-11 (Stromelysin-1, -2 and -3)

Stromelysins are metallo proteases that are capable of cleaving extracellular matrix proteins but are incapable of cleaving triple helical fibrillar collagens. There are three members in this group are MMP-3, MMP-10, and MMP-11. Collagen II, III, IV, IX, and X are degraded by MMP-3. MMP-10 can cleave collagen III, IV, and V

along with non-collagenous connective tissue macromolecules with proteoglycans, laminin, and fibronectin [82]. MMP-3 and MMP-10 are involved in proteolysis and tissue remodeling through participating in pro-MMP activation and digests ECM molecules. However, MMP-11 demonstrates weak activity with the ECM molecules. In chronic wound, keratinocytes and fibroblast cells prominently express MMP-3. MMP-10 has a unique pattern of expression produced by basal keratinocytes at the edge of migrating cells. Though gelatinases are commonly found in acute wound, they are observed in large quantities in chronic wound and ulcers. Unregulated expression of MMP-10 can leads to irregularity in wound healing. Aberration in expression can cause proteolysis of newly formed matrix, disorganization of migrating epithelium, deviation in cell–cell contacts of the migrating keratinocytes, and an increased rate of cell death of wound edge keratinocytes. Other factors, which control expression, are the cytokines EGF, TGF-β1, and TNF-α. Alternatively, MMP-3 plays a vital role in wound contraction regulating the rate of wound healing.

7.4 Matrilysins (MMP 7, MMP 26, and MMP 28)

Matrilysins are capable of cleaving ECM components and cell surface molecules like Fas-ligand, pro-α-defensin, pro-tumor necrosis factor α and E-cadherin (Parks 2004) [66]. Two members of this group are MMP-7 and MMP-26. They are mostly expressed by injured mucosal epithelia [83]. MMP-7 has established an integral role in re-epithelialization. It can cleave aggrecan, fibronectin, type IV collagen, laminin, and entactin. There are reports indicating that re-epithelialization of tracheal wounds are almost completely abolished in absence of MMP-7 [59, 84]. MMP-7 regulates re-epithelialization by cleavage of E-cadherin within the adherens junction, which enables the migration of epithelial cells away from the wound edge. Moreover, loss of MMP-7 can lead to impaired E-cadherin cleavage in vivo following bleomycin injury [84]. Another MMP in matrilysin group is MMP-26, also called endometase. Matrilysin-2 is a small protein of the MMP-family expressed in some epithelial carcinomas and normal tissues. It is up-regulated in keratinocytes during wound healing. It can degrade type IV collagen, fibronectin, fibrinogen, vitronectin, denaturated collagen types I–IV, α1-antitrypsin, α2-macroglobulin in vitro, and insulin-like growth factor-binding protein 1 (IGFBP-1) and activates pro-MMP-9 [85, 86].

MMP-28 (epilysin) is a new member of MMP-family, a caseinolytic enzyme which is expressed by proliferating basal keratinocytes distally from the wound edge and is not expressed by migrating keratinocytes. This pattern of expression suggests a possible role of MMP-28 in re-epithelialization through reorganizing the basement membrane or by cleaving cellular adhesion molecules and thus, giving more cells for the migrating front [61, 87]

7.5 Other MMPs

There is other MMPs play an important role in re-epithelialization. For example, MMP-14 (MT1-MMP) exhibit a vital role in remodeling of extracellular matrix (ECM) and enhancing cell migration during wound healing. Its expression is localized in the migrating front of the wound which is expressed by keratinocytes. It also cleaves various types of ECM proteins, comprising collagens, gelatin, laminins, and fibronectin along with its ligands, the integrins [88]. In addition to cleaving ECM proteins, it can also activate other MMPs like MMP-2 through coordinated process with TIMP-2 thereby intensifying the process of proteolysis [89]. It is important in the migration of cells in type I collagen and even required during angiogenesis [90]. Growth factors such as VEGF and HGF (hepatocyte growth factor) induces MT1-MMPs. Earlier citation also discussed activation of a number of intracellular signaling, pathways, such as the MAPK family pathway, focal adhesion kinase (FAK), Src family, Rac, and CD44, during cell migration and tumor invasion by MMP-14 [91]. They also regulate and induce epithelial cell proliferation and migration in vitro by varying the expression of the KGF receptor through the cleavage of syndecan-1, CD44 and laminin-332, respectively, during wound healing. In MMP-8 deficient mice, wound healing got delayed which is thought because of sustained inflammation to reduced neutrophil apoptosis and clearance.

7.6 Tissue Inhibitors of Metalloproteinases

The MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs). They are classified under four family members, TIMP-1, -2, -3, and -4. They regulate the action of MMPs and inhibit them in a 1:1 stoichiometry by interacting active site of the MMP with their N-terminal domain although their affinities may differ [92]. Two TIMPs among four, play crucial roles by regulating several MMPs. TIMP-1 is present in epithelial cells and fibroblasts and inhibits MMP (-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, and -16). Studies indicated the role of TIMP-2 in modulating cell migration and inhibits MMP-1, -2, -3, -7, -8, -9, -10, -13, -14, -15, -16, and -19) [93]. Similarly, TIMP-3 plays a significant role in controlling ECM remodeling during wound healing and inhibits MMP-1, -2, -3, -7, -9, -13, -14, and -15.

8 Serine Proteases

Along with MMPs, there are numerous serine proteases involved in wound healing such as neutrophil elastase (human leukocyte elastase), urokinase-type plasminogen activator, possibly cathepsin, and plasmin among with predominance of human neutrophil elastase [51]. These proteases regulate the inflammatory process.

Elevated levels of serine proteases like neutrophil elastase (human leukocyte elastase), urokinase-type plasminogen activator, plasmin, and possibly cathepsin are found in chronic wounds. In this case, the activity of neutrophil-derived elastase dominates. Its presence supports the notion that healing is delayed in chronic wounds because the wound is stuck in the inflammatory phase of repair. Elastase and cathepsin are capable of cleaving a variety of extracellular matrix proteins including elastin, fibronectin, laminin, vitronectin, and collagen IV [94]. Recent investigation has recommended that the activity of these proteases could be used as an early biomarker to detect wound infection due to their high elevation by neutrophils during infection. Plasminogen also exhibits significant role in the re-epithelialization process.

9 Level of Protease Activity in Acute and Chronic Wound

Several investigations indicated variation in proteolytic activity between acute and chronic wounds [44–47]. It is widely accepted that an imbalance between proteases and their inhibitors in chronic wounds obstructs healing. High level of proteases activity destroys growth factors and their receptors, inhibits angiogenesis and degrades granulation tissue, resulting in tissue damage. In normal healing, proteolytic activity is properly regulated but this is not followed in chronic wounds. Increased levels of various MMPs and serine proteases are observed in fluids from chronic wounds. These variations in acute surgical and chronic wounds were initially studied by Trengove and his team in 1999 using substrate assays and gelatine-zymography. Level of MMP activity is higher within chronic wound fluids as compared to acute wound fluids. Chronic wounds are characterized by elevated levels and activities of collagenases (MMP-1 and MMP-8) and gelatinases (MMP-2 and MMP-9), serine proteases such as neutrophil elastase and abnormally low levels of TIMPs along with higher degradation of the growth factors like epidermal growth factors. Unregulated high level of serine proteases degrade fibronectin, an essential protein involved in the remodeling of the ECM resulting in delay in wound healing. In vitro studies suggest that under such conditions certain growth factors are also degraded by proteases [95].

10 Factors Affecting Protease Activity

Protease activity is the best biomarker for predicting the healing of any wound. It is recommended that clinicians should identify the level of protease appropriate for debridement. The specific point on the healing trajectory where protease activity becomes imbalanced should be detected. There are several factors which can affect protease activity to halt healing of wounds. For example, sometimes factors related to the patient also contribute in obstructing healing of wounds, such as age,

hormone levels, and diabetes. The other factors are production and regulation of proteases, which are complex. MMPs are produced by tissue cells involved in healing, e.g., neutrophils, fibroblasts, endothelial cells, and epithelial cells. They are also produced by immune cells as part of the inflammatory process or in response to infection. Likewise, MMPs are activated by neutrophil elastases and other proteases. TIMPs are produced by a variety of tissue cells; inhibit the activation of pro-MMPs and the activity of activated MMPs. But the main inhibitor of elastase is α-1 protease inhibitor which is secreted by macrophages and liver cells. Similarly, infection may increase protease activity because presence of bacteria in wounds induces an inflammatory response that stimulates protease production. This is in addition to production of proteases by the bacteria themselves [96]. Therefore, slight abnormality in any of the cascades can lead to imbalance in protease level resulting tissue damage.

11 Detection of Elevated Protease Activity

There are several evidence suggesting that elevated level of proteases activities particularly MMPs and human neutrophil elastase obstruct wound healing. Generally, in the course of normal healing, the level of proteases rises gradually and remains at peak for about three days and start reducing by day five. The reasons for generation of imbalance between elevated level of protease and a lack of protease inhibition in non-healing or chronic wounds are not completely understood. However, regular monitoring of protease activities during treatment can give an idea for appropriate wound care management. Earlier researchers used techniques like gelatine-zymography that primarily detects MMP-2 and MMP-9. Alternative method is ELISAs which use antibodies to measure levels of proteases and assays. Studies were consistent about low levels of proteases in acute wounds and high levels in stalled or poorly healing wounds that decrease when the wounds begin to heal. However, this laboratory evaluation of protease activity is not always feasible. Recent development in tissue viability offered better protocol to detect abnormally elevated levels of protease activity in about 15 min (WoundChek™ Protease Status, Systagenix, see http://www.woundchek.com). WoundChek™ Protease Status is a point of care test that evaluates wound fluid swabbed from chronic wounds.

12 Protease Modulating Dressings

Several dressings are commercially available that modulate protease activity. They act by various means, such as absorbing exudate, removing cofactors, or releasing inhibitors. Protease modulators are a new range of interactive dressings that not only create a moist wound healing environment but also regulating protease activity directly by binding or inactivating proteases to promote wound healing [96].

There are various categories of technology and modality of dressings. For example, starch-based matrix which can modulate the wound pH, fibrous-based consist of superabsorbent polymers or oxidized regenerated cellulose (ORC)/collagen and have a gel-blocking action which creates a soft gel when in contact with exudate such as Promogran. Protease modulating matrix composed of 55% collagen and 45% oxidized regenerated cellulose (ORC) is an advanced topical treatment for chronic wounds. It has the potential to modify the wound environment and stimulate healing. This can help to improve consequences for patients with static or hard-to-heal wounds [97–100]. While doing protease-modulating dressings, it is always necessary to regularly monitor and regulate the duration of treatment as per clearly documented with a review date. Because every patient has different needs that should be considered while choosing the correct dressing protocol. Thus, selecting dressing for any patients, following question should be addressed. Amount of exudate volume produced in a particular wound. If a chronic wound is generating moderate-to-high volumes of exudate, a protease-modulating dressing may be required. Moisture management is vital to sustain a moist wound healing thereby controlling wound proteases so that they do not exceed their required levels of activity and prolong the inflammatory phase of healing. Second question should be types of wound for protease-modulating dressings because these dressings can only be done on wound that is failing to heal over a period of time. However, not all wounds with delayed healing have elevated protease activity. Therefore, researchers should identify which wounds have elevated protease activity in which protease modulating strategies will be effective. There are no visual signs specific to elevated protease activity. They should know at what level protease activity is probably started to cause harm and to be able to easily identify affected wounds. Earlier it was suggested an eight-week period without evidence of healing whereas currently the time frame extended to 12 weeks [5, 101]. Third question should be about the fragility of the skin of the patient according to which adhesive fixation will be low or high. Similarly, other questions which should be addressed are needed to have dressings to reduce antimicrobial activity as the wound is infected or should be done under compression. Applying compression may be a problem for wounds that produce heavy volumes of exudate, which is often where protease-modulating dressings are used in order to absorb protease-rich wound fluid. An international consensus suggested that protease modulating dressings should be used for small courses of 2–4 weeks, followed by a full reassessment. There are other MMP modulators attracted researchers for regulating MMPs level in non-healing or delayed wound such as signaling molecules, microRNA regulation, and peptides. Expression of MMP is regulated by signal transduction mechanisms. Recently, a member of the FAK family that is proline-rich protein tyrosine kinase 2 (Pyk2) exhibited an important role in wound healing by regulating of both keratinocyte migration and MMP expression. Currently, MiR noncoding RNAs also showed in regulating gene expression of MMPs. MMP-1 expression can be regulated by down-regulation of MiR-199a-5p which induces wound angiogenesis. Likewise, there are reports on peptides which can regulate MMPs. Lipocalin-2 present in the

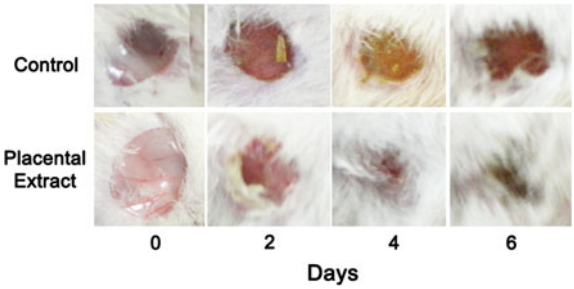

Fig. 3 Wound contraction in rat after treatment with an aqueous extract of placenta. Pictures indicated that 5 mm wound was contracted to 3.75 mm on 6th day of wounding in control whereas placental extract treated set, healing of wound was prominent with contraction of wound to 1.50 mm along with hair growth

venom of the *Lonomia obliqua* caterpillar, can bind and stabilize MMP-9 promoting cell migration and wound healing [102–104].

There are also natural products used as potent wound healer. One of our ongoing endeavors is to characterize an aqueous extract of human placenta which is used as a wound healer. NADPH, fibronectin type III-like peptide that stabilizes trypsin activity and an ubiquitin-like protein which exhibits collagenase activity have been detected in the extract. The extract also exhibited antimicrobial activity against common disease causing microorganisms, which together with induction of NO by mouse peritoneal macrophages together with enhancement of their adhesion property [105–111]. These findings collectively support wound healing potency of the extract. Figure 3 demonstrate such activity on wound using a sterile biopsy punch of 5 mm diameter on inner thigh surface of rats (Namrata Singh, Unpublished results).

13 Proteases and Immunity

Besides significant attribution in wound healing, proteases are also discussed in immunity. Proteases are capable of recognizing and eliminating invading pathogens, alter host cells through different activation routes thereby activating immune cells. Previous studies showed that trypsin, papain, and other proteolytic enzymes can degrade existing pathogenic immune complexes and even prevent their formation augmenting lymphatic drainage. This action stimulates immune system. Proteolytic enzymes also increase the potency of natural killer cells. While these immune complexes are a normal part of the immune response, when they occur in excess, it can be the cause of certain kidney diseases, nerve inflammations and a number of rheumatologic diseases. Serine proteases act as a central component in complement system. Proteolytic products, e.g., anaphylatoxins of complement system trigger inflammatory reactions by activating the cellular elements of the

immune system like leukocytes and endothelial cells. Prior findings indicated that TIMP-3 and ADAM-17 act as regulators of key inflammatory, proliferative, and developmental pathways. They provide stimulus to expand our understanding of this important family of enzymes in mammalian signal transduction. There are several studies suggested that the innate and adaptive immune system utilize endocytic protease activity to promote functional immune responses. Cysteine and aspartic proteases (cathepsins) constitute a subset of endocytic proteases, the immune function of which has been described extensively. Earlier these studies have focused on the role of proteases in antigen presentation and zymogen processing within the endocytic compartment but currently, investigation indicated a crucial role in other intracellular compartments, and within the extracellular environment. Moreover, the coagulation system is activated in response to infection by a variety of different viruses, such as HIV, CVB3, Dengue virus, and Ebola virus. This response likely evolved as host defenders [112–115].

14 Conclusion and Future Directions

Regulatory roles of proteases in wound healing and immunity are well established. Enhancement of proteolytic activity could be achieved by genetically overproduction of the enzyme itself or conversion of proenzyme to the active form by proteolysis. Presence of an inhibitor in variable amount may also regulate a protease activity. From the perspective of enzyme functioning, the inhibitors are likely to be an analog of its substrate i,e, proteins or peptides. However, inhibitors whose structures are completely unrelated to proteins/peptides are emerging. Heparin can reversibly inactivate Proteinase K [116] while cholesterol on cell surface can reversibly bind to the bacterial protease Proteinase K forming an inactive entity [117]. In either set, autodigestion of the proteases could be reduced. It is hypothesized that the cell bound Proteinase K may act in defending the second serge of microbial infection by destroying proteins of the invading pathogens. It appears that more biomolecules of unrelated structures to that of the substrates will be attributed as inhibitor of proteases. Proteomic studies including interactions with small biomolecules could unravel complete pathway of proteases functioning.

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References

1. Hutchinson J (1992) The wound programme. Centre for Medical Education: Dundee
2. Leaper DJ, Harding KG (1998) Wounds: biology and management. Oxford University Press, Oxford
3. Enoch S, Price P (2004) Cellular, molecular and biochemical differences in the pathophysiology of healing between acute wounds, chronic wounds and wounds in the aged. World wide Wounds

4. Cooper P (2005) A review of different wound types and their principles of management in Wound Healing: a systematic approach to advanced wound healing and management. Cromwell Press, Wiltshire
5. Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Pecoraro RE, Rodeheaver G, Robson MC (1994) Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol 130:489–493
6. Bischoff M, Kinzl L, Schmelz A (1999) The complicated wound. Unfallchirurg 102: 797–804
7. Robson MC, Steed DL, Franz MG (2001) Wound healing: biologic features and approaches to maximize healing trajectories. Curr Probl Surg 38:72–140
8. Szycher M, Lee SJ (1992) Modern wound dressings: a systematic approach to wound healing. J Biomater Appl 7:142–213
9. Vanwijck R (2001) Surgical biology of wound healing. Bull Mem Acad R Med Belg 115:175–184
10. Moreo Kathleen (2005) Understanding and overcoming the challenges of effective case management for patients with chronic wounds. The Case Manager 16:62–63
11. Glat PM, Longaker MT (1997) Wound healing. In: Aston SJ, Beasley RW, Thorne CH (eds) Grabb and Smith's Plastic Surgery, 5th edn. Lippincott-Raven, Philadelphia, pp 3–12
12. Hunt TK, Hopf H, Hussain Z (2000) Physiology of wound healing. Adv Skin Wound Care 13:6–11
13. Attinger CE, Janis JE, Steinberg J, Schwartz J, Al-Attar A, Couch K (2006) Clinical approach to wounds: debridement and wound bed preparation including the use of dressings and wound-healing adjuvants. Plast Reconstr Surg 117:72S–109S
14. Broughton G, Janis JE, Attinger CE (2006) Wound healing: an overview. Plast Reconstr Surg 117:1e-S–32e-S
15. Myers BA (2008) Wound management principles and practice, 2nd edn. Pearson, Upper Saddle River, NJ
16. You HJ, Han S (2014) Cell therapy for wound healing. J Korean Med Sci 29:311–319
17. Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA (2012) Concise review: role of mesenchymal stem cells in wound repair. Stem Cells Transl Med 1:142–149
18. Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. Nature 453:314–321
19. Grose R, Werner S (2004) Wound-healing studies in transgenic and knockout mice. Mol Biotechnol 28:147–166
20. Martin P, Leibovich SJ (2005) Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol 15:599–607
21. Werner S, Grose R (2003) Regulation of wound healing by growth factors and cytokines. Physiol Rev 83:835–870
22. Robson MC (2003) Cytokine manipulation of the wound. Clin Plast Surg 30:57–65
23. Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. J Pathol 200:500–503
24. Scott N, Estes M (1999) Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair. J Pediatr Surg 34:218–223
25. Beanes SR, Dang C, Soo C, Ting K (2004) Skin repair and scar formation: the central role of TGF-beta. Expert Rev Mol Med 5:1–11
26. Armstrong DG, Jude EB (2002) The role of matrix metalloproteinases in wound healing. J Am Podiatr Med Assoc 92:12–18
27. Edwards JV, Howley P, Cohen IK (2004) In vitro inhibition of human neutrophil elastase by oleic acid albumin formulations from derivatized cotton wound dressings. Int J Pharm 284:1–12
28. Ovington L (2003) Bacterial toxins and wound healing. Ostomy Wound Manag 49:8–12
29. Edwards R, Harding KG (2004) Bacteria and wound healing. Curr Opin Infect Dis 17:91–96

30. Eming SA, Krieg T, Davidson JM (2007) Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol 127:514–525
31. Wolcott RD, Rhoads DD, Dowd SE (2008) Biofilms and chronic wound inflammation. J Wound Care 17:333–341
32. Ben-Porath I, Weinberg RA (2005) The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 37:961–976
33. Mast BA, Schultz GS (1996) Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. Wound Repair Regen 4:411–420
34. Lauer G, Sollberg S, Cole M, Flamme I, Stürzebecher J, Mann K, Krieg T, Eming SA (2000) Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. J Invest Dermatol 115:12–18
35. Hartley S (1960) Proteolytic enzymes. Annu Rev Biochem 29:45–72
36. Menon AS, Goldberg AL (1987) Protein substrates activate the ATP-dependent protease La by promoting nucleotide binding and release of bound ADP. J Biol Chem 262:14929–14934
37. Rawlings ND, Barrett AJ (1994) Families of serine peptidases. Methods Enzymol 244:19–61
38. Caley MP, Martins VL, O'Toole EA (2015) Metalloproteinases and Wound Healing. Adv Wound Care (New Rochelle) 4:225–234
39. Rogers AA, Burnett S, Moore JC, Shakespeare PG, Chen WY (1995) Involvement of proteolytic enzymes—plasminogen activators and matrix metalloproteinases— in the pathophysiology of pressure ulcers. Wound Repair Regen 3:273–283
40. Weckroth M, Vaheri A, Lauharanta J, Sorsa T, Konttinen YT (1996) Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. J Invest Dermatol 106:1119–1124
41. Yager DR, Nwomeh BC (1999) The proteolytic environment of chronic wounds. Wound Repair Regen 7:433–441
42. Trengove NJ, Stacey MC, MacAuley S (1999) Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen 7:442–452
43. Lobmann R, Zemlin C, Motzkau M, Reschke K, Lehnert H (2006) Expression of matrix metalloproteinases and growth factors in diabetic foot wounds treated with a protease absorbent dressing. J Diabetes Complicat 20:329–335
44. McCarty SM, Percival SL (2013) Proteases and delayed wound healing. Adv Wound Care (New Rochelle) 2:438–447
45. Baker EA, Leaper DJ (2000) Proteinases, their inhibitors and cytokine profiles in acute wound fluid. Wound Repair Regen 8:392–398
46. Yager DR, Zhang LY, Liang HX, Diegelmann RF, Cohen IK (1996) Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. J Invest Dermatol 107:743–748
47. Overall CM, López-Otín C (2002) Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer 2:657–672
48. Bennett NT, Schultz GS (1993) Growth factors and wound healing: Part II. Role in normal and chronic wound healing. Am J Surg 166:74–81
49. Rawlings ND, Barrett AJ, Bateman A (2010) MEROPS: the peptidase database. Nucleic Acids Res 38:D227–D233
50. Beidler SK, Douillet CD, Berndt DF, Keagy BA, Rich PB, Marston WA (2008) Multiplexed analysis of matrix metalloproteinases in leg ulcer tissue of patients with chronic venous insufficiency before and after compression therapy. Wound Repair Regen 16:642–648
51. Iuonut AMM, Dindelegan GC, Ciuce C (2011) Proteases as biomarkers in wound healing. TMJ 61:1–2
52. Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8:221–233
53. Edwards DR, Handsley MM, Pennington CJ (2008) The ADAM metalloproteinases. Mol Aspects Med 29:258–289
54. Verma RP, Hansch C (2007) Matrix metalloproteinases (MMPs): chemical-biological functions and (Q) SARs. Bioorg Med Chem 15:2223–2268

55. Woessner JR (1994) The family of matrix metalloproteinases. Ann N Y Acad Sci 732:11–21
56. McCarty SM, Cochrane CA, Clegg PD, Percival SL (2012) The role of endogenous and exogenous enzymes in chronic wounds: a focus on the implications of aberrant levels of both host and bacterial proteases in wound healing. Wound Repair Regen 20:125–136
57. Gill SE, Parks WC (2008) Metalloproteinases and their inhibitors: regulators of wound healing. Int J Biochem Cell Biol 40:1334–1347
58. Inoue M, Kratz G, Haegerstrand A, Ståhle-Bäckdahl M (1995) Collagenase expression is rapidly induced in wound-edge keratinocytes after acute injury in human skin, persists during healing, and stops at re-epithelialization. J Invest Dermatol 104:479–483
59. Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC (1998) Matrilysin expression and function in airway epithelium. J Clin Invest 102:1321–1331
60. Pilcher BK, Wang M, Qin XJ, Parks WC, Senior RM, Welgus HG (1999) Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. Ann N Y Acad Sci 878:12–24
61. Saarialho-Kere U, Kerkela E, Jahkola T, Suomela S, Keski-Oja J, Lohi J (2002) Epilysin (MMP-28) expression is associated with cell proliferation during epithelial repair. J Invest Dermatol 119:14–21
62. Corry DB, Rishi K, Kanellis J, Kiss A, Song Lz LZ, XuJ Feng L, Werb Z, Kheradmand F (2002) Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. Nat Immunol 3:347–353
63. Corry DB, Kiss A, Song LZ, Song L, Xu J, Lee SH, Werb Z, Kheradmand F (2004) Overlapping and independent contributions of MMP2 and MMP9 to lung allergic inflammatory cell egression through decreased CC chemokines. FASEB J 18:995–997
64. Salmela MT, Pender SL, Karjalainen-Lindsberg ML, Puolakkainen P, Macdonald TT, Saarialho-Kere U (2004) Collagenase-1 (MMP-1), matrilysin-1 (MMP-7) and stromelysin-2 (MMP-10) are expressed by migrating enterocytes during intestinal wound healing. Scand J Gastroenterol 39:1095–1104
65. Warner RL, Bhagavathula N, Nerusu KC, Lateef H, Younkin E, Johnson KJ, Varani J (2004) Matrix metalloproteinases in acute inflammation: induction of MMP-3 and MMP-9 in fibroblasts and epithelial cells following exposure to pro-inflammatory mediators in vitro. Exp Mol Pathol 76:189–195
66. Parks WC, Wilson CL, López-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immuno 4:617–629
67. Kawaguchi M, Hearing VJ (2011) The roles of ADAMs family proteinases in skin diseases. Enzyme Res. 482498
68. Ala-aho R, Kähäri VM (2005) Collagenases in cancer. Biochimie 87:273–286
69. Ravanti L1, Kähäri VM (2000) Matrix metalloproteinases in wound repair (review). Int J Mol Med 6:391–407
70. Danielsen PL, Holst AV, Maltesen HR, Bassi MR, Holst PJ, Heinemeier KM, Olsen J, Danielsen CC, Poulsen SS, Jorgensen LN, Agren MS (2011) Matrix metalloproteinase-8 overexpression prevents proper tissue repair. Surgery 150:897–906
71. Klein T, Bischoff R (2011) Physiology and pathophysiology of matrix metalloproteases. Amino Acids 41:271–290
72. Salo T, Mäkelä M, Kylmäniemi M, Autio-Harmainen H, Larjava H (1994) Expression of matrix metalloproteinase-2 and -9 during early human wound healing. Lab Invest 70: 176–182
73. Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V (1997) Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science 277:225–228
74. Lechapt-Zalcman E, Prulière-Escabasse V, Advenier D, Galiacy S, Charrière-Bertrand C, Coste A, Harf A, d'Ortho MP, Escudier E (2006) Transforming growth factor-beta1

increases airway wound repair via MMP-2 upregulation: a new pathway for epithelial wound repair. Am J Physiol Lung Cell Mol Physiol 290:1277–1282
75. Buisson AC, Zahm JM, Polette M, Pierrot D, Bellon G, Puchelle E, Birembaut P, Tournier JM (1996) MMP9 is involved in the in vitro wound repair of human respiratory epithelium. J Cell Physiol 166:413–426
76. Mohan R, Chintala SK, Jung JC, Villar WV, McCabe F, Russo LA, Lee Y, McCarthy BE, Wollenberg KR, Jester JV, Wang M, Welgus HG, Shipley JM, Senior RM, Fini ME (2002) Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. J Biol Chem 277:2065–2072
77. Kyriakides TR, Wulsin D, Skokos EA, Fleckman P, Pirrone A, Shipley JM, Senior RM, Bornstein P (2009) Mice that lack matrix metalloproteinase-9 display delayed wound healing associated with delayed re-epithelization and disordered collagen fibrillogenesis. Matrix Biol 28:65–73
78. Kobayashi T, Kim H, Liu X, Sugiura H, Kohyama T, Fang Q, Wen FQ, Abe S, Wang X, Atkinson JJ, Shipley JM, Senior RM, Rennard SI (2014) Matrix metalloproteinase-9 activates TGF-β and stimulates fibroblast contraction of collagen gels. Am J Physiol Lung Cell Mol Physiol 306:L1006–L1015
79. Mulholland B, Tuft SJ, Khaw PT (2005) Matrix metalloproteinase distribution during early corneal wound healing. Eye (Lond) 19:584–588
80. Betsuyaku T, Fukuda Y, Parks WC, Shipley JM, Senior RM (2000) Gelatinase B is required for alveolar bronchiolization after intratracheal bleomycin. Am J Pathol 157:525–535
81. Heljasvaara R, Nyberg P, Luostarinen J, Parikka M, Heikkilä P, Rehn M, Sorsa T, Salo T, Pihlajaniemi T (2005) Generation of biologically active endostatin fragments from human collagen XVIII by distinct matrix metalloproteases. Exp Cell Res 307:292–304
82. Murphy G, Cockett MI, Ward RV, Docherty AJ (1991) Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). Biochem J 277:277–279
83. Chen P, McGuire JK, Hackman RC, Kim KH, Black RA, Poindexter K, Yan W, Liu P, Chen AJ, Parks WC, Madtes DK (2008) Tissue inhibitor of metalloproteinase-1 moderates airway re-epithelialization by regulating matrilysin activity. Am J Pathol 172:1256–1270
84. McGuire JK, Li Q, Parks WC (2003) Matrilysin (matrix metalloproteinase-7) mediates E-cadherin ectodomain shedding in injured lung epithelium. Am J Pathol 162:1831–1843
85. Uría JA, López-Otín C (2000) Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer Res 60:4645–4751
86. Marchenko GN, Ratnikov BI, Rozanov DV, Godzik A, Deryugina EI, Strongin AY (2001) Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin. Biochem J 356:705–718
87. Lohi J, Wilson CL, Roby JD, Parks WC (2001) Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. J Biol Chem 276:10134–10144
88. Chakraborti S, Mandal M, Mandal A, Chakraborti T (2003) Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253:269–285
89. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase: Isolation of the activated form of the membrane metalloprotease. J Biol Chem 270:5331–5338
90. Itoh Y (2006) MT1-MMP: a key regulator of cell migration in tissue. IUBMB Life 58: 589–596
91. Joo CK, Seomun Y (2008) Matrix metalloproteinase (MMP) and TGFβ1-stimulated cell migration in skin and cornea wound healing. Cell Adh Migr 2:252–253
92. Brew K, Dinakarpandian D, Nagase H (2000) Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 1477:267–283

93. Vaalamo M, Leivo T, Saarialho-Kere U (1999) Differential expression of tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4) in normal and aberrant wound healing. Human Pathol 30:795–802
94. Wilgus TA, Roy S, McDaniel JC (2013) Neutrophils and wound repair: positive actions and negative reactions. Adv Wound Care (New Rochelle) 2:379–388
95. Enoch S, Price P (2004) Cellular, molecular and biochemical differences in the pathophysiology of healing between acute wounds, chronic wounds and wounds in the aged. World Wide Wounds 13:1–17
96. Young T (2012) Using a protease test to inform wound care treatments decisions. Wounds UK 8:74–80
97. Vin F, Teot L, Meaume S (2002) The healing properties of PROMOGRAN in venous leg ulcers. J Wound Care 11:335–341
98. Veves A, Sheenan P, Pham HT (2002) A randomized, controlled trial of Promogran (a collagen/oxidised regenerated cellulose dressing) vs standard treatment in the management of diabetic foot ulcers. Arch Surg 137:822–827
99. Gottrup F, Cullen BM, Karlsmark T, Bischoff-Mikkelsen M, Nisbet L, Gibson MC (2013) Randomized controlled trial on collagen/oxidized regenerated cellulose/silver treatment. Wound Repair Regen 21:216–225
100. Smeets R, Ulrich D, Unglaub F, Wöltje M, Pallua N (2008) Effect of oxidized regenerated cellulose/collagen matrix on proteases in wound exudate of patients with chronic venous ulceration. Int Wound J 5:195–203
101. Mustoe TA, O'Shaughnessy K, Kloeters O (2006) Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. Plast Reconstr Surg 117:35S–41S
102. Yan L, Borregaard N, Kjeldsen L, Moses MA (2001) The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL) modulation of MMP-9 activity by NGAL. J Biol Chem 276:37258–37265
103. Koppel AC, Kiss A, Hindes A, Burns CJ, Marmer BL, Goldberg G, Blumenberg M, Efimova T (2014) Delayed skin wound repair in proline-rich protein tyrosine kinase 2 knockout mice. Am J Physiol Cell Physiol 306:C899–C909
104. Chan YC, Roy S, Huang Y, Khanna S, Sen CK (2012) The microRNA miR-199a-5p down-regulation switches on wound angiogenesis by de repressing the v-ets erythroblastosis virus E26 oncogene homolog 1-matrix metalloproteinase-1 pathway. J Biol Chem 287:41032–41043
105. Chakraborty PD, Bhattacharyya D (2005) In vitro growth inhibition of microbes by human placental extract. Curr Sci 88:782–786
106. Chakraborty PD, Bhattacharyya D (2005) Isolation of fibronectin type III like peptide from human placental extract used as wound healer. J Chromatogr B 818:67–73
107. Chakraborty PD, Bhattacharyya D, Pal S, Ali N (2006) In vitro induction of nitric oxide by mouse peritoneal macrophages treated with human placental extract. Int Immunopharmacol 6:100–107
108. Chakraborty PD, Bhattacharyya D (2012) Aqueous extract of human placenta as a therapeutic agent in Recent Advances in Research on the Human Placenta. In Tech Publishers, Rijeka, Croatia, pp, 77–92
109. Chakraborty PD, De D, Bandyopadhyay S, Bhattacharyya D (2009) Human aqueous placental extract as a wound healer. J Wound Care 18:462–467
110. De D, Chakraborty PD, Bhattacharyya D (2010) Regulation of trypsin activity by peptide fraction of an aqueous extract of human placenta used as wound healer. J Cell Physiol 226:2033–2040
111. De D, Chakraborty PD, Mitra J, Sharma K, Mandal S, Das A, Chakrabarti S, Bhattacharyya D (2013) Ubiquitin-like protein from human placental extract exhibits collagenase activity. PLoS ONE 8:e59585

112. Bird PI, Trapani JA, Villadangos JA (2009) Endolysosomal proteases and their inhibitors in immunity. Nat Rev Immunol 9:871–882
113. Sim RB, Tsiftsoglou SA (2004) Proteases of the complement system. Biochem Soc Trans 32:21–27
114. Pavan R, Jain S, Shraddha Kumar A (2012) Properties and therapeutic application of bromelain: a review. Biotechnol Res Int. 2012:976203
115. Antoniak S, Mackman N (2014) Multiple roles of the coagulation protease cascade during virus infection. Blood 123:2605–2613
116. Sharma K, Mukherjee C, Roy S, De D, Bhattacharyya D (2014) Human placental extract mediated inhibition of proteinase K: implications of heparin and glycoproteins in wound physiology. J cell physiol 229:1212–1223
117. Singh N, Bhattacharyya D (2017) Cholesterol and Its Derivatives Reversibly Inhibit Proteinase K. J Cell Physiol 232:596–609

9 Translating the Knowledge of Functional Dynamics Toward Designing Inhibitors of BACE1, a Key Aspartate Protease in Alzheimer's Disease

Sandipan Chakraborty and Soumalee Basu

Abstract

Beta-site APP cleaving enzyme 1 (BACE1) which plays a major role in Alzheimer's disease (AD) is being currently considered as a promising target in AD therapeutics by designing inhibitors against it. But there exists very little information regarding the physiological function of the protein. Many highly potent BACE1 inhibitors failed in the clinical trials due to undesired side effects and toxicity. Thus more research to explore the physiological role of BACE1 is an urgent need. The protein is highly flexible and exhibits significant ligand-induced flexibility which poses a serious challenge in computer-aided drug designing. Recent advances in simulation methods as well as increase in computational resources shed novel insight into the ligand recognition process of BACE1, which stimulates the field of research that deals with devising novel methods to incorporate receptor flexibility in BACE1 for computational drug discovery. This chapter aims to provide up-to-date information of the physiological role of BACE1 as well as its localization and sorting pathways and discuss in details the inherent structural flexibility and ligand-induced motion of BACE1. Also, novel drug designing strategies developed targeting this receptor flexibility has been discussed in critical details which paves the path for developing novel method/algorithm in computational drug discovery of BACE1 inhibitors for AD.

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Alzheimer's disease · β-secretase · Conformational flexibility · Computational drug discovery · BACE1 inhibitor

1 Introduction

β-secretase 1 (BACE1) is an aspartyl protease in human, that is believed to play prime role in Alzheimer's disease (AD). In the diseased condition, the protein is over-expressed and competes with α-secretase to initiate the cleavage of APP at the β-site. The cleavage by γ-secretase follows leading to the secretion of a highly aggregate-prone Aβ peptide [1, 2]. Aβ aggregation stimulates many toxic pathways leading to the extensive neuronal loss in the region of brain that controls memory and cognition. BACE1 is currently considered a lucrative target in AD therapeutics due to the fact that BACE1 knockout mice lack amyloid production without any noticeable toxicity [3]. Physiologically, BACE1 is known to play a major role in the formation of myelin sheaths in peripheral nervous system during developmental stages [4, 5]. This observation does not hold back development of CNS (Central Nervous System) drugs in AD therapeutics as AD is primarily a disease of older people. But very little information is available regarding other physiological roles of BACE1, if any, and also how inhibition of BACE1 would influence other signaling cascades. Thus, different functions of the protein need to be understood more extensively to eliminate mechanism-based toxicity associated with BACE1 inhibitors. Another challenge in computational designing of BACE1 inhibitors is the flexibility of the protein. The protein is highly flexible and undergoes significant induced fit motion after ligand binding. Not much is known about this functional motion, which needs to be explored in critical details in order to design strategies, incorporating receptor flexibility in structure-based drug design. Hit prediction during virtual screening can certainly be improved if the flexibility of the receptor can be mimicked properly. This chapter briefly describes the cell biology and trafficking of the protease and also provides up-to-date information regarding its biological substrates identified so far. Functional motions of the protein and how the protein recognizes its substrate have been discussed in critical details along with different strategies/methods that have been developed to incorporate receptor flexibility.

2 BACE1: Identification of the Enzyme

Emergence of the amyloid hypothesis as the principal pathogenesis paradigm of AD prompted the research of identification of novel proteases involved in aberrant processing of amyloid precursor protein (APP) and generation of amyloid β

thereafter. These resulted in the identification of the β-site APP cleavage enzyme simultaneously by five different groups within 1999–2000, although the denotations of the enzyme are different in different groups like BACE, Asp2, or memapsin 2 [6–10]. Different approaches, namely genomic or biochemical strategies used to characterize the protein always, led to the same primary sequence of the protein. The protein was finally termed BACE1. Interestingly, its paralogous counterpart BACE2 is functionally different and behaves more like "α-secretase" by promoting the non-amyloidogenic processing of APP [11].

BACE1 is a 501-amino-acid-long peptide, while BACE2 is composed of 518 amino acids. Sequence alignment reveals that the primary sequences of both the proteins are 45.8% identical and up to 61% similar (Fig. 1a). Structurally, both the proteins are highly similar and share the same structural fold. Structural superposition of inhibitor bound crystal structure of the active site domain of BACE1 (PDB ID: 4BEK) and BACE2 (PDB ID: 2EWY) is shown in Fig. 1b. Both the proteins have highly similar secondary and tertiary structure, and structural superposition reveals that the Cα root-mean-square deviation is only 0.826 Å.

Among these two proteins, BACE1 satisfies all previously determined characteristics of β-secretase involved in APP proteolysis [6]. The pattern and level of expression of BACE1 is highly consistent with the reported activity of β-secretase in cells and tissues. Maximal β-secretase activity was observed in neural tissue and neuronal cell lines, although some activity was detected in majority of body tissues [6]. Interestingly, astrocytes exhibited less β-secretase activity than neuronal cells (Fig. 2).

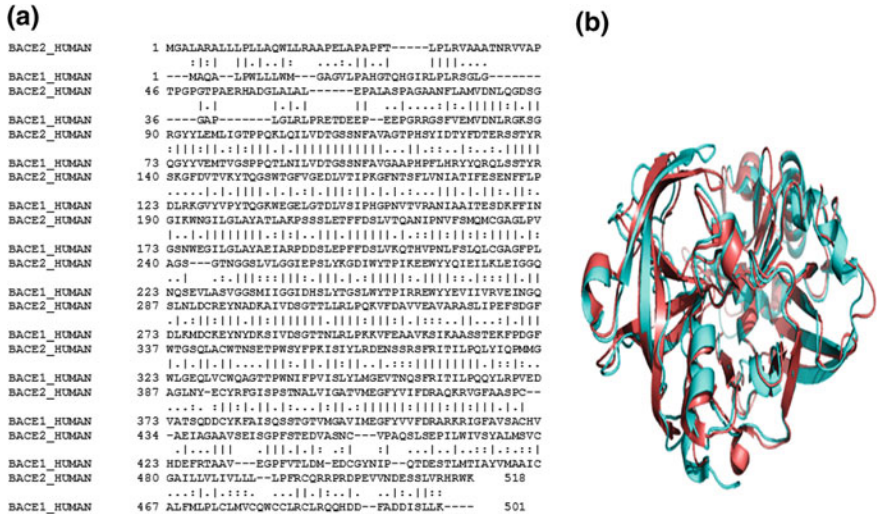

Fig. 1 Sequence and structural alignment of BACE1 and BACE2. **a** Pair-wise sequence alignment of human BACE1 and BACE2 sequences. **b** Structural superposition of the active site domain of BACE1 (PDB ID: 4BEK) and BACE2 (PDB ID: 2EWY). Both the proteins are shown in cartoon representation and colored cyan for BACE1 and deep salmon for BACE2

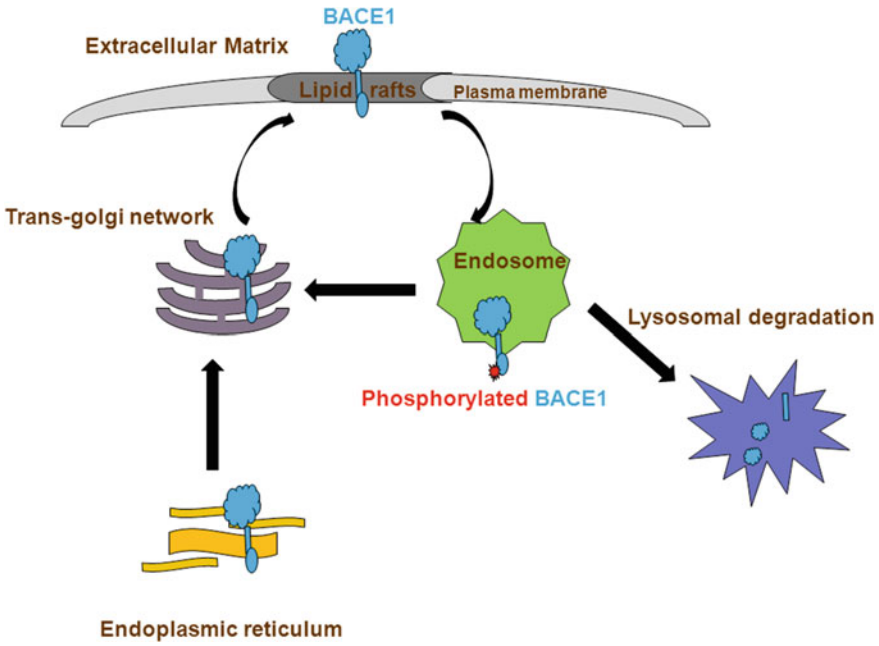

Fig. 2 Schematic representation of cellular sorting pathway of BACE1

BACE1 mRNA is highly expressed in brain and pancreas, whereas the expression is significantly less in most other body tissues [12]. Moreover, BACE1 mRNA is highly expressed in neurons but little is found in resting glial cells [13]. Northern blot analysis of human BACE1 mRNA shows low levels of expression in peripheral tissues, while brain sub-regions show uniform, moderately high expression across all the regions [6]. In situ hybridization probes the strongest signal of the protein in hippocampus, cortex, and cerebellum. Overall, the pattern of BACE1 expression is consistent with the expected pattern of expression of β-secretase involved in APP proteolysis [6]. Overexpression of BACE1 shows increased production of β-secretase cleavage products. The paradoxically high BACE1 mRNA level in pancreatic cells was resolved when a splice variant of BACE1 mRNA with missing major portion of exon 3 was identified [14, 15]. This splice variant encodes a BACE1 isoform devoid of β-secretase activity, but the functional relevance of this pancreas-specific splice variant still remains elusive.

Till date, the functional role of BACE2 in AD pathogenesis is somewhat controversial. Some studies demonstrated that BACE2 cleavage occurs around the α-site [11], whereas there are some reports revealing that the enzyme cleaves at the β-site of APP [16]. The BACE1 gene is localized on chromosome 11q23.3, whereas the BACE2 gene is located on chromosome 21q22.3. Genotyping shows no association between AD and the intronic polymorphism in BACE2. Tissue expression of BACE2 is also markedly different with respect to its homolog,

BACE1. BACE2 mRNA is nearly undetectable in human adult or fetal brain [17]. However, in kidney, prostate, placenta, colon, and pancreas, BACE2 mRNA is highly expressed [17]. Studies also demonstrated that BACE1 and BACE2 are transcriptionally regulated differently. The BACE2 gene is controlled by a TATA-less promoter and transcription factor prediction showed little similarity between the promoters of the two proteins. A comparative analysis of the transcription factor-binding sites of both BACE1 and BACE2 genes was carried out by Sun et al. [18]. All these observations symbolize BACE1 as the probable β-secretase candidate in brain that is involved in APP processing.

3 BACE1: Cell Biology of the Protein

Many years of pain-staking research has shed some light on the cellular sorting mechanism of BACE1. The protein is synthesized in the endoplasmic reticulum as a zymogen [19]. Acetylation of seven lysine residues at the N-terminal domain of nascent BACE1 dictates its trafficking from ER to trans-Golgi network [20]. Pro-BACE1 matures into the active form in Golgi. Here, the pro-peptide domain is cleaved between Arg45 and Glu46. Furin, a ubiquitous proprotein convertase (PC), present in the Golgi, plays an essential role in the peptidic cleavage [21]. Glycosylation of four asparagine residues and palmitoylation of three cytosolic cysteine residues of BACE1 occur during the final maturation of the protein which directs its intracellular trafficking [22]. In Golgi lumen, the seven lysine residues are de-acetylated after the maturation of BACE1. Mature BACE1 mostly localizes within cholesterol-rich noncaveolar lipid rafts in plasma membrane [23]. What is fascinating is that, within lipid rafts, BACE1 co-localizes with APP and γ-secetase. BACE1 has reportedly shown optimal activity in the acidic pH (pH 4.5–5.5) [24]. Therefore, within plasma membrane, BACE1 shows very low activity. Also, it has been demonstrated that in normal physiological condition, BACE1 is unable to readily bind to APP within lipid rafts. It is noteworthy that BACE1 in its lifetime shuttles many times between the cell surface and early endosome, where it is activated due to the low pH. Interestingly, APP co-localizes with BACE1 within the endocytic compartment, where most of the proteolysis take place. Particularly, the dileucine motif of BACE1 and the YENPTY motif of APP in the cytoplasmic domain dictate their co-localization into endosomes [22]. Several adapter proteins play crucial roles in appropriate sorting of BACE1. Although the co-localizations of BACE1 and APP occur in early endosome, whether they follow similar trafficking pathway or not is highly debatable. It appears that APP and BACE1 are internalized through distinct sorting pathways which converge in early endosome. From endosomes, BACE1 is either sorted in Golgi using a retrograde pathway or toward lysosome for degradation. Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding (GGA) proteins dictate the trafficking of BACE1 between the late Golgi and early endosomes [25, 26]. These proteins recognize the DXXLL motif of the C-terminal domain of BACE1. GGA1 negatively regulates the residence time of

BACE1 in endosome through a complicated pathway that are not fully characterized. However, it has been demonstrated that GGA1 recognizes the dileucine motif of BACE1 and its binding is highly dependent on the phosphorylation status of BACE1. On the other hand, GGA3 regulates the trafficking of ubiquitinated BACE1 between early endosomes and lysosome [27]. In AD brains, GGA3 expression is significantly reduced [26] and reduced GGA3 levels increase localization of BACE1 to early endosomes by preventing the trafficking of BACE1 to lysosomes where it is degraded. A significant population of BACE1 is also recycled back to endosome from the cell surface in Rab11-dependent pathway [28]. Recent advances in live cell imaging techniques provide evidence that trafficking of BACE1 from the dendrite surface to the soma in hippocampal neuron occurs through EHD1/3-dependent pathway, whereas axonal BACE1 undergoes Rab11-dependent endosomal recycling pathway [29, 30]. Thus, a balance between Rab11 and EDH proteins fine tunes the sorting of BACE1 toward endosomes and impairing this balance can lead to an increased pool of active BACE1 within neuron capable of producing more Aβ.

An interesting note for BACE1 is that unlike zymogens of other related proteases, proBACE1 demonstrates considerable β-secretase activity and can cleave APP to produce an intracellular pool of Aβ early in the cellular trafficking pathway. It has been demonstrated that intracellular Aβ is more neurotoxic than its extracellular counterpart [31].

Another interesting observation, although minor, is that BACE1 undergoes ectodomain shedding [32]. Co-expression of APP and the soluble ectodomain of BACE1 increase generation of Aβ which indicate that ectodomain shedding of BACE1 increases the amyloidogenic processing of APP and the process is suppressed by palmitoylation. Another intriguing observation is that, soluble, functionally active BACE1 has been detected in the cerebrospinal fluid (CSF) of AD brains.

4 BACE1: Physiological Function of the Protein

Initial reports demonstrated that BACE1 knockout mice live with normal physiology without any associated morbidity [3]. Recent studies, however, revealed a complex phenotypic behavior of BACE1 knockout mice, which implicate functional role of BACE1. However, it may be mentioned that a consensus about the physiological role of BACE1 is yet to be achieved. BACE1-/- mice exhibit significant evidence of hypomyelination of peripheral nerves [33]. The phenotype is very similar to that observed in type III NRG1 mutation. Type III NRG1 (neuregulin1) functions as a signaling molecule and stimulates the production of myelin sheaths by the accompanying Schwann cells, which ensheath the nerve axons. NRG1 contains an epidermal growth factor (EGF) domain. A proteolytic cleavage releases the EGF domain to interact with ErbB receptor and thereby activate the protein. It is now believed that BACE1 might be one of the proteases involved in the ectodomain shedding of the type III NRG1 [34]. Thus, BACE1 is

required for myelination and correct bundling of axons by Schwann cells, probably through processing of type III NRG1.

It has also been reported that BACE1 can cleave the P-selectin glycoprotein ligand 1 (PSGL-1) to generate a soluble ectodomain and a C-terminal trans-membrane fragment which mediates leukocyte adhesion during inflammatory reactions [35]. Alongside, research evidence clearly indicates a functional role of BACE1 in neuronal activity through the regulation of voltage-dependent sodium channels. Neuronal action potentials are highly dependent on the voltage-dependent sodium channel Nav1.1. The voltage-gated sodium channel (Nav) β2-subunit is a type I membrane protein that covalently binds to Nav1 α-subunit. Interestingly, it was demonstrated that the β2-subunit is sequentially processed by BACE1 and γ-secretase leading to the secretion of a fragment which regulates the transcription of the α-subunit through a feedback mechanism [36, 37]. As a result the α-subunits accumulate inside the cell. Decrease of functional sodium channels at the cell surface results in the lowering of sodium current densities. However, the status of sodium current densities in BACE1 knockout mice is yet to be explored.

Researchers also demonstrated that the neural cell adhesion molecule L1 or close homolog of L1 (CHL1) is also another possible substrate of BACE1. CHL1 is a type I membrane protein which is cleaved by BACE1 to liberate a soluble ectodomain fragment that binds to neuropilin-1 and semaphorin 3A [38]. These two proteins play crucial role in axonal guidance. Mice model study demonstrated that BACE1 null mice possess a significant amount of mis-targeted axons in the olfactory bulb and hippocampus region.

Recently, high-throughput OMICS approach has been adopted to identify novel BACE1 substrates. Hemming et al. used an unbiased quantitative proteomic approach on two human epithelial cell lines, HEK and HeLa, stably expressing BACE1 to identify novel BACE1 substrates [39]. They have identified 68 putative substrates and a number of them were further validated in cell culture system. On the other hand, Kuhn et al. used novel secretome protein enrichment with click sugars (SPECS) method to identify novel BACE1 targets. Interestingly, 34 substrates of BACE1 have been identified in primary neurons. Among them seizure protein 6, L1, CHL1, and contactin-2 were validated in brains of BACE1 inhibitor-treated and BACE1 knockout mice [40].

5 BACE1: Role of the Protein in Alzheimer's Disease

Autopsy of the brain of AD patients shows extensive neuronal loss in the region of the brain that controls memory and cognition. Interestingly, the presence of extracellular aggregates of proteinaceous debris, known as "amyloid plaques" and intracellular filamentous "neurofibrillary tangles", has been noticed. Plaques are composed of aggregates of amyloid-β peptide (Aβ), mostly insoluble. On the contrary, tangles are intracellular insoluble twisted fibers of the tau protein [41, 42]. Neurons possess an internal support system called microtubules which guide the

flow of nutrients and molecules from the body of the cell down to the axon. Tau, a microtubule-associated protein, is known to stabilize microtubules. In AD patients, tau has been found to be hyper-phosphorylated. The hyper-phosphorylated tau rapidly aggregates as paired helical filaments known as neurofibrillary tangles (NFT) [43]. Interestingly, it has been demonstrated that NFT formation is induced upon Aβ treatment in transgenic mice model. Studies on APP-transgenic mice model demonstrated that mutation in the tau gene causes autosomal dominant frontotemporal lobe dementia with tau pathology and symptoms as observed in AD but without the appearance of Aβ plaques [44, 45]. Thus, fibrillar Aβ might alter the phosphorylation state of tau, which in turn facilitates NFT formation. Therefore, "amyloid hypothesis" emerges as the principal paradigm in AD pathology. According to this hypothesis, the central pathogenic event is the formation and aggregation of Aβ. Aβ is generated due to the proteolysis of a larger integral type I trans-membrane glycoprotein, amyloid precursor protein (APP). Generally, three proteases, namely α, β, γ, proteases are involved in APP proteolysis (Fig. 3).

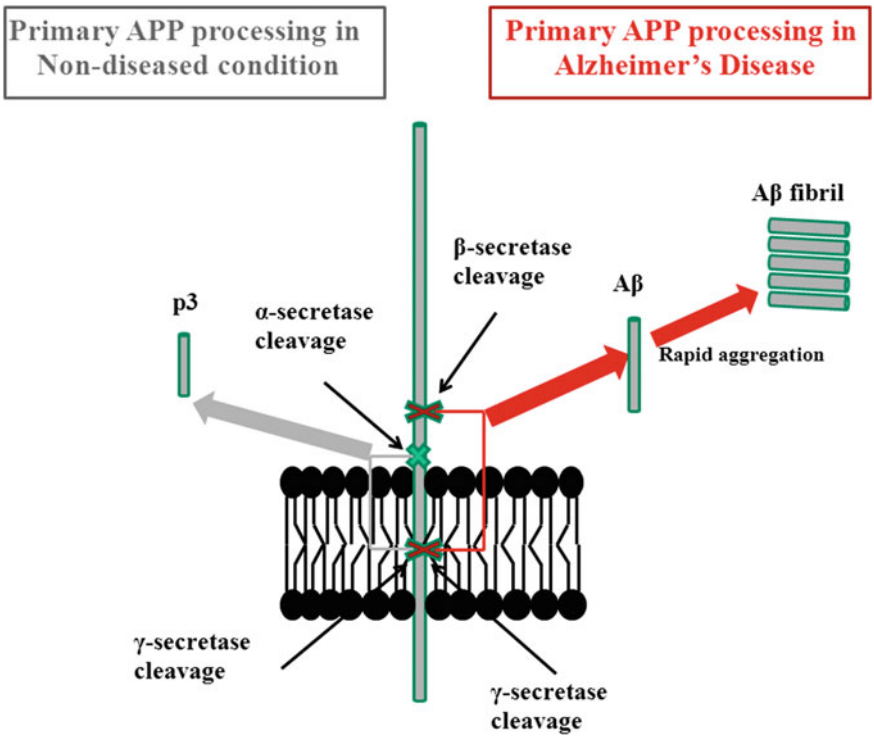

Fig. 3 Schematic representation of the processing of the amyloid precursor protein (APP) in the normal condition and amyloid fibril formation as observed in the diseased (AD) condition. In normal physiology, APP processing is primarily composed of successive cleavage by α-secretase and γ-secretase leading to the secretion of a 3 kDa peptide, p3. In the amyloidogenic pathway, cleavage by β-secretase, followed by γ-secretase, releases the aggregate-prone peptide, Aβ, which rapidly aggregates in a nucleation-dependent manner to form "amyloid fibril" that has the characteristic highly stable cross β-sheeted arrangement

In non-diseased condition, α-secretase cleaves APP to produce α-APP and an 83-residue COOH-terminal fragment (CT83). γ-secretase acts on CT83 to generate a 3-kDa peptide called p3 and the AICD peptide. In AD, β-secretase competes with α-secretase and cuts APP at the NH_2-terminus of the Aβ peptide domain to produce a soluble extracellular APP (β-APP) and a 99-residue fragment (CT99). γ-secretase then acts on CT99 to produce Aβ and amyloid intracellular domain (AICD) peptide [46].

It is to be noted that in the diseased condition, Aβ is generated by the sequential cleavage of APP by β and γ-secretase producing peptide fragments ranging from 39 to 43 residues. Among these several fragments, Aβ42 is highly aggregate-prone and the most abundant component in cerebral plaques observed in AD brains. Moreover, AD-causing mutations in APP near the β and γ secretase cleavage sites increase the production of Aβ. Site-directed mutagenesis shows that the substitution of the Met residue at P1 by a larger hydrophobic amino acid Leu (found in the Swedish FAD mutation) improves the efficiency of β-secretase cleavage. Conversely, substitution at the same position by Val, a smaller hydrophobic amino acid, inhibits cleavage [47]. Thus β-secretase recognition is highly sequence-specific. Interestingly, a number of Aβ species with different N-terminus residues have been identified. Although the predominant cleavage component of APP begins at the Asp+1 residue of Aβ, other minor Aβ species beginning at Val-3, Ile-6, and Glu+11 too have been reported. Inhibitor-binding studies suggest that the Val-3 and Ile-6 species are generated by a protease that is different from β-secretase but Glu+11 species is produced in parallel with Asp+1 species of Aβ fragment which implies that β-secretase is responsible for cleaving at both these positions [48–50].

6 BACE1: A Therapeutic Target in AD

In AD, BACE1 initiates the proteolysis of APP. Although initial reports demonstrate that BACE1 knockout mice live with normal physiology without any noticeable morbidity, later studies confirm the functional role of BACE1 on myelination of the peripheral nervous system. However, the process is activated during the developmental stage. Therefore, hypomyelination is not a limitation for BACE1-targeted therapies in AD which is primarily an age-related disorder. But recent advances on high-throughput proteomics and genomics technologies indicate functional role of BACE1 in nervous system. New physiological targets have been identified for BACE1, which certainly limit the application of BACE1-based therapy in AD. However, a recent interest is to explore the possibilities of partial inhibition of BACE1 activity such that optimum CNS beneficial effects may be achieved while limiting mechanism-based toxicities. Studies on *APPswe*; *PS1DE9*; *BACE1+/–* mice interestingly demonstrated a significant reduction in amyloid burden without any noticeable lethal phenotype as compared to that of *APPswe*; *PS1DE9*; *BACE1+/+* mice. This suggests exciting possibility of partial inhibition of

BACE1 in AD therapeutics which can effectively reduce Aβ deposition without any mechanism-based toxicity [51].

All these results intensify the urge for the identification of novel inhibitors of BACE1 for AD therapeutics. The biological target of BACE1 is a peptide; therefore, initial interest was to design/synthesize non-cleavable peptide-based transition state analogs or peptidomimetics inspired by the BACE1 recognition motif in APP. The first inhibitor in this category is P10–P4'StatVal which shows IC_{50} value of 30 nM. The second one is known as OM99-2 which exhibits an IC_{50} value of 1.6 nM [52]. These compounds show high efficacies in vitro but their unfavorable in vivo pharmacological properties restrict their potential as drug candidate. Therefore, the focus was shifted toward designing small-molecule BACE1 inhibitors. Several classes of small molecular scaffolds were lately reported as BACE1 inhibitors that include imidazolidinone analogs, hydroxymethylcarbonyl isosteres, pyridinium-based derivatives, flavonoids, and acyl guanidines [53–57]. But till date there are only few phase III clinical trials of BACE1 inhibitors, which implicate difficulty in the development of inhibitors targeting BACE1. Effects of two CNS drugs, pioglitazone and rosiglitazone, on cognition in AD patients were not positive. Currently, CTS-21166, an oral drug targeting BACE1, is under phase I trial and it has been documented that it is well tolerated and exhibits reduced plasma Aβ concentrations [58]. Another compound LY2886721, developed by Eli Lilly pharmaceutical company, demonstrated encouraging result in phase I clinical trial [59]. The drug significantly reduced both plasma and CSF Aβ40 as well as Aβ42 levels with significant increase in CSF sAPPα level. There was no noticeable mechanism-based toxicity during the course of the 14 day study. But the drug failed in phase II trial where the drug showed liver toxicity in a small group of volunteers. A BACE1 inhibitor, MK-8931, developed by Merck pharmaceutical company exhibits promising result in phase I clinical trial. MK-8931 noticeably reduced Aβ levels in the CSF in a dose-dependent manner. A single oral dose of 100/550 mg of the drug decreased CSF Aβ40 levels by 75 and 92%, respectively. During the randomized, double-blind, placebo-controlled phase I clinical trial, the drug was reported to be safe and well tolerated with no serious adverse effects. Phase II clinical studies of MK-8931 are also very encouraging [60, 61]. In both the EPOCH study and NCT01739348, the drug was well tolerated and highly effective. The results are waiting to be validated further in phase III clinical trials which presumably has started in 2017. Another BACE1 inhibitor, AZD3293, developed by AstraZeneca has been demonstrated to be safe, well tolerated and with promising pharmacokinetics in phase I randomized, double-blind, placebo-controlled, SAD (single-ascending dose), and MAD (multiple-ascending dose) studies [62]. The drug significantly reduced plasma and CSF Aβ levels in healthy young (18–55 years) and elderly (55–80 years) subjects. The drug now will be under phase II clinical trials.

Failure of many BACE1 inhibitors in clinical trials due to safety as well as cross-reactivity with other targets shifts the focus to develop BACE1 inhibitors from natural origin. Polyphenols are of particular interest as BACE1 inhibitor. Shimmyo et al. demonstrated that five flavonoids, namely, myricetin, kaempherol,

morin, quercetin, and apigenin, inhibit BACE1 as evident from in vitro and cell culture-based assays [63]. Chitosan derivatives from crab shell display weak β-secretase inhibitory activity [64], while green tea catechins [65], ellagic acid from pomegranate [66], and hispidin from mycelial cultures of *Phellinus linteus* have shown moderate β-secretase inhibitory activity [67]. Oral administration of tannic acid for 6 months improves cognitive function in transgenic PSAPP mouse model [68]. Recently, hesperidin has been found to be nM active as BACE1 inhibitor, in vitro [69]. Prevalence of these compounds in commonly consumed food items and beverages makes them viable alternative in conventional AD therapeutics as well as their applicability as functional food.

Experimental identification of inhibitors using synthesis followed by in vitro validation is both time consuming and expensive. State-of-the-art computational drug discovery methods have been widely used to discover novel compounds. They are broadly classified as ligand-based and structure-based drug designing methods. The ligand-based designing process is particularly useful in such cases where little or no structural information of the receptor is available and information of known active compounds is used to mine novel inhibitors. Pharmacophore modeling and quantitative structure activity relationship (QSAR) methods are widely used in this category. But the success of such modeling is highly dependent on the dataset used to derive the model and therefore, for unknown compounds these procedures can predict reliably if it falls within the "domain of applicability", i.e., the chemical and physical space where the model makes reliable prediction. In structure-based drug designing, receptor–inhibitor interaction features are used to mine novel inhibitors. Molecular docking is the most widely used method in this category. Most of the docking programs ignore receptor flexibility; they only consider the ligand flexibility. Incorporation of both receptor and ligand flexibility increases the complexity of the search problem. Thus mimicking receptor flexibility during structure-based drug designing has ample opportunity in computational drug discovery. This is particularly important for flexible enzyme like BACE1. BACE1 happens to be a difficult target for structure-based drug design. Not a single BACE1 inhibitor was found in a library containing more than 1800 renin inhibitors, despite the fact that both BACE1 and renin are pepsin-like enzymes [70]. Flexibility of BACE1 is a prime challenge that needs to be addressed during discovery of inhibitors, computationally.

7 BACE1: Flexibility of the Receptor as Evident from Crystal Structures

Currently, there are more than 350 crystal structures of BACE1 available in the public structural repository, protein data bank (PDB). Analyses of the crystal structures of BACE1 show that the protein possesses a bilobate structure composed of a single polypeptide chain. The catalytic dyad consists of two conserved aspartic acid residues (Asp), one from each lobe. Comparison of inhibitor free and bound BACE1 crystal structure reveals high degree of flexibility of the protein. Effects are particularly

pronounced in the "flap region", a β-hairpin (residue 67–77), which covers the opening of the catalytic cavity of BACE1. In the presence of a bound ligand, the flap moves closer to the catalytic Asp dyad to tightly retain the ligand within the active site, representing a closed conformation of BACE1. In the APO form, the flap generally moves away from the catalytic dyad, representing an open conformation.

Interestingly, the orientation and exposure of flap opening greatly varies in different reported crystal structures. Comparison of several inhibitor bound BACE1 structures reveals variety of flap conformations. Also it shows that the position and orientation of the flap depends on the volume of the bound inhibitor. Furthermore, recently, Xu et al. reported two crystal structures of BACE1 bound to the same inhibitor belonging to different space groups and exhibiting differential flap opening [71]. Hydrogen-bonding interactions between main-chain atoms of flap residues and the bound ligand lock the flap in the closed conformation. When this hydrogen bonding is absent, the bound ligand resists flap closing due to steric effect leading to an open conformation of the protein. An interesting observation is that most of the crystal structures of BACE1 inhibitor complexes belonging to space group P_{6122} exhibit open flap conformation [71]. Thus, there is strong influence of crystal packing on the flap orientation of the BACE1 inhibitor complexes. A completely conserved tyrosine residue, Tyr71, close to the flap tip has an unusual orientation in an open BACE1 crystal structure, where it forms a hydrogen bond with a lysine residue, Lys107, outside the ligand binding site. Usually, Tyr71 is involved in hydrogen-bonding interaction with the bound ligand, thus tightly retaining the flap in a closed conformation upon inhibitor binding. In addition, significant flexibility in other loop regions of the active site (Fig. 4b) such as "10s loop" (residues 9–14)

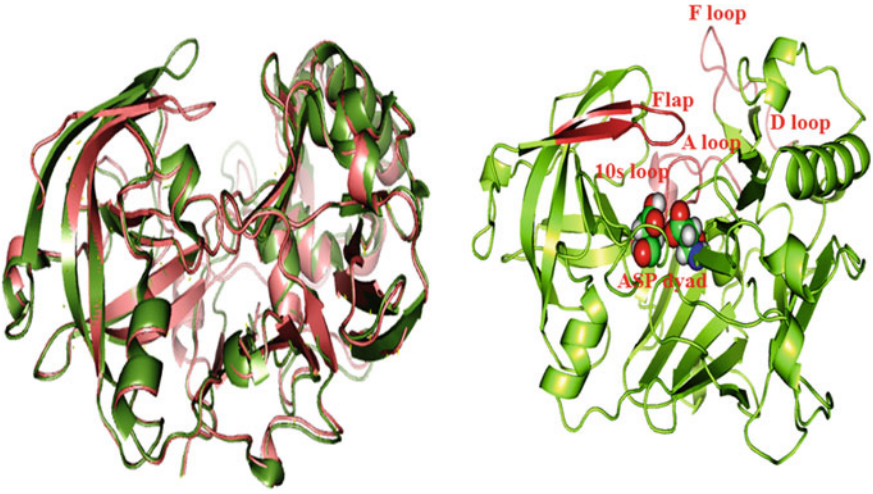

Fig. 4 Structure of BACE1 in open and closed form. *Left panel* Superimposed structure of BACE1 in open (PDB ID: 1W50, *green*) and closed form (PDB ID: 1FKN, *deep salmon*) in cartoon representation. *Right panel* Structure of BACE1 as green cartoon where different flexible loops that line the active site cavity are labeled and colored in *red*. Catalytic aspartate dyad is represented in CPK representation

located at the base of the S3 subpocket, "A loop" (residues 158–167), "F loop" (residues 311–318), and the "D loop" (residues 270–273) also has been reported [72, 73]. Their concomitant movement places the ligand appropriately within the active site cavity of the protein.

Optimal BACE1 activity is reported at ~pH 4.5–5. In this pH range the catalytic Asp dyad is probably mono-protonated which facilitates the water-mediated nucleophilic attack. Shimizu et al. determined the crystal structure of the active form of BACE1 and inferred that the activation of BACE1 in low pH is related with the conformational switching of the protein from the inactive to the active form [74]. These observations implicate that the catalytic properties of BACE1 are modulated by large-scale conformational changes between the active and inactive forms. Interestingly, two conserved water molecules, Wat1 and Wat2, play important role in the catalytic process [75]. Wat1 is involved in a hydrogen-bonding network with the catalytic aspartate dyad, Asp32 and Asp228 of BACE1. This water takes part in the proteolysis directly through an acid–base reaction. After substrate binding, the Asp dyad activates Wat1 by forming a hydrogen bond with it and this activated water nucleophilically attacks the carbonyl carbon to form a germinal diol. Finally, decomposition of the scissile C–N bond is accompanied by the transfer of a proton from Asp to the leaving amino group. The second water molecule, Wat2, indirectly facilitates this nucleophilic reaction. Wat2 is involved in a hydrogen-bonding network, Wat2-Ser35-Asp32-Wat1-Asp228, which stabilizes the substrate APP and reaction intermediates [75].

8 BACE1: Functional Dynamics of the Protein Explored by Molecular Dynamics Simulation

Crystal structure reveals the static picture of a protein. But proteins are not static in solution rather they exhibit small motions like residue side chain rotations, backbone dihedral bending to large-scale motions that include domain motion. Recent advances in computational architecture and development of novel algorithm allow one to explore the dynamics of a protein in solution and also to explore the ligand-induced protein motion using molecular dynamics simulation. Molecular simulations were employed to explore the dynamics of BACE1 both in its free and inhibitor bound form [72, 73, 75]. Coarse grain and also all-atom molecular dynamics simulations were employed to explore the atomistic details of the inherent flexibility of BACE1. Simple computationally inexpensive elastic network model was used to explore the collective dynamics of BACE1, which revealed that the low-frequency mode is able to capture the conformational transition of the protein [72]. In its open conformation, BACE1 possesses a large catalytic cavity that allows binding of wide range of substrates. In the open conformation, D and F loops frame the C-terminal lining of the cavity and 10 s loop is included within the cavity. The intermediate half-open-half-closed (HOHC) conformations have intermediate cavity opening where the 10 s loop moves upward and lines the cavity side, the F loop

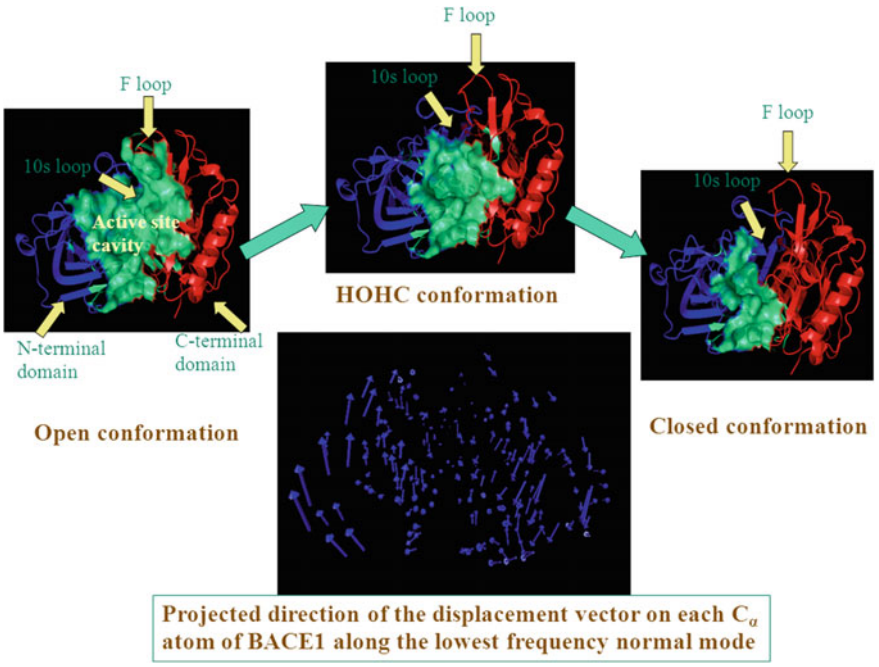

Fig. 5 Changes in the active site cavity of BACE1 during the conformational transition of the protein from open to HOHC (half-open-half-closed) to closed form. Lower panel shows projection vector on each Cα atom obtained from normal mode analysis

detaches away from the catalytic cavity thus reducing the cavity volume. In the closed conformation the upward movement of the 10 s loop further squeezes the cavity (Fig. 5).

Interestingly, this conformational transition of the protein is associated with a large-scale domain motion. A hinge motion has been observed where the two domains move in an anti-correlated motion with respect to each other leading to the cavity closure (Fig. 5, lower panel). Equilibrium molecular dynamics simulation reveals that free BACE1 can visit both open and closed conformations during simulation timescale [73]. Distance between Thr72 at the flap region and Thr329 at the other side of the active site cleft has been used as a criterion for flap opening [73, 75]. Throughout the simulation of free BACE1, the cavity opening varies constantly, never settling to any particular cavity opening distance. Simulation indicates that free BACE1 is very dynamic in nature and can visit numerous conformations with varied cavity exposure. This is so as the energy landscape of the protein around its native structure minima is relatively flat and there are many local minima close to its native minima with small energy barrier. Therefore, free BACE1 is polymorphic in solution. Interestingly, when substrate APP binds to the protein, there is a conformational stabilization. The cavity opening distances of the BACE1–APP complex are distributed within a narrower region and the complex is

highly stable throughout the simulation. During APP recognition, the flexibility of BACE1, particularly the flap region, reduces significantly. Simulation reveals that the flap and its opposite loop region move toward each other and also the D loop, F loop, and 113 loop regions move toward bound APP, thus allowing maximum surface complementarity between the receptor and bound APP. All these concerted movement enable APP to tightly bind in the BACE1 active site cavity forming a BACE1–APP closed complex [73]. It is worth mentioning that BACE1 is capable of binding a wide range of compounds that include peptide, peptidomimetics to small molecule. Simulation data suggest that binding of ligand to BACE1 is a conformational selection process.

Currently, two most probable hypotheses of substrate recognition are accepted: one is conformational selection and the other is induced fit [76, 77]. In the conformational selection approach, a ligand binds to preexisting ensemble of conformations and substrate recognition leads to an equilibrium shift toward the most stable protein–ligand complex. Choosing the receptor conformation depends on the volume of the ligand. Larger ligands choose wider cavity conformations while small ligands choose small cavity conformation of the receptor. On the other hand, induced fit approximation is generally associated with substrate-induced structural changes in the receptor. Free BACE1 in solution constantly fluctuates between open and closed conformations. Binding of ligand induces a population shift of the protein toward a stable closed complex where maximum surface complementarity is observed. The process is summarized in Fig. 6.

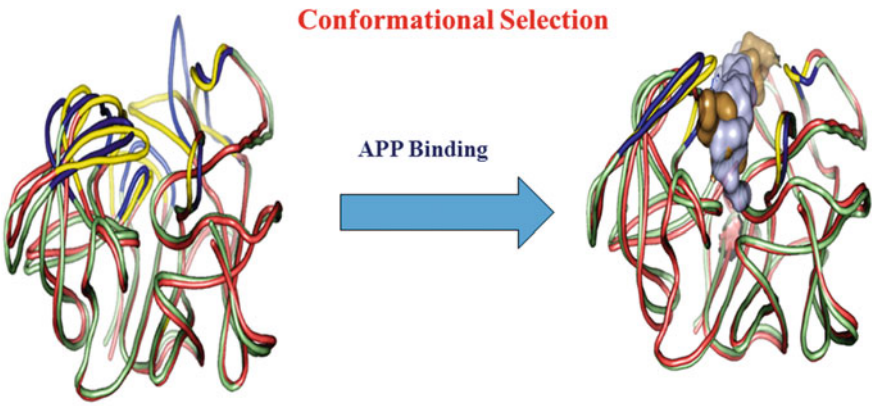

Fig. 6 A schematic representation of the substrate recognition mechanism of BACE1. *Left panel* Free BACE1 and *Right panel* BACE1-APP complex. Highly fluctuating loops in two different conformers of BACE1 (*green* and *deep salmon*) are colored differently with *yellow* and *blue*

9 BACE1: Mimicking Receptor Flexibility in Inhibitor Design

In silico virtual screening is now becoming an integral part of computational drug design, because identification of new inhibitors by random synthesis and experimental validation is time consuming and expensive too. In this process, a structural library of ligands is used to dock to the protein of interest for the identification of novel lead compounds and the receptor structure is used to predict/optimize the binding of small molecule as potential drug candidate. The success of such process depends on the choice of receptor conformation for proper elucidation of pharmacophoric feature of receptor–ligand interaction. Ligand-induced flexibility of the protein is the most critical issue that needs to be addressed during structure-based drug designing. Molecular dynamics simulation can be applied to probe ligand-induced protein flexibilities. However, large-scale protein motions are difficult to explore using all-atom molecular dynamics simulations and it is worth mentioning that the biologically relevant functional motion of a protein occurs on a timescale inaccessible to standard simulation using existing computational architecture. Moreover, simulations are computationally highly expensive and, therefore, inapplicable in the virtual screening of large compound database. Recently, several methods have been proposed to incorporate protein flexibility into the docking process, but those processes can only incorporate limited flexibility of few residues in the active site within reasonable computational demands. Thus, large-scale conformational plasticity of the receptor during ligand binding is still an unsolved puzzle in structure-based drug design. Ensemble docking emerges as the most popular solution to incorporate receptor flexibility in molecular docking of BACE1 [69, 78, 79]. Molecular dynamics simulation reveals that the ligand recognition by BACE1 is a conformational selection process. Therefore, construction of a proper receptor ensemble of BACE1 is a prime challenge in structure-based designing of the protein. The number of conformation in the receptor ensemble must be small enough to reduce the cost of computation, and simultaneously the ensemble must be diverse enough to capture the collective dynamics of the active site cavity. Chakraborty et al. in the recent past showed the applicability of NMA (Normal Mode Analysis)-derived multiple receptor conformation (MRC) of BACE1 to in silico virtual screening [72, 78]. An ensemble of 11 BACE1 conformations representing open-to-closed conformational transition obtained by simulating the protein in its lowest frequency normal mode using an elastic network model has been generated. Most suitable receptor conformation of BACE1 for small-molecule docking has been selected based on the prediction statistics of a developed hybrid QSAR model using a combination of ligand and structure-based descriptors from structurally diverse set of compounds. The 11th conformation of BACE1 was most suited for docking since it significantly enhanced the prediction ability of the designed equation [78]. The hybrid QSAR model based on the 11th conformation of BACE1 was then subsequently used to screen an *in-house* developed phytochemical structural library of 731 compounds. Of late, a unique hybrid-designing strategy has

been developed encompassing receptor flexibility during virtual screening of a phytochemical library [78]. A combination of structure- and ligand-based approach has been adopted. In the structure-based design, an ensemble of two crystal structures of BACE1 (PDB ID: 3IND and 3TPP) was considered during ensemble docking and it was found that this small number yet diverse conformations of the receptor successfully docked the seven structurally distinct inhibitors within the BACE1 cavity and reproduced crystallographic contacts as observed in the seven original BACE1 inhibitor crystal structure [69]. In the ligand-based designing strategy, a 2-D QSAR of BACE1 inhibition has been derived using heuristic approach from 20 structurally diverse BACE1 inhibitors and validated on a test set comprising 10 known BACE1 inhibitors. The model showed excellent prediction ability judged by R^2 and R^2_{CV}. Both structure-based and ligand-based models were then simultaneously used to mine an in-house developed phytochemical library. As a result several potential inhibitors were identified with predicted activity in the nM range. The efficacy of a potential hit, hesperidin, was then validated using several in vitro bioassays [69]. The flow of the work carried out has been summarized in Fig. 7.

Ensemble docking is frequently used in the structure-based designing of BACE1 inhibitors. Limongelli et al. successfully predicted the binding modes of six potent BACE1 inhibitors using an ensemble of five different BACE1 crystal structures [79]. Since the molecular recognition of BACE1 is a conformational selection

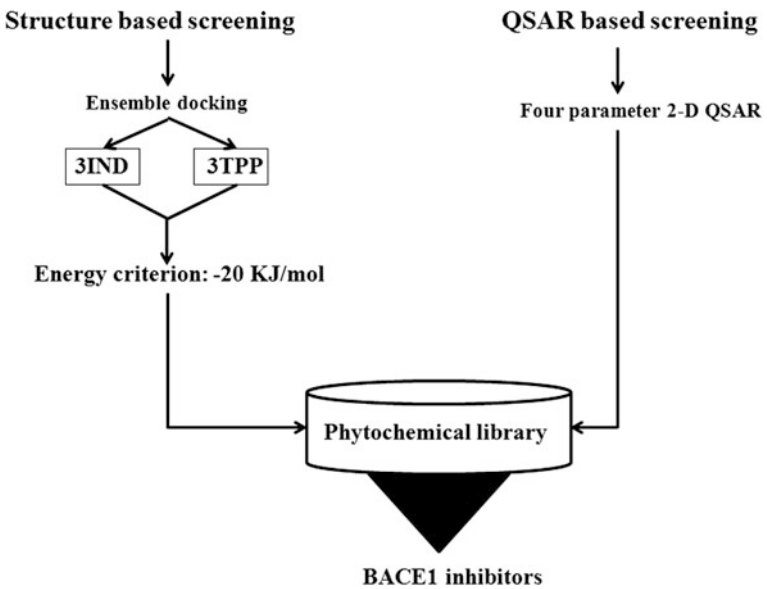

Fig. 7 Schematic representation of the mixed structure and ligand-based designing model for BACE1 inhibitor design

process, this observation justifies the success of ensemble docking in inhibitor screening using docking. But the prime challenge is to select a set of conformation of the receptor that explains the functional dynamics of the protein. In a benchmark study, application of molecular docking in the virtual screening of BACE1 has been performed using two different docking methods, FlexX and FlexX-Pharm with five different scoring functions, Dock, Gold, Chem, PMF, and FlexX, on open and closed conformations of the protein [80]. Interestingly, both open and closed conformations of BACE1 were found to be suitable for virtual screening. In addition, it has been demonstrated that the protonation states of the catalytic dyad, Asp32 and Asp228, play a crucial role in docking. Vijayan et al., on the other hand, employed a novel algorithm based on the active site pharmacophore feature in rapid overlay of chemical structure within the structure-based docking process implemented in GOLD [81]. During the virtual screening of a ligand structural library, it was found that the method possesses higher degree of prediction ability compared to standard single receptor docking process. Kacker et al. applied both quantum mechanical calculations in combination with molecular dynamics simulation and receptor ensemble concept to construct a drug designing pipeline for BACE1 inhibitors [82]. Forty seven high-resolution BACE1 inhibitor crystal structures were clustered into seven groups based on structural diversity of bound inhibitors. Then density functional theory calculation on a representative structure from each cluster was performed to identify most favorable dyad protonation. Five different protonation states were considered. Four mono-protonated states were 32i, 32o, 228i, and 228o (where 32 and 228 represent the catalytic Asp dyad residue numbers and i and o represent the inner and outer oxygen conformations), and the fifth one is the di-deprotonated state. Using QM and MD simulation it was observed that 32i is the most stable BACE1 catalytic dyad configuration, in general. Interestingly, when the DFT predicted protonation state was used for a particular cluster, docking methods were successfully reproducing the near-native inhibitor conformation in 85% cases during self-docking procedure. In cross-docking procedure, in general, using the 32i protonation state significant accuracy was shown in producing the native binding conformation as observed in the crystal structure.

10 Conclusion and Future Perspective

BACE1, which is over-expressed in the diseased condition, accelerates amyloid production and thus plays a major role in Alzheimer's disease. Designing BACE1 inhibitors is currently being considered as one of the promising drug development strategies in AD. Plenty of BACE1 inhibitors are now in phase I and phase II clinical trials. Many promising drug candidates failed in the clinical trials due to toxicity and cross-reactivity. One of the main limitations of drug development targeting BACE1 is that very little information is available of the physiological function of the protease. BACE1 probably plays a role in myelination of the

peripheral nervous system during the developmental stage. Therefore, BACE1-targeted therapies in AD may be a lucrative strategy since AD is primarily an age-related disorder. But physiological role of BACE1 needs to be explored more extensively to eliminate mechanism-based toxicity associated with BACE1 inhibitors. More recently, possibilities of drug development based on partial inhibition of BACE1 activity have been explored such that maximum CNS beneficial effects have been achieved while limiting mechanism-based toxicities. Mice model data suggest promising aspects of this approach.

Another challenge in drug discovery based on BACE1 inhibition is the cross-reactivity. BACE1 belongs to the pepsin family of aspartyl protease and its structural organization is very similar with other proteases of this family which play crucial role in several biochemical and signaling pathways. Thus designing inhibitors that specifically target and bind BACE1 is a challenge. Computational approaches reveal novel insight into the functional dynamics of the protein. Particularly, ligand recognition by BACE1 has been found to be a conformational selection process. This knowledge makes ensemble docking a promising approach to incorporate receptor flexibility in computational drug discovery of BACE1 inhibitors. Different approaches have been adopted to design a suitable conformational ensemble of BACE1 that significantly increases the hits during virtual screening. But more development in terms of devising novel algorithm and strategy to incorporate protein flexibility is an urgent need on BACE1-targeted therapy in AD.

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References

1. Hardy J (2009) The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. J Neurochem 110:1129–1134
2. Suh YH, Checler F (2002) Amyloid precursor protein, presenilins, and α-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. Pharmacol Rev 54:469–525
3. Roberds SL, Anderson J, Basi G, Bienkowski MJ, Branstetter DG, Chen KS, Freedman S, Frigon NL, Games D, Hu K, Johnson-Wood K, Kappenman KE, Kawabe TT, Kola I, Kuehn R, Lee M, Liu W, Motter R, Nichols NF, Power M, Robertson DW, Schenk D, Schoor M, Shopp GM, Shuck ME, Sinha S, Svensson KA, Tatsuno G, Tintrup H, Wijsman J, Wright S, McConlogue L (2001) BACE knockout mice are healthy despite lacking the primary β-secretase activity in brain: implications for Alzheimer's disease therapeutics. Hum Mol Gen 10:1317–1324
4. Hu X, He W, Diaconu C, Tang X, Kidd GJ, Macklin WB, Trapp BD, Yan R (2008) Genetic deletion of BACE1 in mice affects remyelination of sciatic nerves. FASEB J 22:2970–2980
5. Willem M, Lammich S, Haass C (2009) Function, regulation and therapeutic properties of beta-secretase (BACE1). Semin Cell Dev Biol 20:175–182
6. Vassar R, Bennett BD, Babu-Khan S et al (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735–741

7. Sinha S, Anderson JP, Barbour R et al (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature 402:537–540
8. Hussain I, Powell D, Howlett DR et al (1999) Identification of a novel aspartic protease (Asp 2) as beta-secretase. Mol Cell Neurosci 14:419–427
9. Yan R, Bienkowski MJ, Shuck ME et al (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature 402:533–537
10. Lin X, Koelsch G, Wu S, Downs D, Dashti A, Tang J (2000) Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein. Proc Natl Acad Sci USA 97:1456–1460
11. Yan R, Munzner JB, Shuck ME, Bienkowski MJ (2001) BACE2 functions as an alternative alpha-secretase in cells. J Biol Chem 276:34019–34027
12. Mowrer KR, Wolfe MS (2008) Promotion of BACE1 mRNA alternative splicing reduces amyloid β-peptide production. J Biol Chem 283:18694–18701
13. Cole SL, Vassar R (2007) The Alzheimer's disease β-secretase enzyme, BACE1. Mol Neurodegen 2:22
14. Bodendorf U, Fischer F, Bodian D, Multhaup G, Paganetti P (2001) A splice variant of β-secretase deficient in the amyloidogenic processing of the amyloid precursor protein. J Biol Chem 276:12019–12023
15. Ehehalt R, Michel B, De Pietri Tonelli D, Zacchetti D, Simons K, Keller P (2002) Splice variants of the beta-site APP-cleaving enzyme BACE1 in human brain and pancreas. Biochem Biophys Res Commun 293:30–37
16. Farzan M, Schnitzler CE, Vasilieva N, Leung D, Choe H (2000) BACE2, a b-secretase homolog, cleaves at the b site and within the amyloid-b region of the amyloid-b precursor protein. Proc Natl Acad Sci USA 97:9712–9717
17. Bennett BD, Babu-Khan S, Loeloff R, Louis JC, Curran E, Citron M, Vassar R (2000) Expression analysis of BACE2 in brain and peripheral tissues. J Biol Chem 275:20647–20651
18. Sun X, Wang Y, Qing H, Christensen MA, Liu Y, Zhou W, Tong Y, Xiao C, Huang Y, Zhang S, Liu X (2005) Distinct transcriptional regulation and function of the human BACE2 and BACE1 genes. FASEB J 19:739–749
19. Benjannet S, Elagoz A, Wickham L et al (2001) Post-translational processing of beta-secretase (beta-amyloid-converting enzyme) and its ectodomain shedding. The pro- and transmembrane/cytosolic domains affect its cellular activity and amyloid-beta production. J Biol Chem 276:10879–10887
20. Jonas MC, Costantini C, Puglielli L (2008) PCSK9 is required for the disposal of non-acetylated intermediates of the nascent membrane protein BACE1. EMBO Rep 9:916–922
21. Bennett BD, Denis P, Haniu M, Teplow DB, Kahn S, Louis JC, Citron M, Vassar R (2000) A furin-like convertase mediates propeptide cleavage of BACE, the Alzheimer's beta-secretase. J Biol Chem 275:37712–37717
22. Vassar R, Kuhn PH, Haass C, Kennedy ME, Rajendran L, Wong PC, Lichtenthaler SF (2014) Function, therapeutic potential and cell biology of BACE proteases: current status and future prospects. J Neurochem 130:4–28
23. Kalvodova L, Kahya N, Schwille P, Ehehalt R, Verkade P, Drechsel D, Simons K (2005) Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro. J Biol Chem 280:36815–36823
24. Fukumoto H, Cheung BS, Hyman BT, Irizarry MC (2002) Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch Neurol 59:1381–1389
25. He X, Li F, Chang WP, Tang J (2005) GGA proteins mediate the recycling pathway of memapsin 2 (BACE). J Biol Chem 280:11696–11703
26. Tesco G, Koh YH, Kang EL et al (2007) Depletion of GGA3 stabilizes BACE and enhances beta-secretase activity. Neuron 54:721–737
27. Kang EL, Cameron AN, Piazza F, Walker KR, Tesco G (2010) Ubiquitin regulates GGA3-mediated degradation of BACE1. J Biol Chem 285:24108–24119

28. Udayar V, Buggia-Prevot V, Guerreiro RL et al (2013) A Paired RNAi and RabGAP overexpression screen identifies Rab11 as a regulator of beta-amyloid production. Cell Rep 5:1536–1551
29. Buggia-Prevot V, Fernandez CG, Udayar V et al (2013) A function for EHD family proteins in unidirectional retrograde dendritic transport of BACE1 and Alzheimer's disease Aβ production. Cell Rep 5:1552–1563
30. Buggia-Prevot V, Fernandez CG, Riordan S, Vetrivel KS, Roseman J, Waters J, Bindokas VP, Vassar R, Thinakaran G (2014) Axonal BACE1 dynamics and targeting in hippocampal neurons: a role for Rab11 GTPase. Mol Neurodegener 9:1
31. Cerpa W, Dinamarca MC, Inestrosa NC (2008) Structure-function implications in Alzheimer's disease: effect of Aβ oligomers at central synapses. Curr Alzheimer Res 5:233–243
32. Hussain I, Hawkins J, Shikotra A, Riddell DR, Faller A, Dingwall C (2003) Characterization of the ectodomain shedding of the β-site amyloid precursor protein-cleaving enzyme 1 (BACE1). J Biol Chem 278:36264–36268
33. Willem M, Garratt AN, Novak B et al (2006) Control of peripheral nerve myelination by the beta-secretase BACE1. Science 314:664–666
34. Fleck D, Bebber FV, Colombo A, Galante C, Schwenk BM, Rabe L, Hampel H et al (2013) Dual cleavage of neuregulin 1 type III by BACE1 and ADAM17 liberates its EGF-like domain and allows paracrine signaling. J Neurosci 33:7856–7869
35. Lichtenthaler SF, Dominguez DI, Westmeyer GG, Reiss K, Haass C, Saftig P, Strooper BD, Seed B (2003) The cell adhesion protein P-selectin glycoprotein ligand-1 is a substrate for the aspartyl protease BACE1. J Biol Chem 278:48713–48719
36. Kim DY, Gersbacher MT, Inquimbert P, Kovacs DM (2011) Reduced sodium channel Na(v) 1.1 levels in BACE1-null mice. J Biol Chem 286:8106–8116
37. Kim DY, Carey BW, Wang H et al (2007) BACE1 regulates voltage-gated sodium channels and neuronal activity. Nat Cell Biol 9:755–764
38. Zhou L, Barão S, Laga M, Bockstael K, Borgers M, Gijsen H, Annaert W et al (2012) The neural cell adhesion molecules L1 and CHL1 are cleaved by BACE1 protease in vivo. J Biol Chem 287:25927–25940
39. Hemming ML, Elias JE, Gygi SP, Selkoe DJ (2009) Identification of β-secretase (BACE1) substrates using quantitative proteomics. PLoS ONE 4:e8477
40. Kuhn PH, Koroniak K, Hogl S, Colombo A, Zeitschel U, Willem M, Volbracht C et al (2012) Secretome protein enrichment identifies physiological BACE1 protease substrates in neurons. EMBO J 31:3157–3168
41. Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. Neuron 6:487–498
42. Walsh DM, Selkoe DJ (2007) Aβ oligomers—a decade of discovery. J Neurochem 101:1172–1184
43. Wille H, Drewes G, Biernat J, Mandelkow EM, Mandelkow E (1992) Alzheimer-like paired helical filaments and antiparallel dimers formed from microtubule-associated protein tau in vitro. J Cell Biol 118:573–584
44. Götz J, Schild A, Hoerndli F, Pennanen L (2004) Amyloid-induced neurofibrillary tangle formation in Alzheimer's disease: insight from transgenic mouse and tissue-culture models. Intl J Dev Neurosci 22:453–465
45. Busciglio J, Lorenzo A, Yeh J, Yankner BA (1995) β-Amyloid fibrils induce tau phosphorylation and loss of microtubule binding. Neuron 14:879–888
46. Neve RL, Robakis NK (1998) Alzheimer's disease: a re-examination of the amyloid hypothesis. Trends Neurosci 21:15–19
47. Vassar R (2002) β-Secretase (BACE) as a drug target for Alzheimer's disease. Adv Drug Deliv Rev 54:1589–1602
48. Haass C, Schlossmacher MG, Hung AY, Vigo-Pelfrey C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Schenk D, Teplow DB, Selkoe DJ (1992) Amyloid beta-peptide is produced by cultured cells during normal metabolism. Nature 359:322–325

49. Citron M, Diehl TS, Capell A, Haass C, Teplow DB, Selkoe DJ (1996) Inhibition of amyloid β-protein production in neural cells by the serine protease inhibitor AEBSF. Neuron 17:171–179
50. Gouras GK, Xu H, Jovanovic JN, Buxbaum JD, Wang R, Greengard P, Relkin NR, Gandy S (1998) Generation and regulation of β-Amyloid peptide variants by neurons. J Neurochem 71:1920–1925
51. Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, Wen H et al (2005) BACE1, a major determinant of selective vulnerability of the brain to amyloid-β amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. J Neurosci 25:11693–11709
52. Luo X, Yan R (2010) Inhibition of BACE1 for therapeutic use in Alzheimer's disease. Intl J Clin Expt Path 3:618–628
53. Pandey A, Mungalpara J, Mohan CG (2010) Comparative molecular field analysis and comparative molecular similarity indices analysis of hydroxyethylamine derivatives as selective human BACE-1 inhibitor. Mol Div 14:39–49
54. Al-Nadaf A, Sheikha GA, Taha MO (2010) Elaborate ligand-based pharmacophore exploration and QSAR analysis guide the synthesis of novel pyridinium-based potent β-secretase inhibitory leads. Bioorg Med Chem 18:3088–3115
55. Jain P, Jadhav HR (2013) Quantitative structure activity relationship analysis of aminoimidazoles as BACE-I inhibitors. Med Chem Res 22:1740–1746
56. Li Z, Zhou M, Wu F, Li R, Ding Z (2011) Self-organizing molecular field analysis on human β-secretase nonpeptide inhibitors: 5, 5-disubstituted aminohydantoins. Eur J Med Chem 46:58–64
57. Cumming JN, Le TX, Babu S, Carroll C, Chen X, Favreau L, Gaspari P, Guo T, Hobbs DW, Huang Y, Iserloh U (2008) Rational design of novel, potent piperazinone and imidazolidinone BACE1 inhibitors. Bioorg Med Chem Lett 18:3236–3241
58. Yiannopoulou KG, Papageorgiou SG (2013) Current and future treatments for Alzheimer's disease. Ther Adv Neurol Dis 6:19–33
59. Martenyi F, Dean RA, Lowe S, Nakano M, Monk S, Willis BA, Gonzales C, Mergott D, Leslie D, May P, James A, Gevorkyan H, Jhee S, Ereshefsky L, Citron M (2012) BACE inhibitor LY2886721 safety and central and peripheral PK and PD in healthy subjects (HSs). Alzheimers Dem 8:P583–P584
60. Forman M, Palcza J, Tseng J, Leempoels J, Ramael S, Han D, Jhee S, Ereshefsky L, Tanen M, Laterza O, Dockendorf M, Krishna G, Ma L, Wagner J, Troyer M (2012) The novel BACE inhibitor MK-8931 dramatically lowers cerebrospinal fluid Aβ peptides in health subjects following single- and multiple-dose administration. Alzheimers Dem 8:P704
61. Forman M, Kleijn H, Dockendorf M, Palcza J, Tseng J, Canales C, Egan M, Kennedy M, Laterza O, Ma L, Scott J, TanenM Apter J, Backonja M, Ereshefsky L, Gevorkyan H, Jhee S, Rynders R, Zari A, Bryan E, Wagner J, Troyer M, Stone J (2013) The novel BACE inhibitor MK-8931 dramatically lowers CSF beta-amyloid in patients with mild-to-moderate Alzheimer's disease. Alzheimers Dem 9:P139
62. Alexander R, Budd S, Russell M, Kugler A, Cebers G, Ye N, Olsson T, Burdette D, Maltby J, Paraskos J, Elsby K, Han D, Goldwater R, Ereshefsky L (2014) AZD3293 a novel bace1 inhibitor: safety, tolerability and effects on plasma and CSF Ab peptides following single- and multiple-dose administration. Neurobiol Aging 35:S2
63. Shimmyo Y, Kihara T, Akaike A, Niidome T, Sugimoto H (2008) Flavonols and flavones as BACE-1 inhibitors: structure–activity relationship in cell-free, cell-based and in silico studies reveal novel pharmacophore features. Biochim Biophys Acta 1780:819–825
64. Je JY, Kim SK (2005) Water-soluble chitosan derivatives as a β-secretase inhibitor. Bioorg Med Chem 13:6551–6555
65. Jeon SY, Bae KH, Seong YH, Song SK (2003) Green tea catechins as a β-secretase inhibitor. Bioorg Med Chem Lett 13:3905–3908

66. Kwak HM, Jeon SY, Sohng BH, Kim JG, Lee JM, Lee KB (2005) β-secretase (BACE1) inhibitors from pomegranate (*Punica granatum*) husk. Arch Pharm Res 28:1328–1332
67. Park IH, Jeon SY, Lee HJ, Kim SI, Song KS (2004) A β-secretase (BACE1) inhibitor hispidin from the mycelial cultures of *Phellinus linteus*. Planta Med 70:143–146
68. Mori T, Rezai-Zadeh K, Koyama N, Arendash GW, Yamaguchi H, Kakuda N, Horikoshi-Sakuraba Y, Tan J (2012) Town T (2012) Tannic acid is a natural β-secretase inhibitor that prevents cognitive impairment and mitigates Alzheimer-like pathology in transgenic mice. J Biol Chem 287:6912–6927
69. Chakraborty S, Bandyopadhyay J, Chakraborty S, Basu S (2016) Multi-target screening mines hesperidin as a multi-potent inhibitor: implication in Alzheimer's disease therapeutics. Eur J Med Chem. doi:10.1016/j.ejmech.2016.03.057
70. Gruninger-Leitch F, Schlatter D, Kung E, Nelbock P, Dobeli H (2002) Substrate and inhibitor profile of BACE (β-secretase) and comparison with other mammalian aspartic proteases. J Biol Chem 277:4687–4693
71. Xu Y, Li MJ, Greenblatt H, Chen W, Paz A, Dym O, Peleg Y, Chen T, Shen X, He J, Jiang H, Silman I, Sussman JL (2011) Flexibility of the flap in the active site of BACE1 as revealed by crystal structures and molecular dynamics simulations. Acta Crystal D: Biol Crystal 68:13–25
72. Chakraborty S, Kumar S, Basu S (2011) Conformational transition in the substrate binding domain of β-secretase exploited by NMA and its implication in inhibitor recognition: BACE1—myricetin a case study. Neurochem Intl 58:914–923
73. Chakraborty S, Basu S (2015) Structural insight into the mechanism of amyloid precursor protein recognition by β-secretase 1: A molecular dynamics study. Biophys Chem 202:1–12
74. Shimizu H, Tosaki A, Kaneko K, Hisano T, Sakurai T, Nukina N (2008) Crystal structure of an active form of BACE1, an enzyme responsible for amyloid β protein production. Mol Cell Biol 28:3663–3671
75. Gorfe AA, Caflisch A (2005) Functional plasticity in the substrate binding site of β-secretase. Structure 13:1487–1498
76. Grant BJ, Gorfe AA, McCammon JA (2010) Large conformational changes in proteins: signaling and other functions. Curr Opin Struct Biol 20:142–147
77. Sperandio O, Mouawad L, Pinto E, Bruno OV, Perahia D, Miteva MA (2010) How to choose relevant multiple receptor conformations for virtual screening: a test case of Cdk2 and normal mode analysis. Eur Biophys J 39:1365–1372
78. Chakraborty S, Ramachandran B, Basu S (2014) Encompassing receptor flexibility in virtual screening using ensemble docking-based hybrid QSAR: discovery of novel phytochemicals for BACE1 inhibition. Mol Biosys 10:2684–2692
79. Limongelli V, Marinelli L, Cosconati S, Braun HA, Schmidt B, Novellino E (2007) Ensemble-docking approach on BACE-1: pharmacophore perception and guidelines for drug design. ChemMedChem 2:667–678
80. Polgár T, Keserü GM (2005) Virtual screening for β-secretase (BACE1) inhibitors reveals the importance of protonation states at Asp32 and Asp228. J Med Chem 48:3749–3755
81. Vijayan RSK, Prabu M, Mascarenhas NM, Ghoshal N (2009) Hybrid structure-based virtual screening protocol for the identification of novel BACE1 inhibitors. J Chem Inform Model 49:647–657
82. Kacker P, Masetti M, Mangold M, Bottegoni G, Cavalli A (2012) Combining dyad protonation and active site plasticity in BACE-1 structure-based drug design. J Chem Inform Model 52:1079–1085

Serine Proteases and Their Inhibitors in Human Health and Disease

10

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Abstract

Serine proteases and their inhibitors are being extensively studied in the past few decades, accentuating their pivotal role in diverse biological processes. In this chapter, we have discussed about their role as drug targets, associated pathologies and therapeutic interventions. Fine tune equilibrium between proteolytic enzymes and their respective inhibitors enables normal functions of the body. The upregulation or downregulation of this class of molecules is deleterious and results in various diseased conditions like inflammation, cancer, skin diseases, atherosclerosis, immunological disorders, coagulation abnormalities, pulmonary and neuronal disorders, and other pathologies. Several approaches to illustrate this relationship are comprehended with consequent stress on how these findings apply to pathologies that are the outcome of malfunction of serine proteases or their inhibitors. We have outlined the history and classification of proteases and their inhibitors as therapeutics and drug targets. Also an overview of their current clinical applications and approaches to improve and expand their use is discussed.

Keywords

Serine proteases · Serine protease inhibitors · Pathologies · Drug targets · Therapeutics · Classification

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1 Introduction

Cellular life is orchestrated by equilibrium between proteolytic activity and their respective inhibitors as well as the concentration and state of the enzymes. Proteases, the enzymes that hydrolyse proteins, are therefore critical elements of the genome. Proteolytic enzymes are encoded by 2–4% of genes in a typical genome [1]. In some of the physiological processes like complement activation, phagocytosis, immune responses, tissue reorganization, blood clotting, fibrinolysis, blood pressure regulation, food digestion, defence mechanisms, etc. proteases serve a promising role. The human degradome consists of at least 1449 proteases and homologues, of which 399 are serine proteases (MEROPS release 10.0) [2]. The cascade of activation and deactivation mechanisms of proteases needs to be controlled at every point of regulation, for example at mRNA translation, transcription of protease gene, activation of zymogen, etc. Undesired proteolysis leading to various disease states is prevented by plethora of protease inhibitors with diverse specificities. Increase in proteolytic activity in certain instances mediates damage of cartilage in diarthrodial joints [3]. In certain diseases like tumor invasion, gingivitis, emphysema and other inflammatory ailments it is thought that proteases are the cause for tissue injury [3]. Fibrous proteins such as elastin and collagen can be solubilized by some serine proteases like cathepsin G, collagenase and elastase that are the culprits in extracellular matrix damage [4].

If the specificity of proteases is known with respect to their amino acid residues, there is every probability to inhibit those enzymes that are involved in various pathologies. Inhibitors with prospective inhibitory potential can be developed as new therapeutic agents. It is known that there are specific protease inhibitors against serine proteases of mammals, which are isolated from various plants and animals. This provides an excellent opportunity for developing novel medicines. Hence, the extracts of PIs from various sources are key in developing non-toxic drugs [3]. The future studies will mostly be concentrated on testing the specificity of protease inhibitors at clinical level against inflammation, cancer, dermatitis and emphysema. A good number of proteases are potential drug targets or are versatile molecules of consideration as diagnostic and prognostic biomarkers [5].

2 Serine Proteases

2.1 Introduction and Classification

Nature has provided abundant sources of serine proteases which are distributed among all living cells. The presences of nucleophilic Serine residue in the active site that attacks the carbonyl moiety of substrate, derives its name as serine protease. Ser proteases are endoproteases and catalyse polypeptide bond hydrolysis in

the middle of the chain. A similar feature can be observed in several other exoprotease families.

Barrett and Rawlings classified all known proteolytic enzymes based on sequence similarity and structure into clans and families and termed this database as MEROPS [6].

Three useful methods of grouping peptidases are currently in use as shown in Table 1.

1. Based on catalytic mechanism
2. Based on reaction catalysed and
3. Based on molecular structure and homology.

2.1.1 Based on Catalytic Mechanism

Enzymes exhibiting proteolytic activity are classified as cysteine, glutamic, serine, threonine, aspartic, asparagine or metalloproteases. Depending upon the catalytic type, the clans and families of proteolytic enzymes are named as C, G, S, T, A, M, N and U in the MEROPS database. Plus, P is given to proteases which are of mixed type [7].

2.1.2 Based on Reaction Catalysed

Though peptidases are known to catalyse peptide bond, no single type of enzyme catalyses all peptide bonds. They are specific to certain polypeptide chains of the substrate. Based on the polypeptide chain specificity they are classified into exopeptidases, endopeptidases, aminopeptidases, carboxypeptidases, omega-peptidases, dipeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases and tripeptidyl-peptidases [8].

2.1.3 Based on Homology and Molecular Structure

With the advent of high throughput technologies, classification based on homology and molecular structure is the latest. Classification by Rawlings and Barrett assigns individual peptidases into families and further into clans. In order to develop the MEROPS database, such scheme was designed which was further extended to store the information about proteins that inhibit peptidases [8].

2.2 Serine Proteases as Therapeutics

Despite many studies on proteases, their physiologically specific substrates are unknown. Their activity in a particular tissue in the human body also differs. The characteristics manifested by most proteases also differ in disease. Dysregulation of hydrolysis of proteins is a common trait identified in different diseases and in inflammatory responses. Tumor metastasis, invasion and growth in different types of cancer are commonly associated with upregulation of proteolysis [7]. In using

Table 1 Serine proteases classification and catalytic mechanism

Clan	Family	Families in humans	Prevalent	Catalytic residues	Counts of peptidase homologues	Activation mechanism
PA	14	S1	E	Asp, His, Ser	199	Cleavage of an N-terminal propeptide that permits folding to the active configuration Regulation of peptidase activity
SB	2	S8	B, Ar, Pr, F, Pl, An, V	Asp, His, Ser	20	Process the proteins along secretion pathway Catalyses the proteolytic activation of sterol regulatory element-binding protein Have an ability to function at acidic pH
		S53	B, Ar, Pr, F, Pl, An	Glu, Asp, Asp, Ser	5	
SC	6	S9	B, Ar, Pr, F, Pl, An, V	Ser, Asp, His	58	Associated with pro-specific N-terminal, process peptides and proteins Degrades biologically active peptides Have an ability to function at acidic pH Serine carboxypeptidases are synthesized as preproenzymes Degrades caseins and removes N-terminal pro and hydroxyproline residues from peptides Catalyse hydrolysis of epoxide bonds into diols and play a role in detoxification or cell signalling
		S10	B, Ar, Pr, F, Pl, An		7	
		S15	B, Ar, Pr, F, Pl, An		3	
		S28	B, Ar, Pr, F, Pl, An, V		9	
		S33	B, Ar, Pr, F, Pl, An, V		24	
		S37	B	Not been determined	–	Processes the transglutaminase precursor from *Streptomyces mobaraensis*
SE	3	S12	B, Ar, Pr, F, Pl, An, V	Ser, Lys	2	Carboxypeptidase B is involved in the synthesis and remodelling of bacterial cell walls
SF	2	S26	B, Ar, Pr, F, Pl, An, V	Ser, Lys or His	10	Removes the signal peptides and facilitate secretion
SH	5	S21, S73, S77, S78, S80	B, Ar, Pr, F, Pl, An, V	His, Ser, His	–	–
SJ	3	S16	B, Ar, Pr, F, Pl, An, V	Ser, Lys	5	It is ATP-dependent proteolysis and ability to act as protein-activated ATPases

(continued)

Table 1 (continued)

Clan	Family	Families in humans	Prevalent	Catalytic residues	Counts of peptidase homologues	Activation mechanism
SK	3	S14	B, Ar, Pr, F, Pl, An, V	Ser, His, Asp	2	Play important role in both protein quality control and the regulatory degradation of specific proteins Responsible for the degradation of nascent polypeptides Degradation of incorrectly synthesized proteins
		S41	B, Ar, F, Pl, An		3	
SO	1	S74	B, An, V	Ser, Lys	–	–
SP	1	S59	B, Ar, Pr, F, Pl, An	His, Ser	2	Autolytic processing to generate nucleoporins
SR	1	S60	Ar, Pl, An	Lys, Ser	9	Proteolytic activity towards a number of proteins
SS	1	S66	B, Ar, Pr, F, Pl, An	Ser, His, Glu	–	–
ST	1	S54	B, Ar, Pr, F, Pl, An, V	Ser, His	14	Hydrolyze peptide bonds within a phospholipid bilayer Cleaves the single span transmembrane proteins near the periplasmic edge of the membrane
Unassigned	7	S68	An	His, Ser	2	C-terminal fragments translocate from the cytoplasm to the nucleus where they lead to activation of cell survival or apoptotic pathways
		S71	An	Ser	15	–
		S72	B, An	Ser	1	–
		S79	An	Ser	3	–

E Eukaryotes, *B* bacteria, *Ar* archaea, *Pr* protozoa, *F* fungi, *Pl* plants, *An* animal, *V* viruses

proteases as therapeutics, one needs to understand the regulatory mode of their activity in both intra and extra cellular regions [8].

2.2.1 Thrombolytic Therapeutic u-PA (Urokinase-Type Plasminogen Activator)

Thrombolytic therapy based on enzymes is one of the best alternatives for surgical treatment and the first protease drug approved by FDA is u-PA (urokinase). u-PA is in vogue since 1978 for its efficacy in reviving patency of clogged blood vessels and catheters clearing. u-PA is obtained from cultures of primary neonatal kidney cells and known to possess three domains. u-PA exhibits lower affinity for fibrin

relative to other molecules known for fibrinolytic activity. Localized administration of u-PA to the site of thrombosis minimizes the adverse side effects and enhances focused activity. u-PA is a potential drug for both cancer treatment and diagnosis due to its association with the degeneration of matrix proteins in cancer cell proliferation [9]. Though newer agents with similar properties are coming up u-PA continues to be the choice for catheters cleaning due to its low cost [10].

t-PA is the second protease to be marketed as drug for treatment of thrombotic disease [11]. t-PA also hydrolyses plasminogen to form active enzyme plasmin. Plasmin exhibits property of digestion of blood clots by degrading fibrin mesh. The difference between u-PA and t-PA is their specificity towards fibrin [12]. Of the five domains of t-PA, the EGF2 and fibronectin finger domains, binds explicitly to fibrin. Characteristically t-PA acts locally, i.e. at the site of fibrin mesh formation contrary to streptokinase and u-PA. Systemic fibrinolysis is prevented by preferential activation of plasminogen by t-PA. First generation product of t-PA is alteplase marketed by Genentech as Activase®. In addition to alteplase, reteplase (Retavase®; Boehringer Mannheim) and tenecteplase (TNKase®, TNK-tPA; Genentech) are the bi-variants of t-PA which are in current usage.

The t-PA's truncated form reteplase is also an approved drug for treatment of thrombolytic AMI (Acute Myocardial Infarction). Protease affinity is reduced on fibrin due to deletion of EGF, Kringle and N-terminal fibronectin, which increases the clearance rate. Thus, half-life of reteplase is increased in plasma. Rather than infusing reteplase, it can be directed by double bolus, which is cost-effective and lowers time of administration. Genetic engineering methods like manipulation of carbohydrate content and site-directed mutagenesis are employed to improve tenecteplase half-life [13].

Thrombolytic therapy is the frontline method for treatment of stroke and AMI. There is still a significant need in development for improved pharmacodynamics and pharmacokinetics. Continuous efforts are made for developing next generation t-PAs and simultaneously for developing formulation of plasmin, the serine protease formed in vivo by t-PA activity. Genetic engineering techniques are in vogue for increasing the life span of therapeutic proteases [14].

2.2.2 Coagulation Factors

Many of the bleeding disorders are nowadays treated by employing proteases and proteins of coagulation cascade, as well as in combination also. FVII, FVIII and FIX are employed in treating haemophilia A, B and C, respectively. FVII and FIX are serine protease zymogens whereas FVIII is a bulky protein which on activation figures in as the cofactor for FIXa. Replacement of missing coagulation factors extracted from plasma is the mode of treatment for haemophilia in earlier days [15]. But in the recent past numerous plasma-derived biologicals having proteases are accepted. Safe recombinant versions of coagulation factors are the need of the day especially in AIDS and hepatitis C cases. Risk of contamination by prions and human viruses is a major challenge in plasma-derived biologicals. However, viral

inactivation and screening can be done to remove contaminants. Genetic engineering is the favoured method for the production of proteases [16].

Thrombin

Thrombin is the heart of coagulation cascade converting fibrinogen to fibrin [17]. It also activates factor V, VIII, PAR-1 and even PAR-4 in severe injury [18]. It also exhibits anticoagulant activity by complexing with thrombomodulin there by activating protein-C. This dual nature facilitated the production of anticoagulant molecules that lack procoagulant functionality [19].

Prothrombin an inactive thrombin consists of a serine protease domain, N-terminal Gla domain and two kringle domains [20]. Removal of calcium- and membrane-binding Gla domain by FXa bound to FVa allows thrombin to diffuse locally to the site of its action. This leads to the development of Recothrom® (Zymogenetics) a topical recombinant human thrombin which helps in stopping small blood vessels bleeding after surgery. It was approved by FDA in 2008 and it is less immunogenic compared to bovine thrombin [21].

Activated Protein C (APC)

APC a serine protease is associated with both inflammation and blood clotting; this property augments its use as a therapeutic molecule [22]. APC removes cofactors function from FVa and FVIIIa in the presence of the cofactor Protein-S and down regulates the coagulation response [23]. The pleiotropic effects exhibited by APC limits its use clinically through its interaction with endothelial Protein-C receptor. APC functions as anti-apoptotic and anti-inflammatory agent [24]. Riewald and his co-workers elucidated the cytoprotective effect of APC [25]. The gene expression profile towards the anti-inflammatory and anti-apoptotic pathways is due to downstream signalling through PAR-1 [26]. Activation by thrombin is in paradox to that of PAR-1 [27]. Downstream signalling via APC is independent of PAR-1 but is through apolipoprotein-E receptor [28] and integrins β3 and β1 [29]. Severe sepsis is treated by recombinant human APC and it is marketed as Xigris® [drotrecogin alfa, (EliLilly) and was approved in 2001].

2.2.3 In Dermatology—Penzyme

Proteinases from lysosomes are under active consideration for the treatment of scar tissue and for restoration of healthy tissue. A combination of chymotrypsin and trypsin, the Penzyme digests the outer layer of skin for the treatment of psoriasis, in addition to treatment of dermatological conditions. Penzyme is isolated from the alimentary canal of North Atlantic Cod and shows promise [8].

Owing to the multifaceted physiological roles and undesired emanations, therapeutic usage of proteases should be meticulously executed. For example, Lanetoplase an isoform of t-PA was developed for reduced dosage regime and extended half-life, but was found to exhibit adverse effects like intracranial haemorrhage [30]. The pharmacological and physiological role at biological level is needed to be understood for use of proteases as therapeutics. Development of proteins via

engineering with site specific protease activity is inevitable for future course of research and development.

2.3 Serine Proteases as Drug Targets

Proteases play a vital role in development of drugs which are articulated to act upon the proteases and proteasome involved in dysregulation and tumor suppression. Irregular protease signalling pathways lead to cancer, cardiovascular, neurodegenerative and pulmonary diseases. Substrates that are specific to upregulated proteases are used as prodrugs and the prodrug is activated into therapeutic by substrate catalysation. At present, a number of proteases are recognized as diagnostic and prognostic biomarkers. Activation of pro-MMPs is initiated by urokinase plasminogen activator (u-PA), membrane-type matrix metalloproteinase (MMP) and cathepsin B. Extracellular serine proteases u-PA, urokinase plasminogen activator receptor (u-PAR), plasminogen and MMPs activate extracellular matrix (ECM) degradation and also initiate, invasiveness, cellular motility and tumor growth factors cascade [31, 32].

2.3.1 Kallikreins in Relation to Cancer

Kallikreins are serine protease secretions of epithelial cells from skin, brain, breast, pancreas and prostate. They are also found in saliva, cerebrospinal fluid, sweat and seminal plasma milk. These are conventionally linked to clinical prognosis of human carcinoma. Human kallikrein 3 (hK3) is the most often used diagnostic biomarker for prostate cancer and hk3 thereby is also known as prostate specific antigen. Amino acids serine, histidine and aspartic acid present in serine proteases in proximity to hKs and also to one another bring about substrate cleavage. Sex-steroid hormones regulate the expression of tissue kallikrein gene (KLK) [31], like androgen regulation of KLK2 and KLK3 [33]. In some diseased conditions like cancer, dysregulation of hKs occur. Upregulation of 12 KLK genes take place in ovarian cancer [31]. Based on the type of tissue and hormone balance, tumor progression is either inhibited or promoted by hKs [34]. hK3, hK8, hK9, hK10, hK13 and hK14 play role in tumor suppression [35]. hKs are overexpressed in various cancers and are the choice for drug delivery.

Various hKs are involved in cancer progression, with its interaction through other serine proteases like u-PA and u-PAR. hK3 endorses prostate tumor growth by instigating growth factors and proteolytic surge to vitiate the extracellular matrix. Binding protein proteases hK3 and hK2 are recognized as insulin-like growth factors (IGF) [36]. Bioavailability of IGF is increased, when binding proteases degrade the binding protein in the tumor fuelling proliferation of prostate cancer. Moreover, the inactive plasminogen activator inhibitor-1 form active u-PA proteolytic cascade by hK2 and hK4 [37]. In the absence of inhibitor, activation of plasminogen to plasmin takes place via u-PA and its receptor. Plasmin further

activates pro-MMPs and causes the discharge of growth factors like EGF, augment angiogenesis and ECM degradation [38]. hK3 influences the activity of TFGβ (tumor growth factor β) and can also block FGF-2 (fibroblast growth factor-2) [39]. In addition, ECM degrades MMPs like type IV collagenases through activation of hKs [40]. u-PA–u-PAR pathway offer potential drug targets such as PSA, hK2, u-PA, plasmin and MMPs (proteases) for therapeutic applications in cancer [41]. Upregulation of u-PA and other serine proteases was reported in different cancers like gastric [42], prostate [43], cervical [9] and colorectal [44]. PAR (Protease-activated receptor) G-protein coupled receptor is expressed both in cancer and tumor cells. PAR signalling is another pathway that has been implicated in different cancers. Trypsin and thrombin activate PAR by cleaving its extracellular domain and mediate signals in the cell that trigger cancer cell proliferation [45].

2.3.2 Epidermal Serine Proteases

Matriptase and Prostasin

Profilaggrin plays a significant role in epidermal barrier function of skin. It is converted into filaggrin monomers at stratum granulosum/stratum corneum (SG/SC) interphase. Filaggrins form macro fibrils by crosslinking and maintain hydration of SC and act as natural moisturizing factors (NMFs) [46]. Human genetic studies have underscored profilaggrin proteolysis in maintaining epidermal architecture and hydration. It was also reported that ichthyosis vulgaris, asthma and atopic dermatitis are caused due to loss-of-functional mutations in profilaggrin, which disturb epidermal barrier and allow the free entry of infection causing agents and allergens [47].

Serine proteases prostasin, a membrane anchored glycosyl-phosphatidyl-inositol, and type II transmembrane matriptase are essential for initiation of profilaggrin processing. Arnett et al., has reported that in cascade of zymogen activation, the auto-activated protease matriptase acts upstream to that of prostasin. Autoactivating protease matriptase regulates terminal epidermal differentiation and is required for prostasin zymogen activation [48]. Epidermal appendages, incomplete terminal differentiation of epidermis and oral epithelium are associated with reduced expression of matriptase [49]. Recent reports also suggest that mutations in matriptase gene cause icthyosis [50]. Matriptase is present in the skin and when exposed to acidic pH it gets activated immediately, which suggests that its activation is a response of direct exposure to proton [51]. The proteolytic cascade of matriptase-prostasin, is regulated either by the hepatocyte growth factor activator inhibitor-1 (HAI-1) or by prostasin activation. These mechanisms rapidly inhibit both prostasin and matriptase, which provides the opportunity for matriptase and prostasins to act on their respective substrates [52]. The role of these membrane-bound proteins was thought to be limited only for skin homeostasis. However, recent studies suggest that matriptase has ability to activate kallikrein-related proteases which play a significant role in conditions causing skin inflammation [53].

Kallikreins

One of the major family in tryptic–chymotryptic serine protease cluster is kallikrein-related proteases (KLKs) encoded by 15 different genes located on chromosome 19q13.4. Keratinocytes of the stratum granulosum (SG) present in skin produces KLKs and are liberated into upper SG and lower SC. The present knowledge on serine protease activity in SC is ascribed to KLKs of human tissues [54]. In healthy skin tissues around eight different KLKs are known to be expressed, among those KLK14, KLK8, KLK7 and KLK5 are observed to be most important [55]. These KLKs are extensively studied and their putative functions were determined [55, 56]. There are a number of reports which suggest that KLK5 and KLK7 have proteolytic function in SC. Previously KLK5 and KLK7 proteases were termed as 'stratum corneum tryptic enzyme (SCTE)' and 'stratum corneum chymotryptic enzyme (SCCE)' respectively. In ex vivo conditions it is known that serine protease inhibitors are capable of inhibiting shedding of corneocytes, hence KLK5 and KLK7 are very important in desquamation process [57]. They are most commonly found to be expressed in SG, and located in SC interstices. Both KLK5 and KLK7 are thought to have self-activation capabilities to form a proteolytic cascade [46]. It is believed that these two enzymes are capable of hydrolysing DSG-1, corneodesmosin and desmocollin-1 when they are active as suggested in in vitro studies. Recent studies have also shown certain evidences that other KLKs also have role in desquamation, as about half of the total proteolytic activity in SC is carried out by KLK14. The reason behind this may be that KLK14 can catalyse and also can be triggered by KLK5.

KLK8 is also known for its role in the cascade of proteolytic activity controlling desquamation. KLK8 is synthesized abundantly and is co-localized with other KLKs of sweat glands and human epidermis. KLKs play an important role in barrier function of SC and are transported and exocytosed into the SG/SC interface by lamellar bodies. The recombinant KLK8 plays an important part in the upper SG where in the pH is normal and the optimum activity is at pH 8.5 [56]. KLK8 activity was also identified in extracts of SC and sweat where, kininase II and KLK1 were known to be active. Invariably, this suggests its prospective role in desquamation of skin, although there is lot to know about its physiological substrates.

Neutrophil Serine Proteases

The primary cell infiltrates are neutrophils, during skin infection causing 'neutrophilic dermatoses'. Pustule formation can be observed in the epidermis when there is massive infiltrate. At the time of infection, microbes are phagocytosed by neutrophils within the phagolysosome. This is done by the α-defensins, ROS generating systems, as well as by proteases and is liberated from 1° to 2° granules [58]. High levels of cathepsin-G, protease-3 and human leukocyte elastase are found only in primary granules. Phagocytosis alone is not responsible for the release of these enzyme, also 'frustrating phagocytosis' and development of NETs (neutrophil extracellular traps) comprising neutrophil-derived DNA [59]. In patients with psoriasis (neutrophilic dermatosis) the HLE activity is observed on the surface

of skin lesion [60]. Hence, neutrophil serine proteases are said to be important in innate immune regulation [61].

2.3.3 Serine Protease in Synaptic Function and Behaviour

A large number of studies suggest that the activity of proteases, the corresponding receptors and inhibitors are co-opted by brain to regulate various synaptic activities.

Thrombin

The normal functioning of brain is associated with synthesis of thrombin from prothrombin in brain [62, 63]. Thrombin has a profound impact on both glial cells and neurons. The neurite outgrowth inhibition with growth cone collapse [64] was observed in cultured neurons and neuroblastoma cells when thrombin was applied [65]. A number of responses in Astrocytes like reversal of stellate morphology and stimulating proliferation are induced by thrombin [66]. Administration of high amounts of thrombin for therapy exhibits programmed cell death in astrocytes and neurons [67]. These observations emphasise a significant role of thrombin in neuronal development and maintenance. The deleterious effects on cognitive function of brain are most likely associated with high levels of thrombin. It was observed that when rats were treated with thrombin via intra cerebroventricular infusion, it resulted in higher memory errors and task completion potency in 8 arm radial maze [68]. Numerous neuropathological changes can be observed with such behavioural deficits which include increased astrogliosis, cell death and expanded cerebral ventricles. Even increase in levels of apolipoprotein-E (ApoE) and phosphorylated neurofilament proteins were also observed. These findings also predict the role of thrombin in cognitive decline and neuropathology related to Alzheimer's disease [62, 63, 66, 68].

Tissue Plasminogen Activator (t-PA)

Tissue plasminogen activator (t-PA) is one of the most widely studied serine protease associated with CNS. Endothelial cell, Neurons and Glial cells synthesize and release t-PA in brain, and are expressed highly in hippocampus, amygdala cerebellum and cortex [69]. Literature available on t-PA suggests it to be a synaptic activity modulator, but this mechanism is under debate [70]. It is also reported that NMDAR (*N*-Methyl-D-Aspartate receptor) signalling is enhanced by cleavage of the GluN1 subunit and is associated with proteolytic activity of t-PA [71].

2.3.4 Serine Proteases in Human Immune System

The human immune system consists of cells known as endosomal vesicles which are responsible for expression of chymase and tryptase in mast cells, proteases in granulocytes and granzymes in lymphocytes. Serine proteases from endosomal vesicles are associated with inflammation, tissue remodelling, apoptosis and phagocytosis. Increase in serine protease activity contributes to pathology in allergy, auto-immune disorders and in cancer proliferation.

Granzymes

Apoptosis via granule associated enzymes (granzymes) and by death receptor pathway are the two mechanisms employed by CTLs (cytotoxic T lymphocytes) and NK cells (natural killer cells) to efficiently combat virus-infected cells and tumor cells [67]. Apoptosis induced by granzyme relies on a pore-forming protein called perforin. Perforins are localized in the same granules and helps in delivering granzymes to target [72]. Granzymes are efficient in activating the apoptotic pathways in cytoplasm in two ways, by targeting proteins responsible for integrity of mitochondria and DNA, secondly by cleavage of caspases.

In humans, five variants of granzymes are known which include Gzm A, B, H, K and M. All the variants have exclusive pattern of expression and possess substrate specificity, despite structural similarities among them. The best-studied and most abundant members among human granzyme family are GzmA and GzmB. Both GzmA and GzmB granzymes are expressed in effector $CD8^+$ T cells and CD56dim NK cells. Additionally, with GzmA expression GzmK is also expressed simultaneously within the granules of memory T cells [73]. Expression of GzmA and GzmB is higher with activation of T and NK cells but GzmK levels are unaltered [74]. After activation Regulatory T cells expresses only granzymes. GzmA are most prominently expressed by activated natural Treg cells whereas GzmB levels are highly expressed by adaptive Treg cells. Perforin-dependent apoptosis can be induced by both the Treg subtypes in autologous target cells. This property suggests that granzymes are utilized by Treg cells to exert their anti-proliferative effects [75]. Non-cytolytic cell types like basophils, mast cells and chondrocytes lack perforin but expresses GzmB supporting alternative function of granzymes i.e. inducing cell death [76]. Several studies suggest that granzymes are important in immuno-pathological processes. Increased numbers of positive granzyme lymphocytes was observed during various immune-mediated disorders which include transplant rejection, systemic lupus erythematosus and rheumatoid arthritis [77].

Proteases from Neutrophils

Neutrophils are the first cells from bodies defence mechanism that turn up in the vicinity of inflammation. Neutrophils through acquiescent action of proteases, free radicals and antimicrobial peptides successfully mortify microorganisms. Three serine proteases are identified in neutrophil granules: (1) Neutrophil elastase (NE), (2) Proteinase-3 (PR3) and (3) Cat-G. They exhibit intra and extracellular activities. Cat-C cleaves them into active forms as they are synthesized as zymogens [78]. G-CSF, C5a and TNF trigger neutrophil degranulation and remarkably augment inflammation by interacting with cellular surfaces and extracellular matrix [79]. Neutrophil proteases inside the phagolysosome are implicated in phagocytosis and tissue injury. Neutrophils are meant for first line of defence and are essential during infections. However, in some cases imbalance in its activity contribute to certain diseases. The reason for imbalance of neutrophils may be due to lack of control on neutrophil proteases or may be because of over-exposure to proteolytic activity in the vicinity of inflamed region. Inflammatory bowel disease (IBD), chronic obstructive pulmonary disease (COPD) and inflammation of genital tract exhibit

increased levels of neutrophil elastase [80]. This strong parallel correspondence between neutrophil elastase and activation has attracted scientists to study the usage of neutrophil elastase as a biomarker. This will be a breakthrough in diagnosing and monitoring inflammation in episodes like COPD [81]. However, lack of specificity dissuades elastases from neutrophils and like serine proteases as biomarkers for diagnosis in the clinic. More or less, same NE levels are observed in sputum of healthy smokers and COPD patients. But serine protease can be of use in monitoring disease progression and reflects the standing of immune cells. Neutrophil proteases in blood can be utilized for diagnosis of the origin of the disease. Neutropenia is caused by massive neutrophil activation. Low levels of neutrophil elastase combined with neutropenia indicate defective production and survival of neutrophils.

Another example of imbalance of neutrophil proteases leading to disease is cystic fibrosis (CF), which is characterized by gentle and persistent breakdown of airway architecture. Viscosity of the mucus increases paving way for bacteria to invade small airways and colonize. The colonized bacteria trapped in the mucus layer draw good number of neutrophils that eventually undergoes necrosis. The release of pro-inflammatory compounds and toxic substances initiates epithelial damage; reduce mucus clearance and inflammatory cell infiltration [82]. This is a vicious cycle of events and the activity of protease from neutrophils in CF makes serine proteases as potential drug targets (Fig. 1).

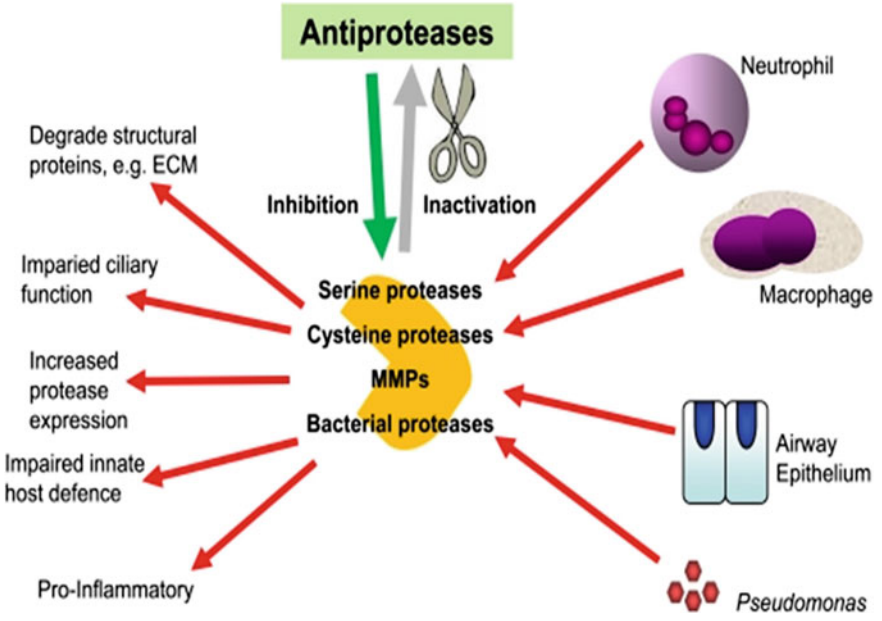

Fig. 1 The importance of the balance between proteases and anti-proteases in CF airways

NE induces Chemokine IL-8 production in epithelial cells of lungs and can inactivate the innate defence system by inhibiting surfactant D molecule which thereby enhances the colonization of bacteria and hence NE are said to be fundamental mediators of inflammatory responses [83]. The expression of CD40, CD80 and CD86 is downregulated when neutrophil elastase from sputum of COPD patients or purified neutrophil elastase is incubated with dendritic cells (DC). DC antigen-presenting function and maturation also is impaired by NE. Collectively, this outcome advocates that NE disables DC and restricts ample immune responses towards microbial infections. There is reduced expression of CD8 and CD4 on $CD3^+$ T cells when incubated with purified NE or Cat-G which renders these cells less cytotoxic [84]. Hence it is clear that, sputum from patients suffering from CF exhibit lower $CD4^+$ and $CD8^+$ T cells and increased Cat-G and NE levels. Neutrophil proteases induce T-cell dysfunction need to be targeted to combat CF.

Wegener's granulomatosis is another example of imbalance of neutrophil proteases. The disease characterized by granulomatous inflammation affects several organs like kidneys and lungs. PR3 combating antibodies known as anti-neutrophils are present in most of the patients. As observed in the literature such antibodies can activate neutrophils [85].

Proteases from Mastocytes

Mastocytes (Mast cells) is synonymous with reactions to allergens. Mast cells are loaded with granules containing cytokines, histamine, proteases and proteoglycans, chymase and tryptase as zymogens. Stimulation of mastocytes leads to degranulation [86]. Degranulation releases granular contents initiating inflammation, vasoconstriction, blood coagulation and extracellular matrix degradation [87]. Expression of GzmB and Cat-G depends on the activation status and/or localization of mastocytes [88]. Pathogen clearance is efficiently enabled by the action of mastocyte proteases.

Mastocytes are associated with a host of IMID (Immune-Mediated Inflammatory Diseases); e.g. allergic diseases, IBD (Inflammatory Bowel disease) and atherothrombosis allied with the activity of proteases from mastocytes [89]. Mast cell degranulation is evidenced by increased levels of tryptase in vivo and suggests IgE-mediated response [90]. Mastocytes present in the joints of patients suffering from rheumatoid arthritis and tumor invasion sites account for degradation of neighbouring connective tissue [91]. This phenomenon points to role of serine proteases and are yet to be elucidated for their role in induction and perpetuation of disease. IgA nephropathy and lupus nephritis are inflammatory kidney diseases due to protease activity of mast cells [92]. Tryptase plays a vital role through activated receptors and formation of type I collagen in the genesis of connective tissue fibrosis [93]. Increase of tryptase levels in blood represents mastocyte incitement and is associated with itching in patients undergoing hemodialysis. Mastocytes release various pruritogens and influence of tryptase on subsequent itch development in vivo is difficult to separate [94]. In a nut shell Table 2 depicts serine proteases as potential drug targets.

Table 2 Serine proteases as potential therapeutic targets

Serine proteases	Function	Association with disease
Tryptase	Promotes extracellular pathogen clearance ECM compounds degradation Regulates inflammatory responses Induces release of TGFβ from ECM Promotes IL-8 production Promotes cell signalling Restricts blood coagulation	Promotes atherosclerosis Contributes to allergic reactions Aggravates psoriasis Attributes to inflammatory kidney diseases Correlates with poor prognosis in liver cancer Inflammatory role in asthma and allergic rhinitis
Cathepsin G	ECM degradation, migration, regulation of inflammatory disorders	Inflammation, metastasis
Matriptase	Matrix degradation, regulation of intestinal barrier, ironmetabolism	Pathogenesis of epithelialtissues, tumor growth and progression
Human elastase	Pathogen killing, ECM degradation, inflammatory disorders	Pulmonary disease, inflammation
Chymase	Promotes extracellular pathogen clearance ECM compounds degradation Might promote vasoconstriction Regulates inflammatory responses Activates pro-inflammatory cytokines	Inflammation, asthma, Promotes atherosclerosis Involved in gastric cancer
Proteasome	Protein degradation, cell proliferation, differentiation, angiogenesis and apoptosis	Carcinogenesis, inflammation, neurodegeneration

3 Serine Protease Inhibitors

3.1 Introduction

Fermi and Pernossi in 1894, for the first time, reported the presence and availability of protease inhibitors in nature [95]. They are very important as they regulate the activity of proteolytic enzymes thereby maintaining homeostasis [96]. Proteins proficient in impeding the catalytic activity of proteolytic enzymes, stoichiometrically, competitively and reversibly are categorized as protease inhibitors. Uncontrolled proteolysis by endogenous and exogenous proteases is prevented by these inhibitors. Protease inhibitors specificity is very helpful in targeting some of the proteases which are known for pathogenesis in humans, viz. hepatitis, pancreatitis, cancer, arthritis, AIDS, emphysema, high blood pressure, thrombosis, muscular dystrophy, etc. Having such potential, the new era of drugs and diagnostics associated to protease inhibitors are being emerged [97]. The most abundant and extensively distributed protease inhibitors are from serine protease family [98]. Around 17,451 serine protease inhibitors account from genomes of all five kingdoms [99, 100]. In order to inhibit the targets, serine proteases use specific changes in the conformation [96]. The molecular weight of serine proteases is about 40–60 kDa, having 330–500 amino acid residue and are monomeric protein molecules

[101]. These are homologous proteins exhibiting sundry functions that carry out many physiological and biological activities like fibrinolysis, clot formation, apoptosis, cell growth, inflammation, angiogenesis and tumor suppression [96, 102]. Table 3 shows function and dysfunction of serpins.

Table 3 Serpins function and dysfunction diversity

Clade name	Gene name	Serpin known as	Target	Involvement in disease
α1 proteinase inhibitor anti-trypsin	SERPINA1	α1-anti-trypsin	Neutrophil elastase	Emphysema, cirrhosis
	SERPINA2	Anti-trypsin-related protein	Not characterized	
	SERPINA3	α1-antichymotrypsin	Cathepsin G	Emphysema
	SERPINA4	Kallistatin	Kallikrein	
	SERPINA5	Protein C inhibitor	Active protein C	Angioedema
	SERPINA6	Corticosteroid-binding globulin	Cortisol binding	Chronic fatigue
	SERPINA7	Thyroxine-binding globulin	Thyroxine binding	Hypothyroidism
	SERPINA8	Angiotensinogen	Release of the decapeptide angiotensin I	Hypertension
	SERPINA9	Centerin	Maintenance of naive B cells	
	SERPINA10	Protein Z-dependent proteinase inhibitor	Inhibition of activated factor Z and XI	Venous thromboembolic
	SERPINA11	XP_170754.3	Not characterized	
	SERPINA12	Vaspin	Insulin-sensitizing adipocytokine	
	SERPINA13	XM_370772	Not characterized	
Ov serpins	SERPINB1	Monocyte neutrophil elastase inhibitor	Inhibition of neutrophil elastase	
	SERPINB2	Plasminogen activator inhibitor-2	Inhibition of uPA	
	SERPINB3	Squamous cell carcinoma antigen-1	Inhibition of cathepsins L and V	
	SERPINB4	Squamous cell carcinoma antigen-2	Inhibition of cathepsins G and chymase	
	SERPINB5	Maspin	Inhibition of metastasis	Tumor progression
	SERPINB6	Proteinase inhibitor-6	Inhibition of cathepsin G	
	SERPINB7	Megsin	Megakaryocyte maturation	IgA nephropathy
	SERPINB8	Cytoplasmic antiproteinase 8	Inhibition of furin	
	SERPINB9	Cytoplasmic antiproteinase 9	Inhibition of granzyme B	
	SERPINB10	Bomapin	Inhibition of thrombin and trypsin	

(continued)

Table 3 (continued)

Clade name	Gene name	Serpin known as	Target	Involvement in disease
		Epipin	Not characterized	
	SERPINB12	Yukopin	Inhibition of trypsin	
	SERPINB13	Headpin	Inhibition of cathepsins L and K	
Anti-thrombin	SERPINC1	Anti-thrombin	thrombin and factor Xa inhibitor	Thrombosis
Heparin cofactor	SERPIND1	Heparin cofactor II	Thrombin inhibitor	Thrombotic risk
Plasminogen activator inhibitor 1	SERPINE1	Plasminogen activator inhibitor 1	Inhibitor of thrombin, u-PA, t-PA and plasmin	Abnormal bleeding
	SERPINE2	Protease nexin I	Inhibition of u-PA and tPA	
	SERPINE3	Hs.512272	Not characterized	
Alpha-2 pigment epithelium derived factor	SERPINF1	Pigment epithelium derived factor	Potent anti-angiogenic molecule	
	SERPINF2	Alpha-2-antiplasmin	Plasmin inhibitor	Bleeding
C1 inhibitor	SERPING1	C1 inhibitor	C1 esterase inhibitor	Angioedema
Heat-shock protein	SERPINH1	Heat-shock protein	Chaperone for collagens	
Neuroserpin	SERPINI1	Neuroserpin (PI12)	Inhibitor of t-PA, u-PA and plasmin	Dementia
	SERPINI2	Myoepithelium-derived serine proteinase inhibitor	Inhibition of cancer metastasis	

3.2 Classification

SERPINs represent a superfamily of proteins deriving its terminology from **ser**ine **p**rotease **in**hibitors [103]. MEROPS database currently holds 71 different families accounting for about 17,451 inhibitors based on sequence homology of proteins. Serpins are segregated into 38 clans depending on tertiary structure and this classification is regularly updated [99]. The term proteinase was replaced with peptidase in human gene in 2005. Serpins are classified based on

1. Clade
2. Group

3.2.1 Based on Clade

Serpins are divided into clades, known as clade based classification system. Serpins are categorized into 16 clades as A-P, among them A to I include first nine clades

which are human serpins [104]. The sequence similarity and specific phylogenetic relationships are grouped and determined as clade where as those which cannot be grouped are known as orphans, around 10 orphan sequences are found. Based on the function and inhibitory activity they are classified into inhibitory and non-inhibitory groups [98]. Based on the amino acid sites and gene structure clades further consist of six sub-groups [105]. To understand the nomenclature, for example in SERPINA1, letter A denotes the clade and number 1 denotes the gene number within clade. In eukaryotes, serpins are omnipresent. About 36 functional protein coding human genes are identified, clade A representing extracellular serpins whereas clade B with intracellular serpins. In humans, SERPIN-A the α1 proteinase inhibitor anti-trypsin accounts as the largest group followed by SERPIN-B the ov-like serpins. SERPIN-C and SERPIN-D involve orphan heparin cofactor II (HCF-II) and anti-thrombin (ATIII) [106].

3.2.2 Based on Group

In vertebrates, serpin genes are categorized based on "group-based classification". The criteria of classification is based on gene structure and comprises of 6 groups namely V1–V6 [107]. Serpins are very specific and target mostly serine proteases, in some cases even target caspases known as cathepsins [108], papain-like cysteine proteases [109] and some proteases involved in hormone transport and blood pressure regulation [106]. Serpins play a significant role in corticosteroid binding, blood pressure regulation, coagulation and hormone transport. And in seldom, a non-inhibitory function is also significant, for example, they function as molecular chaperones [110], as hormone transporters [111] and some as tumor suppressors [112]. Inhibitors play a vital role in protease characterization and are used in the pharmaceutical industry [99].

3.3 Serpins Mechanism

Serpins structurally comprise 3-β-sheets viz., A, B and C, 8-9 α-helices and a **R**eactive **C**entre **L**oop (RCL) (Fig. 2). RCL plays an important role in targeting proteases [113]. Based on RCL these protein inhibitors exist in different variants viz., active, latent, cleaved, delta and polymeric. The amino acid terminus of the RCL inserts into the β-A sheet forming a fourth strand, this progression is called stressed (S) to relaxed (R) shift and results eventually into a cleaved form. Increase of potential in inhibition is observed for serpins which bind to cofactors. Serpins are capable of interchanging from active form to latent form and vice versa. However, such shift is not observed in all serpins. The inhibitory activity is not seen in latent form, but through refolding and denaturation it can translate into active form. The secondary structures of serpins have noticeable RCL, which target the protease active site and inhibit its activity. The inhibitory activities of protease indispensably rely on the conformational change of tertiary structure [103]. Ser195, His57 and Asp102 are catalytic triad residues in serpins liable for hydrolysis of amide bond

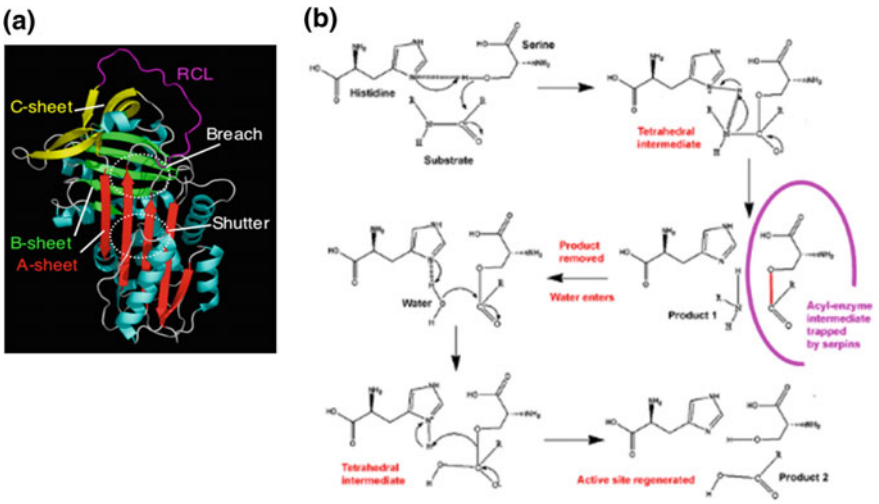

Fig. 2 The five-stranded A-sheet is in *red*, the six-stranded B-sheet in *green*, and the four-stranded B-sheet in *yellow*. α-helices are shown in *cyan*. The RCL is at the top of the molecule in *magenta*. Two functionally important regions of the serpin, the breach and the shutter, are labelled. **a** Structure of human native anti-trypsin. **b** Mechanism of protease inhibition by serpins

(Fig. 3). Serpins are grouped into three major classes which are trypsin-like, elastase-like and chymotrypsin-like [114].

With all these potential functions this class of protease inhibitors are the emerging therapeutics, which are used in treating infections pertaining to fungal—*Candida albicans*, viral—HIV, Hepatitis, Herpes and parasitic—schistosomiasis, malaria and diseases of respiratory, cardiovascular, inflammatory, immunological and neurodegenerative disorders [115].

Sometimes the change in conformation and deficiency of serpins leads to different diseases like emphysema, thrombosis and angioedema. Increase in the levels of serpins in endoplasmic reticulum is defined in diseases like chronic fatigue, hypothyroidism, hypertension, cirrhosis, tumor progression and familial dementia [116].

3.4 Serpins as Therapeutics

In human plasma, next to albumin and immunoglobulins, the third largest functional groups of proteins are plasma protease inhibitors based on the weight of molecule. These inhibitors account for about 10% of plasma proteins which are capable of controlling many critical processes like complement activation, coagulation, connective tissue turnover, inflammatory reactions and fibrinolysis.

α_1PI-neutrophil elastase; α_2-AP-plasmin; α_1Achy-cathepsin G and AT III-thrombin are present in human proteome which are specific of pairing with inhibitor-target. Clr and Cls are the complement proteases which are controlled by

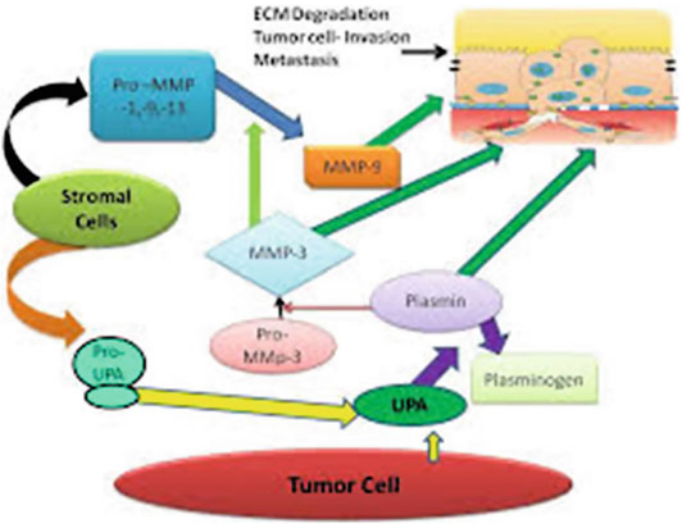

Fig. 3 Role of proteases in ECM degradation, invasion and metastasis

CI-Inh, yet this mechanism needs to be completely understood and that in regulation of mast cell chymase α_1Achy are involved. Human α_2M, functions as fast and effective clearing agent due to its ability to inactivate proteases, whenever they are freely found in circulation. Till date there are no specific reports about the functional role of IαI, β_1AC and αCPI [117].

3.4.1 In Diabetes

Protease inhibitors inhibit the enzymatic degradation of insulin and hence are widely used along with insulin to increase absorption. There are several reports that suggest that in clinical trials, when protease inhibitors and insulin are administered together it had showed better hypoglycemic effect when compared to patients who were administered only with insulin. Contradictory to this even there are some reports, which suggest that concomitant administration of protease inhibitors and insulin has shown no improvement in absorption of insulin. This may be due to short period of exposure time of aprotinin, i.e. 24 h only and even, dose of aprotinin may be low. Nevertheless, their role in insulin therapy still remains uncertain. To understand their positive effect in insulin therapy more clinical studies in larger settings is required [118].

In diabetic nephropathy, mesangial matrix accumulation is associated with reduction in activity of plasmin, MMP-2 (matrix metalloproteinase) and MMP-9. Megsin is the recently identified protein belonging to serpin family and is overexpressed in mesangial cells that progressively induce mesangial proliferation in mice. Hyperglycemia upregulates megsin at translational level in both in vivo model of type II diabetic nephropathy and in vitro model of cultured mesangial cells. The decreased degradation of mesangial matrix was observed when MMP-2

and MMP-9 were inhibited by megsin. Anti-megsin neutralizing antibodies specific to MMPs reinstated MMP activity. Hyperglycemia in diabetes upregulates megsin which in turn inhibits plasmin and MMP activities suggesting the accumulation of mesangial matrix [119].

3.4.2 In Obesity

Vaspin a serine protease inhibitor is recognized in visceral adipose tissue. Vaspin exerts an insulin-sensitizing effect targeted toward visceral white adipose tissues (WATs) in states of obesity. These findings drive the search for identification of potential protease substrate that helps in developing anti-protease inhibitor therapy. This would enhance insulin sensitivity and reverse altered expression of insulin resistance in subjects suffering from diabetes [120].

3.4.3 In Cancer

Human Serpins associated with cancers and role of proteases in ECM degradation, invasion and metastasis are depicted in Table 4 and Fig. 4.

3.4.4 In Central Nervous System

The role of serine proteases in glial and neuronal function is suggestive for their involvement in health and disease of nervous system. Deranged proteolytic balance is identified in many pathological conditions of nervous system. It is known that in cognitive function, the synaptic proteolysis plays a very important role. Further understanding of the association of neurotrypsin in mental retardation is of prime importance. Extrication of molecular basis of other neurological disorders which are not associated with the mutation of neuroserpin or neurotrypsin may involve different components which are associated with the extracellular proteolytic signalling pathway. Understanding such phenotypes associated neurological disorders will help in elucidating novel targets. Identification of novel targets will optimistically contribute to proper management of these disorders [121].

3.4.5 In Skin Diseases

In desquamation and terminal differentiation process, serine proteases play a very crucial role. Stratified epithelium is formed by a complex differentiation mechanism without disturbing the barrier function. Corneocytes are separated from one another by the timely and specially orchestrated proteolytic system. In recent times, it is also known that the proteolytic homeostasis is crucial not only for physical barrier but also for immunological responses. Most of the recent research was focused on epidermal proteases their inhibitors and their role in pathogenesis (Fig. 4). Augmented desquamation, atopy, dry skin and abnormalities of hair, e.g. bamboo hair are the characteristic feature of ichthyosiform skin disease. The symptoms are decreased levels of functional LEKTI (Lympho Epithelial Kazal-Type Related Inhibitor) or the synthesis of abnormal LEKTI forms that are devoid of enzyme inhibiting domains. It is also demonstrated that decreased levels of LEKTIs are inversely correlated to activity of serine protease in Statum Corneum (SC). Specific expression of another Kazal-type inhibitor in hand and foot is an interesting speculation attributed to be a

Table 4 Human serpins associated with cancers

Serpins	Molecular target	Associated cancers	Effects on cancers	Experimental therapy
α1-Anti-trypsin (SERPINA1)	Neutrophil elastate, trypsin, chymotrypsin	Liver Thyroid Cervical Lung	Tumor formation Tumor formation Poor prognosis Invasion	
Kallistatin (SERPINA4)	Kallikrein	Liver		Lung Liver Colon
PAI-2 (SERPINB2)	u-PA, t-PA	Colon	Poor prognosis	Colon
SERPINB3/B4	Granzyme M	Lung Head/neck squamous	Anti-apoptosis	
Maspin (SERPINB5)	GST, HDAC1-integrin, collagen I, III - catenin, EGR1, Rac1, PI3 K/ERK	Gastric Colon Bladder Lung Ovarian Breast Oral Laryngeal Gallbladder Melanoma	Growth retardation Anti-apoptosis, poor prognosis Favourable prognosis Migration inhibition, migration inhibition Less invasive Favourable prognosis Anti-angiogenesis	
Protease inhibitor-9 (SERPINB9)	Granzyme B	Melanoma Breast Cervical Colon	Poor prognosis, immune escape	
SERPINB13		Skin	Anti-angiogenesis	Head/neck squamous
PAI-1 (SERPINE1)	u-PA, t-PA, thrombin, protein C, vitronectin	Lung Colon Breast Fibrosarcoma Thyroid Endometrial Pancreatic	Anti-apoptosis Anti-apoptosis, dissemination, poor prognosis Anti-apoptosis, poor prognosis Anti-apoptosis Poor prognosis Poor prognosis Dissemination	Gastric
Protease nexin-1 (SERPINE2)	Prostasin, t-PA, u-PA, plasmin, trypsin	Prostate Breast Colon Oral Breast Testicular	Inhibition of growth and angiogenesis Enhanced metastasis Tumor formation Enhanced metastasis	

(continued)

Table 4 (continued)

Serpins	Molecular target	Associated cancers	Effects on cancers	Experimental therapy
PEDF (SERPINF1)		Prostate Osteosarcoma Pancreatic Colon Breast Lung Melanoma Ovarian Glioma	Anti-angiogenesis, pro-apoptosis Anti-angiogenesis Anti-angiogenesis Anti-angiogenesis	Prostate Endometrial Colon Lung Brain metastasis Chondrosarcoma Cervical Liver Retinoblastoma Melanoma
Neuroserpin (SERPINI1, protease inhibitor-12)	u-PA, t-PA	Gastric prostate	Growth retardation Poor prognosis	

Courtesy by Zheng et al. [127]

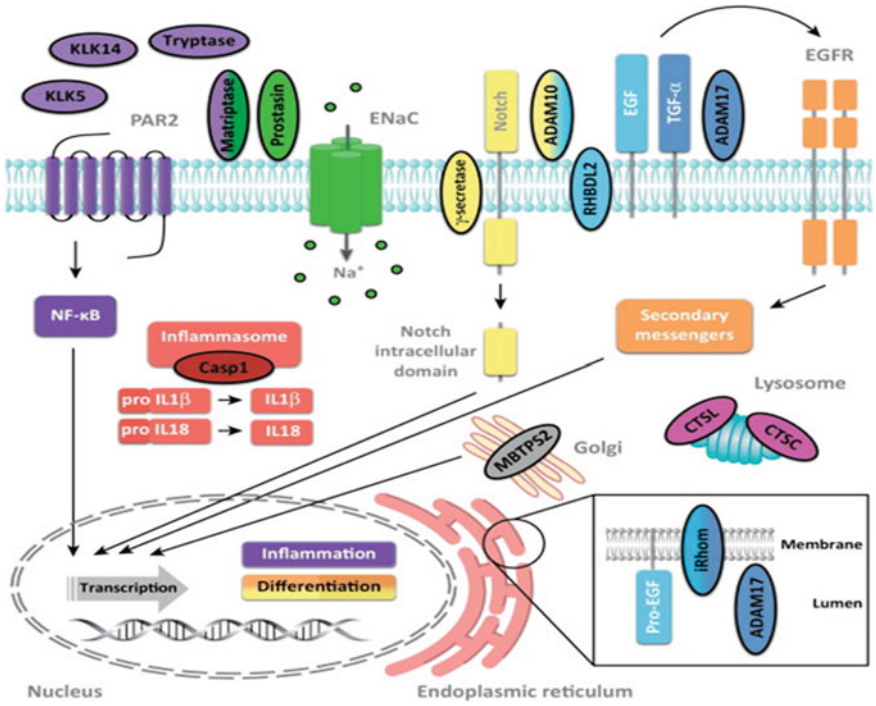

Fig. 4 Proteases the common culprits in human skin disorders

significant factor in eczema of hand and foot. This Kazal-type inhibitor named as LEKTI2/SPINK9 specifically inhibits KLK5. Meyer-Hoffert and Wiedow in 2011 identified SPINK6 in skin which is a selective inhibitor of KLKs [61]. SPINK6 was identified in skin appendages like sweat glands and sebaceous glands in SG of healthy individuals. On the contrary, downregulated expression of SPINK6 is identified in lesions of patients suffering from atopic dermatitis.

Other than LEKTIs a large number of other protease inhibitors are produced by keratinocytes. Of which, one group is termed as 'trappins' (transglutaminase substrate, WAP-domain-containing proteins) [122]. Elafin and secretory leukocyte protease inhibitor (SLPI) are two human epidermal protease inhibitors. Inhibition of elastase and protease-3 by elafin, cathepsin G and elastase by SLPI is suggestive of the efficacy of these two inhibitors against neutrophil serine proteases. All these findings demonstrate the protective role of neutrophil serine proteases during inflammation. In psoriasis, a highly inflammatory disease elafin is upregulated, whereas SLPI gene is a housekeeping gene expressed continuously in the epidermis [123]. Elafin is stored as a proenzyme in lamellar bodies and is secreted at the junction of SG and SC and via transglutamination is crosslinked to the proteinaceous molecules of cornified envelopes. It is reported that in human skin few members of SERPIN family are expressed which include SERPINB13 (headpin/hurpin), SERPINB4 (squamous cell carcinoma antigen-2) and SERPINB3 (squamous cell carcinoma antigen-1). Subtilisin A is inhibited by SERPINB8 and SERPINB9 which suggest that SERPINs has a protective role from tissue proteolysis caused by bacterial proteases.

Dandruff or Seborrheic Dermatitis (SD) is most common affliction of human scalp. Generally, dandruff or SD is caused by dermatophytic fungi, most commonly *Malassezia furfur* and other species of *Malassezia*, which are opportunistic pathogens on human scalp and skin. SD is often characterized by scaling on the scalp and causes inflammation [124]. *Malassezia* degrade sebum and release multiple free fatty acids from available triglycerides. Fungal colonies consume specific saturated fatty acids to proliferate leaving unsaturated fatty acids (USFAs) behind. These USFAs alters the combination of sebaceous secretions and penetrates into stratum corneum, the outermost layer of epidermis, disrupting the skin barrier function, resulting in inflammation, irritation and leads to scalp flaking. Studies found that SNTI, a Soap Nut Trypsin Inhibitor, was effective against dandruff causing fungi compared to the antifungal chemical drugs—fluconazole and ketoconazole [125].

3.4.6 In Human Airway Inflammation

Bronchial asthma, fever and COPD are the symptoms in response to viral replication and release of inflammatory cytokines in influenza. Viral entry and replication is facilitated by serine proteases secreted by epithelial cells of the airway. Surface epithelial cells of the human nasal mucosa, trachea, distal airways, lung and human alveolar epithelial cell line A549 expressed TMPRSS-1 (Transmembrane protease serine S-1), TMPRSS-2, TMPRSS-4 and TMPRSS-11D which belong to serine protease family. Protease inhibitors like aprotinin and camostat are found to reduce replication of influenza virus and release TNF-α (Tumor Necrosis Factor) and IL-6 (InterLeukin) into cell supernatants. Conversion of HA0 the precursor

protein into subunit HA1 in influenza virus is retarded by camostat. These findings authenticate the role of serine proteases in the proteolytic activation of influenza virus in human tracheal epithelial cells. Thus, serpins are coveted therapeutics candidates in treating viral influenza [126].

3.4.7 In Blood Clotting Abnormalities

To curb excessive thrombin activity a few anticoagulant strategies are in vogue which in turn control hyper-coagulation. Warfarin, agonist of vitamin K is being used since 1950, but its administration requires careful monitoring because of its interactions with different drugs and food and also because of its narrow therapeutic range. Heparin and LMWH (Low Molecular Weight Heparin) are used to enhance anti-thrombin activity and are incapable to inhibit clot-bound thrombin. Based on these elements, over the last twenty years' enormous efforts are put forward to design low molecular weight, orally bioavailable and selective thrombin inhibitors [115]. Table 5 portrays the clinical status of serine protease inhibitors as therapeutics.

Table 5 Serine protease inhibitor drugs in clinic

Target	Drug name	Indication	Clinical status
Thrombin	Ximelagatran	Venous thrombosis	Launched
	Melagatran	Thrombosis, general	Pre-registration
	Argatroban	Arterial thrombosis	Launched
	BIBR-1048	Venous thrombosis	Phase III
	MCC-977	Thrombosis	Phase II
	TGN-167, TGN-255	Thrombosis	Phase I
	SSR-182289	Thrombosis	Phase I
	AZD-0837	Thrombosis	Phase I
	E-5555	Thrombosis	Phase I
	LB-30870	Venous thrombosis	Preclinical
Factor Xa	DX-9065a	Thrombosis, angina	Phase II
	DPC-906	Venous thrombosis	Phase II
	CI-1031	Thrombosis	Phase II
	JTV-803	Venous thrombosis	Phase II
NS3-protease	BILN-2061	Hepatitis C virus infection	Phase II
	VX-950	Hepatitis C virus infection	Phase I
Elastase	Sivelestat, Elaspol	SIRS, inflammation	Launched
	Midesteine	COPD	Pre-registration
	AE-3763	COPD	Preclinical
	R-448	COPD	Phase I
Broad-spectrum	Nafamostat, FUT-175	Pancreatitis, inflammation	Launched
	Camostat mesilate	Pancreatits	Launched
Urokinase	WX-UK1	Cancer, Gastrointestinal	Phase II
Chymase	NK-3201	Restenosis	Preclinical
DPP IV	LAF-237	Diabetes type II	Phase III
	MK-0431	Diabetes	Phase II
	P32/98 (P3/01)	Diabetes	Phase I
	T-6666	Diabetes	Phase I
	NN-7201	Diabetes	Phase I

4 Conclusion

Serine proteases account to about one-third of all the known proteases. Large and diversified clusters of serine proteases and their inhibitors are encoded by human genome. Search for low molecular weight inhibitors to regulate proteases and their activity is attracting pharmaceutical industry. However, it is an uphill task in view of the expression of closely related proteins in the genome. Active site recognition enables the regulation of multiple protease targets and focuses on active site-directed therapies. But now other regions are also being targeted. Macromolecular recognition is envisaged by exosites and allosteric communication between these regions and the active site resulting in conformational changes. This interplay is crucial in biological system as exemplified by proteases in coagulation and immune system. Disturbance in the delicate balance between serine protease and serpins is the reason for a wide range of pathologies. Early reports of such imbalance are identified in blood coagulation in which there was deficiency of factor IX. On the contrary, overexpression of immune system serine proteases culminates in inflammatory states. So also, imbalance in serine protease inhibition affects multiple biological systems. Emphysema, cirrhosis and thrombosis result from aberrant conformations and belong to proteinopathies. Unravelling the molecular interactions in the regulatory pathways of proteolysis in vivo continues to be a puzzling and intuitive venture to alleviate human well-being. Pharmaceutical, biotechnological industries, academic researchers and their financial backers consider serine proteases and serpins as future medicine worthy for clinical trials in human applications.

This is a humble endeavour to elucidate the progress of protease and protease inhibitors, predict their future and some of the hurdles overcome till date. They remain to be the challenging molecules that are to be expounded as a promising class of new drug targets and therapeutic agents.

References

1. Puente XS, Sánchez LM, Gutiérrez-Fernández A et al (2005) A genomic view of the complexity of mammalian proteolytic systems. Biochem Soc Trans 33:331–334
2. Rawlings ND, Barrett AJ, Finn R (2015) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 44:343–350
3. Royston D (1996) Preventing the inflammatory response to open-heart surgery: the role of aprotinin and other protease inhibitors. Int J Cardiol 53:S11–S37
4. Berquin IM, Sloane BF (1996) Cathepsin B expression in human tumors. Adv Exp Med Biol 389:281–294
5. Turk B (2006) Targeting proteases: successes, failures and future prospects. Nat Rev Drug Discov 5:785–799
6. Rawlings ND, Morton FR, Kok CY, Kong JBA (2007) MEROPS: the peptidase database. Nucleic Acids Res 36:D320–D325
7. Duffy M, McGowan P, Gallagher W (2008) Cancer invasion and metastasis: changing views. J Pathol 214:283–293
8. Craik CS, Page MJ, Madison EL (2011) Proteases as therapeutics. Biochem J 435:1–16

9. Dass K, Ahmad A, Azmi AS et al (2008) Evolving role of uPA/uPAR system in human cancers. Cancer Treat Rev 34:122–136
10. Dillon PW, Jones GR, Bagnall-Reeb HA et al (2004) Prophylactic urokinase in the management of long-term venous access devices in children: a Children's Oncology Group study. J Clin Oncol 22:2718–2723
11. Rijken DC, Lijnen HR (2009) New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost 7:4–13
12. Hoylaerts M, Rijken DC, Lijnen HR, Collen D (1982) Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J Biol Chem 257:2912–2919
13. Semba CP, Sugimoto K, Razavi MK, Society of Cardiovascular and Interventional Radiology (SCVIR) (2001) Alteplase and tenecteplase: applications in the peripheral circulation. Tech Vasc Interv Radiol 4:99–106
14. Ambrus JL, Ambrus CM, Back N et al (1957) Clinical and experimental studies on fibrinolytic enzymes. Ann N Y Acad Sci 68:97–137
15. Eley RC, Green AA, McKhann CF et al (1936) The use of a blood coagulant extract from the human placenta in the treatment of hemophilia. J Pediatr 8:135–147
16. Pipe SW, Kaufman RJ (2000) A chamber of hope for hemophilia. Nat Biotechnol 18:264–265
17. Di Cera E (2008) Thrombin. Mol Aspects Med 29:203–254
18. Coughlin SR (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost 3:1800–1814
19. DI Cera E (2007) Thrombin as procoagulant and anticoagulant. J Thromb Haemost 5:196–202
20. Van De Locht A, Stubbs MT, Bauer M, Bode W (1996) Crystallographic evidence that the F2 kringle catalytic domain linker of prothrombin does not cover the fibrinogen recognition exosite. J Biol Chem 271(7):3413–3416
21. Bowman LJ, Anderson CD, Chapman WC (2010) Topical recombinant human thrombin in surgical hemostasis. Semin Thromb Hemost 36:477–484
22. Esmon CT (2006) The endothelial protein C receptor. Curr Opin Hematol 13:382–385
23. Kisiel W (1979) Human plasma protein C: isolation, characterization, and mechanism of activation by alpha-thrombin. J Clin Invest 64:761–769
24. Esmon CT (2006) Inflammation and the activated protein C anticoagulant pathway. Semin Thromb Hemost 32(Suppl 1):49–60
25. Riewald M, Petrovan RJ, Donner A et al (2002) Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science 296:1880–1882
26. Joyce DE, Gelbert L, Ciaccia A et al (2001) Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 276:11199–11203
27. Nakanishi-Matsui M, Zheng YW, Sulciner DJ et al (2000) PAR3 is a cofactor for PAR4 activation by thrombin. Nature 404:609–613
28. Yang XV, Banerjee Y, Fernández JA et al (2009) Activated protein C ligation of ApoER2 (LRP8) causes Dab1-dependent signaling in U937 cells. Proc Natl Acad Sci U S A 106:274–279
29. Elphick GF, Sarangi PP, Hyun Y-M et al (2009) Recombinant human activated protein C inhibits integrin-mediated neutrophil migration. Blood 113:4078–4085
30. Bhana N, Spencer CM (2000) Lanoteplase. BioDrugs 13:217–224
31. Borgoño CA, Diamandis EP (2004) The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer 4:876–890
32. Gabriel D, Zuluaga MF, van den Bergh H et al (2011) It is all about proteases: from drug delivery to in vivo imaging and photomedicine. Curr Med Chem 18:1785–1805

33. Cleutjens KB, van Eekelen CC, van der Korput HA et al (1996) Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J Biol Chem 271:6379–6388
34. Yousef GM, Diamandis EP (2001) The new human tissue kallikrein gene family: structure, function, and association to disease. Endocr Rev 22:184–204
35. López-Otín C, Matrisian LM (2007) Emerging roles of proteases in tumor suppression. Nat Rev Cancer 7:800–808
36. Réhault S, Monget P, Mazerbourg S et al (2001) Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hK2 and hK3. Eur J Biochem 268:2960–2968
37. Takayama TK, McMullen BA, Nelson PS et al (2001) Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. Biochemistry 40:15341–15348
38. Pepper MS (2001) Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 21:1104–1117
39. Fortier AH, Nelson BJ, Grella DK, Holaday JW (1999) Antiangiogenic activity of prostate-specific antigen. J Natl Cancer Inst 91:1635–1640
40. Desrivières S, Lu H, Peyri N et al (1993) Activation of the 92 kDa type IV collagenase by tissue kallikrein. J Cell Physiol 157:587–593
41. Foekens JA, Peters HA, Look MP et al (2000) The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. Cancer Res 60:636–643
42. Duffy MJ, Maguire TM, McDermott EW, O'Higgins N (1999) Urokinase plasminogen activator: a prognostic marker in multiple types of cancer. J Surg Oncol 71:130–135
43. Miyake H, Hara I, Yamanaka K et al (1999) Elevation of serum levels of urokinase-type plasminogen activator and its receptor is associated with disease progression and prognosis in patients with prostate cancer. Prostate 39:123–129
44. Stephens RW, Nielsen HJ, Christensen IJ et al (1999) Plasma urokinase receptor levels in patients with colorectal cancer: relationship to prognosis. J Natl Cancer Inst 91:869–874
45. Morris DR, Ding Y, Ricks TK et al (2006) Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells. Cancer Res 66:307–314
46. Ovaere P, Lippens S, Vandenabeele P, Declercq W (2009) The emerging roles of serine protease cascades in the epidermis. Trends Biochem Sci 34:453–463
47. Sandilands A, O'Regan GM, Liao H, Zhao Y, Terron-Kwiatkowski A, Watson RM, Cassidy AJ, Goudie DR, Smith FJ, McLean WH, Irvine AD (2006) Prevalent and rare mutations in the gene encoding filaggrin cause ichthyosis vulgaris and predispose individuals to atopic dermatitis. J Invest Dermatol 126:1770–1775
48. Netzel-Arnett S, Currie BM, Szabo R et al (2006) Evidence for a matriptase-prostasin proteolytic cascade regulating terminal epidermal differentiation. J Biol Chem 281:32941–32945
49. Bugge TH, List K, Szabo R (2007) Matriptase-dependent cell surface proteolysis in epithelial development and pathogenesis. Front Biosci 12:5060–5070
50. Alef T, Torres S, Hausser I et al (2009) Ichthyosis, follicular atrophoderma, and hypotrichosis caused by mutations in ST14 is associated with impaired profilaggrin processing. J Invest Dermatol 129:862–869
51. Tseng I-C, Xu H, Chou F-P et al (2010) Matriptase activation, an early cellular response to acidosis. J Biol Chem 285:3261–3270. doi:10.1074/jbc.M109.055640
52. Chen Y-W, Wang J-K, Chou F-P et al (2010) Regulation of the matriptase-prostasin cell surface proteolytic cascade by hepatocyte growth factor activator inhibitor-1 during epidermal differentiation. J Biol Chem 285:31755–31762. doi:10.1074/jbc.M110.150367

53. Sales KU, Masedunskas A, Bey AL et al (2010) Matriptase initiates activation of epidermal pro-kallikrein and disease onset in a mouse model of Netherton syndrome. Nat Genet 42:676–683
54. Borgoño CA, Michael IP, Komatsu N et al (2007) A potential role for multiple tissue kallikrein serine proteases in epidermal desquamation. J Biol Chem 282:3640–3652
55. Lundwall A, Brattsand M (2008) Kallikrein-related peptidases. Cell Mol Life Sci 65:2019–2038
56. Eissa A, Amodeo V, Smith CR, Diamandis EP (2011) Kallikrein-related peptidase-8 (KLK8) is an active serine protease in human epidermis and sweat and is involved in a skin barrier proteolytic cascade. J Biol Chem 286:687–706
57. Lundström A, Egelrud T (1988) Cell shedding from human plantar skin in vitro: evidence of its dependence on endogenous proteolysis. J Invest Dermatol 91:340–343
58. Faurschou M, Borregaard N (2003) Neutrophil granules and secretory vesicles in inflammation. Microbes Infect 5:1317–1327
59. Brinkmann V, Reichard U, Goosmann C et al (2004) Neutrophil extracellular traps kill bacteria. Science 303:1532–1535
60. Wiedow O, Wiese F, Streit V, Kalm CCE (1992) Lesional elastase activity in psoriasis, contact dermatitis, and atopic dermatitis. J Invest Dermatol 99:306–309
61. Meyer-Hoffert U, Wiedow O (2011) Neutrophil serine proteases: mediators of innate immune responses. Curr Opin Hematol 18:19–24
62. Turgeon VL, Houenou LJ (1997) The role of thrombin-like (serine) proteases in the development, plasticity and pathology of the nervous system. Brain Res Rev 25:85–95
63. Turgeon VL, Salman N, Houenou LJ et al (2000) Thrombin. Thromb Res 99:417–427
64. de La Houssaye BA, Mikule K, Nikolic D, Pfenninger KH (1999) Thrombin-induced growth cone collapse: involvement of phospholipase A(2) and eicosanoid generation. J Neurosci 19:10843–10855
65. Gill JS, Pitts K, Rusnak FM et al (1998) Thrombin induced inhibition of neurite outgrowth from dorsal root ganglion neurons. Brain Res 797:321–327
66. Rohatgi T, Sedehizade F, Reymann KG, Reiser G (2004) Protease-activated receptors in neuronal development, neurodegeneration, and neuroprotection: thrombin as signaling molecule in the brain. Neuroscientist 10:501–512
67. Chávez-Galán L, Arenas-Del Angel MC, Zenteno E et al (2009) Cell death mechanisms induced by cytotoxic lymphocytes. Cell Mol Immunol 6:15–25
68. Mhatre M, Nguyen A, Kashani S et al (2004) Thrombin, a mediator of neurotoxicity and memory impairment. Neurobiol Aging 25:783–793
69. Lochner JE, Honigman LS, Grant WF et al (2006) Activity-dependent release of tissue plasminogen activator from the dendritic spines of hippocampal neurons revealed by live-cell imaging. J Neurobiol 66:564–577
70. Samson AL, Medcalf RL, Baranes D et al (2006) Tissue-type plasminogen activator: a multifaceted modulator of neurotransmission and synaptic plasticity. Neuron 50:673–678
71. Samson AL, Nevin ST, Croucher D et al (2008) Tissue-type plasminogen activator requires a co-receptor to enhance NMDA receptor function. J Neurochem 107:1091–1101
72. Cullen SP, Martin SJ (2008) Mechanisms of granule-dependent killing. Cell Death Differ 15:251–262
73. Bratke K, Kuepper M, Bade B et al (2005) Differential expression of human granzymes A, B, and K in natural killer cells and during $CD8^+$ T cell differentiation in peripheral blood. Eur J Immunol 35:2608–2616
74. Bade B, Lohrmann J, ten Brinke A et al (2005) Detection of soluble human granzyme K in vitro and in vivo. Eur J Immunol 35:2940–2948
75. Grossman WJ, Verbsky JW, Barchet W et al (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 21:589–601
76. Romero V, Andrade F (2008) Non-apoptotic functions of granzymes. Tissue Antigens 71:409–416

77. Grassi M, Capello F, Bertolino L et al (2009) Identification of granzyme B-expressing CD-8-positive T cells in lymphocytic inflammatory infiltrate in cutaneous lupus erythematosus and in dermatomyositis. Clin Exp Dermatol 34:910–914
78. Méthot N, Rubin J, Guay D et al (2007) Inhibition of the activation of multiple serine proteases with a cathepsin C inhibitor requires sustained exposure to prevent pro-enzyme processing. J Biol Chem 282:20836–20846
79. Nathan C (2006) Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 6:173–182
80. Langhorst J, Elsenbruch S, Koelzer J et al (2008) Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. Am J Gastroenterol 103:162–169
81. Tzortzaki EG, Lambiri I, Vlachaki E, Siafakas NM (2007) Biomarkers in COPD. Curr Med Chem 14:1037–1048
82. Roghanian A, Sallenave J-M (2008) Neutrophil elastase (NE) and NE inhibitors: canonical and noncanonical functions in lung chronic inflammatory diseases (cystic fibrosis and chronic obstructive pulmonary disease). J Aerosol Med Pulm Drug Deliv 21:125–144
83. Cooley J, McDonald B, Accurso FJ et al (2008) Patterns of neutrophil serine protease-dependent cleavage of surfactant protein D in inflammatory lung disease. J Leukoc Biol 83:946–955
84. Döring G, Frank F, Boudier C et al (1995) Cleavage of lymphocyte surface antigens CD2, CD4, and CD8 by polymorphonuclear leukocyte elastase and cathepsin G in patients with cystic fibrosis. J Immunol 154:4842–4850
85. Kallenberg CG (2008) Pathogenesis of PR3-ANCA associated vasculitis. J Autoimmun 30:29–36
86. Pejler G, Åbrink M, Ringvall M, Wernersson S (2007) Mast cell proteases. Adv Immunol 95:167–255
87. Sakai K, Ren S, Schwartz LB (1996) A novel heparin-dependent processing pathway for human tryptase. Autocatalysis followed by activation with dipeptidyl peptidase I. J Clin Invest 97:988–995
88. Strik MCM, de Koning PJA, Kleijmeer MJ et al (2007) Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation. Mol Immunol 44:3462–3472
89. Kovanen PT (2007) Mast cells: multipotent local effector cells in atherothrombosis. Immunol Rev 217:105–122
90. Schwartz LB, Bradford TR, Rouse C et al (1994) Development of a new, more sensitive immunoassay for human tryptase: use in systemic anaphylaxis. J Clin Immunol 14:190–204
91. Hartveit F, Thoresen S, Tangen M, Maartmann-Moe H (1984) Mast cell changes and tumor dissemination in human breast carcinoma. Invasion Metastasis 4:146–155
92. Blank U, Essig M, Scandiuzzi L et al (2007) Mast cells and inflammatory kidney disease. Immunol Rev 217:79–95
93. Frungieri MB, Weidinger S, Meineke V et al (2002) Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPAR gamma: possible relevance to human fibrotic disorders. Proc Natl Acad Sci U S A 99:15072–15077
94. Erin EM, Leaker BR, Zacharasiewicz A et al (2006) Effects of a reversible beta-tryptase and trypsin inhibitor (RWJ-58643) on nasal allergic responses. Clin Exp Allergy 36:458–464
95. Fermi C, Pernossi L (1894) Ueber die Enzyme. Zeitschrift für Hyg und Infekt 18:83–127
96. Huntington JA, Read RJ, Carrell RW (2000) Structure of a serpin-protease complex shows inhibition by deformation. Nature 407:923–926
97. Vijaya Rachel K, Sirisha Gandreddi V D (2014) A review of protease inhibitors from different sources. Int J Appl Phys Bio-Chem Res 4:1–18
98. Law RHP, Zhang Q, McGowan S et al (2006) An overview of the serpin superfamily. Genome Biol 7:216

99. Rawlings ND (2010) Peptidase inhibitors in the MEROPS database. Biochimie 92:1463–1483
100. Potempa J, Korzus E, Travis J (1994) The serpin superfamily of proteinase inhibitors: structure, function, and regulation. J Biol Chem 269:15957–15960
101. van Gent D, Sharp P, Morgan K, Kalsheker N (2003) Serpins: structure, function and molecular evolution. Int J Biochem Cell Biol 35:1536–1547
102. Carrell RW, Pemberton PA, Boswell DR (1987) The serpins: evolution and adaptation in a family of protease inhibitors. Cold Spring Harb Symp Quant Biol 52:527–535
103. Heit C, Jackson BC, McAndrews M et al (2013) Update of the human and mouse serpin gene superfamily. Hum Genomics 7:22
104. Irving JA, Pike RN, Lesk AM, Whisstock JC (2000) Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res 10:1845–1864
105. Ragg H, Lokot T, Kamp PB et al (2001) Vertebrate serpins: construction of a conflict-free phylogeny by combining exon-intron and diagnostic site analyses. Mol Biol Evol 18:577–584
106. Silverman GA, Bird PI, Carrell RW et al (2001) The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem 276:33293–33296
107. Kumar A, Ragg H, Silverman G et al (2008) Ancestry and evolution of a secretory pathway serpin. BMC Evol Biol 8:250
108. Ray CA, Black RA, Kronheim SR et al (1992) Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. Cell 69:597–604
109. Irving JA, Pike RN, Dai W et al (2002) Evidence that serpin architecture intrinsically supports papain-like cysteine protease inhibition: engineering alpha(1)-antitrypsin to inhibit cathepsin proteases. Biochemistry 41:4998–5004
110. Nagata K (1996) Hsp47: a collagen-specific molecular chaperone. Trends Biochem Sci 21:22–26
111. Pemberton PA, Stein PE, Pepys MB et al (1988) Hormone binding globulins undergo serpin conformational change in inflammation. Nature 336:257–258
112. Zou Z, Anisowicz A, Hendrix MJ et al (1994) Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science 263:526–529
113. Nakashima T, Pak SC, Silverman GA et al (2000) Genomic cloning, mapping, structure and promoter analysis of HEADPIN, a serpin which is down-regulated in head and neck cancer cells. Biochim Biophys Acta 1492:441–446
114. Hedstrom L (2002) Serine protease mechanism and specificity. Chem Rev 102:4501–4524
115. Abbenante G, Fairlie DP (2005) Protease inhibitors in the clinic. Med Chem (Shāriqah (United Arab Emirates)) 1:71–104
116. Page MJ, Cera E Di, Page MJ, Di Cera E (2008) Serine proteases and serine protease inhibitors. Wiley Encycl Chem Biol
117. Travis J, Salvesen GS (1983) Human plasma proteinase inhibitors. Annu Rev Biochem 52:655–709
118. Ansari MJ (2015) Role of protease inhibitors in Insulin therapy of Diabetes: are these beneficial? Bull Environ Pharmacol Life Sci 4:01–08
119. Ohtomo S, Nangaku M, Izuhara Y et al (2008) The role of megsin, a serine protease inhibitor, in diabetic mesangial matrix accumulation. Kidney Int 74:768–774
120. Hida K, Wada J, Eguchi J et al (2005) Visceral adipose tissue-derived serine protease inhibitor: a unique insulin-sensitizing adipocytokine in obesity. Proc Natl Acad Sci 102:10610–10615
121. Molinari F, Meskanaite V, Munnich A et al (2003) Extracellular proteases and their inhibitors in genetic diseases of the central nervous system. Hum Mol Genet R195–R200

122. Schalkwijk J, Wiedow O, Hirose S (1999) The trappin gene family: proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core. Biochem J 340:569–77. doi:10.1042/bj3400569
123. Wiedow O, Schröder JM, Gregory H et al (1990) Elafin: an elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence. J Biol Chem 265:14791–14795
124. Ranganathan S, Mukhopadhyay T (2010) Dandruff: the most commercially exploited skin disease. Indian J Dermatol 55:130
125. Rachel KV, Vimala Y, Apta Chaitanya D (2013) A trypsin inhibitor-SNTI with antidandruff activity from Sapindus trifoliatus. Indian J Appl Res 3:3–5
126. Yamaya M, Shimotai Y, Hatachi Y, Morio H, Nishimura H (2016) Serine proteases and their inhibitors in human airway epithelial cells: effects on influenza virus replication and airway serine proteases and their inhibitors in human airway epithelial cells: effects on influenza virus replication and airway inflammation. Clin Microbiol 5:1–10
127. Zheng D, Chen H, Davids J et al (2013) Serpins for diagnosis and therapy in cancer. Cardiovasc Hematol Disord Drug Targets 13:123–132

Metalloproteases and Human Diseases: The Astacin Family

11

Sibani Chakraborty, Asim K. Bera, Ankur Chaudhuri and Satyajit Sen

Abstract

Astacins are a group of zinc metalloproteases characterized by a signature sequence of 18 amino acid residues (HExxHxxGxxHxxxRxDR) and a conserved SXMHY domain containing the Met-turn characteristic of the metzincins. They are synthesized as inactive zymogens whereby they are activated after the post-translational removal of the amino-terminal pro-segments. Astacins are widely spread in bacteria and throughout animal kingdom. They are multi-domain, glycosylated complex endopeptidases which may be membrane-bound or secretory in nature. The astacins are known to play diverse role in bone morphogenesis, tissue differentiation, wound healing, digestion and in diseases like fibrosis, cancer, neurodegenerative and Alzheimer's disease. The homologous protease domain containing about 200 residues and two conserved disulphide bridges present in most astacins is the common thread linking all the members of this family. Though similar in structure the astacins differ widely in their substrate specificity. In humans, six astacins have been reported. They are meprin-α and-β, bone morphogenetic protein-1 (BMP-1) with its major splice variant mammalian

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tolloid, mammalian tolloid-like enzymes and ovastacin. Among them meprins α and β and bone morphogenetic protein1 have been found to be expressed at elevated level in certain diseases and hence important druggable targets. Several small molecule inhibitors and macromolecular inhibitors are known to control the activity of the astacins thereby contributing to the control of several diseases.

Keywords
Astacins · Subsites · Meprins · Bone morphogenetic protein1 · Inhibitors

1 Introduction

The astacin family represents a group of zinc metalloproteases having a unique 18 amino acid signature sequence (HExxHxxGxxHxxxRxDR) which is a part of an approximately 200 amino acid protease/catalytic domain. The astacins are a subfamily of the metzincin superfamily of metalloproteases. All astacins contain a catalytic domain with an amino acid sequence similarity of 29–99%. The prototype of the astacin family was first isolated as digestive enzyme from the fresh water crayfish *Astacus astacus* [1]. Structural studies indicate that the zinc atom is penta-coordinately bound to all the astacins except in bone morphogenetic protein-1 (BMP-1), where the tyrosine residue does not participate in zinc binding to the active site cleft of the protease domain. The astacins are structurally similar to other members of the metalloprotease family, i.e., serralysins, matrixins and snake-venom metalloproteases but distinctly differ from the thermolysin-like metalloproteases. The astacins are complex, glycosylated, multi-domain proteins which may be membrane-bound or secretory in nature and are expressed in tissue and species-specific manner in mature organisms and in a temporal and spatial specific manner in developmental systems. They have been identified to perform a various role in mature and developmental systems such as bone morphogenesis, tissue differentiation, hatching process, digestive function as well as several disease including cancer [2]. Eukaryotic members contain a pre- and pro-domain sequence at the amino-terminal and one or two copies of the epidermal growth factor (EGF)-like domains and complement-like domains (Clr/Cls) at the carboxy-terminal whereas prokaryotic member contains only the protease domain. These metalloprotease have been detected in animal kingdom as well as in bacteria.

Meprins and bone morphogenetic protein-1 (BMP-1) belonging to the astacin family are responsible for several diseases in humans. Meprins reported in vertebrates are found in small intestine, skin and kidney where they proteolytically degrade biologically active peptides such as cytokines and different constituents of the extracellular matrix [2]. BMP-1/tolloid-like proteinases (BTPs) have been detected in species from *Drosophila* to human. Procollagen C-proteinase or bone morphogenetic protein-1 of the astacin family cleaves procollagen and non-collagen

substrates into the insoluble fibrillar collagen. It also processes prolysyl oxidase to its active form which is responsible for catalyzing the cross-linking of collagen fibrils thereby contributing to the structural stability of collagen [3]. This chapter reviews the recent advances on the structure, function and diverse role of the disease causing metalloproteases of the astacin family. Special attention will be focused on discussing the meprins and bone morphogenetic protein-1 (BMP-1) as these metalloproteases of the astacin family are important therapeutic targets for combating several diseases in human. Astacin family is widely spread in bacteria and throughout animal kingdom. Table 1 describes the different members of the astacin family reported so far in animal kingdom.

The astacin family ranges in molecular weight of 23–110 kDa. The smallest is astacin from fresh water crayfish *A. astacus* and the largest is meprin β-subunit. The common thread linking all members of the astacin family is the homologous protease domain comprising of about 200 amino acid residues. The domain

Table 1 Members of the astacin family in animal kingdom

Protease	Species/tissue	UniProt ID	Total length	Length of protease domain	Identity (%)
HCE1	Fish hatching gland cells	P31580	270	71–270 (200)	34.8
HCE2	Fish hatching gland cells	P31581	279	80–279 (200)	36.2
LCE	Fish hatching gland cells	P31579	271	72–271 (200)	36.4
Humepα	Kidney, intestine, embryo	Q16819	746	66–263 (198)	30.5
Humepβ	Kidney, intestine, embryo	Q16820	701	64–259 (196)	36.1
Mumepα	Kidney, intestine, embryo	P28825	747	65–262 (198)	30.5
Mumepβ	Kidney, intestine, embryo	Q61847	704	63–260 (198)	34.5
QuCAM1	Quail embryos	P42662	310	1–183 (184)	36.9
HMP1	Hydra tentacles	Q25174	285	52–241 (190)	33.2
Hubmp1	Embryos, tissues	P13497	986	121–321 (201)	35.3
Mubmp1	Mouse embryos	P98063	991	126–326 (201)	35.3
Xebmp1	Frog embryos	P98070	707	84–284 (201)	35.8
Tll1	Embryos, tissues	O43897	1031	148–348 (201)	37.1
Tll2	Embryos, tissues	Q9Y6L7	866	150–350 (201)	36.6

Note Sequence identity of protease domain with astacin
HCE High choriolytic enzyme; *LCE* low choriolytic enzyme; *Hu* human; *Mu* mouse; *Xe* Xenopus; *QuCAM1* quail chorioallantoic membrane-1; *HMP* hydra metalloprotease-1; *BMP-1* bone morphogenetic protein-1; *Tll1* tolloid-like 1; *Tll2* tolloid-like 2

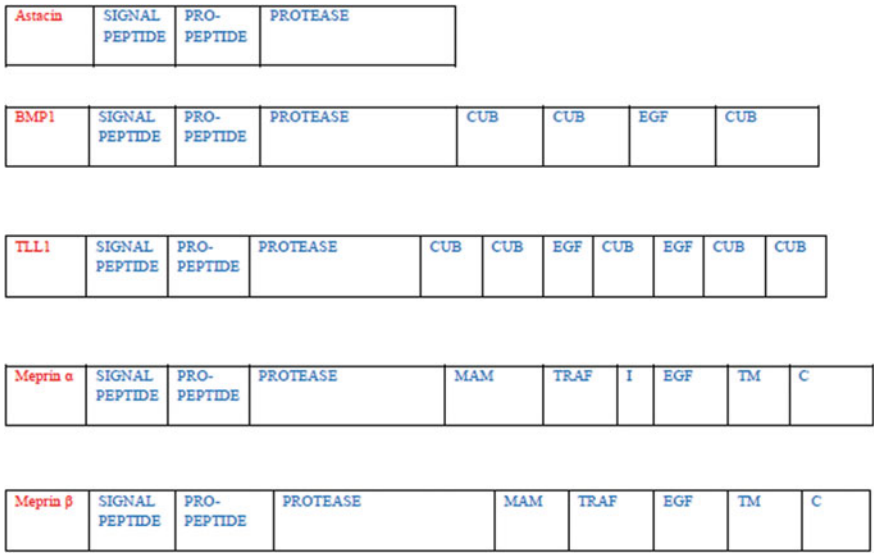

Fig. 1 Domain structure of astacins. N-terminal signal peptide, pro-peptide, catalytic protease domain, EGF (epidermal growth factor-like), CUB (embryonic sea urchin Uegf, and BMP1), MAM (meprin, A5 receptor protein, tyrosine phosphatase μ), TRAF (intracellular signaling proteins), C (cytosolic domain), I (inserted domain), and TM (transmembrane domain)

composition of some known astacin family members is shown in Fig. 1. Unique zinc-binding motif and presence of an unusually conserved methionine refers astacins as metzincins. In humans six astacins are known, namely meprin-α and -β [1], bone morphogenetic protein-1 (BMP-1) with its major splice variant mammalian tolloid, mammalian tolloid-like enzymes [4] and ovastacin [5]. Their diverse role in embryogenesis, bone morphogenesis, digestion, tissue differentiation and also in certain diseases like cancer, renal and neurodegenerative diseases identify them as important therapeutic targets. Thus regulating activity of astacins by specific inhibition may be a suitable approach in the treatment of certain pathological conditions.

2 Structure of the Protease Domain of Astacin Family Members

The common thread linking different members of the astacin family is the homologous catalytic domain consisting of approximately 200 amino acid residues (Fig. 2). This domain in most astacins contain four conserved cysteine residues contributing to two disulphide bridges Cys^{42}–Cys^{198} and Cys^{64}–Cys^{84} imparting stability to the proteolytic unit. BMP-1 contains an additional disulphide bond

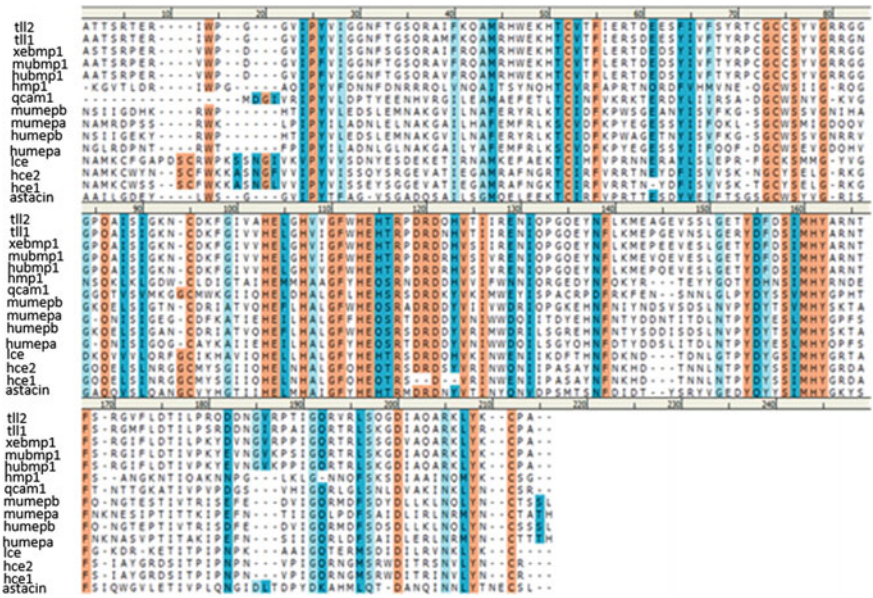

Fig. 2 Multiple sequence alignment of protease domain of represented astacin family members. Identical (conserved), strong and weak residues represented as *orange*, *blue* and *cyan* color respectively. The abbreviation for species are the same as used in Table 1

between Cys^{62} and Cys^{65}. The catalytic unit is compact kidney-shaped ellipsoid with dimensions of about 55 × 45 × 35 Å. The active site cleft sub-divided the catalytic domain into the amino-terminal and carboxy-terminal domains of the protein. The deep active site cleft is buried within the two domains in the structure.

2.1 Primary Structure

The amino acid sequences of all members of the astacin family except for the digestive enzyme astacin from *A. astacus* were deduced from their cDNA sequences [6]. All eukaryotic members of the astacin family contain the 18 amino acid signature sequence extending from His^{92} to Arg^{109}. In addition to the signature sequence, they also contain the SXMHY sequence, Ser^{145} to Tyr^{149} which is also conserved as in metzincins [7]. Each of the eukaryotic astacins contain two disulphide bridges [8] comprising of four cysteine residues, Cys^{42}–Cys^{198} and Cys^{64}–Cys^{84} respectively (All residue numbering as in astacin). The prokaryotic astacin isolated from *Flavobacterium meningosepticum* called flavastacin differ from the eukaryotic members in that it lacks the four conserved cysteine residues. In addition the conserved SXMHY region also contains Met in place of His. Presence

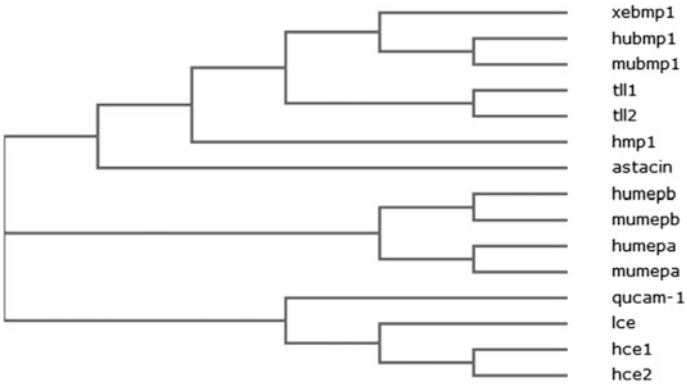

Fig. 3 Phylogenetic tree based on the sequence alignment of the protease domain of astacins

of Ile instead of Phe in the signature sequence also distinguishes the prokaryotic astacin from its eukaryotic members [9]. The phylogenetic tree constructed based on the sequence alignment of protease domains has three major branches. The fish enzymes (hce1, hce2 and lce) and qcam-1 from quail form one branch, meprins form other branch and bmp1, tolloid-like enzymes and hmp1 form another branch (Fig. 3).

2.2 X-ray Structure

The first solved structure of the protease domain is that of the digestive enzyme astacin from fresh water crayfish *A. astacus* at 1.8 Å resolution [10, 11]. A kidney-shaped structure with active site located in a deep cleft has been observed (Fig. 4). The NH_2-terminal domain contains a five-stranded pleated β-sheet as well as two extended α-helices. The COOH-terminal domain is mainly organized in irregular turns but contains a large number of hydrophobic residues important for folding. The zinc atom is penta-coordinately connected to three His residues (His^{92}, His^{96} and His^{102}) present in the signature sequence. The fourth and fifth coordination is with Tyr^{149} and a water molecule bound to the carboxylate side chain of Glu^{93}. The four amino acids which participate in coordination at the active site of astacin with catalytic zinc are conserved in BMP-1. The catalytic zinc of BMP-1 is bound with His^{92}, His^{96}, and His^{102} but not to Tyr^{149} in contrast to astacin [12]. Astacins contain two to three disulphide bridges in their catalytic domain. Most common to all structures are the two disulphide bonds between Cys^{42} and Cys^{198} and between Cys^{64} and Cys^{84}. BMP-1 has an additional disulphide bridge between Cys^{62} and Cys^{65}.

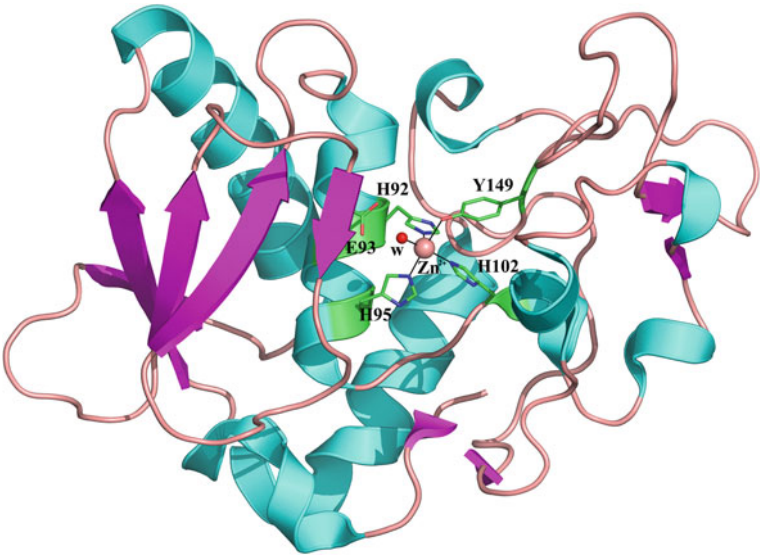

Fig. 4 X-ray structure of astacin from fresh water crayfish *A. astacus*. The active site zinc ion (*orange*) of astacin is trigonal bipyramidally coordinated by three histidine residues, a water molecule (*red*) and a tyrosine residue. His^{92}, His^{96}, His^{102} and the water-linked Glu^{93} are part of the consensus sequence HExxHxxGxxH. Tyr^{149} is located in the conserved Met-turn motif (SXMHY)

3 Substrate Specificity

Chemical reactants that bind to a specific enzyme are termed substrates. There may be more than one substrate for each type of enzyme, depending on the particular chemical reaction. The enzyme's active site binds to the substrate and the binding or active site is composed of a unique combination of amino acid residues. The positions, sequences, structures and properties of the amino acids create a specific chemical environment at the binding site. A specific chemical substrate binds to the active site in an enzyme-specific manner. This particular property of an enzyme makes them very important as research and diagnostic tools. Astacins are Zn^{+2} dependent metalloproteinases though they exhibit arylamidase activity, which is not typical for metalloproteinases. Substrate specificity and domain composition of the astacin family enzymes are more variable than other thermolysin or matrix metalloproteinase families. Substrate specificities of the astacin super family differ especially with respect to P′-site of substrate molecule. Moreover, specificity of metalloproteinase binding to substrate or inhibitor is determined by specific sequence of S′-site of the enzyme though proteolytic domains share structural

similarity throughout the family. This possibly prevents enzyme inhibition by reaction product containing free carboxyl group, which can bind zinc. Blasting available astacins sequences with meprin-α revealed at least seven residues form a wide substrate-binding core. We have seen both broad and narrow substrate specificity in this family. Extreme examples are crab *Paralithodes camtschatica* metalloproteinase [13] and flavastacin [14] respectively. Flavastacin cleaves only Xaa-Asp bonds and does not cleave peptide bonds formed by amino groups of other amino acids including asparagine and glutamine, though it is less active in hydrolysis of Xaa-Glu bonds. Another super-specific substrate is dimethylaminonaphthalene sulfonyl-PKFAPWV for crayfish astacin and it homologs. Only F-A is cleavable by these enzymes, which differentiate them from matrix metalloproteinases. Astacin prefers alanine at the P1′ position over other short aliphatic side chains (i.e. serine, threonine and glycine). Human meprin-α subunit forms bond with aromatic amino acid at P1′-position is determined by interaction of substrate with Tyr-199, whereas preference of β-subunit for Asp or Glu is determined by substrate interaction with the Lys-185 residue. β-meprin is characterized by affinity for substrates containing Glu residues at positions from P2 to P2′. Mouse kidney meprin-α cleaves arginine, lysine or phenylalanine containing substrate at P1′ site more than 10^3 times faster than astacin. Literature suggests substrate specificity of astacin and meprin is possibly determined by Pro-176 of astacin molecule, which causes substantial steric hindrances for binding of residues containing larger side chain. Pro-176 is substituted by Gly-176 in meprin and forms a deep cleft. In meprin, Leu-178 and Tyr-149 is involved into substrate binding in absence of Tyr at 177th position as in astacin structure [10]. Phenylalanine at 165th position is crucial for binding of P2′ amino acid residue of astacin substrates. Binding of proline residue localized at P3 of astacin substrates involves interaction with Trp and Tyr residues. Tyr-177 and/or Trp-158 side chains of astacin molecule favor binding of aromatic amino acid residues at P3′. Absence of Tyr at the 177th position and substitution of Trp to Asn at 158th position in meprin is observed. Amino acid substitutions at P1′ and P1-position influence the kinetic property of meprin-α. BMP-1 substrates have an [conserved] aspartic acid residue at P1′ subsite. Analysis of crystal structure has reported an arginine at the S1′ site of BMP-1. A bulky vicinal disulphide contributes to a restricted S1 pocket, explaining the preference of small aliphatic residues in substrate P1 positions [4, 12]. Lysines (87 and 176) are involved in substrate recognition and binding for all BMP-1 proteinases. The loop containing Lys-176 at S1′ subsite has been found only in astacin proteinases related to the BMP-1 and its homologues [15]. Though we talked about residues at the core substrate-binding pocket, moreover, substitutions of specific residues located distantly from cleavable bond also influence binding and hydrolysis of substrates, thus suggesting formation of an extended system of non-covalent interactions (Table 2).

Table 2 Amino acid preference for specificity in astacin family

Enzyme	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'
Astacin		P	K, R, N, Y	K, R, N, Y	A	P	HP[a]	HP[a]
Meprin	A, V	A, S, T	V, S, P	E, Y, N, L, R	E, Q, T, V, S	E	T	S, V
BMP-1	S, A, F	Y, M, A	Y, M, Q	G, A, D, S	D, Q	D, E, V	A, M	G, D
Nephrosin	P	P	P	F				
Fundulus Choriolysin			P	A				
Choriolysin H			V	Y				

[a]*HP* Hydrophobic

4 Role of Metal Ions

Astacin family proteases require Zn^{2+} for their catalysis. Zn^{2+} ion's coordination with active site residues is essential for catalysis. Zn^{2+} ion preference over other divalent ion is due to its tolerance to oxidants and reductants present in the organism, which provide the possibility for interaction of this cation with ligands of various nature. Crayfish astacin, meprins, choriolysins, and BP10 proteinase from the *Paracentrotus lividus* sea urchin were active after substitution of active site zinc cation by copper or cobalt cation. Some enzymes of the astacin family may additionally bind cations stabilizing structure and/or acting as effectors of the enzymatic reaction [16]. Meprin-α and choriolysins contain calcium ions other than Zn^{2+} to maintain structure. Thermolysin family as well as matrix metalloproteinases require calcium ion for structural stabilization. Interestingly crayfish astacins do not require any extra ion to stabilize the protein structure; disulfide bonds do the job [17]. Disulfide bonds are formed by conserved cysteine residues, and suggest their important role in stabilization of tertiary structure of other astacin proteinases. The only exception is flavastacin [14].

Proteolytic activity of some astacin proteinases depends on Ca^{2+} concentration. BMP-1 and mTld as well as related sea urchin BP10 proteinases cleave substrates more effectively in the presence of Ca^{2+}. Activity of mTLL belonging to the same subfamily of astacin proteinases does not depend on Ca^{2+} ion. Human meprin activity decreases if Ca^{2+} concentration exceeds 1 mM. Mutant BMP-1 and mTld lacking EGF-domains (which may bind Ca^{2+}) are also calcium-dependent enzymes. BMP-1 is more sensitive to inhibition by zinc salts: 0.1 mM $ZnCl_2$ caused almost total inhibition of this enzyme. Cadmium and copper chlorides are highly effective inhibitors of BMP-1. Zinc salts (1–500 μM) activate *T. spiralis* metalloproteinase. More than 1 mM Zn^{2+} salts inhibit human meprin and *T. spiralis* metalloproteinase.

5 Biological Roles of Astacins

Astacins are involved into various physiological processes including digestion, processing of extracellular proteins, morphogenesis, embryonic hatching. Specifically, astacin proteases are involved in hydrolysis of dietary proteins, extracellular matrix formation, morphogenesis and development of organisms.

5.1 Development and Morphogenesis

Astacins play an important role in several physiological functions which include digestion, hatching, morphogenesis and pattern formation. These metalloproteases exits as either membrane bound or secretary in nature. Therefore, two main subgroups of astacins are reported, one contains secreted enzymes and the other is membrane-bound. The secretary astacins are involved in proteolytic processing which occurs during developmental stage of early invertebrates and vertebrates. They also play an important role in digestion. Involvement of astacins in morphogenesis in cnidarians have also been reported and a meprin-like astacin was found to be involved in the morphogenesis of hydra foot. In addition, meprins play an important role in tissue differentiation and signaling [16].

5.2 Matrix Assembly and Wound Healing

Among the astacins, meprin metalloproteases are important enzymes needed for fibrillar collagen assembly. It has been proved that both meprins-α and meprin-β help in the maturation of procollagen by cleaving of the C- and N-terminal propeptides of collagen I and III. The prteolytic maturation of procollagen is needed to start fibrillar assembly [18–20]. Bone morphogenic protein 1 can cleave off only the C-terminal and ADAMTS proteases can cleave only the N-terminal propeptides of procollagen which is insufficient for collagen assembly [4, 21]. Both meprins on the other hand on incubation with procollagen I led to spontaneous fibril formation as studied by transmission electron microscopy [22]. The process of collagen formation is essential in wound healing as well as it is an important phenomena in the formation of extracellular matrix (ECM).

5.3 Hatching

Development of embryos of many aquatic invertebrates like crustaceans, fishes, amphibians and reptiles require cleaving off of the embryonic shell after a certain stage of embryosis by proteolytic enzymes, thereby releasing the young larva in free ambient water. This process known as “hatching” requires astacin metalloproteases. Some of the astacins in the process include crayfish embryonic astacin [23], low

chorolytic enzymes and high chorolytic enzymes [24] or corresponding proteases of amphibians from the frog *Xenopus laevis* [25]. The hatching process has been extensively studied for the chorolytic enzymes. The high chorolytic enzymes cleave off the zona pellucida proteins at specific sites allowing the envelope to swell. Subsequently the low chorolytic enzymes completely digest the egg envelope [26] releasing the young larva.

5.4 Tissue Differentiation

Meprins play an important for in tissue differentiation and pericellular signaling. A variety of meprin substrates have been reported [27] which include biologically active peptides such as gastrin and cholecystokinin, substance P, cytokines, and chemokines. Astacins in general, are involved in proteolytic cleaving of precursors of extracellular matrix constituents and growth factors including their antagonists and cell surface receptors during embryonic patterning and cell differentiation. In late developmental stage and in the adult, BMP-1, mTLD, and mTLL1 were required for bone formation and connective tissue differentiation, since they are involved in specific activation and trimming of a variety of procollagens.

5.5 Digestion

Astacins with digestive function are mostly found in decapods crustacean species [28, 29]. The first enzyme isolated from the astacin family with digestive property was from the crayfish *A. astacus*. Astacin isolated from the hepatopancreas of the crayfish was found to play an important role in the digestion of the organism.

6 Astacins and Disease

The involvement of astacin metalloproteases in a wide range of diseases in human have been reported. Since meprins and Procollagen C-Proteinase (also known as bone morphogenetic protein 1) have been shown to be responsible for a number of diseases in human being, both of these astacins can be used as important druggable targets.

Meprins, are a sub-group of the astacin family belonging to the "metzincin" superfamily which are confined to vertebrates and found in abundance in the small intestine, kidney and skin, where they are thought to cleave biologically active peptides, cytokines and components of the extracellular matrix [1, 30]. Meprins are oligomeric zinc endopeptidases and exists as two subunits α and/or β [1]. Structurally meprin-α differs from meprin-β in having an inserted element that can be

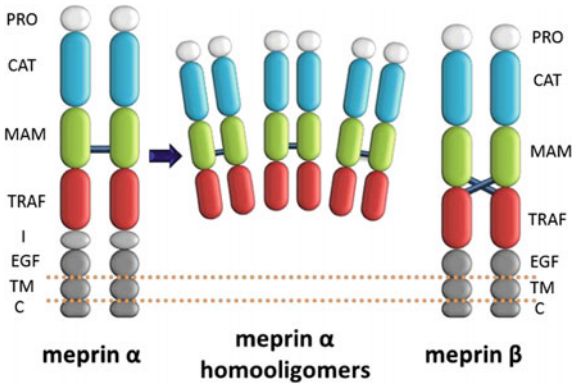

Fig. 5 Domain composition of meprins. Meprin-α and meprin-β are multi-domain enzymes, building dimers linked by one intermolecular disulfide bond between the MAM domains. Meprins are expressed as zymogens with a pro-peptide (PRO) N-terminal to the protease domain (CAT) that must be cleaved off proteolytically to gain full activity. Only meprin-α contains an inserted domain (I) which is cleaved by furin during the secretary pathway, resulting in secretion of the protein and further oligomerization. *C* Cytosolic; *TM* transmembrane

cleaved by furin (Fig. 5). Meprin-α exist as a homo-oligomeric secretory protein or hetero-oligomeric membrane bound α/β protein and is known as the largest secretory protease in nature. Meprin-β is present as a homodimeric membrane bound protein and is secretory in nature when it is present as a α/β heterodimeric structure. Meprins-α and -β participate in the processing and degradation of peptides and proteins at the cell surface. Meprins may also be involved in the remodeling of the extracellular matrix in response to renal injury [31]. Meprin-β dimer exhibits a compact shape whose catalytic domain undergoes major rearrangement upon activation. Meprin-β is involved in the sheddase function of membrane-bound cytokines and growth factors, thereby contributing to inflammatory diseases, angiogenesis and tumor progression [2]. Though meprin-α and-β share 44% sequence identity they exhibit strikingly differences in activation and substrate specificity. They differ in their substrate specificity because of preferences in binding at active site. Meprin-α prefers neutral aliphatic and aromatic side chains in the P1′ position, whereas meprin-β prefers negatively charged amino acid residues [32].

Bone morphogenetic protein 1 (BMP-1), a sub-group within the astacin family co-purified with TGF β-like growth factors, was found in vertebrates as a bone-inducing factor, illustrating the range of physiological functions associated with these proteases. The BMP-1 is also known as the procollagen C-proteinase is a multi-domain, glycosylated, secretory, zinc endopeptidase. Each member of BMP-1 has an astacin-like protease domain, CUB protein–protein interaction domains and EGF motifs [1, 33]. In mammals there are four BMP-1-like proteases: BMP-1 and mTLD (mammalian tolloid), which are alternative spliced products of the same gene [34] and also mTLL-1 and mTLL-2 (mammalian tolloid-like1 and 2) [3, 35].

Structurally BMP-1 is closely related to the digestive enzyme astacin from the crayfish *A. astacus* with a sequence identity of 35%. Crystal structure of BMP-1 indicates it has a deep active site cleft within which is the three conserved His residues binding to the catalytic zinc. But unlike the prototypical protease astacin and other members of the family it differs in that a conserved tyrosine does not participate in zinc binding [12]. Like other astacin-like proteases, BMP-1 consists of a 100 residue N-terminal domain and a C-terminal domain separated by the active site cleft. BMP1s are characterized by a cysteine rich loop comprising residues Pro^{61}–Cys–Gly–Cys–Cys–Ser^{66} which correspond to the β-edge strand of matrix metalloproteases [12]. The N-terminal domain contains a five-stranded β-sheet and two long helices. The C-terminal domain contains few regular secondary structures. The two disulphide bonds present in the other astacin members is conserved in BMP-1 (Cys^{64}–Cys^{84} and Cys^{42}–Cys^{198}) with another additional disulphide bridge between Cys^{62} and Cys^{65}.

6.1 Neurodegenerative and Alzheimer Disease

Amyloid precursor protein is cleaved by meprin β in vivo. The subsequent release of amyloid β peptides is reported to be one of the major causes of Alzheimer's disease [2].

6.2 Keloid Formation and Overgrowth of Fibrous Tissue or Fibrosis

Both the α and β subunits of meprin are responsible for maturation of procollagen III and are overexpressed in fibrotic overgrowth where fibrillar collagen is accumulated in excess. In vertebrates, the BMP-1/Procollagen C-Proteinase catalyzes the cleavage of the solubilizing C-terminal globular domain from procollagen resulting in the formation of insoluble fibrillar collagen responsible for fibrosis. This group of metalloproteinases are important in regulating extracellular matrix (ECM) formation by biosynthetic processing of various precursor proteins into mature functional enzymes, structural proteins, and proteins involved in initiating mineralization of the ECM of hard tissues [36, 37]. The production of extracellular matrix (ECM) is a tightly regulated process required for bone morphogenesis, normal wound healing and repair of bone fractures in adult. Excessive accumulation of ECM, particularly fibrillar collagen results in a variety of chronic fibrotic conditions including pulmonary, renal and liver fibrosis and scleroderma. Inhibition of Procollagen C-Proteinases/BMP-1 may interfere in the progression of fibrosis and are thus important druggable targets as excessive fibrillogenesis of collagen can lead to a number of diseases ranging from keloids, to the formation of surgical adhesions, to deep-seated fibroses of organs.

6.3 Kidney Disease and Inflammatory Bowel Disease

Meprins are normally found in kidneys and intestine. In intestine meprin is activated by the pancreatic enzyme trypsin. Enhanced level of meprins can be seen in kidney damage. Treatment of animals with inhibitor actinonin has been shown to prevent kidney damage. The intestinal mucosa is continuously undergoing damage-inflammation-repair cycle due to toxic peptides and bacteria which involves different inflammatory mediators such as growth factors, cytokines, etc. Meprins can degrade toxic peptide and components of bacteria cell surface and may play a role in this process.

6.4 Cancer

Meprin α and β genes are expressed in various cancer cells. Meprin expression may play a role in tumor cell invasion and migration and in doing so may be involved in tumor progression. In colorectal tumor meprin α subunit is detected into the stroma of the tumor in contrast to normal colon [38]. The role of astacins in fibrosarcoma is yet to be established; however, astacins have been identified to be responsible for overgrowth of fibrous tissue. Recently it has been shown that BMP-1 is involved in modulation of angiogenesis through processing of prolactin and growth hormone [39]. So the role of BMP-1 in genesis of cancer is to be explored.

The involvement of meprins in cancer progression, inflammatory bowel disease and acute/chronic renal disease indicate the potential of meprins as drug targets for therapy with the development of potent and specific inhibitors [38].

7 Inhibitors of Astacins

Astacin family proteinases are insensitive to inhibitors of serine, cysteine and aspartyl proteinases. But metal chelating inhibitors of metalloproteinases from other families may also inhibit astacin proteinases, though inhibition efficiency is determined by the structure of substrate-binding region of these enzymes. Thus inhibitory mechanism depends on particular site of the enzyme molecule involved into interaction with inhibitor. Peptide and protein inhibitors have also been found for some astacin metalloproteinases. Activities of some astacin proteinases are also regulated by endogenous inhibitors.

7.1 Small-Molecular Inhibitors

Small molecule inhibitors like hydroxamates, actinonin, batimastat, galardin, NNGH, TAPI-0, TAPI-2 (tumor necrosis factor alpha protease inhibitors), Ro 32-7315, captopril are potent inhibitors of meprins [40]. Inhibition of Procollagen

C-Proteinases/BMP-1 may interfere in the progression of fibrosis and are thus important druggable targets as excessive fibrillogenesis of collagen can lead to a number of diseases ranging from keloids to the formation of surgical adhesions, to deep-seated fibroses of organs. Some small molecules functioning as inhibitors of PCP have been reported. Small molecule inhibitors such as EDTA, 1,10-phenanthroline [41] *N*-ethylmaleimide (NAM) [42], UK3863 [43] and glutamic acid/diamino acid derived hydroxamates also inhibit BMP-1. Hydroxamate derivatives of several diamino acids were described as potent inhibitors of PCP [44]. As a part of the anti-fibrosis program several non peptidic inhibitors containing different zinc chelating groups like hydroxamates, thiols, carboxylic acids have been designed [45]. It is reported that the hydroxamate bearing scaffolds present as the zinc chelating group as BMP-1 inhibitor has the most potent binding [45]. The hydroxamate inhibitor utilizing a *N*-arylsulphonamide aspartic acid core showed greater potency compared to the initial peptidyl mimetic. Hydroxamate-containing compounds can functions as systemic drugs for other zinc metalloproteases [46–48].

7.2 Macromolecular Inhibitors

Different macromolecular inhibitors inhibit astacins. A plasma protein fetuin-A inhibits the proteolytic activity of both meprins [49]. Interestingly, cystatin C inhibits human meprin-α and not meprin-β [50]. Fetuin-A and cystatin C, members of cystatin superfamily, are both involved in inhibition, thereby pointing to a common role played by both these proteins in the regulation of meprins. This finding is corroborated by a fetuin A homolog from carp, the nephrosin inhibitor which shows inhibition towards both meprin metalloproteases [51]. Hall and co-worker reported that N-terminal of the cystatin C is of major importance for its tight binding with the proteinase [52]. Mannan-binding protein has been reported to inhibit meprins in mice [53], but the inhibitory potential has not yet been reported for the human enzymes [51]. A natural inhibitor of astacin is scavenger α 2-macroglobulin. Modified form of α 2-macroglobulin also shows inhibition for bone morphogenetic protein 1 [54–56]. Nephrosin, a fish astacin in complex with "nephrosin inhibitor," circulates in blood stream of carp [57]. Fish fetuin contains cystatin-like domains similar to fetuin A. The exogenous inhibitor *Xenopus* sizzled tightly binds human BMP-1 (K_i of 1.5 ± 0.5 nM) and is reported to strongly inhibits mTLD and mTLL-1 [58].

8 Conclusion and Future Perspectives

Astacins isolated from different organisms show diverse biological functions and hence studies using this group of metalloproteases are very promising in future studies. They have important roles in pharmaceutical and medicine industry due to

its involvement in several diseases and hence important targets for the drug industry. Astacins have high implication in biotechnology applications and can also be used as bio tools for research purpose.

This chapter has reviewed the diverse roles of astacins on the basis of mechanisms and regulation. The astacin family metalloproteases represent important drug targets in several diseases like cardiovascular disease, inflammatory diseases, neurodegeneration and cancer. While the search to identify potential targets are going on, there are numerous metalloproteases whose substrates and functions remain unknown and which may provide new novel therapeutic opportunities. Knowledge on the intricate interrelationships of metalloproteases and their substrates and inhibitors is yet to reveal.

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References

1. Bond JS, Beynon RB (1995) The astacin family of metalloendopeptidases. Protein Sci 4:1247–1261
2. Claudia B, Christoph B (2013) The metalloproteases meprin alpha and meprin beta: unique enzymes in inflammation, neurodegeneration, cancer and fibrosis. Biochem J 450:253–264
3. Takahara K Brevard R, Hoffman GG, Suzuki N, Greenspan DS (1996) Characterization of a novel gene product (mammalian tolloid-like) with high sequence similarity to mammalian tolloid/bone morphogenetic protein-1. Genomics 34:157–165
4. Hopkins DR, Keles Sand Greenspan DS (2007) The bone morphogenetic protein 1/Tolloidlike metalloproteinases. Matrix Biol 26:508–523
5. Quesada V, Sanchez LM, Alvarez J, Lopez-Otin C (2004) Identification and characterization of human and mouse ovastacin: a novel metalloproteinase similar to hatching enzymes from arthropods, birds, amphibians, and fish. J Biol Chem 279:26627–26634
6. Titani K, Torff HJ, Hormel S, Kumar S, Walsh KA, Rodl J, Neurath H, Zwilling R (1987) Amino acid sequence of a unique protease from the crayfish Asiacus fluviatilis. Biochemistry 26:222–226
7. Jiang W, Bond JS (1992) Families of metalloendopeptidases and their relationships. FEES Lett 312:110–114
8. Stocker W, Gomis-Ruth FX, Bode Wand Zwilling R (1993) Implications of the three-dimensional structure of astacin for the structure and function of the astacin family of zinc-endopeptidases. Eur J Biochem 214:215–231
9. Tarentino AL, Quinones G, Grimwood BG, Hauer CR, Plummer TH (1995) Molecular cloning and sequence analysis of flavastacin: An 0-glycosylated prokaryotic zinc metalloendopeptidase. Arch Bochem Biophys 319:281–285
10. Bode W, Gomis-Rüth FX, Huber R, Zwilling R, Stöcker W (1992) Structure of astacin and implications for activation of astacins and zinc-ligation of collagenases. Nature 358:164–167
11. Gomis-Rüth FX, Stöcker W, Huber R, Zwilling R, Bode W (1993) Refined 1.8 Å X-ray crystal structure of astacin, a zinc endopeptidase from the crayfish *Astacus astacus* L. Structure determination, refinement, molecular structure and comparison with thermolysin. J Mol Biol 229:945–968
12. Mac Sweeney A, Gil-Parrado S, Vinzenz D, Bernardi A, Hein A, Bodendorf U, Erbel P, Logel C, Gerhartz B (2008) Structural basis for the substrate specificity of bone morphogenetic protein 1/tolloid-like metalloproteases. J Mol Biol 384:228–239

13. Semenova SA, Rudenskaya GN, Rebrikov DV, Isaev VA (2006) cDNA cloning, purification and properties of *Paralithodes camtschatica* metalloproteases. Protein Pept Lett 13:571–575
14. Tarentino AL, Barrett AJ, Rawlings ND, Woessner JF (eds) (1998) Handbook of proteolytic enzymes. Academic, London, pp 1241–1242
15. Garrigue-Antar L, Barker C, Kadler KE (2001) Identification of amino acid residues in bone morphogenetic protein-1 important for procollagen C-proteinase activity. J BiolChem 276:26237–26242
16. Semenova SA, Rudenskaya GN (2009) The astacin family of metalloproteinases. Biochem (Moscow) Suppl Ser B: Biomed Chem 3:17–32
17. Soler D, Nomizu T, Brown WE, Shibata I, Auld DS (1995) Matrilysin: expression, purification, and characterization. J Protein Chem 14:511–520
18. Hulmes DJ (2002) Building collagen molecules, fibrils, and suprafibrillar structures. J Struct Biol 137:2–10
19. Bourhis JM, Vadon-Le Goff S, Afrache H, Mariano N, Kronenberg D, Thielens N, Moali CandHulmes DJ (2013) Procollagen C-proteinase enhancer grasps the stalk of the C-propeptidetrimer to boost collagen precursor maturation. Proc Natl Acad Sci USA 110:6394–6399
20. Bourhis JM, Mariano N, Zhao Y, Harlos K, Exposito JY, Jones EY, Moali C, Aghajari N, Hulmes DJ (2012) Structural basis of fibrillar collagen trimerization and related genetic disorders. Nat Struct Mol Biol 19:1031–1036
21. Apte SS (2009) A disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif (ADAMTS) superfamily: functions and mechanisms. J Biol Chem 284:31493–31497
22. Prox J, Arnold P, Becker-Pauly C (2015) Meprin α and meprin β: procollagen proteinases in health and disease. Matrix Biol 44–46:7–13
23. Geier G, Zwilling R (1998) Cloning and characterization of a cDNA coding for *Astacus* embryonic astacin, a member of the astacin family of metalloproteases from the crayfish *Astacus astacus*. Eur J Biochem 253:796–803
24. Yasumasu S, Shimada H, Inohaya K, Yamazaki K, Iuchi I, Yasumasu I, Yamagami K (1996) Different exon-intron organizations of the genes for two astacin-like proteases, high choriolytic enzyme (choriolysin H) and low choriolytic enzyme (choriolysin L), the constituents of the fish hatching enzyme. Eur J Biochem 237:752–758
25. Fan TJ, Katagiri C (2001) Properties of the hatching enzyme from *Xenopus laevis*. Eur J Biochem 268:4892–4898
26. Sano K, Kawaguchi M, Yoshikawa M, Iuchi I, Yasumasu S (2010) Evolution of the teleostean zona pellucida gene inferred from the egg envelope protein genes of the Japanese eel, *Anguilla japonica*. FEBS J 277:4674–4684
27. Bertenshaw GP, Turk BE, Hubbard SJ, Matters GL, Bylander JE, Crisman JM, Cantley LC Bond JS (2001) Marked differences between metalloproteases meprin A and B in substrate and peptide bond specificity. J BiolChem 276:13248–13255
28. Möhrlen F, Hutter H, Zwilling R (2003) The astacin protein family in *Caenorhabditis elegans*. Eur J Biochem 270:4909–4920
29. Stöcker W, Zwilling R (1995) Astacin. Methods Enzymol 248:305–325
30. Stöcker W, Grams F, Baumann U, Reinemer P, Gomis-Rüth FX, McKay DB, Bode W (1995) The metzincins—topological and sequential relations between the astacins, adamalysins, serralysins and matrixins (collagenases) define a superfamily of zinc-peptidases. ProtSci 4:823–840
31. Claudia B, Philipp A, Sandrine VLG, Moritz AK, Kerstin B, Stefan M, Overall CM, Judith SB, Tomas K, Andreas T, Hulmes DJS, Catherine M, Christoph B (2013) Metalloproteases meprin alpha and meprin beta are C- and N-procollagen proteinases important for collagen assembly and tensile strength. Proc Natl Acad Sci USA 110:14219–14224

32. Villa JP, Bertenshaw GP, Bond JS (2003) Critical amino acids in the active site of meprin metalloproteinases for substrate and peptide bond specificity. J BiolChem 278:42545–42550
33. Greenspan DS (2005) Biosynthetic processing of collagen molecules. Top Curr Chem 247:149–183
34. Takahara K, Lyons GE, Greenspan DS (1994) Bone morphogenetic protein-1 and a mammalian tolloid homologue (mTld) are encoded by alternatively spliced transcripts which are differentially expressed in some tissues. J Biol Chem 269:32572–32578
35. Scott IC, Blitz IL, Pappano WN, Imamura Y, Clark TG, Steiglitz BM, Thomas CL, Maas SA, Takahara K, Cho KW, Greenspan DS (1999) Mammalian BMP-1/Tolloid-related metalloproteinases, including novel family member mammalian Tolloid-like 2, have differential enzymatic activities and distributions of expression relevant to patterning and skeletogenesis. Dev Biol 213:283–300
36. Hojima Y, van der Rest M, Prockop DJ (1985) Type I procollagen carboxyl-terminal proteinase from chick embryo tendons. Purification and characterization. J Biol Chem 260:15996–16003
37. Medeck RJ, Sosa S, Morris N, Oxford JT (2003) BMP-1-mediated proteolytic processing of alternatively spliced isoforms of collagen type XI. Biochem J 376:361–368
38. Sterchi EE, Stocker Wand Bond JS (2008) Meprins, membrane-bound and secreted astacin metalloproteinases. Mol Aspects Med 29:309–328
39. Ge GX, Fernandez CA, Moses MA, Greenspan DS (2007) Bone morphogenetic protein 1 processes prolactin to a 17-kDa antiangiogenic factor. Proc Natl Acad Sci USA 104:10010–10015
40. Kruse MN, Becker C, Lottaz D, Köhler D, Yiallouros I, Krell HW, Sterchi EE, Stöcker W (2004) Human meprin alpha and beta homo-oligomers: cleavage of basement membrane proteins and sensitivity to metalloprotease inhibitors. Biochem J 378:383–389
41. Leung MK, Fessler LI, Greenberg DB, Fessler JH (1979) Separate amino and carboxyl procollagen peptidases in chick embryo tendon. J Biol Chem 254:224–232
42. Kessler E, Adar R, Goldberg B, Niece R (1986) Partial purification and characterization of a procollagen C-proteinase from the culture medium of mouse fibroblasts. Coll Relat Res 6:249–266
43. Allan GA, Gedge JI, Nedderman AN, Roffey SJ, Small HF, Webster R (2006) Pharmacokinetics and metabolism of UK-383,367 in rats and dogs: a rationale for long-lived plasma radio activity. Xenobiotica 36:399–418
44. Robinson LA, Wilson DM, Delaet NG, Bradley EK, Dankwardt SM, Campbell JA, Martin RL, Van Wart HE, Walker KA, Sullivan RW (2003) Novel inhibitors of procollagen *C*-Proteinase. Part 2: glutamic acid hydroxamates. Bioorg Med Chem Lett 13:2381–2384
45. Turtle E, Chow N, Yang C, Sosa S, Bauer U, Brenner M, Solow-Cordero D, Ho WB (2012) Design and synthesis of procollagen C-proteinase inhibitors. Bioorg Med Chem Lett 22:7397–7401
46. Hajduk PJ, Shuker SB, Nettesheim DG, Craig R, Augeri DJ, Betebenner D, Albert DH, Guo Y, Meadows RP, Xu L, Michaelides M, Davidsen SK, Fesik SW (2002) NMR-based modification of matrix metalloproteinase inhibitors with improved bioavailability. J Med Chem 45:5628–5639
47. Becker DP, Barta TE, Bedell LJ, Boehm TL, Bond BR, Carroll J, Carron CP, Decrescenzo GA, Easton AM, Freskos JN, Funckes-Shippy CL, Heron M, Hockerman S, Howard CP, Kiefer JR, Li MH, Mathis KJ, McDonald JJ, Mehta PP, Munie GE, Sunyer T, Swearingen CA, Villamil CI, Welsch D, Williams JM, Yu Y, Yao J (2010) Orally active MMP-1 sparing α-tetrahydropyranyl and α-piperidinyl sulfone matrix metalloproteinase (MMP) inhibitors with efficacy in cancer, arthritis, and cardiovascular disease. J Med Chem 53:6653–6680
48. Flipo M, Charton J, Hocine A, Dassonneville S, Deprez BandDeprez-Poulain R (2009) Hydroxamates: relationships between structure and plasma stability. J Med Chem 52:6790–6802

49. Becker-Pauly C, Barre O, Schilling O, Auf dem Keller U, Ohler A, Broder C, Schutte A, Kappelhoff R, Stocker W, Overall CM (2011) Proteomic analyses reveal an acidic prime side specificity for the astacin metalloprotease family reflected by physiological substrates. Mol Cell Proteomics 10(M111):009233
50. Jefferson T, Auf dem Keller U, Bellac C, Metz VV, Broder C, HedrichJ Ohler A, Maier W, Magdolen V, Sterchi E et al (2013) The substrate degradome of meprin metalloproteases reveals an unexpected proteolytic link between meprin β and ADAM10. Cell Mol Life Sci 70:309–333
51. Hedrich J, Lottaz D, Meyer K, Yiallouros I, Jahnen-Dechent W, Stöcker Wand Becker-Pauly C (2010) Fetuin-A and cystatin C are endogenous inhibitors of human meprin metalloproteases. Biochemistry 49:8599–8607
52. Hall A, Hakanssn K, Mason RW, Grubb A (1995) Structural basis for the biological specificity of cystatin C. Identification of leucine 9 in the N-terminal binding region as a selectivity-conferring residue in the inhibition of mammalian cysteine peptidases. J Biol Chem 270:5115–5521
53. Hirano M, Ma BY, Kawasaki N, Okimura K, Baba M, Nakagawa T, Miwa K, Oka S, Kawasaki T (2005) Mannan-binding protein blocks the activation of metalloproteases meprin alpha and beta. J Immol 175:3177–3185
54. Zhang Y, Ge G, Greenspan DS (2006) Inhibition of bone morphogenetic protein 1 by native and altered forms of alpha2-macroglobulin. J Biol Chem 281:39096–39104
55. Stöcker W, Breit S, Sottrup-Jensen L, Zwilling R (1991) alpha2-Macroglobulin from hemolymph of the freshwater crayfish *Astacus astacus*. Comp Biochem Physiol B 98:501–509
56. Marrero A, Duquerroy S, Trapani S, Goulas T, Guevara T, Andersen GR, Navaza J, Sottrup-Jensen L, Gomis-Rüth FX (2012) The crystal structure of human α2-macroglobulin reveals a unique molecular cage. Angew Chem Int Ed Engl 51:3340–3344
57. Tsai PL, Chen CH, Huang CJ, Chou CM, Chang GD (2004) Purification and cloning of an endogenous protein inhibitor of carp nephrosin, an astacin metalloproteinase. J Biol Chem 279:11146–11155
58. Bijakowski C, Vadon-Le Goff S, Delolme F, Bourhis JM, Lécorché P, Ruggiero F, Becker-Pauly C, Yiallouros I, Stöcker W, Dive V, Hulmes DJS, Moali C (2012) Sizzled is unique among secreted frizzled-related proteins for its ability to specifically inhibit bone morphogenetic protein-1 (BMP-1)/tolloid-like proteinases. J Biol Chem 287:33581–33593

Proteases of Parasitic Helminths: Their Metabolic Role in Establishment of Infection in the Host

12

Veena Tandon, Bidyadhar Das and Shakti Kumar

Abstract

Proteases catalyze hydrolysis of peptide bonds in proteins and play an important role in the survival of living organisms, encoded by about 2% of the whole genome in all kind of organisms. Mostly they are nonspecific, while some are highly specific toward a peptide bond. Generally, proteases are grouped into different clan, family, and type, depending on kinds of reaction they catalyze, mechanism of catalysis, and their molecular structure and homology. Proteases control many biological processes in living organisms including helminths. There are about 1828 sequences that pertain to 25 genera of helminth parasites. In this chapter, we have discussed various types of proteases found in helminth parasites, like aspartic-, cysteine-, metallo-, and serine proteases, and their possible role in these parasites and their hosts.

Keywords

Protease · Parasite · Helminths

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1 Introduction

Proteases are the biological scissors, which hydrolyze the peptide bonds in proteins, and may have appeared in biological systems during early periods of protein evolution. Protein degradation plays an important role in the survival of all living organisms as it is involved in food digestion and defense mechanism against the pathogen. Activation and inactivation of proteins by degrading some specific portion of them regulate some physiological and cellular processes, thus preventing the accumulation of unwanted or abnormal proteins within the cells [1]. Proteases are found in almost all living organisms from viruses to human, and they are encoded by about 2% of the whole genome in all kind of organisms [2]. Most of these proteases are relatively nonspecific for their substrates, like proteinase K, while some are highly specific toward a particular peptide bond, like angiotensin-converting enzyme; the later group, thus, launched a new and exciting filed of protease research [3]. The proteases also vary in their molecular sizes from simple catalytic units ($\sim$20 kDa) to sophisticated molecular machines, like the proteasome and meprin metalloproteinase isoforms ($\sim$6000 kDa) [4]. Till today, even after 100 years of their discovery, these enzymes remain at the cutting edge of research in most laboratories around the globe.

Generally proteases are classified into endopeptidases, which target internal peptide bonds, and exopeptidases, which catalyze the terminal NH_2 (aminopeptidases) or COOH (carboxypeptidases) bonds. However, the availability of structural and mechanistic information on these enzymes facilitates new classification schemes [5]. According to recent information, proteolytic enzymes may be grouped depending on the kinds of reaction they catalyze (like endopeptidases, omega peptidases, exopeptidases, aminopeptidases, carboxypeptidases, dipeptidyl peptidases, tripeptidyl peptidases, peptidyl dipeptidases, and dipeptidases), or on the mechanism of catalysis (such as aspartic-, glutamic-, cysteine-, serine-, threonine-, and metalloproteases) [6], or on their molecular structure and homology (such as clan, family, and type) [7].

Proteases control many biological processes in living organisms, like regulation of proteins, creation of new bioactive molecules, processing of cellular information, and molecular signaling [8–16]. Hence, alterations in proteolytic systems in human cause many diseases such as cancer, neurodegenerative disorders, and inflammatory and cardiovascular diseases. Accordingly, many proteases are of special attention for the pharmaceutical industry as potential drug targets or as biomarkers [17]. Unswerving with other higher animals, in parasitic helminths too, proteases play crucial roles, like tissue penetration, digestion of host's tissue for nutrition and evasion of host's immune response, and thus determine the establishment and survival of the pathogen in its host [18].

In "protease.lib" of the latest MEROPS-10.0 database (http://merops.sanger.ac.uk), a huge number (362,652) of protease sequences (including protease inhibitors) are found. Of these, only 1828 sequences pertain to 25 genera of helminth parasites (Table 1). In class/clan-wise categorization, the protease sequences in helminths fall

Table 1 Genus-wise distribution of 1828 proteases found in helminths

S. No.	Helminth parasite (Genus)	CsP	AsP	MtP	SeP	MxP	UsC	Total
Trematodes								
1.	*Clonorchis*	60	34	50	20	16	0	180
2.	*Fasciola*	41	2	2	0	0	0	45
3.	*Metagonimus*	7	0	0	0	0	0	7
4.	*Opisthorchis*	6	1	0	1	1	0	9
5.	*Paragonimus*	12	0	1	0	0	0	13
6.	*Schistosoma*	130	40	173	71	58	0	472
7.	*Trichobilharzia*	6	0	0	0	0	0	6
Cestodes								
8.	*Echinococcus*	56	6	81	43	33	9	228
9.	*Spirometra*	2	0	0	0	0	0	2
10.	*Taenia*	4	0	1	0	0	0	5
Nematodes								
11.	*Ancylostoma*	7	3	14	0	0	0	24
12.	*Angiostrongylus*	4	1	1	0	0	0	6
13.	*Anisakis*	1	1	0	0	1	0	3
14.	*Ascaris*	40	15	94	40	16	0	205
15.	*Brugia*	58	13	77	30	22	0	200
16.	*Dirofilaria*	1	0	1	1	0	0	3
17.	*Gnathostoma*	1	0	1	0	1	0	3
18.	*Loa*	60	12	117	19	10	0	218
19.	*Necator*	5	2	1	1	0	0	9
20.	*Onchocerca*	2	1	1	2	0	0	6
21.	*Strongyloides*	1	10	1	1	0	0	13
22.	*Toxocara*	3	0	0	0	0	0	3
23.	*Trichinella*	38	10	67	17	37	0	169
24.	*Trichuris*	1	0	1	0	0	0	2
25.	*Wuchereria*	0	1	0	0	0	0	1
Total		546	152	681	245	195	9	1828

under six protease groups; cysteine protease (CsP-546 sequences), aspartic protease (AsP-152 sequences), metalloprotease (MtP-681 sequences), serine protease (SeP-245 sequences), mixed protease (MxP-including cysteine, aspartic, and serine clans; 195 sequences) and unassigned protease (UsP-9 sequences) (Fig. 1). Using Database of Essential Genes (eDEG; http://tubic.tju.edu.cn/deg/) and applying a two-layered filtering approach via protein–protein BLAST against (mammalian) host proteases, highly diverse and essential proteases could be sieved out of the total sequences identified to exist among helminths (Fig. 2). Out of 1828 sequences, only 108 are predicted as highly diverse and essential proteases, of which 36, 12, 26, 1, 24, and 9 sequences belong to AsP, CsP, MtP, SeP, MxP, and UsP,

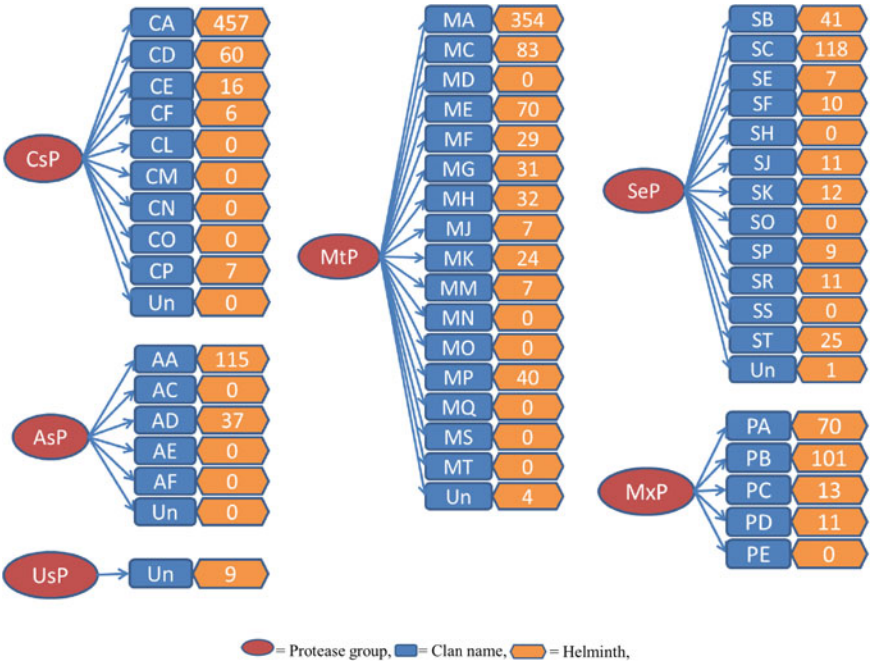

Fig. 1 Total protease sequences available in "protease.lib" of MEROPS-10.0. Available type of proteases and their clan-wise distribution in helminth parasites

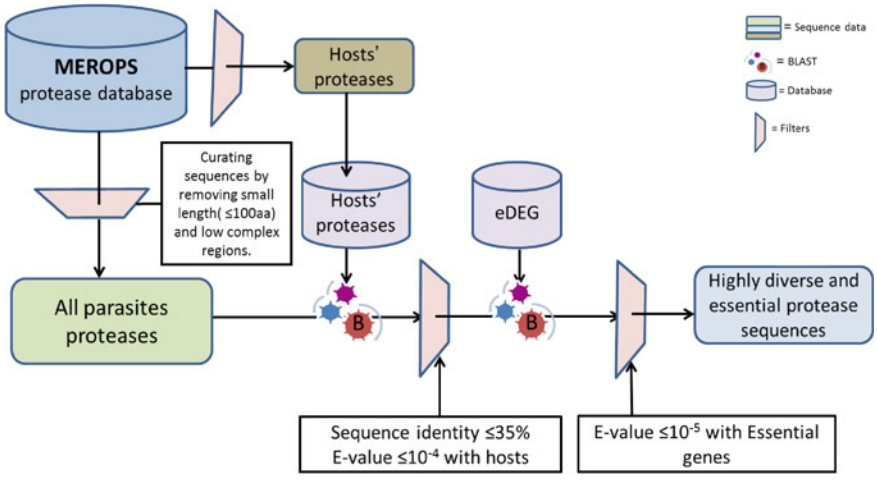

Fig. 2 Two-level filtering approach for finding highly diverse and essential proteases. Two-level-filtering approach means two times protein–protein BLAST program was used with mammalian (human and cattle) proteases and database of essential genes

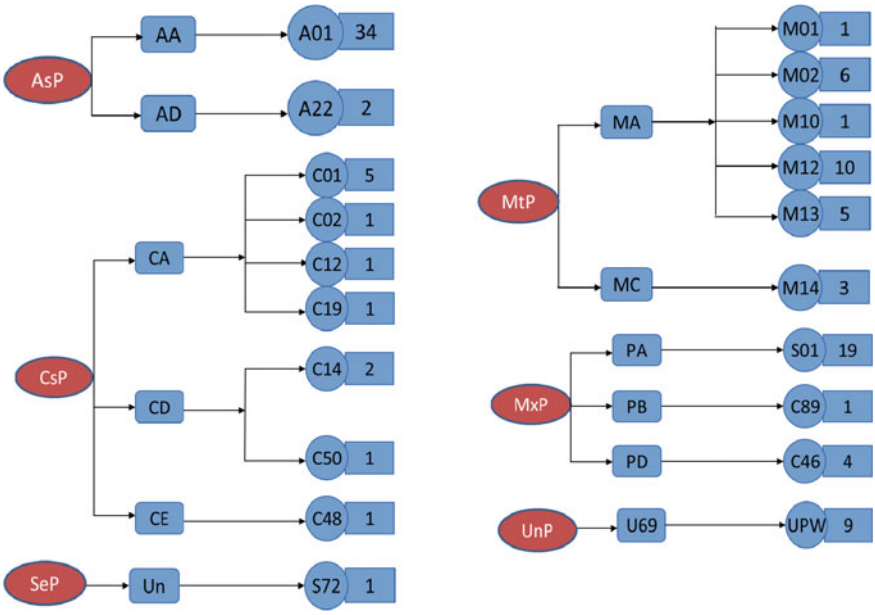

Fig. 3 Types and clan-wise distribution of 108 protease sequences after two-level filtration by BLAST with mammalian (human and cattle) proteases and database of essential genes (DEG)

respectively (Fig. 3). It is noteworthy that protease sequences of glutamine (GuP) and asparagine (AnP) groups are conspicuously missing in helminths group (http://merops.sanger.ac.uk).

The fact that many infectious microorganisms require proteases for replication or use them as virulence factors has facilitated the development of protease-targeted therapies for diseases of great relevance to human life [3]. Besides, proteases are also important tools of the biotechnological industry because of their usefulness as biochemical reagents [19]. Like microbes, macropathogens such as helminths also possess proteases, which have diverse functions in parasite biology. These enzymes are secreted by helminth parasites and used for their entry into the host as well as feeding and migration. In this chapter, we focus on various types of proteases in parasitic helminths and their metabolic roles in establishment of infection in the host.

2 Role of Proteases in Parasitic Helminths

Besides therapeutic importance of proteases in various types of cancers, respiratory and cardiovascular disorders, they have also been considered as potent therapeutic targets for various infections. When a parasite infects a host, it survives by escaping from the host immune system; parasites must have sufficient mechanisms by which

they can cross various barriers, such as epithelial cell wall, connecting tissues and extracellular matrix to reach their final destination [20]. The roles of various proteases in helminth parasites are discussed under.

2.1 Aspartic proteases are associated with digestion of host hemoglobin in blood-feeding nematodes such as the trichostrongylids, *Haemonchus contortus*, hookworms (*Ancylostoma caninum* and *Necator americanus*), and *Angiostrongylus costaricensis* [21, 22]. Consequently, many aspartic proteases are being used to develop vaccines against trichostrongylids and hookworm infections [23, 24]. It has been observed that aspartic proteases of hookworms are also capable to degrade skin macromolecules and aid skin penetration, suggesting that their role in nematode parasitism is not limited to digestion of hemoglobin but they also function in intestinal digestion and tissue degradation of the host [25, 26]. Substrate specificity of hemoglobin-degrading proteases employed by blood-feeding helminth parasites influences the parasite–host species range. The differences in amino acid sequences in the catalytic sites of these proteases interact less or more efficiently with hemoglobin of permissive or nonpermissive hosts [27]. In the human liver fluke, *Opisthorchis viverrini,* cathepsin D-like aspartic protease, Ov-APR-1, is expressed in the gut and reproductive tissues of the mature hermaphroditic parasite. This is also present in the developing larval miracidium stage within the eggshell, and in the excretory/secretory products (ESPs) of the cultured adult flukes. The presence of Ov-APR-1 in all developmental stages and ESPs of *O. viverrini* shows its indispensable role in the host–parasite relationship [26].

2.2 Cysteine proteases are the most widely reported class of proteases from parasitic nematodes and are secreted by larval and adult parasites for tissue invasion, feeding and defense against effector mechanisms of the host immune response. Major cysteine proteases belong to the papain superfamily (Family C1) and are common in nematodes. Cathepsins B of *H. contortus* are expressed in the intestine of adult worms that are capable of digesting hemoglobin (Hb), fibrinogen, collagen, and immunoglobulin-G. Similarly, cysteine proteases in ES products of *N. americanus* have lytic activity against Hb, fibrinogen and antibodies [28, 29]. Comparative analysis of the transcriptome of various life-cycle stages of *Brugia malayi* shows that cathepsin-like cysteine proteases (including Bm-cpl-1, 4 and 5) are expressed at every stage, though more prominently in MF, L3, and L4 stages [30]. The expression of cathepsin B, AcCP-2, was more abundant in eggs and larval developmental stages of *A. caninum*, showing that cathepsin B might play a role in the early development of the dog hookworm [31]. Similarly, intestinal expression of four distinct Cathepsin B (Na-CP-2, -3, -4, -5) from the human hookworm, *N. americanus*, shows that these cysteine proteases and likely to be involved in nutrient acquisition [32].

The whole-genome analysis of various trematode parasites, for example *Schistosoma japonicum* and other *Schistosoma* species reveals that cysteine proteases are the largest and most important protease family. A total of 102 cysteine protease sequences have been identified from the whole genome of *S. japonicum* and all are assigned to 17 subtypes. Among them, the cathepsins B, C, F, L, K, and S have a pivotal role in schistosome feeding and nutrition, as well as in migration through

human tissues [33]. Analysis of the expressed sequence tags (ESTs) of *Clonorchis sinensis* revealed that proteases are the largest proportion of the protein population of this fluke, which are essential for stage transition, nutrient uptake, and immune evasion [34, 35]. Furthermore, the partially purified cysteine proteases from ESPs of *C. sinensis* adult worms show cytotoxic effects on cultured cells, and the endogenous cysteine proteases of the metacercaria appear to be involved in excystation from the cyst [36]. Several cysteine proteases were identified in *C. sinensis* and are phylogenetically more close to the mammalian cathepsin F enzymes [37]. Recently, the published draft genome of *C. sinensis* shows that the largest part of protease members belong to cysteine protease superfamily. Some cysteine proteases are also reported for the first time and probably contribute to the catabolism of bilirubin and other host proteins [38].

Various developmental stages of the liver fluke *Fasciola hepatica* also express different types of cysteine proteases. The transcriptome analysis of the invasive juvenile stage of *F. hepatica* shows that cathepsins L3, L4, and L6 are specifically identified in the juvenile ESTs, while these are not present in the adult stage. Some isoforms of various cathepsins are expressed in different developmental stages. For example, the secretome analysis of the adult *F. hepatica* shows that FhCL1 and FhCL2 peptidases are the most abundant proteins, comprising 67 and 27%, respectively of the total cathepsin Ls. A total 31% cysteine peptidases are made up of the total proteins secreted by the newly excysted juvenile (NEJ), in which various isoforms of cathepsin Ls; L3, L4, and L6 (37%), Cathepsin B (45%), and Asparaginyl endopeptidases (18%) have been identified [39, 40]. The total RNA analysis of the adult form of another liver fluke, *O. viverrini*, the causative agent of cholangiocarcinoma, shows two novel cysteine proteases—cathepsin F (Ov-CF-1) and cathepsin B1 (Ov-CB-1). Ov-CF-1 is secreted as an inactive zymogen that autocatalytically processes and activates itself to mature enzyme at pH 4.5 via an intermolecular cleavage at the prosegment–mature domain junction. Also, Ov-CB-1 is secreted as a zymogen but, in contrast to Ov-CF-1, is fully active against peptide and macromolecular substrates despite retaining the N-terminal prosegment [41].

The cysteine proteases of the lung fluke *Pargonimus westermani* newly excysted metacercariae (PwNEM) play a role in host tissue invasion. Two isoforms (PwMc27 and PwMc28) of a particular type of cysteine protease enzyme having 27 and 28 kDa molecular weight and purified from PwNEM ESPs, preferentially degrade fibrillar proteins, but not globular proteins [42].

In the case of *Echinococcus multilocularis* metacestodes, two cDNA clones, encoding cathepsin L-like (EmCLP1 and EmCLP2) and cathepsin B—like (EmCBP1 and EmCBP2) cysteine proteases, are isolated from ESPs and the extract of the metacestodes. These are suggested to play a key role during protein digestion for the parasite's nutrition and in parasite–host interactions [43, 44]. *Taenia solium* and other species of *Taenia* are the cause of neurocysticercosis in human and other animals, respectively. Immunoglobulin degradation by cysteine proteinases of the pathogenic *Taenia* species is suggested to play a key role in escaping from the host immune system and thus could be employed as a target for chemotherapy [45]. Another cysteine protease, cathepsin L-like peptidase, is secreted by *Taenia*

species, which can be utilized as immunodiagnostic antigen for cysticercosis treatment [46]. In another tapeworm, *Spirometra erinaceieuropaei*, a cysteine protease (SeCP), recognized in the sparganum ES proteins by early infection sera and identified by MALDI-TOF/TOF-MS, is a 336-amino acid long chain. In SeCP, 15 potential antigenic epitopes and 19 HLA-I restricted epitopes are computationally predicted, giving insights on the diagnostic antigens and target molecular sites of antisparganum drugs [47].

2.3 Metalloproteases comprise a heterogeneous group of proteolytic enzymes whose main characteristic is the utilization of a metal ion to polarize a water molecule for performing hydrolytic reactions. A major group of metalloproteases includes zinc-dependent endopeptidases, which have the ability to cleave one or more extracellular matrix components as well as non-matrix proteins [48]. According to their substrate specificity, MMPs can be categorized as collagenases, gelatinases, elastases, stromelysins, and membrane-type. In nematodes, metalloproteases including collagenases, gelatinases, and elastases play an important and essential role in larval and adult migration and invasion through host's connective tissues [48]. A novel astacin-like metalloprotease (Ac-MTP-1) is characterized in ESP of *A. caninum* L3 larvae. Ac-MTP-1 has significant sequence similarity with Zinc-metalloprotease and is exclusively expressed in L3 stage, indicating its role in host tissue invasion [49]. More recently, an ortholog of Ac-MTP-1, known as Ay-MTP-1, has also been identified in *Ancylostoma ceylanicum* and is believed to be a plausible protein target for vaccine development to prevent larval migration through tissues [50]. Recently, two metalloproteases, a 175 kDa collagenase and another leucine aminopeptidase (LAP), have been purified and characterized from adult female *Setaria cervi* (a filarial parasite of Indian buffalo). In vivo study for these enzymes reveals that collagenase plays an important role in host immune evasion and immunoprotection by specifically cleaving human IgG in vitro [51].

2.4 Serine proteases, secreted by the intestinal nematode parasites, have the ability to change the properties of the mucus barrier, making it more porous by degrading the mucin component of the mucus gel [52]. The parasitoid nematode, *Steinernema carpocapsae*, is capable of killing its insect host within 48 h. The ESP of the parasitic stage of this worm shows high proteolytic activity; a chymotripsin-like serine protease, Sc-SP-3, participates in degradation of extracellular proteins and is thus involved in nematode pathogenesis [53, 54]. Serine proteases derived from ESPs of in vitro cultures of *Trichinella spiralis* L1 muscle larval have been shown to participate in hydrolysis of collagens and elastin proteins [55].

3 Proteases as Drug Targets in Helminths

From the foregoing account, it clearly emerges that proteases have multifarious functions in the biology and pathogenesis of parasitic organisms. They are unusually immunogenic and have been exploited as serodiagnostic markers and vaccine targets. Although host homologs exist, parasite proteases have distinct structural and

biochemical properties including optimum pH and stability, alteration in peptide loops or domain extensions, diverse substrate specificity, and cellular location. The disparate nature of parasite proteases compared to the host orthologous proteins has opened opportunities for chemotherapy [56]. For comprehensive understanding of the role proteases in molecular and biochemical mechanisms for survivability, nutrition, metabolism, host-dependent development and maturation, immune evasion and evolution, numerous parasite whole genomes have been sequenced in recent years and some are still in the pipeline [57, 58]. Depending on the chemical groups in their active sites, proteases found in helminths parasites are grouped as four major classes (aspartic-, cysteine-, metallo-, and serine proteases), which are discussed below.

3.1 Aspartic proteases, like pepsin proteases (Clan AA and Family A01), digest the ingested food. In blood-feeding nematodes, aspartic proteases, along with cysteine proteases, are involved in degrading the host's blood in a multienzyme cascade manner [59]. Aspartic proteases in adult *O. viverrini* and *Heligmosomoides polygyrus* secretome are found to play a key role in hemoglobin degradation [60, 61]. Comparative genomic analysis of aspartic proteases in eight parasitic platyhelminths reveals that aspartic protease members of family A01 are prevalent in schistosomes than in cestodes. Proteases of family A22 are evolutionarily highly conserved among all the parasites, and one retroviral-like AP in family A28 shares a highly similar predicted 3D structure with the HIV protease, thus implying its potential to be inhibited by HIV inhibitor-like molecules [62]. Analysis of secretory cDNA of *C. sinensis* reveals a 425 amino acids-long Cathepsin D-like Aspartic protease that may be potential to diagnose the antigen and the drug target of clonorchiasis [63].

3.2 Cysteine proteases are the most studied proteases in helminth parasites and involved many aspects of hosts–parasite relationship. These proteases are mainly intracellular in higher organisms, but are often extracellular proteases in helminths parasites [64]. Such diverse utility of this group of proteases reveals their therapeutic importance against helminth infections. Recently, K11777, a potent cysteine protease inhibitor has been developed and its clinical trial is being tested [64]. A recent study about Cathepsin F (member of Cysteine protease group) of *Trichinella* spp has shown that it is a major virulence factor for parasitic helminths, and it may be a potential anthelmintic drug target and vaccine candidate of trichinellosis, a reemerging infectious disease [65]. Whole genome sequencing of the whipworm *Trichuris* and its comparative analysis to find new predicted drug targets based on transcript-level expression, essentiality of protein homologs. It has been shown that Cathepsin B and gut-specific cysteine protease-1, and -2 are novel drugable targets in the parasite [66]. Phylogenetic analysis using Bayes approach of *T. solium* genome shows their functional divergence among regulatory cysteine- and serine proteases from their hosts, hence, these proteases can be used for drug targets [67]. Analysis of the ESPs of the encysted progenetic metacercariae of the digenetic trematode parasite, *Euclinostomum heterostomum,* has shown that cysteine proteases, being a major component, can be exploited as serodiagnostic markers and therapeutic and vaccine targets [68]. Similar study of *C. sinensis* ESPs shows the presence of four *C. sinensis* cathepsin B cysteine proteases (CsCB1, CsCB2, CsCB3, and CsCB4), and immunological and biochemical studies reveal

their potential to be vaccine candidates and drug targets in *C. sinensis* prevention [69]. *S. mansoni* cathepsin B1 (SmCB1) is the most abundant papain-like cysteine peptidase in the parasite gastrodermis, gut lumen, and probably in caeca and protonephridia [70]. Immunological studies of schistosomiasis patients suggest that SmCB1 is an immunodominant target of the immune response during pre-patent schistosome infection. It has been demonstrated that SmCB1 is targeted by IgG- and IgE-specific antibodies. In the first situation, SmCB1 significantly reduces the worm burden (by 66–73%), eggs in the liver (51%), and in the small intestine (25%). However, when SmCB1 is incubated with the proteinase-inhibitor prior to immunization, levels of protection decrease significantly. This study points out the importance of the peptidase activity in protective potential. Therefore, SmCB1 has been considered a strong candidate for drug development and vaccine designing [71]. Some natural and synthetic compounds are being developed as cysteine protease inhibitors for pathogenic helminths [72–75].

3.3 Metalloproteases (like aspartic- and cysteine proteases) also play an important role in survival of the parasite in the host system. From bioinformatics analysis, 26 metalloproteases are mined from MEROPS database which are considered highly diverse and essential, and thus, potent therapeutic drug targets. Two novel leucine aminopeptidases (CsLAP1 and CsLAP2), identified from *C. sinensis*, are suggestedly involved in the digestion of intestinal peptides of the host [76]. Another study of the ESPs of *C. sinensis* suggested that methionine aminopeptidase 2 (MAP-2), belonging to metallopeptidase family M24, is highly expressed in eggs, metacercaria, and adult stages of *C. sinensis*. These proteases have been considered as potential drug targets in immunotherapy of clonorchiasis and biliary diseases [77]. The differential expression profile of proteases in *S. japonicum* degradome reveals that 14 putative M8 family members are surface proteases. Their annotation reveals similarity with leishmanolysin protease of protozoan parasites, which plays a crucial role in host body invasion. It has been speculated that leishmanolysin (invadolysin) may also contribute to tissue invasion by schistosome cercaria; thus leishmanolysin inhibition could serve as a novel intervention strategy for schistosomiasis [78]. In protease analysis of *T. solium* genome, it has been revealed that 27% of total metalloprotease are membrane bound. It has been speculated that their inhibition will be effective in eradication of *T. solium* infection [67]. Neurocysticercosis (NCC) is the central nervous system (CNS) infection caused by the larva of *T. solium* tapeworm. Its matrix metalloprotease (MMP) expression plays a crucial role in the differential breakdown of the blood–brain barrier (BBB), and inhibition of this enzyme has been considered its therapeutic importance [79]. Inhibition of metalloprotease by selected chemical compounds shows prolongation of the moulting process in nematode larvae [80]. In *A. cantonensis*, the L3 larva releases heavily secreted proteases, in which metalloproteases are dominantly found. The study shows the capability of *A. cantonensis* secretory metalloproteases to degrade human metalloproteinase (MMP-9). This analysis has revealed the therapeutic importance of the metalloprotease of *A. cantonensis* [81]. The third-stage larvae of *Strongyloides stercoralis* have an ability to migrate through

tissues at a speed of 5–15 cm per hour. This process of migration is facilitated by a metalloproteinase with elastase activity, which is determined by inhibition study [82]. The parasitic virulence factor may be guided by metalloproteinase and could be a molecule for therapeutic purpose [23].

3.4 Serine proteases also play an important role in a broad range of biological processes, such as intra- and extracellular protein metabolism, digestion, blood coagulation, regulation of development, and fertilization. Therefore, this group of enzymes is also therapeutically important like aspartic-, cysteine-, and metalloproteinases. Expression of different types of serine proteases is observed in some nematodes parasites. In *T. spiralis* a novel serine protease (i.e., TsSerP) is found during all life-cycle stages, whereas two TsSP-1 and TsSP-2 are expressed only during the invasive larvae stage in the host muscle [83]. Recently, a newborn larval stage-specific serine protease gene (NBL1) has been identified via a subtractive cDNA library of *T. spiralis* newborn larvae. Based on the high immunogenicity of its C-terminal domain, it is speculated that it may be important therapeutic target [84]. Novel serine protease was isolated from the infective larvae of *Anisakis simplex,* which is similar to the extracellular serine protease of the pathogenic bacterium *Dichelobacter nodosus*, which can degrade elastin, keratin, and collagen [85]. Blisterase, a subtilisin-like serine protease, is expressed in *Onchocerca volvulus,* and plays an important role in the nematode biology including the cuticle production and maintenance, neural signaling, and development. Thus, it is a potential drug target for controlling the parasite infection [86]. Three neutral serine proteases, two trypsin-like proteases (198 and 104 kDa) and one chymotrypsin-like protease (36 kDa), have been extracted from the plerocercoid larvae (spargana) of *S. mansoni*. These purified proteins elicit strong antibody responses in infected patients, suggesting that they could be potential antigens in serologic diagnosis of human sparganosis [87].

A novel antigen 5 (Ag5) proteins that harbors two subunits has been characterized by RT-PCR from *Echinococcus granulosus*. One of them, the 22-kDa subunit, contains a highly conserved glycosaminoglycan-binding motif, and the other is a 38 kDa subunit that shows high similarity to serine proteases of the trypsin family. But, biochemical tests reveal that in native purified Ag5 neither the proteolytic activity nor the binding to protease inhibitors could be found. This intriguing feature of Ag5 suggests that it could be a new drug target [88]. In a recent study, a trypsin-like serine protease TsAg5 protein has been identified from *T. solium*, which has high homology with the *E. granulosus* antigen Ag5. Detected from the cyst fluid and ES of the cysticercus, TsAg5 seems to be a potential candidate for immunodiagnostic and drug designing [89].

A secreted dipeptidylpeptidase (DPP) (a type of serine protease) has been isolated from *F. hepatica* and characterized [90]. Recently, serine proteases like proteins (Pic and PII) have been partially purified from *Fasciola gigantica* [91]. Genomes and transcriptomes analysis of blood flukes, *S. mansoni*, *S. japonicum S. haematobium,* and *S. douthitti,* reveal that the skin invasion is facilitated by secretions from the acetabular and head glands, which contains cercarial elastase, a chymotrypsin-like protease. Disruption of the enzyme activity by specific drugs/vaccines may provide therapeutic benefits in schistosomiasis [33, 92–95].

4 Conclusions

Finally, detailed analyses of complex protease-mediated processes in helminths and their role in parasitic metabolic activities will help in better understanding the establishment of infection in their host. However, proteolytic regulation of the transcription factor activity, protein ectodomain shedding, and regulated intra-membrane proteolysis are challenges to be addressed in the near future. Hopefully, this chapter will provide a current view various types of proteases found in helminth parasites.

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References

1. Rawlings ND, Salvesen GS (Eds.) (2012) Handbook of proteolytic enzymes (Vol. 1) Academic press, Cambridge
2. Barrett AJ, Tolle DP, Rawlings ND et al (2003) Managing peptidases in the genomic era. Biol Chem 384:873–882
3. López-Otín C, Bond JS (2008) Proteases: multifunctional enzymes in life and diseases. J Biol Chem 283:30433–30437
4. Bertenshaw GP, Norcum MT, Bond JS et al (2003) Structure of homo- and hetero-oligomeric meprin metalloproteases. Dimers, tetramers, and high molecular mass multimers. J Biol Chem 278:2522–2532
5. Rawlings ND, Barrett AJ, Bateman A et al (2012) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 40:D343–D350
6. Hartley BS (1960) Proteolytic enzymes. Annu Rev Biochem 29:45–72
7. Rawlings ND, Barrett AJ (1993) Evolutionary families of peptidases. Biochem J 290:205–218
8. López-Otín C, Overall CM (2002) Protease degradomics: a new challenge for proteomics. Nat Rev Mol Cell Biol 3:509–519
9. Ehrmann M, Clausen T (2004) Proteolysis as a regulatory mechanism. Annu Rev Genet 38:709–724
10. Sauer RT, Bolon DN, Burton BM et al (2004) Sculpting the proteome with AAA(+) proteases and disassembly machines. Cell 119:9–18
11. Siegel RM (2006) Caspases at the crossroads of immune-cell life and death. Nat Rev Immunol 6:308–317
12. Oikonomopoulou K, Hansen KK, Saifeddine M et al (2006) Proteinase-mediated cell signalling: targeting proteinase-activated receptors (PARs) by kallikreins and more. Biol Chem 387:677–685
13. Urban S (2006) Rhomboid proteins: conserved membrane proteases with divergent biological functions. Genes Dev 20:3054–3068
14. Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8:221–233
15. Mariño G, Uría JA, Puente XS et al (2003) Human autophagins, a family of cysteine proteinases potentially implicated in cell degradation by autophagy. J Biol Chem 278:3671–3678
16. Ciechanover A (2005) Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol 6:79–87

17. Turk B (2006) Targeting proteases: successes, failures and future prospects. Nat Rev Drug Discov 5:785–799
18. Yong Y, Yun JW, Ya NC et al (2015) Serine proteases of parasitic helminths. Korean J Parasitol 53:1–11
19. Saeki K, Ozaki K, Kobayashi T et al (2007) Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. J Biosci Bioeng 103:501–508
20. McKerrow JH, Caffrey C, Kelly B et al (2006) Proteases in parasitic diseases. Annu Rev Pathol 1:497–536
21. Williamson AL, Brindley PJ, Abbenante G et al (2002) Cleavage of hemoglobin by hookworm cathepsin D aspartic proteases and its potential contribution to host specificity. FASEB J 16:1458–1460
22. Rebello KM, Siqueira CR, Ribeiro EL et al (2012) Proteolytic activity in the adult and larval stages of the human roundworm parasite *Angiostrongylus costaricensis*. Mem Inst Oswaldo Cruz 107:752–759
23. Bethony J, Brooker S, Albonico M et al (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367:1521–1532
24. Loukas A, Bethony J, Brooker S et al (2006) Hookworm vaccines: past, present, and future. Lancet Infect Dis 6:733–741
25. Athauda SB, Nomura H, Inoue H et al (2003) Two distinct types of aspartic proteases in the filarial parasite *Brugia malayi*: Molecular cloning and tissue distribution. Biomed Res 24:269–276
26. Suttiprapa S, Mulvenna J, Huong NT et al (2009) Ov-APR-1, an aspartic protease from the carcinogenic liver fluke, *Opisthorchis viverrini*: functional expression, immunolocalization and subsite specificity. Int J Biochem Cell Biol 41:1148–1156
27. Koehler JW, Morales ME, Shelby BD et al (2007) Aspartic protease activities of schistosomes cleave mammalian hemoglobins in a host-specific manner. Mem Inst Oswaldo Cruz 102:83–85
28. Shompole S, Jasmer DP (2001) Cathepsin B-like cysteine proteases confer intestinal cysteine protease activity in *Haemonchus contortus*. J Biol Chem 276:2928–2934
29. Baig S, Damian RT, Peterson DS (2002) A novel cathepsin B active site motif is shared by helminth bloodfeeders. Exp Parasitol 101:83–89
30. Choi YJ, Ghedin E, Berriman M et al (2011) A deep sequencing approach to comparatively analyze the transcriptome of lifecycle stages of the filarial worm Brugia malayi. PLoS Negl Trop Dis 5:e1409
31. Yang Y, Qin W, Wei H et al (2011) Characterization of cathepsin B proteinase (AcCP-2) in eggs and larvae stages of hookworm *Ancylostoma caninum*. Exp Parasitol 129:215–220
32. Ranjit N, Zhan B, Stenzel DJ et al (2008) A family of cathepsin B cysteine proteases expressed in the gut of the human hookworm, *Necator americanus*. Mol Biochem Parasitol 160:90–99
33. Zhou Y, Zheng H, Chen Y et al (2009) The *Schistosoma japonicum* genome reveals features of host-parasite interplay. Nature 460:345–351
34. Cho PY, Lee MJ, Kim TI et al (2006) Expressed sequence tag analysis of adult *Clonorchis sinensis*, the Chinese liver fluke. Parasitol Res 99:602–608
35. Cho PY, Kim TI, Whang SM et al (2008) Gene expression profile of *Clonorchis sinensis* metacercariae. Parasitol Res 102:277–282
36. Li S, Chung YB, Chung BS et al (2004) The involvement of the cysteine proteases of *Clonorchis sinensis* metacrcariae in excystment. Parasitol Res 93:36–40
37. Kim TI, Na BK, Hong SJ (2009) Functional genes and proteins of *Clonorchis sinensis*. Korean J Parasitol 47:S59–S68
38. Wang X, Chen W, Huang Y et al (2011) The draft genome of the carcinogenic human liver fluke *Clonorchis sinensis*. Genome Biol 12:R107
39. Cancela M, Ruétalo N, Dell'Oca N et al (2010) Survey of transcripts expressed by the invasive juvenile stage of the liver fluke *Fasciola hepatica*. BMC Genom 11:227

40. McVeigh P, Maule AG, Dalton JP et al (2012) *Fasciola hepatica* virulence-associated cysteine peptidases: a systems biology perspective. Microbes Infect 14:301–310
41. Sripa J, Laha T, To J et al (2010) Secreted cysteine proteases of the carcinogenic liver fluke, *Opisthorchis viverrini*: regulation of cathepsin F activation by autocatalysis and trans-processing by cathepsin B. Cell Microbiol 12:781–795
42. Na BK, Kim SH, Lee EG et al (2006) Critical roles for excretory-secretory cysteine proteases during tissue invasion of *Paragonimus westermani* newly excysted metacercariae. Cell Microbiol 8:1034–1046
43. Sako Y, Nakaya K, Ito A (2011) *Echinococcus multilocularis*: identification and functional characterization of cathepsin B-like peptidases from metacestode. Exp Parasitol 127:693–701
44. Sako Y, Yamasaki H, Nakaya K et al (2007) Cloning and characterization of cathepsin L-like peptidases of *Echinococcus multilocularis* metacestodes. Mol Biochem Parasitol 154:181–189
45. Baig S, Damian RT, Molinari JL et al (2005) Purification and characterization of a metacestode cysteine proteinase from *Taenia solium* involved in the breakdown of human IgG. Parasitology 131:411–416
46. Zimic M, Pajuelo M, Rueda D et al (2009) Utility of a protein fraction with cathepsin L-Like activity purified from cysticercus fluid of *Taenia solium* in the diagnosis of human cysticercosis. Am J Trop Med Hyg 80:964–970
47. Liu LN, Cui J, Zhang X et al (2013) Analysis of structures, functions, and epitopes of cysteine protease from *Spirometra erinaceieuropaei* spargana. Biomed Res Int 2013:198250
48. Lai SC, Jiang ST, Chen KM et al (2005) Matrix metalloproteinases activity demonstrated in the infective stage of the nematodes, *Angiostrongylus cantonensis*. Parasitol Res 97:466–471
49. Williamson AL, Lustigman S, Oksov Y et al (2006) *Ancylostoma caninum* MTP-1, an Astacin-like metalloprotease secreted by infective hookworm larvae, is involved in tissue migration. Infect Immun 74:961–967
50. Mendez S, Zhan B, Goud G et al (2005) Effect of combining the larval antigens *Ancylostoma* secreted protein 2 (ASP-2) and metalloprotease 1 (MTP-1) in protecting hamsters against hookworm infection and disease caused by *Ancylostoma ceylanicum*. Vaccine 23:3123–3130
51. Pokharel DR, Rai R, Kumar P et al (2006) Tissue localization of collagenase and leucine aminopeptidase in the bovine filarial parasite *Setaria cervi*. Filaria J 5:7
52. Hasnain SZ, McGuckin MA, Grencis RK et al (2012) Serine protease(s) secreted by the nematode *Trichuris muris* degrade the mucus barrier. PLoS Negl Trop Dis 6:e1856
53. Toubarro D, Lucena-Robles M, Nascimento G et al (2009) An apoptosis-inducing serine protease secreted by the entomopathogenic nematode *Steinernema carpocapsae*. Int J Parasitol 39:1319–1330
54. Toubarro D, Lucena-Robles M, Nascimento G et al (2010) Serine protease-mediated host invasion by the parasitic nematode *Steinernema carpocapsae*. J Biol Chem 285:30666–30675
55. Todorova VK (2000) Proteolytic enzymes secreted by larval stage of the parasitic nematode *Trichinella spiralis*. Folia Parasitol (Praha) 47:141–145
56. Sajid M, McKerrow JH (2002) Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 120:1–21
57. Brindley PJ, Mitreva M, Ghedin E et al (2009) Helminth genomics: the implications for human health. PLoS Negl Trop Dis 3:e538
58. Holroyd N, Sanchez-Flores A (2012) Producing parasitic helminth reference and draft genomes at the Wellcome Trust Sanger Institute. Parasite Immunol 34:100–107
59. Williamson AL, Lecchi P, Turk BE et al (2004) A multi-enzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. J Biol Chem 279:35950–35957
60. Mulvenna J, Sripa B, Brindley PJ et al (2010) The secreted and surface proteomes of the adult stage of the carcinogenic human liver fluke *Opisthorchis viverrini*. Proteomics 10:1063–1078
61. Hewitson JP, Maizels RM (2014) Vaccination against helminth parasite infections. Expert Rev Vaccines 13:473–487

62. Wang S, Wei W, Luo X et al (2015) Comparative genomic analysis of aspartic proteases in eight parasitic platyhelminths: insights into functions and evolution. Gene 559:52–61
63. Hu FY, Zhao JH, Hu XC et al (2009) Bioinformatics analysis of the full-length cathepsin D-like aspartic protease gene from *Clonorchis sinensis* [J]. J Univ South China (Medical Edition) 1
64. Vermeire JJ, Lantz LD, Caffrey CR et al (2012) Cure of hookworm infection with a cysteine protease inhibitor. PLoS Negl Trop Dis 6:e1680
65. Qu ZG, Ma XT, Li WH et al (2015) Molecular characterization of a cathepsin F-like protease in *Trichinella spiralis*. Parasit Vectors 8:1–10
66. Foth BJ, Tsai IJ, Reid AJ et al (2014) Whipworm genome and dual-species transcriptome analyses provide molecular insights into an intimate host-parasite interaction. Nat Genet 46:693–700
67. Yan HB, Lou ZZ, Li L et al (2014) Genome-wide analysis of regulatory proteases sequences identified through bioinformatics data mining in *Taenia solium*. BMC Genom 15:1
68. Shareef PA, Abidi SM (2014) Cysteine protease is a major component in the excretory/secretory products of *Euclinostomum heterostomum* (Digenea: *Clinostomidae*). Parasitol Res 113:65–71
69. Chen W, Wang X, Lv X et al (2014) Characterization of the secreted cathepsin B cysteine proteases family of the carcinogenic liver fluke *Clonorchis sinensis*. Parasitol Res 113:3409–3418
70. Sajid M, McKerrow JH, Hansell E et al (2003) Functional expression and characterization of *Schistosoma mansoni* cathepsin B and its trans-activation by an endogenous asparaginyl endopeptidase. Mol Biochem Parasitol 131:65–75
71. Skelly PJ, Shoemaker CB (2001) *Schistosoma mansoni* proteases Sm31 (cathepsin B) and Sm32 (legumain) are expressed in the cecum and protonephridia of cercariae. J Parasitol 87 (5):1218–1221
72. Figueiredo BC, Ricci ND, de Assis NR et al (2015) Kicking in the guts: *Schistosoma mansoni* digestive tract proteins are potential candidates for vaccine development. Front Immunol 6:22
73. Murkin AS, Moynihan MM (2014) Transition-state-guided drug design for treatment of parasitic neglected tropical diseases. Curr Med Chem 21:1781–1793
74. Fonseca NC, da Cruz LF, da Silva Villela F et al (2015) Synthesis of a sugar-based thiosemicarbazone series and structure-activity relationship versus the parasite cysteine proteases rhodesain, cruzain, and *Schistosoma mansoni* cathepsin B1. Antimicrob Agents Chemother 59:2666–2677
75. Jones BD, Tochowicz A, Tang Y et al (2015) Synthesis and evaluation of oxyguanidine analogues of the cysteine protease inhibitor WRR-483 against cruzain. ACS Med Chem Lett 7:77–82
76. Kang JM, Ju HL, Ju JW et al (2012) Comparative biochemical and functional properties of two leucine aminopeptidases of *Clonorchis sinensis*. Mol Biochem Parasitol 182:17–26
77. Zheng M, Hu K, Liu W et al (2013) Proteomic analysis of different period excretory secretory products from *Clonorchis sinensis* adult worms: molecular characterization, immunolocalization, and serological reactivity of two excretory secretory antigens—methionine aminopeptidase 2 and acid phosphatase. Parasitol Res 112:1287–1297
78. Liu S, Cai P, Piao X et al (2014) Expression profile of the *Schistosoma japonicum* degradome reveals differential protease expression patterns and potential anti-schistosomal intervention targets. PLoS Comput Biol 10:e1003856
79. Bruschi F, Pinto B (2013) The significance of matrix metalloproteinases in parasitic infections involving the central nervous system. Pathogens 2:105–129
80. Ondrovics M, Silbermayr K, Mitreva M et al (2013) Proteomic analysis of *Oesophagostomum dentatum* (Nematoda) during larval transition, and the effects of hydrolase inhibitors on development. PLoS ONE 8:e63955

81. Adisakwattana P, Nuamtanong S, Yenchitsomanus PT et al (2012) Degradation of human matrix metalloprotease-9 by secretory metalloproteases of *Angiostrongylus cantonensis* infective stage. Southeast Asian J Trop Med Public Health 43:1105–1113
82. Greaves D, Coggle S, Pollard C et al (2013) *Strongyloides stercoralis* infection. BMJ 347:f4610
83. Ros-Moreno RM, Vázquez-López C, Giménez-Pardo C et al (2000) A study of proteases throughout the life cycle of *Trichinella spiralis*. Folia Parasitol (Praha) 47:49–54
84. Wang B, Wang ZQ, Jin J, Ren HJ, Liu LN, Cui J (2013) Cloning, expression and characterization of a *Trichinella spiralis* serine protease gene encoding a 35.5 kDa protein. Exp Parasitol 134:148–154
85. Morris SR, Sakanari JA (1994) Characterization of the serine protease and serine protease inhibitor from the tissue-penetrating nematode *Anisakis simplex*. J Biol Chem 269:27650–27656
86. Poole CB, Jin J, McReynolds LA et al (2003) Cloning and biochemical characterization of blisterase, a subtilisin-like convertase from the filarial parasite, *Onchocerca volvulus*. J Biol Chem 278:36183–36190
87. Kong Y, Chung YB, Cho SY et al (1994) Characterization of three neutral proteases of *Spirometra mansoni* plerocercoid. Parasitology 108:359–368
88. Lorenzo C, Salinas G, Brugnini A et al (2003) *Echinococcus granulosus* antigen 5 is closely related to proteases of the trypsin family. Biochem J 369:191–198
89. Rueda A, Sifuentes C, Gilman RH et al (2011) TsAg5, a *Taenia solium* cysticercus protein with a marginal trypsin-like activity in the diagnosis of human neurocysticercosis. Mol Biochem Parasitol 180:115–119
90. Carmona C, McGonigle S, Dowd AJ et al (1994) A dipeptidylpeptidase secreted by *Fasciola hepatica*. Parasitology 109:113–118
91. Mohamed SA, Fahmy AS, Mohamed TM et al (2005) Proteases in egg, miracidium and adult of *Fasciola gigantica*. Characterization of serine and cysteine proteases from adult. Comp Biochem Physiol B: Biochem Mol Biol 142:192–200
92. Dvořák J, Mashiyama ST, Braschi S et al (2008) Differential use of protease families for invasion by schistosome cercariae. Biochimie 90:345–358
93. Young ND, Jex AR, Li B et al (2012) Whole-genome sequence of *Schistosoma haematobium*. Nat Genet 44:221–225
94. Ingram JR, Rafi SB, Eroy-Reveles AA et al (2012) Investigation of the proteolytic functions of an expanded cercarial elastase gene family in *Schistosoma mansoni*. PLoS Negl Trop Dis 6:e1589
95. Aslam A, Quinn P, McIntosh RS et al (2008) Proteases from *Schistosoma mansoni* cercariae cleaves IgE at solvent exposed interdomain regions. Mol Immunol 45:567–574

Natural Polyphenols as Prospective Inhibitors for MMPs Remodeling in Human Diseases

13

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Abstract

ECM composition and turnover is carefully regulated by various proteases, among which the most important are matrix metalloproteinases (MMPs). Many MMP inhibitors have been followed as clinical applicants for targeting MMPs, but most of these studies have failed during clinical trials due to side effects resulting from broad-spectrum inhibition and inefficiency of inhibitors and regulation of MMPs during diseases is very complicated. Development of MMPs inhibitors from natural products showed beneficial effects in various diseases like cancer, cardiovascular, skin-aging, neurodegenerative diseases, and other. These natural products include secondary metabolites of edible and nonedible plants, such as polyphenols, monophenols, and others. Polyphenols are ample micronutrients in our diet and its beneficial effects depend on the quantity consumed and on their bioavailability. In this chapter, the various types of polyphenols, such as flavonoids, curcuminoids, phenolic acids, etc. and their biological activity to regulate the MMPs in cancer, cardiovascular and other diseases are reviewed.

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1 Introduction

Matrix metalloproteinases (MMPs), major players of extracellular matrix (ECM) biology, are involved in different biological processes at extracellular, intracellular, and at the cellular membrane interfaces [1]. As discussed earlier, MMPs are a family of 23 structurally correlated, zinc-dependent, and multifunctional proteases that are present in both soluble and membrane bound states. MMPs are responsible for the degradation and turnover of ECM components [2, 3]. MMPs are secreted as inactive zymogen having four conserved modular structures namely a pro-peptide and catalytic domain; hinge region; and a hemopexin-like C-terminal domain [4]. MMP genes have been found in humans and have roles in several physiological and pathological processes, mainly tissue remodeling, wound healing, embryonic development, and bone resorption [5, 6]. Although ECM is always undergoing remodeling process thereby regulating cell differentiation but dysregulation of ECM dynamics may also lead to tissue fibrosis and cancer [7, 8]. MMPs are found to be upregulated in many diseases including cancer, inflammatory, and vascular diseases, where they are mainly responsible for reorganization of tissues, wound healing, cancer cell invasion, etc. [9]. Figure 1 briefly displays the list of different diseases associated with improper upregulation of different classes of MMPs in humans.

The recent advances in the MMP domain has gone together with the expansion of different MMP inhibitors where targeting MMPs as therapeutics has been expanded significantly in the past decade. Recent advances in drug delivery methods and the expanded knowledge of compounds binding outside the active sites further contributes to the progress of the field [10].

Current literature reports suggest that synthetic MMP inhibitors shows disappointing results in clinical trials and very few inhibitors only accomplish into new therapies [11]. At present, there is only one approved therapeutic agent for inhibiting MMPs in cancerous cells, doxycycline (Periostat®), which is chemically modified tetracycline antibiotic and inhibit MMPs by a mechanism unrelated to its antimicrobial effects and most useful against the collagenases, but the drug (doxycycline) concentration's lower half is enough for inhibition [12].

This leads to a need of exploring natural sources to discover MMP inhibitors with better mechanism of action and reduced associated side effects.

Fig. 1 Different classes of MMPs and their involvement in human diseases

In this chapter, we discuss the natural polyphenols-based MMP inhibitors, their sources, structure, and mechanism of action in detail. We also highlight the role of MMPs in major human diseases including cardiovascular diseases and cancer, and clinical trials for MMP inhibitors.

2 Polyphenols and Their Classification

Polyphenols, groups of polyhydroxylated phytochemicals, are structured molecules with several hydroxyl groups present on aromatic rings and abundant micronutrients in our diet which are having antioxidant properties [13]. They are found ubiquitously in plants; mostly in edible plants (known as secondary metabolites) and their great dietary consumption in our body decreased risk of many diseases, such as various form of cancer, cardiovascular, and neurodegenerative diseases [14]. The basic properties of polyphenols are that they are water soluble compounds and having 500–4000 Da in weight with more than 12 phenolic hydroxyl groups and 5–7 aromatic rings per 1000 Da. Polyphenols can be classified into many classes according their structures based on the number of phenolic rings present, the

structure elements binding the rings and other substituents linked to the rings. There are three major classes of polyphenols; flavonoids, stilbenoids, and phenolic acids. These polyphenols are found in plants as free aglycones or in the esterified form with glucose and other carbohydrates [15]. There are some other polyphenols such as dietary polyphenols; these are isolated from plants like berries, apple, cherries citrus, etc. Vegetables like onion, herbs, roots, wine, black tea, and spices like turmeric, etc. Polyphenols are potential antioxidants, pro-oxidants, antitoxic, antibacterial, antiviral, antiallergic, anti-inflammatory, anti-ischemic, free radicals, lipid lowering, enzyme inhibitors, anti-mutagenic, multidrug resistant, and anti-carcinogenic and they reduce toxicity, cytotoxicity, oxidative stress, and apoptosis [16]. Research on animals and in vitro has been extensively demonstrated and various positive effects of polyphenols were analyzed as human health benefits against many diseases. Figure 2 shows the broad classification of polyphenols and the major polyphenols are discussed in detail in the following sections.

2.1 Flavonoids

This class of polyphenols contains two or more number of aromatic rings with each ring having one or more phenolic hydroxyl groups, and connected by a carbon bridge. One ring is connected to the second by a carbon bridge of three carbon atoms. The formed structure becomes cyclic as a result of the connection of two

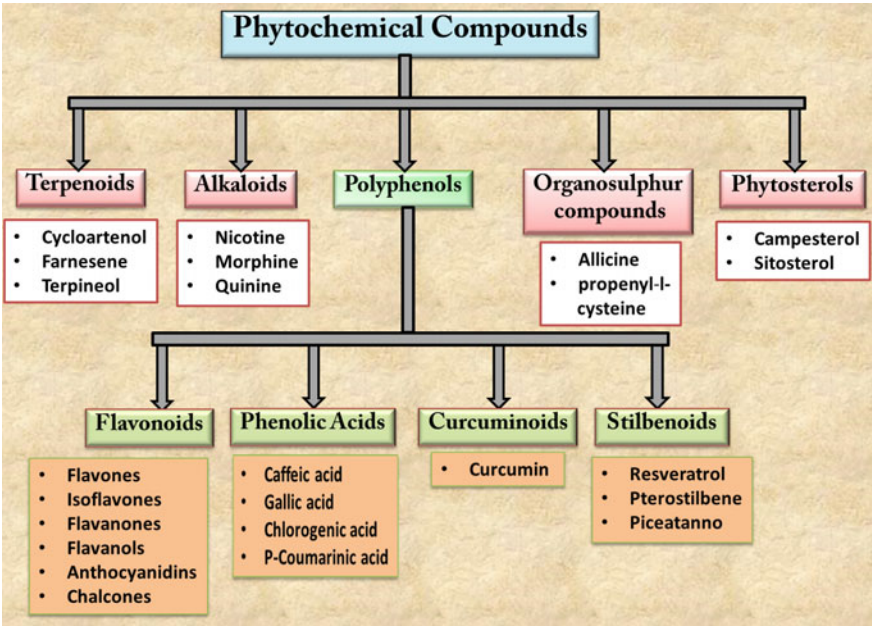

Fig. 2 Different classes of MMPs and their involvement in human diseases

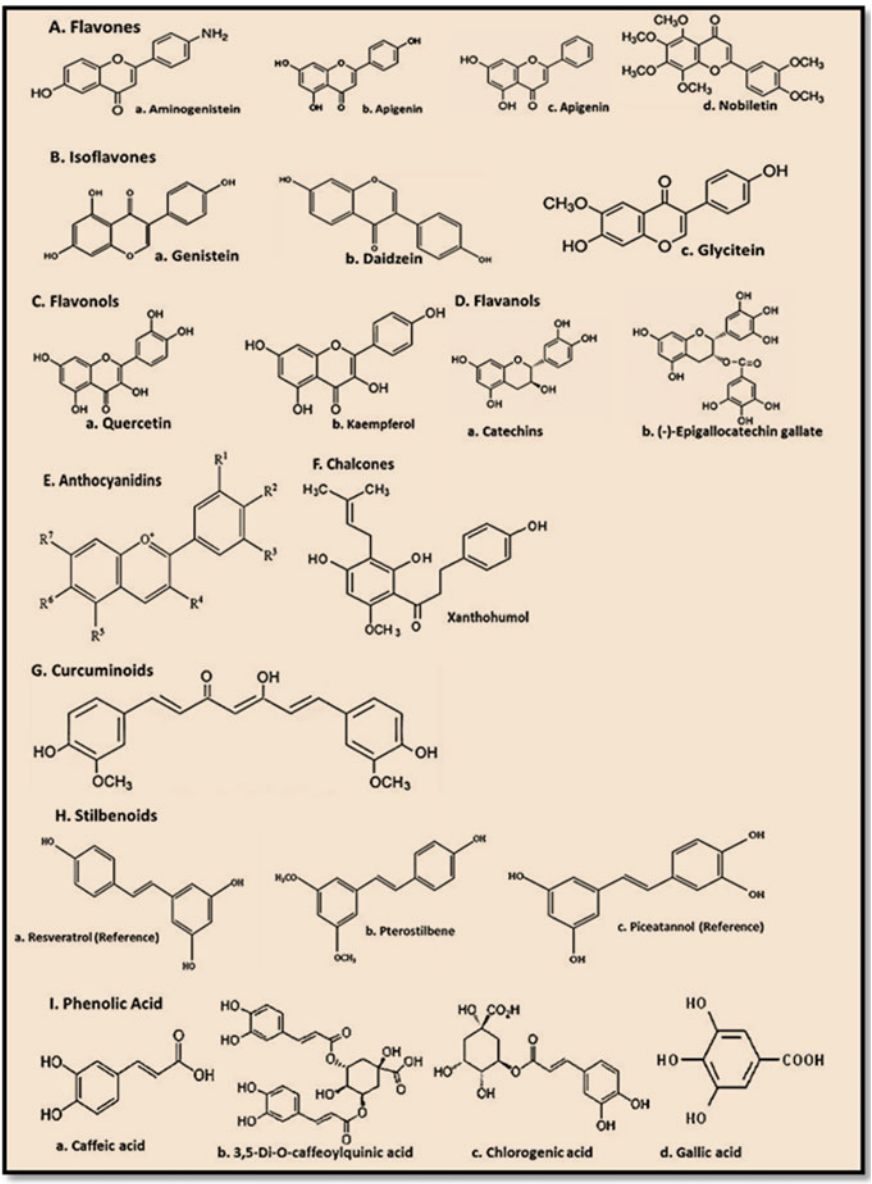

Fig. 3 Chemical structures of different subtypes of polyphenols

3-carbon chain and most of the flavonoids carry this type of phenylbenzenepyrane structure. Flavonoids are further subdivided into seven classes; Flavones, Isoflavones, Flavonones, Flavonols, Flavanols, Anthocyanidins, and Chalcones, and their structures are shown in Fig. 3.

2.1.1 Flavones

Flavones are those flavonoids when the two or more aromatics rings are adhered on a carbon bridge of two carbon atoms. These are the methylated compounds and they are generally more active than non-methylated compounds. There are 10 commonly used flavones which are used to regulate various diseases following as:

(a) **Aminogenistein** is also known as 4′-Amino-6-hydroxyflavone and it is the inhibitor of p56lck protein-tyrosine kinase [17].
(b) **Apigenin** is also known as 4′,5,7-Trihydroxyflavone and it is synthetic flavonoid which is having chemopreventive and antitumor activity. Apigenin inhibits expression of MMP-9 TNFα- triggered activation of Akt, signaling of p38 MAPK, JNK, and HIF-1, and vascular endothelial growth factor expression [18].
(c) **Chrysin** is also known as 5,7-Dihydroxyflavone. It is an antioxidant flavonoid which displays anti-inflammatory and antitumor properties. It also inhibits HIF-1α but induces apoptosis. Chrysin showed the anticancer effect by regulating MMP-9 expression through suppression of AP-1 activity through blockage of the JNK1/2 and ERK1/2 signaling pathways in gastric cancer AGS cells [19].
(d) **Nobiletin** is a citrus polymethoxy flavonoid and a novel MEK inhibitor and displays antitumor metastasis in human fibrosarcoma cells. It is an antioxidant and anti-inflammatory compound and inhibits mitogen-activated protein kinase MEK. It also suppresses the expression of MMP 1, 3, and 9. It is also shown to suppress the NF-κB transcriptional activation, nitric oxide (NO) and PGE2 production, inducible nitric oxide (NOS; NOS II), and cyclooxygenase-2 (COX-2) expression [20].

2.1.2 Isoflavones

Isoflavones are flavonoids with structural similarities with estrogens and not similar to steroids. Hydroxyl groups are present at the positions 7 and 4′ in a configuration analogous like estradiol molecule. They are most found in the part of leguminous in plants. The main source of isoflavones in the human diet is soya and its processed products. The three main molecules are genistein, daidzein, and glycitein, present in ratio of 1:1:0.2 and found in four forms: aglycone, 7-O-glucoside, 7-O-glucoside, 6-O-acetyl-, and 6-O-malonyl-7-O-glucoside [21].

(a) **Genistein** is also known as 6,7,4′-trihydroxyisoflavone. It is a major metabolite of daidzein. It inhibits tyrosine protein kinase thereby inhibiting the phosphorylation of EGFR kinase, topoisomerase II, and tumor cell proliferation. It results in cell cycle arrest and apoptosis. It is also a direct inhibitor in adipocytes for insulin-induced glucose uptake.
(b) **Daidzein** is also known as 4′,7-Dihydroxyisoflavone and a synthetic polyphenol. It shows anti-inflammatory effect as it is an inactive analog of tyrosine kinase inhibitor.

(c) **Glycitein** is also known as 4′,7-Dihydroxy-6-methoxyisoflavone that mainly accounts for the total isoflavones present in soy food products. It is a phytoestrogen with weak estrogenic activity as compared to the other soy isoflavones. It has shown to inhibit Jurkat cell invasion at comparable levels of genistein. Genistein and glycitein have also shown to downregulate the MMP-13 proteolytic activity and MMP-8 expression significantly. It is also reported that the combination of genistein and caffeine could block G2/M arrest, but could not block the inhibition of invasion by genistein and glycitein [22].

2.1.3 Flavonols

Flavonols are the subclass of flavonoids, structurally similar to flavones, having a double bond between positions 2 and 3, andn oxygen of a ketone group in position 4, but they do differ in the presence of a hydroxyl group at the position 3 and hence, flavonols' skeleton is 3-hydroxyflavone. Flavonols are present in high concentrations in apples, apricots, beans, broad beans, broccoli, cherry tomatoes, chives, cranberries, kale, leeks, pear, onions, red grapes, sweet cherries, and white currants [23]. Glycosides of quercetin, kaempferol, myricetin, and isorhamnetin mainly represent the flavonols. Derivatives of quercetin and kaempferol are commonly found ubiquitous compounds, and both the molecules have around 280–350 different glycosidic combinations, respectively [24].

(a) **Quercetin** is also known as 3,3′,4′,5,7-Pentahydroxyflavone. $2H_2O$. It is found in different plant and food items including red wine, onions, green tea, apple, and berries. Buckwheat tea is rich in quercetin amount. Epidemiological studies have shown a contrasting association between the consumption of diet rich in flavonoids and atherosclerosis risk. Gliomas are considered as the most common and malignant primary brain tumors. They are associated with a poor prognosis despite the presence of various therapeutic options. Recent studies showed that the treatment of glioblastoma and glioma cell lines with quercetin inhibits cell viability in a dose-dependent manner [25].

(b) **Kaempferol** is also known as 3,4′,5,7-Tetrahydroxyflavone, an antioxidant flavonoid. Studies have shown that kaempferol protects against cardiovascular disease and cancer. It induces apoptosis and reversible inhibitor of fatty acid synthase [FAS].

2.1.4 Flavanols

Flavanols are also known as flavan-3-ols are the derivatives of flavones having 2-phenyl-3, 4-dihydro-2H-chromen-3-ol skeleton. Flavanols majorly includes catechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and proanthocyanidins.

(a) **Catechins** is also known as (±)-3,3′,4′,5,7-Flavanpentol and it is antioxidant flavonoid and free scavenger which shows chemopreventive and antitumor properties [26].

(b) **(−)-Epigallocatechin gallate (EGCG)** or epigallocatechin-3-gallate is the ester of epigallocatechin and gallic acid and isolated from green tea. Green tea has many beneficial actions including anti-mutagenic, antibacterial, hypocholesterolemic, antioxidant, and cancer preventive properties. It is also involved in cell cycle regulation, apoptotic cellular death, angiogenesis, and metastasis. It inhibits MAP kinase signaling, angiogenesis telomerase activity, and DNA methyltransferase thereby preventing DNA damage [27]. Angiogenesis is a life-threatening event for tumor growth and metastasis [28]. EGCG functions as a pro-oxidant and directly interacts with the plasma membrane components thereby regulating signal transduction pathways and displays the beneficial biological actions [29]. EGCG competitively inhibits the enzyme dihydrofolate reductase.

2.1.5 Anthocyanidins

Anthocyanidins are the sugar-free counterparts of anthocyanins. Chloride is mostly the counterion of the flavylium cation and results in the dissimilarity of the anthocyanidins differ from other flavonoids. The anti-metastatic effects, major anthocyanins extracted from black rice have shown to inhibit the invasion and motility of SKHep-1 cancer cells. This effect was shown to be associated with a reduced expression of MMP-9 and urokinase-type plasminogen activator (u-PA). These major anthocyanins—Peonidin 3-glucoside and cyanidin 3-glucoside, also inhibited the DNA binding activity and AP-1 nuclear translocation. As reported in various studies, these compounds also exert an inhibitory effect of cell invasion in various cancer cells including SCC-4, Huh-7, and HeLa [30]. Most common examples of anthocyanidins are Cyanidin chloride, Delphinidin chloride, Malvidin chloride, Pelargonidin chloride, and Peonidin chloride.

2.1.6 Chalcones

Chalcones are subtype of flavonoids and plant polyphenols. They are potent antioxidant and anti-inflammatory agents. Xanthohumol is the most common chalcones falvonoids which is isolated from hops. It is a potential inhibitor of diacylglycerol acetyltransferase and s DNA polymerases and human P450 enzymes [31]. It has anti-proliferative and cytotoxic effects in human carcinoma cell lines. It induces quinone reductase to inhibit the expression of HIF-1α and VEGF in hypoxic environment [32]. Butein is another subtype of chalcones which is activator of human decetylase SIRT1 and it inhibits aromatase which directly inhibits IKK and shows chemopreventive properties [33].

2.2 Curcuminoids

Curcuminoids are natural phenols, structurally linear diarylheptanoids, present in turmeric and mustard and gives a prominent yellow color to these plants. Curcumin is a natural polyphenol present in the rhizomes of turmeric plant, and well known

for its antimicrobial, antifungal, anti-inflammatory, antioxidant, and anticarcinogenic characteristics [34–37]. Curcumin has been a part of the diet from more than 200 years in many countries demonstrates its safe consumption [38, 39]. Curcumin has shown to display anti-metastatic properties in cancer cells by regulating MMP and TIMP expressions.

2.3 Phenolic Acids

Phenolic acids are derived from benzoic acids and are generally characterized into two main subparts: Hydroxycinnamic acids and hydroxybenzoic acids. They are rarely found in the free form, except in processed food that has undergone various processing steps including freezing, sterilization, or fermentation. The bound forms of phenolic acids are either glycosylated or esters of quinic acid, shikimic acid, and tartaric acid. The most common representative isolated phenolic compounds are ferulic acid, chlorogenic acid, gallic acid, caffeic acid, gentisic acid, and p-coumaric acid.

2.3.1 Caffeic Acid

Caffeic acid or 3-(3,4-Dihydroxyphenyl)-2-propenoic acid is a synthetic polyphenol and displays antitumor, antiviral, antioxidant, and anti-inflammatory effects. It is the chief inhibitor of 5- and 12-lipoxygenase. It is a potential inhibitor for leukotriene biosynthesis [40]. Caffeic acid derivatives also display in vitro and in vivo anti-inflammatory properties.

2.3.2 3,5-Di-O-Caffeoylquinic Acid

3,5-Di-O-caffeoylquinic acid is also known as 3,5-CQA; Isochlorogenic acid. Its source is *Cynara scolymus* and displays antioxidant and anti-proliferative activities. *In vitro* antioxidative effects and tyrosinase inhibitory activities of hydroxycinnamoyl derivatives in green coffee beans have been reported [41].

2.3.3 Chlorogenic Acid

Chlorogenic acid is also known as 3-O-Caffeoylquinic acid. It is an analog of caffeic acid and shows antioxidant, analgesic, antipyretic, and chemopreventive activity [42].

2.3.4 Gallic Acid

Gallic Acid is also known as 3,4,5-trihydroxybenzoic acid. It is universally present as a component of tannins, known as gallotannins. Few natural products are rich in gallic acid content like bananas, strawberries, pineapples, lemons, gallnuts, red and white wines, tea leaves, apple peels, and oak bark. Gallic acid has antibacterial, antiviral, anti-inflammatory properties along with anti-melanogenic activity with the inhibition of tyrosinase activity [43].

2.4 Stilbenoids

Stilbenoids are an eminent subclass of natural polyphenols, they are chemically characterized because of their structure which is 1,2-diphenylethylene. Stilbenoids are mostly stress metabolites, produced in plants and shows antifungal phytoalexins. They are reported as potential cancer chemo-protective compounds, which are able to inhibit number of cellular events, involved in carcinogenesis, and includes tumor initiation to progression [44].

2.4.1 Resveratrol

Resveratrol, also known as trans-3,4′,5-Trihydroxystilbene, is a polyphenol present in red wine having range of unique biological properties. It is a natural polyphenolic compound present in various types of plants and food products. The beneficial properties include antioxidant, antithrombogenic, anti-inflammatory, cardioprotective, neuroprotective, and cancer preventive activities. MMPs appear to be responsible for much of the ECM degradation observed in cancer progression, aging, and inflammation. Resveratrol in combination of Zn is reported as an enhancer of the intracellular altered free zinc in epithelial cells of human prostrate [45]. Additionally, resveratrol is shown to influence Zn homeostasis via enhancing intracellular zinc accumulation [46].

2.4.2 Piceatannol

Piceatannol is also known as 3, 4, 3′, 5′-Tetrahydroxy-trans-stilbene. Piceatannol occurs in both natural as well as in synthetic forms and found in in berries, peanuts, sugarcane, wine, and grape skins. It is a biotransformed metabolite from resveratrol, and displays anti-inflammatory, anticancerous, and cardioprotective effects. Its pharmacological properties suggests that it might be a potential biomolecule for prostate cancer prevention; however, more detailed research on its bioavailability and toxicity is required in humans [47].

2.4.3 Pterostilbene

Pterostilbene is also known as trans-3,5-dimethoxy-4-hydroxystilbene and is a well-known antioxidant that is mainly found in barriers and grapes. It also displays chemopreventive and potential therapeutic effects for different types of cancers [48]. Smooth muscle cells migration and MMP-2 activation are two important processes in atherosclerosis [49]. It is also further demonstrated to play a novel role in the treatment of prostate cancer and cardiac diseases.

3 Targeting MMPs in Different Human Diseases

MMPs comprise the major share of the cellular microenvironment and hence, become an important player in the pathogenesis of numerous diseased states as well as a promising therapeutic target.

3.1 Cancer

Cancer research generally involves the study of mutations in proliferative and survival pathways in the cancerous cells. Cancer therapies include the destruction of tumors, but the focus has recently been diverted toward the microenvironment of cancer cells, mainly ECM and its components. Studies have shown that MMPs are present in mostly all human cancers where their expression has been witnessed by the fibroblasts cells thereby promoting angiogenesis, tumor growth, and metastasis [50]. Based on the preclinical studies, MMPs have been found as a prevailing factor for cancer progression and emerged as a potential therapeutic target for treating cancers [51]. Studies on MMPs and TIMPS in cancer research have provided a novel platform for developing anti-metastatic drugs.

Apart from the chemical-based MMP inhibitors, dietary polyphenols has been examined for their anticancerous potential in wide range of tumors [52]. Dietary polyphenols are nontoxic natural components significantly modulating signaling pathways. They are considered as idyllic and effective chemopreventive agents as they are involved in epigenetic changes, microRNA expressions and posttranslational modifications associated with the cancer cells [53].

Genistein inhibited on MMP-2 and -9 gene expressions in prostate cancer in vitro in PC-3 and DU 145 cell lines. Genistein reduced significantly the expression of MMP-9 in both cell lines but only diminished expression of MMP-2 in DU-145 cell line [54]. The expression of various MMPs in human prostate cancer cells was shown to be inhibited by genistein treatment [55].

In brain tumor, glycitein was reported as potential inhibitor of the expression of MMP-3 and MMP-9 at transcriptional and translational levels in PMA-stimulated U87MG human astroglioma cells. The glycitein-mediated inhibition of MMP-3 and MMP-9 also suppressed the in vitro invasiveness of glioma cells. Few mechanistic mechanism of glycitein revealed that it also suppresses the DNA binding and other transcriptional activities of NFκB and AP-1, both transcription factors are very important for MMP-3 or MMP-9 gene expression. Additionally, it controls PMA-induced phosphorylation of three different MAP kinases, which are upstream signaling molecules in NFκB and AP-1 activities and MMPs gene expressions in glioma cells. Therefore, glycitein-based inhibition of MMP-3 and MMP-9 expression may do have therapeutic regulatory effects for invasiveness of malignant gliomas.

Quercetin is a competitive inhibitor of MMP-9 and could downregulate the expression of MMP-9 and TGF-β1, which plays a crucial role in A549 apoptosis. Lung cancer growth and metastasis of A549 lung cancer cells have been shown to be inhibited by quercetin induction with the activation of apoptosis mediated events. It was a reversible competitive inhibitor of MMP-9 and with the increase in quercetin concentration, the levels of MMP-9 and TGF-β1 were decreased, and the number of tumor cells on wear filter membrane was reduced. The combination of quercetin (at low concentrations) with TIMP-1 showed synergistic inhibitory effect on the A549 cells growth [58]. The protein levels of p-AKT, p-ERK, Bcl-2, MMP-9, and fibronectin were also shown to be significantly reduced by quercetin

treatment. Hence, Quercetin also might be a potential candidate for glioma therapeutics [56].

Pterostilbene, polyphenol found in berries and grapes, is known for its antioxidative and chemopreventive potential. It has shown to induce prostrate-specific antigens in human androgen-responsive LNCaP cells thereby leading to apoptosis and cell cycle arrest [57]. It significantly suppressed 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced invasion, migration, and metastasis of human hepatoma cells (HepG2 cells) where TPA-treated HepG2 cells displayed the increased enzyme activity, higher MMP-9 protein, and mRNA levels were found to be blocked by pterostilbene [58]. The MMP-2 and urokinase-type plasminogen activator (u-PA) activities as well as protein levels were also found to be inhibited in the presence of pterostilbene. These inhibitory effects were associated with the upregulation of MMP-2 tissue inhibitor, plasminogen activator inhibitor-1, and the downregulation of the major transcription factors [59]. Additionally, it was found that TPA-induced expression of vascular endothelial growth factor, epidermal growth factor, and its receptor can also be inhibited by pterostilbene in HepG2 cells [56]. Recently, a study also showed that pterostilbene can also act as a chemopreventive agent in oral cancer metastasis as it significantly inhibited the migration and invasion capacities of SCC-9 cells in vitro.

EGCG inhibits MT1-MMP, resulted in suppressed tumor and inhibited urokinase-type plasminogen activator that is responsible for ECM degradation. The activities of MMP-2 and -9 were also inhibited by EGCG and reduced mRNA expression of MMP-2 and MMP-9 was reported in HT1080 fibrosarcoma cells [60]. EGCG modulates any of NF-κB/MAPK/IGFR/COX-2 signaling pathways and inhibits prostate carcinogenesis, many protein kinases and suppressing the activation of numerous transcription factors which are involved in the pathways [61]. Recent studies showed that EGCG induced prostate cancer cell death by three mechanism; downregulation of ID2 and upregulation and stabilization of p53 [62]. EGCG also stimulated apoptosis with increased expression of CASP-9a (both alone and in combination with cisplatin) splice variants in prostate cancer cells, [63, 64]. EGCG suppressed proinflammatory cytokines and MMPs specially MMP-2 and MMP-9 in prostrate cancer cells, and protects inflammation [65].

Anticancerous property of Gallic acid has been reported in oral tumor, leukemia, and esophageal cancer cells [66]. This polyphenol is reported as potential inhibitor of the tumor growth in DU145 and 22Rv1 prostate cancer xenografts in nude mice [67]. Penta-O-galloyl-beta-D-glucose (5GG), other substitute, and form of Gallic acid induce apoptosis and inhibit fatty acid synthase (FAS) expression, lipopolysaccharide-induced NFκB activation, and by reducing MMP-9 expression, it suppressed cell invasion [68]. 5GG's was found as a novel inhibitor of DNA polymerases where the studies showed that 5GG blocked DNA replication by inducing PCa S-phase arrest and induced G1 arrest via cyclin D1 downregulation [69].

Resveratrol induced the expression of type II collagen and sex-determining region and MMP-9 and the production of sulfated proteoglycans, but it is inhibited the expression of MMP-2 in HTB94 chondrosarcoma cells [70]. It inhibited cancer

metastasis both in vitro and in vivo, likely reduced MMP-9 activity caused by resveratrol. It also suppresses the heme oxygenase mediated NFκB pathway activation and positively downregulated many MMPs expression level, which results reduced lung adenocarcinoma cell metastasis [71]. Resveratrol effects are enhanced further by inhibition of MMP-2 and MMP-9 with MMP inhibitor III. Additionally, it was shown to significantly reduce the phosphorylation of JNK and induced phosphorylation of p38 in HTB94 cells [72].

The anticancerous effects of Piceatannol observed in prostate cancer cells, where it was found that Piceatannol is QR2-dependent and Piceatannol-mediated inhibition of proliferation takes place. The activity of QR2 was observed very less in QR2-knockdown cells relative to QR2 expressing cells. This study suggested prostate cancer prevention by Piceatannol is more recognized than resveratrol [73]. Earlier, there was one more study that showed the inhibitory effect of Piceatannol on the expression of MMP-9 that further reduced the invasive potential of DU145 cells. Piceatannol suppressed MMP-9 activation via Akt-mediated NFκB pathways, resulted inhibition of TNF-α-induced invasion by suppression in DU145 PCa cells [74]. It was also reported that oral dose of Piceatannol inhibits tumor formation and growth, and able to diminished cell colonization in LNCaP prostrate cancer xenografts [75]. Piceatannol is a polyphenol present in fruits including peanuts, rhubarb, sugar cane, wine, berries etc., is a biotransformed metabolite of resveratrol and has been shown to have anticancerous, anti-inflammatory and cardio-preventive effects [76]. Piceatannol has been shown to inhibit MMP-9 activation and decrease the TNF-α-induced invasion of tumor in prostate cancer cell line by Akt-mediated NFκB pathways [74].

Ellagic acid, polyphenolic compound from fruits and berries has anti-mutagenic and antioxidant properties [77]. It has shown to have protease activities against MMP-2 secretion in prostate cancer cell line where nontoxic doses of Ellagic acid was shown to inhibit the invasion and motility of cancer cells. It also significantly decreased the collagenase and gelatinase proteolytic activity in PLS-10 cell line thereby inhibiting the invasive potential of prostate cancer cells [78]. Penta-O-galloyl-beta-D-glucose, a gallic acid derivative, have shown reduced MMP-9 expression and suppressed cancer cell invasion along with G1 arrest and initiation of apoptosis. Mechanistic studies of MMP-9 regulation by Penta-O-galloyl-beta-D-glucose showed reduced EGFR expression thereby suppressing tumor invasion and growth in vivo [79].

Concentration-dependent treatment of kaempferol inhibits the invasion and adhesion of U-2 osteosarcoma (OS) cells. Treatment of kaempferol with optimized dose decreased the DNA binding activity of AP-1, which results in the reduced expression of MMP-2, MMP-9, and uPA. Kaempferol also diminished the MAPK signaling pathways and ERK, JNK, and p38 pathways, resulted in the reduced DNA binding ability of AP-1. Therefore, the expression and enzymatic activities of MMP-2, MMP-9, and uPA is downregulated which contributes in the inhibition of metastasis of U-2 OS cells [80]. This polyphenol also showed the anti-metastatic effects in human tongue squamous cell carcinoma SCC4 cells [81]. It could also inhibit adhesion, migration, and invasion of other cancer cells like MDA-MB-231

human breast carcinoma cells which headed to the reduced activity and expression of MMP-2 and MMP-9. Treated cells with this polyphenol showed the inhibition of the activation of many transcription factors such as activator protein-1 (AP-1) and MAPK signaling pathway. The reduced expression and activity of PMA-induced MMP-9 was studied through suppressing the translocation of protein kinase Cδ (PKCδ) and MAPK signaling pathway in kaempferol treated cancer cells [82]. Taken all kaempferol studies together, it is concluded that this polyphenol could inhibit cancer cell invasion by blocking the PKCδ/MAPK/AP-1 cascade and following MMP-9 expression and its activity, and might act as a therapeutic potential compound for cancer metastasis.

Curcumin, polyphenol from turmeric, has been shown to regulate cell metastasis by downregulating MMP-2 and -9 expressions, and upregulating TIMP-1 and -4 gene expressions in vitro in breast cancer cell lines [83].

3.2 Cardiovascular Diseases

MMPs have been drawn in numerous cardiovascular pathologies and circulating levels of MMPs are found in heart patients with acute myocardial infraction and atherosclerotic plaques. The higher activation of MMPs is seems to be involved in the resistance of the plaque to rupture. The increased expression of MMPs was also observed after coronary angioplasty, even with stent placement. In a murine model of cardiac infarction, the in vivo upregulation of MMP-2 and MMP-9 was showed the maximal expression at 1–2 weeks, persevering to 4 weeks following induction of the infarct by utilizing an activated fluorescent probes upon proteolytic cleavage [84]. With the point of the higher risk gradation and clinical administration of coronary artery disease patients, the enhanced levels of MMP3 in circulating plasma were observed in these patients [85].

In earlier studies, the consumption of many polyphenols has been demonstrated which makes the limits to incidence of cardiac diseases. Daidzein inhibits in vitro casein kinase II activity, but does not hinder MAP2 kinase activity. Activation of casein kinase II by polylysine enhances the activity of MAP2 kinase in fibroblast cells, suggesting that daidzein blocked G1 phase cell cycle progression by inhibiting the action of casein kinase II [86].

Quercetin competitively binds to plasminogen which shows anticlotting abilities and controls the plasmin concentration via urokinase plasminogen activator modulation. Its anti-proliferative effect on vascular smooth muscle cells occurs chiefly by inhibiting JNK and AP-1 signaling pathways [87, 88]. Quercetin acts primarily to modulate Ang II and then reduced ventricular hypertrophy [89, 90]. Quercetin prevents telomere shortening thereby reducing cardiac myocyte apoptosis [91]. Quercetin is also shown to inhibit MMP-9 activity by interacting with the MMP-9 active site [92].

The anti-atherosclerotic property of pterostilbene and its underlying mechanism was reported recently in the rat smooth muscle cell (SMC) A7r9 cell lines. The pterostilbene treatment successively inhibited migration level in A7r9 cell. The

decreased activity and expression of MMP-2 was also found in A7r9 cell by the treatment of pterostilbene. In the MAPK signaling pathway, the upregulated phosphorylation of extracellular-signal-regulated kinase (Erk)1/2 and inhibition of Erk1/2 by pterostilbene and other specific inhibitors may significantly terminated the pterostilbene-dependent decreased expression of MMP-2 and migration capacities. These findings concludes that pterostilbene inhibited Erk1/2 phosphorylation mediated SMC migration and that MMP-2 activation [93].

Prominent in red wine is resveratrol can maintain mitochondrial integrity, reduce insulin like growth factor-1, activate Sir2/SIRT1, and increase lifespan from yeast to mammals [94, 95]. Resveratrol also improves outcome after ischemic episodes in the heart and brain [96] and requires SIRT1 to mediate ischemic protection [97, 98]. Resveratrol stimulates the NAD-dependent deacetylase SIRT1 that is an important subcellular target of resveratrol. SIRT1 is a major modulator of pathways downstream of calorie restraint that produces various beneficial effects on glucose homeostasis and insulin sensitivity thereby affecting the lifespan. The higher sugar level induced significant increases in oxidative stress and inflammation in the hearts and kidneys just after 3 months. Resveratrol was found to attenuated these diabetic cardiorenal pathogenic changes by downregulated the expression of NF-kB, upregulated the expression of Nrf2 and its downstream antioxidants [99]. There is one case study from France where the population of France consumes foods high in saturated fats, yet experiences less cardiovascular pathology, known as the “French paradox” and may be partially explained by their daily consumption of red wine [100]. Many clinical trials are reported of patients with stable coronary artery diseases where the effect of a daily oral intake of 10 mg resveratrol capsule for 3 months showed extreme increase in flow-mediated vasodilation [101].

Anthocyanins are very much involved in the preclusion of cardiovascular diseases which is highly linked to defence against ROS induced stress. Few years back a study was reported where four anthocyanins was extracted from elderberries, they were further transfected into the plasma, lemma, and cytosol of endothelial cells in vitro to directly finding their role and the results were indicated that anthocyanins produce significant oxidative stress protection if it can be directly incorporated into endothelial cells [102].

If normal human consume catechin-rich tea (EGCG), it is positively affects body weight, body-mass-index, body weight, waist circumference, body fat mass, and subcutaneous fat of individuals [103]. There are various epidemiological observations which spotted the association between the ratio of heavy tea consumption and the superficial risk reduction in cardiovascular disease [104]. High intake of green tea has made the incidence of decrease in triglycerides, total and LDL cholesterol. It has also resulted in increasing the HDL cholesterol in cardiovascular diseases. Epidemiological studied the relation between green tea intake and cardiovascular risk and the results revealed the significant benefits of green tea consumption [105].

The polyphenol curcumin is the active component of turmeric, commonly found as Indian spice. A dose of 500 mg of curcumin could decrease lipid peroxides and total serum cholesterol, and increased serum HDL levels in CVDs patients [106].

3.3 Others

MMPs play a critical role in the degrading collagen in skin, thereby rushing the aging process of skin. In a study, the effect of 6,7,4′-THIF was investigated against solar UV (sUV)-induced MMPs in normal human dermal fibroblasts. MMP-1 function is modulated by numerous signaling pathways such as the mitogen-activated protein/extracellular-signal-regulated kinase (MEK)/ extracellular-signal-regulated kinase, mitogen-activated protein kinase (MKK) 3/6/p38 and MKK4/c-Jun N-terminal kinases signaling pathways and their activation by sUV was significantly reduced by 6,7,4′-THIF pretreatment [107]. Oral administration of EGCG resulted in inhibition of UVB-induced expression of matrix degrading MMPs significantly in hairless mouse skin. This study suggested that the GTP as a dietary supplement could be beneficial to reduce solar UVB light-induced premature skin -aging [108]. Few Studies also revealed the EGCG levels found in tissue and blood samples of people who drank green tea were sufficient to bind DHFR, induce apoptosis, inhibit the growth of cells, and reduce tumor growth [109]. Nitric oxide was found as key player in the neuropathogenesis, it get induced during brain ischemia/reperfusion and hypoxia. The hypoxic rats treated with high dosages of EGCG (25 or 50 mg/kg) showed significant reduced expression of NADPH-d/nNOS, its suggesting that EGCG may reduce oxidative stress by following acute hypoxia [110].

4 Conclusion

Recent studies deliver generous evidences suggesting the huge potential of polyphenols to prevent various human diseases, but better understanding of bioavailability is required for exploring the health effects of polyphenols, whatever the approach used. Given, the potential cancer, cardiovascular, neurodegenerative and diseases preventing activities of these natural compounds as regulating MMPs and other disease responsible genes, we would expect to observe these beneficial activities in human populations. Future developed drug or synthetic compounds from polyphenols may afford actual, modified therapy affecting a subgroup of MMPs specific to a particular disease condition with minimal adverse side effects in patients.

References

1. Sang QXA, Jin Y, Newcomer RG, Monroe SC et al (2006) Matrix metalloproteinase inhibitors as prospective agents for the prevention and treatment of cardiovascular and neoplastic diseases. Curr Top Med Chem 6(4):289–316
2. Chelladurai P, Seeger W, Pullamsetti SS (2012) Matrix metalloproteinases and their inhibitors in pulmonary hypertension. Euro Res J 40(3):766–782
3. Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat rev Mol cell Bio 8(3):221–233

4. Iyer S, Visse R, Nagase H et al (2006) Crystal structure of an active form of human MMP-1. J Mol Bio 362(1):78–88
5. Tardif G, Hum D, Pelletier JP et al (2009) Regulation of the IGFBP-5 and MMP-13 genes by the microRNAs miR-140 and miR-27a in human osteoarthritic chondrocytes. BMC musculoskelet disord 10(1):1
6. Chhabra A, Kohli S, Rani V (2014) MMPs in cardiovascular diseases: emerging pharmacological targets in role of proteases in cellular dysfunction. Springer Publishing Company, New York, pp 407–426
7. Lu P, Takai K, Weaver VM et al (2011) Extracellular matrix degradation and remodeling in development and disease. NY Cold Spring Harbor Persp Biol 3(12):a005058– a005068
8. Rohani MG, Parks WC (2015) Matrix remodeling by MMPs during wound repair. Mat Bio 44:113–121
9. Barrett AJ, Rawlings ND, Woessner JF (1998) Handbook of proteolytic enzymes. Academic Press London, San Diego
10. Liao C, Sitzmann M, Pugliese A et al (2011) Software and resources for computational medicinal chemistry. Fut Med Chem 3(8):1057–1085
11. Hu J, Van den Steen PE, Sang QXA et al (2007) Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. Nat rev Drug Dis 6(6):480–498
12. Cathcarta J, Pulkoski-Grossa A, Caob J (2015) Targeting matrix metalloproteinases in cancer: bringing new life to old ideas. Genes Dis 2(1):26–34
13. Dai J, Mumper RJ (2010) Plant Phenolics:Extraction, Analysis and Their Antioxidant and Anticancer Properties. Mol 15:7313–7352
14. Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2(5):270–278
15. Tsao R (2010) Chemistry and Biochemistry of Dietary Polyphenols. Nutrients 2(12):1231–1246
16. Lobo V, Patil A, Phatak A et al (2010) Free radicals, antioxidants and functional foods: impact on human health. Pharmaco Rev 4(8):118–126
17. Knethen V, Abts H, Kube D et al (1997) Expression of p56lck in B-cell neoplasias. Leuk Lymphom 5(6):551–562
18. Palmieri D, Perego P, Palombo D (2012) Apigenin inhibits the TNFα-induced expression of eNOS and MMP-9 via modulating Akt signalling through oestrogen receptor engagement. Mol Cell Biochem 371(1–2):129–136
19. Xia Y, Lian S, Khoi PN et al (2015) Chrysin inhibits tumor promoter-induced MMP-9 expression by blocking AP-1 via suppression of ERK and JNK pathways in gastric cancer cells. PLoS ONE 10(4):1–13
20. Miyata Y, Sato T, Imada K et al (2008) A citrus polymethoxyflavonoid, nobiletin, is a novel MEK inhibitor that exhibits antitumor metastasis in human fibrosarcoma HT-1080 cells. Biochem Biophys Res Commun 366(1):168–173
21. Vanella L, Barbagallo I, Acquaviva R et al (2013) Ellagic acid: cytodifferentiating and antiproliferative effects in human prostatic cancer cell lines. Cur pharmaceut des 19 (15):2728–2736
22. Kim MH, Gutierrez AM, Goldfarb RH (2001) Different mechanisms of soy isoflavones in cell cycle regulation and inhibition of invasion. Antican Res 22(6C):3811–3817
23. Aherne SA, O'Brien NM (2002) Dietary flavonols: chemistry, food content, and metabolism. Nutrition 18(1):75–81
24. Rösch D, Krumbein A, Mügge C et al (2004) Structural investigations of flavonol glycosides from sea buckthorn (*Hippophaë rhamnoides*) pomace by NMR spectroscopy and HPLC-ESI-MS(n). J Agri Food Chem 52(13):4039–4046
25. Zhao X, Zhang J (2015) Mechanisms for quercetin in prevention of lung cancer cell growth and metastasis. J Cent South Uni Medi sci 40(6):592–597
26. López-Lázaro M (2002) Flavonoids as anticancer agents: structure-activity relationship study. Curr Med Chem Anticancer Agents 2(6):691–714

27. Khan N, Afaq F, Saleem M et al (2006) Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3-gallate. Cancer Res 66(5):2500–2505
28. Wang Z, Dabrosin C, Yin X et al (2015) December Broad targeting of angiogenesis for cancer prevention and therapy. Semi Cancer 35:S224–S243
29. Kim HS, Michael J, Quon et al (2014) New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. Redox Bio 2:187–195
30. Chen PN, Kuo WH, Chiang CL et al (2006) Black rice anthocyanins inhibit cancer cells invasion via repressions of MMPs and u-PA expression. Chem Bio Interac 163(3):218–229
31. Stevens JF, Page JE (2004) Xanthohumol and related prenylflavonoids from hops and beer: to your good health. Phytochem 65(10):1317–1330
32. Yang JY, Della-Fera MA, Rayalam S et al (2007) Effect of xanthohumol and isoxanthohumol on 3T3-L1 cell apoptosis and adipogenesis. Apoptosis 12(11):1953–1963
33. Pandey MK, Sandur SK, Sung B et al (2007) Butein, a tetrahydroxychalcone, inhibits nuclear factor (NF)-kappaB and NF-kappaB-regulated gene expression through direct inhibition of Ikappa Balpha kinase beta on cysteine 179 residue. J Bio Chem 282 (24):17340–17350
34. Alrawaiq NS, Abdullah A (2014) A review of antioxidant polyphenol curcumin and its role in detoxification. Int J Pharm Tech Res 6(1):280–289
35. De R, Kundu P, Swarnakar S et al (2009) Antimicrobial activity of curcumin against helicobacter pylori isolates from India and during infections in mice. Antimicrob agents and Chemo 53(4):1592–1597
36. Menon VP, Sudheer AR (2007) Antioxidant and anti-inflammatory properties of curcumin. In: The molecular targets and therapeutic uses of curcumin in health and disease. Springer, US 105–125
37. Sikora E, Scapagnini G, Barbagallo M (2010) Curcumin, inflammation, ageing and age-related diseases. Imm Ageing 7(1):1–10
38. Aggarwal BB, Sung B (2009) Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends pharmaco sci 30(2):85–94
39. Sharma RA, Gescher AJ, Steward WP (2005) Curcumin: the story so far Euro. J Cancer 41 (13):1955–1968
40. Boudreau LH, Maillet J, LeBlanc LM et al (2012) Caffeic acid phenethyl ester and its amide analogue are potent inhibitors of leukotriene biosynthesis in human polymorphonuclear leukocytes. PLoS ONE 7(2):1–8
41. Iwai K, Kishimoto N, Kakino Y et al (2004) In vitro antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans. J Agri Food Chem 52(15):4893–4898
42. Rani A, Sharma A (2013) The genus vitex: a review. Pharmaco Rev 7(14):188–198
43. Badhani B, Sharma N, Kakkar R (2015) Gallic acid:a versatile antioxidant with promising therapeutic and industrial applications. RSC Adv 5:27540–27557
44. Burns J, Yokota T, Ashihara H et al (2002) Plant foods and herbal sources of resveratrol. J Agri Food Chem 50(11):3337–3340
45. Zhang JJ, Wu M, Schoene NW et al (2009) Effect of resveratrol and zinc on intracellular zinc status in normal human prostate epithelial cells. Am J Cell Physio 297(3):C632–C644
46. Kavas GÖ, Aribal-Kocatürk P, Büyükkağnici DI (2007) Resveratrol:is there any effect on healthy subject? Biol Trace Element Res 118(3):250–254
47. Piotrowska H, Kucinska M, Murias M (2012) Biological activity of piceatannol:leaving the shadow of resveratrol Mutation. Res/Rev Mutat Res 750(1):60–82
48. McCormack D, McFadden D (2012) Pterostilbene and cancer: current review. J Surgical Res 173(2):e53–e56
49. Lee T, Esemuede N, Sumpio BE et al (2003) Thrombospondin-1 induces matrix metalloproteinase-2 activation in vascular smooth muscle cells. J Vasc Surg 38(1):147–154
50. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6(5):40–392

51. Gialeli C, Theocharis AD, Karamanos NK (2011) Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 278(1):16–27
52. Lall RK, Syed DN, Adhami VM et al (2015) Dietary polyphenols in prevention and treatment of prostate cancer. Int J Mol Sci 16(2):3350–3376
53. Leiva B, Carrasco I, Montenegro I et al (2015) Equol and daidzein decrease migration, invasion and matrix metalloproteinase (MMPs) gene expression in prostate cancer cell lines, DU-145 and PC-3. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 14(3):251–262
54. Li Y, Che M, Bhagat S et al (2004) Regulation of gene expression and inhibition of experimental prostate cancer bone metastasis by dietary genistein. Neoplasia 6(4):354–363
55. Lee EJ, Kim SY, Hyun JW et al (2010) Glycitein inhibits glioma cell invasion through down-regulation of MMP-3 and MMP-9 gene expression. Chemico-Biolo Interac 185(1):18–24
56. Pan MH, Chiou YS, Chen WJ et al (2009) Pterostilbene inhibited tumor invasion via suppressing multiple signal transduction pathways in human hepatocellular carcinoma cells. Carcinogenesis 30(7):1234–1242
57. Wang TT, Schoene NW, Kim YS et al (2010) Differential effects of resveratrol and its naturally occurring methylether analogs on cell cycle and apoptosis in human androgen-responsive LNCaP cancer cells. Mol Nutr Food Res 54(3):335–344
58. Li K, Dias SJ, Rimando AM et al (2013) Pterostilbene acts through metastasis-associated protein 1 to inhibit tumor growth, progression and metastasis in prostate cancer. PLoS ONE 8(3):1–13
59. Lin CW, Chou YE, Chiou HL et al (2014) Pterostilbene suppresses oral cancer cell invasion by inhibiting MMP-2 expression. Expert Opin Therap Targets 18(10):1109–1120
60. Oku N, Matsukawa M, Yamakawa S et al (2003) Inhibitory Effect of green tea polyphenols on membrane-Type 1 matrix metalloproteinase, MT1-MMP1. Biologi Pharmaceut Bulletin 26(9):1235–1238
61. Khan N, Adhami VM, Mukhtar H (2009) Review: green tea polyphenols in chemoprevention of prostate cancer: preclinical and clinical studies. Nutr Cancer 61(6):836–884
62. Luo KL, Luo JH, Yu YP (2010) (−)-Epigallocatechin-3-gallate induces Du145 prostate cancer cell death via downregulation of inhibitor of DNA binding 2, a dominant negative helix-loop-helix protein. Cancer Sci 101(3):707–712
63. Hagen RM, Chedea VS, Mintoff CP et al (2013) Epigallocatechin-3-gallate promotes apoptosis and expression of the caspase 9a splice variant in PC3 prostate cancer cells. Int J Onco 43(1):194–200
64. Wang H, Li GX, Yang Z et al (2011) Cancer prevention by tea: evidence from laboratory studies. Pharmac Res 64(2):113–122
65. Mukherjee S, Siddiqui MA, Dayal S et al (2014) Epigallocatechin-3-gallate suppresses proinflammatory cytokines and chemokines induced by toll-like receptor 9 agonists in prostate cancer cells. J Inflam Res 7:89–101
66. Inoue M, Sakaguchi N, Isuzugawa K et al (2000) Role of reactive oxygen species in gallic acid-induced apoptosis. Biologi Pharmaceut Bulletin 23(10):1153–1157
67. Kaur M, Velmurugan B, Rajamanickam S et al (2009) Gallic acid, an active constituent of grape seed extract, exhibits anti-proliferative, pro-apoptotic and anti-tumorigenic effects against prostate carcinoma xenograft growth in nude mice. Pharmaceut Res 26(9):2133–2140
68. Ho LL, Chen WJ, Lin-Shiau SY et al (2002) Penta-O-galloyl-β-d-glucose inhibits the invasion of mouse melanoma by suppressing metalloproteinase-9 through down-regulation of activator protein-1. Euro J pharmaco 453(2):149–158
69. Hu H, Zhang J, Lee HJ et al (2009) Penta-O-galloyl-beta-D-glucose induces S-and G1-cell cycle arrests in prostate cancer cells targeting DNA replication and cyclin D1. Carcinogenesis 30(5):818–823
70. Gweon EJ, Kim SJ (2014) Resveratrol attenuates matrix metalloproteinase-9 and -2-regulated differentiation of HTB94 chondrosarcoma cells through the p38 kinase and JNK pathways. Onco Rep 32(1):71–78

71. Liu M, Wilk SA, Wang A et al (2010) Resveratrol inhibits mTOR signaling by promoting the interaction between mTOR and DEPTOR. J Bio Chem 285(47):36387–36394
72. Wu XP, Xiong M, Xu CS et al (2015) Resveratrol induces apoptosis of human chronic myelogenous leukemia cells in vitro through p38 and JNK-regulated H2AX phosphorylation. Act Pharmacologica Sinica 36(3):353–361
73. Pal HC, Hunt KM, Diamond A et al (2016) Phytochemicals for the management of melanoma. Mini Rev Med Chem 16(12):953–979
74. Jayasooriyar RGPT, Lee YG, Kang CE et al (2013) Piceatannol inhibits MMP-9-dependent invasion of tumor necrosis factor-α-stimulated DU145 cells by suppressing the Akt-mediated nuclear factor-κB pathway. Onco Lett 5(1):341–347
75. Dias SJ, Li K, Rimando AM et al (2013) Trimethoxy-resveratrol and Piceatannol administered orally suppress and inhibit tumor formation and growth in prostate cancer xenografts. Prostate 73(11):1135–1146
76. Roupe KA, Remsberg CM, Yáñez JA et al (2006) Pharmacometrics of stilbenes: segueing towards the clinic. Curr Clinic Pharmaco 1(1):81–101
77. Bell C, Hawthorne S (2008) Ellagic acid, pomegranate and prostate cancer—a mini review. J Pharm Pharmaco 60(2):139–144
78. Pitchakarn P, Chewonarin T, Ogawa K et al (2013) Ellagic acid inhibits migration and invasion by prostate cancer cell lines. Asian Pac J Cancer Prev 14(5):2859–2863
79. Madlener S, Saiko P, Vonach, C et al (2010) Multifactorial anticancer effects of digalloyl-resveratrol encompass apoptosis, cell-cycle arrest, and inhibition of lymphendothelial gap formation in vitro. British J Cancer 102(9):1361–1370
80. Chen HJ, Lin CM, Lee CY et al (2013) Kaempferol suppresses cell metastasis via inhibition of the ERK-p38-JNK and AP-1 signaling pathways in U-2 OS human osteosarcoma cells. Onco Reports 30(2):925–932
81. Lin CW, Chen PN, Chen MK et al (2013) Kaempferol reduces matrix metalloproteinase-2 expression by down-regulating ERK1/2 and the activator protein-1 signaling pathways in oral cancer cells. PLoS ONE 8(11):1–13
82. Li C, Zhao Y, Yang D et al (2014) Inhibitory effects of kaempferol on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-9. Biochem Cell Bio 93(1):16–27
83. Hassan ZK, Daghestani MH (2012) Curcumin effect on MMPs and TIMPs genes in a breast cancer cell line. Asian Paci J Cancer Prev 13(7):3259–3264
84. Chen J, Tung Allport CH et al (2005) Near-infrared fluorescent imaging of matrix metalloproteinase activity after myocardial infarction. Circulation 111(14):1800–1805
85. Wu TC, Leu HB, Lin WT et al (2005) Plasma matrix metalloproteinase-3 level is an independent prognostic factor in stable coronary artery disease. Euro J Clinic Inves 35 (9):537–545
86. Higashi K, Ogawara H (1994) Daidzein inhibits insulin-or insulin-like growth factor-1-mediated signaling in cell cycle progression of Swiss 3T3 cells. Biochimica et Biophysica Acta (BBA)-Mol. Cell Res 1221(1):29–35
87. Perez-Vizcaino F, Bishop-Bailley D, Lodi F et al (2006) The flavonoid quercetin induces apoptosis and inhibits JNK activation in intimal vascular smooth muscle cells. Biochem Biophys Res Commun 346(3):919–925
88. Yoshizumi M, Tsuchiya K, Suzaki Y et al (2002) Quercetin glucuronide prevents VSMC hypertrophy by angiotensin II via the inhibition of JNK and AP-1 signaling pathway. Biochem Biophys Res Commun 293(5):1458–1465
89. Qin TC, Chen L, Yu LX et al (2001) Inhibitory effect of quercetin on cultured neonatal rat cardiomyocytes hypertrophy induced by angiotensin. Acta Pharmacol Sin 22(12):1103–1106
90. Jalili T, Carlstrom J, Kim S et al (2006) Quercetin-supplemented diets lower blood pressure and attenuate cardiac hypertrophy in rats with aortic constriction. J Cardiovas Pharmaco 47 (4):531–541

91. Sheng R, Gu ZL, Xie ML (2013) Epigallocatechin gallate, the major component of polyphenols in green tea, inhibits telomere attrition mediated cardiomyocyte apoptosis in cardiac hypertrophy. Int J cardio 162(3):199–209
92. Saragusti AC, Ortega MG, Cabrera JL et al (2010) Inhibitory effect of quercetin on matrix metalloproteinase 9 activity Molecular mechanism and structure–activity relationship of the flavonoid–enzyme interaction. Euro J Pharmaco 644(1):138–145
93. Lin HC, Hsieh MJ, Peng CH et al (2015) Pterostilbene Inhibits vascular smooth muscle cells migration and matrix metalloproteinase-2 through modulation of MAPK pathway. J Food Sci 80(10):H2331–H2335
94. Yang Y, Duan W, Li Y et al (2013) Novel role of silent information regulator 1 in myocardial ischemia. Circulation 128(20):2232–2240
95. Morris KC, Lin HW, Thompson JW et al (2011) Pathways for ischemic cytoprotection:role of sirtuins in caloric restriction, resveratrol, and ischemic preconditioning. J Cereb Blood Flow Metab 31(4):1003–1019
96. Koronowski KB, Perez-Pinzon MA (2015) Sirt1 in cerebral ischemia Brain. Circulation 1(1):69
97. Chen J, Bai Q, Zhao Z et al (2015) Resveratrol improves delayed r-tPA treatment outcome by reducing MMPs. Acta Neurologica Scandin 134:54–60
98. Della-Morte D, Dave KR, DeFazio RA et al (2009) Resveratrol pretreatment protects rat brain from cerebral ischemic damage via a sirtuin 1–uncoupling protein 2 pathway. Neuroscience 159(3):993–1002
99. Liu FC, Tsai HI, Yu HP (2015) Organ-protective effects of red wine extract, resveratrol, in oxidative stress-mediated reperfusion injury. Oxid Med Cell Longev 2015:1–15
100. Renaud SD, de Lorgeril M (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. The Lancet 339(8808):1523–1526
101. Magyar K, Halmosi R, Palfi A et al (2012) Cardioprotection by resveratrol: a human clinical trial in patients with stable coronary artery disease. Clin Hemorheol Microcirc 50(3):179–187
102. Youdim KA, McDonald J, Kalt W et al (2002) Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. J Nutr Biochem 13(5):282–288
103. Nagao T, Komine Y, Soga S et al (2005) Ingestion of a tea rich in catechins leads to a reduction in body fat and malondialdehyde-modified LDL in men. Am J Clinic Nutr 81 (1):122–129
104. Kuriyama S, Shimazu T, Ohmori K et al (2006) Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. JAMA 296 (10):1255–1265
105. Kuriyama S (2008) The relation between green tea consumption and cardiovascular disease as evidenced by epidemiological studies. J Nutr 138(8):1548S–1553S
106. Soni KB, Kuttan R (1992) Effect of oral curcumin administration on serum peroxides and cholesterol levels in human volunteers. In J Phys Pharma 36:273–283
107. Lim TG, Kim JE, Lee SY et al (2014) The daidzein metabolite, 6, 7, 4'-Trihydroxyisoflavone, is a novel inhibitor of PKCα in suppressing solar UV-induced matrix metalloproteinase 1. Int J Mol Sci 15(11):21419–21432
108. Vayalil PK, Mittal A, Hara Y et al (2004) Green tea polyphenols prevent ultraviolet light-induced oxidative damage and matrix metalloproteinases expression in mouse skin. J Inves Derma 122(6):1480–1487
109. Mitrica R, Dumitru I, Lavinia L et al (2012) The Dual Action of Epigallocatechin Gallate (EGCG), the Main Constituent of Green Tea, against the Deleterious Effects of Visible Light and Singlet Oxygen-Generating Conditions as Seen in Yeast Cells. Molecules 17:10355–10369
110. Wei IH, Wu YC, Wen CY et al (2004) Green tea polyphenol (−)-epigallocatechin gallate attenuates the neuronal NADPH-d/nNOS expression in the nodose ganglion of acute hypoxic rats. Brain Res 999(1):73–80

Autophagic Proteases: Functional and Pathophysiological Aspects

14

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Abstract

Autophagy is a ubiquitous eukaryotic cellular process, in which cells degrade their own cytoplasmic components by hydrolases within the lysosome. Among many processes, the proteolytic processing of autophagy mediator protein Atg8 is catalyzed by a cysteine protease Atg4, to form Atg8—phosphatidylethanolamine (PE) conjugation, one of the most important step for autophagosome formation. Deconjugation of existing Atg8-PE is also catalyzed by Atg4 facilitates the release of Atg8 from the autophagosome membrane to recycle autophagy progression. Lysosomal hydrolases, familiar as cathepsins, divided into diverse number of enzyme subtypes namely cysteine, serine and aspartic proteases contribute to autophagy by catalyzing the cleavage of peptide bonds of autophagic substrates and thus help disposing the autophagic flux, albeit with the help of many other factors. Even though the cathepsins are implicated in autophagic processes, cathepsin A shows contrasting effect by reducing the rate of chaperone-mediated autophagy through proteolytic processing of lysosome-associated membrane protein type 2a (Lamp2a). Moreover, other families of proteases, such as calpains and caspases, may cleave autophagy-related proteins, negating the execution of autophagic processes. In this review, the overall focus is on the functional role of proteases in autophagy mainly, lysosomal hydrolases known as cathepsins, the cysteine protease Atg4 in yeast and the four orthologs of yeast protease Atg4 in mammalian system termed as "autophagins." The present review also highlights the fundamental processes of autophagy including dysregulation of Atg4 protease in diverse pathological conditions such as cancer, cardiac diseases, neurodegenerative and infectious

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diseases. The overall approach of this article has also been extended with a view to emphasize on therapeutic strategies by targeting dysregulation of Atg4 protease associated with various diseases.

Keywords
Atg4 · Atg8 · Autophagy · Cathepsins · Pathogenesis · Protease · Therapeutic approach

1 Introduction

Autophagy is an intracellular degradation process in lysosome which involves the engulfment of cytoplasmic constituents essential for maintaining cell survival under various stress conditions [1]. The concept of autophagy comes sequentially, first, through the observation of "dense bodies" by Clark and Novikoff. They examined mitochondria from mouse kidneys within membrane-bound compartments and termed as "dense bodies" [2]. Ashford and Porter observed that membrane-bound vesicles contain semi-digested mitochondria and endoplasmic reticulum in rat hepatocyte cells that have been exposed to glucagon [3]. They further confirmed that the same bodies also carried lysosomal hydrolases. Before 1960s, Belgian cytologist Christian de Duve proposed that for nonspecific bulk segregation and digestion of own cytoplasmic constituents in the lysosomes, most (not all) living cells utilize a mechanism preferentially at the requirement of selective proteolysis which acts on abnormal cellular proteins or organelles [4]. Later, in 1963, at the Ciba Foundation conference on lysosomes, Duve first coined the term 'autophagy' which comes from the Greek words "auto" meaning "self" and "phagy" means "eat" [5]. He is also known as the discoverer of lysosome and awarded Nobel Prize in 1974 [6]. In 1973, Bolender and Weibel confirmed that the smooth endoplasmic reticulum can be engulfed by autophagic process [7]. Veenhuis also revealed that in *Hansenula polymorpha* yeast system, superfluous peroxisomes are selectively degraded by autophagy [8]. In 1998, Lemasters et al. suggested that autophagy is also induced by mitochondrial membrane potential change [9]. It is also reported that in yeast, nitrogen starvation triggers the accumulation of autophagic bodies in the vacuoles, but deficiency of other stimulus such as carbon, nucleic acids, even sulfate can also induce autophagy, though with less efficiency [10]. In plant cells, autophagy is induced not only by the nitrogen starvation but also by the depletion of carbon [11]. In many types of cultured cells, autophagy is significantly persuaded by depletion of total amino acid but the effects of individual amino acids are different [12]. In mammalian cells, degradation processes of most long-lived cytoplasmic proteins usually occur within the lysosomes [13] (Table 1).

Table 1 Main phenomena of cellular death

Cell death	Apoptosis	Programmed cell death Associated with moderate stress Involving two main pathways
	Autophagy	Mild stress associated self-eating catabolic process involving lysosomes and several hydrolytic enzymes
	Necrosis	Death caused by external factors like extreme stress and toxins Not programmed

Therefore, autophagy is evolutionary conserved catalytic machinery, which strictly regulates lysosomal pathway that degrades cytoplasmic constituents. Depending upon the mode of cargo delivery to the lysosome, autophagy process is categorized into three types namely, microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy [14]. In microautophagy, the cytoplasmic components are directly engulfed by lysosomal membrane, whereas in CMA for the delivery of cytoplasmic protein to the lysosome first chaperone protein binds to its cytosolic target substrate and then to receptor of lysosomal membrane where unfolding of proteins takes place. The unfolded cytosolic target protein is then directly translocated into the lysosome for degradation. Macroautophagy, the predominant phenomenon, involves in the formation of a cytoplasmic double-membrane vesicle, called autophagosomes, which eventually fuse with lysosomes to form autolysosomes, where cytoplasmic cargos are degraded. The resulting degradation products are then returned to the cytoplasm as basic building blocks for reuse by the cell to synthesize new biomolecules.

Proteases are engaged in several different steps of autophagy mainly coordinated by complex molecular systems, comprising the ubiquitin-like protein (Ubl) conjugation system and the cysteine protease Atg4 are involved in the formation, elongation and fusion of autophagic vesicles [15]. Moreover, in the final degradative stages of the autophagic process a huge number of lysosomal proteases are also involved [16]. The autophagy machinery proteins are encoded by the autophagy-related genes (ATGs) and the autophagosome formation is mediated by a set of autophagy-related proteins (Atg), which are highly conserved from yeast to mammals [17].

Among the numerous Atg proteinases identified in yeast, cysteine protease, Atg4 and four mammalian orthologs of yeast protease Atg4, termed as “autophagins” have paramount role and are directly implicated in autophagy. Atg4 plays a key role in the proteolytic processing of a particular protein Atg8 resulting the formation of autophagosome. In plant cells, these ubiquitin-like processing of protein Atg8 is also mediated by cysteine protease Atg4 [18].

Apart from cysteine protease Atg4, lysosomal hydrolases known as cathepsins are also active members in autophagic events. These cathepsins catalyze the peptide bonds cleavage of autophagic substrates and also help in the formation of autophagic flux along with other factors [19]. Initially, the autophagy process was very perplexing as the cells itself destroy its own unwanted biomolecules by virtue of the

process. Later, it was thought that these self-eating mechanisms are nothing but the process of disposing cellular rubbish, for example unwanted or misfolded proteins, damaged organelles or invading microorganisms. But still then, the real consequence of autophagic events is still under debate. Since last 15 years, accumulated evidence suggested that in variety of physiological and pathophysiological conditions autophagy plays important roles, although the mechanisms of which rather appear sometimes complex, particularly in aging process, cancer, immune response, neurodegenerative diseases [20]. So, autophagy encompasses the regulation of an array of cellular responses by proteolytic modifications involving different proteases.

2 Cysteine Protease Atg4 in Yeast System

Autophagy is a dynamic membrane phenomenon by which large amount of proteins is degraded in membrane-bound vacuoles like lysosomes by different types of proteases. The first step of autophagy (macroautophagy) process is the formation of autophagosome which is a double-membrane-bound vacuole containing segregated parts of the cytoplasm. In keeping with traditional point of view, once autophagosomes are fused with lysosomes, the internal proteins are subjected to degradation by different types of hydrolytic enzymes through different cellular pathways [21]. According to recent report, Atg4 plays very active roles in several steps of autophagy processes [22]. In the yeast system, Atg4 cleaves the C-terminus part of Atg8, a unique ubiquitin-like (Ubl) protein synthesized as an inactive soluble protein and consists of a single member in yeast and multiple homologues in higher eukaryotes [23]. Atg8 is post-translationally modified to facilitate the covalent attachment with phosphatidylethanolamine (PE) molecule from the preautophagosomal membrane [24]. In this process Atg4 plays a central role in the activation of Atg8 through C-terminal cleavage to form $Atg8^{G116}$ by exposing a glycine residue. Subsequently, resulting $Atg8^{G116}$ involves in the formation of autophagosomes by a ubiquitin-like conjugation reactions catalyzed by other additional autophagic proteins like Atg7 (E1-ubiquitin ligase like enzyme), Atg3 (E2-ubiquitin ligase like enzyme) and Atg12-Atg5-Atg16 complex (E3 like enzyme). These interactions of autophagic proteins lead to the final conjugation of Atg8 with the amino group of PE (Fig. 1). Atg8-PE employs Atg12-Atg5-Atg16 complex to the membrane to assemble in a scaffold formation that is crucial for phagophore biogenesis [25, 26].

This proteolytic conjugation of Atg8-PE was found to be essential regulators of autophagic vesicle formation [17]. Finally, Atg4 attacks Atg8 proteins which are associated with the outer membrane of autophagosome and deconjugated the protein from its target lipid and released back to the cytosol [12, 16]. Thus, Atg4 makes the lipidation process reversible. In this way the liberated Atg8 recycled and participates in a new conjugation reaction, whereas Atg8-PE entrapped within the

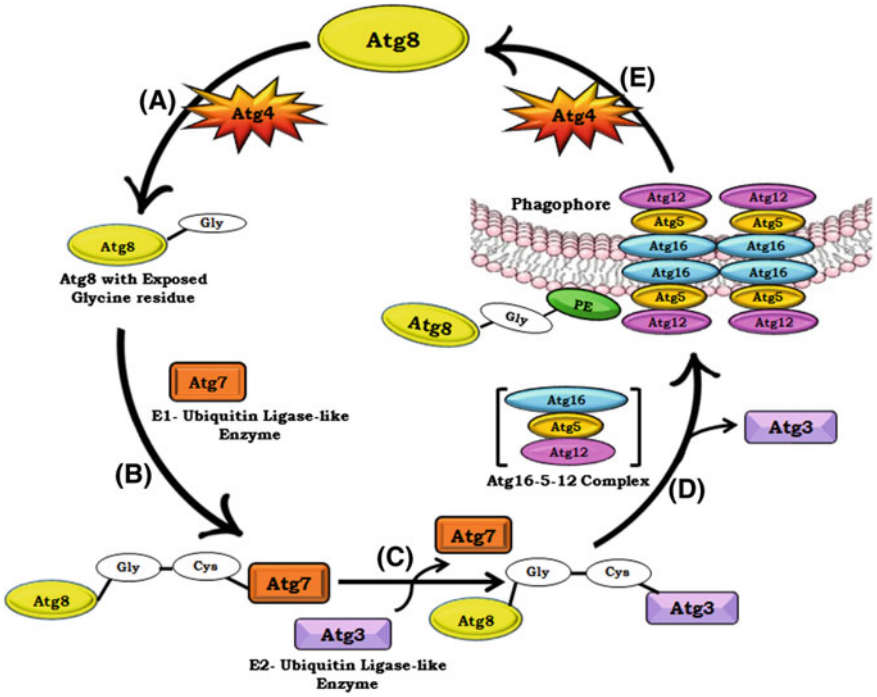

Fig. 1 Processing of Atg8 in autophagosome formation: the following steps are involved in the processing of Atg8; *A* cysteine protease Atg4 cleaves Atg8 exposing C-terminal glycine residue (Gly), *B* formation of thioester bond between exposed Gly residue of Atg8 and catalytic cysteine (Cys) residue of Atg7, *C* activated Atg8 conjugates with Atg3 through amide bond, *D* Finally, Gly residue of Atg8 binds with PE through another amide bond, *E* deconjugation of Atg8-PE with the help of Atg4 forming free Atg8

autophagosome is degraded following fusion with the lysosome. Thus, both the conjugation and deconjugation activities of Atg4 are required for normal progression of autophagy.

3 Atg4 Protease Family in Mammals

Four mammalian Atg4 orthologs were identified as "Autophagins," which show sequence similarity to single yeast Atg4. These are autophagin-1/Atg4B, autophagin-2/Atg4A, autophagin-3/Atg4C, and autophagin-4/Atg4D [27]. These four proteinases (autophagins) target at least six distinct substrates in Atg4-Atg8 proteolytic systems. Generally, it is assumed that in the mammalian system, proteolysis by Atg4 autophagic protease to its corresponding substrate is more complex when compared to the yeast system where one single protease cleaves a sole substrate. But, this cysteine proteolysis is very important for Atg8 processing for

the initiation of macroautophagy. In yeast single Atg8 expresses, where as the mammalian Atg8 homologs compose of three subfamilies those have different structural and functional features in the autophagosome formation. These three subfamilies include microtubule-associated protein 1 light-chain 3 (MAP1-LC3) subfamily (consisting MAP1-LC3A, MAP1-LC3B, and MAP1-LC3C), which participate in the elongation of initial phagophore. Other two subfamilies γ-aminobutyric acid receptor-associated protein (GABARAP) and Golgi associated ATPase enhancer of 16 kDa (GATE-16) (including GABARAP, GABARAPL1/Atg8L and GABARAPL2/GATE16) are involved in the final maturation of double-membrane vesicles [15, 28–31].

In the proteolytic system these Atg8 homologues are differentially cleaved by the different Atg4 family members of which Atg4B (autophagin-1) representing the broadest substrate spectrum with similar affinity and catalytic efficiency towards the Atg8 substrates [32]. Atg4A (autophagin-2) is the second best enzyme among four autophagins with the ability to target GABARAPL2 (belongs to subfamily of GABARAP), where as Atg4C and Atg4D represents the minimal activity in terms of substrate specificity and can only process MAP1-LC3B and GABARAPL2 [32]. Thus, these proteolysis are involved in the processing of Atg8 proteins, and are essential for the initial steps of macroautophagy by acting as important regulators of autophagic vesicle formation.

For all these proteolytic activities Atg4 itself needs activation by proteases which are governed by caspase dependent or independent manner. For example Cleavage of human Atg4D is assisted by caspase-3 to increase the activity towards GABARAPL1 which belongs to subfamily of GABARAP [33]. Not only Atg4D, in vitro caspase mediated activation of Atg4C is also facilitated due to the presence of canonical cleavage site DEVD [34]. It is important to note that Atg4A and Atg4B do not show any caspase-mediated activation due to lack of such motif. In cleavage process of Atg4D, a BH3-like domain is exposed which facilitates the recruitment of the protein to the mitochondria, where it induces apoptosis. Therefore, Atg4D seems to be a central component in the crosstalk between autophagy and apoptosis.

4 Lysosomal Proteases in Autophagy Process

Lysosomes are single membrane-bound cytoplasmic organelles present almost in all eukaryotic cells. They are the major degradative compartment of the endosomal/lysosomal system and the terminal part of the endocytic pathway, where various macromolecules, such as proteins, glycoconjugates, lipids, and nucleic acids, are degraded [35]. The degradation is carried out by a number of acid hydrolases (phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases, lipases, etc.), which are capable of digesting all major cellular macromolecules. The best-studied lysosomal hydrolases are the cathepsins. Cathepsins are the cluster of proteases in the endosomal proteolytic system which is originated from the Greek term meaning “to digest.” The general function of cathepsins is

considered to be in disintegrating large proteins by irreversibly cleaving peptide bonds. They are classified according to their active site amino acids, into three sub-groups i.e. cysteine (B, C, H, F, K, L, O, S, V, W and X), serine (A and G) and aspartate cathepsins (D and E) [36].

4.1 Cysteine Family Cathepsins

Cysteine cathepsins belong to the members of the family of papain-like cysteine proteases localized in lysosomes [37]. Primarily, cysteine cathepsins were regarded as intracellular enzymes which are responsible for the nonspecific, bulk proteolytic action in the acidic environment of the endosomal/lysosomal compartment, where they degrade intracellular and extracellular proteins [38]. However, this view is changing rapidly with the clear evidence of their localization in other cellular compartments [37, 38]. Not only they are implicated in protein degradation but also cysteine cathepsins are believed to be important regulators and signalling molecules of a large number of biological processes as well as a vital participant in terminal protein degradation during necrotic and autophagic cell death [39]. In humans, there are 11 cysteine cathepsins have been identified till now, including cathepsins B, C, F, H, K, L, O, S, V, X and W [40]. Pucer et al. suggested that cathepsins B and L are involved in autophagy-associated cell death induced by arsenic trioxide in U87 human glioblastoma cells [41]. The cathepsin L is considerably upregulated with other autophagy-related proteins, such as LC3 and BNIP3 (Bcl-2 19 kilodalton interacting protein 3; a pro-apoptotic BH3 protein and potent inducer of autophagy) by the transcription factor FoxO3 dependent pathway followed by high levels of oxidative stress [42]. Similarly, other research showed cathepsin B-deficient cells fuses with LC3-containing autophagic vacuoles with lysosomes, does not show any defect. Another observation by Takahashi et al. has implicated the importance of cathepsins in autophagy by applying inhibitors of cathepsin B (CA-074) and cathepsin L (CAA0225) that substantially reduced the degradation of long-lived proteins. Furthermore, several results suggest that cathepsin B is associated with the degradation of the whole autophagosomes, whereas cathepsin L is involved in the degradation of GABARAP-II and LC3-II autophagosomal membrane markers [43–47].

4.2 Serine Protease Family Cathepsins

In mammalian cells, two cathepsins (cathepsin A and cathepsin G) are expressed with serine protease activity. Cathepsin A with serine carboxypeptidase activity plays a vital role in the regulation of chaperone-mediated autophagy (CMA) through cleavage of the lysosomal receptor. Cuervo and colleagues reported that the lysosome-associated membrane protein type 2a (Lamp2a) is a substrate of cathepsin A [48]. Lamp2a is one of the three forms of lamp2 that originates by alternative splicing of mRNA from a single gene [49], acts as a receptor for CMA substrates in chaperone-mediated autophagy. Triggering of lamp2a degradation by

this protease cathepsin A appears very significant because the amount of receptor in the lysosomal membrane is a rate-limiting step of CMA [50]. In cathepsin A defective cells degradation rate of lamp2a is diminished, higher lamp2a levels and higher the rates of CMA. Restoration of cathepsin A protease activity increases rates of lamp2a degradation, reduces levels of lysosomal lamp2a and reduces rates of CMA. Cathepsin A interacts with lamp2a on the lysosomal membrane and this interaction promotes the cleavage of lamp2a close to the border line between the luminal and transmembrane domains [51]. Thus, role of cathepsin A is indispensible in the regulation of various physiological processes where CMA is involved, for example the immune response, cellular response to oxidative stress, aging and general cell homeostasis [52]. Additionally, cathepsin A also takes part in a protective function towards two lysosomal glycosidases namely, β-D-galactosidase and N-acetyl-α-neuraminidase and regulates their lysosomal activity as well as their stability also by targeting lysosomes in the case of α-neuraminidase [53, 54]. The second serine protease, cathepsin G, an endopeptidase expressed in promyelocytes regulates a critical role in antigen presenting and autoantigen processing [55, 56]. But, the function of Cathepsin G in autophagy process awaits further investigation.

4.3 Aspartic Protease Family Cathepsins

Cathepsin E which belongs to endolysosomal aspartic proteinase of the pepsin superfamily mainly expressed in immune-related cells [57]. Cathepsin E shared substrate specificity with cathepsin D that was totally involved in endolysosomal pathway, whereas its deficiency leads to macrophage lysosomal storage disorder. The disorder is associated with accumulation of lysosomal membrane sialoglycoproteins, LAMP-1 and LAMP-2, resulting in elevation of pH in lysosome, without changing in vacuolar-type H(+)-ATPase activity [58]. It was further noted that cathepsin E showed cell-type specificity [59, 60]. Tsukuba et al. also proposed that deficiency of Cathepsin E causes impairment of autophagic proteolysis in macrophages [61].

Additionally, aspartyl protease also plays a major role in imparting fungal resistance in plants by autophagic cleavage of BAG6 [62]. The Bcl-2-associated athanogene (BAG) family is an evolutionarily conserved group of cochaperones that adjust numerous cellular processes. It is also reported that autophagy is induced through the coupling of an aspartyl protease with a molecular cochaperone to trigger autophagy, which is associated with plant defense system, providing a key link between fungal recognition and the induction of cell death and resistance [62, 63].

5 Inhibition of Autophagy Process by Proteases

Unlike cathepsins that are involved in autophagy execution, other proteases like caspases and calpains are also engaged in the inhibition of macroautophagy, by altering autophagy-related proteins. Atg proteins are truncated by caspases which

are cysteine aspartate-specific proteases. This proteolytic cleavage leads to form a switch between autophagy to apoptosis by inactivating autophagy signal. It is reported that two caspase-3 cleavage sites are present in Beclin-1, i.e., downstream of the BH3 domain (aa 108–127) at D133 and D149. After cleavage, this truncated Beclin-1 may produce apoptotic amplification loop inside mitochondria [64]. Additionally, caspases-3 may also cleave Atg4D [33] to is truncated form DeltaN63, which stimulates delipidation mammalian Atg8 paralog, gamma-aminobutyric acid receptor-associated protein-like 1 (GABARAP-L1) and reduces GABARAP-L1 dependent autophagosome formation. Apart from caspase-3, Beclin-1 is also targeted by caspase-6. Furthermore, caspase-3, -6 and -8 truncate human Atg3 whereas Atg9, Atg7 and Atg4 may be cleaved merely by caspase-3 [34]. Additionally, LC3-binding protein p62, another critical modulator of autophagy, undergoes proteolytic modification by caspase-3 and caspase-8. Moreover, both Beclin-1 and PI3KC3 (phosphatidylinositol-3-kinase class-3) two vital components of the autophagy-inducing complex are lacking autophagy-inducing capacity [64, 65] when truncated by caspase mediated proteolysis and promote apoptosis.

In recent times, various reports suggested that calpain-1, a non-lysosomal calcium-dependent cysteine protease cleaves majority of human Atg proteins [34]. Calpain-1 plays a crucial role in controlling autophagy, by regulating the intensity of Atg5–Atg12 conjugation [66]. It is proposed that calpain mediated cleavage of Atg5 attenuates the level of Atg5–Atg12 conjugation [67]. The truncated form of Atg5 is translocated from the cytosol to mitochondria, where it combines with the Bcl-XL anti-apoptotic protein, eliciting the release of cytochrome c and activation of caspases. Similarly, the Ras oncogene activates the GTPase RhoA, which endorses activation of calpain followed by Beclin-1 degradation [68] and this phenomenon induces the increase of malignant intestinal epithelial cells. Calpain-2 mediated degradation of Atg7 and Beclin-1 damages mitochondrial autophagy, which may cause mitochondria permeability-dependent hepatocyte death after anoxia [69]. In CVB4 virus infected neurons, calpains elicit similar function by hindering viral replication, where the calpain inhibitors prominently condensed autophagosome development [70]. In addition, reports show that MEFs (mouse embryonic fibroblasts) from Capns1 (a regulatory subunit of calpain1 and calpain2 proteases, encoded by the calpain gene) knockout mice and human cells with depleted Capns1 demonstrate disrupted macroautophagy [71]. Accordingly, during starvation of these cells, the level of lysosomal activity and degradation of long-lived proteins was considerably reduced.

Therefore, although cathepsins actively participate in the completion or in the breakdown phases of autophagy, caspases and calpains are equally important and involved in the Atg cleavage process leading to inactivation of autophagic events. So, it has been established that Atg proteins have a crucial role in the regulation of autophagy and thus its proteolytic cleavage may constitute a switch between autophagy to apoptosis [19].

6 Dysfunction of Atg4 Protease Family in Pathological Conditions

Autophagy is prompted by different stress signals both in mammalian and yeast cells as evidenced by damaged organelles, pathogenic infection and/or a variety of disease conditions [72]. Recent studies have shown that basal level of autophagy is crucial for maintaining normal physiological processes in humans while abnormal autophagy is associated with different disease conditions. Dysregulation of autophagic processes has been reported to be associated with various pathologies including cancer, cardiac diseases, neurological disorder, microbial infections and many other diseases [73–77]. Aberrant autophagy is mainly related to dysregulation of Atg4 cysteine protease family during the beginning or development of these diseases. The functions of this protease are controlled by a number of different regulatory transcription factors such as p53, EGR1, Foxo3 and some micro RNAs namely miR-376b, miR-101 and miR-34a. Moreover, Atg4 proteases are also regulated by the ROS-dependent oxidation [78–83].

Current evidence has indicated that aberrant expression of Atg4 protein occurs in several types of human cancer and that may be related to the development and suppression of tumor and resistance against tumor chemotherapy [84]. Some experimental evidence has been suggested that during tumor development autophagic activity is reduced. During development of prostate cancer accumulation of ROS is higher and it causes the inhibition of Atg4 [85, 86]. In the contrary, autophagy also plays a crucial role in the survival of the existing tumor, e.g., in several cancer cell lines, ROS can induce the autophagy and increases the survival of cancerous cells [87]. In various malignancies such as ovarian cancer, uterine carcinosarcoma, adrenocortical carcinoma structural alteration of ATG4 genes with higher copy number has been found [88].

The Crohn's disease is a chronic inflammatory bowel disease where ATG4A and ATG4D genes mutation is responsible for the outcome of this disease [89]. Mutations in ATG4A, ATG4B, and ATG4D genes are also connected to some other inflammatory bowel diseases (IBDs). Experimental evidence suggested that ATG4B expression increases in experimental mice model of colitis [90].

During the developmental stage or in the neurodegenerative diseases neuronal cell death occurs and this type of the neuronal death involves the autophagic processes. It is reported that patients with various neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's diseases where the neuronal death occurs due to the autophagic cell death. Conversely, severe oxidative stress and increasing amounts of ROS may activate signalling pathways that promote autophagy-induced cell death in the neuronal cell [91].

Cardiac diseases are associated with the increased levels of reactive oxygen species and other oxidative stresses. Elevation of ROS in the cell may cause cell survival or death and the fate of this situation depends on the severity of the stress arises in the specific disease [92]. In myocardial infarction xanthine oxidase mediated ROS generation exacerbates autophagy and leads to the death of

cardiomyocytes [93]. Oxidative stress-mediated autophagic cellular death is also observed during cardiac tissue remodeling [94].

Different autophagic proteins including Atg4 are involved in many infectious diseases. In HIV-1 infected host cell, viruses can escape the pathway by which host cells can eliminate the viruses leading to an increase in viral population. This subversion process occurs by autophagy due to overexpression of ATG4 gene [95]. In the case of Hepatitis C virus, it evades host cell defense mechanism and develops autophagy by using autophagic components such as Atg4B (autophagin-1), which initiates their replication [96]. Recent evidence has shown that parasites including *Trypanosoma cruzi*, responsible for Chagas disease and *Leishmania major* which cause leishmaniasis exploit the autophagy machinery [97]. Genome database searches have revealed that most of the proteins that are involved in the autophagy pathways in *S. cerevisiae* have orthologs in the trypanosomatids [98, 99]. In trypanosomatids, two types of Atg4 protein were found. In the case of *Leishmania major*, LmATG4.1 is necessary for the proteolytic processing of the Atg8B and Atg8C, and LmATG4.2 is also engaged for the proteolytic processing of the Atg8A. In fact, the activity of their Atg4 orthologs is fundamental for their survival, differentiation and virulence [100–103].

7 Therapeutic Approach Targeting Atg4 Protease Family

Growing scientific evidence suggested that the dysfunction of mammalian homologs of yeast Atg4 protease occurs in different human pathophysiological conditions. Additionally, many research articles demonstrated that some stimulators could clear autophagosomes that can be used for prevention and/or treatment of infection, cancer, neurodegenerative diseases and some inhibitors also inhibit the autophagy which could be used for cancer resistance therapy. The unique proteolytic process involved in the autophagy has opened a new opportunity for the advancement of treatments by targeting the autophagic cysteine protease particularly Atg4. As Atg4 plays an essential role in the autophagic processes, various specific inhibitors of Atg4 are now being developed for the treatment of human pathologic conditions where the activity of the autophagic protease is excess [104]. The inactive mutant forms of Atg4 proteases that could not cleave their specific substrate can also be employed as another strategy for inhibiting the autophagy [105]. Preferably, conjugation and deconjugation steps might be specifically targeted.

According to the recent findings, attenuated activity of Atg4 protease in different pathological conditions has shown the probable approach of new therapeutics for the diseases. The viability of hepatocellular carcinoma cells is increased by the overexpression of Atg4B [106], whereas their viability has been reduced due to inhibition of autophagy by miR-101 [107]. Moreover, in vitro model of various lung infections, ischemia heart diseases and neurodegenerative diseases such as in Huntington's disease overexpression of this protease is also used as a potential

therapeutic target and stimulation of autophagic response improve the disease condition [108–110].

However, the function of Atg4 proteases in most of the diseases is case dependent, and the consequences of their modulation could differ to a great extent. In the case of cancer, autophagy plays a dual role. Some anti-cancer drug can induce the autophagy. For example, it has been suggested that alteration in the expression of Atg4B in prostate cancer acts as chemotherapy for treatment of prostate cancer [111]. In most of the cases, inhibition of Atg4 proteases in radiation therapies sensitizes resistant carcinoma cells [112].

The dysregulation of the activity of Atg4 is associated with various diseases and its dual roles in cancer arise from the complexity regarding its functional specificities. Thus, the development of novel therapies based on targeting the Atg4 proteases is yet at a very initial stage and hence deserves better understanding of the molecular basis of this protease. To date, so many modulators of this protease have been tested in clinical trials (Phase I/II) and development of specific inhibitors of this protease is still ongoing [113]. Consequently, new proteomic approaches will be essential for the improvement of Atg4-targeted treatments.

8 Conclusions and Future Perspectives

Originally, autophagy is involved in adaptive mechanism of cells to provide nutrients under stress conditions and since it has been related to diverse physiological and pathological processes. It is clear that proteases play a key role in regulation of various steps of autophagy. Cysteine protease Atg4 plays a role in Atg8 processing to be conjugated with PE on autophagosomal membranes, a vital step in autophagosome biogenesis. Lysosomal acidic proteases, viz. cathepsins, also participate in the execution of autophagic events, which are essential for the efficient degradation of autophagic substrates. In this context, it needs to be mentioned that activation of some other proteases like calpains and/or caspases suppress the levels of autophagosome formation through the cleavage of autophagy-related proteins. Additionally, cathepsin A, belonging to serine protease family, also inhibits chaperone-mediated autophagy through the cleavage of its receptor Lamp-2a. Dysregulation of Atg4 protease activity has been connected with various diseases such as cancer, neurodegenarative diseases, inflammatory diseases and parasitic diseases.

Therefore, with overall perspective we can conclude that the autophagic machinery is tightly controlled by Atg4 protease family. Additionally, lysosomal proteases like different classes of cathepsins regulate the machinery of autophagic processes. Overall control of autophagic events by Atg4 protease family makes an emerging target for therapeutic approach of various diseases which are caused by dysregulation of these autophagic proteases. However, many questions remain unanswered regarding the specificity of the Atg4 family proteins and need further

investigation to elucidate better understanding of their roles in pathophysiological consequences in humans to develop appropriate therapies for concerned diseases in future.

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References

1. Yorimitsu T, Klionsky DJ (2005) Autophagy: molecular machinery for self-eating. Cell Death Differ 2:1542–1552
2. Novikoff AB (1959) The proximal tubule cell in experimental hydronephrosis. J Biophys Biochem Cytol 6:136–138
3. Ashford TP, Porter KR (1962) Cytoplasmic components in hepatic cell lysosomes. J Cell Biol 12:198–202
4. de Duve C, Wattiaux R (1966) Functions of lysosomes. Annu Rev Physiol 28:435–492
5. Yang Z, Klionsky DJ (2010) Eaten alive: a history of macroautophagy. Nat Cell Biol 12:814–822
6. Boya P (2012) Lysosomal function and dysfunction: mechanism and disease. Antioxid Redox Signal 17:766–774
7. Bolender RP, Weibel ER (1973) A morphometric study of the removal of phenobarbital-induced membranes from hepatocytes after cessation of treatment. J Cell Biol 56:746–761
8. Veenhuis M, Douma A, Harder W et al (1983) Degradation and turnover of peroxisomes in the yeast *Hansenula polymorpha* induced by selective inactivation of peroxisomal enzymes. Arch Microbiol 134:193–203
9. Lemasters JJ, Nieminen AL, Qian T et al (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1366:177–196
10. Takeshige K, Baba M, Tsuboi S et al (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J Cell Biol 119:301–311
11. Ren C, Liu J, Gong Q (2014) Functions of autophagy in plant carbon and nitrogen metabolism. Front Plant Sci 5:1–5
12. Mizushima N (2007) Autophagy: process and function. Genes Dev 21:2861–2873
13. Appelqvist H, Wäster P, Kågedal K et al (2013) The lysosome: from waste bag to potential therapeutic target. J Mol Cell Biol 5:214–226
14. Chen Y, Klionsky DJ (2011) The regulation of autophagy—unanswered questions. J Cell Sci 124:161–170
15. Shpilka T, Weidberg H, Pietrokovski S et al (2011) Atg8: an autophagy-related ubiquitin-like protein family. Genome Biol 12:1–11
16. Fernández ÁF, López-Otín C (2015) The functional and pathologic relevance of autophagy proteases. J Clin Invest 125:33–41
17. Slobodkin MR, Elazar Z (2013) The Atg8 family: multifunctional ubiquitin-like key regulators of autophagy. Essays Biochem 55:51–64
18. Yoshimoto K, Hanaoka H, Sato S et al (2004) Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell 16:2967–2983
19. Kaminskyy V, Zhivotovsky B (2012) Proteases in autophagy. Biochim Biophys Acta 1824:44–50

20. Mijaljica D, Prescott M, Devenish RJ (2010) Autophagy in disease. Methods Mol Biol 648:79–92
21. Punnonen EL, Autio S, Marjomäki VS et al (1992) Autophagy, cathepsin L transport, and acidification in cultured rat fibroblasts. J Histochem Cytochem 40:1579–1587
22. Glick D, Barth S, Macleod KF (2010) Autophagy: cellular and molecular mechanisms. J Pathol 221:3–12
23. Xie Z, Nair U, Klionsky DJ (2008) Atg8 controls phagophore expansion during autophagosome formation. Mol Biol Cell 19:3290–3298
24. Yang Z, Klionsky DJ (2010) Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 22:124–131
25. Walczak M, Martens S (2013) Dissecting the role of the Atg12-Atg5-Atg16 complex during autophagosome formation. Autophagy 9:424–425
26. Ichimura Y, Kirisako T, Takao T et al (2000) A ubiquitin like system mediates protein lipidation. Nature 408:488–492
27. Marino G, Uria JA, Puente XS et al (2003) Human autophagins, a family of cysteine proteinases potentially implicated in cell degradation by autophagy. J Biol Chem 278:3671–3678
28. Scherz-Shouval R, Sagiv Y, Shorer H et al (2003) The COOH terminus of GATE-16, an intra-Golgi transport modulator, is cleaved by the human cysteine protease HsApg4A. J Biol Chem 278:14053–14058
29. Tanida I, Komatsu M, Ueno T et al (2003) GATE-16 and GABARAP are authentic modifiers mediated by Apg7 and Apg3. Biochem Biophys Res Commun 300:637–644
30. Nath S, Dancourt J, Shteyn V et al (2014) Lipidation of the LC3/GABARAP family of autophagyproteins relies on a membrane-curvature-sensing domain in Atg3. Nat Cell Biol 16:415–424
31. Weidberg H, Shvets E, Shpilka T et al (2010) LC3 and GATE-6/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. EMBO J 29:1792–1802
32. Li M, Hou Y, Wang J et al (2011) Kinetics comparisons of mammalian Atg4 homologues indicate selective preferences toward diverse Atg8 substrates. J Biol Chem 286:7327–7338
33. Betin VM, Lane JD (2009) Caspase cleavage of Atg4D stimulates GABARAP-L1 processing and triggers mitochondrial targeting and apoptosis. J Cell Sci 122:2554–2566
34. Norman JM, Cohen GM, Bampton ET (2010) The in vitro cleavage of the hAtg proteins by cell death proteases. Autophagy 6:1042–1056
35. Turk B, Turk V (2009) Lysosomes as “suicide bags” in cell death: myth or reality? J Biol Chem 284:21783–21787
36. Kirkegaard T, Jäättelä M (2009) Lysosomal involvement in cell death and cancer. Biochim Biophys Acta 1793:746–754
37. Dubey VK, Pande M, Singh BK et al (2007) Papain-like proteases: applications of their inhibitors. Afr J Biotech 6:1077–1086
38. Turk B, Turk D, Turk V (2000) Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta 1477:98–111
39. Turk V, Stoka V, Vasiljeva O et al (2012) Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochim Biophys Acta 1824:68–88
40. Rossi A, Deveraux Q, Turk B et al (2004) Comprehensive search for cysteine cathepsins in the human genome. Biol Chem 385:363–372
41. Pucer A, Castino R, Mirković B et al (2010) Differential role of cathepsins B and L in autophagy-associated cell death induced by arsenic trioxide in U87 human glioblastoma cells. Biol Chem 391:519–531
42. Dobrowolny G, Aucello M, Rizzuto E et al (2008) Skeletal muscle is a primary target of SOD1G93A-mediated toxicity. Cell Metab 8:425–436
43. Ueno T, Takahashi K (2009) A cathepsin L-specific inhibitor preferentially inhibits degradation of autophagosomal LC3 and GABARAP in HeLa and Huh-7 cells. Autophagy 5:878–879

44. Takahashi K, Ueno T, Tanida I et al (2009) Characterization of CAA0225, a novel inhibitor specific for cathepsin L, as a probe for autophagic proteolysis. Biol Pharm Bull 32:475–479
45. Nedelsky NB, Todd PK, Taylor JP (2008) Autophagy and the ubiquitin-proteasome system: collaborators in neuroprotection. Biochim Biophys Acta 1782:691–699
46. Zheng X, Chu F, Mirkin BL et al (2008) Role of the proteolytic hierarchy between cathepsin L, cathepsin D and caspase-3 in regulation of cellular susceptibility to apoptosis and autophagy. Biochim Biophys Acta 1783:2294–2300
47. Bhoopathi P, Chetty C, Gujrati M et al (2010) Cathepsin B facilitates autophagy-mediated apoptosis in SPARC overexpressed primitive neuroectodermal tumor cells. Cell Death Differ 17:1529–1539
48. Cuervo AM, Dice JF (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273:501–503
49. Lichter-Konecki U, Moter SE, Krawisz BR et al (1999) Expression patterns of murine lysosome-associated membrane protein 2 (Lamp-2) transcripts during morphogenesis. Differentiation 65:43–58
50. Cuervo AM, Dice JF (2000) Unique properties of lamp2a compared to other lamp2 isoforms. J Cell Sci 113:4441–4450
51. Cuervo AM, Mann L, Bonten EJ et al (2003) Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor. EMBO J 22:47–59
52. Orenstein SJ, Cuervo AM (2010) Chaperone-mediated autophagy: molecular mechanisms and physiological relevance. Semin Cell Dev Biol 21:719–726
53. d'Azzo A, Hoogeveen A, Reuser AJ et al (1982) Molecular defect in combined β-galactosidase and neuraminidase deficiency in man. Proc Natl Acad Sci USA 79:4535–4539
54. van der Spoel A, Bonten E, d'Azzo A (1998) Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. EMBO J 17:1588–1597
55. Grisolano JL, Sclar GM, Ley TJ (1994) Early myeloid cell-specific expression of the human cathepsin G gene in transgenic mice. Proc Natl Acad Sci USA 91:8989–8993
56. Burster T, Macmillan H, Hou T et al (2010) Cathepsin G: roles in antigen presentation and beyond. Mol Immunol 47:658–665
57. Yamamoto K, Kawakubo T, Yasukochi A et al (2012) Emerging roles of cathepsin E in host defense mechanisms. Biochim Biophys Acta 1824:105–112
58. Yanagawa M, Tsukuba T, Nishioku T et al (2007) Cathepsin E deficiency induces a novel form of lysosomal storage disorder showing the accumulation of lysosomal membrane sialoglycoproteins and the elevation of lysosomal pH in macrophages. J Biol Chem 282:1851–1862
59. Kakehashi H, Nishioku T, Tsukuba T et al (2007) Differential regulation of the nature and functions of dendritic cells and macrophages by cathepsin E. J Immunol 179:5728–5737
60. Matarrese P, Nencioni L, Checconi P et al (2011) Pepstatin A alters host cell autophagic machinery and leads to a decrease in influenza A virus production. J Cell Physiol 226:3368–3377
61. Tsukuba T, Yanagawa M, Kadowaki T et al (2013) Cathepsin E deficiency impairs autophagic proteolysis in macrophages. PLoS ONE 8:e82415
62. Li Y, Kabbage M, Liu W et al (2016) Aspartyl Protease-Mediated Cleavage of BAG6 Is Necessary for Autophagy and Fungal Resistance in Plants. Plant Cell 28:233–247
63. Takayama S, Reed JC (2001) Molecular chaperone targeting and regulation by BAG family proteins. Nat Cell Biol 3:237–241
64. Wirawan E, Walle LV, Kersse K et al (2010) Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. Cell Death Dis 1:e18
65. Luo S, Rubinsztein DC (2010) Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ 17:268–277
66. Xia HG, Zhang L, Chen G (2010) Control of basal autophagy by calpain1 mediated cleavage of ATG5. Autophagy 6:61–66

67. Yousefi S, Perozzo R, Schmid I et al (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol 8:1124–1132
68. Yoo BH, Wu X, Li Y et al (2010) Oncogenic ras-induced down-regulation of autophagy mediator Beclin-1 is required for malignant transformation of intestinal epithelial cells. J Biol Chem 285:5438–5449
69. Kim JS, Nitta T, Mohuczy D et al (2008) Impaired autophagy: a mechanism of mitochondrial dysfunction in anoxic rat hepatocytes. Hepatology 4:1725–1736
70. Yoon SY, Ha YE, Choi JE et al (2008) Coxsackievirus B4 uses autophagy for replication after calpain activation in rat primary neurons. J Virol 82:11976–11978
71. Demarchi F, Bertoli C, Copetti T et al (2007) Calpain as a novel regulator of autophagosome formation. Autophagy 3:235–237
72. Cheng Y, Ren X, Hait WN et al (2013) Therapeutic targeting of autophagy in disease: biology and pharmacology. Pharmacol Rev 65:1162–1197
73. Choi AM, Ryter SW, Levine B (2013) Autophagy in human health and disease. N Engl J Med 368:651–662
74. Green DR, Levine B (2014) To be or not to be? How selective autophagy and cell death govern cell fate. Cell 157:65–75
75. Deretic V, Saitoh T, Akira S (2013) Autophagy in infection, inflammation and immunity. Nat Rev Immunol 13:722–737
76. Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132:27–42
77. Levine B, Mizushima N, Virgin HW (2011) Autophagy in immunity and inflammation. Nature 469:323–335
78. Kenzelmann Broz D, Spano Mello S, Bieging KT et al (2013) Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. Genes Dev 27:1016–1031
79. Chen ZH, Kim HP, Sciurba FC et al (2008) Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. PLoS ONE 3:1–13
80. Mammucari C, Milan G, Romanello V et al (2007) FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab 6:458–471
81. Frankel LB, Wen J, Lees M et al (2011) microRNA-101 is a potent inhibitor of autophagy. EMBO J 30:4628–4641
82. Korkmaz G, le Sage C, Tekirdag KA et al (2012) miR-376b controls starvation and mTOR inhibition-related autophagy by targeting ATG4C and BECN1. Autophagy 8:165–176
83. Scherz-Shouval R, Shvets E, Fass E et al (2007) Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J 26:1749–1760
84. Zhang L, Li J, Ouyang L et al (2016) Unraveling the roles of Atg4 proteases from autophagy modulation to targeted cancer therapy. Cancer Lett 373:19–26
85. Kisen GO, Tessitore L, Costelli P et al (1993) Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. Carcinogenesis 14:2501–2505
86. Pelicano H, Carney D, Huang P (2004) ROS stress in cancer cells and therapeutic implications. Drug Resist Updat 7:97–110
87. Waris G, Ahsan H (2006) Reactive oxygen species: role in the development of cancer and various chronic conditions. J Carcinog 5:1–8
88. Liao YP, Chen LY, Huang RL et al (2014) Hypomethylation signature of tumor-initiating cells predicts poor prognosis of ovarian cancer patients. Hum Mol Genet 23:1894–1906
89. Brinar M, Vermeire S, Cleynen I et al (2012) Genetic variants in autophagy-related genes and granuloma formation in a cohort of surgically treated Crohn's disease patients. J Crohns Colitis 6:43–50
90. Cabrera S, Fernández AF, Mariño G et al (2013) ATG4B/autophagin-1 regulates intestinal homeostasis and protects mice from experimental colitis. Autophagy 9:1188–1200
91. Webb JL, Ravikumar B, Atkins J (2003) Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem 278:25009–25013
92. Eric EE, Flora S (2010) Oxidative stress and autophagy in cardiac disease, neurological disorders, aging and cancer. Oxid Med Cell Longev 3:168–177

93. Matsui Y, Takagi H, Qu X et al (2007) Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP activated protein kinase and beclin 1 in mediating autophagy. Circ Res 100:914–922
94. Cao DJ, Gillette TG, Hill JA (2009) Cardiomyocyte autophagy: remodeling, repairing and reconstructing the heart. Curr Hypertens Rep 11:406–411
95. Wang X, Gao Y, Tan J et al (2012) HIV-1 and HIV-2 infections induce autophagy in Jurkat and CD4 + T cells. Cell Signal 24:1414–1419
96. Dreux M, Gastaminza P, Wieland SF et al (2009) The autophagy machinery is required to initiate hepatitis C virus replication. Proc Natl Acad Sci USA 106:14046–14051
97. Williams RA, Tetley L, Mottram JC et al (2006) Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol Microbiol 61:655–674
98. Herman M, Gillies S, Michels PA et al (2006) Autophagy and related processes in trypanosomatids: insights from genomic and bioinformatic analyses. Autophagy 2:107–118
99. Kiel JAKW (2010) Autophagy in unicellular eukaryotes. Philos Trans R Soc Lond B Biol Sci 365:819–830
100. Alvarez VE, Niemirowicz GT, Cazzulo JJ (2012) The peptidases of Trypanosoma cruzi: digestive enzymes, virulence factors, and mediators of autophagy and programmed cell death. Biochim Biophys Acta 1824:195–206
101. Williams RA, Mottram JC, Coombs GH (2013) Distinct roles in autophagy and importance in infectivity of the two ATG4 cysteine peptidases of Leishmania major. J Biol Chem 288:3678–3690
102. Alvarez VE, Kosec G, Sant'Anna C et al (2008) Autophagy is involved in nutritional stress response and differentiation in Trypanosoma cruzi. J Biol Chem 283:3454–3464
103. Alvarez VE, Niemirowicz GT, Cazzulo JJ (2013) Metacaspases, autophagins and metallocarboxypeptidases: potential new targets for chemotherapy of the trypanosomiases. Curr Med Chem 20:3069–3077
104. Shu CW, Madiraju C, Zhai D et al (2011) High-throughput fluorescence assay for small-molecule inhibitors of autophagins/Atg4. J Biomol Screen 16:174–182
105. Nguyen TG, Honson NS, Arns S et al (2014) Development of fluorescent substrates and assays for the key autophagy related cysteine protease enzyme, ATG4B. Assay Drug Dev Technol 12:176–189
106. Toshima T, Shirabe K, Mastumoto Y et al (2014) Autophagy enhances hepatocellular carcinoma progression by activation of mitochondrial beta-oxidation. J Gastroenterol 49:907–916
107. Xu Y, An Y, Wang Y et al (2013) miR-101 inhibits autophagy and enhances cisplatin-induced apoptosis in hepatocellular carcinoma cells. Oncol Rep 29:2019–2024
108. Singh KK, Yanagawa B, Quan A et al (2014) Autophagy gene fingerprint in human ischemia and reperfusion. J Thorac Cardiovasc Surg 147:1065–1072
109. Wang JH, Ahn IS, Fisher TD et al (2011) Autophagy suppresses age-dependent ischemia and reperfusion injury in livers of mice. Gastroenterology 141:2188–2199
110. Proenca CC, Stoehr N, Bernhard M et al (2013) Atg4b-dependent autophagic flux alleviates Huntington's disease progression. PLoS ONE 8:e68357
111. Tran E, Chow A, Goda T et al (2013) Context-dependent role of ATG4B as target for autophagy inhibition in prostate cancer therapy. Biochem Biophys Res Commun 441:726–731
112. Apel A, Herr I, Schwarz H et al (2008) Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res 68:1485–1494
113. Gorski SM, Ries J, Lum JJ (2012) Targeting autophagy: the Achilles' heel of cancer. Autophagy 8:1279–1280

ADAM and ADAMTS Family of Metalloproteinases: Role in Cancer Progression and Acquisition of Hallmarks

15

Rajakishore Mishra and Siddavaram Nagini

Abstract

The adamalysins, which include the ADAMs and ADAMTSs, are multidomain, multifunctional proteins of the metzincin superfamily of zinc-dependent metalloproteinases that play a key role in extracellular matrix remodeling and regulation of the tissue microenvironment. While ADAMs are mostly membrane-anchored proteinases, the ADAMTSs are secreted proteinases and/or adhesion molecules. A major function of the ADAMs is ectodomain shedding of membrane-bound growth factors, receptors, cytokines, chemokines, and proteoglycans. The adamalysins are also involved in a multitude of biological processes including fertilization, organogenesis, hemostasis, cell adhesion, intracellular signaling, angiogenesis, and ECM assembly and turnover. These metalloproteinases exert both promoting and inhibitory effects on tumorigenesis and serve as biomarkers of cancer progression and prognosis. Dysregulated expression of adamalysins leads to acquisition of cancer hallmarks such as increased cell proliferation, apoptosis evasion, migration, neovascularization, invasion, and metastasis. In addition, aberrant expression of these proteases also results in drug resistance. Of late, the adamalysins have emerged as potential molecular targets for cancer therapeutics. This chapter summarizes current knowledge on the different types of ADAMs and ADAMTSs, their general structure, functions, role in cancer progression, and acquisition of major cancer hallmarks as well as their potential as diagnostic and prognostic aids and therapeutic targets based on available literature.

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1 Introduction

Cancer, a multifactorial, heterogeneous disease, arises due to sequential accumulation of mutations that promote clonal selection of cells characterized by uncontrolled proliferation, apoptosis evasion, invasion of surrounding tissues, and metastasis to other organs [1, 2]. Tumor invasion, an essential prerequisite for cancer metastasis, involves remodeling of the extracellular matrix (ECM), a process initially considered to be the prerogative of the matrix metalloproteinases (MMPs) [3]. It has now become apparent that adamalysins, which include the ADAMs (*A Di*sintegrin *A*nd *M*etalloproteinase) and ADAMTSs (*A Di*sintegrin *A*nd *M*etalloproteinase with *T*hrombospondin motifs), are also key players in ECM homeostasis and regulation of the tissue microenvironment [4–7]. The metalloproteinase system is in turn regulated by *t*issue *i*nhibitors of *m*etalloproteinases (TIMPs) [8] and *r*eversion-inducing *c*ysteine-rich protein with *K*azal motifs (RECK) [9, 10].

The adamalysins are multidomain, multifunctional proteins of the metzincin superfamily of zinc-dependent metalloproteinases [11]. While ADAMs are mostly membrane-anchored proteinases, the ADAMTSs are secreted proteinases and/or adhesion molecules [12]. Although 40 different proteins have been recognized as members of the ADAMs family, only 25 of these are believed to function in humans (Table 1). Of these, only 13 ADAMs display proteolytic activity. Information on ADAMs is constantly updated in http://people.virginia.edu/~jw7g/Table_of_the_ADAMs.html and http://degradome.uniovi.es/). Members of the ADAMs family are localized to specific organs such as, the heart (ADAM9, -17, -19) [13], kidney (ADAM19) [14], lungs (ADAM33) [15], teeth (ADAM28), and pancreas (ADAM-9, -10, -17) [16].

The human family of ADAMTs comprising 19 known members [17] is classified based on their preferred substrates as the aggrecanases or proteoglycanases (ADAMTS1, 4, 5, 8, 9, 15 and 20), the procollagen N-propeptidases (ADAMTS2, 3 and 14), the cartilage oligomeric matrix protein-cleaving enzymes (ADAMTS7 and 12), the von Willebrand Factor proteinase (ADAMTS13), and orphan enzymes (ADAMTS6, 10, 16, 17, 18 and 19). Table 2 lists the various ADAMTs. Data on ADAMTs is available at http://www.lerner.ccf.org/bme/apte/adamts.

The adamalysins play a central role in biological processes including fertilization, organogenesis, hemostasis, cell adhesion, intracellular signaling, angiogenesis, and ECM assembly and turnover. Mutations and aberrant expression of ADAMs and ADAMTs have been implicated in diverse pathologies including thrombotic thrombocytopenic purpura, inflammatory bowel diseases, airway diseases,

Table 1 ADAMs: chromosomal loci, tissue expression and functions

ADAM	Chromosomal locus	Tissue distribution	Function	Reference (s)
ADAM 1a,b	12q24.13	Sperm	Sperm–egg binding and fusion, interaction with the integrins: α6β1 and α9β1	[121, 122]
ADAM2	8p11.22	Sperm	Sperm–egg binding and fusion, interaction with the integrins: α4β1, α6β1 and α9β1	[123, 124]
ADAM6	14q32.33	Testis	Not fully defined	[125]
ADAM7	8p21.2	Testis	Not fully defined but it interacts with the integrins like α4β1, α4β7 and α9β1	[126, 127]
ADAM8	10q26.3	Lung, kidney, brain, macrophage, neutrophils	Cancer cell migration, neutrophil infiltration and shedding of CD23	[128]
ADAM9	8p11.23	Breast, pancreas, lung, stomach, and skin	Promotion of cell adhesion, invasion, binding to integrins, shedding of HB-EGF, tumor necrosis factor-p75 receptor, cleavage of amyloid precursor protein (APP) and digestion of fibronectin and gelatin	[129]
ADAM10	15q22	Brain, breast, liver, oral cavity, ovary, prostate, colon, kidney	Promotion of cell growth and migration, release of TNFα, digestion of collagen IV, gelatin and myelin basic protein; cleavage of delta, APP, L1, and CD44 and shedding of HB-EGF	[130–132]
ADAM11	17q21.3	Brain	Not fully defined but may act as a tumor suppressor	[133]
ADAM12	10q26.3	Liver, stomach, colon, brain, breast, osteoblast, muscle, placenta and chondrocytes	Promotion of cell growth, muscle formation, binding to integrins, insulin-like growth factor binding protein-3 (IGFBP-3) and IGFBP-5, shedding of HB-EGF, digestion of collagen IV, gelatin, and fibronectin	[134]
ADAM15	1q21.3	Brain, prostate, lungs, stomach, endothelium smooth muscle, chondrocyte, and osteoclast	Promotion of cell growth, Expressed in arteriosclerosis, binds to integrins: αVβ3, α5β1 and α9β1 and helps in digestion of collagen IV and gelatin	[135, 136]

(continued)

Table 1 (continued)

ADAM	Chromosomal locus	Tissue distribution	Function	Reference (s)
ADAM17	2p25	Macrophage, ovary, prostate, kidney, colon, and breast tissue	Promotion of cell growth, binding with integrins: shedding of signaling molecules/surface receptors (TNFα, TGF-β, TNF-p75 receptor, ErbB4, TNF-related activation induced cytokine HB-EGF, APP, Notch, L-selectin and CD44)	[137]
ADAM18	8p11.22	Brain, testis, kidney	Not fully defined	[138]
ADAM19	5q32–q33	Testis	Formation of neuron, digestion of neuregulin and interacts with the integrins like α4β1, α5β1	[128]
ADAM20	14q24.1	Testis	Formation of sperm	[139]
ADAM21	14q24.1	Testis	–	[139]
ADAM22	7q21	Brain	–	[140]
ADAM23	2q33	Brain, Heart	Not fully defined but it interacts with the integrins like αVβ3	[140]
ADAM28	8p21.2	Testis, lung, lymphocyte, pancreas, uterus	IGFBP-3 cleavage, promotion of cell growth, binding with integrins: α4β1, α4β7, α9β1; digestion of myelin basic protein and IGFBP-3	[141, 142]
ADAM29	4q34	Testis	–	[143]
ADAM30	1p13–p11	Testis	–	[144]
ADAM32	8p11.23	Testis	–	[145]
ADAM33	20p13	Lung (fibroblast, smooth muscle)	Interactions with integrins; cleavage of APP, Kit-ligand-1 (KL-1) and insulin B chain	[146]

osteoarthritis, atherosclerosis, neurodegeneration, and cancer. Adam-19 KO mouse suffers from developmental defects and embryos died due to abnormalities of the heart and other cardiovascular system disorders [18]. The proteolytic activities, regulation of growth factors and cytokines and the ability to degrade ECM components, suggest that these enzymes may be involved in cell migration, invasion, angiogenesis, and metastatic spread of tumor cells [19, 20]. This chapter summarizes current knowledge on different types of ADAMs and ADAMTSs, their general structure, functions, role in cancer progression, and acquisition of major cancer

Table 2 ADAMTs: chromosomal loci, tissue expression and functions

ADAMT	Chromosomal locus	Tissue distribution	Function	Reference (s)
ADAMTS1	21q21	Ovary, breast, bronchial epithelial cells, fetal lung, placenta, smooth muscle, uterus, adrenal cortex, adipocytes, ciliary ganglion, prostate, olfactory bulb, breast stromal fibroblasts and myoepithelial cells	Promotion of cell growth, cell survival and invasion, Binding to heparin, HB-EGF and AR shedding, digestion of aggrecan and versican, syndecan 4, TFPI-2, semaphorin 3C, nidogen-1, -2, desmocollin-3, dystroglycan, mac-2, gelatin, amphiregulin, TGF-α	[89, 100, 147, 148]
ADAMTS2	5q35	Adipocyte, skeletal muscle, superior cervical ganglion, uterus, placenta, heart, liver, lung, tongue, smooth muscle, breast stromal fibroblasts	Processing of collagen I and II N-propeptides, Glucocorticoids (in monocytes) and IL-6	[149]
ADAMTS3	4q21 (NM014243.1)	Skeletal muscle, tendon, cartilage, bone, breast myoepithelial cells, CD105+ endothelial cells, CD34+ cells and pineal gland	Processing of collagen N-propeptides, fibrillar procollagen type II and biglycan	[150]
ADAMTS4	1q23	Brain, heart, ovary, spinal cord, adrenal cortex, ciliary ganglion, trigeminal ganglion, retina, pancreas (islets), fetal lung and breast myoepithelial cells	Digestion of aggrecan, brevican and versican, reelin, biglycan, matrilin-3, α2-macroglobulin, COMP, IL-1 + oncostatin M, TNFα, S100A8, S100A9, leptin, IL-6	[31, 151, 152]
ADAMTS5	21q21	Brain, adipocyte, uterus, breast myoepithelial cells, uterus, placenta	Promotion of invasion, Digestion of aggrecan, versican, reelin, biglycan, matrilin-4, brevican, α2-macroglobulin cleavage, IL-1, TNFα, S100A8, S100A9, leptin, IL-6	[153–155]

(continued)

Table 2 (continued)

ADAMT	Chromosomal locus	Tissue distribution	Function	Reference (s)
ADAMTS6	5q12	Heart, breast myoepithelial cells, superior cervical ganglion, trigeminal ganglion, appendix	Regulation of TNFα	[151]
ADAMTS7	5q24	Liver, heart, skeletal muscle, trigeminal ganglion, adrenal cortex, intervertebral disc and breast stromal fibroblasts	Regulation of PTHrP, acts on COMP	[156, 157]
ADAMTS8	11q24	Skeletal muscle, heart, lungs, liver, superior cervical ganglion, adrenal cortex, breast stromal fibroblasts and luminal epithelial cells	Inhibitor of angiogenesis, helps in digestion of aggrecan	[102]
ADAMTS9	3p14	Dorsal root ganglion, breast and myoepithelial cells	Digestion of aggrecan, versican, TNFα, IL1 + oncostatin M and leptin	[56, 158]
ADAMTS10	19p13	Brain, uterus, breast stromal fibroblasts and CD8+ T cells	Acts on fibrillin-1	[159]
ADAMTS12		Liver, bone marrow, atrioventricular node, intervertebral disc, breast stromal fibroblasts and myoepithelial cells	Acts on COMP	[160, 161]
ADAMTS13	9q34	Liver, CD71+ early erythroid cells, lung, thyroid, breast myoepithelial cells; prostate, brain	Cleavage of von Willebrand factor (vWF) and IL-1	[162]
ADAMTS14	10q22	Thalamus, brain, uterus, bone marrow, fetal thyroid, adipocyte, cerebellum, bone, skin, fibroblasts, breast myoepithelial and luminal epithelial cells	Processing of collagen N-propeptides such as fibrillar procollagen type I (pNα1 and pNα2 chains)	[163]
ADAMTS15	11q24	Colon, brain, heart, uterus, musculoskeletal system, breast myoepithelial cells, Liver (fetus), Kudney (fetus)	Digestion of aggrecan and versican	[57, 58, 93]

(continued)

Table 2 (continued)

ADAMT	Chromosomal locus	Tissue distribution	Function	Reference (s)
ADAMTS16	5p15	Breast myoepithelial cells	Regulated by follicle stimulating hormone; forskolin cAMP; Transcription factors: Wilm's tumor-1; Egr-1 and Sp1	[164]
ADAMTS17	15q26	Breast myoepithelial cells	–	[165]
ADAMTS18	16q23	Ciliary ganglion, heart, skin, brain and breast myoepithelial cells	–	[166, 167]
ADAMTS19	5q23	Dorsal root ganglion, breast myoepithelial cells	–	[168]
ADAMTS20	2q12	Brain, appendix, heart, liver, skeletal muscle, pituitary, trigeminal ganglion, breast myoepithelial cells	–	[169]

hallmarks as well as their potential as diagnostic aids and therapeutic targets based on available literature.

2 Structure of Adamalysin Family of Proteins

The adamalysins family of proteins shows sequence similarities with the MMP family members as well as the reprolysin family of snake venomases [21, 22]. Based on their structure, the adamalysin family proteins are classified into the membrane-anchored ADAMs and the secreted ADAMTSs (Fig. 1).

The ADAM family members have a complex structure with multiple domains. The structural elements from the amino terminus comprise a *signal peptide* that marks the protein for the secretory pathway, a *prodomain* that ensures accurate folding of the protein and prevents catalytic activity of the metalloproteinase domain via a cysteine-switch mechanism until it is cleaved in the Golgi apparatus, a *metalloproteinase domain*, with the consensus sequence HEXGHXXGXXHD [23], a highly conserved *disintegrin domain* that interacts with integrins and mediates cell adhesion [24, 25], a *cysteine-rich domain* involved in substrate recognition and cell adhesion [26], an *EGF-like domain*, a *transmembrane domain*, and a *cytoplasmic tail* that contains phosphorylation sites and interacts with proteins containing the Src homology domain [27].

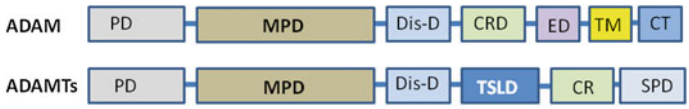

Fig. 1 General structure of adamalysin family of proteins. ADAM family members contain a propeptide domain (PD), a metalloproteinase domain (MPD), disintegrin domain (Dis-D), cysteine-rich domain (CRD), EGF-like domain (ED), transmembrane (TM) and cytoplasmic tail (CT). On the other hand, the secretory ADAMTs do not possess a functional TM, CT and ED but contain a thrombospondin-like domain (TSLD) and spacer domain (SPD)

The activation of ADAMs involves removal of the prodomain from the precursor protein by a proprotein convertase of furin type or by an autocatalytic process [28, 29]. Analysis of the crystal structure revealed that the disintegrin and cysteine-rich domains form a C-shaped structure, restricting accessibility for protein binding. Isoforms of ADAM9, ADAM11, ADAM12, and ADAM28 are secreted proteins that lack the transmembrane and cytoplasmic domains. The ADAM19 isoform lacks the propeptide, metalloproteinase, and disintegrin domains. Splice variants of ADAM15 and ADAM22 have also been identified.

Unlike the ADAMs, the ADAMTS do not possess the EGF-like, transmembrane and cytoplasmic domains [30, 31]. These proteins are characterized by the presence of a thrombospondin type I sequence repeat (TSR) motif. Some of the members contain one or two additional specific C-terminal modules such as a mucin domain (ADAMTS-7, and -12). Members of the ADAMTs family differ in the carboxy-terminal region downstream of the TSR, known as the ancillary domain. The ancillary domains provide substrate-binding specificity and ensure correct tissue compartmentalization, whereas cleavage site specificity is endowed by the protease domain. The ADAMTS differ from the ADAMs in their cysteine signatures. A unique family of seven ADAMTS-like (ADAMTSL) proteins that include ADAMTSL 1–6 and papilin, contain the ancillary domains of ADAMTS but lack the catalytic domains may modulate the activities of the ADAMTSs. The ADAMTS with the exception of ADAMTS4 as well as the ADAMTSL undergo posttranslational modifications that involve the addition of *N*-linked carbohydrate essential for activity.

3 Functions

Like the MMPs, both ADAMs and ADAMTs exhibit catalytic activity. Several ADAMs degrade ECM substrates and insulin-like growth factor binding proteins (IGFBPs). For example, ADAM10 cleaves type IV collagen, ADAM12 cleaves gelatin, type IV collagen and fibronectin, ADAM15 digests type IV collagen and gelatin, and ADAM28 cleaves IGFBP-3. Unlike ADAMs which due to their

membrane localization are predominantly involved in ectodomain shedding of proteins from the cell surface, the ADAMTS being secreted proteases are primarily involved in proteolytic events in the ECM. The ADAMTS1, 2, 3, 4, 5, 7, 8, 9, 14, 15, 16, 18, and 20 have been documented to degrade the ECM. ADAMTS1 remodels the ECM via proteolytic degradation of chondroitin sulfated proteoglycans and collagen [32]. ADAMTS-4 and ADAMTS-5 cleave aggrecan and are referred to as aggrecanases. These proteases also cleave brevican and versican [33, 34], while ADAMTS-2 is known to process procollagen chains [35]. However, in contrast to MMPs, most ADAMTS proteases do not cleave short peptides. Furthermore, proteolysis by ADAMTS may require posttranslational modifications of the substrate. ADAMTS2 processes procollagen efficiently when it is in the triple-helical conformation, but is unable to cleave the heat-denatured form. Mutation of ADAMTS13, a von Willebrand factor-cleaving protease, causes thrombotic thrombocytopenic purpura, a potentially fatal disease.

Considering the fact that only half of the proteins of the ADAMs and ADAMTS family display catalytic activity, it is apparent that the functions of these proteins extend beyond proteolytic activity and ECM remodeling. The first identified ADAMs (ADAM-1 and -2) were shown to induce the fusion of the sperm with the egg through interactions with the disintegrin domain. ADAM-15, a component of adherens junctions, is believed to regulate cell adhesion through interaction with various integrins via the disintegrin domain [36]. ADAM-10 regulates central nervous system development by cleaving the NOTCH protein [37]. ADAMs play a key role in signal transduction by interacting with tyrosin kinases and cytoskeletal components through their cytoplasmic domain [38].

One of the most important functions of the proteolytic ADAMs is their ability to cleave membrane-bound growth factors, receptors, cytokines, chemokines, and proteoglycans, thereby releasing the mature soluble forms, by their *sheddase* activity. ADAM-17, a prototype sheddase that cleaves pro-tumor necrosis factor-α (pro-TNF-α), is also known as TNF-α converting enzyme (TACE) [39–41]. In addition to TNF-α, ADAM-17 is also involved in shedding other membrane proteins such as proTGF-α, pro-amphiregulin and pro-epiregulin. ADAM-17 as well as ADAM9 and ADAM12 are responsible for shedding pro-heparin-binding epidermal growth factor (pro-HB-EGF) thereby regulating cell proliferation. ADAM-10 is responsible for shedding the low-affinity immunoglobulin E receptor CD23 [40, 42]. Some ADAMTS also participate in ectodomain shedding, such as ADAMTS1 which sheds syndecan-4 besides enhancing the shedding of HB-EGF. The sheddase activity of ADAMs is thought to be regulated through the PKC and MAPK pathways [43].

Regulated intramembrane proteolysis (RIP) is a highly conserved signaling process by which membrane-bound signaling proteins are cleaved before being released into the cytoplasm. In most cases, RIP is preceded by ectodomain shedding. The membrane proteins Notch, CD44 and amyloid precursor protein, first undergo ADAM-dependent ectodomain cleavage followed by RIPping by γ-secretase [44].

4 Adamalysins in Cancer

There is substantial evidence to implicate the role of adamalysins in the aetiopathogenesis of various cancer types. The adamalysins exert both promoting and inhibitory effects on tumorigenesis. These dual roles probably reflect the complex interplay between the tumor, the surrounding stroma and the immune system (Figs. 2 and 3).

ADAMs and ADAMTs are primarily involved in processing the ligands of growth factor receptors thereby facilitating extracellular matrix remodeling to promote tumor progression and metastasis. Overexpression of several members of the ADAM family proteins has been reported in diverse malignancies. These include ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, and ADAM28 [22]. ADAM-9 is upregulated in a number of cancers including renal, breast, and prostate cancer [45–47]. Upregulation of ADAM10 expression has been documented in diverse malignancies including cancer of the

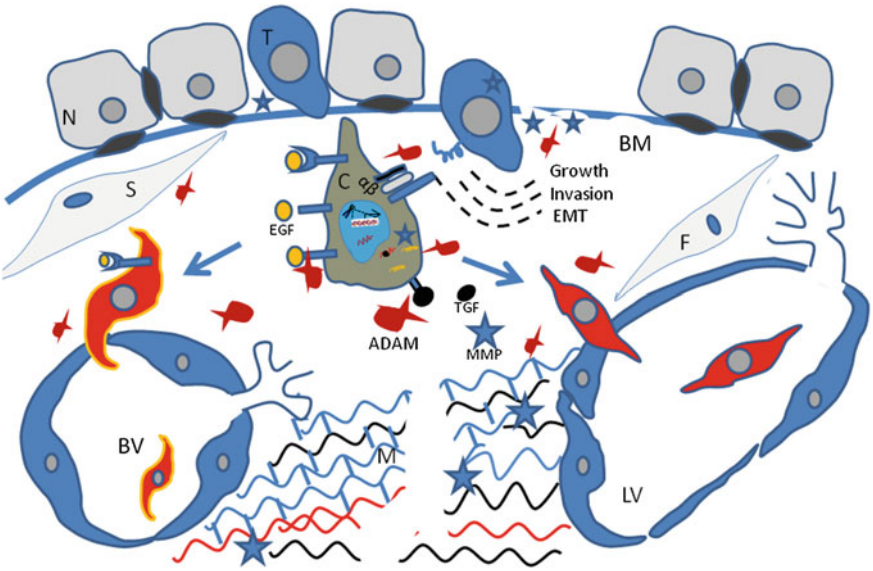

Fig. 2 The role of ADAM family of metalloproteinases in cancer. ADAM-mediated cancer cell proliferation and progression. ProADAMs are activated by furin or matrix metalloproteinases (MMPs). The sheddase activity of ADAMs cleaves and releases the cell surface ligands such as heparin-binding epidermal growth factor (HP-EGF), transforming growth factor TGFα and epidermal growth factor receptor (EGFR) to promote cancer. The interaction of ADAMs with integrins or syndecans on the cells enables cleavage of substrates, enhances invasion/metastasis or promotes proliferation signals. Many membrane-anchored molecules like chemokines, cytokines and their receptors, may interact with various ADAMs and promote cancer cell proliferation, angiogenesis, lymphangiogenesis and thus contribute to cancer cell progression. *N* nontransformed cell, *T* transformed cell, *S* stromal cell, *F* fibroblast cell, *BM* basement membrane, *BV* blood vessel, *LV* lymphatic vessel

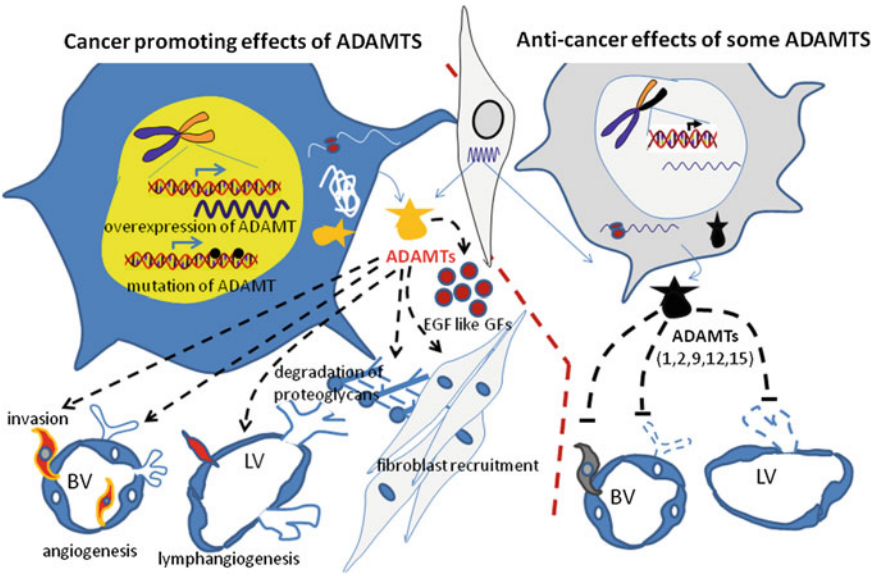

Fig. 3 The pro/anticancer effects mediated by different members of ADAMTS family of proteases. Many of the ADAMTSs family members are produced by stromal or cancer cells. Epigenetic modification of ADAMTS genes is mainly responsible for their expression. Their contribution to cancer progression is not fully understood. While most members exert cancer-promoting effects, other members (including ADAMT-1, 2, 9, 12 and 15) are involved directly or indirectly in inhibiting carcinogenesis. ADAMTs thus have either positive or negative influence on angiogenesis or lymphangiogenesis, or affect cancer-promoting signaling pathways through the degradation of extracellular components such as thrombospondin 1/2, nidogen 1/2, VEGF sequestration, activation of pro-angiogenic factors (HB-EGF, amphiregulin, IGFBP2), digest extracellular matrix components (proteoglycans), and recruitment of fibroblasts involved in cancer growth

stomach, oral cavity, ovary, uterus, colon, prostate as well as leukemia [22, 48]. ADAM15 has been reported to be overexpressed in breast, prostate, stomach, and lung cancer [22]. ADAM-17 expression is increased in breast cancer tissues with higher expression in advanced-grade compared to low-grade tumors. Patients displaying a higher expression of ADAM17 have a shorter overall survival than those with low expression [49]. The increased level of ADAM29 has been suggested to have a significant prognostic value for patients with CLL [50]. Likewise ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS6, and ADAMTS14 are also upregulated in malignant tumors [22, 51].

Several adamalysins are also downregulated in malignant tumors due to loss by mutation or epigenetic silencing. Interestingly most of these belong to the ADAMTS family including ADAMTS1, ADAMTS3, ADAMTS5, ADAMTS8, ADAMTS9, ADAMTS10, ADAMTS15, and ADAMTS18. These proteins apparently function as tumor suppressors [51]. ADAMTS1 is poorly expressed in hepatocellular carcinoma (HCC) [52]. Expression profiling has shown downregulation

of ADAMTS8 gene expression in breast carcinoma, non-small cell lung cancer (NSCLC), and brain cancers [53–55]. Knockdown of ADAMTS-9 and ADAMTS-15 increases the tumorigenic potential of breast, gastric, and colon cancer cells [56, 57]. Clinical studies on patients with breast cancer revealed reduced ADAMTS15 expression that correlates with a higher probability of cancer development and increased mortality [58]. Notably, ADAMTS15 gene is not only epigenetically silenced, but also frequently mutated in colon and pancreatic carcinomas [57, 59, 60]. Studies on cancer cell lines indicated that ADAMTS18 gene is frequently epigenetically silenced that was subsequently confirmed in tissue samples from cancer patients [61].

5 Adamalysins and Cancer Hallmarks

Dysregulated functions and activities of adamalysins lead to acquisition of cancer hallmarks such as increased cell proliferation, apoptosis evasion, migration, invasion, and neovascularization. In addition, aberrant expression of these proteases also results in drug resistance.

5.1 Cell Proliferation and Apoptosis

Apoptosis evasion, a key hallmark capability of cancer, plays a critical role in promoting cell proliferation and cell survival. ADAM family of proteolytic enzymes regulates cell proliferation by cleaving growth factors or cell surface proteins. The EGF receptor ligands (heparin-binding EGF, amphiregulin, betacellulin, epiregulin) are synthesized as transmembrane proteins and all these require ectodomain shedding for their activation [62, 63].

ADAM9 has been reported to facilitate cell proliferation and cell survival by promoting the degradation of E-cadherin. Silencing of ADAM9 reduced ESCC cell proliferation and migration by inhibiting EGF receptor-AKT signaling [64]. ADAM9 has been reported to be involved in the proteolytic cleavage of the HB-EGF precursor and contribute to melanoma progression [65].

ADAM-10 has a broad substrate range and contains the six EGFR ligands, TNF, epireguline, HB-EGF and EGF. It also contributes to E-cadherin shedding [66, 67]. ADAM-10 promotes cell proliferation by modulating β-catenin signaling and regulating cyclin D1 levels [68]. ADAM-10 knockout (KO) embryos suffer from cell growth arrest and apoptosis associated with overexpression of full-length E-cadherin [67]. ADAM10 plays a role in regulated intramembrane proteolysis (RIP), which is part of the Notch/Delta signaling pathway involved in the cleavage of Notch membrane receptor and tumor promotion [69–71]. ADAM12 that promotes HB-EGF shedding was found to be overexpressed in colorectal, breast, liver, and stomach cancer [22]. ADAM-12 has been shown to increase stromal cell apoptosis and decrease tumor cell apoptosis.

ADAM17, a potent inducer of tumor growth and cell division promotes the cleavage of different substrates including TGF-β [72]. ADAM-17 cleavage of amphiregulin enhances proliferation of cancer cells. ADAM-17 cleaves and releases bioactive epigen that serves as a ligand of EGFR and promotes growth and tumorigenesis. ADAM17-catalyzed HB-EGF shedding was demonstrated to induce mitogenic ERK1/2 signaling [73].

The expression of membrane-anchored ADAM28m and secreted-type ADAM28s was found to be significantly higher in breast carcinomas compared to nonneoplastic breast tissues. Treatment of ADAM28-expressing MDA-MB231 breast carcinoma cells with insulin-like growth factor-I (IGF-I) increased cell proliferation, cleavage of IGF binding protein (IGFBP)-3, and IGF-I cell signaling. However, treatment with ADAM inhibitor, anti-ADAM28 antibody or siRNA silencing of ADAM-28, attenuated these processes as well as growth of xenografts in mice. These results suggest that ADAM28 enhances proliferation of breast cancer cells by releasing IGF-I released from the IGF-I/IGFBP-3 complex [74].

ADAMTS1 is reported to play a role in breast cancer development and progression. Induced overexpression of ADAMTS1 results in poor survival and accelerated tumor growth in mouse models of breast cancer. Overexpression of full-length ADAMTS-1 in CHO cells enhances tumor growth [75]. A recent study showed a link between overexpression of ADAMTS1 and tumor growth rate in a fibrosarcoma model.

5.2 Cell Migration, Invasion, and Metastasis

Excessive cell proliferation coupled with apoptosis evasion enables accumulation of mutations that facilitate cell migration, invasion, and metastasis which are the most important events in tumor progression responsible for cancer morbidity and mortality. Adamalysins that play an important role in sculpting the tumor microenvironment are invaluable biomarkers of disease progression and therapeutic outcome.

ADAM9 abrogates cell–cell contact and facilitates cellular migration by promoting the degradation of E-cadherin. ADAM9 was found to reduce cellular migration, invasion, and induction of the epithelial marker E-cadherin in pancreatic cancer [76]. The possible binding of DIS domain of ADAM9 to α6β4 and α2β1 integrins and subsequent proteolytic activity of ADAM9 enables cleavage of laminins and promotes invasion in cancer of the breast, pancreas, stomach, skin, liver, and lung [22]. Phosphorylation of the cytoplasmic domain of ADAM9 by PKCδ has been reported to lead to HB-EGF shedding. Abety et al. (2012) demonstrated increased proliferation and reduced apoptosis in coculture of melanoma cells and ADAM-9(−/−) fibroblasts, as well as in ADAM-9(−/−) mice injected melanoma cells. The data indicate that stromal expression of ADAM-9 influences melanoma development both in vitro and in vivo by targeting TIMP-1 and sTNFR1. Chang et al. (2015; 2016) provided evidence to indicate that ADAM9 is a potential candidate for targeted therapy of non-small cell lung carcinoma

(NSCLC). Downregulation of ADAM9 expression by RNA interference-mediated gene silencing in human A549 NSCLC cells inhibited cell proliferation, migration and invasion, and induced apoptosis. ADAM9 gene silencing also suppressed tumor growth in a mouse model of lung metastasis [77].

ADAM-10 contributes to E-cadherin shedding and subsequent release of soluble E-cadherin in the extracellular environment thereby promoting cell migration. Overexpression of ADAM10 was demonstrated to drive metastasis in various cancers. Knockout of ADAM10 by siRNA enhanced the antitumor activity of the VEGFR inhibitor sorafenib as evidenced by reduced proliferation, migration and invasion, and induction of apoptosis in hepatoma cells in vitro, and suppressed tumor growth in vivo. This was associated with inhibition of PI3K and AKT phosphorylation implying the involvement of ADAM10 in the activation of PI3/Akt signaling pathway [78].

ADAM17 is involved in the proteolysis of collagen IV of the ECM as well as the release of several integrins from the cell surface, suggesting that ADAM17 has a profound influence on the invasive activity of different cancer cells. Furthermore, ADAM17 as a primary upstream component for multiple EGFR pro-ligands may also activate MEK/ERK and PI3K/Akt pathways, which contribute to invasiveness. Primary blood blasts CD13+ CD33+ from patients with acute myeloid leukemia (AML) expressed ADAM17 transcript with higher surface expression in subtype M4 (myelomonocytic) and M5 (monocytic) specimens than in M0 and M1/M2 (early and granulocytic) specimens. Knockdown of CD13 revealed that it is required for downregulation of ADAM17. Interaction of ADAM17 with CD13 is believed to be essential for ADAM17 mediated cell growth, migration, and invasion [79].

ADAMTS-1 is involved in tumor progression and facilitates local invasion and lymph node metastasis. It is overexpressed in pancreatic cancer. Further, the overexpression of a catalytically inactive ADAMTS-1 impedes these events, which strongly suggests a prometastatic role for this metalloprotease mediated by its proteolytic activity. Elevated ADAMTS-1 expression has also been associated with high risk of bone and lung metastasis in breast cancer patients. It has been proposed that ADAMTS-1 could facilitate the spread of tumor cells through the degradation of versican, a predictor of metastatic relapse in human breast cancer. Similarly, ADAMTS5 promotes brain tumor invasion [22].

Overexpression of ADAMTS-1 promotes pulmonary metastasis of TA3 mammary carcinoma and Lewis lung carcinoma cells associated with angiogenesis, invasion, shedding of the transmembrane precursors of heparin-binding epidermal growth factor (EGF) and amphiregulin (AR), and activation of the EGF receptor and ErbB-2. However, the proteinase-dead mutant of ADAMTS-1 (ADAMTS-1E/Q) inhibits metastasis. Overexpression of the NH(2)- and COOH-terminal fragments generated by auto-proteolytic cleavage of ADAMTS-1 also inhibits pulmonary tumor metastasis as well as Erk1/2 kinase activation induced by soluble heparin-binding EGF and AR. These results suggest that the metalloproteinase activity of ADAMTS-1 is essential for its prometastatic activity [80].

5.3 Angiogenesis

Angiogenesis, the formation of new blood vessels from preexisting microvasculature plays a central role in tumor growth, invasion, and metastasis. The transformation from a microscopic prevascular lesion to a rapidly expanding highly vascularized referred to as an "*angiogenic switch*" occurs when the pro-angiogenic factors outweigh the effect of angiostatic molecules. Angiogenesis is a complex and tightly regulated process involving the activation of diverse intracellular signaling pathways, chiefly vascular endothelial growth factor (VEGF) signaling. Specific ADAMTSs play a pivotal role in regulating tumor angiogenesis. Multiple mechanisms have been proposed to explain the inhibition of angiogenesis by members of the ADAM and ADAMTS family.

Transfection of both the full-length ADAMTS1 and catalytic domain-deleted ADAMTS1 (delta ADAMTS1) inhibited endothelial cell proliferation, migration, and tube formation by inducing apoptosis. These effects were abolished following immunoprecipitation of the secreted protein from the medium. Both full ADAMTS1 and delta ADAMTS1 gene transfer into tumor-bearing mice significantly inhibited tumor growth as well as angiogenesis and induced apoptosis. These results demonstrate that the antiproliferative and antiangiogenic effects of ADAMTS1 are independent of its protease activity [81].

ADAM-15 expressed in smooth muscle cells, umbilical vein endothelial cells, and activated endothelial cells is documented to regulate angiogenesis [82]. In ADAM-15-deficient mice, angiogenesis was found to be inhibited [83]. The presence of Arg-Gly-Asp (RGD) sequence in the disintegrin domain that binds integrins has been suggested to play a role in regulating angiogenesis. The recombinant human disintegrin domain (rhdd) of ADAM15 was found to be a potent inhibitor of tumor formation and angiogenesis. ADAM-15 RDD decreases tumor growth associated with reduced vascularization of MDA-MB-231and B16F10 cells [84]. rhddADAM15 inhibited the proliferation of Bel-7402 hepatoma cells via the mitogen-activated protein kinase pathway and reduced the activation of Src. In addition, rhddADAM15 inhibited the proliferation, migration, and tube formation of vascular endothelial EA.hy926 cells in vitro and angiogenesis in zebrafish in vivo [85]. However, contrary to these reports, mice with sufficient or deficient ADAM-15 showed no difference in tumor vascularity between wild-type and mutant mice [83].

ADAMTS-1 and ADAMTS-8 have been established as antiangiogenic factors [86]. The thrombospondin motifs of ADAMTS-1/-8 interact directly with a membrane glycoprotein receptor CD36 of endothelial cells or directly through VEGF binding [87, 88]. The TSP-1 repeats in ADAMTS-1 are believed to contribute to its antiangiogenic activity by trapping VEGF [87, 89].

ADAMTS-2 plays a crucial role in processing fibrillar procollagen to mature collagen. Recombinant ADAMTS-2 reduces proliferation of endothelial cells, inhibits vasculature, and induces apoptosis associated with dephosphorylation of Erk1/2 and MLC. ADAMTS-2 also suppressed growth and vascularization of tumors induced in nude mice by HEK 293-EBNA cells. The antiangiogenic

properties of ADAMTS-2 were shown to be mediated by nucleolin, a receptor found in the nucleus and the cell membrane [90].

ADAMTS5 and 8 ADAMTS-8 have been demonstrated to exert antiangiogenic function in tumors. Overexpression of full-length ADAMTS5 inhibited B16 melanoma growth in mice by suppressing angiogenesis through the central TSR (TSR1) presumably by downregulating the pro-angiogenic factors VEGF, placental growth factor (PlGF), and platelet-derived endothelial growth factor (PD-EGF). This was associated with diminished cell proliferation and enhanced apoptosis. Catalytically active ADAMTS5 proteolytic fragment also suppressed angiogenesis in vitro [91]. ADAMTS8 was shown to block angiogenesis via the inhibition of FGF-induced vascularization and VEGF-induced angiogenesis. Mice with a single silenced Adamts9 allele showed spontaneous neovascularization, thus confirming the antiangiogenic activity of ADAMTS9 [92].

Decreased ADAMTS15 expression correlated with a worse prognosis in mammary carcinoma [58]. Kelwick et al. [93] investigated the effects of ADAMTS15 on MDA-MB-231 and MCF-7 breast cancer cells by stable expression of either a wild-type (wt) or metalloproteinase-inactive (E362A) protein. While neither form influenced cell proliferation or apoptosis, both forms suppressed cell migration on fibronectin or laminin matrices. The wt ADAMTS-15 but not the E362A mutant inhibited endothelial tubulogenesis and angiogenesis indicating that catalytic functionality is essential for antiangiogenic effects. Experimental metastasis assays in nude mice revealed decreased spread to the liver for both the wt and mutant forms, with enhanced lung colonization for cells expressing wt ADAMTS-15 implying tissue niche-dependent effects [93].

Unlike other ADAMTSs, which exert antiangiogenic effects, ADAMTS-4 has been shown to promote angiogenesis in Ewing's sarcoma. It is noteworthy that ADAMTS-4 undergoes an autocatalytic processing similar to that described for ADAMTS-1, which affects the balance between protumorigenic and antitumorigenic functions of this metalloprotease.

6 Adamalysins and Chemoresistance

Resistance to chemotherapeutic drugs, especially multidrug resistance is a major obstacle in cancer treatment. Several adamalysin family members are reported to induce drug resistance given their intricate involvement in proliferation, migration and invasion. Large-scale expression analysis of drug-resistant cells using high-density oligonucleotide microarrays revealed altered expression of 13 genes encoding MMPs, ADAMs, ADAMTSs, and TIMPs in drug-resistant sublines when compared with sensitive MCF-7 breast cancer cells [94]. Recent research is focused on developing strategies to overcome chemoresistance by silencing these molecules.

Increased expression of ADAM-17 that leads to growth factor shedding and growth factor receptor activation is postulated to induce drug resistance. In multidrug-resistant colorectal carcinoma (CRC), an inverse correlation was observed between the expression levels of ADAM-17 and miR-222. Transfection of HCT116/L-OHP and HCT-8/VCR cells with miR-22 mimics reduced ADAM-17 expression and sensitized these cells to apoptosis induced by anticancer drugs. Pharmacological inhibition of ADAM-17 in conjunction with chemotherapy may have greater therapeutic efficacy [95].

Wang et al. [96] reported that hypoxia-induced resistance to cisplatin treatment in Hep3B and HepG2 hepatocarcinoma cells is mediated by upregulation of ADAM-17 via HIF1-α. Furthermore, overexpression of ADAM17 inhibited cisplatin-induced apoptosis and enhanced the phosphorylation of epidermal growth factor receptor (EGFR) and Akt, suggesting that ADAM17 causes cisplatin resistance via the HIF1α/EGFR/PI3K/Akt pathway [96].

Cancer stem cells (CSCs) are known to mediate chemoresistance in patients with metastatic colorectal cancer. Analysis of the effect of ADAM-17 inhibition by siRNA knockdown or by TAPI-2 revealed a role for ADAM17 on cancer stem cell (CSC) phenotype and chemosensitivity to 5-fluorouracil (5-FU) in colorectal cancer via cleavage and release of soluble Jagged-1 and -2 and activation of Notch signaling [97].

ADAM10 is upregulated in several cancers and is associated with advanced tumor stage and grade. Small interfering RNA (siRNA) knockdown of ADAM10 decreased cell proliferation, migration, and invasion and increased cisplatin-induced apoptosis in bladder cancer cell lines indicating that ADAM10 is a candidate therapeutic target [98].

7 Epigenetic Modifications of Adamalysins

Epigenetic mechanisms including aberrant DNA methylation at CpG islands and histone modifications play a fundamental role in the development and progression of cancer. In addition, microRNAs also control target gene expression posttranscriptionally.

Significantly higher methylation of ADAM23 was observed in estrogen receptor (ER) positive breast cancers compared to ER negative cases [99]. The frequency of ADAMTS1 methylation was significantly higher in gastric cancer and positively correlated with depth of tumor invasion and tumor node, metastasis and stage [100]. Downregulation of ADAMTS9 in multiple myeloma was associated with promoter methylation [101]. ADAMTS8, a novel tumor suppressor that inhibits EGFR signaling and phosphorylation of MEK and ERK was frequently silenced by promoter methylation in nasopharyngeal, esophageal squamous cell, gastric, and colorectal carcinomas [102]. Using high-resolution melting (HRM) as a tool for analysis of promoter methylation, higher degree of methylation of ADAMTS9 and ADAMTS18 was observed in several cancers indicating gene silencing [61, 103].

ADAMTS12 promoter is epigenetically silenced in tumor cells by hypermethylation, whereas in the surrounding stromal cells, expression of this protease is higher presumably as a protective response [104]. Methylation of *ADAMTS19* gene promoter was linked to altered in vivo migration and invasion capabilities of CRC cells [105].

ADAM17 was identified as a direct target of miR-145, a tumor suppressor miR that is significantly downregulated in glioma cells. Ectopic expression of miR-145 decreased in vitro proliferation, migration, and invasion of glioma cells as well as the expression of ADAM17 and EGFR [106]. High expression of ADAM9 in bladder cancer was found to correlate inversely with miR-126 and indicated poor prognosis. While knockdown of ADAM9 ameliorated invasiveness of bladder cancer cells, restoration of miR-126 levels suppressed invasion [107].

8 Therapeutic Potential

Adamalysins have emerged as potential molecular targets for cancer therapeutics. Synthetic molecules targeting ADAMs such as KB-R7785, a GM6001-derived hydroxamate have been developed [108]. KB-R7785 is believed to inhibit ADAM17 and block the synthesis of TNF-α, inhibit ADAM10 processing of CD44 and consequent cell migration. Drugs targeting the cysteine-rich region of ADAM-12 have been suggested to inhibit invasion and metastasis [109].

ADAM-17 has been implicated in the development and progression of breast cancer and is an independent predictor of prognosis [49]. Several strategies have been developed to target ADAM-17 including selective low-molecular-weight inhibitors [49, 110, 111]. An inhibitory humanized monoclonal antibody D1(A12), that binds to both the catalytic domain and the disintegrin/cysteine-rich domain of ADAM-17, was found to inhibit the proteolysis of several substrates as well as tumor growth in an animal model of ovarian cancer and in triple-negative breast cancer cell lines [112–114].

The ADAM10 inhibitor GI254023X was shown to suppress proliferation and induce apoptosis of H929 multiple myeloma cells and acute T-lymphoblastic leukemia Jurkat cells by preventing Notch1 activation [115, 116].

Overexpression of the ErbB family of receptors in human tumors is associated with poor prognosis and resistance to therapy. An attractive approach to prevent ErbB-mediated tumor growth and survival is to block sheddase activity. The selective potent, orally bioavailable small-molecule ADAM inhibitor, INCB3619, blocks the shedding of ErbB ligands including heregulin and reduces tumor cell survival. INCB3619 also inhibits gefitinib-resistant HER3 signaling and augments gefitinib blockade of EGFR signaling. Combining INCB3619 with a lapatinib-like dual inhibitor of EGFR and HER-2/neu kinases inhibited growth of MCF-7 and HER-2/neu-transfected MCF-7 human breast cancer cells. The second-generation sheddase inhibitor INCB7839 when combined with lapatinib suppressed the growth of HER-2/neu-positive BT474-SC1 human breast cancer xenografts in vivo.

These findings underscore the scope for ADAM inhibition in pharmacological intervention, either alone or in combination with other drugs [110, 117, 118].

Wiernik et al. [119] tested whether combination treatment through CD16 signaling and targeting CD33 (CD16 × 33 bispecific killer cell engager (BiKE) plus ADAM17 inhibitor could activate NK cells against acute myelogenous leukemia (AML). They found that the combination inhibited CD16 shedding in NK cells, and enhanced NK cell activation highlighting its potential for patients with relapsed AML or for adjuvant antileukemic therapy posttransplantation [119].

9 Conclusion

Adamalysins are relatively new players in cancer biology. Recent evidences suggest their role in cancer cell growth and proliferation [62, 63]; invasion and metastasis [22]; angiogenesis and cancer cell stemness [120]. These enzymes release membrane-bound growth factors, receptors, cytokines, and other molecules by shedding and RIPping, resulting in the activation of key signaling pathways. They also act on integrins or syndecans and influence cell–cell adhesion. These enzymes cleave ECM molecules and facilitate metastasis of cancer cells to metastasize to distant organs. The adamalysis have dual roles, while some members promote tumor development and progression, several others function as tumor suppressors. Although understanding the complex roles of adamalysins in cancer is technically challenging, the emerging knowledge and exciting new discoveries will provide deeper mechanistic insights into the tumor microenvironment besides enabling drug development.

References

1. Greaves M, Maley CC (2012) Clonal evolution in cancer. Nature 481:306–313
2. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674
3. Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52–67
4. Murphy G (2008) The ADAMs: signalling scissors in the tumour microenvironment. Nature Rev Cancer 8:929–941
5. Cal S, Lopez-Otin C (2015) ADAMTS proteases and cancer. Matrix Biol 44–46:77–85
6. Bonnans C, Chou J, Werb Z (2014) Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol 15:786–801
7. Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science 326:1216–1219
8. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circul Res 92:827–839
9. Alexius-Lindgren M, Andersson E, Lindstedt I, Engstrom W (2014) The RECK gene and biological malignancy—its significance in angiogenesis and inhibition of matrix metalloproteinases. Anticancer Res 34:3867–3873
10. Nagini S (2012) RECKing MMP: relevance of reversion-inducing cysteine-rich protein with kazal motifs as a prognostic marker and therapeutic target for cancer (a review). Anti-Cancer Agents Med Chem 12:718–725

11. Rocks N, Paulissen G, El Hour M, Quesada F, Crahay C, Gueders M, Foidart JM, Noel A, Cataldo D (2008) Emerging roles of ADAM and ADAMTS metalloproteinases in cancer. Biochimie 90:369–379
12. Turner SL, Blair-Zajdel ME, Bunning RA (2009) ADAMs and ADAMTSs in cancer. Brit J Biomed Sci 66:117–128
13. Horiuchi K, Zhou HM, Kelly K, Manova K, Blobel CP (2005) Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins beta1 and beta2. Dev Biol 283:459–471
14. Melenhorst WB, van den Heuvel MC, Timmer A, Huitema S, Bulthuis M, Timens W, van Goor H (2006) ADAM19 expression in human nephrogenesis and renal disease: associations with clinical and structural deterioration. Kidney Int 70:1269–1278
15. Haitchi HM, Powell RM, Shaw TJ, Howarth PH, Wilson SJ, Wilson DI, Holgate ST, Davies DE (2005) ADAM33 expression in asthmatic airways and human embryonic lungs. Am J Resp Crit Care Med 171:958–965
16. Asayesh A, Alanentalo T, Khoo NK, Ahlgren U (2005) Developmental expression of metalloproteases ADAM 9, 10, and 17 becomes restricted to divergent pancreatic compartments. Dev Dyn 232:1105–1114
17. Stanton H, Melrose J, Little CB, Fosang AJ (2011) Proteoglycan degradation by the ADAMTS family of proteinases. Biochim Biophys Acta 1812:1616–1629
18. Kurohara K, Komatsu K, Kurisaki T, Masuda A, Irie N, Asano M, Sudo K, Nabeshima Y, Iwakura Y, Sehara-Fujisawa A (2004) Essential roles of Meltrin beta (ADAM19) in heart development. Dev Biol 267:14–28
19. Overall CM, Kleifeld O (2006) Tumour microenvironment- opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nature Rev Cancer 6:227–239
20. Turker KS, Miles TS (1991) Threshold depolarization measurements in resting human motoneurones. J Neurosci Methods 39:103–107
21. van Goor H, Melenhorst WB, Turner AJ, Holgate ST (2009) Adamalysins in biology and disease. J Pathol 219:277–286
22. Mochizuki S, Okada Y (2007) ADAMs in cancer cell proliferation and progression. Cancer Sci 98:621–628
23. Black RA, White JM (1998) ADAMs: focus on the protease domain. Curr Opin Cell Biol 10:654–659
24. Eto K, Huet C, Tarui T, Kupriyanov S, Liu HZ, Puzon-McLaughlin W, Zhang XP, Sheppard D, Engvall E, Takada Y (2002) Functional classification of ADAMs based on a conserved motif for binding to integrin alpha 9beta 1: implications for sperm-egg binding and other cell interactions. J Biol Chem 277:17804–17810
25. Reiss K, Ludwig A, Saftig P (2006) Breaking up the tie: disintegrin-like metalloproteinases as regulators of cell migration in inflammation and invasion. Pharmacol Ther 111:985–1006
26. Huovila AP, Almeida EA, White JM (1996) ADAMs and cell fusion. Curr Opin Cell Biol 8:692–699
27. Stone AL, Kroeger M, Sang QX (1999) Structure-function analysis of the ADAM family of disintegrin-like and metalloproteinase-containing proteins (review). J Protein Chem 18:447–465
28. Endres K, Anders A, Kojro E, Gilbert S, Fahrenholz F, Postina R (2003) Tumor necrosis factor-alpha converting enzyme is processed by proprotein-convertases to its mature form which is degraded upon phorbol ester stimulation. Eur J Biochem 270:2386–2393
29. Schlomann U, Wildeboer D, Webster A, Antropova O, Zeuschner D, Knight CG, Docherty AJ, Lambert M, Skelton L, Jockusch H, Bartsch JW (2002) The metalloprotease disintegrin ADAM8. Processing by autocatalysis is required for proteolytic activity and cell adhesion. J Biol Chem 277:48210–48219
30. Kaushal GP, Shah SV (2000) The new kids on the block: ADAMTSs, potentially multifunctional metalloproteinases of the ADAM family. J Clin Invest 105:1335–1337

31. Tang BL, Hong W (1999) ADAMTS: a novel family of proteases with an ADAM protease domain and thrombospondin 1 repeats. FEBS Lett 445:223–225
32. Tan Ide A, Ricciardelli C, Russell DL (2013) The metalloproteinase ADAMTS1: a comprehensive review of its role in tumorigenic and metastatic pathways. Int J Cancer 133:2263–2276
33. Arner EC (2002) Aggrecanase-mediated cartilage degradation. Curr Opin Pharmacol 2:322–329
34. Nagase H, Kashiwagi M (2003) Aggrecanases and cartilage matrix degradation. Arthritis Res Ther 5:94–103
35. Colige A, Ruggiero F, Vandenberghe I, Dubail J, Kesteloot F, Van Beeumen J, Beschin A, Brys L, Lapiere CM, Nusgens B (2005) Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. J Biol Chem 280:34397–34408
36. Ham C, Levkau B, Raines EW, Herren B (2002) ADAM15 is an adherens junction molecule whose surface expression can be driven by VE-cadherin. Exp Cell Res 279:239–247
37. Lieber T, Kidd S, Young MW (2002) Kuzbanian-mediated cleavage of Drosophila Notch. Genes Dev 16:209–221
38. Seals DF, Courtneidge SA (2003) The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 17:7–30
39. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385:729–733
40. Weskamp G, Ford JW, Sturgill J, Martin S, Docherty AJ, Swendeman S, Broadway N, Hartmann D, Saftig P, Umland S, Sehara-Fujisawa A, Black RA, Ludwig A, Becherer JD, Conrad DH, Blobel CP (2006) ADAM10 is a principal 'sheddase' of the low-affinity immunoglobulin E receptor CD23. Nature Immunol 7:1293–1298
41. Moss ML, Jin SL, Becherer JD, Bickett DM, Burkhart W, Chen WJ, Hassler D, Leesnitzer MT, McGeehan G, Milla M, Moyer M, Rocque W, Seaton T, Schoenen F, Warner J, Willard D (1997) Structural features and biochemical properties of TNF-alpha converting enzyme (TACE). J Neuroimmunol 72:127–129
42. Lemieux GA, Blumenkron F, Yeung N, Zhou P, Williams J, Grammer AC, Petrovich R, Lipsky PE, Moss ML, Werb Z (2007) The low affinity IgE receptor (CD23) is cleaved by the metalloproteinase ADAM10. J Biol Chem 282:14836–14844
43. Peng Y, Lee DY, Jiang L, Ma Z, Schachter SC, Lemere CA (2007) Huperzine A regulates amyloid precursor protein processing via protein kinase C and mitogen-activated protein kinase pathways in neuroblastoma SK-N-SH cells over-expressing wild type human amyloid precursor protein 695. Neuroscience 150:386–395
44. Groot AJ, Vooijs MA (2012) The role of Adams in Notch signaling. Adv Exp Med Biol 727:15–36
45. O'Shea C, McKie N, Buggy Y, Duggan C, Hill AD, McDermott E, O'Higgins N, Duffy MJ (2003) Expression of ADAM-9 mRNA and protein in human breast cancer. Int J Cancer 105:754–761
46. Fritzsche FR, Wassermann K, Jung M, Tolle A, Kristiansen I, Lein M, Johannsen M, Dietel M, Jung K, Kristiansen G (2008) ADAM9 is highly expressed in renal cell cancer and is associated with tumour progression. BMC Cancer 8:179
47. Fritzsche FR, Jung M, Tolle A, Wild P, Hartmann A, Wassermann K, Rabien A, Lein M, Dietel M, Pilarsky C, Calvano D, Grutzmann R, Jung K, Kristiansen G (2008) ADAM9 expression is a significant and independent prognostic marker of PSA relapse in prostate cancer. Eur Urol 54:1097–1106
48. Wu X, Tang H, Liu G, Wang H, Shu J, Sun F (2016) miR-448 suppressed gastric cancer proliferation and invasion by regulating ADAM10. Tumour Biol J Int Soc Onco Dev Biol Med (in press)

49. McGowan PM, McKiernan E, Bolster F, Ryan BM, Hill AD, McDermott EW, Evoy D, O'Higgins N, Crown J, Duffy MJ (2008) ADAM-17 predicts adverse outcome in patients with breast cancer. Ann Oncol 19:1075–1081
50. Oppezzo P, Vasconcelos Y, Settegrana C, Jeannel D, Vuillier F, Legarff-Tavernier M, Kimura EY, Bechet S, Dumas G, Brissard M, Merle-Beral H, Yamamoto M, Dighiero G, Davi F, French Cooperative Group on CLL (2005) The LPL/ADAM29 expression ratio is a novel prognosis indicator in chronic lymphocytic leukemia. Blood 106:650–657
51. Wagstaff L, Kelwick R, Decock J, Edwards DR (2011) The roles of ADAMTS metalloproteinases in tumorigenesis and metastasis. Front Biosci 16:1861–1872
52. Braconi C, Meng F, Swenson E, Khrapenko L, Huang N, Patel T (2009) Candidate therapeutic agents for hepatocellular cancer can be identified from phenotype-associated gene expression signatures. Cancer 115:3738–3748
53. Porter S, Scott SD, Sassoon EM, Williams MR, Jones JL, Girling AC, Ball RY, Edwards DR (2004) Dysregulated expression of adamalysin-thrombospondin genes in human breast carcinoma. Clin Cancer Res 10:2429–2440
54. Heighway J, Knapp T, Boyce L, Brennand S, Field JK, Betticher DC, Ratschiller D, Gugger M, Donovan M, Lasek A, Rickert P (2002) Expression profiling of primary non-small cell lung cancer for target identification. Oncogene 21:7749–7763
55. Dunn JR, Reed JE, du Plessis DG, Shaw EJ, Reeves P, Gee AL, Warnke P, Walker C (2006) Expression of ADAMTS-8, a secreted protease with antiangiogenic properties, is downregulated in brain tumours. Brit J Cancer 94:1186–1193
56. Du W, Wang S, Zhou Q, Li X, Chu J, Chang Z, Tao Q, Ng EK, Fang J, Sung JJ, Yu J (2013) ADAMTS9 is a functional tumor suppressor through inhibiting AKT/mTOR pathway and associated with poor survival in gastric cancer. Oncogene 32:3319–3328
57. Viloria CG, Obaya AJ, Moncada-Pazos A, Llamazares M, Astudillo A, Capella G, Cal S, Lopez-Otin C (2009) Genetic inactivation of ADAMTS15 metalloprotease in human colorectal cancer. Cancer Res 69:4926–4934
58. Porter S, Span PN, Sweep FC, Tjan-Heijnen VC, Pennington CJ, Pedersen TX, Johnsen M, Lund LR, Romer J, Edwards DR (2006) ADAMTS8 and ADAMTS15 expression predicts survival in human breast carcinoma. Int J Cancer 118:1241–1247
59. Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE (2006) The consensus coding sequences of human breast and colorectal cancers. Science 314:268–274
60. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321:1801–1806
61. Li Z, Zhang W, Shao Y, Zhang C, Wu Q, Yang H, Wan X, Zhang J, Guan M, Wan J, Yu B (2010) High-resolution melting analysis of ADAMTS18 methylation levels in gastric, colorectal and pancreatic cancers. Med Oncol 27:998–1004
62. Cauwe B, Van den Steen PE, Opdenakker G (2007) The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. Crit Rev Biochem Mol Biol 42:113–185
63. Blobel CP (2005) ADAMs: key components in EGFR signalling and development. Nat Rev Mol Cell Biol 6:32–43
64. Liu R, Gu J, Jiang P, Zheng Y, Liu X, Jiang X, Huang E, Xiong S, Xu F, Liu G, Ge D, Chu Y (2015) DNMT1-microRNA126 epigenetic circuit contributes to esophageal

squamous cell carcinoma growth via ADAM9-EGFR-AKT signaling. Clin Cancer Res 21:854–863
65. Felli N, Felicetti F, Lustri AM, Errico MC, Bottero L, Cannistraci A, De Feo A, Petrini M, Pedini F, Biffoni M, Alvino E, Negrini M, Ferracin M, Mattia G, Care A (2013) miR-126&126* restored expressions play a tumor suppressor role by directly regulating ADAM9 and MMP7 in melanoma. PLoS ONE 8:e56824
66. Ito K, Okamoto I, Araki N, Kawano Y, Nakao M, Fujiyama S, Tomita K, Mimori T, Saya H (1999) Calcium influx triggers the sequential proteolysis of extracellular and cytoplasmic domains of E-cadherin, leading to loss of beta-catenin from cell-cell contacts. Oncogene 18:7080–7090
67. Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, de Strooper B, Hartmann D, Saftig P (2005) ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. Proc Natl Acad Sci USA 102:9182–9187
68. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96:5522–5527
69. Hartmann D, de Strooper B, Serneels L, Craessaerts K, Herreman A, Annaert W, Umans L, Lubke T, Lena Illert A, von Figura K, Saftig P (2002) The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum Mol Genet 11:2615–2624
70. Qi H, Rand MD, Wu X, Sestan N, Wang W, Rakic P, Xu T, Artavanis-Tsakonas S (1999) Processing of the notch ligand delta by the metalloprotease Kuzbanian. Science 283:91–94
71. Taylor KL, Henderson AM, Hughes CC (2002) Notch activation during endothelial cell network formation in vitro targets the basic HLH transcription factor HESR-1 and downregulates VEGFR-2/KDR expression. Microvasc Res 64:372–383
72. Zhang Q, Thomas SM, Lui VW, Xi S, Siegfried JM, Fan H, Smithgall TE, Mills GB, Grandis JR (2006) Phosphorylation of TNF-alpha converting enzyme by gastrin-releasing peptide induces amphiregulin release and EGF receptor activation. Proc Natl Acad Sci USA 103:6901–6906
73. el Akool S, Gauer S, Osman B, Doller A, Schulz S, Geiger H, Pfeilschifter J, Eberhardt W (2012) Cyclosporin A and tacrolimus induce renal Erk1/2 pathway via ROS-induced and metalloproteinase-dependent EGF-receptor signaling. Biochem Pharmacol 83:286–295
74. Mitsui Y, Mochizuki S, Kodama T, Shimoda M, Ohtsuka T, Shiomi T, Chijiiwa M, Ikeda T, Kitajima M, Okada Y (2006) ADAM28 is overexpressed in human breast carcinomas: implications for carcinoma cell proliferation through cleavage of insulin-like growth factor binding protein-3. Cancer Res 66:9913–9920
75. Kuno K, Bannai K, Hakozaki M, Matsushima K, Hirose K (2004) The carboxyl-terminal half region of ADAMTS-1 suppresses both tumorigenicity and experimental tumor metastatic potential. Biochem Biophys Res Commun 319:1327–1333
76. Hamada S, Satoh K, Fujibuchi W, Hirota M, Kanno A, Unno J, Masamune A, Kikuta K, Kume K, Shimosegawa T (2012) MiR-126 acts as a tumor suppressor in pancreatic cancer cells via the regulation of ADAM9. Mol Cancer Res 10:3–10
77. Chang L, Gong F, Cui Y (2015) RNAi-mediated A disintegrin and metalloproteinase 9 gene silencing inhibits the tumor growth of non-small lung cancer in vitro and in vivo. Mol Med Rep 12:1197–1204
78. Zhang W, Liu S, Liu K, Ji B, Wang Y, Liu Y (2014) Knockout of ADAM10 enhances sorafenib antitumor activity of hepatocellular carcinoma in vitro and in vivo. Oncol Rep 32:1913–1922
79. Bouchet S, Tang R, Fava F, Legrand O, Bauvois B (2014) Targeting CD13 (aminopeptidase-N) in turn downregulates ADAM17 by internalization in acute myeloid leukaemia cells. Oncotarget 5:8211–8222

80. Liu YJ, Xu Y, Yu Q (2006) Full-length ADAMTS-1 and the ADAMTS-1 fragments display pro- and antimetastatic activity, respectively. Oncogene 25:2452–2467
81. Obika M, Ogawa H, Takahashi K, Li J, Hatipoglu OF, Cilek MZ, Miyoshi T, Inagaki J, Ohtsuki T, Kusachi S, Ninomiya Y, Hirohata S (2012) Tumor growth inhibitory effect of ADAMTS1 is accompanied by the inhibition of tumor angiogenesis. Cancer Sci 103:1889–1897
82. Herren B, Raines EW, Ross R (1997) Expression of a disintegrin-like protein in cultured human vascular cells and in vivo. FASEB J 11:173–180
83. Horiuchi K, Weskamp G, Lum L, Hammes HP, Cai H, Brodie TA, Ludwig T, Chiusaroli R, Baron R, Preissner KT, Manova K, Blobel CP (2003) Potential role for ADAM15 in pathological neovascularization in mice. Mol Cell Biol 23:5614–5624
84. Trochon-Joseph V, Martel-Renoir D, Mir LM, Thomaidis A, Opolon P, Connault E, Li H, Grenet C, Fauvel-Lafeve F, Soria J, Legrand C, Soria C, Perricaudet M, Lu H (2004) Evidence of antiangiogenic and antimetastatic activities of the recombinant disintegrin domain of metargidin. Cancer Res 64:2062–2069
85. Hou Y, Chu M, Cai Y, Lei J, Chen Y, Zhu R, Gong X, Ma X, Jin J (2015) Antitumor and anti-angiogenic activity of the recombinant human disintegrin domain of A disintegrin and metalloproteinase 15. Mol Med Rep 12:2360–2366
86. Vazquez F, Hastings G, Ortega MA, Lane TF, Oikemus S, Lombardo M, Iruela-Arispe ML (1999) METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity. J Biol Chem 274:23349–23357
87. Iruela-Arispe ML, Lombardo M, Krutzsch HC, Lawler J, Roberts DD (1999) Inhibition of angiogenesis by thrombospondin-1 is mediated by 2 independent regions within the type 1 repeats. Circulation 100:1423–1431
88. Lawler J (2000) The functions of thrombospondin-1 and-2. Curr Opin Cell Biiol 12:634–640
89. Luque A, Carpizo DR, Iruela-Arispe ML (2003) ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165. J Biol Chem 278:23656–23665
90. Dubail J, Kesteloot F, Deroanne C, Motte P, Lambert V, Rakic JM, Lapiere C, Nusgens B, Colige A (2010) ADAMTS-2 functions as anti-angiogenic and anti-tumoral molecule independently of its catalytic activity. Cell Mol Life Sci 67:4213–4232
91. Kumar S, Sharghi-Namini S, Rao N, Ge R (2012) ADAMTS5 functions as an anti-angiogenic and anti-tumorigenic protein independent of its proteoglycanase activity. Am J Pathol 181:1056–1068
92. Koo BH, Coe DM, Dixon LJ, Somerville RP, Nelson CM, Wang LW, Young ME, Lindner DJ, Apte SS (2010) ADAMTS9 is a cell-autonomously acting, anti-angiogenic metalloprotease expressed by microvascular endothelial cells. Am J Pathol 176:1494–1504
93. Kelwick R, Wagstaff L, Decock J, Roghi C, Cooley LS, Robinson SD, Arnold H, Gavrilovic J, Jaworski DM, Yamamoto K, Nagase H, Seubert B, Kruger A, Edwards DR (2015) Metalloproteinase-dependent and -independent processes contribute to inhibition of breast cancer cell migration, angiogenesis and liver metastasis by a disintegrin and metalloproteinase with thrombospondin motifs-15. Int J Cancer 136:E14–E26
94. Iseri OD, Kars MD, Arpaci F, Gunduz U (2010) Gene expression analysis of drug-resistant MCF-7 cells: implications for relation to extracellular matrix proteins. Cancer Chemother Pharmacol 65:447–455
95. Xu K, Liang X, Shen K, Sun L, Cui D, Zhao Y, Tian J, Ni L, Liu J (2012) MiR-222 modulates multidrug resistance in human colorectal carcinoma by down-regulating ADAM-17. Exp Cell Res 318:2168–2177
96. Wang XJ, Feng CW, Li M (2013) ADAM17 mediates hypoxia-induced drug resistance in hepatocellular carcinoma cells through activation of EGFR/PI3K/Akt pathway. Mol Cell Biochem 380:57–66
97. Wang R, Ye X, Bhattacharya R, Boulbes DR, Fan F, Xia L, Ellis LM (2016) A Disintegrin and Metalloproteinase Domain 17 regulates colorectal cancer stem cells and chemosensitivity via Notch1 signaling. Stem Cells Transl Med 5:331–338

98. Fu L, Liu N, Han Y, Xie C, Li Q, Wang E (2014) ADAM10 regulates proliferation, invasion, and chemoresistance of bladder cancer cells. Tumour Biol 35:9263–9268
99. Fridrichova I, Smolkova B, Kajabova V, Zmetakova I, Krivulcik T, Mego M, Cierna Z, Karaba M, Benca J, Pindak D, Bohac M, Repiska V, Danihel L (2015) CXCL12 and ADAM23 hypermethylation are associated with advanced breast cancers. J Lab Clin Med 165:717–730
100. Chen J, Zhang C, Xu X, Zhu X, Dai D (2015) Downregulation of A disintegrin and metallopeptidase with thrombospondin motif type 1 by DNA hypermethylation in human gastric cancer. Mol Med Rep 12:2487–2494
101. Peng L, Yang Z, Tan C, Ren G, Chen J (2013) Epigenetic inactivation of ADAMTS9 via promoter methylation in multiple myeloma. Mol Med Rep 7:1055–1061
102. Choi GC, Li J, Wang Y, Li L, Zhong L, Ma B, Su X, Ying J, Xiang T, Rha SY, Yu J, Sung JJ, Tsao SW, Chan AT, Tao Q (2014) The metalloprotease ADAMTS8 displays antitumor properties through antagonizing EGFR-MEK-ERK signaling and is silenced in carcinomas by CpG methylation. Mol Cancer Res 12:228–238
103. Zhang C, Shao Y, Zhang W, Wu Q, Yang H, Zhong Q, Zhang J, Guan M, Yu B, Wan J (2010) High-resolution melting analysis of ADAMTS9 methylation levels in gastric, colorectal, and pancreatic cancers. Cancer Genet Cytogenet 196:38–44
104. Moncada-Pazos A, Obaya AJ, Fraga MF, Viloria CG, Capella G, Gausachs M, Esteller M, Lopez-Otin C, Cal S (2009) The ADAMTS12 metalloprotease gene is epigenetically silenced in tumor cells and transcriptionally activated in the stroma during progression of colon cancer. J Cell Sci 122:2906–2913
105. Alonso S, Gonzalez B, Ruiz-Larroya T, Duran Dominguez M, Kato T, Matsunaga A, Suzuki K, Strongin AY, Gimenez-Bonafe P, Perucho M (2015) Epigenetic inactivation of the extracellular matrix metallopeptidase ADAMTS19 gene and the metastatic spread in colorectal cancer. Clin Epigenet 7:124
106. Lu Y, Chopp M, Zheng X, Katakowski M, Buller B, Jiang F (2013) MiR-145 reduces ADAM17 expression and inhibits in vitro migration and invasion of glioma cells. Oncol Rep 29:67–72
107. Jia AY, Castillo-Martin M, Bonal DM, Sanchez-Carbayo M, Silva JM, Cordon-Cardo C (2014) MicroRNA-126 inhibits invasion in bladder cancer via regulation of ADAM9. Brit J Cancer 110:2945–2954
108. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S (2002) Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. Nat Med 8:35–40
109. Nyren-Erickson EK, Jones JM, Srivastava DK, Mallik S (2013) A disintegrin and metalloproteinase-12 (ADAM12): function, roles in disease progression, and clinical implications. Biochim Biophys Acta 1830:4445–4455
110. Fridman JS, Caulder E, Hansbury M, Liu X, Yang G, Wang Q, Lo Y, Zhou BB, Pan M, Thomas SM, Grandis JR, Zhuo J, Yao W, Newton RC, Friedman SM, Scherle PA, Vaddi K (2007) Selective inhibition of ADAM metalloproteases as a novel approach for modulating ErbB pathways in cancer. Clin Cancer Res 13:1892–1902
111. Giricz O, Calvo V, Peterson EA, Abouzeid CM, Kenny PA (2013) TACE-dependent TGFalpha shedding drives triple-negative breast cancer cell invasion. Int J Cancer 133:2587–2595
112. Tape CJ, Willems SH, Dombernowsky SL, Stanley PL, Fogarasi M, Ouwehand W, McCafferty J, Murphy G (2011) Cross-domain inhibition of TACE ectodomain. Proc Natl Acad Sci USA 108:5578–5583
113. Richards FM, Tape CJ, Jodrell DI, Murphy G (2012) Anti-tumour effects of a specific anti-ADAM17 antibody in an ovarian cancer model in vivo. PloSOne 7:e40597

114. Caiazza F, McGowan PM, Mullooly M, Murray A, Synnott N, O'Donovan N, Flanagan L, Tape CJ, Murphy G, Crown J, Duffy MJ (2015) Targeting ADAM-17 with an inhibitory monoclonal antibody has antitumour effects in triple-negative breast cancer cells. Brit J Cancer 112:1895–1903
115. Chen LL, Fan GQ, Zhang ZY, Zhang BY, Yan ZL, Li HJ, Luo JP, Chen C, Yao Y, Xu KL, Li ZY (2015) Effect of ADAM10 Inhibitor GI254023X on proliferation and apoptosis of multiple myeloma H929 cells and its possible mechanisms. J Exp Hematol 23:1628–1632
116. Ma S, Xu J, Wang X, Wu QY, Cao J, Li ZY, Zeng LY, Chen C, Xu KL (2015) Effect of ADAM10 Inhibitor GI254023X on proliferation and apoptosis of acute T-lymphoblastic leukemia Jurkat cells in vitro and its possible mechanisms. J Exp Hematol 23:950–955
117. Zhou BB, Peyton M, He B, Liu C, Girard L, Caudler E, Lo Y, Baribaud F, Mikami I, Reguart N, Yang G, Li Y, Yao W, Vaddi K, Gazdar AF, Friedman SM, Jablons DM, Newton RC, Fridman JS, Minna JD, Scherle PA (2006) Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. Cancer Cell 10:39–50
118. Witters L, Scherle P, Friedman S, Fridman J, Caulder E, Newton R, Lipton A (2008) Synergistic inhibition with a dual epidermal growth factor receptor/HER-2/neu tyrosine kinase inhibitor and a disintegrin and metalloprotease inhibitor. Cancer Res 68:7083–7089
119. Wiernik A, Foley B, Zhang B, Verneris MR, Warlick E, Gleason MK, Ross JA, Luo X, Weisdorf DJ, Walcheck B, Vallera DA, Miller JS (2013) Targeting natural killer cells to acute myeloid leukemia in vitro with a CD16 × 33 bispecific killer cell engager and ADAM17 inhibition. Clin Cancer Res 19:3844–3855
120. Kamarajan P, Shin JM, Qian X, Matte B, Zhu JY, Kapila YL (2013) ADAM17-mediated CD44 cleavage promotes orasphere formation or stemness and tumorigenesis in HNSCC. Cancer Med 2:793–802
121. Nishimura H, Kim E, Nakanishi T, Baba T (2004) Possible function of the ADAM1a/ADAM2 Fertilin complex in the appearance of ADAM3 on the sperm surface. J Biol Chem 279:34957–34962
122. Wysocki R, Clemens S, Augustyniak D, Golik P, Maciaszczyk E, Tamas MJ, Dziadkowiec D (2003) Metalloid tolerance based on phytochelatins is not functionally equivalent to the arsenite transporter Acr3p. Biochem Biophys Res Commun 304:293–300
123. Blobel CP, Wolfsberg TG, Turck CW, Myles DG, Primakoff P, White JM (1992) A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. Nature 356:248–252
124. Gupta SK, Alves K, Palladino LO, Mark GE, Hollis GF (1996) Molecular cloning of the human fertilin beta subunit. Biochem Biophys Res Commun 224:318–326
125. Puente XS, Gutierrez-Fernandez A, Ordonez GR, Hillier LW, Lopez-Otin C (2005) Comparative genomic analysis of human and chimpanzee proteases. Genomics 86:638–647
126. Bates EE, Fridman WH, Mueller CG (2002) The ADAMDEC1 (decysin) gene structure: evolution by duplication in a metalloprotease gene cluster on chromosome 8p12. Immunogenetics 54:96–105
127. Oh J, Woo JM, Choi E, Kim T, Cho BN, Park ZY, Kim YC, Kim DH, Cho C (2005) Molecular, biochemical, and cellular characterization of epididymal ADAMs, ADAM7 and ADAM28. Biochem Biophys Res Commun 331:1374–1383
128. Wildeboer D, Naus S, Amy Sang QX, Bartsch JW, Pagenstecher A (2006) Metalloproteinase disintegrins ADAM8 and ADAM19 are highly regulated in human primary brain tumors and their expression levels and activities are associated with invasiveness. J Neuropathol Exp Neurol 65:516–527
129. Shintani Y, Higashiyama S, Ohta M, Hirabayashi H, Yamamoto S, Yoshimasu T, Matsuda H, Matsuura N (2004) Overexpression of ADAM9 in non-small cell lung cancer correlates with brain metastasis. Cancer Res 64:4190–4196

130. Gutwein P, Oleszewski M, Mechtersheimer S, Agmon-Levin N, Krauss K, Altevogt P (2000) Role of Src kinases in the ADAM-mediated release of L1 adhesion molecule from human tumor cells. J Biol Chem 275:15490–15497
131. Mechtersheimer S, Gutwein P, Agmon-Levin N, Stoeck A, Oleszewski M, Riedle S, Postina R, Fahrenholz F, Fogel M, Lemmon V, Altevogt P (2001) Ectodomain shedding of L1 adhesion molecule promotes cell migration by autocrine binding to integrins. J Cell Biol 155:661–673
132. Amour A, Knight CG, Webster A, Slocombe PM, Stephens PE, Knauper V, Docherty AJ, Murphy G (2000) The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. FEBS Lett 473:275–279
133. Emi M, Katagiri T, Harada Y, Saito H, Inazawa J, Ito I, Kasumi F, Nakamura Y (1993) A novel metalloprotease/disintegrin-like gene at 17q21.3 is somatically rearranged in two primary breast cancers. Nature Genet 5:151–157
134. Kveiborg M, Frohlich C, Albrechtsen R, Tischler V, Dietrich N, Holck P, Kronqvist P, Rank F, Mercurio AM, Wewer UM (2005) A role for ADAM12 in breast tumor progression and stromal cell apoptosis. Cancer Res 65:4754–4761
135. Kuefer R, Day KC, Kleer CG, Sabel MS, Hofer MD, Varambally S, Zorn CS, Chinnaiyan AM, Rubin MA, Day ML (2006) ADAM15 disintegrin is associated with aggressive prostate and breast cancer disease. Neoplasia 8:319–329
136. Karkkainen I, Karhu R, Huovila AP (2000) Assignment of the ADAM15 gene to human chromosome band 1q21.3 by in situ hybridization. Cytogenet Cell Genet 88:206–207
137. Wei S, Kashiwagi M, Kota S, Xie Z, Nagase H, Brew K (2005) Reactive site mutations in tissue inhibitor of metalloproteinase-3 disrupt inhibition of matrix metalloproteinases but not tumor necrosis factor-alpha-converting enzyme. J Biol Chem 280:32877–32882
138. Frayne J, Hurd EA, Hall L (2002) Human tMDC III: a sperm protein with a potential role in oocyte recognition. Mol Human Reprod 8:817–822
139. Hooft van Huijsduijnen R (1998) ADAM 20 and 21; two novel human testis-specific membrane metalloproteases with similarity to fertilin-alpha. Gene 206:273–282
140. Sagane K, Ohya Y, Hasegawa Y, Tanaka I (1998) Metalloproteinase-like, disintegrin-like, cysteine-rich proteins MDC2 and MDC3: novel human cellular disintegrins highly expressed in the brain. Biochem J 334:93–98
141. Tyan YC, Yang MH, Chen SC, Jong SB, Chen WC, Yang YH, Chung TW, Liao PC (2011) Urinary protein profiling by liquid chromatography/tandem mass spectrometry: ADAM28 is overexpressed in bladder transitional cell carcinoma. Rapid Commun Mass Spectr 25:2851–2862
142. Ohtsuka T, Shiomi T, Shimoda M, Kodama T, Amour A, Murphy G, Ohuchi E, Kobayashi K, Okada Y (2006) ADAM28 is overexpressed in human non-small cell lung carcinomas and correlates with cell proliferation and lymph node metastasis. Int J Cancer 118:263–273
143. Wang F, Xu R, Zhu P, Hu J, Ying B, Zhao S, Li C (2001) Preliminarily functional analysis of a cloned novel human gene ADAM29. Life Sci 44:392–399
144. Hu C, Zhang R, Wang C, Wang J, Ma X, Lu J, Qin W, Hou X, Wang C, Bao Y, Xiang K, Jia W (2009) PPARG, KCNJ11, CDKAL1, CDKN2A-CDKN2B, IDE-KIF11-HHEX, IGF2BP2 and SLC30A8 are associated with type 2 diabetes in a Chinese population. PLoS ONE 4:e7643
145. Choi I, Woo JM, Hong S, Jung YK, Kim DH, Cho C (2003) Identification and characterization of ADAM32 with testis-predominant gene expression. Gene 304:151–162
146. Yoshinaka T, Nishii K, Yamada K, Sawada H, Nishiwaki E, Smith K, Yoshino K, Ishiguro H, Higashiyama S (2002) Identification and characterization of novel mouse and human ADAM33s with potential metalloprotease activity. Gene 282:227–236
147. Xiao S, Li Y, Li T, Chen M, Xu Y, Wen Y, Zhou C (2014) Evidence for decreased expression of ADAMTS-1 associated with impaired oocyte quality in PCOS patients. J Clin Endocrinol Metab 99:E1015–E1021

148. Freitas VM, do Amaral JB, Silva TA, Santos ES, Mangone FR, Pinheiro Jde J, Jaeger RG, Nagai MA, Machado-Santelli GM (2013) Decreased expression of ADAMTS-1 in human breast tumors stimulates migration and invasion. Mol Cancer 12:2
149. Colige A, Sieron AL, Li SW, Schwarze U, Petty E, Wertelecki W, Wilcox W, Krakow D, Cohn DH, Reardon W, Byers PH, Lapiere CM, Prockop DJ, Nusgens BV (1999) Human Ehlers-Danlos syndrome type VII C and bovine dermatosparaxis are caused by mutations in the procollagen I N-proteinase gene. Am J Hum Genet 65:308–317
150. Jeltsch M, Jha SK, Tvorogov D, Anisimov A, Leppanen VM, Holopainen T, Kivela R, Ortega S, Karpanen T, Alitalo K (2014) CCBE1 enhances lymphangiogenesis via A disintegrin and metalloprotease with thrombospondin motifs-3-mediated vascular endothelial growth factor-C activation. Circulation 129:1962–1971
151. Kelwick R, Desanlis I, Wheeler GN, Edwards DR (2015) The ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) family. Genome Biol 16:113
152. Gao G, Westling J, Thompson VP, Howell TD, Gottschall PE, Sandy JD (2002) Activation of the proteolytic activity of ADAMTS4 (aggrecanase-1) by C-terminal truncation. J Biol Chem 277:11034–11041
153. Foulcer SJ, Nelson CM, Quintero MV, Kuberan B, Larkin J, Dours-Zimmermann MT, Zimmermann DR, Apte SS (2014) Determinants of versican-V1 proteoglycan processing by the metalloproteinase ADAMTS5. J Biol Chem 289:27859–27873
154. Verma P, Dalal K (2011) ADAMTS-4 and ADAMTS-5: key enzymes in osteoarthritis. J Cell Biochem 112:3507–3514
155. Kintakas C, McCulloch DR (2011) Emerging roles for ADAMTS5 during development and disease. Matrix Biol 30:311–317
156. Wu W, Zhou Y, Li Y, Li J, Ke Y, Wang Y, Zheng J (2015) Association between plasma ADAMTS-7 levels and ventricular remodeling in patients with acute myocardial infarction. Eur J Med Res 20:27
157. Pu X, Xiao Q, Kiechl S, Chan K, Ng FL, Gor S, Poston RN, Fang C, Patel A, Senver EC, Shaw-Hawkins S, Willeit J, Liu C, Zhu J, Tucker AT, Xu Q, Caulfield MJ, Ye S (2013) ADAMTS7 cleavage and vascular smooth muscle cell migration is affected by a coronary-artery-disease-associated variant. Am J Hum Genet 92:366–374
158. Lo PH, Lung HL, Cheung AK, Apte SS, Chan KW, Kwong FM, Ko JM, Cheng Y, Law S, Srivastava G, Zabarovsky ER, Tsao SW, Tang JC, Stanbridge EJ, Lung ML (2010) Extracellular protease ADAMTS9 suppresses esophageal and nasopharyngeal carcinoma tumor formation by inhibiting angiogenesis. Cancer Res 70:5567–5576
159. Kutz WE, Wang LW, Bader HL, Majors AK, Iwata K, Traboulsi EI, Sakai LY, Keene DR, Apte SS (2011) ADAMTS10 protein interacts with fibrillin-1 and promotes its deposition in extracellular matrix of cultured fibroblasts. J Biol Chem 286:17156–17167
160. Cal S, Arguelles JM, Fernandez PL, Lopez-Otin C (2001) Identification, characterization, and intracellular processing of ADAM-TS12, a novel human disintegrin with a complex structural organization involving multiple thrombospondin-1 repeats. J Biol Chem 276:17932–17940
161. Llamazares M, Obaya AJ, Moncada-Pazos A, Heljasvaara R, Espada J, Lopez-Otin C, Cal S (2007) The ADAMTS12 metalloproteinase exhibits anti-tumorigenic properties through modulation of the Ras-dependent ERK signalling pathway. J Cell Sci 120:3544–3552
162. Furlan M, Lammle B (2001) Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. Best Pract Res Clin Haematol 14:437–454
163. Poonpet T, Honsawek S, Tammachote N, Kanitnate S, Tammachote R (2013) ADAMTS14 gene polymorphism associated with knee osteoarthritis in Thai women. Genet Mol Res 12:5301–5309
164. Joe B, Saad Y, Dhindaw S, Lee NH, Frank BC, Achinike OH, Luu TV, Gopalakrishnan K, Toland EJ, Farms P, Yerga-Woolwine S, Manickavasagam E, Rapp JP, Garrett MR, Coe D, Apte SS, Rankinen T, Perusse L, Ehret GB, Ganesh SK, Cooper RS, O'Connor A, Rice T,

Weder AB, Chakravarti A, Rao DC, Bouchard C (2009) Positional identification of variants of Adamts16 linked to inherited hypertension. Hum Mol Genet 18:2825–2838
165. Morales J, Al-Sharif L, Khalil DS, Shinwari JM, Bavi P, Al-Mahrouqi RA, Al-Rajhi A, Alkuraya FS, Meyer BF, Al Tassan N (2009) Homozygous mutations in ADAMTS10 and ADAMTS17 cause lenticular myopia, ectopia lentis, glaucoma, spherophakia, and short stature. Am J Hum Genet 85:558–568
166. Xu B, Zhang L, Luo C, Qi Y, Cui Y, Ying JM, Zhang Q, Jin J (2015) Hypermethylation of the 16q23.1 tumor suppressor gene ADAMTS18 in clear cell renal cell carcinoma. Int J Mol Sci 16:1051–1065
167. Peluso I, Conte I, Testa F, Dharmalingam G, Pizzo M, Collin RW, Meola N, Barbato S, Mutarelli M, Ziviello C, Barbarulo AM, Nigro V, Melone MA, European Retinal Disease C, Simonelli F, Banfi S (2013) The ADAMTS18 gene is responsible for autosomal recessive early onset severe retinal dystrophy. Orphanet J Rare Dis 8:16
168. Knauff EA, Franke L, van Es MA, van den Berg LH, van der Schouw YT, Laven JS, Lambalk CB, Hoek A, Goverde AJ, Christin-Maitre S, Hsueh AJ, Wijmenga C, Fauser BC, Dutch POFC (2009) Genome-wide association study in premature ovarian failure patients suggests ADAMTS19 as a possible candidate gene. Hum Reprod 24:2372–2378
169. Llamazares M, Cal S, Quesada V, Lopez-Otin C (2003) Identification and characterization of ADAMTS-20 defines a novel subfamily of metalloproteinases-disintegrins with multiple thrombospondin-1 repeats and a unique GON domain. J Biol Chem 278:13382–13389

Role of Proteases in Lung Disease: A Brief Overview

16

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Abstract

Proteases play an important role in health and disease of the lung. In the normal lungs, proteases maintain their homeostatic functions that regulate processes like its regeneration and repair. Dysregulation of proteases–antiproteases balance is crucial in the manifestation of different types of lung diseases. Chronic inflammatory lung pathologies are associated with a marked increase in protease activities. Thus, in addition to protease activities, inhibition of anti-proteolytic control mechanisms are also important for effective microbial infection and inflammation in the lung. Herein, we briefly summarize the role of different proteases and to some extent antiproteases in regulating a variety of lung diseases.

Keywords

Protease · Antiprotease · Lung diseases · Infection · Inflammation

1 Introduction

The lung possesses a large number of anti-inflammatory components [1], which fight against microbial infections.

Serine, cysteine, aspartic, and metalloproteases are the principal classes of protease present in the human lung. A good number of evidence suggest that neutrophil serine proteases (NSPs) such as elastase, proteinase 3 (PR3), cathepsin G

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(CatG), and matrix metalloproteases (MMPs) are major pathogenic determinants of chronic inflammatory lung disorders [1]. The lung proteases act in concert with the proteases of invading microbes, inactivate antiproteases, and antimicrobial compounds and thereby play a pivotal role in different types of lung diseases including chronic obstructive pulmonary disease (COPD), asthma, acute respiratory distress syndrome (ARDS), influenza, and cancer [1].

The lung proteases can either intracellularly or extracellularly regulate processes such as tissue remodeling, mucin production, neutrophil chemotaxis, and microbial destruction. Additionally, they regulate infection and inflammation in the lung, for example neutrophil elastase (NE), a serine protease, which plays critical role in the progression of a variety of lung diseases. It can regulate activities of CatB and MMP-2 in alveolar macrophages [2] and also activates proMMP-2, MMP-7, and MMP-9 [3–5], indicating that NE may act as a proinflammatory mediator. In some cases, NE regulates important signaling pathways that modulate innate immunity [6, 7]. NE's multiple roles characterize it as a decisive factor controlling many aspects of infection and inflammation in the lung.

2 Pulmonary Hypertension

Pulmonary hypertension (PAH) occurs due to elevation of pulmonary artery pressure and if it is prolonged, then right ventricular failure may occur with subsequent fatality [8]. PAH often leads to secondary complications of many pulmonary disorders such as COPD, asthma and chronic bronchitis bronchopulmonary displasia, cystic fibrosis, chronic bronchitis, and emphysema [9, 10].

2.1 Serine Protease and Pulmonary Hypertension

The role of oxidants such as hydrogen peroxide, hydroperoxides, superoxide, and peroxynitrite in producing PAH is now well established [11–15]. Administration of the oxidant, tert-butylhydroperoxide (tert-buOOH) to the perfusate of isolated rabbit lungs causes pulmonary vasospasm [16]. Oxidant-induced pulmonary vasoconstriction can be blocked by the cyclooxygenase or thromboxane synthase inhibitor, indomethacin and is closely correlated with the thromboxane level in the effluent perfusate [13, 16–19], suggesting a critical role of thromboxane in pulmonary vasoconstriction. On the other hand, TMB-8, an intracellular Ca^{2+} ($[Ca^{2+}]_i$) antagonists, has been shown to prevent oxidant-mediated pulmonary vasoconstriction [13]. Thus, oxidant-mediated PAH is triggered by an increase in $[Ca^{2+}]_i$. In many occasions PAH occur due to an increase in $[Ca^{2+}]_i$ caused by stimulants such as thromboxane A_2 and endothelin-1 that generates oxidants. Oxidant and Ca^{2+} ionophore-mediated pulmonary hypertension has been observed to be inhibited by serine protease inhibitors, for example, aprotinin [19–21].

The mechanism by which oxidants stimulate production of the arachidonic acid (AA) metabolites has gained considerable interest. A report by Chakraborti et al. [22] indicated that oxidants, e.g., tert-buOOH stimulation of pulmonary artery endothelial and smooth muscle cells caused a marked increase in phospholipase A_2 (PLA_2) activity with subsequent generation of AA. Mepacrine, an inhibitor of PLA_2 inhibits tert-buOOH-induced increase in PLA_2 activity, thromboxane B_2 production, and PAH [19, 22]. Some investigators, considering the analogy of activation of pancreatic phospholipase A_2 [23], suggested that a serine protease might be involved in regulating PLA_2 activity [24]. Chakraborti et al. [25, 26] demonstrated that oxidant-mediated activation of PLA_2 activity in pulmonary endothelial and smooth muscle cells occur with the involvement of proteolytically activated protein kinase Cα (PKCα). They have also demonstrated that oxidants caused increase in $[Ca^{2+}]_i$ in pulmonary endothelial and smooth muscle cells can activate an aprotinin sensitive protease having mol mass of ~43 kDa [26]. The protease then proteolytically activates PKCα resulting in stimulation of $cPLA_2$ (Fig. 1), which generates thromboxane and that has been observed to be important in producing PAH [25, 26]. Oxidants elicit an increase in $[Ca^{2+}]_i$ due to proteolytic activation of

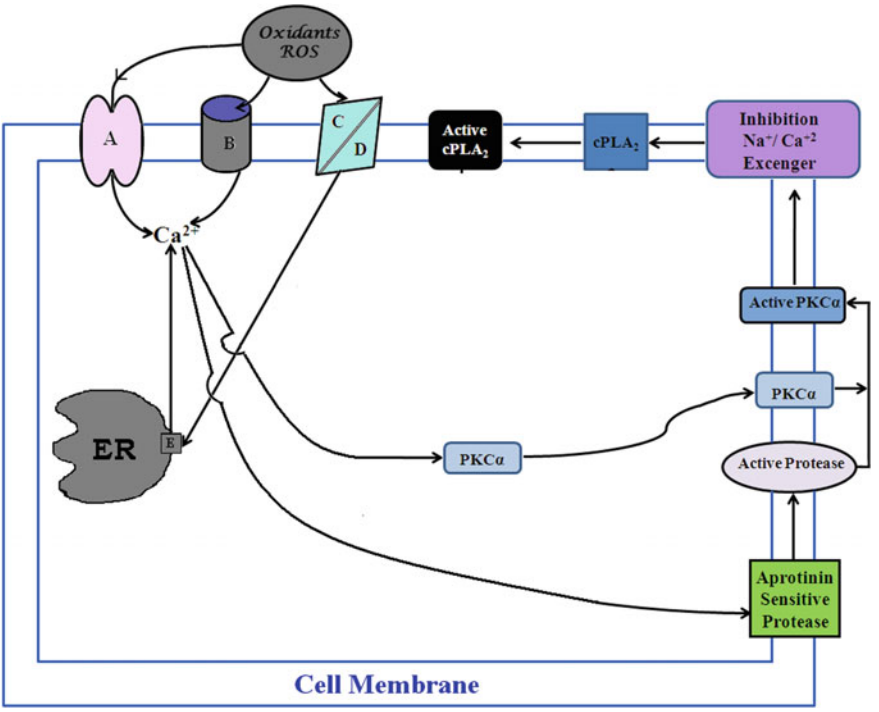

Fig. 1 Schematic representation of the underlying mechanism associated with oxidant (reactive oxygen species: ROS)-mediated $cPLA_2$ activation in pulmonary vascular endothelial and smooth muscle cells. *A* calcium channels; *B* membrane bound Ca^{2+} stores; *C* anion channels; *D* diffusion; *E* inhibition of Na^+ dependent Ca^{2+} uptake

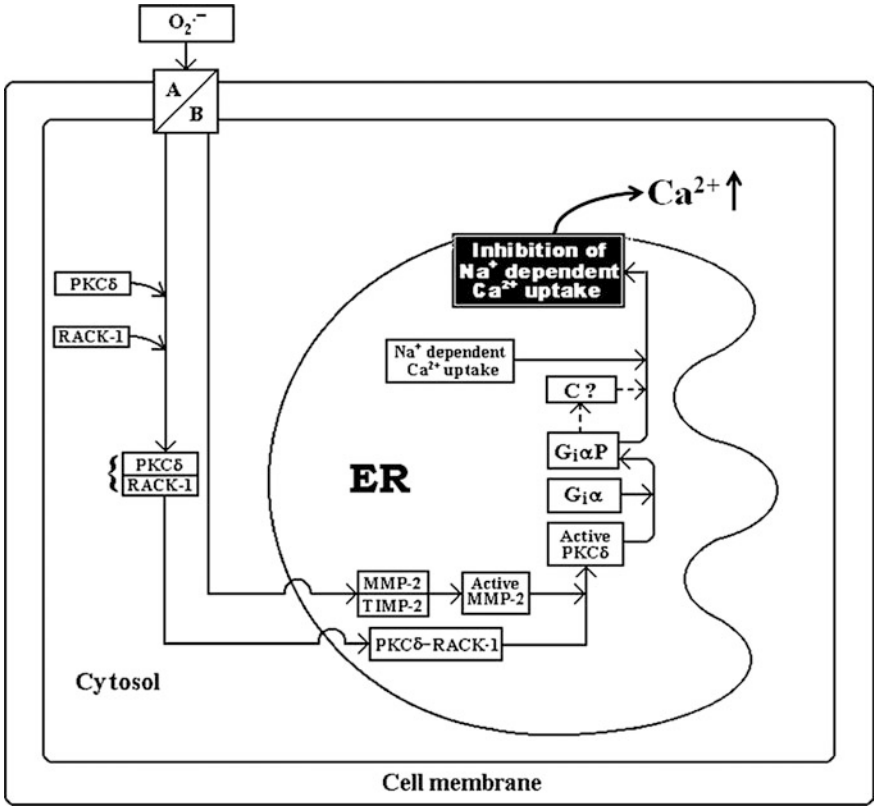

Fig. 2 Schematic representation of the underlying mechanism of oxidant (reactive oxygen species: ROS) triggered inhibition of Na^+ dependent Ca^{2+} uptake in bovine pulmonary smooth muscle ER resulting in an increase in $[Ca^{2+}]_i$. *A* Anion channel; *B* diffusion; *MMP-2* matrix metalloprotease-2; *TIMP-2* tissue inhibitor of metalloprotease-2; *PKCδ* protein kinase C delta; *RACK-1* receptor for activated *C* kinase-1; *Giα* inhibitory G protein α subunit; *GiαP* phosphorylated Giα; *C* Na^+-K^+-ATPase/Na^+-H^+ exchanger. (Taken from Chakraborti et al. (2005) Mol Cell Biochem. 280: 107–117 with permission)

PKC-δ by MMP-2 resulting in phosphorylation of a pertussis toxin sensitive protein (Gi) leading to inhibition of Na^+ dependent Ca^{2+} uptake (Na^+/Ca^{2+} exchanger) in the endoplasmic reticulum (ER) [27–31] (Fig. 2), whereas the role of cell membrane for an increase in $[Ca^{2+}]_i$ has been observed to be due to phosphorylation of G_i via proteolytically activated PKCα by an aprotinin sensitive serine protease leading to inhibition of Na^+ dependent Ca^{2+} efflux (Na^+/Ca^{2+} exchanger) (Fig. 1) in pulmonary vascular cells [24–26].

In many systems, Ca^{2+}-ATPase represents only about 1% of the total proteins [32]. In heart sarcolemmal vesicles, Ca^{2+} uptake via NCX produces maximum transport velocity and that has been demonstrated to be about 30-fold up than that elicited by the sarcoplasmic reticulum {S(ER)} Ca^{2+} pump system [33]. In addition

to the Ca^{2+} pump, Na^{+} dependent Ca^{2+} uptake system is an important mechanism to sequester Ca^{2+} in the ER of pulmonary vascular cells [29–33]. A decrease in Ca^{2+} sequestration by proteolytic inhibition of Na^{+} dependent Ca^{2+} uptake has been observed to measure duration of free $[Ca^{2+}]_i$ transient, which eventually produces vasoconstriction [34, 35]. In different systems, Na^{+}/Ca^{2+} exchanger controls the contractility of smooth muscle cells [34, 35]. For example, contractile dysregulation in the myocardium could be related with activation of proteases [36]. Thus, the role of cell membrane associated aprotinin sensitive protease and ER MMP-2 on Na^{+}/Ca^{2+} exchange in pulmonary artery endothelial and smooth muscle cells under oxidant triggered condition is an important mechanism for the pathological manifestation of pulmonary vasoconstriction [24–26, 29–31].

2.2 MMPs and Pulmonary Hypertension

PAH is characterized by persistent vasoconstriction and remodeling of pulmonary vasculature associated with activation of proteases, for instance, MMPs [34]. Remodeling of pulmonary artery is associated with an alteration of extracellular matrix (ECM) turnover with concomitant change in ECM proteins level. In PAH, dysregulation of ECM turnover has been suggested to play an important role in the pathological remodeling process [35, 36]. ECM degradation occurs by different proteases of which matrix metalloproteases (MMPs) has been shown to play the crucial role [37, 38]. Of the MMPs, MMP-2, and MMP-9 are able to cleave basement membrane associated type IV collagen, which increase remodeling of the pulmonary vasculature in PAH [39]. Given the potency of MMPs, its activity is tightly regulated at the transcriptional and post-translational level, where the tissue inhibitors of MMP (TIMPs) play a pivotal role [40, 41].

IL-1, a potent endogenously generated inducer of PAH, elicits its effect via an increase in the level of TGF and TNF in pulmonary smooth muscle cells. TGF causes an increase in the expression of the 92 kDa proMMP-9 and 72 kDa proMMP-2 mRNAs, while TNF triggers activation of proMMP-9 and proMMP-2 [42].

MMP-2 is produced upon activation of proMMP-2 by a variety of stimuli under different pathophysiological conditions. It has been observed that the activation of proMMP-2 occurs at the cell membrane. Interaction between MT1-MMP and TIMP-2 is an important phenomenon in the activation of proMMP-2. The MT1MMP-TIMP2 associates with proMMP-2 and forms a trimolecular complex, which triggers the activation of proMMP-2 and subsequently generates MMP-2 [41, 42]. The activation of proMMP-2 in pulmonary artery smooth muscle cells occur with the involvement of protein kinase C-α dependent and NF-κB-MT1MMP-mediated signaling mechanism. TNF-α augments mRNA and protein expression of MT1MMP, while the expression level of TIMP-2 diminishes. The increase in TNF-α leads to IKK activation, IB phosphorylation and degradation, and subsequently activation of NF-κB. Upon activation, NF-κB binds to the MT1-MMP promoter, thereby enhancing its expression and subsequently increases

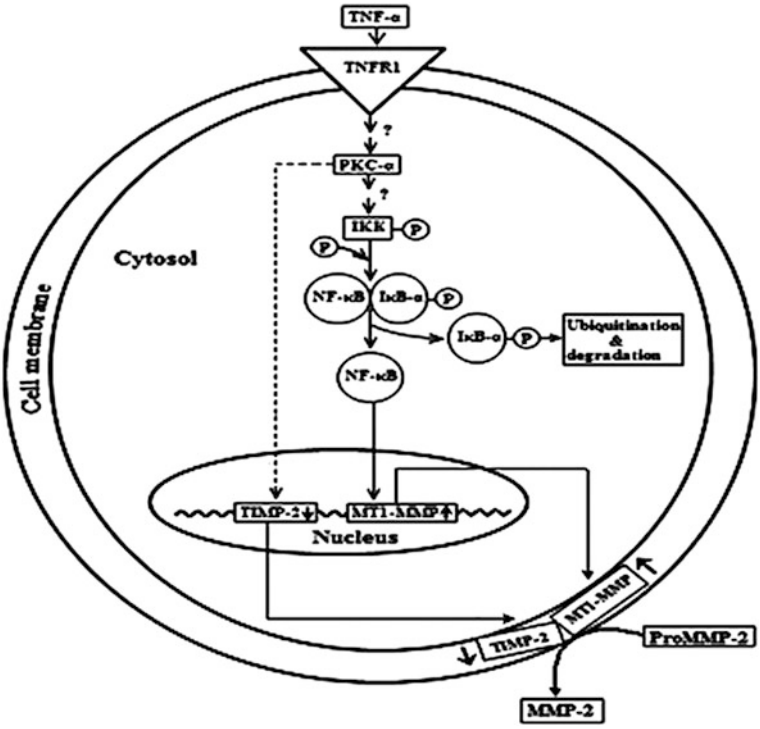

Fig. 3 Schematic representation of TNFα-induced proMMP-2 activation in the SMCs. TNFα binds to cell surface receptor TNFR1. Upon binding, TNFα induces PKCα activation, which subsequently activates IKK by phosphorylation. Activated IKK then phosphorylates IkB-α, which upon phosphorylation is ubiquitinated and degraded in the cytosol. The free NF-κB then translocates to the nucleus and increases the expression of MT1-MMP, which then accumulates on the cell surface. PKC-α, on the other hand, also down regulates TIMP-2 expression by mechanism that is currently unknown. (Taken from Roy et al. (2013) J Biochem 153:289–302 with permission)

proMMP-2 level in association with TIMP-2 that is modulated by protein kinase C-α at the cell membrane [41, 42] (Fig. 3). This indicates therapeutic potentiality of PKC inhibitors in ameliorating the PAH, where activation of proMMP-2 is an important phenomenon.

ProMMP-9 activation by TNF-α has been observed to occur with the involvement of an aprotinin sensitive serine protease [42]. TNF-α was shown to inhibit aprotinin and TIMP-1 mRNA and protein expression, which trigger activation of proMMP-2 resulting in the stimulation of MMP-2 (Fig. 4). Under IL-1β stimulation, the aprotinin sensitive protease was not activated, although a discernible inhibition of TIMP-2 mRNA and protein expression were triggered by TNF-α [42]. Thus, IL-1-induced stimulation of the two progelatinases occurs via different mechanisms.

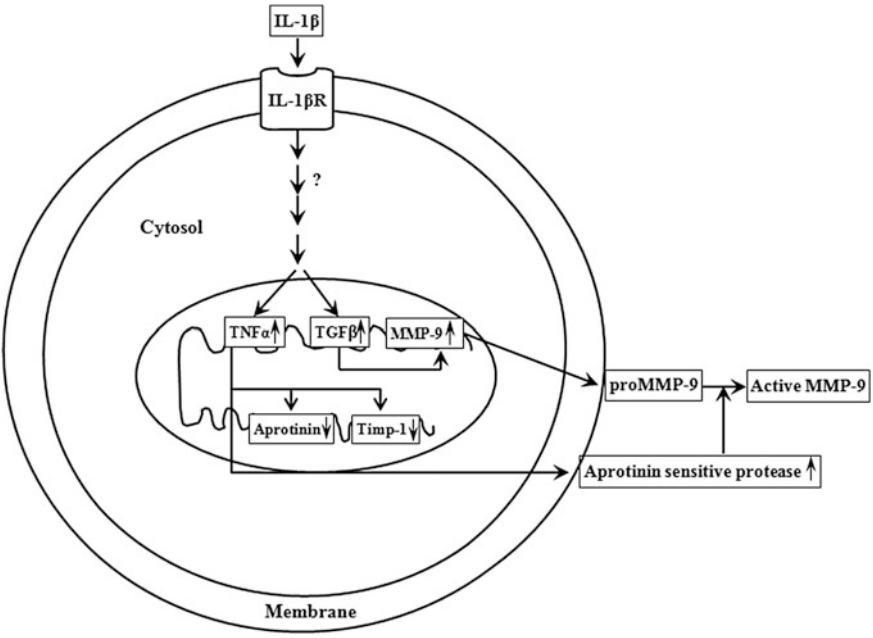

Fig. 4 Schematic representation of proMMP-9 activation by an aprotinin sensitive protease during IL-1β stimulation of pulmonary artery smooth muscle cells. IL-1β treatment to the cells stimulates TGF-β and TNF-α. TGF-β stimulates expression of proMMP-9, while TNF-α activates proMMP-9 via an increase in a ~43 kDa aprotinin sensitive protease concomitant with the down regulation of aprotinin and TIMP-1 expression

3 Influenza

Influenza viruses are highly infectious and trigger acute respiratory diseases with significant morbidity and mortality in humans and other animals [43–46].

Influenza viruses can be classified as A, B, or C. Influenza virus A, found in humans and other mammals and birds, played a nefarious role in causing the three twentieth century major influenza outbreak and also the influenza outbreak of swine origin that occurred in the recent past [47]. Many of the influenza A-related mortalities are attributable to secondary bacterial pneumonia [48, 49].

Haemagglutinin (HA) protein contributes critically to influenza virus-mediated pathogenicity. HA of influenza virus binds to sialic acid containing cell surface receptors. HA upon cleavage by a good number of host of protease(s) forms HA1 and HA2 subunits and that has been fused with host cell membrane, which subsequently initiates the infection process [49–52]. In most cases, the cleavage site of HA of avian and mammalian influenza viruses is a single arginine, albeit a single lysine amino acid has also been observed at the cleavage site in some cases. Cleavage can occur extracellularly by trypsin [53, 54] and proteases such as

plasmin [55–57], tryptase of bronchiolar epithelial and mast cells [58], and also by bacterial proteases [59–61].

Several other proteases expressed in the lung are also able to facilitate influenza virus spread. Böttcher et al. [62] demonstrated that TMPRSS2 and TMPRSS11D, transmembrane serine proteases, (a.k.a. human airway trypsin-like protease: HAT) activate the influenza viruses H1N1 (A/Memphis/14/96), H2N9 (A/Mallard/Alberta/205/98), and H3N2 (A/Texas/6/96) upon cleavage of haemagglutinin (HA) and contribute to the high pathogenicity of these influenza viruses in the lung [63]. In addition to HAT, TMPRSS-2 and -4, the granzymes (Gzm) such as GzmA, GzmB, and GzmE are known to play a key role in the process of cleavage of 1918 H1N1 HA as a part of the progression of the influenza disease [62, 63].

The sites of virus replication in the microenvironments of respiratory tract represent complex extracellular proteases (such as trypsin and tryptase), which activate a family of receptors called protease activated receptors (PARs) [64, 65] and that play an important role in both virus replication and innate immune response [58, 66]. Four PARs (PAR1-4) are known to be activated by different proteases. After cleavage of the receptor(s) by proteases, the newly released amino terminal sequence binds and internally activates the receptor [67]. In the airways of IAV-infected mice, an increase in the level PAR2 upon IFNγ-mediated modulation has been shown to play a crucial role in influenza pathology [68, 69].

Multiple serine protease activities are implicated in mediating influenza virus infection. Inhibition of influenza A virus infection in cultured lung epithelial cells by serine protease inhibitors, for example, aprotinin markedly protects mice from infection [70]. Another serine protease inhibitor, camostat has also been shown to possess anti-influenza (Taiwan/1/86) virus pathology [71].

4 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is associated with the pathological manifestations of emphysema and chronic bronchitis. Emphysema is characterized by a marked destruction of the alveolar septa with concomitant decrease in lung plasticity and that results in gas trapping leading to a marked decrease in pulmonary oxygenation in the lung. Chronic bronchitis usually occurs with inflamed and thickened airways along with an increase in mucus production by the cells in the airways, which leads to a marked increase in cough and difficulty in breathing. Noxious particles present in cigarette smoke and automobile exhaustion have been observed to be an important causative agent of COPD [72, 73].

In the early 1960s, proteases have been shown to produce lung lesions in experimental animals similar to human emphysema. Initial studies in this scenario included metalloproteinases, papain and subsequently serine proteases, for example, porcine pancreatic elastase [74].

4.1 Serine Proteases and COPD

The identification of α1-PI (an endogenous serine protease inhibitor) and subsequent research confirmed that a strong association exists between the development of emphysema and inherited deficiency of the inhibitor [75]. A critical role of serine proteases have been established in the pathobiology of emphysema. Preliminary studies in this genetic condition of inherited α1-PI deficiency indicated that chronic bronchitis is associated with the early onset of the disease [76, 77].

The lung epithelium of normal individuals is protected from the detrimental effects of neutrophil serine proteases (NSPs) by a battery of antiproteases. Large quantities of NSPs (∼20-fold w.r.t. normal subjects) released by neutrophils in acute and chronic inflammatory conditions overpowered antiprotease activities, leading to uncontrolled proteolysis and subsequently lung damage [74–76].

Alpha-1-PI deficiency increases activities of different enzymes secreted by activated neutrophils such as NE, cathepsin G (CatG), and proteinase 3 (PR3), all of which are capable of damaging different components of the ECM such as collagen, laminin, fibrillin, and elastin. However, several evidences suggest that it is the destruction of lung elastin that is important in causing emphysema, which generates COPD pathophysiology in animal model systems [75, 76].

Human neutrophil elastase (HNE), by destroying elastin, plays a critical role in the development of pulmonary emphysema [76, 77]. HNE has many other biological activities. For example, it stimulates mucin production [78], activates MMPs [5], inactivates TIMPs [79], and generates neutrophil chemotactic elastin-derived fragments [80, 81]. PR3 and CatG, two elastase homologues secreted in massive quantities from neutrophils at inflammatory sites, have also been shown to have proinflammatory functions acting through various mechanisms [80–82]. The most abundant is the α1-PI, which targets preferentially HNE. Secretory leukoprotease inhibitor (SLPI), an inhibitor of HNE and CatG, but not of PR3, has been shown to control excess proteolysis in the upper airways [83]. Elafin, derived from trappin-2 (pre-elafin) [84], is an NSP inhibitor that controls the activities of HNE and PR3 [85]. Other NSP inhibitors including 1-antichymotrypsin and monocyte/NE inhibitor (MNEI) were found to play relatively minor role as protease inhibitors [86, 87]. NSPs, therefore, could prove useful as therapeutic target for a number of inflammatory lung diseases.

There are differences in pathological manifestations among smoking and non-smoking α1-PI deficient COPD patients. The smokers with COPD frequently show pulmonary emphysema and bronchitis, while patients of the latter category often show emphysema without bronchitis. Although the chronic smokers usually suffer from the antiprotease inactivation in both the airways and respiratory units as a result of oxidant-mediated inactivation of α1-PI, the non-smoking α1-PI deficient individuals possess diminished antiprotease content primarily in respiring units, which are free of mucus glands and depends upon α1-PI for antiprotease defence [88].

Oxidative stress induced by cigarette smoke in COPD patients may promote the inflammatory state by recruiting additional neutrophils and upregulating the

inflammatory transcription factor, NF-κB and neutralizing TIMPs in addition to α1-PI and SLPI [89].

4.2 Urokinase Plasminogen Activator and COPD

In COPD patients, an increase in urokinase plasminogen activator (uPA) level in the airway epithelial and alveolar cells, and lung macrophages cause destruction of small airways and alveolus of the lung [90]. Urokinase plasminogen activator receptor (uPAR), in addition to functioning as a protease receptor, mediates intracellular signaling [91]. An increase in uPAR level in the macrophages has been observed in patients with COPD, which suggests the critical role of uPAR in inflammation and tissue remodeling including parenchymal destruction and fibrosis of small airways [92].

4.3 MMPs and COPD

MMPs are known to induce morphological changes in the lung that are prevalent in COPD. Several MMPs are known to play important roles in the pathogenesis of COPD [93]. Lung parenchyma and inflammatory cells such as neutrophils and macrophages are the major sources of MMPs in patients with COPD [94].

MMP-12 (a.k.a. macrophage elastase) is known to play an important role in COPD pathogenesis. A marked increase in MMP-12 expression in alveolar macrophages is associated with smoking associated emphysema [95]. In mouse, deletion of MMP-12 gene prevents cigarette smoke-induced inflammation, neutrophil influx, and emphysema in the lung [96, 97]. Genetic analysis of human COPD patients demonstrated that the common serine (codon 357) of the MMP-12 gene plays a crucial role in the pathological manifestations of matrix degradation, which has been observed to be related with the severity of the disease [98, 99].

Analysis of COPD lung tissue indicated an increase in the activity of MMP-1 and MMP-8, but not MMP-13 [100, 101]. An increase in MMP-1 activity was found in type II pneumocytes in patients with emphysema, but not in normal control subjects [101]. Neutrophil-derived MMP-8 levels were markedly increased in patients with COPD in comparison to the normal subjects [101]. Prominent increase in MMP-2 and MMP-9 expression has been observed in the lung of COPD patients [102]. During interleukin-10 (IL-10)-mediated airflow obstruction, an imbalance between MMP-9 and TIMP-1 results in an increase in MMP-9 activity was found in an animal model system [103].

Acrolein, a component of cigarette smoke, has been shown to initiate cleavage of proMMP-9, thereby producing active MMP-9. However, MMP-9 knockout mice do not completely inhibit cigarette smoke-induced emphysema, suggesting that other MMPs also play role in COPD pathogenesis [103]. Importantly, MMP-14, the membrane-type MMP (MT1-MMP), has been observed to be induced by acrolein which upon increase in mucin production leads to COPD [104] (Fig. 5).

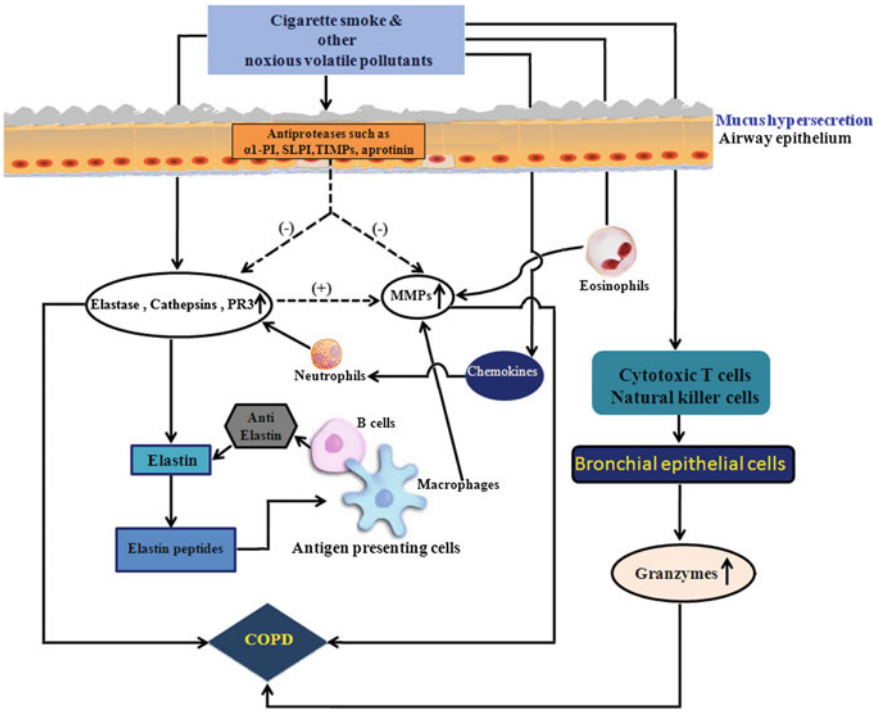

Fig. 5 Cigarette smoke and other noxious volatile components-mediated dysregulation of protease–antiprotease balance resulting in an increase in protease activity for pathological manifestation of COPD

5 Lung Fibrosis

Lung fibrosis (a.k.a. interstitial lung diseases) is a chronic disorder, exemplified by a marked increase in matrix degradation and intra-alveolar fibrosis leading to dyspenea, impaired oxygen transfer and alveolar collapse [105, 106]. Lung fibrosis occurs in the alveolar space and interstitium and is characterized by a widespread accumulation of differentiated fibroblasts (i.e., myofibroblasts) and ECM components.

Fibrotic disorders in the lung are associated with dysregulation of proteolytic activities. A considerable number of reports have suggested the involvement of cathepsins in this scenario. Enhanced proteolytic processing of CatB was observed in the lungs during an increase in TGF-β, suggesting that CatB may participate in fibrogenesis [107].

Microarray studies have revealed that in addition to NE, MMP-7 (a.k.a. matrilysin) is an important COPD marker. MMP-7 degrades decorin, the extracellular proteoglycan, which subsequently releases decorin-bound transforming growth

factor-β (TGF-β) [108] and that subsequently contributes to TGF-β activation, which is known as a critical marker of COPD [109].

6 Silicosis

An increase in the activity of MMP-2, MMP-9, and stromelysin has been demonstrated in alveolar macrophages from silica-treated rats, which contribute to extracellular matrix (ECM) and basement membrane (BM) degradation [110]. Administration of silica particles to mice causes upregulation of cathepsin K (CatK) expression and activity in silicotic lung homogenates compared to control lungs. Lung fibroblasts and macrophages were known as the main CatK-producing cells. Expression of CatK is inversely correlated to the level of TGF-β1, suggesting a protective role of CatK during silicotic process [111]. Mature active CatB, -H, -K, -L, and -S were identified in the broncho alveolar lavage fluids (BALFs) of patients suffering from silicosis. Among them, CatH has been observed to be the most abundant aminopeptidase, while CatB and CatL were mostly found thiol dependant endoproteases. Importantly, an increase in cathepsins/inhibitors ratio has been shown to favor uncontrolled proteolysis during silicosis [110, 111].

7 Cystic Fibrosis

In cystic fibrosis (CF), a marked increase in the activities of proteases could damage the airway architecture and that contributes to progressive bronchiectasis, a condition where the bronchial tubes of lungs are permanently damaged and enlarged due to infection in the bronchi [112, 113]. CF is an autosomal recessive genetic disorder caused by loss of expression or functional mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) [114, 115]. CF affects multiple organs, albeit the pathology associated with CF appears to be due to its effect on the respiratory system. Non-functional CFTR channels in CF patients prevent the regulation of chloride and sodium ions across epithelial membranes leading to an increase in dehydrated mucus secretions in the lungs [112–116].

The key immune cell mediators seen in CF patients are polymorphonuclear neutrophils [115]. Upon recruitment, activated neutrophils release a wide variety of proteases, which induce inflammatory response and subsequently tissue damage [115, 116].

The impairment of mucociliary clearance mainly revolves around the interactions between NE and mucins. Mucins are a family of highly glycosylated proteins produced by epithelial cells and are the main components of the mucus found clogging of the airways in CF patients [117, 118]. NE has been shown to regulate the mucins via activation of TNF—converting enzyme, which upregulates their expression via epidermal growth factor receptor (EGFR) pathway [118–120].

CF patients cannot efficiently clear mucus due to damage by proteases to the cilia structures in the lungs and, therefore, are highly susceptible to chronic bacterial infections [121–123].

MMPs were found to play a crucial role in CF pathogenesis [124]. MMP levels are increased in the BAL of CF individuals [125]. MMPs produce proline-glycine-proline (PGP), a neutrophil chemoattractant derived from extracellular matrix, which regulates the immune response during CF [126].

A marked increase in NE has been shown in CF lung, which causes airway remodeling by degrading ECM proteins such as elastin and fibronectin [127]. The resulting alteration of airway epithelial cell membrane by NE induces neutrophil-mediated inflammation upon increase in the expression of proinflammatory cytokines, for example, IL-8, which results in neutrophil-mediated inflammation by upregulating the proinflammatory MMPs, CatG, and PR3 leading to tissue damage in the CF lung [6].

8 Asthma and Allergy

8.1 Asthma

Asthma is a chronic inflammatory disease of the airways and its occurence and propagation is on the rise. The number of patients with asthma is estimated to attain a staggering figure of 100 million globally by 2025 [128]. Generally, asthma is triggered by the activation of adaptive immune response that upon inducing the lung triggers mucus production, increased IgE level, airway remodeling, and airway hyperactivity [129].

Manifestation of Asthma is characterized by acute inflammatory response and airway obstruction [130]. In both acute and chronic asthma, proinflammatory cells, including neutrophils, eosinophils, mast cells and macrophages enters into the lung tissue [131, 132]. These proinflammatory cells secrete a variety of extracellular proteases of which serine proteases and MMPs are important as these enzymes play prominent role in asthma pathogenesis [133–139].

Plasminogen can be converted to the active enzyme plasmin by tissue type plasminogen activator (PA) or urokinase-type PA (u-PA). Tissue PA and u-PA are associated with the dissolution of fibrin and also in the degradation of ECM components [140]. Plasminogen activator inhibitor-1 (PAI-1) is a major inhibitor of tissue type plasminogen activator and u-PA, and thereby contributes to matrix formation by preventing matrix degradation. Mast cells (MCs) in the airways of patients with asthma are crucial in initiating allergic inflammation [141]. MCs and bronchial epithelial cells (BECs) are the major source of plasminogen activator inhibitor (PAI-1). The interactions between the BECs and the MCs are important in maintaining persistent inflammation and structural changes in asthma [142].

IgE-mediated inflammation is well known for the pathogenesis of asthma. MCs-derived TGF-β upon cross-linking with IgE receptor enhances PAI-1

production in BECs. This increase in the production of PAI-1 has been suggested to play a critical role in the development of fibrosis that occurs adjacent to the epithelium [143]. Conceivably, drugs that inhibit activation of MCs, for example, by anti-IgE may prove useful in preventing airway remodeling in asthma.

8.2 Serine Protease and MMP in Asthma Pathophysiology

Proteolytic enzymes including NE and MMP-9 play important roles in tissue remodeling and repair in the airways [144]. The proteolytic enzymes levels are increased in asthma, which occurs due to an imbalance in the protease–antiprotease system.

In neutrophilic asthma, high levels of active NE and proMMP-9 were observed, whereas only a small amount of MMP-9 has been observed to be active. However, eosinophilic asthma was characterized with high level of active MMP-9 without free elastase. Thus, a differential profile of protease activity has been observed in asthma. A deficiency of antiproteases may explain the differential enzyme activity observed in eosinophilic and neutrophilic asthma. An increase in the level of MMP-9 bound to TIMP-1 in subjects with neutrophilic asthma in comparison to the eosinophilic asthma has been observed. This could explain about the presence of low level of active MMP-9 in neutrophilic asthma. This relative deficiency of TIMP-1 in subjects with eosinophilic asthma in comparison to neutrophilic asthma may explain about the high level of active MMP-9 that exists in the sputum of subjects in this group [144–146].

Alpha1-PI level has been observed to be increased in neutrophilic asthma, but its function was impaired leading to a marked increase in free elastase (NE) activity. Proteolytic inactivation of α1PI may lead to a form, which acts as an activator of neutrophils and that could result in superoxide ($O_2^{\cdot -}$) production [146]. However, role of proteolytic enzymes in specific inflammatory phenotypes of asthma is not clearly known. In contrast, IL-8 (a potent chemoattractant and an activator of neutrophils) plays an important role in eosinophilic asthma [6]. NE can induce production of IL-8 and its potency has been observed to be elevated upon proteolytic processing by MMP-9 [147].

Patients with persistent inflammatory asthma elicit more non-eosinophilic asthma progression than that of the eosinophilic asthma. These exacerbations are not prevented by corticosteroid treatment [148]. COPD and neutrophilic asthmatic patients generally show chronic airway inflammation associated with a marked airway neutrophilia, which are not discernibly responsive to inhaled corticosteroids [148, 149].

8.3 Cytokines and Asthma

Inflammation in asthma has been observed to be mediated by a specific subclass of T-lymphocytes referred primarily to Th2 lymphocytes, which causes inflammation and remodeling via secretion of specific cytokines [150].

Cytokines, for example, IL-11 play primary role in mediating asthma pathophysiology through its receptor (IL-11R). ADAM-10, a matrix metalloprotease, can release the IL-11R ectodomain upon cleavage of IL-11 receptor. Serine proteases such as NE and PR3 can also cleave the IL-11R. The resulting truncated soluble IL-11R (sIL-11R) activates the inflammatory cells. Thus, IL-11 signaling pathology proceeds upon proteolytic cleavage of its receptor [151].

An increase in the numbers of apoptotic airway epithelial cells in COPD has been observed to be associated with secondary necrosis [152, 153]. In severe asthma, conditions associated with increased airway neutrophilia, tissue damage and an increase in apoptosis of airway epithelial and smooth muscle cells have also been demonstrated [154]. Granzymes, a family of serine protease, have a repute to initiate immune-mediated cell death. Cytotoxic T cells and natural killer (NK) via granzyme-mediated pathway induces apoptosis of target cells, e.g., bronchial epithelial cells. Granzymes play critical roles in a variety of age-related chronic inflammatory diseases. There are five human granzymes identified so far in the lungs. These are granzyme A (GzmA-tryptase), granzyme B (GzmB-aspase), granzyme H (GzmH-chymase), granzyme K (GzmK-tryptase), and granzyme M (GzmM-metase). Granzymes, especially granzyme B and perforin, are stored in secretory granules of cytotoxic cells, and are released into the intercellular space following adhesion to the target cells. In presence of Ca^{2+}, perforin pores in the cell membrane enable entry of granzyme B and subsequently induces caspase-dependent apoptosis [155], which may be an important mechanism of lung injury in asthma.

8.4 Allergy

Many aeroallergens like house dust mites and fungal allergens associated proteases play important role in asthma pathophysiology. Epidemiological studies suggested that sensitivity to fungal allergens could be an important cause of allergic asthma [156].

8.5 Alternaria Alternate and Asthma Severity

The fungus *Alternaria alternate* has been observed to cause asthma under certain circumstances [157, 158]. The allergen of the fungus possesses intrinsic proteolytic activities and that upon activating protease activated receptors (PARs) play a prominent role in mediating allergic airway diseases. Interleukin-33 (IL-33) has been observed to be associated with the development of allergic asthma [159]. IL-33 expression in the lung was found to be elevated in the asthmatic subjects and the asthma severity could be positively correlated with the IL-33 expression in the airways [160, 161].

Alternaria driven release of IL-33 occurs with the involvement of a serine protease specific to this aeroallergen. The Alternaria serine proteases cause marked

inflammation because of the capacity of the serine protease to drive IL-33 release, which in turn induces rapid onset of asthma exacerbations. Thus, targeting the protease—IL-33 signaling axis could prove useful as a therapeutic measure in this kind of asthma pathogenesis [162].

8.6 Aeroallergenicity of Acanthamoeba

The free living amoeba, *Acanthamoeba trophozoite*, is found in human airway cavities and possesses high protease activities, which can elicit allergic airway inflammation [163]. Intranasal inoculation of *A. trophozoite* or its excretory secretory (ES) proteins in mice have been shown to elicit allergic airway inflammation. ES proteins with strong protease activities stimulate dendritic cells and also able to enhance the differentiation of early T cells into mature IL-4 secreting T cells. Treatment of ES proteins in the protease activated receptor (PAR-2) knockout mouse showed inhibition of lung airway inflammation and Th2 immune responses with lower IgE level compared with the normal mouse. This suggests a role of PAR-2 in the aeroallergenicity of Acanthamoeba allergens [163].

8.7 Seasonal Rhinitis and Asthma

Allergic diseases like seasonal rhinitis and asthma are generally result from exposure to airborne pollens. Asthmatic patients allergic to pollen have been observed to develop a chronic inflammation of the airways leading to bronchial obstruction and hyper responsiveness. Upon inhalation, pollen grains release a wide variety of allergens with protease activities, which may act as inflammatory mediators and subsequently pathogenesis of respiratory allergies. The proteases were known to inactivate lung regulatory neuropeptides, for example, substance P and vasoactive intestinal peptide (VIP) [164] leading to dysregulation of the contraction-relaxation rhythm of the respiratory airways. The inhaled allergens were processed by dendritic cells (DCs) present at the subepithelial regions, and then present allergen peptides to native T-lymphocytes for stimulation of IgE production. Some pollen allergens exhibit proteases such as aminopeptidase and trypsin-like serine protease activities, which can cleave proteins from junctional complexes between epithelial cells [165]. A 98 kDa aminopeptidase of *Parietaria judaica*, for instance, has been shown to cause detachment of A549 human alveolar epithelial cells by degrading intercellular adhesion proteins from tight junctions and adherens junctions [165, 166]. Pollen proteases can induce degradation of cell junction proteins such as occluding, claudin-1 and E-cadherin, thereby help allergens to cross the epithelial barrier and contact with DCs for intensifying immune response [164–166].

8.8 Organic Dust Allergy

Organic dusts made for agriculture may lead to airway inflammation, which may cause sinusitis and chronic bronchitis to workers in agricultural industries [167–170]. Workers in livestock industries working in concentrated animal feeding operations (CAFOs) are susceptible to chronic airway diseases [171]. Extracts of dust collected from CAFOs are potent stimulators of lung inflammatory responses. Hog dust extract (HDE) contains active proteases, which play a critical role in lung inflammatory processes [172, 173].

Epidermal growth factor receptor (EGFR) signaling has been shown to play an important role in the proinflammatory response of bronchial epithelial cells (BECs) to HDE [172]. The proinflammatory effect of HDE has been suggested to be due to the proteolytic activation of PARs, especially PAR-2 [173]. In lung epithelial cells, actions of fungal, cockroach and dust mite allergen proteases are mediated by the cleavage and activation of protease activated receptor-2 (PAR-2) [174–176]. However, PAR-1 is unable to mediate the effects of these proteases. PARS play an important role in bronchial fibroblast proliferation; epithelial cell wound healing and hypersecretion of mucus [177, 178]. By inhibiting HDE proteases or abrogating activation of epithelial cells, PAR-2 could inhibit HDE-induced inflammatory indexes in bronchial epithelial cells (BECs) and subsequently inhibition of the allergic response [179]. Thus, targeting the protease activity of organic dusts made for agricultural usage and other air borne dusts may prove useful as a strategy for preventing airway inflammation in agricultural workers, who are generally exposed to dusty agricultural environments.

8.9 Cockroach Allergy

Bernton and Brown [180] first observed skin rashes upon exposure of cockroach over the skin of allergic patients. Subsequently, a considerable number of evidence have confirmed that exposure to cockroach can induce allergy. Proteases associated with cockroach can produce bleb formation to the skin, which could play a critical role in the development of allergic disease [181–183].

Cockroach allergens such as saliva, feces, cast skins, and dead bodies contain serine protease activities, however, feces (frass) was found to be the prominent source of allergens, which contains serine protease activity [184, 185]. Sensitization of wild type mice to German cockroach frass (GC frass) has been shown to increase allergic hyperactivity (AHR) due to a marked increase in serum IgE and also production of cytokines such as IL-13, IL-4, IL-5, and IL-17 [186]. Sensitization of PAR-2 deficient mice with GC frass, however, did not show a discernible increase in allergic airway inflammation indicating a role of PAR-2 in mediating allergic airway inflammation [185].

9 Lung Cancer

Lung cancer is one the most prevalent and lethal diseases worldwide. Despite recent advances in chemotherapy, the molecular basis of its progression to a metastatic disease remains unclear.

Exposure of bronchial epithelial cells of smokers produce oxidants and reactive oxygen species (ROS) with consequent cellular responses to activate NF-κB and other transcription factors that regulate inflammation-related genes and initiates several signaling pathways depending on both genetic and epigenetic factors and manifest COPD and lung cancer [187, 188].

9.1 MMPs and Lung Cancer

Lung cancers are of two major types: (i) small cell lung carcinoma (LC); and (ii) non-small cell lung carcinoma (NSCLC). ECM and basement membrane components are proteolytically cleaved by MMPs. These components play a pivotal role in cancer progression. Lung cancers express high levels of MMPs. MMP-7 and MMP-9 expressions were found to be markedly high in NSCLC in comparison to the normal tissue [189]. MMP-1 and MMP-3 promoter polymorphisms are associated in modifying susceptibility to NSCLC and also to an increase in the risk of lymphatic metastasis of these tumors [190]. MMP-2 and MMP-9 activities have been found to be associated with an increase in tumor spread [191].

Treatment of mice with CH1104I (dual inhibitor of MMP-2 and -9) has been shown to markedly inhibit metastasis of lung carcinoma, which suggests that inhibition of MMP-2 and -9 could significantly inhibit tumor invasion and metastasis [192]. MMP-2 and -9 expressions may have prognostic implications in patients with NSCLC [193]. Over expression of MMP-1 has been observed to induce the formation of lung metastases [194, 195].

9.2 High Temperature Requirement A (HtrA) Serine Protease and Lung Cancer

HtrA (a.k.a. DegP) is a heat shock-induced serine protease that is active in the periplasm of *Escherichia coli*. Homologues of HtrA were found in a wide range of bacteria and in eukaryotes. Till date, four human homologues of the bacterial serine protease HtrA have been described, which are named as HtrA-1, -2, -3, and -4 [196, 197]. They have a variety of functions including cancer [198].

HtrA1 has been observed to be downregulated in lung cancers [198]. In human cancer cells, HtrA1 over expression prevents cancer cell growth and proliferation suggesting HtrA1 as a tumor suppressor. A modest expression of HtrA1 has been observed in primary tumors and lymph node metastases. However, the exact functions of HtrA1 in cancer are mostly unknown. A previous report suggested that

HtrA1 elicits its function by inhibiting TGF-β pathway [199]. The role of TGF-β in cancer progression is well documented [200]. Accordingly, the TGF-β signaling pathway has been considered as both a tumor suppressor and promoter pathway of tumor progression and invasion. It, therefore, seems probable that activation of TGF-β signaling pathway occurs when HtrA1 is downregulated, thereby contributing to the cancer progression. Alternatively, over expression of HtrA1 has been shown to induce apoptosis [201]. Thus, loss of HtrA1 expression alters regulation of apoptosis and could lead to cancer progression [201].

HtrA1 degrades tubulin by disrupting microtubules (MTs), which suggest that HtrA1 could play an important role in regulating MT and tubulin stability and MT-associated cellular functions [245]. HtrA1 also regulates cell migration and offers a potential role in regulating MT organization associated with cell migration. However, the exact mechanism(s) by which HtrA1 regulates cell migration is currently unclear. Active HtrA1 upon removal of N-terminal Kazal-type trypsin inhibitory domains contributes to cell death through caspase dependent, as well caspase-independent mechanisms [202].

The role of HtrA2 protease in stress responses and apoptosis in lung cells has been established and a previous report suggested its involvement in cisplatin-induced death of renal cells [203]. Over expression of HtrA2 by cisplatin has been observed to follow the release of HtrA2 from mitochondria to the cytosol and it degrades anti-apoptotic proteins. This mechanism seems to be obligatory to trigger mitochondrial permeabilization for HtrA2 to participate in cell death [203, 204]. Therefore, HtrA2 plays a vital role in programmed cell death upon eliminating the caspase inhibitory activity of apoptosis [205, 206]. However, the detail mechanism(s) by which HtrA2 regulates lung cancer is currently unknown.

Smoking is a critical factor for lung cancers and the HtrA3 has been shown to be associated with smoking-induced lung cancer. HtrA3 expression has been found to be markedly downregulated in lung cancer cell lines and also primary lung tumors isolated from heavy smokers [207]. HtrA3, in contrast to the steady HtrA1 and HtrA2 expression, has been identified as a probable target for cigarette smoke-induced changes in normal human bronchial epithelial cells [207]. It has also been suggested that cigarette smoke-induced methylation of HtrA3 may play an important role in the etiology of smoking-linked lung cancer [207].

Studies on HtrA3 exon in lung cancer cell lines indicates that it possesses core xenobiotic response element (XRE) consensus sequence, 5-TNGCGTG-3 and is a target for methylation at CpG. XRE is located in the promoter region of the genes involved in metabolizing xenobiotic carcinogens, mainly aryl hydrocarbons from cigarette smoke and environmental pollutants, for instance, automobile exhaust [208, 209]. These compounds upregulate the gene products of XRE via aryl hydrocarbon receptors and multitude of other transcription factors [210, 211]. The degree of methylation of HtAr3 is similar when studied in A549 and H157 lung epithelial cells, when treated with NNK (Nicotine-derived nitrosamine ketone), a.k.a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, an important tobacco-specific nitrosamines, which play key role in carcinogenesis [208]. NNK suppressed the expression of HtAr3, whereas 5-azo-dc has been shown to induce it [208].

Differential expressivity of HtAr3 may be controlled by other cross-talking mechanisms such as histone deacetylation, micro RNA activation, loss of heterozygosity and genetic mutation. Along with that HTAr3, HtAr1, and HtAr2 are also believed to be upregulated by xenobiotic stress [208, 212]. However, more research is needed to ascertain them as therapeutic targets pertaining to lung cancer for epigenetic therapies as well as prognosis to forecast tumor response.

10 Tuberculosis

Upregulation of CatG, but not NE, has been shown to induce cell death of activated *Mycobacterium tuberculosis* infected macrophages. The substrate specificity of CatG and NE is distinct. CatG cleaves the C-terminus of aromatic or positively charged amino acid residues, while NE cleaves the C-terminus of small hydrophobic amino acid residues [213, 214]. Enhanced necrosis in infected macrophages by CatG may result from proteolysis of specific target sequences, which are currently unknown. It has been shown that serpinb3a inhibition of CatG is necessary to prevent necrosis induced by IFN-γ in *M. tuberculosis* infected macrophages [214].

Role of matrix metalloproteinases (MMPs) as an important mediator of tissue destructive response in TB has now been clearly known [215, 216]. MMPs can cleave ECM components [217]. In humans, MMP-1 cleaves fibrillar components (types I and III collagen) of ECM. The level of MMP-3, the activator of MMP-1 is high in respiratory secretions of TB patients than control subjects [216–218]. In rabbits, MMP-1, -3, -7, -12, and -13 expressions are elevated in granulomatous and cavitary pathologies in human respiratory secretions observed in vivo model systems of TB [218–220]. In a human monocyte infection model, a marked reduction in TIMP-3 has been observed to be correlated with TB pathogenesis [221]. In a mice model, TIMP-3 has been observed to be associated with cavity formation and subsequently ECM degradation, which are important for TB pathogenesis [221, 222].

11 Acute Lung Injury

Severe acute respiratory distress syndrome (SARS) was identified in 2003, which triggered death of thousands of people worldwide [223, 224]. A new type of coronavirus has been identified and found responsible for the SARS, which produces pneumonia and associated high fever and severe dyspnea and subsequently acute respiratory distress syndrome (ARDS) followed by death due to acute lung injury (ALI) [224, 225]. ARDS is characterized by accumulation of inflammatory cells and severe hypoxia that leads to pulmonary edema [226].

Renin-angiotensin system (RAS) has been observed to play a critical role in SARS. In animal studies, a prominent role of angiotensin converting enzyme (ACE) in the pathogenesis of ARDS has been suggested [227, 228]. ACE2, a homologue of ACE, was shown to be a key regulator for coronovirus infection that produces SARS. ACE2 has been shown to be expressed in the lungs of both healthy and diseased humans, and it protects against SARS-induced ALI [229–231]. Therefore, it seems conceivable that ACE2 might prove a novel therapeutic target for SARS-coronovirus-induced ARDS that develops in emerging lung infectious diseases including influenza [231].

12 Elafin and ALI

Elafin, a serine protease inhibitor having mol wt of 6 kDa, found in lung secretions. Elafin is formed by proteolytic cleavage of its precursor protein, trappin-2 [232]. The antiprotease activity of elafin is located in the C-terminal domain having specificity for NE and proteinase 3. The N-terminus transglutaminase substrate binding motif (GQDPVK) of elafin cross-links with extracellular matrix proteins [233, 234].

In ALI, the protease–antiprotease balance alters in favor of proteases leading to an increase in protease activity, and this protease burden can produce pulmonary edema [235]. A decrease in plasma elafin level has been observed to be correlated with altered elafin gene expression and seems a critical component for an increase in acute respiratory distress syndrome (ARDS) [236–238].

In ALI patients, the 20S proteasome was observed to be markedly higher compared with normal subjects [239], but elafin level was shown to be decreased with consequent proteolytic degradation of antiproteases by the 20S proteasome in lung patients with ALI. This decrease may contribute to an increase in NE activity in ALI regulation and expression; however, its biological role in the lung is currently unknown. The cleavage of elafin by 20S proteosome suggests that the increment of antiprotease levels in ALI patients could prove clinically beneficial in attenuating uncontrolled activity of NE. Elafin's multifunctional properties could prove useful as the therapeutic target for ALI [239–241].

13 Elane and ALI

Elane has a potential catalytic activity to hydrolyze elastin. Under physiological conditions, lungs are protected from this enzyme by endogenous inhibitors such as α1-PI, α2-macroglobulin, and SLPI. However, in the course of ALI, the balance between elane and its endogenous inhibitors is disregulated in favor of the enzyme [242–244] leading to massive infiltration of neutrophils into the lungs and

subsequently tissue injury. Therefore, peptidic and non-peptidic elane inhibitors may prove useful for treating ALI associated with systemic inflammation [245, 246].

14 Age-Related Pulmonary Diseases

Granzymes, especially granzyme A and granzyme B, are the most abundant granzymes involving the membrane perforating molecule, perforin, which induce cell death [247]. Perforin facilitates granzymes entry into the target cell and that subsequently induces cell death [248]. Granzyme A has originally been thought to induce caspase-independent cell death; however, recent findings suggest that granzyme A may be involved in immune regulation of age-related lung disorders [249, 250]. In contrast, granzyme B induces apoptosis through caspase-dependent and -independent pathways [251]. An increase in granzyme A and B activities is known to promote generation of proinflammatory cytokines. ECM degradation and formation of autoantigens may exacerbate the inflammatory response [251]. Chronic inflammation is a hallmark of age-related cardiovascular and lung diseases. Thus, granzyme A and B may serve as important agent in promoting a positive feedback cycle that may be common to many persistent age-related disorders [251, 252]. However, role of other granzymes such as GzmH, GzmK, and GzmM in age-related ARDS are currently unknown.

15 Aspergillosis

Invasive pulmonary aspergillosis (IPA) elicited by the filamentous members of the genus *Aspergillus* can have devastating role in immune compromised individuals [253, 254]. *Aspergillus fumigatus* is responsible for the majority of IPA infections. Normal individuals are at little risk because of the effectiveness of their lung defences. However, inhalation of conidia becomes life threatening to subjects having weak immunity, which could allow the conidia to germinate into invasive hyphae in the lung [255].

The populations at greatest risk for IPA are patients with cancer, solid organ transplants, bone marrow transplants and those with advanced AIDS [256]. *A. fumigatus* secretes proteases like an alkaline serine protease (ALP), a metallo-protease (Mep), an aspartic protease (Pep) and a prolyl endopeptidase in the lungs [257–260]. *A. fumigates* secreted proteases are expressed in the lung during infection [261, 262].

A. fumigates binds to ECM proteins with the involvement of polysaccharides and glycoproteins of the conidial cell wall [263]. Several agents secreted from fungus such as proteases and toxins have been shown to influence its infection in host lung tissue [263].

16 Systemic Sclerosis

Systemic sclerosis (SSc) is an autoimmune disease, which could occur due to vascular injuries and fibrosis in skin and certain internal organs [264]. Some cytokines and growth factors like transforming growth factor-β (TGF-β) have been observed to stimulate fibroblast proliferation [264]. The disintegrin and metalloprotease-12 (ADAM-12) possess the extracellular cell binding functions. ADAM-12 is expressed in two alternative forms: (i) membrane-anchored form (ADAM12-L); and (ii) a short secreted form (ADAM12-S). An increase in the level of serum ADAM12-S level plays an important role in the pathological events of diffuse cutaneous systemic sclerosis (dcSSc) [264, 265].

ADAM-12 plays a critical role in fibrotic process. ADAM12-S has the ability to degrade physiological substrates such as the ECM substrates: fibronectin, type IV collagen [265] and also insulin-like growth factor binding protein (IGFBPs) [266, 267]. Degradation of IGFBPs augments the association between insulin-like growth factors, for example, IGF-I and its receptors. IGF-I down regulates collagenase activity with consequent increase in collagen production [268], which indicates that IGF-I could be an important mediator in the progression of fibrosis. Additionally, ADAM-12 has been observed to be upregulated in chronic wound suggesting that ADAM-12 could be related to the fibrotic process [269].

17 Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) usually occurs in prematurely born infants. Due to deficiency of lung development, BPD patients require prolonged medical ventilation for oxygen. BPD causes an increase in morbidity and mortality in preterm infants. BPD is characterized by chronic inflammation, alveolar hypoplasia and respiratory infections [270, 271]. In an animal model of BPD, a significant high elastase activity along with excessive proteolytic degradation of the elastic fibers due to a marked decrease in the levels of endogenous protease inhibitors are usually observed in the lung secretion [272]. Additionally, a discernible increase in mRNA and protein expression and also activities of cathepsins-B, -H, -K, -L, and -S have been observed in new borne BPD tracheal aspirates [273]. BAL fluid of newborn preterm infants with BPD showed elevation of MMP-9 and a decrease in free TIMP-1 level [274, 275].

18 Lymphangioleiomyomatosis

Lymphangioleiomyomatosis, a rare and progressive lung disease, usually affects women of pre-menopausal age. This disease is characterized by the infiltration of smooth muscle cells that express contractile proteins, for example, desmin.

Immunohistochemical studies have demonstrated a strong expression of CatK restricted to lymphangioleiomyomatosis cells. CatK has been suggested as a marker for diagnosis of lymphangioleiomyomatosis [276].

19 Bronchiolitis Obliterans Syndrome

Bronchiolitis obliterans syndrome (BOS) is a complication, which usually occurs during chronic rejection of lung transplant patients. Elevated levels of MMP-8 and MMP-9 were observed in obliterative bronchiolitis patients after a few years of lung transplantation [277]. A marked elevation in gelatinase activity was found in BAL fluid from BOS patients that could be due to MMP-9 secretion by local neutrophils [278]. In lung transplant model, inhibition of matrix metalloproteases in the donor and recipient, respectively, before lung harvest and after lung transplantation have been shown to improve oxygenation and markedly decreased PMN leukocyte influx into the isograft [279]. Both MMP-8 and MMP-9 deficient mice were observed to be protected from BOS as evidenced by a marked decrease in neutrophil influx and collagen deposition [280, 281].

MMPs and TIMPs were suggested to play a crucial role important role during lung allograft rejection. While TIMP-1 and TIMP-2 over expression have not been observed to elicit consistent effect on the level of cytokines or rejection pathology, MMP inhibition via systemic administration of MMP inhibitors were shown to reduce lung allograft rejection [282].

20 Lung Surfactant Proteins and Proteases

Lung surfactants are a mixture of lipids and proteins complex, which form a thin film in the lung alveoli and that plays a vital role in respiratory function especially gas exchange [283]. Additionally, the surfactant also shows the first line of innate immune defence in the lung. Its mode of action appears to lie in the inhibition of microbial infectivity and attenuation of inflammatory responses [284]. SP-A, SP-B, SP-C, and SP-D are the major surfactant proteins, which elicit important roles to trigger immune response in the lung [285–289]. SP-A has been demonstrated to be an important surfactant component having relevant functional immune response during *Staphylococcus aureus* infection [290].

Surfactant protein D (SP-D) is an important target of numerous proteases present in the CF lung. Host defence appears to be impaired due to proteolysis of SP-D and may contribute to the supportive lung disease in CF. SP-D, a glycoprotein of the collecting family, is produced and secreted by alveolar type II cells and non-ciliated bronchial epithelial cells [291].

SP-D has been observed to be protective against a wide variety of pathogens such as *Pseudomonas aeruginosa*, Haemophilus *influenza,* and *A. fumigates* [291–293]. Upon binding with SP-D, these pathogens trigger their agglutination, enhanced killing and clearance [294, 295]. SP-D has been shown to cause a number of secretions, which present larger protection in the body. An important characteristic of CF is chronic neutrophil-mediated inflammation in the airways mainly with an increase in the levels of HLE and PR3 [296–298]. Proteolytic degradation of some proteins such as SP-A and SP-D was found in BALF of CF patients [299].

Proteases have been observed to modulate surfactant activity in addition to its action on mucus proteins. Secretory proteases of *P. aeruginosa* can degrade SP-A and SP-B from lipid–protein complexes [300]. Purified elastase or secretory protease IV of *P. aeruginosa* supernatants have also been shown to degrade SP-A and SP-D [301–303]. The protease IV-mediated degradation of SP-A and SP-D may cause a discernible loss of bacterial aggregation or increase bacterial phagocytosis by alveolar macrophages [304]. NSPs like NE, PR3 and CatG can cleave the surfactant proteins [305]. These proteases may also cleave SP-D within the conserved sub-region of the C-terminal lectin domain with the generation of a ~35-kDa fragment, which decreases bacterial aggregation and mannan binding of SP-D [306]. *P. aeruginosa* elastase digestion of SP-D has also been shown to produce the 35-kDa fragment that retain the N-terminal collagen tail, albeit devoid of functional C-terminal globular lectin domain, which consequently elicit loss of innate immune functions [300, 303, 304].

In CF, COPD and asthma, like chronic lung airway diseases, an increase in the epidermal growth factor receptor (EGFR) could be the mechanism for mucus production [307, 308]. EGFR phosphorylation has been observed to activate mitogen activated protein kinases (MAPKs)-dependent signaling pathways, which in turn stimulates MMPs, for example, ADAM-17 and also NSPs leading to the production of mucins [309–312].

Human airway trypsin-like protease (HAT) has been observed to enhance the synthesis of mucus glycoconjugates in airway epithelial cells [313]. HAT is a natural ligand for PAR-2 present in bronchial cells [314] and HAT-dependent upregulation of mucin genes have also been shown to occur via PAR-EGFR signaling pathway [313].

21 Particulate Matter

Particulate matter (PM) having diameter of about 10 nm (PM10) is a complex mixture of metals, polycyclic aromatic hydrocarbons, nitrates, sulfates and other chemicals [315], where traffic and industrial activities have an important impact on that composition. Adverse effects of PM10, especially on alveolar epithelia were related to inflammation triggered by phagocytic cells upon PM10 internalization [316]. An immediate response is to augment generation of cytokines and chemokines such as IL-1, IL-6, and IL-8, TNF-α [317, 318].

Airborne PM10 is a risk factor for the development of a variety of lung diseases including cancer [315, 316]. In vitro, treatment of PM10 induces an increase in MMP-2 and MMP-9 activities, which causes ECM degradation during acute lung injury [319]. PM10 was found to be responsible for lung diseases such as tuberculosis [320], emphysema [321] and COPD [322]. In A549 lung epithelial cells, PM10 causes a marked decrease in E-cadherin/β-catenin expression and subsequently induces potentially invasive characteristics and thereby could contribute to cancer development [323].

22 Conclusion and Future Perspective

In order to fight against infections, the lung is orchestrated with different antiproteases and anti-inflammatory components. In pulmonary diseases like COPD and asthma, the balance between host proteases and their secreted endogenous inhibitors shift toward the proteases. Proteases such as NSPs, MMPs, and cathepsins are known to act along with the bacterial proteases and play important role in the manifestation of a variety of pulmonary diseases. Thus, agents that restore lung protease–antiprotease balance by upregulating endogenous protease inhibitors and/or down regulating host protease activities, appear important to control excessive inflammatory responses in the lung.

Inflammatory cells, which are rich in oxidants and proteases cause proteolytic inactivation of protein inhibitors of proteases. SLPI and trappin-2/elafin are relatively stable; even though they may be cleaved and inactivated by proteases, for example, by cathepsins at their N-terminal end, albeit it does not affect their inhibitory potency [324]. In contrast, cleavage of elafin by *P. aeruginosa* proteases may inactivate its antiprotease activity [325]. To overcome the unwanted proteolysis of antiproteases, encapsulations of protease inhibitors within liposomes have been suggested [326]. Aerosol delivery of liposomes entrapped antiproteases may prove useful since it has several features such as sustained release and relatively high loading capacities.

NE has been chosen as a target for inhibition by synthetic compounds because it is a widely recognized serine protease, which has been shown to be associated with a variety of lung diseases including CF [327]. DX-890, a small protein inhibitor of NE, has been observed to be tolerable in rats and humans after phase 1 clinical trial [328]. This compound was shown to be involved in IL-8 release from CF neutrophils and to reduce neutrophil transmigration through the epithelial barrier. Outcome of phase 3 clinical trials will reveal the usefulness of DX-890 as a therapeutic measure for a variety of lung diseases.

In a guinea pig model system, the dual MMP9/MMP12 inhibitor, AZ11557272 was found to be protective toward cigarette smoke-induced emphysema [329]. This compound markedly reduces number of inflammatory cells in bronchoalveolar lavage (BAL) fluid and has also been shown to decrease smoke-induced air space enlargement, which suggests that MMP-2 and MMP-12 could be the potential

targets for therapeutic intervention in COPD. AS112108, another dual MMP-9/MMP-12 inhibitor, has been shown to inhibit the early inflammatory responses with a decrease in neutrophil numbers [330]. AS111793, a selective MMP-12 inhibitor, elicited dose-dependent inhibition in the levels of neutrophils and macrophages in bronchoalveolar lavage (BAL) fluid, and also on the concentration of several inflammation markers that express after cigarette smoke exposure [331]. However, it did not inhibit lung inflammation observed by lipopolysaccharide (LPS). Understanding the mechanism of these antiproteases will eventually lead us to gain an insight into the basic biochemical mechanisms that regulate COPD.

MMP-1 has been considered as one of the target proteases in lung cancer [332]. The generation of MMP-1 deficient mouse model suggested pro-tumorigenic role of the enzyme [333]. Further studies on MMP-1 and other MMPs in knockout mice will be required to evaluate the functional redundancy and relative relevance of MMP-1 and other MMPs in cell proliferation, regulation of inflammatory cells, and different stages of cancer progression.

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References

1. Lecaille F, Lalmanach G, Andrault PM (2016) Antimicrobial proteins and peptides in human lung diseases: a friend and foe partnership with host proteases. Biochimie 122:151–168
2. Geraghty P, Rogan MP, Greene CM et al (2007) Neutrophil elastase up-regulates cathepsin B and matrix metalloprotease-2 expression. J Immunol 178:5871–5878
3. Imai K, Yokohama Y, Nakanishi I et al (1995) Matrix metalloproteinase 7 (matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymic properties. J Biol Chem 270:6691–6697
4. Ferry G, Lonchampt M, Pennel L et al (1997) Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. FEBS Lett 402:111–115
5. Shamamian P, Schwartz JD, Pocock BJ et al (2001) Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: a role for inflammatory cells in tumor invasion and angiogenesis. J Cell Physiol 189:197–206
6. Nakamura H, Yoshimura K, McElvaney NG et al (1992) Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. J Clin Invest 89:1478–1484
7. Benarafa C, Priebe GP, Remold-O'Donnell E (2007) The neutrophil serine protease inhibitor serpinb1 preserves lung defense functions in *Pseudomonas aeruginosa* infection. J Exp Med 204:1901–1909
8. D'Alonzo GE, Barst RJ, Ayres SM et al (1991) Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. Ann Intern Med 115:343–349
9. Perkett EA, Lyons RM, Moses HL et al (1990) Transforming growth factor-beta activity in sheep lung lymph during the development of pulmonary hypertension. J Clin Invest 86:1459–1464
10. Meyrick B, Reid L (1983) Pulmonary hypertension. Anatomic and physiologic correlates. Clin Chest Med 4:199–217
11. Archer SL, Nelson DP, Weir EK (1989) Detection of activated O_2 species in vitro and in rat lungs by chemiluminescence. J Appl Physiol 67:1912–1921

12. Farrukh IS, Sciuto AM, Spannhake EW et al (1986) Leukotriene D4 increases pulmonary vascular permeability and pressure by different mechanisms in the rabbit. Am Rev Respir Dis 134:229–232
13. Farrukh IS, Michael JR, Summer WR et al (1985) Thromboxane-induced pulmonary vasoconstriction: involvement of calcium. J Appl Physiol 58:34–44
14. Freeman BA, Topolosky MK, Crapo JD (1982) Hyperoxia increases oxygen radical production in rat lung homogenates. Arch Biochem Biophys 216:477–484
15. Gurtner GH, Michael JR, Farrukh IS et al (1985) Mechanism of hyperoxia-induced pulmonary vascular paralysis: effect of antioxidant pretreatment. J Appl Physiol 59:953–958
16. Farrukh IS, Michael JR, Peters SP et al (1988) The role of cyclooxygenase and lipoxygenase mediators in oxidant-induced lung injury. Am Rev Respir Dis 137:1343–1349
17. Feddersen CO, Chang S, Czartalomna J et al (1990) Arachidonic acid causes cyclooxygenase-dependent and -independent pulmonary vasodilation. J Appl Physiol 68:1799–1808
18. Gurtner GH, Knoblauch A, Smith PL et al (1983) Oxidant- and lipid-induced pulmonary vasoconstriction mediated by arachidonic acid metabolites. J Appl Physiol 55:949–954
19. Gurtner GH, Burke-Wolin T (1991) Interactions of oxidant stress and vascular reactivity. Am J Physiol 260:L207–L211
20. Seeger W, Wolf H, Graubert E et al (1983) Influence of aprotinin and gabexate mesilate on arachidonic acid release by the Ca-ionophore A 23187 in the lung. Adv Exp Med Biol 156:553–567
21. White RP (1988) Pharmacodynamic effects of tosyl-arginine methyl ester (TAME) on isolated human arteries. Gen Pharmacol 19:387–392
22. Chakraborti S, Gurtner GH, Michael JR (1989) Oxidant-mediated activation of phospholipase A_2 in pulmonary endothelium. Am J Physiol 257:L430–L437
23. de Haas GH, Postema NM, Nieuwenhuizen W, van Deenen LL (1968) Purification and properties of an anionic zymogen of phospholipase A from porcine pancreas. Biochim Biophys Acta 159(1):118–129
24. Chakraborti S, Roy S, Mandal A et al (2012) Role of PKCα-p38MAPK-Giα axis in NADPH oxidase derived O_2—mediated activation of cPLA2 under U46619 stimulation in pulmonary artery smooth muscle cells. Arch Biochem Biophys 523:169–180
25. Chakraborti T, Das S, Chakraborti S (2005) Proteolytic activation of protein kinase Calpha by peroxynitrite in stimulating cytosolic phospholipase A_2 in pulmonary endothelium: involvement of a pertussis toxin sensitive protein. Biochemistry (USA) 44:5246–5257
26. Chakraborti S, Chowdhury A, Chakraborti T (2015) Cross-talk between p(38)MAPK and G iα in regulating cPLA 2 activity by ET-1 in pulmonary smooth muscle cells. Mol Cell Biochem 400:107–123
27. Chakraborti S, Mandal A, Das S, Chakraborti T (2004) Inhibition of Na+/Ca^{2+} exchanger by peroxynitrite in microsomes of pulmonary smooth muscle: role of matrix metalloproteinase-2. Biochim Biophys Acta 1671:70–78
28. Chakraborti S, Mandal A, Das S, Chakraborti T (2005) Role of MMP-2 in PKCdelta-mediated inhibition of Na^+ dependent Ca^{2+} uptake in microsomes of pulmonary smooth muscle: involvement of a pertussis toxin sensitive protein. Mol Cell Biochem 280:107–117
29. Chakraborti T, Ghosh SK, Michael JR et al (1996) Role of an aprotinin-sensitive protease in the activation of Ca^{2+}-ATPase by superoxide radical $O_2^{-\bullet}$ in microsomes of pulmonary vascular smooth muscle. Biochem J 317:885–890
30. Ghosh SK, Chakraborti T, Michael JR et al (1996) Oxidant-mediated proteolytic activation of Ca^{2+}-ATPase in microsomes of pulmonary smooth muscle. FEBS Lett 387:171–174
31. Chakraborti T, Das S, Mandal M et al (2002) Role of Ca^{2+} dependent metalloprotease-2 in stimulating Ca^{2+} ATPase activity under peroxynitrite treatment in bovine pulmonary artery smooth muscle cell membrane. IUBMB Life 53:167–173

32. Bouchard RA, Bose D (1992) Contribution of sarcolemmal sodium-calcium exchange and intracellular calcium release to force development in isolated canine ventricular muscle. J Gen Physiol 99:931–960
33. Blaustein MP (1988) Sodium/calcium exchange and the control of contractility in cardiac muscle and vascular smooth muscle. J Cardiovasc Pharmacol 5:S56–S68
34. Vieillard-Baron A, Frisdal E, Raffestin B et al (2003) Inhibition of matrix metalloproteinases by lung TIMP-1 gene transfer limits monocrotaline-induced pulmonary vascular remodeling in rats. Hum Gene Ther 14:861–869
35. Stenmark KR, Mecham RP (1997) Cellular and molecular mechanisms of pulmonary vascular remodeling. Ann Rev Physiol 59:89–144
36. Stamenkovic I (2003) Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 200:448–464
37. Woessner JF Jr (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5:2145–2154
38. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T (2003) Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253:269–285
39. Mandal M, Das S, Chakraborti T, Mandal A, Chakraborti S (2003) Identification, purification and partial characterization of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in bovine pulmonary artery smooth muscle. Mol Cell Biochem 254:145–155
40. Mandal M, Mandal A, Das S, Chakraborti T, Chakraborti S (2003) Identification, purification and partial characterization of tissue inhibitor of matrix metalloproteinase-2 in bovine pulmonary artery smooth muscle. Mol Cell Biochem 254:275–287
41. Roy S, Chakraborti T, Chowdhury A, Chakraborti S (2013) Role of PKC-α in NF-κB-MT1-MMP-mediated activation of proMMP-2 by TNF-α in pulmonary artery smooth muscle cells. J Biochem 153:289–302
42. Roy S, Samanta K, Chakraborti T et al (2011) Role of TGF-β1 and TNF-α in IL-1β mediated activation of proMMP-9 in pulmonary artery smooth muscle cells: involvement of an aprotinin sensitive protease. Arch Biochem Biophys 513:61–69
43. Palese P (2004) Influenza: old and new threats. Nat Med 10:S82–S87
44. Yewdell J, Garcia-Sastre A (2002) Influenza virus still surprises. Curr Opin Microbiol 5:414–418
45. Horimoto T, Kawaoka Y (2005) Influenza: lessons from past pandemics, warnings from current incidents. Nat Rev Microbiol 3:591–600
46. Garten RJ, Davis CT, Russell CA et al (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197–201
47. Blanquer J, Blanquer R, Borra's R et al (1991) Aetiology of community acquired pneumonia in Valencia, Spain: a multicentre prospective study. Thorax 46:508–511
48. Lauderdale TL, Chang FY, Ben RJ et al (2005) Etiology of community acquired pneumonia among adult patients requiring hospitalization in Taiwan. Respir Med 99:1079–1086
49. Garten W, Klenk HD (2008) Cleavage activation of the influenza virus hemagglutinin and its role in pathogenesis. In Avian influenza: monographs in virology, vol 27. Karger, Basel, Switzerland
50. Klenk HD, Garten W (1994) Host cell proteases controlling virus pathogenicity. Trends Microbiol 2:39–43
51. Steinhauer DA (1999) Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology 258:1–20
52. Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69:531–569
53. Klenk HD, Rott R, Orlich M et al (1975) Activation of influenza A viruses by trypsin treatment. Virology 68:426–439
54. Lazarowitz SG, Choppin PW (1975) Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 68:440–454

55. Goto H, Kawaoka Y (1998) A novel mechanism for the acquisition of virulence by a human influenza A virus. Proc Natl Acad Sci U S A 95:10224–10228
56. Lazarowitz SG, Goldberg AR, Choppin PW (1973) Proteolytic cleavage by plasmin of the HA polypeptide of influenza virus: host cell activation of serum plasminogen. Virology 56:172–180
57. LeBouder F, Morello E, Rimmelzwaan GF et al (2008) Annexin II incorporated into influenza virus particles supports virus replication by converting plasminogen into plasmin. J Virol 82:6820–6828
58. Kido H, Okumura Y, Yamada H et al (2007) Proteases essential for human influenza virus entry into cells and their inhibitors as potential therapeutic agents. Curr Pharm Des 13:405–414
59. Tashiro M, Ciborowski P, Klenk HD et al (1987) Role of Staphylococcus protease in the development of influenza pneumonia. Nature 325:536–537
60. Scheiblauer H, Reinacher M, Tashiro M et al (1992) Interactions between bacteria and influenza A virus in the development of influenza pneumonia. J Infect Dis 166:783–791
61. Bahgat MM, Błazejewska P, Schughart K (2011) Inhibition of lung serine proteases in mice: a potentially new approach to control influenza infection. Virol J 8:27
62. Böttcher E, Matrosovich T, Beyerle M et al (2006) Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. J Virol 80:9896–9898
63. Chaipan C, Kobasa D, Bertram S et al (2009) Proteolytic activation of the 1918 influenza virus hemagglutinin. J Virol 83:3200–3211
64. Steinhoff M, Buddenkotte J, Shpacovitch V et al (2005) Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocr Rev 26:1–43
65. Vergnolle N (2008) Proteinase-activated receptors (PARs) in infection and inflammation in the gut. Int J Biochem Cell Biol 40:1219–1227
66. Riteau B, de Vaureix C, Lefevre F (2006) Trypsin increases pseudorabies virus production through activation of the ERK signalling pathway. J Gen Virol 87:1109–1112
67. Chignard M, Pidard D (2006) Neutrophil and pathogen proteinases versus proteinase-activated receptor-2 lung epithelial cells: more terminators than activators. Am J Respir Cell Mol Biol 34:394–398
68. Lan RS, Stewart GA, Goldie RG et al (2004) Altered expression and in vivo lung function of protease-activated receptors during influenza A virus infection in mice. Am J Physiol 286: L388–L398
69. Khoufache K, LeBouder F, Morello E et al (2009) Protective role for protease-activated receptor-2 against influenza virus pathogenesis via an IFN-gamma-dependent pathway. J Immunol 182:7795–7802
70. Ovcharenko AV, Zhirnov OP (1994) Aprotinin aerosol treatment of influenza and paramyxovirus bronchopneumonia of mice. Antiviral Res 23:107–118
71. Lee MG, Kim KH, Park KY et al (1996) Evaluation of anti-influenza effects of camostat in mice infected with non-adapted human influenza viruses. Arch Virol 141:1979–1989
72. Manzano-Leon N, Quintana R, Sanchez B (2013) Variation in the composition and in vitro proinflammatory effect of urban particulate matter from different sites. J Biochem Mol Toxicol 27:87–97
73. Heijink IH, de Bruin HG, Dennebos R et al (2016) Cigarette smoke-induced epithelial expression of WNT-5B: implications for COPD. Eur Respir J ERJ-01541-2015
74. Chadwick D, Goode JA (eds) (2001) Chronic obstructive pulmonary disease: pathogenesis to treatment. Novartis Foundation Symposium 234, vol 234. Novartis Foundation. ISBN: 0-471-49437-2
75. Stockley JA, Stockley RA (2016) Pulmonary physiology of chronic obstructive pulmonary disease, cystic fibrosis, and alpha-1 antitrypsin deficiency. Ann Am Thorac Soc 2:S118–S122

76. Zuo L, Pannell BK, Zhou T et al (2016) Historical role of alpha-1-antitrypsin deficiency in respiratory and hepatic complications. Gene 589:118–122
77. Ganrot PO, Laurell CB, Eriksson S (1967) Obstructive lung disease and trypsin inhibitors in α1-antitrypsin deficiency. Scand J Clin Lab Invest 19:205–208
78. Kueppers F, Briscoe WA, Bearn AG (1964) Hereditary deficiency of serum α-l-antitrypsin. Science 146:1678–1679
79. Okada Y, Watanabe S, Nakanishi I et al (1988) Inactivation of tissue inhibitor of metalloproteinases by neutrophil elastase and other serine proteinases. FEBS Lett 229:157–160
80. Hunninghake GW, Davidson JM, Rennard S (1981) Elastin fragments attract macrophage precursors to diseased sites in pulmonary emphysema. Science 212:925–927
81. Kessenbrock K, Dau T, Jenne DE (2010) Tailor-made inflammation: how neutrophil serine proteases modulate the inflammatory response. J Mol Med 89:23–28
82. Korkmaz B, Horwitz MS, Jenne DE et al (2011) Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev 62:726–759
83. Pham CT (2008) Neutrophil serine proteases fine-tune the inflammatory response. Int J Biochem Cell Biol 40:1317–1333
84. Kramps JA, Franken C, Dijkman JH (1984) ELISA for quantitative measurement of low-molecular-weight bronchial protease inhibitor in human sputum. Am Rev Respir Dis 129:959–963
85. Vogelmeier C, Hubbard RC, Fells GA et al (1991) Anti-neutrophil elastase defense of the normal human respiratory epithelial surface provided by the secretory leukoprotease inhibitor. J Clin Invest 87:482–488
86. Schalkwijk J, Wiedow O, Hirose S (1999) The trappin gene family: proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core. Biochem J 340:569–577
87. Hochstrasser K, Albrecht GJ, Schonberger OL et al (1981) An elastase-specific inhibitor from human bronchial mucus: isolation and characterization. Hoppe-Seyler's Z Physiol Chem 362:1369–1375
88. Takubo Y, Guerassimov A, Ghezzo H et al (2002) Alpha1-antitrypsin determines the pattern of emphysema and function in tobacco smoke-exposed mice: parallels with human disease. Am J Respir Crit Care Med 166:1596–1603
89. Janoff A, Carp H, Lee DK (1980) Inactivation of alpha 1-proteinase inhibitor and bronchial mucous proteinase inhibitor by cigarette smoke in vitro and in vivo. Bull Eur Physiopathol Respir 16:321–340
90. Simon RH, Gross TJ, Edwards JA et al (1992) Fibrin degradation by rat pulmonary alveolar epithelial cells. Am J Physiol 262:L482–L488
91. Blasi F, Carmeliet P (2002) uPAR: a versatile signaling orchestrator. Nat Rev Mol Cell Biol 3:932–943
92. Zhang Y, Xiao W, Jiang Y et al (2012) Levels of components of the urokinase-type plasminogen activator system are related to chronic obstructive pulmonary disease parenchymal destruction and airway remodelling. J Int Med Res 40:976–985
93. Ostridge K, Williams N, Kim V et al (2016) Relationship between pulmonary matrix metalloproteinases and quantitative CT markers of small airways disease and emphysema in COPD. Thorax 71:126–132
94. Navratilova Z, Kolek V, Petrek M (2016) Matrix metalloproteinases and their inhibitors in chronic obstructive pulmonary disease. Arch Immunol Ther Exp (Warsz) 64:177–193
95. Churg A, Wang RD, Tai H et al (2003) Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. Am J Respir Crit Care Med 167:1083–1089
96. Lagente V, Le Quement C, Boichot E (2009) Macrophage metalloelastase (MMP-12) as a target for inflammatory respiratory diseases. Expert Opin Ther Targets 13:287–295

97. Aida Y, Shibata Y, Abe S et al (2014) Inhibition of elastase-pulmonary emphysema in dominant-negative MafB transgenic mice. Int J Biol Sci 10:882–894
98. Haq I, Lowrey GE, Kalsheker N et al (2011) Matrix metalloproteinase-12 (MMP-12) SNP affects MMP activity, lung macrophage infiltration and protects against emphysema in COPD. Thorax 66:970–976
99. Navratilova Z, Zatloukal J, Kriegova E et al (2012) Simultaneous up-regulation of matrix metalloproteinases 1, 2, 3, 7, 8, 9 and tissue inhibitors of metalloproteinases 1, 4 in serum of patients with chronic obstructive pulmonary disease. Respirology 17:1006–1012
100. Raulo SM, Sorsa TA, Kiili MT et al (2001) Evaluation of collagenase activity, matrix metalloproteinase-8, and matrix metalloproteinase-13 in horses with chronic obstructive pulmonary disease. Am J Vet Res 62:1142–1148
101. Roderick JT, Cheryl LF, Laura M et al (2006) Matrix metalloproteinases promote inflammation and fibrosis in asbestos-induced lung injury in mice. Am J Respir Cell Mol Biol 35:289–297
102. Deshmukh HS, Shaver C, Case LM et al (2008) Acrolein-activated matrix metalloproteinase-9 contributes to persistent mucin production. Am J Respir Cell Mol Biol 38:446–454
103. Lim S, Roche N, Oliver BG et al (2000) Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. Am J Respir Crit Care Med 162:1355–1360
104. Deshmukh HS, McLachlan A, Atkinson JJ et al (2009) Matrix metalloproteinase-14 mediates a phenotypic shift in the airways to increase mucin production. Am J Respir Crit Care Med 180:834–845
105. Quan TE, Cowper SE, Bucala R (2006) The role of circulating fibrocytes in fibrosis. Curr Rheumatol Rep 8:145–150
106. Katzenstein AL, Myers JL (1998) Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. Am J Respir Crit Care Med 157:1301–1315
107. Moles A, Tarrats N, Fernández-Checa JC et al (2009) Cathepsins B and D drive hepatic stellate cell proliferation and promote their fibrogenic potential. Hepatology 49:1297–1307
108. Imai K, Hiramatsu A, Fukushima D et al (1997) Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. Biochem J 322:809–814
109. van den Brûle S, Misson P, Bühling F et al (2005) Overexpression of cathepsin K during silica-induced lung fibrosis and control by TGF-beta. Respir Res 6:84
110. Scabilloni JF, Wang L, Antonini JM et al (2005) Matrix metalloproteinase induction in fibrosis and fibrotic nodule formation due to silica inhalation. Am J Physiol Lung Cell Mol Physiol 288:L709–L717
111. Perdereau C, Godat E, Maurel MC et al (2006) Cysteine cathepsins in human silicotic bronchoalveolar lavage fluids. Biochim Biophys Acta 1762:351–356
112. Rowe SM, Miller S, Sorscher EJ (2005) Cystic fibrosis. N Engl J Med 352:1992–2001
113. Gibson GJ, Loddenkemper R, Lundbäck B (2013) Respiratory health and disease in Europe: the new European Lung White Book. Eur Respir J 42:559–563
114. Tilly BC, Winter MC, Ostedgaard LS et al (1992) Cyclic AMP-dependent protein kinase activation of cystic fibrosis transmembrane conductance regulator chloride channels in planar lipid bilayers. J Biol Chem 267:9470–9473
115. Downey DG, Bell SC, Elborn JS (2009) Neutrophils in cystic fibrosis. Thorax 64:81–88
116. Kreda SM, Davis CW, Rose MC (2012) CFTR, mucins, and mucus obstruction in cystic fibrosis. Cold Spring Harb Perspect Med 2:a009589
117. Voynow JA, Rubin BK (2009) Mucins, mucus, and sputum. Chest 135:505–512
118. Shao MX, Nadel JA (2005) Neutrophil elastase induces MUC5AC mucin production inhuman airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-alpha-converting enzyme. J Immunol 175:4009–4016

119. Song JS, Cho KS, Yoon HK et al (2005) Neutrophil elastase causes MUC5AC mucin synthesis via EGF receptor, ERK and NF-kB pathways in A549 cells. Korean J Intern Med 20:275–283
120. Fischer BM, Voynow JA (2002) Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. Am J Respir Cell Mol Biol 26:447–452
121. Amitani R, Wilson R, Rutman A (1991) Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. Am J Respir Cell Mol Biol 4:26–32
122. Mall MA (2008) Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. J Aerosol Med Pulm Drug Deliv 21:13–24
123. Åstrand AB, Hemmerling M, Root J et al (2015) Linking increased airway hydration, ciliary beating, and mucociliary clearance through ENaC inhibition. Am J Physiol Lung Cell Mol Physiol 308:L22–L32
124. Gaggar A, Hector A, Bratcher PE et al (2011) The role of matrix metalloproteinases in cystic fibrosis lung disease. Eur Respir J 38:721–727
125. Gaggar A, Li Y, Weathington N et al (2007) Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients. Am J Physiol Lung Cell Mol Physiol 293: L96–L104
126. Gaggar A, Jackson PL, Noerager BD et al (2008) A novel proteolytic cascade generates an extracellular matrix-derived chemoattractant in chronic neutrophilic inflammation. J Immunol 180:5662–5669
127. Matthew S, Twigg SB, Philip L et al (2015) The role of serine proteases and antiproteases in the cystic fibrosis lung. Mediat Inflamm 2015:10
128. Kim HY, DeKruyff RH, Umetsu DT (2010) The many paths to asthma: phenotype shaped by innate and adaptive immunity. Nat Immunol 11:577–584
129. Braman SS (2006) The global burden of asthma. Chest 130:4S–12S
130. Barnes PJ (2008) Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 8:183–192
131. Holgate ST (2008) Pathogenesis of asthma. Clin Exp Allergy 38:872–897
132. Agrawal DK, Shao Z (2010) Pathogenesis of allergic airway inflammation. Curr Allergy Asthma Rep 10:39–48
133. Broide DH (2008) Immunologic and inflammatory mechanisms that drive asthma progression to remodeling. J Allergy Clin Immunol 121:560–570
134. Cataldo D, Munaut C, Noel A et al (2000) MMP2 and MMP9 linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. Int Arch Allergy Immunol 123:259–267
135. Corry DB, Rishi K, Kanellis J et al (2002) Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2 deficiency. Nat Immunol 3:347–353
136. Greenlee KJ, Corry DB, Engler DA et al (2006) Proteomic identification of in vivo substrates for matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. J Immunol 177:7312–7321
137. Greenlee KJ, Werb Z, Kheradmand F (2007) Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiol Rev 87:69–98
138. McMillan SJ, Kearley J, Campbell JD (2004) Matrix metalloproteinase9 deficiency results in enhanced allergen induced airway inflammation. J Immunol 172:2586–2594
139. Tsai YS, Tseng YT, Chen PS et al (2016) Protective effects of elafin against adult asthma. Allergy Asthma Proc 37:15–34
140. Rijken DC, Sakharov DV (2001) Basic principles in thrombolysis: regulatory role of plasminogen. Thromb Res 103:S41–S49

141. Pesci A, Foresi A, Bertorelli G et al (1993) Histochemical characteristics and degranulation of mast cells in epithelium and lamina propria of bronchial biopsies from asthmatic and normal subjects. Am Rev Respir Dis 147:684–689
142. Cho SH, Tam SW, Demissie-Sanders S et al (2000) Production of plasminogen activator inhibitor-1 by human mast cells and its possible role in asthma. J Immunol 165:3154–3161
143. Cho SH, Lee SH, Kato A et al (2015) Cross-talk between mast cells and bronchial epithelial cells in plasminogen activator inhibitor-1 production via transforming growth factor β1. Am J Respir Cell Mol Biol 52:88–95
144. Vignola AM, Riccobono L, Mirabella A et al (1998) Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlated with airflow obstruction in asthma and chronic bronchitis. Am J Respir Crit Care Med 158:1945–1950
145. Mautino G, Henriquet C, Gougat C et al (1999) Increased expression of tissue inhibitor of metalloproteinase-1 and loss of correlation with matrix metalloproteinase-9 by macrophages in asthma. Lab Invest 79:39–47
146. Gibson PG, Simpson JL, Saltos N (2001) Heterogeneity of airway inflammation in persistent asthma. Chest 119:1329–1336
147. Van Den Steen PE, Proost P, Wuyts A et al (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO—and leaves RANTES and MCP-2 intact. Blood 96:2673–2681
148. George L, Brightling CE (2016) Eosinophilic airway inflammation: role in asthma and chronic obstructive pulmonary disease. Ther Adv Chronic Dis 7:34–51
149. Renauld JC (2001) New insights into the role of cytokines in asthma. J Clin Pathol 54:577–589
150. Hodge S, Hodge G, Holmes M et al (2005) Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. Eur Respir J 25:447–454
151. Lokau J, Nitz R, Agthe M et al (2016) Proteolytic cleavage governs interleukin-11 trans signaling. Cell Rep 14:1761–1773
152. Trautmann A, Kruger K, Akdis M et al (2005) Apoptosis and loss of adhesion of bronchial epithelial cells in asthma. Int Arch Allergy Immunol 138:142–150
153. Kuwano K (2007) Epithelial cell apoptosis and lung remodeling. Cell Mol Immunol 4:419–429
154. Solarewicz-Madejek K, Basinski TM, Crameri R et al (2009) T cells and eosinophils in bronchial smooth muscle cell death in asthma. Clin Exp Allergy 39:845–855
155. Lieberman J (2003) The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 3:361–370
156. Lopez M, Salvaggio JE (1985) Mold-sensitive asthma. Clin Rev Allergy 3:183–196
157. Agarwal R (2011) Severe asthma with fungal sensitization. Curr Allergy Asthma Rep 11:403–413
158. Downs SH, Mitakakis TZ, Marks GB et al (2001) Clinical importance of Alternaria exposure in children. Am J Respir Crit Care Med 164:455–459
159. Kouzaki H, Iijima K, Kobayashi T et al (2011) The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. J Immunol 186:4375–4387
160. Prefontaine D, Nadigel J, Chouiali F et al (2010) Increased IL-33 expression by epithelial cells in bronchial asthma. J Allergy Clin Immunol 125:752–754
161. Prefontaine D, Lajoie-Kadoch S, Foley S et al (2009) Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. J Immunol 183:5094–5103
162. Snelgrove RJ, Gregory LG, Peiró T et al (2014) Alternaria-derived serine protease activity drives IL-33-mediated asthma exacerbations. J Allergy Clin Immunol 134:583–592
163. Park MK, Cho MK, Kang SA et al (2014) Acanthamoeba protease activity promotes allergic airway inflammation via protease-activated receptor 2. PLoS ONE 9:e92726

164. Widmer F, Hayes PJ, Whittaker RG et al (2000) Substrate preference profiles of proteases released by allergenic pollens. Clin Exp Allergy 30:571–576
165. Hammad H, Lambrecht BN (2008) Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. Nat Rev Immunol 8:193–204
166. Matheson N, Schmidt J, Travis J (1995) Isolation and properties of an angiotensin II-cleaving peptidase from mesquite pollen. Am J Respir Cell Mol Biol 12:441–448
167. Medical Section of the American Lung Association (1998) The official conference report of the American Thoracic Society and Approved by the ATS board of Directors. Respiratory health hazards in agriculture. Am J Respir Crit Care Med 158:S1–S76
168. Kirkhorn SR, Garry VF (2000) Agricultural lung diseases. Environ Health Perspect 108:705–712
169. Langley RL (2011) Consequences of respiratory exposures in the farm environment. N C Med J 72:477–480
170. Poole JA, Romberger DJ (2012) Immunological and inflammatory responses to organic dust in agriculture. Curr Opin Allergy Clin Immunol 12:126–132
171. May S, Romberger DJ, Poole JA (2012) Respiratory health effects of large animal farming environments. J Toxicol Environ Health B Crit Rev 15:524–541
172. Dodmane PR, Schulte NA, Heires AJ et al (2011) Airway epithelial epidermal growth factor receptor mediates hogbarn dust-induced cytokine release but not Ca2 response. Am J Respir Cell Mol Biol 45:882–888
173. Romberger DJ, Heires AJ, Nordgren TM et al (2015) Proteases in agricultural dust induce lung inflammation through PAR-1 and PAR-2 activation. Am J Physiol Lung Cell Mol Physiol 309:L388–L399
174. Kouzaki H, O'Grady SM, Lawrence CB et al (2009) Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J Immunol 183:1427–1434
175. Page K, Strunk VS, Hershenson MB (2003) Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease activated receptor (PAR)-2 and extracellular-signal-regulated kinase. J Allergy Clin Immunol 112:1112–1118
176. Post S, Heijink IH, Petersen AH et al (2014) Protease-activated receptor-2 activation contributes to house dust mite-induced IgE responses in mice. PLoS ONE 9:e91206
177. Liu C, Li Q, Zhou X et al (2013) Human airway trypsin-like protease induces mucin5AC hypersecretion via a protease activated receptor 2-mediated pathway in human airway epithelial cells. Arch Biochem Biophys 535:234–240
178. Matsushima R, Takahashi A, Nakaya Y et al (2006) Human airway trypsin-like protease stimulates human bronchial fibroblast proliferation in a protease-activated receptor-2-dependent pathway. Am J Physiol Lung Cell Mol Physiol 290:L385–L395
179. Asokananthan N, Graham PT, Stewart DJ et al (2002) House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Derp 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. J Immunol 169:4572–4578
180. Bernton H, Brown H (1964) Insect allergy-preliminary studies of the cockroach. J Allergy 35:506–513
181. Arruda LK, Vailes LD, Ferriani VPL et al (2001) Cockroach allergens and asthma. Curr Rev Allergy Clin Immunol 107:419–428
182. Crain EF, Walter M, O'Connor GT et al (2002) Home and allergic characteristics of children with asthma in seven U.S. urban communities and design of an environmental intervention: the inner-city asthma study. Env Health Persp 110:939–945
183. Bhat RK, Page K, Tan A et al (2003) German cockroach extract increases bronchial epithelial cell interleukin-8 expression. Clin Exp Allergy 33:35–42
184. Hughes VS, Page K (2007) German cockroach frass proteases cleave promatrix metalloproteinase-9. Exp Lung Res 33:135–150

185. Page K, Ledford JR, Zhou P et al (2010) Mucosal sensitization to German cockroach involves protease-activated receptor-2. Respir Res 11:62
186. Page K (2012) Role of cockroach proteases in allergic disease. Curr Allergy Asthma Rep 12:448–455
187. Young RP, Hopkins RJ (2011) How the genetics of lung cancer may overlap with COPD. Respirology 16:1047–1055
188. Brzóska K, Bartłomiejczyk T, Sochanowicz B et al (2014) Matrix metalloproteinase 3 polymorphisms as a potential marker of enhanced susceptibility to lung cancer in chronic obstructive pulmonary disease subjects. Ann Agric Environ Med 21:546–551
189. Safranek J, Pesta M, Holubec L et al (2009) Expression of MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in lung tissue of patients with non-small cell lung cancer (NSCLC) and benign pulmonary disease. Anticancer Res 29:2513–2517
190. Fang S, Jin X, Wang R et al (2005) Polymorphisms in the MMP1 and MMP3 promoter and non-small cell lung carcinoma in North China. Carcinogenesis 26:481–486
191. Turpeenniemi-Hujanen T (2005) Gelatinases (MMP-2 and-9) and their natural inhibitors as prognostic indicators in solid cancers. Biochimie 87:287–297
192. Chen MH, Cui SX, Cheng YN et al (2008) Galloyl cyclic-imide derivative CH1104I inhibits tumour invasion through suppressing matrix metalloproteinase activity. Anticancer Drugs 19:957–965
193. Qian Q, Wang Q, Zhan P et al (2010) The role of matrix metalloproteinase 2 on the survival of patients with non-small cell lung cancer: a systematic review with meta-analysis. Cancer Invest 28:661–669
194. Pulukuri SM, Rao JS (2008) Matrix metalloproteinase-1 promotes prostate tumour growth and metastasis. Int J Oncol 32:757–765
195. Houghton AM, Grisolano JL, Baumann ML et al (2006) Macrophage elastase (matrix metalloproteinase-12) suppresses growth of lung metastases. Cancer Res 66:6149–6155
196. Nie GY, Hampton A, Li Y et al (2003) Identification and cloning of two isoforms of human high-temperature requirement factor A3 (HtrA3), characterization of its genomic structure and comparison of its tissue distribution with HtrA1 and HtrA2. Biochem J 371:39–48
197. Clausen T, Southan C, Ehrmann M (2002) The HtrA family of proteases: implications for protein composition and cell fate. Mol Cell 10:443–455
198. Esposito V, Campioni M, De Luca A et al (2006) Analysis of HtrA1 serine protease expression in human lung cancer. Anticancer Res 26:3455–3459
199. Oka C, Tsujimoto R, Kajikawa M et al (2004) HtrA1 serine protease inhibits signaling mediated by TGF, family proteins. Development 131:1041–1053
200. Hirotaka O, Takahashi T (2002) Genetic alterations of multiple tumor suppressors and oncogenes in the carcinogenesis and progression of lung cancer. Oncogene 21:7421–7434
201. Shen ZT, Shen JS, Ji XQ et al (2016) TGF-β1 -rs1982073 polymorphism contributes to radiation pneumonitis in lung cancer patients: a meta-analysis. J Cell Mol Med. doi:10.1111/jcmm.12933
202. Chien J, Ota T, Aletti G et al (2009) Serine protease HtrA1 associates with microtubules and inhibits cell migration. Mol Cell Biol 2009:4177–4187
203. Cilenti L, Kyriazis GA, Soundarapandian MM et al (2005) Omi/HtrA2 protease mediates cisplatin-induced cell death in renal cells. Am J Physiol Renal Physiol 288:371–379
204. Egger L, Schneider J, Rhême C et al (2003) Serine proteases mediate apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions. Cell Death Differ 10:1188–1203
205. Leist M, Jaattela M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2:589–598
206. Wolf BB, Green DR (2002) Apoptosis: letting slip the dogs of war. Curr Biol 5:R177–R179
207. Jorgensen ED, Dozmorov I, Frank MB et al (2004) Global gene expression analysis of human bronchial epithelial cells treated with tobacco condensates. Cell Cycle 3:1154–1168

208. Beleford D, Liu Z, Rattan R et al (2010) Methylation induced gene silencing of HtrA3 in smoking-related lung cancer. Clin Cancer Res 16:398–409
209. Fujii-Kuriyama Y, Mimura J (2005) Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes. Biochem Biophys Res Commun 338:311–317
210. Safe S, Wang F, Porter W et al (1998) Ah receptor agonists as endocrine disruptors: antiestrogenic activity and mechanisms. Toxicol Lett 102:343–347
211. Hockings JK, Thorne PA, Kemp MQ et al (2006) The ligand status of the aromatic hydrocarbon receptor modulates transcriptional activation of BRCA-1 promoter by estrogen. Cancer Res 66:2224–2232
212. Chien J, Campioni M, Shridhar V et al (2009) HtrA serine proteases as potential therapeutic targets in cancer. Curr Cancer Drug Targets 9:451–468
213. Korkmaz B, Moreau T, Gauthier F (2008) Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions. Biochimie 90:227–242
214. Reece ST, Loddenkemper C, Askew DJ et al (2010) Serine protease activity contributes to control of *Mycobacterium tuberculosis* in hypoxic lung granulomas in mice. J Clin Invest 120:3365–3376
215. Elkington P, Shiomi T, Breen R et al (2011) MMP-1 drives immunopathology in human tuberculosis and transgenic mice. J Clin Invest 121:1827–1833
216. Walker NF, Clark SO, Oni T et al (2012) Doxycycline and HIV infection suppress tuberculosis-induced matrix metalloproteinases. Am J Resp Crit Care Med 185:989–997
217. Elkington PT, Armiento JM, Friedland JS (2011) Tuberculosis immunopathology: the neglected role of extracellular matrix destruction. Sci Transl Med 3:71–76
218. Seddon J, Kasprowicz V, Walker NF et al (2013) Procollagen III N terminal propeptide and desmosine are released by matrix destruction in pulmonary tuberculosis. J Infect Dis 208:1571–1579
219. Ugarte-Gil CA, Elkington P, Gilman RH et al (2013) Induced sputum MMP-1, -3 and -8 concentrations during treatment of tuberculosis. PLoS ONE 8:e61333
220. Singh S, Saraiva L, Elkington PT et al (2014) Regulation of matrix metalloproteinase-1, -3, and -9 in *Mycobacterium tuberculosis*-dependent respiratory networks by the rapamycin-sensitive PI3K/p70(S6K) cascade. FASEB J 28:85–93
221. Leco KJ, Waterhouse P, Sanchez OH et al (2001) Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). J Clin Invest 108:817–829
222. Kübler A, Luna B, Larsson C et al (2015) Mycobacterium tuberculosis dysregulates MMP/TIMP balance to drive rapid cavitation and unrestrained bacterial proliferation. J Pathol 235:431–444
223. Peiris JS, Yuen KY, Osterhaus AD et al (2003) The severe acute respiratory syndrome. N Engl J Med 349:2431–2441
224. Peiris JS, Guan Y, Yuen KY (2004) Severe acute respiratory syndrome. Nat Med 10:S88–S97
225. Hudson LD, Milberg JA, Anardi D et al (1995) Clinical risks for development of the acute respiratory distress syndrome. Am J Respir Crit Care Med 151:293–301
226. Ware LB, Matthay MA (2000) The acute respiratory distress syndrome. N Engl J Med 342:1334–1349
227. Raiden S, Nahmod K, Nahmod V et al (2002) Nonpeptide antagonists of AT1 receptor for angiotensin II delay the onset of acute respiratory distress syndrome. J Pharmacol Exp Ther 303:45–51
228. Marshall RP, Webb S, Bellingan GJ et al (2002) Angiotensin converting enzyme insertion/deletion polymorphism is associated with susceptibility and outcome in acute respiratory distress syndrome. Am J Respir Crit Care Med 166:646–650

229. Jerng JS, Yu CJ, Wang HC et al (2006) Polymorphism of the angiotensin-converting enzyme gene affects the outcome of acute respiratory distress syndrome. Crit Care Med 34:1001–1006
230. Hamming I, Timens W, Bulthuis ML et al (2004) Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol 203:631–637
231. Kuba K, Imai Y, Rao S et al (2005) A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. Nat Med 11:875–879
232. Guyot N, Zani ML, Berger P et al (2005) Proteolytic susceptibility of the serine protease inhibitor trappin-2 (pre-elafin): evidence for tryptase-mediated generation of elafin. Biol Chem 386:391–399
233. Molhuizen HO, Alkemade HA, Zeeuwen PL et al (1993) SKALP/elafin: an elastase inhibitor from cultured human keratinocytes. Purification, cDNA sequence, and evidence for transglutaminase cross-linking. J Biol Chem 268:12028–12032
234. Nara K, Ito S, Ito T et al (1994) Elastase inhibitor elafin is a new type of proteinase inhibitor which has a transglutaminase-mediated anchoring sequence termed 'cementoin'. J Biochem 115:441–448
235. Petty TL (1991) Protease mechanisms in the pathogenesis of acute lung injury. Ann N Y Acad Sci 624:267–277
236. Wang Z, Beach D, Su L et al (2008) A genome-wide expression analysis in blood identifies pre-elafin as a biomarker in ARDS. Am J Respir Cell Mol Biol 38:724–732
237. Wang Z, Chen F, Zhai R et al (2009) Plasma neutrophil elastase and elafin imbalance is associated with acute respiratory distress syndrome (ARDS) development. PLoS ONE 4: e4380
238. Tejera P, Wang Z, Zhai R et al (2009) Genetic polymorphisms of peptidase inhibitor 3 (elafin) are associated with ARDS. Am J Respir Cell Mol Biol 41:696–704
239. Sixt SU, Adamzik M, Spyrka D et al (2009) Alveolar extracellular 20S proteasome in patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 179:1098–1106
240. Roth GA, Moser B, Krenn C et al (2005) Heightened levels of circulating 20S proteasome in critically ill patients. Eur J Clin Invest 35:399–403
241. Kerrin A, Weldon S, Chung AHK et al (2013) Proteolytic cleavage of elafin by 20S proteasome may contribute to inflammation in acute lung injury. Thorax 68:315–321
242. Merritt TA, Cochrane CG, Holcomb K et al (1983) Elastase and alpha 1-proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome. Role of inflammation in the pathogenesis of bronchopulmonary dysplasia. Clin Invest 72:656–666
243. Janoff A (1985) Elastases and emphysema. Current assessment of the protease antiprotease hypothesis. Am Rev Respir Dis 132:417–433
244. Taggart CC, Greene CM, Carroll TP et al (2005) Elastolytic proteases: inflammation resolution and dysregulation in chronic infective lung disease. Am J Respir Crit Care Med 171:1070–1076
245. Kawabata K, Suzuki M, Sugitani M et al (1991) ONO-5046, a novel inhibitor of human neutrophil elastase. Biochem Biophys Res Commun 177:814–820
246. Inoue Y, Omodani T, Shiratake R et al (2009) Development of a highly water-soluble peptide-based human neutrophil elastase inhibitor; AE-3763 for treatment of acute organ injury. Bioorg Med Chem 17:7477–7486
247. Chowdhury D, Lieberman J (2008) Death by a thousand cuts: granzyme pathways ofprogrammed cell death. Annu Rev Immunol 26:389–420
248. Boivin WA, Cooper DM, Hiebert PR et al (2009) Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. Lab Invest 89:1195–1220
249. Metkar SS, Menaa C, Pardo J et al (2008) Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity 29:720–733

250. Hendel A, Hiebert PR, Boivin WA et al (2010) Granzymes in age-related cardiovascular and pulmonary diseases. Cell Death Differ 17:596–606
251. Pardo J, Aguilo JI, Anel A et al (2009) The biology of cytotoxic cell granule exocytosis pathway: granzymes have evolved to induce cell death and inflammation. Microbes Infect 11:452–459
252. García-Laorden MI, Stroo I, Blok DC et al (2016) Granzymes A and B regulate the local inflammatory response during *Klebsiella pneumoniae* pneumonia. J Innate Immun 8:258–268
253. Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310–350
254. Segal BJ (2009) Aspergillosis. N Engl J Med 360:1870–1884
255. Woodcock AA, Steel N, Moore CB et al (2006) Fungal contamination of bedding. Allergy 61:140–142
256. Moutaouakil M, Monod M, Prevost MC et al (1993) Identification of the 33-kDa alkaline protease of *Aspergillus fumigates* in vitro and in vivo. J Med Microbiol 39:393–399
257. Kunert J, Kopecek P et al (2000) Multiple forms of the serine proteinase ALP of *Aspergillus fumigatus*. Mycoses 43:339–347
258. Monod M, Paris S, Sanglard D et al (1993) Isolation and characterization of a secreted metalloprotease of *Aspergillus fumigatus*. Infect Immun 61:4099–5104
259. Reichard U, Eiffert H, Ruchel R (1994) Purification and characterization of an extracellular aspartic proteinase of *Aspergillus fumigatus*. J Med Vet Mycol 32:427–436
260. Kolattukudy PE, Lee JD, Rogers LM et al (1993) Evidence for possible involvement of an elastolytic serine protease in aspergillosis. Infect Immun 61:2357–2368
261. Markaryan A, Morozova I, Yu H et al (1994) Purification and characterization of an elastinolytic metalloprotease from *Aspergillus fumigatus* and immunoelectron microscopic evidence of secretion of this enzyme by the fungus invading the murine lung. Infect Immun 62:2149–2157
262. McDonagh A, Fedorova ND, Crabtree J et al (2008) Sub-telomere directed gene expression during initiation of invasive aspergillosis. PLoS Pathog 4:e1000154
263. Bouchara JP, Sanchez M, Esnault K et al (1999) Interactions between *Aspergillus fumigatus* and host matrix protein. Contrib Microbiol 2:167–181
264. Asano Y (2010) Future treatments in systemic sclerosis. J Dermatol 37:54–70
265. Roy R, Wewer UM, Zurakowski D et al (2004) ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem 279:51323–51330
266. Shi Z, Xu W, Loechel F et al (2000) ADAM 12, a disintegrin metalloprotease, interacts with insulin-like growth factor-binding protein-3. J Biol Chem 275:18574–18580
267. Loechel F, Fox JW, Murphy G et al (2000) ADAM 12-S cleaves IGFBP-3 and IGFBP-5 and is inhibited by TIMP-3. Biochem Biophys Res Commun 278:511–515
268. Jones JI, Clemmons DR et al (1995) Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3–34
269. Harsha A, Stojadinovic O, Brem H et al (2008) ADAM12: a potential target for the treatment of chronic wounds. J Mol Med (Berl) 86:961–969
270. Coalson JJ (2003) Pathology of new bronchopulmonary dysplasia. Semin Neonatol 8:73–81
271. Madurga A, Mizíková I, Ruiz-Camp J et al (2013) Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 305:893–905
272. Bruce MC, Schuyler M, Martin RJ et al (1992) Risk factors for the degradation of lung elastic fibers in the ventilated neonate. Implications for impaired lung development in bronchopulmonary dysplasia. Am Rev Respir Dis 146:204–212
273. Altiok O, Yasumatsu R, Bingol-Karakoc G et al (2006) Imbalance between cysteine proteases and inhibitors in a baboon model of bronchopulmonary dysplasia. Am J Respir Crit Care Med 173:318–326

274. Davies PL, Spiller OB, Beeton ML et al (2010) Relationship of proteinases and proteinase inhibitors with microbial presence in chronic lung disease of prematurity. Thorax 65:246–251
275. Bry K, Hogmalm A, Backstrom E (2010) Mechanisms of inflammatory lung injury in the neonate: lessons from a transgenicmouse model of bronchopulmonary dysplasia. Semin Perinatol 34:211–221
276. Chilosi M, Pea M, Martignoni G et al (2009) Cathepsin-k expression in pulmonary lymphangioleiomyomatosis. Mod Pathol 22:161–166
277. Smith GN Jr, Mickler EA, Payne KK et al (2007) Lung transplant metalloproteinase levels are elevated prior to bronchiolitis obliterans syndrome. Am J Transplant 7:1856–1861
278. Riise GC, Ericson P, Bozinovski S et al (2010) Increased net gelatinase but not serine protease activity in bronchiolitis obliterans syndrome. J Heart Lung Transplant 29:800–807
279. Iwata T, Chiyo M, Yoshida S et al (2008) Lung transplant ischemia reperfusion injury: metalloprotease inhibition down-regulates exposure of type V collagen, growth-related oncogene-induced neutrophil chemotaxis, and tumour necrosis factor-a expression. Transplantation 85:417–426
280. Khatwa UA, Kleibrink BE, Shapiro SD et al (2010) MMP-8 promotes polymorphonuclear cell migration through collagen barriers in obliterative bronchiolitis. J Leukocyte Biol 87:69–77
281. Fernandez FG, Campbell LG, Liu W et al (2005) Inhibition of obliterative airway disease development in murine tracheal allografts by matrix metalloproteinase-9 deficiency. Am J Transplant 5:671–683
282. Yoshida S, Iwata T, Chiyo M et al (2007) Metalloproteinase inhibition has differential effects on alloimmunity, autoimmunity, and histopathology in the transplanted lung. Transplantation 83:799–808
283. King RJ, Clements JA (1972) Surface active materials from dog lung. I. Method of isolation. Am J Physiol 223:707–714
284. LeVine AM, Kurak KE, Wright JR et al (1999) Surfactant protein-a binds group b streptococcus enhancing phagocytosis and clearance from lungs of surfactant protein-a-deficient mice. Am J Respir Cell Mol Biol 20:279–286
285. Crouch E, Hartshorn K, Ofek I (2000) Collectins and pulmonary innate immunity. Immunol Rev 173:52–65
286. Hartshorn KL, Crouch E, White MR et al (1998) Pulmonary surfactant proteins a and d enhance neutrophil uptake of bacteria. Am J Physiol 274:L958–L969
287. Sano H, Sohma H, Muta T et al (1999) Pulmonary surfactant protein a modulates the cellular response to smooth and rough lipopolysaccharides by interaction with cd14. J Immunol 163:387–395
288. Garcia-Verdugo I, Descamps D, Chiganard M et al (2010) Protease/anti-protease net work and modulation of mucus production and surfactant activity. Biochimie 92:1608–1617
289. Murakami S, Iwaki D, Mitsuzawa H et al (2002) Surfactant protein A inhibits peptidoglycan-induced tumor necrosis factor-alpha secretion in u937 cells and alveolar macrophages by direct interaction with toll-like receptor 2. J Biol Chem 277:6830–6837
290. Kantyka T, Pyrc K, Gruca M et al (2013) *Staphylococcus aureus* proteases degrade lung surfactant protein A potentially impairing innate immunity of the lung. J Innate Immun 5:251–260
291. Reid KBM (1998) Interactions of surfactant protein D with pathogens, allergens and phagocytes. Biochem Biophys Acta 1408:290–295
292. Restrepo CI, Dong Q, Savov J et al (1999) SP-D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. Am J Respir Cell Mol Biol 21:576–585
293. Crouch EC (1998) Structure, biological properties, and expression of surfactant protein D (SP-D). Biochem Biophys Acta 1408:278–289

294. Kuan SF, Rust K, Crouch EC (1992) Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an *Escherichia coli*-binding protein in bronchoalveolar lavage. J Clin Invest 90:97–106
295. Madan T, Eggleton P, Kishore U et al (1997) Binding of pulmonary surfactant protein A and D to *Aspergillus fumigatus* conidia enhance phagocytosis and killing by human neutrophils and alveolar macrophages. Infect Immun 65:3171–3179
296. Persson A, Chang D, Crouch E (1990) Surfactant protein D is a divalent cation-dependent carbohydrate-binding protein. J Biol Chem 265:5755–5760
297. Pikaar JC, Voorhut WF, van Golde LM et al (1995) Opsonic activities of surfactant protein A and D in phagocytosis of gram-negative bacterias by alveolar macrophages. J Infect Dis 172:481–489
298. Birrer P, McElvaney G, Rüdelberg A et al (1994) Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. Am J Respir Crit Care Med 151:207–213
299. von Bredow C, Birrer P, Griese M (2001) Degradation of surfactant protein A and other bronchoalveolar lavage fluid proteins in patients with cystic fibrosis. Eur Respir J 17:716–722
300. Beatty AL, Malloy JL, Wright JR (2005) *Pseudomonas aeruginosa* degrades pulmonary surfactant and increases conversion in vitro. Am J Respir Cell Mol Biol 32:128–134
301. Malloy JL, Veldhuizen RA, Thibodeaux BA et al (2005) *Pseudomonas aeruginosa* protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions. Am J Physiol Lung Cell Mol Physiol 288:409–418
302. Alcorn JF, Wright JR (2004) Degradation of pulmonary surfactant protein D by *Pseudomonas aeruginosa* elastase abrogates innate immune function. J Biol Chem 279:30871–30879
303. Mariencheck WI, Alcorn JF, Palmer SM et al (2003) *Pseudomonas aeruginosa* elastase degrades surfactant proteins A and D. Am J Respir Cell Mol Biol 28:528–537
304. Hirche TO, Crouch EC, Espinola M et al (2004) Neutrophil serine proteinases inactivate surfactant protein D by cleaving within a conserved subregion of the carbohydrate recognition domain. J Biol Chem 279:27688–27698
305. Liau DF, Yin NX, Huang J et al (1996) Effects of human polymorphonuclear leukocyte elastase upon surfactant proteins in vitro. Biochim Biophys Acta 1302:117–128
306. Griese M, Wiesener A, Lottspeich F et al (2003) Limited proteolysis of surfactant protein D causes a loss of its calcium-dependent lectin functions. Biochim Biophys Acta 1638:157–163
307. Burgel PR, Nadel JA et al (2004) Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. Thorax 59:992–996
308. Burgel PR, Nadel JA et al (2008) Epidermal growth factor receptor-mediated innate immune responses and their roles in airway diseases. Eur Respir J32:1068–1081
309. Hewson CA, Edbrooke MR, Johnston SL et al (2004) PMA induces the MUC5AC respiratory mucin in human bronchial epithelial cells, via PKC, EGF/TGF-alpha, Ras/Raf, MEK, ERK and Sp1-dependent mechanisms. J Mol Biol 344:683–695
310. Wu DY, Wu R, Chen Y et al (2007) PMA stimulates MUC5B gene expression through an Sp1-based mechanism in airway epithelial cells. Am J Respir Cell Mol Biol 37:589–597
311. Lundgren JD, Rieves RD, Mullol J et al (1994) The effect of neutrophil proteinase enzymes on the release of mucus from feline and human airway cultures. Respir Med 88:511–518
312. Kohri K, Ueki IF, Nadel JA et al (2002) Neutrophil elastase induces mucin production by ligand-dependent epidermal growth factor receptor activation. Am J Physiol Lung Cell Mol Physiol 283:531–540
313. Chokki M, Yamamura S, Eguchi H et al (2004) Human airway trypsin-like protease increases mucin gene expression in airway epithelial cells. Am J Respir Cell Mol Biol 30:470–478
314. Miki M, Nakamura Y, Takahashi A et al (2003) Effect of human airway trypsin-like protease on intracellular free Ca^{2+} concentration in human bronchial epithelial cells. J Med Invest 50:95–107
315. Englert N (2004) Fine particles and human health—a review of epidemiological studies. Toxicol Lett 149:235–242

316. Mukae H, Hogg JC, English D et al (2000) Phagocytosis of particulate air pollutants by human alveolar macrophages stimulates the bone marrow. Am J Physiol Lung Cell Mol Physiol 279:L924–L931
317. van Eeden SF, Tan WC, Suwa T et al (2001) Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM(10)). Am J Respir Crit Care Med 164:826–830
318. Sun J (2010) Matrix metalloproteinases and tissue inhibitor of metalloproteinases are essential for the inflammatory response in cancer cells. J Signal Transduct 2010:985132
319. Wang J, Zhang H, Su C et al (2014) Dexamethasone ameliorates H_2S-induced acute lung injury by alleviating matrix metalloproteinase-2 and -9 expression. PLoS ONE 9:e94701
320. Ong CW, Elkington PT, Friedland JS (2014) Tuberculosis, pulmonary cavitation and matrix metalloproteinases. Am J Respir Crit Care Med 190:9–18
321. Ishii T, Abboud RT, Wallace AM et al (2014) Alveolar macrophage proteinase/antiproteinase expression in lung function and emphysema. Eur Respir J 43:82–91
322. Kumar M, Phougat N, Ruhil S et al (2013) Genomics of chronic obstructive pulmonary disease (COPD); exploring the SNPs of protease–antiprotease pathway. Curr Genomics 14:204–213
323. Morales-Bárcenas R, Chirino YI, Sánchez-Pérez Y et al (2015) Particulate matter (PM_{10}) induces metalloprotease activity and invasion in airway epithelial cells. Toxicol Lett 237:167–173
324. Taggart CC, Lowe GJ, Greene CM et al (2001) Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. J Biol Chem 276:33345–33352
325. Quinn DJ, Weldon S, Taggart CC (2010) Antiproteases as therapeutics to target inflammation in cystic fibrosis. Open Respir Med J 4:20–31
326. Gibbons A, McElvaney NG, Cryan SA (2010) A dry powder formulation of liposome-encapsulated recombinant secretory leukocyte protease inhibitor (rSLPI) for inhalation: preparation and characterisation. AAPS Pharm Sci Tech 11:1411–1421
327. Delacourt C, erigault SH, Delclaux C et al (2002) Protection against acute lung injury by intravenous or intratracheal pretreatment with EPI-HNE-4, a new potent neutrophil elastase inhibitor. Am J Respir Cell Mol Biol 26:290–297
328. Dunlevy FK, Martin SL, de Courcey F et al (2012) Anti-inflammatory effects of DX-890, a human neutrophil elastase inhibitor. J Cyst Fibros 11:300–304
329. Churg A, Wang R, Wang X et al (2007) Effect of an MMP-9/MMP-12 inhibitor on smoke-induced emphysema and airway remodelling in guinea pigs. Thorax 62:706–713
330. le Quémen C, Lagente V, Guénon I, Muzio V, Gillon J-Y, Boichot E (2008) Anti-inflammatory properties of MMP inhibitors in experimental models of chronic obstructive pulmonary disease and lung inflammation. In: Lagente V, Boichot E (eds) Matrix metalloproteinases in tissue remodelling and inflammation. Springer, New York, pp 57–69
331. Le Quément C, Guénon I, Gillon JY et al (2008) The selective MMP-12 inhibitor, AS111793 reduces airway inflammation in mice exposed to cigarette smoke. Br J Pharmacol 154:1206–1215
332. Jawad MU, Garamszegi N, Garamszegi SP et al (2010) Matrix metalloproteinase 1: role in sarcoma biology. PLoS ONE 5:e14250
333. Fanjul-Fernández M, Folgueras AR, Fueyo A et al (2013) Matrix metalloproteinase MMP-1a is dispensable for normal growth and fertility in mice and promotes lung cancer progression by modulating inflammatory responses. J Biol Chem 288:14647–14656

Caspases: Moonlighting Proteins with Theranostic Potential

17

Siddavaram Nagini and Satwinderjeet Kaur

Abstract

Apoptosis evasion, a major hallmark of cancer, promotes tumour development and progression as well as chemoresistance and angiogenesis. Cancer cells resist apoptosis through a variety of strategies including downregulation of death receptors, overexpression of anti-apoptotic proteins, reduced expression of pro-apoptotic proteins, and dysregulation of caspases. Caspases are highly versatile, multifunctional proteolytic enzymes that are involved in both initiation and execution phases of apoptotic cell death. These enzymes are regulated by members of the inhibitor of apoptosis proteins (IAP) family and cellular FLICE inhibitory protein. Although caspases function as tumour suppressors in general, mutations, polymorphisms and their non-apoptotic roles render the involvement of caspases in tumorigenesis more complex and context-specific. Caspases have been extensively exploited as a therapeutic platform to reinstate apoptosis signalling as well as in chemo- and radio-sensitization of malignant tumours. A number of approaches and molecules have been developed to activate caspases for cancer therapeutics. These include SMAC mimetics, caspase activators, proteasomal and histone deacetylase inhibitors among several others. Although targeting caspases has shown promise, it is not clear whether they can be used in diverse malignancies given the pro-survival functions of these enzymes. Deeper insights into the varied functions of caspases will enable development of more effective and safer treatment options for diverse malignancies.

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1 Introduction

Apoptosis, a distinct, genetically programmed form of cell death is a fundamental process vital for normal embryonic development, maintenance of adult tissue homeostasis, execution of immune effector functions, and elimination of unwanted and damaged cells from an organism. The process involves characteristic morphological and biochemical changes that include cell shrinkage, membrane blebbing, chromatin compaction, cytoplasmic condensation, formation of apoptotic bodies, DNA fragmentation, phosphatidylserine externalization and cleavage of cellular proteins such as poly(ADP-ribose) polymerase (PARP). Accumulating evidence indicates apoptosis avoidance during tumour development and progression as well as in blunting therapeutic responses [1]. Of late, apoptosis is increasingly recognized to cause oncogenic effects [2]. It is therefore imperative to maximize tumour cell kill while minimizing adverse pro-tumorigenic effects in order to fully exploit the therapeutic benefits of apoptosis. In this context, regulation of caspases, which play a critical role in apoptotic signalling pathways has evolved as a promising strategy to reinstate apoptosis in tumour cells.

This chapter summarizes the major pathways of apoptosis, the mechanisms of apoptosis evasion in cancer as well as the pro-oncogenic effects of apoptosis. The major focus is on the emerging functions of caspases, their role in tumour suppression and caspase-based strategies to selectively reinstate apoptosis in tumour cells.

2 Pathways of Apoptosis

Classically, apoptosis is transduced via two major pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway [3]. Both these pathways activate proteases, termed caspases, that degrade a variety of cellular components resulting in apoptotic cell death. Figure 1 gives a schematic representation of the two major pathways of apoptosis.

The extrinsic pathway is initiated by extracellular ligands of the tumour-necrosis factor (TNF) cytokine family, Fas ligand (FasL), TRAIL (TNF-related apoptosis-inducing ligand) that bind to their cognate receptors on the cell surface. FasL and TRAIL induce apoptosis via formation of the death-inducing signalling complex (DISC) that activates the initiator caspases-8 and -10, which in turn

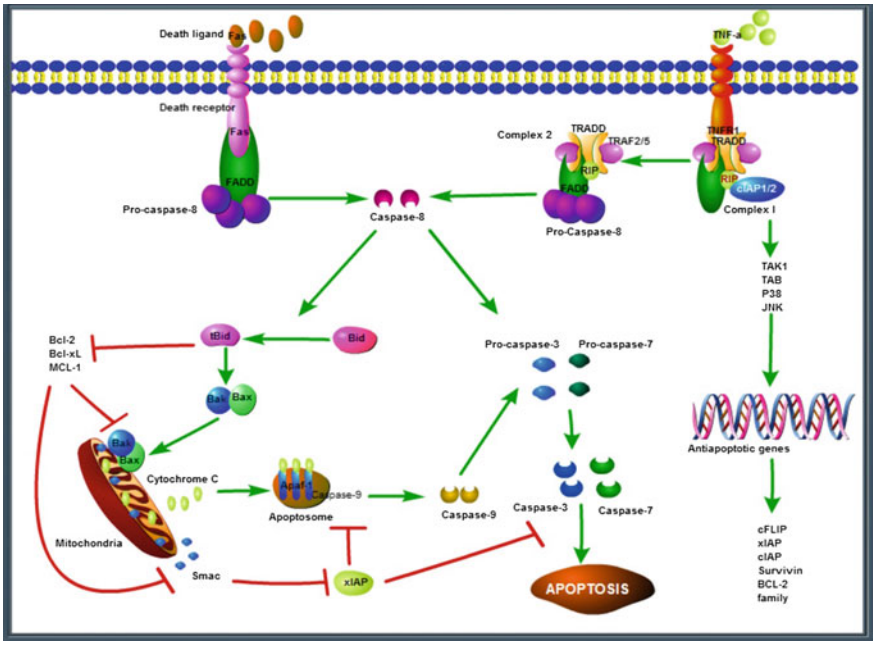

Fig. 1 Apoptosis signalling pathways. Apoptosis can be transduced either via the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway. Stimulation of the extrinsic pathway by FasL leads to the formation of DISC with activation of the caspase cascade. TNF-α on the other hand can trigger the prosurvival pathway through complex I formation. Dissociation of RIP1 and TRADD from the complex I is followed by formation of complex II that promotes apoptosis. The intrinsic pathway is regulated by the interactions between members of the BCL-2 family proteins. Oligomerization of BAX and/or BAK leads to MOMP, release of cytochrome C into the cytosol, formation of the apoptosome, and caspase activation resulting in cell death. Crosstalk between the two pathways occurs via BID. The anti-apoptotic proteins BCL-2 and BCL-X_L inhibit the release of apoptogenic molecules from the mitochondria. IAPs prevent apoptosis by binding to caspases and antagonizing SMAC

activate the effector caspases-3 and -7 that eventually execute the death programme [4]. TNF-α, on the other hand is capable of triggering either the prosurvival pathway or the apoptotic pathway depending on the type of complex formed. Binding of TNF-α to its cognate receptor TNFR1 recruits TNFR1-associated death domain (TRADD), TNF receptor-associated factor-2 (TRAF-2), TRAF5, baculoviral IAP repeat containing 2 (cIAP1/2) and ribosome-inactivating protein (RIP) to form complex 1, which stimulates the prosurvival nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK) and p38 signalling pathways. Deubiquitination of RIP leads to dissociation of RIP1 and TRADD from the complex, which then bind to Fas-associated death domain (FADD) and caspase-8/-10 to form complex 2 that promotes apoptosis.

The intrinsic pathway triggered by a variety of stimuli including DNA damage, radiation, cytokine deprivation, endoplasmic reticulum (ER) stress and cytotoxic agents is regulated by the interactions between members of the BCL-2 family proteins. The BCL-2 family comprises three major subfamilies: the anti-apoptotic proteins such as BCL-2 and BCL-X_L, pro-apoptotic BAX and BAK, and the BH-3 only proteins such as BID and BIM. Oligomerization of BAX and/or BAK causes mitochondrial outer membrane permeabilization (MOMP) with efflux of several inter-membrane space proteins such as cytochrome C and second mitochondria-derived activator of caspases (SMAC) into the cytosol [5, 6]. Cytochrome C released into the cytosol forms an apoptosome complex with apoptotic protease activating factor 1 (APAF1) that activates caspase-9. Active caspase-9 in turn activates the effector caspases-3 and -7 that cleave a number of substrates resulting in cell death. Crosstalk between the two pathways occurs via caspase-8 catalysed cleavage of the pro-apoptotic BCL-2 family member, BID to form a 15 kDa protein termed truncated BID (tBID) that induces MOMP and intrinsic apoptosis [7, 8].

The anti-apoptotic proteins BCL-2 and BCL-X_L inhibit the release of apoptogenic molecules from the mitochondria. They function by binding directly to BH3-only proteins or to BAX–BAK heterodimer. The inhibitors of apoptosis (IAP) family of proteins prevent apoptosis by binding to caspases and antagonizing SMAC [9, 10].

3 Evasion of Apoptosis

Apoptosis evasion, a major hallmark of cancer, promotes tumour development and progression as well as chemoresistance. Apoptosis avoidance enables cancer cells to survive, acquire mutations and facilitate neovascularization. Cancer cells resist apoptosis through a variety of strategies including downregulation of death receptors, overexpression of anti-apoptotic proteins, reduced expression of pro-apoptotic proteins, inactivating mutations of caspases and p53 as well as epigenetic modifications [11–14].

Signalling via the death receptor pathway is impaired in several human cancers through downregulation of death receptors believed to be mediated by promoter hypermethylation or through the expression of decoy receptors that competitively bind to CD95 and TRAIL ligands thereby blocking transduction of apoptotic signals. Aberrant expression of proteins containing death effector domains such as cellular FLICE Inhibitory Protein (c-FLIP) are recruited into the DISC complex in place of caspase-8 due to sequence homology thereby blocking activation of caspase-8 [4].

Evasion of intrinsic apoptosis occurs as a result of a shift in the balance of the anti- and pro-apoptotic members of the BCL-2 family proteins. These include overexpression of BCL-2 and BCL-X_L, mutational inactivation of BAX and BAK, and phosphorylation of BAD that renders it anti-apoptotic. Overexpression of IAPs,

in particular survivin, and mutational inactivation of p53 endorse apoptosis evasion in a wide range of malignancies. Post-mitochondrial changes that affect mitochondrial apoptosis include promoter hypermethylation or loss of heterozygosity at chromosome 12q22-23 that impairs apoptosome formation. In addition, dysregulated expression of caspases due to mutations, promoter hypermethylation and phosphorylation also confer resistance to apoptosis [5, 7, 13].

There is overwhelming evidence to indicate that aberrant caspase activation as well as mutations and polymorphisms are central for apoptosis evasion in cancer [15, 16].

4 Caspases

Caspases are proteolytic enzymes that belong to the clan CD, family C14. These enzymes cleave their substrates after specific aspartate residues in the tetrapeptide motifs (P4–P3–P2–P1) where P1 is an aspartate residue. The term caspase is a contraction of cysteine-dependent aspartate-specific protease. Till date, 15 caspase family members that recognize 400 different substrates have been identified in mammals. These enzymes process hundreds of protein substrates leading to loss or gain of function and change in subcellular localisation [17].

Although all caspases cleave after aspartate residues, preferential recognition sequences have been identified for some caspases. While the consensus recognition motif for caspase-8 is isoleucine-glutamic acid-threonine-aspartic acid, it can also cleave other tetrapeptide sequences, including aspartic acid-glutamic acid-valine-aspartic acid (DEVD), the target sequence for effector caspases.

4.1 Caspases: Structure and Classification

Caspases are synthesized as inactive zymogens consisting of four domains: an N-terminal prodomain, a large subunit, a C-terminal small subunit and a short linker region that separates the large and small subunits. Activation of caspases separates the large and small subunits generating a hetero-tetrameric complex comprising two large (p20) and two small (p10) subunits [8].

Based on their functions, caspases are classified into two broad groups: those involved in inflammation (caspase-1, -4, -5, -11, -12), and those involved in apoptosis (caspase-2, -3, -8, -9, -10). The inflammatory caspases play a role in cytokine maturation. The apoptotic caspases are further subgrouped into initiator (caspase-2, -8, -9, and -10), and effector (caspase-3, -7 and -6) caspases. Initiator caspases synthesized as monomers undergo dimerization, followed by association with activation complexes, leading to conformational change and cleavage. On the other hand, effector caspases are synthesized as dimers that are proteolytically activated by initiator caspases [8, 15].

The initiator caspases that are first activated in response to apoptotic signals contain N-terminal modular regions known as the death effector domain (DED; caspase-8 and -10) or caspase recruitment domain (CARD; caspase-2, -9, and -11), which mediate their dimerization and interaction with adaptor proteins. The initiator caspases subsequently activate the downstream effector caspases that directly mediate the cascade of events leading to apoptotic cell death. Activation of effector caspases is also mediated by non-caspase proteases such as cathepsins, calpains, and granzymes [8, 15]. Figure 2 illustrates the functional classification and domain structure of mammalian caspases.

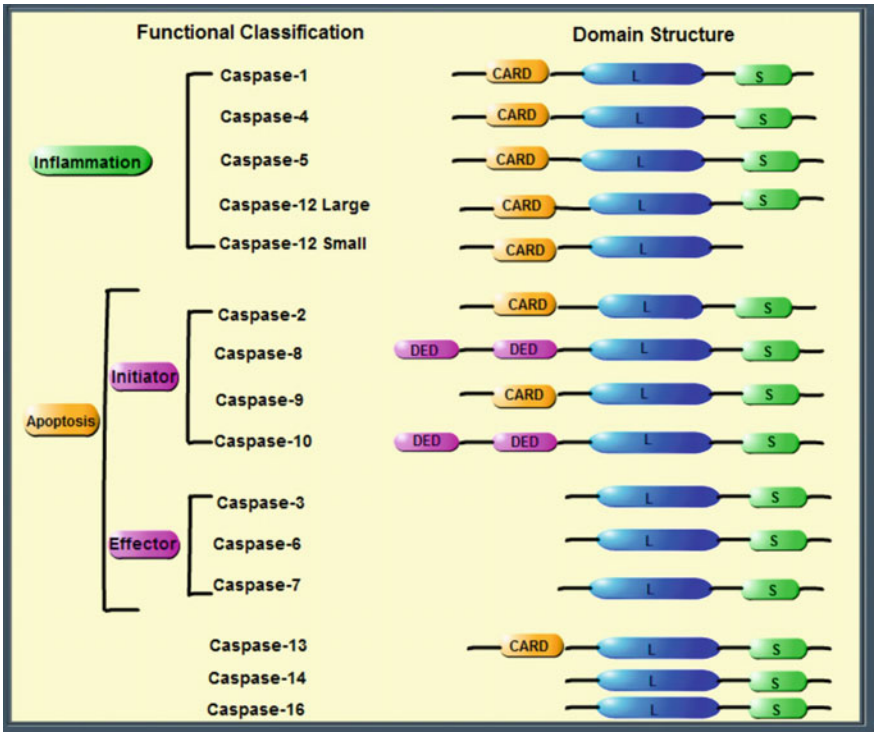

Fig. 2 Functional classification and domain structure of mammalian caspases. Based on their function, caspases are classified into inflammatory caspases (Caspase-1, -4, -5, -11 and -12) and apoptotic caspases. The apoptotic caspases are subgrouped into initiator (Caspase-2, -8, -9 and -10) and effector (Caspase-3, -6 and -7) caspases. Caspase-13 is of bovine origin. Caspase-14 is involved in cornification of the skin. Caspase-16 is believed to be a pseudogene. *CARD* Caspase recruitment domain; *DED* death effector domain; *L* large subunit; *S* small subunit

4.2 Caspases: Archetypal Moonlighting Proteins

Caspases are increasingly recognized as highly versatile, multifunctional proteins that perform opposing, autonomous cellular functions and constitute striking examples of *moonlighting proteins*. The role of caspases in apoptosis has been extensively documented. The initiator caspases such as caspase-8 and -9 are involved in transducing the apoptotic signal in the intrinsic and extrinsic pathways, respectively, during which process the effector caspases are activated. Following activation, the effector caspase-3 and -7 cleave caspase-activated DNase (CAD) which translocates to the nucleus and cleaves DNA. The effector caspases also cleave several vital structural and regulatory proteins including ICAD 1 (inhibitor of CAD), Rho kinase 1 (ROCK 1), PARP, actin, fodrin, lamin, gelsolin, oncoproteins such as mdm2 and tumour suppressor gene products such as retinoblastoma proteins eventually leading to cell disassembly and cell death. Substrates of caspase-3 also include cyclin-dependent kinase (CDK) inhibitors such as p27 and p21 [18].

Beyond their well-defined role in apoptosis, caspases are also involved in other forms of cell death including pyroptosis, necroptosis and autophagy. In particular, interplay between these modes of cell death has been observed in cancer. Caspases-3, -7 and -8 are involved in autophagy, a lysosomal mediated process that degrades long-lived proteins and damaged organelles. Cleavage of Beclin-1 and Atg5 that play a crucial role in autophagy generates truncated proteins that are capable of activating caspase-mediated apoptosis [19].

In addition to cell death, caspases are known to play a role in proliferation, migration, tumour suppression, differentiation and genomic stability. Caspases also play a key role in normal tissue homeostasis, tissue specialization, cytokine maturation during inflammation, metabolism, neural development and aging. Caspases have been documented to modulate intracellular signalling by regulating kinases, phosphatases and other signalling molecules [15, 20].

The executioner caspase-3 has been increasingly reported to be pro-tumorigenic, based on its crucial role in DNA damage-induced genomic instability and in the generation of the prosurvival factor prostaglandin E2 (PGE2) that promotes tumour cell repopulation. During apoptosis, caspases cleave and activate calcium-independent phospholipase A2 (iPLA2) generating PGE2, a key mediator of apoptosis-induced proliferation [21]. The secreted PGE2 was shown to stimulate tumour cell proliferation and repopulation by activating Wnt/β-catenin signalling [20].

Caspase-8 functions in several cellular processes independent of its role in extrinsic apoptosis. Phosphorylation of caspase-8 by SRC kinase on tyrosine 380 was shown to inhibit its proteolytic activation uncoupling it from apoptosis and channelising it to cell migration and adhesion. In addition, caspase-8 promotes cell survival via NF-κB activation, regulates autophagy and alters endosomal trafficking. Caspase-8 has also been reported to be involved in epidermal growth factor (EGF) signalling and extracellular-regulated kinase (ERK) activation. Thus, depending on the cellular context, caspase-8 functions as a tumour suppressor or promoter [22].

The levels of intracellular reactive oxygen species (ROS) may act as a switch between the apoptotic and non-apoptotic functions of caspase-8. It has been suggested that ROS mobilize sequestered caspase-8 from the mitochondria followed by binding to the mitochondrial surface via Bifunctional Apoptosis Regulator (BAR) [23]. ROS also inhibit transcription of NF-κB target genes, including mitochondrial superoxide dismutase [24], which limits ROS accumulation in the mitochondria.

4.3 Regulation of Caspases

The IAP family consists of eight members of which X-linked IAP (XIAP) and survivin function as direct caspase inhibitors [10, 25]. These proteins contain E3 ligases that can block apoptosome formation through binding directly to APAF-1 or caspase-9, thus inhibiting caspase-9 activation [26]. The mechanism involves monoubiquitination of the proenzyme, and ubiquitination and proteasomal degradation of the active enzyme. XIAP, the most potent of the IAPs, also binds directly to caspase-3 preventing its activation, and in addition, facilitates the transfer of ubiquitin, thereby tagging the caspases for degradation by the 26S proteasome [27]. Survivin, a prototype IAP, functions by inhibiting caspases and sequestering SMAC [28]. The IAPs in turn are counteracted by mitochondria-derived SMAC, Omi/HtrA2, and XIAP-associated factor-1 (XAF1) enabling caspase-mediated cell death. Thus cell survival or cell death is determined by a fine and intricate balance of a plethora of proteins.

The cellular FLICE inhibitory protein (c-FLIP), a catalytically inactive homologue of caspase-8 and -10, prevents their activation by blocking binding sites on the DISC. Expression of c-FLIPL, one of the isoforms of c-FLIP was found to be enhanced in several malignant tumours. Following recruitment to the DISC, caspase-8 undergoes successive cleavage, first at aspartate 384 in the p10 subunit that generates a p43/41 intermediate, and a second cleavage at aspartate residues 210, 216 with subsequent release of caspase-8 from the DISC into the cytosol. c-FLIP inhibits caspase-8 recruitment to the DISC as well as its activation [29]. In addition, XIAP and cIAP also block caspase-8 activation [30]. Caspase-8 polyubiquitination mediated by E3 ligases incorporates caspase-8 into an aggresome [31].

4.4 Role of Individual Caspases in Cancer

Since caspases are involved in cell death, it follows that loss of caspases would lead to malignant transformation. However, since caspases also have several non-apoptotic functions, they have been implicated in proliferation, migration and invasion. While caspases function as tumour suppressors in general, mutations, polymorphisms and their non-apoptotic roles render the involvement of caspases in tumorigenesis more complex and context-specific [32]. Sequence analysis across a

wide spectrum of tumours revealed high frequency of mutations in caspase-8, followed by caspase-3 and -7 [16, 33].

4.4.1 Caspase-1

The tumour suppressor function of caspase-1 has been extensively documented. It is frequently downregulated in cancer [20, 34].

4.4.2 Caspase-2

Caspase 2 has a tumour suppressor function and loss of even one allele leads to increased cell proliferation and loss of G2/M checkpoint control. Reduced expression of caspase-2 was found to correlate with poor prognosis in several malignancies. High caspase-2 expression was associated with better survival in leukaemias, whereas in neuroblastomas, it correlated with poor survival indicating that the tumour suppressor function of caspase-2 is tissue- and context-specific [35].

4.4.3 Caspase-3

Although caspase-3 expression was found to be reduced in malignant tumours, high CASP3 levels coincident with increased apoptosis were also observed in certain tumours. In patients with breast cancer, caspase-3 levels correlated with worse treatment outcome [36]. Caspase 3s, a splice variant of the CASP-3 gene, has anti-apoptotic function. Both caspase-3 and its splice variant are coexpressed in tumour cell lines and their relative expression determines chemosensitivity [37]. Variant alleles of CASP3 are associated with increased risk of squamous cell carcinomas of the head and neck (SCCHN), endometrial cancer, non-Hodgkin lymphoma and multiple myeloma [38–40]. The CASP3 rs4647601:TT variant was associated with an increased risk of SSCHN compared with the GG genotype [38]. In a study involving 128 multiple myeloma cases, subjects with the CC genotype of CASP3 Ex8 had a fivefold lower risk compared with the TT genotype [41]. Single nucleotide polymorphisms (SNP) were reported in cancers of the lung and head and neck [20].

4.4.4 Caspase-6

Caspase-6 is an effector caspase that is activated downstream of caspase-3 and -7. It is more relevant in neurodegenerative disorders than in cancer [15].

4.4.5 Caspase-7

Caspase-7, an effector caspase, shares many similarities with caspase-3 in terms of substrate specificity and role in apoptosis and is therefore believed to be functionally redundant. A study involving 720 lung cancer patients, polymorphisms in CASP7 were associated with increased susceptibility to lung cancer [42]. SNPs in CASP7 were associated with altered risk of endometrial cancer. Although mutations in CASP6 and CASP7 are rare, they have been detected in some tumours.

4.4.6 Caspase-8

According to the Human Protein Atlas, loss of caspase-8 is rare in epithelial cancers. However, loss of caspase-8 expression is frequently seen in malignant neuroendocrine tumours. A number of malignant tumours express high levels of caspase-8 [43].

Somatic CASP8 mutations were detected in 5% of invasive colorectal carcinomas and advanced gastric cancers associated with reduced apoptosis [44]. In hepatocellular carcinomas (HCCs), a frameshift mutation in CASP8 with loss of function was detected that was caused by a two base-pair deletion (1225_1226delTG) [45, 46]. Hepatocyte-specific deletion of caspase-8 ($Casp8^{\Delta hepa}$) was demonstrated to protect against hepatocarcinogenesis in $NEMO^{\Delta hepa}$ mice [47]. Frameshift and missense mutations in caspase-8 that induce changes in the amino acid sequence of the DED domain critical for its recruitment to the DISC diminished apoptosis in various tumours [15, 20].

A six-nucleotide deletion (-652 6N del) in the CASP8 promoter has been linked to the decreased susceptibility to multiple cancers [48]. Likewise, CASP8 D302H polymorphism is associated with a reduced risk of breast cancer [49]. Silencing of CASP8 by gene deletion or promoter methylation was associated with amplification of the MYCN oncogene. In neuroblastomas, caspase-8 is silenced by CpG hypermethylation in the 5′ promoter region, as well as loss of heterozygosity allelic deletion [50]. The caspase-8 promoter is upregulated by interferon-γ (IFN-γ) signalling owing to the presence of IFN-γ responsive elements within its promoter [51].

4.4.7 Caspase-9

Caspase-9β a caspase-9 mutant that lacks the large active subunit prevents caspase-3 activation [52]. Phosphorylation of caspase-9 at Thr 129 prevents caspase-9 recruitment to the apoptosome and its activation [53]. SNPs in caspase-3 have also been reported.

4.4.8 Caspase-10

Inactivating CASP10 mutations have been documented in diverse malignancies [54]. Myeloma cells were found to require caspase-10 for survival.

4.4.9 Caspase-14

Reduced expression of caspase-14 is associated with more advanced cancers [15, 20].

5 Targeting Apoptosis Pathways for Cancer Treatment

Strategies that induce tumour cell apoptosis are considered central in designing effective anticancer drugs. Several points in the extrinsic and intrinsic apoptotic signalling pathways are amenable for therapeutic intervention. Many tumours are highly sensitive to TRAIL-induced apoptosis due to high expression of TRAILRs. Recombinant human TRAIL (rhTRAIL) that binds to both TRAIL-R1 and

TRAIL-R2 was demonstrated to induce DISC formation and selective apoptosis of cancer cells. Agonistic TRAIL receptor (TRAIL-R1/R2) antibodies were however, more effective than rhTRAIL due to longer bioavailability and specific targeting of receptors. Addition of rhTRAIL or agonistic TRAILR1/R2 antibodies together with compounds that activate and stabilize caspase-8 was found to be more effective in triggering apoptosis [3, 13, 55, 56].

Approaches to induce the intrinsic pathway involve targeting the anti-apoptotic proteins of the BCL-2 family either by using specific inhibitors or BH3 mimetics. The BCL-2 inhibitor ABT-199 was successful as a single agent in chronic lymphocytic leukaemia (CLL). BH3-only protein mimetics containing the pro-apoptotic BH3 domain capable of binding to and neutralizing anti-apoptotic proteins, and agonistic mimetics that target the prosurvival BH4 domain have been developed. Paradoxically in some cancers such as CLL, increased expression of anti-apoptotic proteins correlated with better prognosis. Furthermore, these tumours were highly sensitive to anti-apoptotic therapies [57, 58].

6 Caspase-Based Therapeutics

Caspases have been extensively exploited as a therapeutic platform to reinstate apoptosis signalling as well as in chemo- and radio-sensitization of malignant tumours. A number of approaches and molecules have been developed to activate the initiator and effector caspases and inhibit the IAPs [15, 20].

Targeting caspase-8 is believed to improve therapeutic outcome in tumours manifesting gene dosage effects or hypermethylation of caspase-8 promoter. The DNA methyltransferase inhibitor 5′aza2′deoxycytidine (decitabine) was found to restore caspase 8 in neuroblastoma [59]. The expression of caspase-8 facilitates tumour cell apoptosis by chemotherapeutic agents. Caspase-8 mediated killing may be influenced by the status of cellular tubulin. While microtubule-stabilizing agents such as taxanes, promote caspase-8 mediated apoptosis, microtubule disrupting agents such as colchicine protect against FAS-mediated killing [22, 60].

Retinoic acid (RA) and its derivatives induce tumour cell apoptosis and chemosensitization by paracrine release of TRAIL associated with phospho-CREB mediated upregulation of caspase-8 [61, 62]. Proteasomal inhibitors such as bortezomib also increase caspase-8 expression. Clinically, the retinoic acid fenretinide and interferon-γ are promising because they increase caspase-8 expression and activate cell death. Derivatives from the traditional Chinese herb *Justicia procumbens* are documented to induce apoptosis via caspase-8 activation, upregulation of BID and subsequent release of cytochrome c [63]. 6-hydroxy justicidin A (JR6), a novel compound from *J. procumbens*, that shares a common molecular nucleus with the tubulin destabilizing anticancer drug podophyllotoxin induces apoptosis by ROS generation and caspase activation in human bladder cancer cells [64]. FKBP12, a caspase-9 fusion protein that induces caspase-9 dimerization was demonstrated to exert anti-angiogenic effects in mouse models [65].

Use of caspase activators may potentiate apoptosis of tumour cells that already have sublethal caspase activity MacCorkle et al. [66] have developed a 'death switch' by which caspases are selectively activated in cancer cells. There has been considerable interest in turning on caspases using selective small-molecule activators that could bypass upstream mutational events which prevent apoptosis. Several approaches have been used including protein fragment complementation assays and chemical-induced dimerization (CID). In particular, CID strategies enable testing different activation modes for apoptotic caspases, oligomerization and processing. Conditional control of caspase activation and use of small molecules to selectively kill cancer cells have been attempted. Procaspase-activating compound 1 (PAC-1) is a zinc chelator that activates procaspase-3 by relieving it from zinc inhibition. Compound-1541 that activates both caspase-3 and -7, self-assembles into nanofibrils that colocalize caspase-3 with procaspase-3 bringing about activation. Compound-42 activates procaspase-3 by releasing the inter-subunit linker from the dimer interface, allowing the enzyme to auto-process this linker in *trans* [17].

Induction of caspases by demethylating agents and transcriptional activators such as inhibitors of histone deacetylases (HDACs) has been attempted. Inhibitors of HDAC that increase the transcriptional availability of caspase-3 promoters reinstate apoptosis besides enhancing sensitization to TRAIL-, radiation- and chemotherapeutic-induced apoptosis of cancer cells [67, 68].

Apoptin encoded by the chicken anaemia virus (CAV) selectively induces apoptosis of tumour cells by activating caspase-3 and -7 and releasing cytochrome c. A triplet sequence of aspartic acid residues that maintains procaspase-3 in an inactive state in resting cells is regarded as a target for intervention. A series of molecules capable of activating caspase-3 such as α-(trichloromethyl)-4-pyridineethanol (PETCM), gambonic acid, and the gambonic acid derivative MX-2060 have been identified by high throughput screening (HTS) projects (reviewed in [69, 70]). Small peptides with the RGD motif (arginine-glycine-aspartate) stimulate apoptosis by inducing auto-processing of procaspase-3 [71]. *Immunocasp-3*, a fusion protein containing a single-chain anti-erbB2/HER2 antibody and active caspase-3, was demonstrated to cause tumour regression in mouse xenografts of HER2-positive tumour cells by autoactivation of caspase-3 [72]. Replacement of defective caspases in cancer cells with their normal counterparts by gene therapy have been attempted using Ad-G/iCasp3, a replication-incompetent adenoviral vector carrying the caspase-3 gene [73]. Agents that target both caspase-3 and -9 such as *Brucea javanica* oil, niflumic acid–ciglitazone and tetrandrine were found to induce apoptosis [74–76].

SMAC mimetics that enhance caspase activity by inhibiting IAPs and by inducing ubiquitin-mediated degradation of cIAPs have been designed. The SMAC mimetic BV6 was found to be effective in combination with glucocorticoids in childhood acute lymphocytic leukaemia (ALL) [77]. RMT5265.2HCL, a SMAC mimetic, not only functions as an IAP inhibitor but also stimulates release of cytochrome c from the mitochondria [78]. A novel SMAC mimetic, SM-1200 was demonstrated to inhibit growth of MDA-MB-231 breast cancer xenograft models

[79]. The antisense oligonucleotide LY2181308 reduces survivin mRNA and restores caspase-3 mediated apoptosis [80]. YM155 (sepantronium bromide), an imidazolium-based small-molecule inhibitor of survivin induced caspase-mediated apoptosis and sensitized tumour cells to radiation and platinum-based chemotherapeutics [81, 82].

Extensive investigations from this laboratory provide compelling evidence that phytochemicals from medicinal plants and dietary agents induce caspase-mediated apoptosis by modulating the expression of a multitude of molecules involved in the apoptotic signalling cascade [83–88].

7 Targeting Apoptosis for Cancer Therapy—The Flip Side

Although targeting proteins in the apoptosis signalling pathways has shown promise in preclinical trials, disruption of the non-apoptotic functions of these proteins leads to on-target adverse effects and/or off-target toxicity, limiting their translation to the clinical setting, especially in combinations. Significant hepatotoxicity due to TRAIL treatment was reported probably due to its role in cell proliferation and migration. Agents activating TRAIL signalling have also been shown to induce mutations and develop more aggressive tumours [2, 89, 90]. The BCL-2/BCL-xL antagonist ABT-263 (Navitoclax) was found to induce platelet death leading to thrombocytopenia. Inhibition of cIAPs increases inflammatory cytokines that promote tumour progression [91].

Several studies have shown that cells that survive despite caspase activity sustain damage. In particular, TRAIL-induced apoptosis causes limited caspase activation and DNA fragmentation catalysed by caspase-activated DNase (CAD) resulting in mutations and genomic instability in surviving cells. Likewise, sublethal doses of stresses that induce MOMP in limited number of mitochondria without killing the cell, a phenomenon termed *minority MOMP* can also cause CAD-dependent DNA damage and genomic instability. In addition to CAD, caspase-3 is also known to release endonuclease G (ENDOG) from the mitochondria to promote radiation-induced DNA damage and transformation. Failed apoptosis can also induce DNA damage during inflammation and promote acquired resistance to apoptosis-inducing anticancer therapies [2].

Tumour cells that respond to apoptosis can provide a vacant niche into which more competitive tumour cells can proliferate leading to tumour repopulation. Apoptotic cells release molecules to signal phagocytosis, such as fractalkine (FKN), ATP and lactotransferrin (LTF). These signals transform tumour-associated macrophages (TAMs) into a pro-oncogenic state facilitating angiogenesis, metastasis and evade immune surveillance. To target cancer cells for therapy, it is important to block the prosurvival function of caspase-3 as well as PGE2 release. Administration of celecoxib, a pharmacological inhibitor of cyclooxygenase-2 (COX-2) that mediates PGE2 production, was found to attenuate tumour cell repopulation [92].

One method of achieving tumour cell apoptosis without causing oncogenic effects is to inhibit caspase activity. Inhibition of caspase function does not ultimately protect against cell death following MOMP. Caspase inhibitors have been used in combination with chemotherapy and radiotherapy. The caspase inhibitors Z-VAD-FMK and M867 were demonstrated to sensitize tumour cells to irradiation both in vitro and in vivo. However, this can lead to undesirable effects such as activation of the stimulator of interferon genes (STING) signalling pathway and blockade of extrinsic apoptosis [93, 94].

8 Conclusions

Regulation of caspase-mediated apoptosis is essential for maintaining tissue homeostasis and protecting against neoplastic transformation. Given that caspases are moonlighting proteins that perform multiple, autonomous, often opposing and unrelated functions, reinstating apoptosis in tumour cells while preventing the tumour promoting effects of key players in apoptosis is of prime concern. It is important to achieve apoptosis without disrupting the non-apoptotic pathways influenced by these proteins that could render cancer cells more aggressive or resistant to treatment. Powerful bioinformatics, proteomics and imaging tools will help predict the sensitivity of tumours to caspase-based therapeutics. Deeper insights into the varied functions of caspases will enable development of more effective and safer treatment options for diverse malignancies.

References

1. Elliott MR, Ravichandran KS (2016) The dynamics of apoptotic cell clearance. Dev Cell 38:147–160
2. Ichim G, Tait SW (2016) A fate worse than death: apoptosis as an oncogenic process. Nat Rev Cancer 16:539–548
3. Fox JL, MacFarlane M (2016) Targeting cell death signalling in cancer: minimising 'Collateral damage'. Br J Cancer 115:5–11
4. Dickens LS, Powley IR, Hughes MA, MacFarlane M (2012) The 'complexities' of life and death: death receptor signalling platforms. Exp Cell Res 318:1269–1277
5. Hatok J, Racay P (2016) Bcl-2 family proteins: master regulators of cell survival. Biomol Concepts 7:259–270
6. Czabotar PE, Lessene G, Strasser A, Adams JM (2014) Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol 15:49–63
7. Pradelli LA, Bénéteau M, Ricci JE (2010) Mitochondrial control of caspase-dependent and—independent cell death. Cell Mol Life Sci 67:1589–1597
8. Shi V (2004) Caspase activation, inhibition, and reactivation: a mechanistic view. Protein Sci 13:1979–1987
9. Vasudevan D, Ryoo HD (2015) Regulation of cell death by IAPs and their antagonists. Curr Top Dev Biol 114:185–208
10. Altieri DC (2010) Survivin and IAP proteins in cell-death mechanisms. Biochem J 430:199–205

11. Fulda S (2010) Evasion of apoptosis as a cellular stress response in cancer. Int J Cell Biol 370835
12. Kelly GL, Strasser A (2011) The essential role of evasion from cell death in cancer. Adv Cancer Res 111:39–96
13. Koff JL, Ramachandiran S, Bernal-Mizrachi L (2015) A time to kill: targeting apoptosis in cancer. Int J Mol Sci 16:2942–2955
14. Kiraz Y, Adan A, Yandim MK, Baran Y (2016) Major apoptotic mechanisms and genes involved in apoptosis. Tumour Biol 37:8471–8486
15. Olsson M, Zhivotovsky B (2011) Caspases and cancer. Cell Death Differ 18:1441–1449
16. Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, Bus CJ, Kadkhoda K, Wiechec E, Halayko AJ, Los M (2009) Apoptosis and cancer: mutations within caspase genes. J Med Genet 46:497–510
17. Demon D, Van Damme P, Vanden Berghe T, Vandekerckhove J, Declercq W, Gevaert K, Vandenabeele P (2009) Caspase substrates: easily caught in deep waters? Trends Biotechnol 27:680–688
18. Fiandalo MV, Kyprianou N (2012) Caspase control: protagonists of cancer cell apoptosis. Exp Oncol 34:165–175
19. Jain MV, Paczulla AM, Klonisch T, Dimgba FN, Rao SB, Roberg K, Schweizer F, Lengerke C, Davoodpour P, Palicharla VR, Maddika S, Łos M (2013) Interconnections between apoptotic, autophagic and necrotic pathways: implications for cancer therapy development. J Cell Mol Med 17:12–29
20. Shalini S, Dorstyn L, Dawar S, Kumar S (2015) Old, new and emerging functions of caspases. Cell Death Differ 22:526–539
21. Atsumi G, Tajima M, Hadano A, Nakatani Y, Murakami M, Kudo I (1998) Fas-induced arachidonic acid release is mediated by Ca^{2+}-independent phospholipase A2 but not cytosolic phospholipase A2, which undergoes proteolytic inactivation. J Biol Chem 273:13870–13877
22. Stupack DG (2013) Caspase-8 as a therapeutic target in cancer. Cancer Lett 332:133–140
23. Zhang H, Xu Q, Krajewski S, Krajewska M, Xie Z, Fuess S, Kitada S, Pawlowski K, Godzik A, Reed JC (2000) BAR: an apoptosis regulator at the intersection of caspases and Bcl-2 family proteins. Proc Natl Acad Sci USA 97:2597–2602
24. Bubici C, Papa S, Pham CG, Zazzeroni F, Franzoso G (2004) NF-kappaB and JNK: an intricate affair. Cell Cycle 3:1524–1529
25. Salvesen GS, Duckett CS (2002) IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol 3:401–410
26. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17:2215–2223
27. Schile AJ, Garcia-Fernandez M, Steller H (2008) Regulation of apoptosis by XIAP ubiquitin ligase activity. Genes Dev 22:2256–2266
28. Athanasoula KCh, Gogas H, Polonifi K, Vaiopoulos AG, Polyzos A, Mantzourani M (2014) Survivin beyond physiology: orchestration of multistep carcinogenesis and therapeutic potentials. Cancer Lett 347:175–182
29. Scaffidi C, Schmitz I, Krammer PH, Peter ME (1999) The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem 274:1541–1548
30. Kruidering M, Evan GI (2000) Caspase-8 in apoptosis: the beginning of "the end"? IUBMB Life 50:85–90
31. Peng C, Cho YY, Zhu F, Zhang J, Wen W, Xu Y, Yao K, Ma WY, Bode AM, Dong Z (2011) Phosphorylation of caspase-8 (Thr-263) by ribosomal S6 kinase 2 (RSK2) mediates caspase-8 ubiquitination and stability. J Biol Chem 286:6946–6954
32. Jager R, Zwacka RM (2010) The enigmatic roles of caspases in tumor development. Cancers (Basel) 2:1952–1979

33. Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, Meyerson M, Gabriel SB, Lander ES, Getz G (2014) Discovery and saturation analysis of cancer genes across 21 tumour types. Nature 505:495–501
34. Ummanni R, Lehnigk U, Zimmermann U, Woenckhaus C, Walther R, Giebel J (2010) Immunohistochemical expression of caspase-1 and -9, uncleaved caspase-3 and -6, cleaved caspase-3 and -6 as well as Bcl-2 in benign epithelium and cancer of the prostate. Exp Ther Med 1:47–52
35. Krumschnabel G, Manzl C, Villunger A (2009) Caspase-2: killer, savior and safeguard-emerging versatile roles for an ill-defined caspase. Oncogene 28:3093–3096
36. O'Donovan N, Crown J, Stunell H, Hill AD, McDermott E, O'Higgins N, Duffy MJ (2003) Caspase 3 in breast cancer. Clin Cancer Res 9:738–742
37. Vegran F, Boidot R, Oudin C, Riedinger JM, Bonnetain F, Lizard-Nacol S (2006) Overexpression of caspase-3s splice variant in locally advanced breast carcinoma is associated with poor response to neoadjuvant chemotherapy. Clin Cancer Res 12:5794–5800
38. Chen K, Zhao H, Hu Z, Wang LE, Zhang W, Sturgis EM, Wei Q (2008) CASP3 polymorphisms and risk of squamous cell carcinoma of the head and neck. Clin Cancer Res 14:6343–6349
39. Lan Q, Morton LM, Armstrong B, Hartge P, Menashe I, Zheng T, Purdue MP, Cerhan JR, Zhang Y, Grulich A, Cozen W, Yeager M, Holford TR, Vajdic CM, Davis S, Leaderer B, Kricker A, Schenk M, Zahm SH, Chatterjee N, Chanock SJ, Rothman N, Wang SS (2009) Genetic variation in caspase genes and risk of non-Hodgkin lymphoma: a pooled analysis of 3 population-based case-control studies. Blood 114:264–267
40. Xu HL, Xu WH, Cai Q, Feng M, Long J, Zheng W, Xiang YB, Shu XO (2009) Polymorphisms and haplotypes in the caspase-3, caspase-7, and caspase-8 genes and risk for endometrial cancer: a population-based, case-control study in a Chinese population. Cancer Epidemiol Biomarkers Prev 18:2114–2122
41. Hosgood HD, Baris D, Zhang Y, Zhu Y, Zheng T, Yeager M, Welch R, Zahm S, Chanock S, Rothman N, Lan Q (2008) Caspase polymorphisms and genetic susceptibility to multiple myeloma. Hematol Oncol 26:148–151
42. Lee WK, Kim JS, Kang HG, Cha SI, Kim DS, Hyun DS, Kam S, Kim CH, Jung TH, Park JY (2009) Polymorphisms in the Caspase7 gene and the risk of lung cancer. Lung Cancer 65:19–24
43. Ponten F, Jirstrom K, Uhlen M (2008) The Human Protein Atlas—a tool for pathology. J Pathol 216:387–393
44. Kim KW, Moretti L, Lu B (2008) M867, a novel selective inhibitor of caspase-3 enhances cell death and extends tumor growth delay in irradiated lung cancer models. PLoS ONE 3:e2275
45. Soung YH, Lee JW, Kim SY, Jang J, Park YG, Park WS, Nam SW, Lee JY, Yoo NJ, Lee SH (2005) CASPASE-8 gene is inactivated by somatic mutations in gastric carcinomas. Cancer Res 65:815–821
46. Soung YH, Lee JW, Kim SY, Sung YJ, Park WS, Nam SW, Kim SH, Lee JY, Yoo NJ, Lee SH (2005) Caspase-8 gene is frequently inactivated by the frameshift somatic mutation 1225_1226delTG in hepatocellular carcinomas. Oncogene 24:141–147
47. Kaldenbach M, Cubero FJ, Erschfeld S, Liedtke C, Trautwein C, Streetz K (2014) Hepatic tissue environment in NEMO-deficient mice critically regulates positive selection of donor cells after hepatocyte transplantation. PLoS ONE 9:e100786
48. Sun T, Gao Y, Tan W, Ma S, Shi Y, Yao J, Guo Y, Yang M, Zhang X, Zhang Q, Zeng C, Lin D (2007) A six-nucleotide insertion-deletion polymorphism in the CASP8 promoter is associated with susceptibility to multiple cancers. Nat Genet 39:605–613
49. Ji GH, Li M, Cui Y, Wang JF (2014) The relationship of CASP 8 polymorphism and cancer susceptibility: a meta-analysis. Cell Mol Biol 60:20–28
50. Lázcoz P, Muñoz J, Nistal M, Pestaña A, Encío I, Castresana JS (2006) Frequent promoter hypermethylation of RASSF1A and CASP8 in neuroblastoma. BMC Cancer 6:254
51. Casciano I, De Ambrosis A, Croce M, Pagnan G, Di Vinci A, Allemanni G, Banelli B, Ponzoni M, Romani M, Ferrini S (2004) Expression of the caspase-8 gene in neuroblastoma

cells is regulated through an essential interferon-sensitive response element (ISRE). Cell Death Differ 11:131–134
52. Srinivasula SM, Ahmad M, Guo Y, Zhan Y, Lazebnik Y, Fernandes-Alnemri T, Alnemri ES (1999) Identification of an endogenous dominant-negative short isoform of caspase-9 that can regulate apoptosis. Cancer Res 59:999–1002
53. Allan LA, Clarke PR (2009) Apoptosis and autophagy: Regulation of caspase-9 by phosphorylation. FEBS J 276:6063–6073
54. Oh JE, Kim MS, Ahn CH, Kim SS, Han JY, Lee SH, Yoo NJ (2010) Mutational analysis of CASP10 gene in colon, breast, lung and hepatocellular carcinomas. Pathology 42:73–76
55. Bucur O, Gaidos G, Yatawara A, Pennarun B, Rupasinghe C, Roux J, Andrei S, Guo B, Panaitiu A, Pellegrini M, Mierke DF, Khosravi-Far R (2015) A novel caspase 8 selective small molecule potentiates TRAIL-induced cell death. Sci Rep 5:9893
56. Lim B, Allen JE, Prabhu VV, Talekar MK, Finnberg NK, El-Deiry WS (2015) Targeting TRAIL in the treatment of cancer: new developments. Expert Opin Ther Targets 19:1171–1185
57. Tam CS, Seymour JF, Roberts AW (2016) Progress in BCL2 inhibition for patients with chronic lymphocytic leukemia. Semin Oncol 43:274–279
58. Han B, Park D, Li R, Xie M, Owonikoko TK, Zhang G, Sica GL, Ding C, Zhou J, Magis AT, Chen ZG, Shin DM, Ramalingam SS, Khuri FR, Curran WJ, Deng X (2015) Small-molecule Bcl2 BH4 antagonist for lung cancer therapy. Cancer Cell 27:852–863
59. Geiger K, Hagenbuchner J, Rupp M, Fiegl H, Sergi C, Meister B, Kiechl-Kohlendorfer U, Müller T, Ausserlechner MJ, Obexer P (2012) FOXO3/FKHRL1 is activated by 5-aza-2-deoxycytidine and induces silenced caspase-8 in neuroblastoma. Mol Biol Cell 23:2226–2234
60. Feng G, Kaplowitz N (2000) Colchicine protects mice from the lethal effect of an agonistic anti-Fas antibody. J Clin Invest 105:329–339
61. Ghobrial IM, Witzig TE, Adjei AA (2005) Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin 55:178–194
62. Hensley P, Mishra M, Kyprianou N (2013) Targeting caspases in cancer therapeutics. Biol Chem 394:831–843
63. Su CL, Huang LL, Huang LM, Lee JC, Lin CN, Won SJ (2006) Caspase-8 acts as a key upstream executor of mitochondria during justicidin A-induced apoptosis in human hepatoma cells. FEBS Lett 580:3185–3191
64. He XL, Zhang P, Dong XZ, Yang MH, Chen SL, Bi MG (2012) JR6, a new compound isolated from *Justicia procumbens*, induces apoptosis in human bladder cancer EJ cells through caspase-dependent pathway. J Ethnopharmacol 144:284–292
65. Nör JE, Hu Y, Song W, Spencer DM, Núñez G (2002) Ablation of microvessels in vivo upon dimerization of iCaspase-9. Gene Ther 9:444–451
66. MacCorkle RA, Freeman KW, Spencer DM (1998) Synthetic activation of caspases: artificial death switches. Proc Natl Acad Sci USA 95:3655–3660
67. Sonnemann J, Hartwig M, Plath A, Saravana Kumar K, Müller C, Beck JF (2006) Histone deacetylase inhibitors require caspase activity to induce apoptosis in lung and prostate carcinoma cells. Cancer Lett 232:148–160
68. Sonnemann J, Kumar KS, Heesch S, Müller C, Hartwig C, Maass M, Bader P, Beck JF (2006) Histone deacetylase inhibitors induce cell death and enhance the susceptibility to ionizing radiation, etoposide, and TRAIL in medulloblastoma cells. Int J Oncol 28:755–766
69. McIlwain DR, Berger T, Mak TW (2015) Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 7
70. Zhang HZ, Kasibhatla S, Wang Y, Herich J, Guastella J, Tseng B, Drewe J, Cai SX (2004) Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay. Bioorg Med Chem 12:309–317

71. Buckley CD, Pilling D, Henriquez NV, Parsonage G, Threlfall K, Scheel-Toellner D, Simmons DL, Akbar AN, Lord JM, Salmon M (1999) RGD peptides induce apoptosis by direct caspase-3 activation. Nature 397:534–539
72. Liu JJ, Lin M, Yu JY, Liu B, Bao JK (2011) Targeting apoptotic and autophagic pathways for cancer therapeutics. Cancer Lett 300:105–114
73. Shariat SF, Desai S, Song W, Khan T, Zhao J, Nguyen C, Foster BA, Greenberg N, Spencer DM, Slawin KM (2001) Adenovirus-mediated transfer of inducible caspases: a novel 'death switch' gene therapeutic approach to prostate cancer. Cancer Res 61:2562–2571
74. Lou GG, Yao HP, Xie LP (2010) *Brucea javanica* oil induces apoptosis in T24 bladder cancer cells via upregulation of caspase-3, caspase-9, and inhibition of NF-kappa B and COX-2 expressions. Am J Chin Med 38:613–624
75. Kim BM, Maeng K, Lee KH, Hong SH (2011) Combined treatment with the Cox-2 inhibitor niflumic acid and PPARc ligand ciglitazone induces ER stress/caspase-8-mediated apoptosis in human lung cancer cells. Cancer Lett 300:134–144
76. Li X, Su B, Liu R, Wu D, He D (2011) Tetrandrine induces apoptosis and triggers caspase cascade in human bladder cancer cells. J Surg Res 166:e45–e51
77. Belz K, Schoeneberger H, Wehner S, Weigert A, Bönig H, Klingebiel T, Fichtner I, Fulda S (2014) Smac mimetic and glucocorticoids synergize to induce apoptosis in childhood ALL by promoting ripoptosome assembly. Blood 124:240–250
78. Ramachandiran S, Cain J, Liao A, He Y, Guo X, Boise LH, Fu H, Ratner L, Khoury HJ, Bernal-Mizrachi L (2012) The Smac mimetic RMT5265.2HCL induces apoptosis in EBV and HTLV-I associated lymphoma cells by inhibiting XIAP and promoting the mitochondrial release of cytochrome C and Smac. Leuk Res 36:784–790
79. Sheng R, Sun H, Liu L, Lu J, McEachern D, Wang G, Wen J, Min P, Du Z, Lu H, Kang S, Guo M, Yang D, Wang S (2013) A potent bivalent Smac mimetic (SM-1200) achieving rapid, complete, and durable tumor regression in mice. J Med Chem 56:3969–3979
80. Carrasco RA, Stamm NB, Marcusson E, Sandusky G, Iversen P, Patel BK (2011) Antisense inhibition of survivin expression as a cancer therapeutic. Mol Cancer Ther 10:221–232
81. Kumar B, Yadav A, Lang JC, Cipolla MJ, Schmitt AC, Arradaza N, Teknos TN, Kumar P (2012) YM155 reverses cisplatin resistance in head and neck cancer by decreasing cytoplasmic survivin levels. Mol Cancer Ther 11:1988–1998
82. Murakami Y, Matsuya T, Kita A, Yamanaka K, Noda A, Mitsuoka K, Nakahara T, Miyoshi S, Nishimura S (2013) Radiosynthesis, biodistribution and imaging of [11C] YM155, a novel survivin suppressant, in a human prostate tumor-xenograft mouse model. Nucl Med Biol 40:221–226
83. Nagini S, Vidya Priyadarsini R (2012) Apoptosis induction by nimbolide, a limonoid from *Azadirachta indica*: molecular targets and signaling networks. In: Chen GC, Lai PBS (eds) Novel apoptotic regulators in carcinogenesis. Springer, Berlin, pp 27–44
84. Kavitha K, Kowshik J, Kranthi Kiran Kishore T, Baba AB, Nagini S (2013) Astaxanthin inhibits NF-κB and Wnt/β-catenin signaling pathways via inactivation of Erk/MAPK and PI3K/Akt to induce intrinsic apoptosis in a hamster model of oral cancer. Biochim Biophys Acta 1830:4433–4444
85. Anitha P, Vidya Priyadarsini R, Kavitha K, Thiyagarajan P, Nagini S (2013) Ellagic acid coordinately attenuates Wnt/β-catenin and NF-κB signaling pathways to induce intrinsic apoptosis in an animal model of oral oncogenesis. Eur J Nutr 52:75–84
86. Nagini S, Palitti F, Natarajan AT (2015) Chemopreventive potential of chlorophyllin: a review of the mechanisms of action and molecular targets. Nutr Cancer 67:203–211
87. Vidya Priyadarsini R, Nagini S (2012) Aberrant signaling pathways in cancer: modulation by the dietary flavonoid, quercetin. In: Shankar S, Srivastava R (eds) Nutrition diet and cancer. Springer, New York, pp 1–21
88. Nagini S, Senthil Murugan R (2013) Cancer chemoprevention by black tea polyphenols: emerging evidence and molecular targets. In: Preedy VR (ed) Tea in health and disease prevention. Academic Press, London, pp 737–750

89. Jeon YJ, Middleton J, Kim T, Lagana A, Piovan C, Secchiero P, Nuovo GJ, Cui R, Joshi P, Romano G, Di Leva G, Lee BK, Sun HL, Kim Y, Fadda P, Alder H, Garofalo M, Croce CM (2015) A set of NF-kappaB-regulated microRNAs induces acquired TRAIL resistance in lung cancer. Proc Natl Acad Sci USA 112:E3355–E3364
90. Lovric MM, Hawkins CJ (2010) TRAIL treatment provokes mutations in surviving cells. Oncogene 29:5048–5060
91. Infante JR, Dees EC, Olszanski AJ, Dhuria SV, Sen S, Cameron S, Cohen RB (2014) Phase I dose-escalation study of LCL161, an oral inhibitor of apoptosis proteins inhibitor, in patients with advanced solid tumors. J Clin Oncol 32:3103–3110
92. Mirzayans R, Andrais B, Kumar P, Murray D (2016) The growing complexity of cancer cell response to DNA-damaging agents: caspase 3 mediates cell death or survival? Int J Mol Sci 17:E708 (pii)
93. Kim HS, Lee JW, Soung YH, Park WS, Kim SY, Lee JH, Park JY, Cho YG, Kim CJ, Jeong SW, Nam SW, Kim SH, Lee JY, Yoo NJ, Lee SH (2003) Inactivating mutations of caspase-8 gene in colorectal carcinomas. Gastroenterology 125:708–715
94. Kudelova J, Fleischmannova J, Adamova E, Matalova E (2015) Pharmacological caspase inhibitors: research towards therapeutic perspectives. J Physiol Pharmacol 66:473–482

Functional Aspects of Activated Protein C (APC) in Regulating Homeostasis and Disease

18

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Abstract

Activated protein C is an intrinsic cytoprotective, anti-inflammatory, and anticoagulant factor. It acts as an active therapeutic molecule against the progression of number of systemic disorders. APC performs majority of its cell survival and homeostatic activities through binding with EPCR and activation of PAR1. Future research on the multifunctionality of APC protein may provide a novel insight into the mechanisms and interrelation of various coagulation and systemic disorders to provide an improved pathophysiological therapeutic approach to diseases.

Keywords

APC · EPCR · PAR · TM

1 Introduction

In cell system, the balance between coagulant and anticoagulant factors is very crucial to sustain life during normal cellular homeostasis. Whenever there is a vascular or endothelial cell rupture/injury, the activation of various coagulant protease factors leads to blood clot formation in order to prevent blood flow. Being a multifunctional homeostatic protein, thrombin regulates its anticoagulant effect by activating protein C and initiating the anticoagulant pathway of protein C. Protein C pathway has become a primary focus of attention in the past decade because of its

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critical role in maintaining hemostasis [1, 2]. In normal hemostasis, the conversion of prothrombin to thrombin occurs through a negative feedback control manner. As protein C is present in the circulatory system (bloodstream) in zymogen form (inactive), it gets activated to form APC (activated protein C) after the formation of active thrombin–thrombomodulin complex. It has been reported that EPCR (endothelial cell protein C receptor) accelerates the generation of activated protein C, when it interacts with thrombin–thrombomodulin complex [3]. Recent studies performed in last few years have shown that APC-mediated cytoprotective signaling is solely dependent on interaction with EPCR. EPCR is a type I transmembrane protein that shares structural homology with the major histocompatibility complex class1/CD1 family of molecules. APC/protein C binding site for EPCR is mediated almost entirely by the vitamin K-dependent GLA domain. Detailed structural investigation of EPCR reveals that EPCR carries a tightly bound phospholipid along with it in the antigen presenting groove and this phospholipid plays a crucial role in protein C binding. Experimental evidence also suggests that removal of lipid from EPCR groove could result in the loss of protein C binding [4]. Therefore, EPCR binding with APC has important functional aspects in regulating APC function. Based on various animal model studies of sepsis, stroke, and ischemic injury, it has been found that APC activation also reduces organ or tissue damages [5, 6]. Apart from its anticoagulant activity, APC in the presence of EPCR also mediates various cellular events like, (a) cytoprotective effects, (b) endothelial barriers protection, (c) anti-inflammatory activities, (d) angiogenic activity through PAR1 activation. The study performed by Chen et al. suggests that APC administration in low dose have neuroprotective effects, which is independent of its anticoagulant activity. APC protects brain cells from ischemic injury directly by blocking p53-mediated apoptosis pathway [7]. In this chapter, we will focus mainly on the implications of APC and its pathophysiological role in the cell system.

2 Anticoagulant Activity of APC

Protein C (PC) is a 461 amino acids single polypeptide serine protease, which is synthesized predominantly in the liver and circulates in the blood in zymogen form. Recent studies suggest that protein C is also synthesized by other cell types like keratinocytes, endothelial, male reproductive tissue, and some hematopoietic cell types [8]. PC is a co-translational and posttranslational modified protein, in which N-terminal region contains vitamin K-dependent posttranslationally modified residues. Structurally, PC consists of four domains; GLA (gamma-carboxyl glutamate-rich residue) (residues 1–37), EGF1 (residues 46–92), EGF2 (residues 93–136) (epidermal growth factor-like domain), and SP (serine protease) (residues 170–419) domain [9] as shown in Fig. 1. In the presence of negatively charged phospholipid (phosphatidylserine/phosphatidylethanolamine) and Ca^{2+} ions, GLA domain of APC/PC binds with membrane surface to show anticoagulant activity. During activation, on endothelilal surface thrombin forms thrombin–

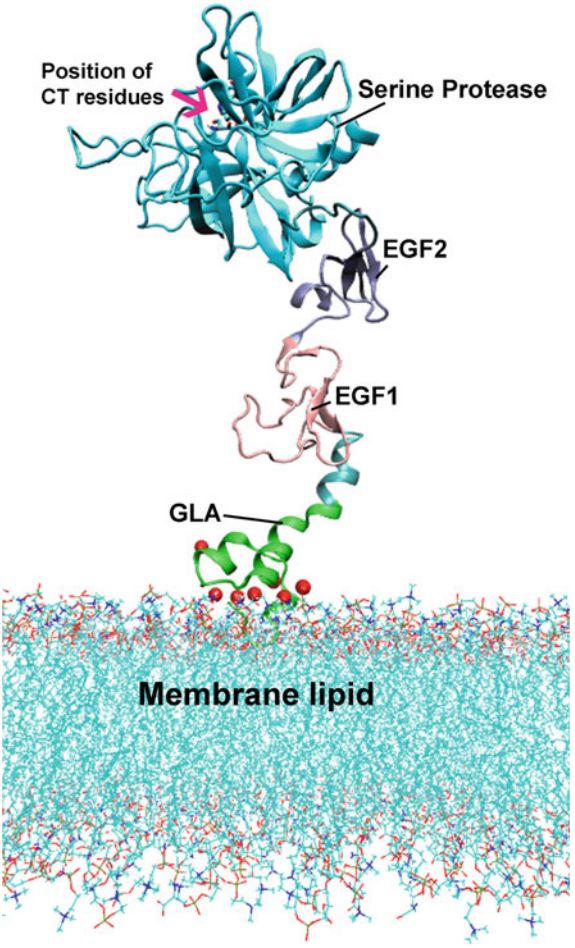

Fig. 1 Structure of activated protein C on lipid bilayer. Ribbon representation of APC showing four major domains, GLA (*green*), EGF1 (*pink*), EGF2 (*blue*), and SP (*cyan*). Calcium ions are shown by *red color* ball representation. Catalytic triad (*CT*) residues (His57, Ser195, and Asp102) of serine protease domain are shown in stick representation

thrombomodulin complex and cleaves the PC activation peptide (residues 158–169) to generate APC. However, in the presence of its receptor EPCR, APC activation gets enhanced several times. Like other serine proteases, it also contains catalytic triad; $His211^{(57)}$, $Asp257^{(102)}$, and $Ser360^{(195)}$ in the serine protease domain (the chymotrypsin numbering is denoted in superscript with parentheses) [9].

In the presence of various cofactors like protein S, negatively charged membrane lipid (phosphatidylserine, cardiolipin), high-density lipoprotein and glycosphingolipids (glucosylceramide), and Ca^{2+} ions, APC prevents the thrombin generation, which is mediated by the inactivation of FVa and FVIIIa, as shown in Fig. 2. This leads to the selective inhibition of prothrombinase and FXase complexes, resulting APC anticoagulant function in circulatory system [10]. In this way, body maintains normal homeostasis by regulating anticoagulant APC and procoagulant thrombin formation. In addition, APC also promotes the conversion of plasminogen to plasmin by binding with plasminogen activator inhibitor and thus promoting fibrinolysis [11].

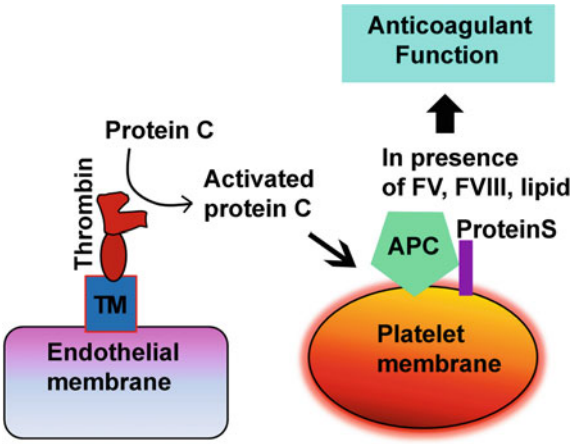

Fig. 2 Activation of protein C to perform the anticoagulant function

3 APC-Mediated Cytoprotective Signaling

APC-mediated cytoprotective signaling is dependent on APC, EPCR, and protease-activated receptor 1 (PAR-1). Although the cleavage site of the receptor is identical, activation of PAR-1 by EPCR-APC complex evokes different signaling pathways in comparison to thrombin-mediated PAR-1 activation [12]. This differential response is due to binding of protein C with EPCR, which leads to the migration of EPCR out of the lipid rafts. Eventually, caveolin-1 is replaced with PAR-1; therefore, APC causes PAR-1 activation, in which PAR-1 couples to a G-protein to initiate cytoprotective signaling. In case of thrombin, PAR-1 activation results to different signaling phenomenon, which promote inflammatory response, involving platelet activation, increase of vascular permeability, NFκβ activation, and an abrupt increase in the level of cytokines responsible for inflammatory processes [13–15].

To mediate the cytoprotective effect, APC in the presence of EPCR, cleaves PAR-1 (proteinase-activated receptor) and activates Gi protein. Gi activation causes phosphorylation of sphingosine to sphingosine-1-phosphate. APC further accomplishes to generate the transactivation of sphingosine-1-phosphate (S1P) receptor, via Rac1 activation through β-arrestin [13, 16–19]. This signaling further causes inhibition of inflammatory gene expression as shown in Fig. 3. Recent in vivo and in vitro studies reveal that APC cytoprotective effects can cause alteration of various gene expressions that includes anti-apoptotic gene, anti-inflammatory gene and genes responsible for endothelial barrier protection [20, 21]. It has been found that APC-treated cells show downregulation of pro-apoptotic (BIRC5, TP53) and pro-inflammatory (B2M, CALR, LTB) gene expression. On the other hand, it also upregulates the expression profile of anti-inflammatory (like CCL2, CXCL2, NOS3, PTGS2) and anti-apoptotic (like BCL2A1, NR4A1, SMAD3, TNFAIP3) to perform anti-inflammatory and anti-apoptotic activities [12, 22–25]. Both in vitro

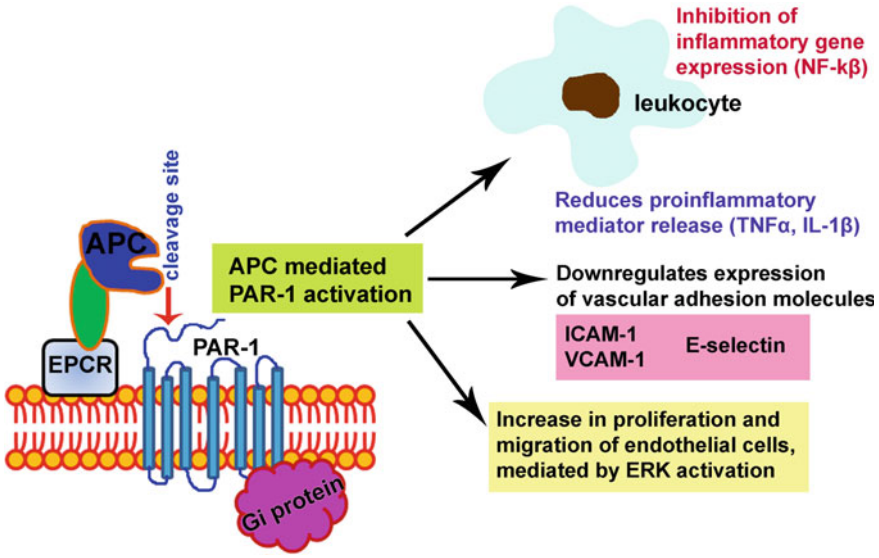

Fig. 3 The cytoprotective protein C pathway for activated protein C anti-inflammatory activity

and in vivo experiments have shown that APC in the presence of its receptor, EPCR and PAR-1 manifests anti-apoptotic activity [7, 23, 24]. APC downregulates pro-apoptotic Bax protein expression level and thereby maintains anti-apoptotic Bcl-2 protein levels. In this process, APC also reduces the degradation of DNA, activation of caspase-3 and translocation of PS to the outer membrane surface, hence, reduces the intrinsic apoptotic pathway [7, 24, 26]. During hypoxic condition of human brain endothelial cells, APC is involved in the reduction of p53 at both protein and mRNA level; thereby inhibiting apoptotic pathway of cells as shown in Fig. 4. These various observations illustrate the role of APC in anti-apoptotic activity; further work is needed to know whether this anti-apoptotic activity is limited to APC-specific cellular signaling, various gene expression profiling, or specific receptors dependent phenomenon.

In addition, APC suppresses the transcription factors related to inflammation, like AP-1 (activator protein-1) family c-Fos and FosB. APC also downregulates vascular adhesion molecules expression (ICAM-1, VCAM-1, and E-selectin), chemotaxis and cytokine production, thereby limiting the adhesion and infiltration of leukocytes on the endothelium surface [25, 27–29], as shown in Fig. 3. This limitation of leukocyte adhesion is probably mediated by decrease in thrombin-dependent movement of selectins, which leads to decrease in the monocyte chemotactic protein-1 synthesis; however, the detailed mechanisms of APC action on leukocytes are yet to be resolved. Several studies also suggest that in addition to barrier stabilization on endothelial cells, other receptors of APC include several integrins, Tie2, PAR3, S1P1 (sphingosine-1-phosphate receptor1), ApoER2 (apolipoprotein E receptor2), glycoprotein lb [30–34]. It has been reported that

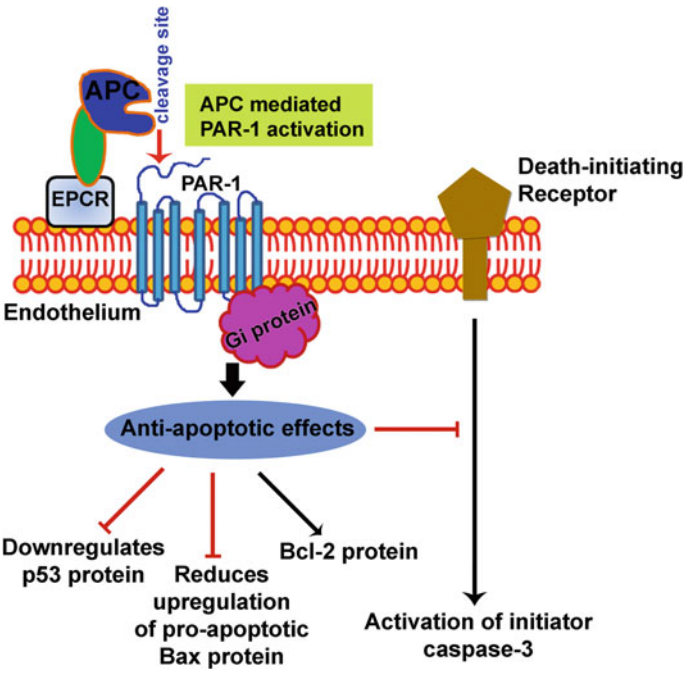

Fig. 4 Involvement of cytoprotective pathway of protein C in anti-apoptotic activity

APC treatment inhibits LPS-mediated release of pro-inflammatory factors in monocytes [35]. Besides it also interacts with β1 and β3 integrins directly through RGD (Arg-Gly-Asp) sequence leading to the inhibition of neutrophil migration [36]. APC treatment also shows anti-inflammatory effect involving PI3 K-Akt survival pathway in U937 cell line via activation of Src family kinases [30, 32]. Together all these observations suggest how APC acts as a key regulatory molecule in maintaining homeostasis by its cytoprotective, anti-apoptotic, and anti-inflammatory effect.

4 Involvement of APC in Angiogenesis (Formation of New Blood Vessels) and Wound Healing

Natural anticoagulant APC has also been demonstrated as an effective pro-angiogenic molecule. It induces the activation of both ERK1/2 and MEK1/2; and found responsible for increasing DNA synthesis and proliferation of cultured HUVECs [37]. It also activates eNOS by PI3K/AKT pathway, which leads to increase in the level of nitric oxide (NO) required for signaling activation and proliferation in endothelial cells. It has also been shown that EPCR bound with APC induces phosphorylation of MAPK via PAR1 activation. Furthermore, APC induces

the upregulation of endothelial cytokines, such as IL-6, IL-8, or chemokines such as MCP-1, which induces wound healing and promotes the endothelial cell migration and proliferation [23, 38, 39]. Studies reveal that APC effectively stimulates keratinocyte proliferation and migration, through the degradation of extracellular matrix by activating matrix metalloproteinase-2 (MMP-2) [40]. In summary, APC appears as a promising molecule for enhancing the blood vessel formation by gelatinase-A activation and wound healing potential of cells [41].

5 Role of APC in Pathophysiological/Disease Condition

In general, protein C is present in plasma in 70 nM concentration having a half-life of 8 h. However, plasma also contains a minimal level of APC (<40 pM) [42]. In the context of several diseases, both homozygous and heterozygous APC deficiencies have been reported. Symptomatic heterozygous deficiencies of APC are associated with pulmonary embolism and deep vein thrombosis, however, homozygous deficiencies are linked with purpura fulminans in newborns and fatal systemic disseminated intravascular thrombosis conditions [43]. The function of APC is completely dependent on the pattern of EPCR and PAR-1 expression. It will be very interesting to establish the relation between APC response and its association with co-receptor, depending on a particular cell's location, condition, and receptor profile. Recently, investigators often presumed in a clinical trial that APC has the ability to reduce mortality in severe sepsis condition in comparison with other anticoagulants like antithrombin or tissue factor pathway inhibitor. APC variants are also useful to reduce endotoxemia-induced death in mice, in addition to its neuroprotective effect. Due to distinct cytoprotective and anticoagulant actions in relation to bleeding risks, the development of APC variant with improved profile could provide a new insight to understand the mechanism of maintaining homeostasis. Several pieces of evidences suggest that APC has therapeutic implications in a number of pathological/disease condition, which is mention as below.

5.1 Sepsis/Endotoxemia

Sepsis is a severe disease condition in which body's response to external infection (gram-negative bacteria) harms its own organs and tissues. In these conditions, blood contains a low amount of oxygen and high amount of lactate. The importance of PC pathway has been widely investigated in animal models and humans in such disease conditions [44–46]. Experiments performed on endotoxemia (LPS)-based animal model of sepsis have established an intricate relationship between hemostasis and inflammation. LPS is the ligand of Toll-like receptor-4, its treatment upregulates TF (tissue factor), leading to increase in coagulation activity and especially increase in the cytokines level of TNF-α, IL-8, IL-6, and IL-1. This increase in cytokine activity is NF-κβ nuclear translocation dependent. These

cytokines reduce the potential ability of the endothelial cells to activate PC into APC by downregulating thrombomodulin in endothelial cells. Thus, increased level of cytokines in sepsis condition diminishes the anticoagulant pathways and enhances coagulation. Patients suffering from acute sepsis having a high risk of death due to organ dysfunction, can be treated with recombinant form of human APC [47], commercially marketed by Eli Lilly at Xigris. Whereas, APC administration is still not accepted as an authentic drug for sepsis, due to excessive bleeding complications in patients especially children. Till date there are various relevant medical issues need to be addressed.

5.2 Stroke Protection and Brain Injury

In addition to anti-thrombotic and anti-inflammatory properties, APC has also a neuroprotective effect for stroke conditions. Studies reveal that APC administration reduces brain damage and cerebrovascular injury in focal cerebral ischemic condition in murine model [5, 31, 48]. The mechanism of neuroprotective effect in a hypoxic condition of human brain endothelium is mediated by blocking p53-mediated apoptosis by APC [7]. APC inhibits the transcription of tumor suppressor protein p53, it reduces the level of caspase-3 (vital for apoptosis) and it also attenuates the level of proapoptotic protein ratio of Bax/Bcl-2. Cytoprotective activity of APC in brain endothelium is both EPCR and PAR1-dependent. The involvement of APC in neuroprotective effect gets enhanced due to its ability to cross the blood–brain barrier and blood-spinal cord barrier [49]. In the presence of APC, the expression of superoxide dismutase-1 gets attenuated in microvessels, motor neurons, neuronal cells, and microglial cells [50, 51]. In ischemic injury condition, APC protects brain cells by the inhibition of TNF-α production and hence downregulates the inflammatory gene production. APC can also regulate the tissue plasminogen activator's neurotoxic effects in stroke and hence reduce neuronal damage [52, 53]. Altogether, these findings collectively indicate that APC has a wide range of clinical applications in protecting stroke and brain injury.

5.3 Rheumatoid Arthritis (RA)

Progressive articular damage due to consistent inflammation of multiple synovial joints is an important characteristic of RA. Commonly in endothelial and synovial cells, the expression level of APC is higher and it is found colocalized with MMP-2 in these cells, suggesting its vital role in tissue remodeling [54]. Expression level of MMP-9 gets directly inhibited at the gene and protein level after addition of recombinant APC, it directly suppresses the production of TNF-α and activation of NF-$\kappa\beta$ [55]. Due to reduced TNF-α and blockade of MMP-9, APC emerges as a beneficial molecule for the prevention of inflammation and joint pain relief in

patients with RA. Studies have concluded that in developing and arthritic joints, but not in normal cartilage, APC may play a pivotal role in the progressive degradation of cartilage by activating MMPs [54].

5.4 Coronary Reperfusion Injury

In ischemic condition, due to limited oxygen supply, tissues suffer from reperfusion injury. APC shows cardio-protective effects on cardiomyocytes by inhibition of apoptosis and the expression of inflammatory cytokines after myocardial ischemia. Further studies reveal that APC directly stimulates the AMP-activated protein kinase (APMK) signaling pathway. AMPK acts as an energy sensor protein, gets activated in response to ATP depletion. AMPK mediates the translocation of glucose transporter protein (GLUT4) for enhancing the glucose uptake. As mentioned previously, APC upregulates AMPK activity and attenuates the reperfusion injury by down-regulating the JNKinase and NF-κβ pathways [22, 56], hence inhibiting the inflammatory cytokines (like TNFα and IL-6) in the cardiac ischemic condition [57].

5.5 Asthma

Asthma is airway's chronic inflammatory disease, in which structural alteration of the airway wall takes place due to hypertrophy and hyperplasia of smooth muscle cells. Recent studies demonstrate that PC pathway is also involved in the remodeling and inflammation of airway cell in asthma [58, 59]. Increased levels of thrombin in asthmatic patients induce extensive production of mucus by epithelial cells and stimulate the proliferation of smooth muscle cells. Further in vitro and in vivo studies confirmed that administration of APC decreases thrombin-induced goblet cells hyperplasia and development of allergic inflammation [59, 60]. Moreover, APC has also been found as an endogenous anticoagulant which enhances fibrinolysis and decreases the obstruction caused by fibrin-deposition. Anti-inflammatory activity of APC is partially the result of its capacity to block the transcription of certain inflammatory cytokines, e.g., NFκβ-dependent cytokines (IL-4, IL-5, IL-13, and TNF-α), which leads to the infiltration of leukocytes at the site of inflammation of airway wall [28, 61, 62]. APC also significantly inhibits bronchial hyper responsiveness and the expression level of T-helper 2 cytokines, immunoglobulin E, and eosinophilic inflammation [59]. More evidently, APC inhalation reduces the nuclear translocation of NFκβ p50 and other members of its family. In support of this, NFκβ p50-deficient mice have reduced allergic airway inflammation, confirming that blockade of this transcription factor is an essential step in the resolution of inflammatory response [63, 64]. Activation of PC has also

been found reduced in the sputum of patients with bronchial asthma. The imbalance between insufficient activation of PC system and increased thrombin leads to enhanced coagulation and exacerbates inflammation of airway cells.

5.6 Diabetes

Apoptosis of kidney cells is one of the characteristic features of diabetes. In the patients with persistent hyperglycemia, decreased level of thrombomodulin (TM) was observed, accompanied by impaired APC formation. Low levels of APC in the glomeruli of animals with diabetes, elevates blood coagulation activation, and fibrin deposition in kidney cells, conciliating the glomerular barrier function [65, 66]. Elevated endogenous APC expression have beneficial effects with diabetic nephropathy by inhibiting apoptosis of endothelial and podocyte cells with improved kidney function in mouse model of Type1 diabetes, thereby preventing hyperglycemia-induced renal dysfunction and enhances glomerular barrier function [67]. In response to renal injury caused by ischemia/reperfusion, APC has the potential to cure renal damage by inhibiting leukocyte activation. Simultaneous administration of soluble TM (sTM) and increased expression of APC are found to be highly effective against diabetic complications. Altogether, these studies evidently support the importance of APC and sTM in curing tubular inflammation and histological damage.

5.7 Tumor Adhesion and Propagation

It has already been established that cancer cells have an activated blood coagulation cascade ultimately resulting in thrombin generation, which plays an exceptional role in providing metastatic property to it. But the ability of APC to abate thrombin formation may have a pivotal role in compromising cancer metastasis. Studies have reported that APC inhibits cancer cells extravasation by targeting VE-cadherin expression, hence preserving endothelial cell-to-cell junction stability [68, 69]. Interestingly, patients having certain types of chemotherapy treatment have been observed with APC deficiency. Additionally, the anticoagulant treatment to the cancer patient with thrombotic complications significantly affects the endogenous APC generation. It has been also found in a murine model of hematogenous B16-F10 melanoma model that APC/EPCR axis helps in reducing tumor cell adhesion and transmigration [68]. It is also been confirmed in EPCR-overexpressing and recombinant human APC (rhAPC) administered mice, that activation of PC pathways inhibits the development of tumor cell metastasis and transmigration by inhibiting P-selectin in the lung [68]. Lastly, the concept of utilizing rhAPC protein against tumor cell metastasis and adhesion has sufficient evidence to establish the role of APC as a potential therapeutic agent.

6 Conclusion

Besides having excellent anticoagulant properties, APC also demonstrates a pivotal role in maintaining a conscientious balance between coagulation, inflammatory and cytoprotective processes during normal homeostasis. In vitro and preclinical data also suggest the pharmacological importance of APC in curing severe sepsis and inflammatory diseases. APC directly affects the leukocytes and endothelial cells, depending on the signaling through EPCR/PAR1 axis. Although the therapeutic use of recombinant human APC was approved in the case of severe sepsis patients with high death risk in 2002 (APACHEII > 25), but due to excessive bleeding complications in few individuals its use in sepsis is still a matter of controversy. Nonetheless, the increased perception of the mechanism of APC activity has extensively contributed to meticulously understand the complicated pathophysiology of acute sepsis and inflammatory complications in several disorders. In future, various forms of active APC molecules (recombinant human APC, its variants, and conjugates) may provide a remarkable assurance for future preparation of commercial therapeutic anticoagulants and anti-inflammatory drugs. These diverse properties of APC to maintain homeostasis in circulation and the ability of EPCR to initiate PC signaling support the persistent development of APC-mediated strategies that are focused to attenuate the cellular process, whereby coagulation aggravates inflammation and vice versa.

References

1. Esmon CT, Ding W, Yasuhiro K et al (1997) The protein C pathway: new insights. Thromb Haemost 78:70–74
2. Esmon CT (1992) The protein C anticoagulant pathway. Arterioscler Thromb 12:135–145
3. Xu J, Esmon NL, Esmon CT (1999) Reconstitution of the human endothelial cell protein C receptor with thrombomodulin in phosphatidylcholine vesicles enhances protein C activation. J Biol Chem 274:6704–6710
4. Oganesyan V, Oganesyan N, Terzyan S et al (2002) The crystal structure of the endothelial protein C receptor and a bound phospholipid. J Biol Chem 277:24851–24854
5. Shibata M, Kumar SR, Amar A et al (2001) Anti-inflammatory, antithrombotic, and neuroprotective effects of activated protein C in a murine model of focal ischemic stroke. Circulation 103:1799–1805
6. Griffin JH, Zlokovic B, Fernández JA (2002) Activated protein C: potential therapy for severe sepsis, thrombosis, and stroke. Semin Hematol 39:197–205
7. Cheng T, Liu D, Griffin JH et al (2003) Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. Nat Med 9:338–342
8. Xue M, Campbell D, Jackson CJ (2007) Protein C is an autocrine growth factor for human skin keratinocytes. J Biol Chem 282:13610–13616
9. Griffin JH, Fernández JA, Gale AJ, Mosnier LO (2007) Activated protein C. J Thromb Haemost 5(Suppl 1):73–80
10. Grinnell BW, Yan SB (1998) Novel antithrombotics based on modulation of the protein C pathway. Coron Artery Dis 9:89–97
11. Esmon CT (2001) The normal role of activated protein C in maintaining homeostasis and its relevance to critical illness. Crit Care 5:S7–S12

12. Riewald M, Ruf W (2005) Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. J Biol Chem 280:19808–19814
13. Bae J-S, Yang L, Rezaie AR (2007) Receptors of the protein C activation and activated protein C signaling pathways are colocalized in lipid rafts of endothelial cells. Proc Natl Acad Sci U S A 104:2867–2872
14. Bae J-S, Yang L, Rezaie AR (2008) Lipid raft localization regulates the cleavage specificity of protease activated receptor 1 in endothelial cells. J Thromb Haemost 6:954–961
15. Bae J-S, Yang L, Manithody C, Rezaie AR (2007) The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. Blood 110:3909–3916
16. Russo A, Soh UJK, Paing MM et al (2009) Caveolae are required for protease-selective signaling by protease-activated receptor-1. Proc Natl Acad Sci U S A 106:6393–6397
17. Bae J-S, Rezaie AR (2009) Thrombin inhibits nuclear factor kappa B and Rho A pathways in cytokine-stimulated vascular endothelial cells when EPCR is occupied by protein C. Thromb Haemost 101:513–520
18. Soh UJK, Trejo J (2011) Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through β-arrestin and dishevelled-2 scaffolds. Proc Natl Acad Sci U S A 108:E1372–E1380
19. Russo A, Soh UJK, Trejo J (2009) Proteases display biased agonism at protease-activated receptors: location matters! Mol Interv 9:87–96
20. Feistritzer C, Riewald M (2005) Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. Blood 105:3178–3184
21. Finigan JH, Dudek SM, Singleton PA et al (2005) Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. J Biol Chem 280:17286–17293
22. Joyce DE, Grinnell BW (2002) Recombinant human activated protein C attenuates the inflammatory response in endothelium and monocytes by modulating nuclear factor-kappa B. Crit Care Med 30:S288–S293
23. Riewald M, Petrovan RJ, Donner A et al (2002) Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science 296:1880–1882
24. Joyce DE, Gelbert L, Ciaccia A et al (2001) Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 276:11199–11203
25. Franscini N, Bachli EB, Blau N et al (2004) Gene expression profiling of inflamed human endothelial cells and influence of activated protein C. Circulation 110:2903–2909
26. Mosnier LO, Griffin JH (2003) Inhibition of staurosporine-induced apoptosis of endothelial cells by activated protein C requires protease-activated receptor-1 and endothelial cell protein C receptor. Biochem J 373:65–70
27. Hirose K, Okajima K, Taoka Y et al (2000) Activated protein C reduces the ischemia/reperfusion-induced spinal cord injury in rats by inhibiting neutrophil activation. Ann Surg 232:272–280
28. Murakami K, Okajima K, Uchiba M et al (1997) Activated protein C prevents LPS-induced pulmonary vascular injury by inhibiting cytokine production. Am J Physiol 272:L197–L202
29. Murakami K, Okajima K, Uchiba M et al (1996) Activated protein C attenuates endotoxin-induced pulmonary vascular injury by inhibiting activated leukocytes in rats. Blood 87:642–647
30. White TC, Berny MA, Tucker EI et al (2008) Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. J Thromb Haemost 6:995–1002
31. Guo H, Liu D, Gelbard H et al (2004) Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. Neuron 41:563–572

32. Yang XV, Banerjee Y, Fernández JA et al (2009) Activated protein C ligation of ApoER2 (LRP8) causes Dab1-dependent signaling in U937 cells. Proc Natl Acad Sci U S A 106: 274–279
33. Xue M, Chow S-O, Dervish S et al (2011) Activated protein C enhances human keratinocyte barrier integrity via sequential activation of epidermal growth factor receptor and Tie2. J Biol Chem 286:6742–6750
34. Zhong Z, Ilieva H, Hallagan L et al (2009) Activated protein C therapy slows ALS-like disease in mice by transcriptionally inhibiting SOD1 in motor neurons and microglia cells. J Clin Invest 119:3437–3449
35. Schmidt-Supprian M, Murphy C, While B et al (2000) Activated protein C inhibits tumor necrosis factor and macrophage migration inhibitory factor production in monocytes. Eur Cytokine Netw 11:407–413
36. Elphick GF, Sarangi PP, Hyun Y-M et al (2009) Recombinant human activated protein C inhibits integrin-mediated neutrophil migration. Blood 113:4078–4085
37. Uchiba M, Okajima K, Oike Y et al (2004) Activated protein C induces endothelial cell proliferation by mitogen-activated protein kinase activation in vitro and angiogenesis in vivo. Circ Res 95:34–41
38. Hooper WC, Phillips DJ, Renshaw MA et al (1998) The up-regulation of IL-6 and IL-8 in human endothelial cells by activated protein C. J Immunol 161:2567–2573
39. Hooper WC, Phillips DJ, Renshaw MA (2001) Activated protein C induction of MCP-1 in human endothelial cells: a possible role for endothelial cell nitric oxide synthase. Thromb Res 103:209–219
40. Xue M, Thompson P, Kelso I, Jackson C (2004) Activated protein C stimulates proliferation, migration and wound closure, inhibits apoptosis and upregulates MMP-2 activity in cultured human keratinocytes. Exp Cell Res 299:119–127
41. Nguyen M, Arkell J, Jackson CJ (2000) Activated protein C directly activates human endothelial gelatinase A. J Biol Chem 275:9095–9098
42. Gruber A, Griffin JH (1992) Direct detection of activated protein C in blood from human subjects. Blood 79:2340–2348
43. Marlar RA, Mastovich S (1990) Hereditary protein C deficiency: a review of the genetics, clinical presentation, diagnosis and treatment. Blood Coagul Fibrinolysis 1:319–330
44. van Ruler O, Schultz MJ, Reitsma JB et al (2009) Has mortality from sepsis improved and what to expect from new treatment modalities: review of current insights. Surg Infect (Larchmt) 10:339–348
45. Loubele STBG, Spronk HMH, Ten Cate H (2009) Activated protein C: a promising drug with multiple effects? Mini Rev Med Chem 9:620–626
46. Eve RL, Duffy MR (2009) Should we be using activated protein C to treat severe sepsis? Br J Hosp Med (Lond) 70:484
47. Martí-Carvajal AJ, Solà I, Lathyris D, Cardona AF (2012) Human recombinant activated protein C for severe sepsis. Cochrane Database Syst Rev CD004388
48. Fernández JA, Xu X, Liu D et al (2003) Recombinant murine-activated protein C is neuroprotective in a murine ischemic stroke model. Blood Cells Mol Dis 30:271–6
49. Feistritzer C, Mosheimer BA, Kaneider NC et al (2004) Thrombin affects eosinophil migration via protease-activated receptor-1. Int Arch Allergy Immunol 135:12–16
50. Castellino FJ, Liang Z, Volkir SP et al (2002) Mice with a severe deficiency of the endothelial protein C receptor gene develop, survive, and reproduce normally, and do not present with enhanced arterial thrombosis after challenge. Thromb Haemost 88:462–472
51. Deane R, LaRue B, Sagare AP et al (2009) Endothelial protein C receptor-assisted transport of activated protein C across the mouse blood-brain barrier. J Cereb Blood Flow Metab 29:25–33
52. Cheng T, Petraglia AL, Li Z et al (2006) Activated protein C inhibits tissue plasminogen activator-induced brain hemorrhage. Nat Med 12:1278–1285

53. Liu D, Cheng T, Guo H et al (2004) Tissue plasminogen activator neurovascular toxicity is controlled by activated protein C. Nat Med 10:1379–1383
54. Buisson-Legendre N, Smith S, March L, Jackson C (2004) Elevation of activated protein C in synovial joints in rheumatoid arthritis and its correlation with matrix metalloproteinase 2. Arthritis Rheum 50:2151–2156
55. Xue M, March L, Sambrook PN et al (2007) Endothelial protein C receptor is overexpressed in rheumatoid arthritic (RA) synovium and mediates the anti-inflammatory effects of activated protein C in RA monocytes. Ann Rheum Dis 66:1574–1580
56. Mosnier LO, Zlokovic BV, Griffin JH (2007) The cytoprotective protein C pathway. Blood 109:3161–3172
57. Taylor FB, Stearns-Kurosawa DJ, Kurosawa S et al (2000) The endothelial cell protein C receptor aids in host defense against *Escherichia coli* sepsis. Blood 95:1680–1686
58. Kanazawa H, Yoshikawa T (2007) Up-regulation of thrombin activity induced by vascular endothelial growth factor in asthmatic airways. Chest 132:1169–1174
59. Yuda H, Adachi Y, Taguchi O et al (2004) Activated protein C inhibits bronchial hyperresponsiveness and Th2 cytokine expression in mice. Blood 103:2196–2204
60. Yasui H, Gabazza EC, Tamaki S et al (2001) Intratracheal administration of activated protein C inhibits bleomycin-induced lung fibrosis in the mouse. Am J Respir Crit Care Med 163:1660–1668
61. Tanaka A, Minoguchi K, Chen X et al (2008) Activated protein C attenuates leukocyte elastase-induced lung injury in mice. Shock 30:153–158
62. White B, Schmidt M, Murphy C et al (2000) Activated protein C inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor kappa B (NF-kappa B) and tumour necrosis factor alpha (TNF-alpha) production in the THP-1 monocytic cell line. Br J Haematol 110:130–134
63. Das J, Chen CH, Yang L et al (2001) A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat Immunol 2:45–50
64. Yang L, Cohn L, Zhang DH et al (1998) Essential role of nuclear factor kappa B in the induction of eosinophilia in allergic airway inflammation. J Exp Med 188:1739–1750
65. Borcea V, Morcos M, Isermann B et al (1999) Influence of ramipril on the course of plasma thrombomodulin in patients with diabetes mellitus. VASA Zeitschrift für Gefässkrankheiten 28:172–180
66. Fujiwara Y, Tagami S, Kawakami Y (1998) Circulating thrombomodulin and hematological alterations in type 2 diabetic patients with retinopathy. J Atheroscler Thromb 5:21–28
67. Isermann B, Vinnikov IA, Madhusudhan T et al (2007) Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. Nat Med 13:1349–1358
68. Bezuhly M, Cullen R, Esmon CT et al (2009) Role of activated protein C and its receptor in inhibition of tumor metastasis. Blood 113:3371–3374
69. Van Sluis GL, Niers TMH, Esmon CT et al (2009) Endogenous activated protein C limits cancer cell extravasation through sphingosine-1-phosphate receptor 1-mediated vascular endothelial barrier enhancement. Blood 114:1968–1973

Part II
Role of Proteases in Cardiovascular Diseases

Role of Calpains (Calcium-Dependent Proteases) on Coronary Artery Disease and Metabolic Syndrome

19

Brittany A. Potz, M. Ruhul Abid and Frank W. Sellke

Abstract

Coronary artery disease and metabolic syndrome together are two major causes of death in the United States and the numbers of people afflicted by these diseases are increasing around the world. Overactivation of calpain has been shown to contribute to cardiovascular disease and its associated comorbidities. Therefore, calpain inhibition may offer a novel potential medical therapy for treating not only coronary artery disease but also the individual aspects of the associated comorbidities.

Keywords

Coronary artery disease · Metabolic syndrome · Calpain · Pharmacologic therapy

1 Introduction

Metabolic syndrome is defined as having the presence of at least three of five risk factors for coronary artery disease (insulin resistance, obesity, hypertension, elevated triglycerides, and low high-density lipoprotein). Mortality from coronary vascular disease is increased in patients with metabolic syndrome [1]. The prevalence of metabolic syndrome in the adult US population is approximately 24% and is currently increasing [1]. Research has attempted to find a pharmacological therapy to reduce the incidence of coronary disease in patients with metabolic

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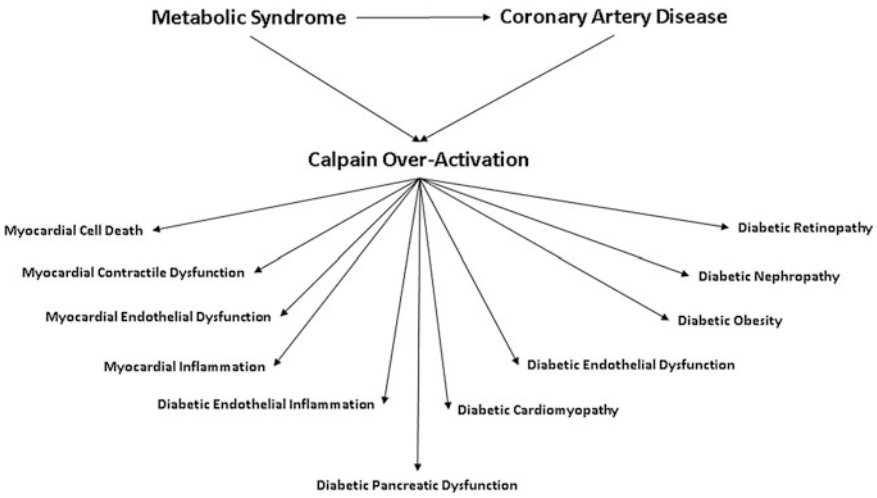

Fig. 1 Metabolic syndrome and coronary artery disease cause overactivation of calpain which has detrimental effects on various organs. Calpain is overactivated in various tissues during times of stress. This over activation of calpain has been shown to contribute to the organ dysfunction seen in patients with metabolic syndrome and coronary vascular disease

syndrome but so far the mechanism through which arterial disease is being accelerated in this group of patients has not been identified. Calpain is a protease whose overactivity has been found to promote coronary disease, insulin resistance, retinopathy, renal disease, and obesity. Overactivation of calpain has been shown to contribute to cardiovascular disease and its associated comorbidities [2–6] (Fig. 1). Moderate calpain inhibition in the setting of hypercholesterolemia and chronic myocardial ischemia has been found to improve proangiogenic protein expression, microvascular relaxation, and myocardial perfusion [5–7]. Therefore, calpain inhibition may be a mechanism through which to treat the coronary vascular disease associated with metabolic syndrome.

2 Calpains

Calpains are a class of calcium activated, intracellular, non-lysosomal cysteine proteases which play a key role in maintaining cellular homeostasis through their proteolytic activity [8]. In conjunction with all cysteine proteases, calpains contain the amino acid cysteine in their active site. The three major groups of cysteine proteases include caspases, cathepsins, and calcium-dependent calpains [4]. When not active, calpain is predominantly situated in the cytosol. Once calpain becomes activated it translocates to the membrane. Calpain works through cleavage of its substrates which results in proteolysis of several cytoskeletal proteins, membrane proteins, enzymes, cytokines, and transcription factors [9]. Excessive activation

of calpain has been found to be a part of the pathophysiology leading to several disorders including ischemia reperfusion injury, trauma, diabetes mellitus, coronary vascular disease, and inflammation [4, 8]. Therefore, an effective calpain inhibitor may serve as a beneficial potential medical therapy for patients suffering from a number of diseases including coronary artery disease and metabolic syndrome.

The calpain family is similar in many different species (ranging from fungi to humans). In mammals, there are 15 isoforms of calpain. Some calpains are expressed ubiquitously (calpains 1, 2, 4, 5, 7, and 10) while others are thought be found in specific tissues. For example, calpain 1 and 2 are thought to be located in endothelial cells, calpain 3 in skeletal muscle, calpain 6 in placenta, calpain 8 in smooth muscle, calpain 9 in the stomach, calpain 11 in the testes, calpain 12 in the skin and calpain 13 in the testes and lung [9, 10]. Ten of the 15 calpain isoforms are thought have been found to be expressed in the heart. Calpain 1 (U-calpain) and calpain 2 (m-calpain) are the most well studied of the calpains [4].

Among the 15 isoforms of calpains found in mammals, 14 are large subunit members (80-kDa catalytic subunit), and there is 1 small subunit member (30-kDA subunit). One endogenous inhibitor (calpastatin) also exists [9]. Calpains can be divided into two groups based on the structure of domain IV (Typical and Atypical). Typical calpains (1, 2, 3, 8, 9, 11, 12, 14) contain a penta-EF motif in domain IV that can bind calcium, the calpain small subunit or calpastatin. Only calpains 1, 2, and 9 have the ability to bind to another calpain small subunit and "dimerize" Atypical calpains (5, 6, 7, 10, 12, and 15) do not have a penta-EF motif in domain IV. They are therefore unable to dimerize or be inhibited by calpastatin [9].

In general, calpains contain four structural domains. In some of the typical calpains (1, 2, and 9), domain I is cleaved after calcium activation. This cleavage is termed autolysis and it leads to autoactivation of the protease. In calpain 10, a calpain found to be a key player in diabetes, domain 1 contains a mitochondrial targeting sequence. The function of domain 1 for the atypical calpains is unknown [9]. Domain II contains the catalytic active site. This active site is described as the "catalytic triad" which consists of cysteine, asparagine, and histidine. The catalytic active site is the functional unit of the protease and it is present in both typical and atypical calpains (except for calpain 6 which lacks any proteolytic activity). Domain II also has the ability to assist in autoactivation by binding two atoms of calcium ion. Domain III contains a phospholipid-binding motif and two Ca^{2+} binding sites. These structures are also present in both typical and atypical calpains (except Calpain 10). Domain III also plays a role in substrate recognition and works to regulate calpain activity through specific electrostatic interactions [9] (Fig. 2). As mentioned, domain IV contains the penta-EF domain that can bind calcium, the calpain small subunit or calpastatin. As mentioned, atypical calpains have the same general structure for domain I, II, and III but they do not have a penta-EF motif on domain IV. Due to these differences in structure, it is thought that atypical domains have different activation and inhibition requirements than typical domains [9].

Calpain 4 has a unique structure consisting of domain V and VI. Calpain 4 is a small calpain subunit that dimerizes with domain IV of the typical calpains. Domain V binds the C-terminus region of domain IV on the large typical calpain

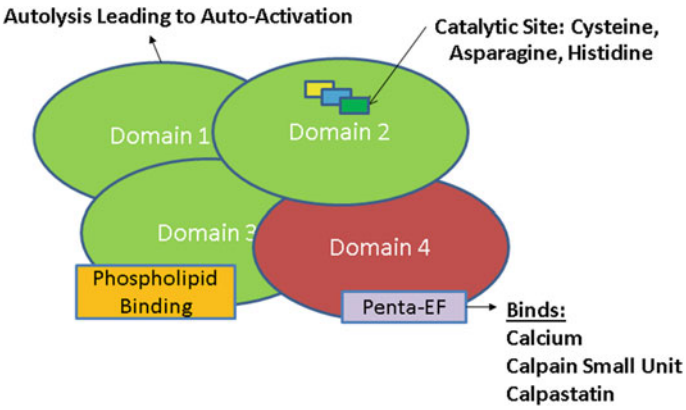

Fig. 2 Schematic of typical calpain structure. Most calpain isoforms contain four structural domains. Calpains are divided into two groups (typical and atypical) based on the structure of domain IV. Typical calpains contain a penta-EF motif in domain IV that can bind calcium, the calpain small subunit or calpastatin. Atypical calpains do not have a penta-EF motif in domain IV. Most calpain isoforms contain four structural domains. In some of the typical calpains, domain I is cleaved after calcium activation a term called autolysis which leads to autoactivation. Domain II contains the catalytic active site known as the "catalytic triad" of cysteine, asparagine and histidine. Domain III contains a phospholipid-binding motif and two Ca^{2+} binding sites

subunits. Domain VI contains a penta-EF domain that, similar to the penta-EF domain on domain IV of the typical calpains, can bind to dimerize with another calpain or bind calcium [9].

3 Calpastatin

Calpastatin is an endogenous protein calpain inhibitor. It is believed to be specific to typical calpains. Interestingly, calpastatin preferentially binds to calcium-activated calpains. This suggests that calpastatin inhibition does not interfere with basal calpain activity which is required for normal homeostasis [11]. Calpastatin has eight splice variants ranging in size from 18.7 to 85 kDA. The largest spice variant contains six domains (I, II, III, IV, L, and XL). Domains I–IV contain subdomains (A–C) that play an essential role calpain inhibition. These domains range in effectiveness as follows: I > IV > III > II. Domain XL contains three protein kinase A phosphorylation sites. The function of domain L is unknown. Calpastatin binds to domain II, IV or VI therefore it only inhibits typical calpains [9].

4 Calpain and Coronary Artery Disease

4.1 Calpain Overactivity and Myocardial Cell Death

In normal metabolic conditions, calpain 1 is active in myocytes. Calpain is responsible for preventing abnormal accumulation of proteins via the ubiquitin/proteasome protein degradation pathway. Overactivation of calpain is an important component of the mechanism that leads to detrimental mitochondrial permeability, aberrant apoptotic cell death, and ischemia/reperfusion injury in the heart [9].

Myocardial ischemia results in dysregulation of calcium homeostasis which leads to calpain overactivation and results in myocardial cell injury. Calpain activity is mainly regulated by calcium binding and that calcium binding is required for its proteolytic activity. It is also known that calcium overload causes auto proteolysis of the N-terminal peptide of calpain contributing to its overactivation [9]. However, studies also suggest that calpain activity is regulated by other means. For example, nicotinamide adenine dinucleotide phosphate (NADPH) is a co-factor used in anabolic reactions. NADPH oxidase has been found to induce calpain activation in stressed cardiomyocytes via upregulation of reactive oxygen species [11].

The mechanism through which calpain inhibition has a beneficial effect on the heart in the setting of stress is not fully known but there are many theories. One thought is that calpain cleaves proteins involved in apoptotic cell signaling including caspase 3, 7, 8, 9, 12, Bcl-2, Bcl-xl, Bid, Bax, and NF-kB [6, 11]. In the presence of a calpain inhibitor, the expression levels of these proteins are modulated and apoptosis is decreased [6, 11]. Additional calpain substrates include the transcription factors c-mos, YY1, c-fos, and c-jun and pro-apoptotic protein p53 [12, 13]. Specifically calpain cleaves the transcription factor YYI which decreases myogenic transcription [14].

Calpains also play a role in regulating mitochondrial function. In the setting of calcium overload (or mitochondrial stress), calpains located in the mitochondrion have been reported to cleave of Apoptosis Inducing Factor (AIF). This cleavage allows it to translocate to the nucleus where it functions to induce DNA degradation. In the presence of a calpain inhibitor, this process is reduced leading to reduced cardiac cell death [15].

4.2 Calpain Overactivity and Myocardial Contractile Dysfunction

Calpain overactivity has been found to cause cardiac dysfunction. Upregulation of calpain has been found to exacerbate the remodeling that takes place during myocardial infarction. This remodeling is associated with contractile dysfunction, chamber dilatation, and reduced overall function. Interestingly, one study found that calpain-mediated proteolysis is increased in the chronic phase (7 days or later) but

not in the acute phase (before 24 h) after a myocardial infarct and that calpain activity seemed to be located in the border zone of ischemia [8]. Calpain has also been implicated in the remodeling that leads to development of the atrial fibrillation seen in patients with diabetes and valvular heart disease [16]. There are a number of mechanisms through which this remodeling of cardiac tissue may occur.

In diseased myocardial tissue, ischemia induces changes to cytoskeletal, contractile, and myofibrillar proteins. Calpain has been found to cleave myofibrillar specific proteins including N-cadherin, connexin 43, troponin T, troponin I, troponin T, titin, α-fodrin, and desmin in cardiomyocytes [8, 11, 13, 17]. In the ischemic myocardial territory after a myocardial infarct, research has found that there is decreased expression of the important cytoskeletal protein N-cadherin and upregulation of molecular markers for cardiac hypertrophy and fibroses. Importantly, calpain is known to cleave N-cadherin and calpain overexpression is assocaited with decreased expression levels other cytoskeletal markers including B-catenin and connexin 43. These changes are attenuated in the presence of a calpain inhibitor [8].

Troponin I is a cardiac subunit of the troponin complex and is a component of cardiac muscle contraction. Calpain induces breakdown of troponin I in chronically ischemic cardiomyocytes [13]. This breakdown has been found to be associated with systolic dysfunction. Interestingly, overexpression of calpastatin and the presence of a calpain inhibitor have been found to improve this contractile dysfunction [13].

Intracoronary infusion of high calcium levels leads to overload-induced cardiac dysfunction through proteolysis of α-fodrin. A-fodrin is a member of the sarcolemma membrane. Calpain inhibition downregulates this proteolysis and protects the heart from ischemia/reperfusion injury possibly through a conformational change to the L-type calcium channel receptor on the cell membrane which results in protection of left ventricular function [2, 18].

4.3 Calpain Overactivity and Myocardial Endothelial Cell Dysfunction

Endothelial cells are integral to multiple homeostatic functions including blood vessel formation, coagulation, vascular tone, angiogenesis, inflammation, and cell–cell barrier. Calpain plays an integral role in modulating endothelial cell function [19]. Interestingly, calpain is known to cleave numerous cytoskeletal proteins of the endothelial cell including filamin, talin, paxillin, spectrin, vinculin, α tubulin, and vimentin [9, 11, 12, 20]. Angiotensin II is an effector peptide in the renin–angiotensin system which is often dysregulated in the setting of arterial disease and metabolic syndrome. Angiotensin II release induces matrix metalloproteinase-2 (MMP-2) and calpain-1 expression and activity in the smooth muscle of the arterial wall. It is thought that the calpain-1 overactivity induces MMP-2 activation which modulates extracellular matrix remodeling and causes increased collagen I and III production leading to vascular calcification [21]. Calpain is also thought to lead to the increase of transforming growth factor beta 1 (TGF-β1) expression which is a

major profibrotic factor causing the activation of downstream signaling pathways including collagen I synthesis [22].

Additionally, oxidized low-density lipoprotein (oxLDL), which is present in many patients with vascular disease and metabolic syndrome, induces apoptosis in endothelial cells leading to the development of atherosclerosis. OxLDL activates calpain which cleaves proteins on the cytoskeletal membrane and many apoptotic proteins. Inhibition of calpain reverses these negative effects in endothelial cells [23].

In hypoxic retinal endothelial cells, hypoxia-induced calpain activity has been found to lead to a disorganized actin cytoskeleton which leads to neovessel formations that are defective both functionally and architecturally. Moderate calpain inhibition has been found to reduce these architectural abnormalities, reduce vascular leakage, and ultimately lead to a more functional vessel that reduces overall hypoxia. It is thought that this calpain inhibition works by stabilizing and organizing the actin cytoskeletal in the retinal endothelial cells that are undergoing capillary morphogenesis. The result is improved actin cables within the new blood vessels [19, 24]. Interestingly, while moderate calpain inhibition has been shown to be beneficial, complete suppression results in nonfunctional endothelial cells. The optimal amount of calpain inhibition appears to be about 30–35% inhibition [19, 24].

Vascular Endothelial Grown Factor-A (VEGF-A) therapy has been found to induce angiogenesis, however this new vascular growth has been found to be highly abnormal in pathological settings. Interestingly, this vascular growth has been associated with increased calpain activity in endothelial cells. Calpain inhibition has been found to lead to a functional angiogenesis which reduces myocardial infarct size and improves contractile function and hemodynamics in large animal model of cardiac ischemia/reperfusion [25].

A large animal model of chronic myocardial ischemia in the setting of metabolic syndrome shows that calpain activity is increased in the ischemic myocardium. Calpain inhibition improves collateral dependent perfusion, improves endothelial-dependent microvessel relaxation and increases expression of proteins involved in vasodilatation. Furthermore, calpain inhibition promotes expression of the survival proteins, decreases oxidative stress and inhibits apoptotic pathways in animals with metabolic syndrome and chronic myocardial ischemia [5, 6].

4.4 Calpain Overactivity and Myocardial Inflammation

Calpain activity also promotes inflammatory pathways [20]. The proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) is released after myocardiali schemia/reperfusion injury possibly leading to cardiomyocyte apoptosis [11]. In the setting of sepsis, calpain activation is associated with overexpression of TNF-α and nitric oxide (NO) which leads to depressed myocardial contractile function [4, 11].

Calpain cleaves and leads to the degradation of IkBα. This degradation allows transcription factor NF-κB to translocate from the cytosol to the nucleus [20, 26]. NF-kB then binds to the promoter region of genes which serve as inflammatory mediators including interleukin-1 (Il-1), interleukin-6 (IL-6), vascular molecule-1

(VCAM-1), inducible nitric oxide synthase (iNOS), TNF-α and cyclooxygenase-2 (Cox-2) [4, 9].

In cultured rat cardiomyocytes, lipopolysaccharide induces calpain overactivity and is associated with increased TNF-α expression. The same study showed that calpastatin attenuates this TNF-α expression and improves cardiac function in a small animal model of endotoxemia [11].

5 Calpain and Metabolic Syndrome

Interestingly, calpain overactivation has been implicated in the pathogenesis of many of the aspects of metabolic syndrome including diabetes, obesity, diabetic nephropathy and diabetic retinopathy [10].

5.1 Calpain Overactivity and Diabetic-Induced Inflammatory Pathways

Overactivation of the inflammatory response causes damage to blood vessels and surrounding tissues. The endothelial cells of the vascular tract direct leukocytes to the site of injury. This is referred to as leukocyte trafficking. Pathologic leukocyte trafficking leads to vascular disease [20]. The inappropriate activation of calpain has been found to change platelet function, partially degrade proteins resulting in hyperaggregability and increase leukocyte trafficking in the microcirculation of NIDDM (noninsulin-dependent DM). Interestingly, inhibition of calpain activity attenuates leukocyte–endothelium interactions [10]. Therefore, inhibition of calpain activity may improve myocardial function by the means of the attenuation of tissue leukocyte infiltration and endothelial cell activation in the inflamed coronary vasculature [4].

5.2 Calpain Overactivity and Diabetic Pancreatic Dysfunction

Glucose metabolism triggers the entry of calcium into the pancreatic islets of Langerhans β-cells leading to exocytosis of insulin granules. Hyperglycemia in type 2 diabetes is caused by both impaired insulin secretion from these β-cells and insulin resistance. Patients with diabetes demonstrate not only a deficit in β-cell function, but they also experience an increase in β-cell apoptosis.

Calpain-10 is the first diabetic gene identified. Calpain 10 plays a role in the actin reorganization required for glucose-stimulated insulin release from the pancreatic β-cells. Calpain 10 regulates both insulin-stimulated glucose metabolism and insulin secretion in pancreatic islet cells [27]. Calpain inhibition has been found to lead to decreased insulin secretion [28, 29]. Calpain also plays a key role in the pathophysiology of beta-cell death in patients with type 2 diabetes [30]. It is

thought that elevated plasma-free fatty acids lead to β-cell apoptosis via an interaction between calpain-2 and endoplasmic reticulum stress-induced apoptotic factor (CHOP) [31].

5.3 Calpain Overactivity and Diabetic Cardiomyopathy

Diabetic cardiomyopathy is distinct from the coronary vascular disease that develops in patients with metabolic syndrome. Diabetic cardiomyopathy is defined as the ventricular dysfunction that occurs in patients with diabetes [32]. Calpain activity is increased in the acute inflammatory processes associated with the cardiac hypertrophy of diabetic cardiomyopathy [20]. Elevated glucose levels lead to increased levels of free fatty acids and growth factors. This results in dysregulation of substrate supply, calcium levels and lipid metabolism. Elevated glucose levels also increase production of reactive oxygen species causing oxidative stress leading to cardiomyocyte apoptosis. Reactive oxygen species also lead to stimulation of connective tissue growth factor which causes myocardial fibrosis and the formation of glycation end products. Together this leads to the increased cardiac stiffness associated with diabetic cardiomyopathy [32]. Calpain inhibition is associated with decreased (1) left ventricular hypertrophy, (2) perivascular inflammation and (3) fibrosis in myocardial tissue in mouse models of diabetes [33, 34].

5.4 Calpain Overactivity and Diabetic Endothelial Dysfunction

Calpain is overexpressed in diabetic vasculature. Inhibition of this overactivity attenuates the vascular dysfunction associated with chronic diabetic mellitus [20, 35].

High glucose levels lead to calcium overload and overactivation of calpain in endothelial cells. This is associated with the formation of reactive oxygen species, mitochondrial superoxide generation, cellular apoptosis, and endothelium-dependent vascular dysfunction in the setting of diabetes. Inhibition of calpain prevents glucose-induced reactive oxygen species expression and production of mitochondrial superoxide generation. This leads to decreased endothelial apoptosis and improved endothelium-dependent relaxation [35, 36].

A proposed mechanism of this beneficial effect on endothelial cells is that calpain inhibition leads to the increased expression of endothelial nitric oxide (NO) and attenuates expression of Intercellular Adhesion Molecule 1 (ICAM-1) and VCAM-1 in the diabetic vasculature [20, 37]. In diabetic rats there is decreased expression levels of endothelial heat shock protein 90 (hsp90) and endothelial nitric oxide synthase (enols). It is thought that calpain regulates hsp90 which serves as a dock site for eNOS activation by AKT. Inhibition of calpain activity in these vessels restores eNOS/hsp90 interaction, increases NO release and attenuates leukocyte trafficking [20, 37].

5.5 Calpain overactivity and Obesity

Calpain 10 plays an important role in regulating diabetic obesity. This obesity has been partially contributed to inadequate diet and sedentary lifestyle. However, one study has shown that in obese patients there is a significant association between having the genotype 1/1 (SNP19 of Calpain10) and the phenotype of excess weight. This relationship exists even in patients who have an active lifestyle [38]. Similarly, calpain expression is greater in obese rats with diabetes than in lean rats with diabetes. Exercise and weight reduction have been found to be associated with decreased calpain expression in the skeletal muscle of diabetic rats [10]. In fact, exercise reduces calpain mRNA expression in a rat model of diabetes. Finally, calpain activity is thought to reduce glucose transport turnover in skeletal muscle. Therefore, calpain may modulate muscle glucose equilibrium and mass [10].

5.6 Calpain overactivity and Diabetic Nephropathy

Diabetic nephropathy is the leading cause of end stage renal disease [27]. The kidney is responsible for maintaining ion, water, and metabolic substrate homoeostasis. Diabetic nephropathy is characterized by the loss of the charge barrier and the expansion of the molecular matrix of the glomerular basement membrane [27, 39]. Calpains degrade cytoskeletal proteins leading to increased plasma membrane permeability, water influx, and eventual necrosis in renal proximal tubular cells [9, 15, 40–43].

Calpain overactivation is also associated with acute renal cell death [15]. In the late phase of renal proximal tubule cell injury there is an influx of calcium into the cell. This influx leads to the overactivation of calpain. Inhibition of calpain blocks this renal proximal tubule cell death [40]. Interestingly, calpain 10 protein expression is decreased in the kidney of aging rats. This decreased expression is attenuated by caloric restriction. In humans, calpain 10 protein and mRNA levels decrease linearly in kidney samples with age as renal function decreases [44]. This contributes to the idea that basal calpain expression is necessary for appropriate cell function but overactivity of calpain in the setting of stressful cellular conditions is detrimental.

5.7 Calpain Overactivity and Ischemic Retinopathy

Retinopathy caused by endothelial dysfunction is a serious comorbidity of both coronary artery disease and metabolic syndrome. Diabetic retinopathy is a leading cause of blindness worldwide [45]. Patients with diabetic retinopathy develop diabetic macular edema, proliferative retinopathy, and retinal blood vessel dysfunction [46]. Calpain hyperactivation has been implicated in this retinal pathology. Calpain expression has been found to be increased in patients with diabetic retinopathy and cataract formation [47]. In a mouse model of ischemic retinopathy

vascular endothelial growth factor induced neovessel formation was not found to relieve hypoxia. However, in the same mouse model, the addition of moderate calpain inhibition normalized pathological retinal neovessel formation and relieved the underlying hypoxia. Calpain inhibition functions by (1) improving the architecture and function of the new vessels and (2) improving vascular regrowth. A mechanism through which calpain inhibition is thought to work is by improving the organization of the endothelial tau protein and actin cytoskeleton in the newly formed vessels [19, 24]. In cultured monkey cells, hypoxia/reoxygenation was found to lead to apoptotic rod, cone, and Muller cell death by activation of calpain. This retinal cell death was inhibited by adding a calpain inhibitor [48].

6 Conclusion

Coronary artery disease and metabolic syndrome together are two major causes of death in the United States and the number of people afflicted by these diseases is increasing around the world [10]. Calpain overactivity is implicated in the pathophysiology of coronary artery disease and its associated comorbidities (obesity, hypertension, hyperlipidemia, insulin intolerance). Currently, there are no medications available that will treat both the components of metabolic syndrome and its associated coronary artery disease. In fact, there is limited evidence that treatment with metformin has a beneficial effect on the risk factors for the development of atherosclerosis [49]. Therefore, calpain inhibition may offer a novel potential medical therapy for treating not only coronary artery disease but also the individual aspects of the associated comorbidities.

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References

1. Malik S (2004) Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. Circulation 110(10):1245–1250
2. Yoshikawa TMY, Hagihara H, Ohga Y et al (2004) S Calpain inhibitor-1 protects the rat heart from ischemia-reperfusion injury: analysis by mechanical work and energetics. AJP Heart Circ Physiol 288(4):H1690–H1698
3. Hayashi M, KasaiY, Kawashima S (1987) Preferential localization of calcium-activated neutral protease in epithelial tissues. Biochem Biophys Res Commun 148(2):567–574
4. Tissier S, Lancel S, Marechal X et al (2004) Calpain inhibitors improve myocardial dysfunction and inflammation induced by endotoxin in rats. Shock 21(4):352–357
5. Sabe AA, Potz BA, Elmadhun NY et al (2016) calpain inhibition improves collateral dependent perfusion in a hypercholesterolemic swine model of chronic myocardial ischemia. J Thorac Cardiovasc Surg 151(1):245–252

6. Potz BA, Sabe AA, Elmadhun NY et al (2015) Calpain inhibition decreases myocardial apoptosis in a swine model of chronic myocardial ischemia. Surgery 158(2):445–452
7. Potz BA, Sabe AA, Abid MR et al (2016) Calpains and coronary vascular disease. Circ J80(1):4–10
8. Kudo-Sakamoto Y, Akazawa H, Ito K et al (2014) Calpain-dependent cleavage of N-cadherin is involved in the progression of post-myocardial infarction remodeling. J Biol Chem 289(28):19408:19419
9. Smith MA, Schnellmann RG (2012) Calpains, mitochondria, and apoptosis. Cardiovasc Res 96(1):32–37
10. Pandurangan M, Hwang I, Orhirbat C, Jieun Y et al (2014) The calpain system and diabetes. Pathophysiology 21(2):161–167
11. Li X, Li Y, Shan L et al (2009) Over-expression of calpastatin inhibits calpain activation and attenuates myocardial dysfunction during endotoxaemia. Cardiovasc Res 83(1):72–79
12. Kubbutat MH, Vousden KH (1997) Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. Mol Cell Biol 17(460):468
13. Kositprapa C, Zhang B, Berger S et al (2000) Calpain-mediated proteolytic cleavage of troponin I induced by hypoxia or metabolic inhibition in cultured neonatal cardiomyocytes. Mol Cell Biochem 214(1–2):47–55
14. Walowitz JL, Bradley ME, Chen S et al (1998) Proteolytic regulation of the zinc finger transcription factor YY1, a repressor of muscle-restricted gene expression. J Biol Chem 273(12):6656–6661
15. Liu X, Rainey JJ, Harriman JF et al (2001) Calpains mediate acute renal cell death : role of autolysis and translocation. Am J Physiol Ren Phsiology 281(4):728–738
16. Zhao Y, Cui G, Zhou N et al (2016) Calpain-calcineurin-nuclear factor signaling and the development of atrial fibrillation in patients with valvular heart. Dis Diab J Diab Res 1:7
17. Streng AS, De Boer D, van der Velden J et al (2013) Posttranslational modifications of cardiac troponin T: an overview. J Mol Cell Cardiol (63):47–56
18. Takeshita D, Tanaka M, Mitsuyama S et al (2012) A new calpain inhibitor protects left ventricular dysfunction induced by mild ischemia-reperfusion in in situ rat hearts. J Physiol Sci 113:123
19. Hoang MV, Nagy JA, Fox JE et al (2010) Moderation of calpain activity promotes neovascular integration and lumen formation during VEGF-induced pathological angiogenesis. PLoS One 5(10)
20. Stalker TJ, Skvarka CB, Scalia R (2003) A novel role for calpains in the endothelial dysfunction of hyperglycemia. FASEB J 17(11):1511–1513
21. Jiang L, Zhang J, Monticone RE et al (2012) Calpain-1 regulation of matrix metalloproteinase 2 activity in vascular smooth muscle cells facilitates age-associated aortic wall calcification and fibrosis. Hypertension 60(5):1192–1199
22. Li FZ, Cai PC, Song LJ et al (2015) Crosstalk between calpain activation and TGF-β1 augments collagen-I synthesis in pulmonary fibrosis. Biochim Biophys Acta 1852(9):1796–1804
23. Porn-Ares MI, Saido TC, Andersson T et al (2013) Oxidized low-density lipoprotein induces calpain-dependent cell death and ubiquitination of caspase 3 in HMEC-1 endothelial cells. Biochem J 411(374):403–411
24. Hoang MV, Smith LE, Senger DR (2011) Calpain inhibitors reduce retinal hypoxia in ischemic retinopathy by improving neovascular architecture and functional perfusion. Biochim Biophys Acta 1812(64):997, 1549–1557
25. Khalil PN, Neuhof C, Huss R et al (2005) Calpain inhibition reduces infarct size and improves global hemodynamics and left ventricular contractility in a porcine myocardial ischemia/reperfusion model. Eur J Pharmacol 528(1–3):124–131
26. Letavernier E, Zafrani L, Perez J et al (2012) The role of calpains in myocardial remodelling and heart failure. Cardiovasc Res 96(1):38–45
27. Wan TT, Li XF, Sun YM et al (2015) Role of the calpain on the development of diabetes mellitus and its chronic complications. Biomed Pharmacother 74(187):190
28. Pablo P, Salazar AM, Burns AL et al (2014) Role of calpain-10 in the development of diabetes mellitus and its complications. Arch Med Res 45(2):103–115

29. Derbel S, Doumaguet C, Hubert D et al (2006) Calpain 10 and development of diabetes mellitus in cystic fibrosis. J Cyst Fibros 5(1):47–51
30. Huang CJ, Gurlo T, Haataja L et al (2010) Calcium-activated calpain-2 is a mediator of beta cell dysfunction and apoptosis in type 2 diabetes. J Biol Chem 285(1):339–348
31. Cui W, Ma J, Wang X et al (2013) Free fatty acid induces endoplasmic reticulum stress and apoptosis of beta-cells by Ca^{2+}/Calpain-2 pathways. PLoS One 8(3)
32. Falcão-Pires I, Leite-Moreira AF (2012) Diabetic cardiomyopathy: understanding the molecular and cellular basis to progress in diagnosis and treatment. Heart Fail Rev 17(3):325–344
33. Letavernier E, Perez J, Bellocq A et al (2008) Targeting the calpain/calpastatin system as a new strategy to prevent cardiovascular remodeling in angiotensin II-induced hypertension. Circ Res 102(6):720–728
34. Li Y, Ma J, Zhu H et al (2011) Targeted inhibition of calpain reduces myocardial hypertrophy and fibrosis in mouse models of type 1 diabetes. Diabetes 60(11):2985–2994
35. Chen B, Zhao Q, Ni R et al (2014) Inhibition of calpain reduces oxidative stress and attenuates endothelial dysfunction in diabetes. Cardiovasc Diabetol 13(88):1–12
36. Kumar SS, Kain V, Sitasawad SL (2012) High glucose-induced Ca^{2+} overload and oxidative stress contribute to apoptosis of cardiac cells through mitochondrial dependent and independent pathways. Biochim Biophys Acta 1820(7):907–920
37. Stalker TJ, Gong Y, Scalia R (2005) The calcium-dependent protease calpain causes endothelial dysfunction in type 2 diabetes. Diabetes 54 (4):1132–1140
38. Orozco AC, Muñoz AM, Velásquez CM et al (2014) Variant in CAPN10 gene and environmental factors show evidence of association with excess weight among young people in a Colombian population. Biomedica 34(4):546–555
39. Hovind P, Rossing P, Tarnow L et al (2001) Progression of diabetic nephropathy. Kidney Int 59(2):702–709
40. Waters SL, Sarang SS, Wang KKW et al (1997) Calpains mediate calcium and chloride influx during the late phase of cell injury 2. J Pharmacol Exp Ther 283(3):1177–1184
41. Harriman JF, Waters-williams, Chu DL et al (2000) Efficacy of novel calpain inhibitors in preventing renal cell death. J Pharmacol Exp Ther 294(3):1083–1087
42. Harriman JF, Liu XL, Aleo MD et al (2002) Endoplasmic reticulum Ca^{2+} signaling and calpains mediate renal cell death. Cell Death Differ 9(734):741
43. Liu X, Schnellmann RG (2003) Calpain mediates progressive plasma membrane permeability and proteolysis of cytoskeleton-associated paxillin, talin, and vinculin during renal cell death. J Pharmacol Exp Ther 304(1):63–70
44. Covington MD, Arrington DD, Schnellman RG (2009) Calpain 10 is required for cell viability and is decreased in the aging kidney. Am Physiol Ren Physiol 296(3):478–448
45. Ahsan H (2015) Diabetic retinopathy—biomolecules and multiple pathophysiology. Diabetes Metab Syndr 9(1):51–54
46. Lois N, McCarter RV, O'Neill C et al (2014) Endothelial progenitor cells in diabetic retinopathy. Front Endocrinol (Lausanne) 2014(5):44
47. Ahn YJ, Kim MD, Chung DK (2016) Calpain and caspase-12 expression in lens epithelial cells of diabetic cataracts. Am J Ophthalmol 167(31):37
48. Nakajima E, Hammond KB, Rosales JL et al (2011) Calpain, not caspase, is the causative protease for hypoxic damage in cultured monkey retinal cells. Investig Ophthalmol Vis Sci 52(10):7059–7067
49. El S, Rongen GA, De Boer RA et al (2011) The cardioprotective effects of metformin. Curr Opin Lipidol 22(445):453

Role of Matrix Metalloproteinases in the Development and Progression of Atherosclerosis

20

Karina Di Gregoli and Jason Lee Johnson

Abstract

Atherosclerosis underlies the majority of cardiovascular diseases and is accepted as a primary cause of mortality worldwide. Matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) perform complex roles during the progression and development of atherosclerosis and subsequent plaque instability. Proposed actions of MMPs include extracellular matrix remodeling alongside regulation of vascular cell proliferation, migration and apoptosis including cell types such as monocytes, macrophages and vascular smooth muscle cells. As such, a large body of evidence from both in vitro and in vivo studies has shown that individual MMPs and TIMPs are utilized by distinct cell types to regulate their behavior. Consequently, it is now accepted that some MMPs promote the growth and development of advanced atherosclerotic plaques in experimental models whilst others do not. Similarly, human genetic and pathological findings reveal some MMPs correlate with vulnerable atherosclerotic plaque phenotypes, whereas others associate with stable lesions. Furthermore, broad-spectrum MMP inhibition in both mouse and man has proved ineffective at protecting from atherosclerotic plaque progression and instability. Considering the divergent effects MMPs exert on atherosclerotic lesions, selectively targeting individual deleterious MMPs may serve as a more efficacious therapeutic strategy. For example, our recent data demonstrate that a selective MMP-12 inhibitor retards atherosclerotic plaque progression in the apolipoprotein E (Apoe) mouse atherosclerosis model, whilst also promoting plaque stabilization through reducing monocyte recruitment into plaques whilst augmenting fibrosis. Similar studies have been conducted assessing MMP-13

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inhibition. Accordingly, as our knowledge of the complex roles MMPs play during the development, progression and rupture of atherosclerotic plaques expands, new impetus is required for clinical trials evaluating the therapeutic potential of selective MMP inhibition, especially in the context of atherosclerosis.

Keywords
MMPs · Macrophages · Atherosclerosis · Vascular smooth muscle cells · Plaque rupture

1 Introduction

Atherosclerotic plaque development and progression is the principal underlying cause of cardiovascular disease, now reported as the primary cause of mortality and morbidity in developed countries [1]. Atherosclerosis is characterized by the accumulation of lipids (atheroma) and fibrous elements (sclerosis) within major arteries sustaining the heart (coronary arteries) and the brain (carotid arteries) [1]. It has been defined as a chronic, autoimmune-like disease, which develops in the presence of elevated circulating lipid levels [2]. Atherosclerotic plaque formation and progression is usually clinically silent. However, plaque rupture followed by thrombus formation and subsequent vessel occlusion can precipitate several clinical events including myocardial infarction, stroke, and peripheral vascular disease. The main underlying trigger for plaque rupture is ascribed to the loss of extracellular matrix (ECM) proteins, such as elastin and collagen, alongside decreased smooth muscle cells content within the plaques, which commonly corresponds to areas of marked inflammation [3]. These areas are characterized by the presence of foam cell macrophages, B- and T-cells, mast cells and smaller amounts of other white bloods cells [4]. Over the last quarter of a century, a large number of pathological and experimental studies have been conducted in this field to elucidate the pathophysiology of atherosclerotic lesion development, progression, and rupture. One of the principal goals in cardiovascular research is to find suitable targets to allow the development of new therapies, aimed at specific cell types or select molecules, attributed deleterious roles in atherosclerotic disease onset and progression. Matrix metalloproteinases (MMPs) have been implicated in all the stages of atherosclerosis, from plaque development to plaque rupture, through a large body of published work [5, 6]. Elevated expression levels of MMPs including MMP-1, -2, -7, -8, -9, -11, -12, -13, and -14 have been identified in human atherosclerotic plaques (see Table 1) [7–15]. Moreover, the majority of increased MMP expression within atherosclerotic lesions is specifically located to macrophages-rich areas (shoulder regions and around the lipid core) suggesting that macrophage-derived MMPs may

Table 1 MMPs up-regulated in human atherosclerotic plaques compared to normal arteries

MMP#	Cell type	Principal reference
MMP-1	Mø, VSMC, EC, and T cell	[7]
MMP-2	Mø and VSMC	[8]
MMP-3	Mø, VSMC, EC, and T cell	[9]
MMP-7	Mø	[10]
MMP-8	Mø, VSMC, and EC	[11]
MMP-9	Mø, VSMC, EC, and T cell	[7]
MMP-11	Mø, VSMC, and EC	[12]
MMP-12	Mø	[10]
MMP-13	Mø	[13]
MMP-14	Mø and VSMC	[14]
MMP-16	Mø and VSMC	[15]

Mø Macrophage, *VSMC* vascular smooth muscle cell, *EC* endothelial cell

play a key role in atherosclerotic plaque progression. Furthermore, considering MMPs have been proposed to induce plaque rupture in a dual manner: by direct degradation of ECM proteins (such as elastin and collagen) and by promoting the death of vascular smooth muscle cells (VSMCs), the main cell type responsible for ECM synthesis within the plaque [16]; many studies have focused their attention on inhibitors of MMPs as a therapeutic strategy to stabilize and perhaps induce regression of atherosclerosis [17].

2 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs), also named matrixins, are a large family of at least 24 proteolytic enzymes having a role is several physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, wound healing and remodeling. For these reasons, MMPs are involved in several pathologies including cancer progression and atherosclerosis, highlighting them as key therapeutic targets for medical research. The MMPs are multi-domain enzymes capable to degrade both ECM components and several non-ECM molecules. MMPs share similarity with two other proteinase families: ADAMs (a disintegrin and metalloproteinase family) and ADAMTSs (ADAM with thrombospondin motifs), as they all contain a zinc atom and a conserved methionine in the catalytic domain, and collectively consist the Metzincin family [18]. Due to its destructive capabilities, MMP activity is tightly regulated by a family of endogenous inhibitors named the tissue inhibitors of metalloproteinases (TIMPs) that, together with MMPs, are responsible the maintenance and balance of ECM homeostasis during physiological and pathological conditions. This equilibrium contributes to multiple other processes such as differentiation, growth, inflammation, migration, and apoptosis due in part to the

capacity of MMPs to target non-ECM substrates. MMP and TIMP expression, which exhibit distinct tissue/cell, temporal and spatial differences, are tightly regulated by numerous molecules including inflammatory cytokines, hormones, growth factors, and physical cell–cell and cell–matrix interactions (as reviewed by [19]).

3 MMP Classification and Structure

MMPs share some structural homology. Usually they present:

(i) ***Signal peptide*** *at the N-Terminus*: a hydrophobic sequence of 18–30 residues responsible for intracellular trafficking from the Golgi apparatus to the cell membrane which is cleaved during secretion [19].
(ii) ***Pro-peptide***: a highly conserved motif responsible for pro-MMP latency [19].
(iii) ***Catalytic Domain***: which contains a zinc-binding site responsible for the endopeptidase activity of MMPs.
(iv) ***Hinge Domain***: known also as a linker peptide, it is situated between the catalytic domain and the hemopexin-like domain. It stabilizes the collagenolytic activity due to the presence of several proline residues.
(v) ***Hemopexin-like Domain***: positioned at the C-terminus, it has strong sequence similarity to the serum protein hemopexin and an extensive range of roles amongst diverse MMPs [18].

Nevertheless, there are notable structural differences between MMPs that confer diverse biological properties. Based on their domain organization, MMPs can be divided into six groups [20] (see Fig. 1).

(i) MMPs presenting the pro-domain and the catalytic domain. This group includes MMP-7 and MMP-26, also known as Matrilysins.
(ii) MMPs containing the pro-domain, the catalytic domain, the hinge domain, and the hemopexin-like domain. This group contains several MMPs with diverse substrate specificities; MMP-1, -8, -13 (Collagenases), MMP-3, -10, -11 (Stromelysins), MMP-12 (Metalloelastase), MMP-20 (Enamelysin), MMP-19, MMP-22, and MMP-28.
(iii) MMPs comprising the pro-domain, a catalytic domain containing fibronectin-like repeats, the hinge domain and the hemopexin-like domain. In this group there are MMP-2 and -9, also named Gelatinases for their affinity to degrade gelatin.
(iv) The transmembrane type I MMPs are a group of MMPs that present, together with the pro-domain, the catalytic domain, hinge domain and the hemopexin-like domain, a transmembrane domain at the N-terminus. This domain allows this group of MMPs to localize on to the cell membrane,

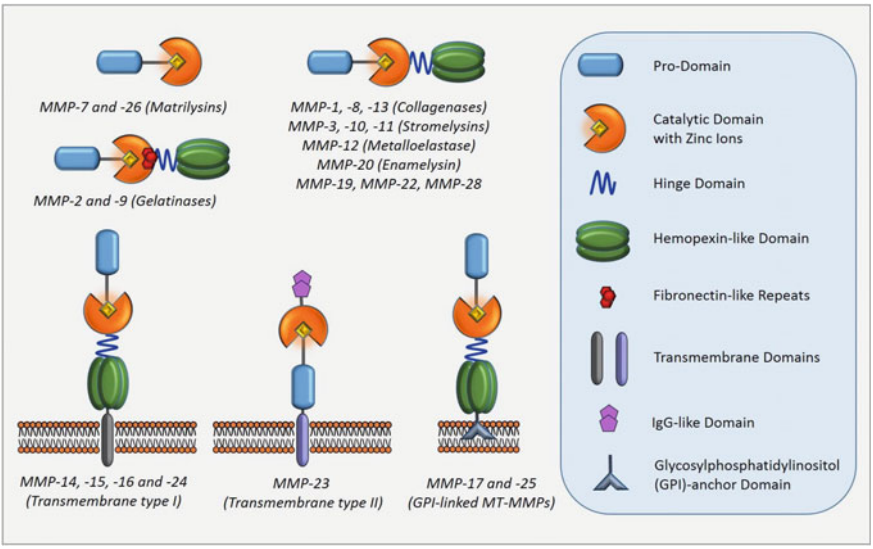

Fig. 1 Domain structure for the major classes of MMPs. Diagram illustrating the differing domain structures of the major MMP classes, including the pro-domain, catalytic domain with the active site zinc (Zn) bound to cysteine residues within this domain and "cysteine switch-residue" in the pro-domain, the hinge domain, the hemopexin-domain, the fibronectin-like type II repeats, and in some cases for MT-MMPs, either a transmembrane domain or a glycophosphatidylinositol (GPI)-anchor domain

projecting toward the extracellular space. This characteristic facilitates pericellular matrix degradation, and hence plays a prominent role in directing cell migration [21]. This group includes MMP-14, -15, -16, and -24.

(v) The transmembrane type II MMPs include one single MMP, MMP-23. Differently from the type I, type II MMP has the transmembrane domain at the C-terminus and additionally present an IgG-like domain.

(vi) There is a third group of membrane-type MMPs localized to the cell membrane via glycosylphosphatidyl inositol (GPI) anchor on the N-terminus. This group includes MMP-17 and -25.

4 MMP Activation and Inhibition

MMPs are produced as zymogens; the interaction between the pro-domain and the catalytic domain keep the MMP in an inactive conformation. In order to achieve full activation of these enzymes the pro-domain has to be cleaved, an essential regulatory step toward MMP activation [19]. Activation of the biologically inactive MMP (pro-form) follows a multi-step sequence of events also known as 'stepwise

activation'. First, the cleavage of a 'proteinase susceptible bait region' through the action of plasma or bacterial proteinases, destabilise the cysteine–Zn^{2+} negative interaction within the pro-domain, resulting in a MMP intermediate form. To achieve full activation, the *in-trans* activity of other intermediary or active MMPs is required in order to fully remove the inhibitory pro-domain [19]. Some MMPs are completely activated intracellularly by furin or other pro-protein convertases and then either translocated to the cell membrane or secreted, as active enzymes. MMP activity is closely regulated by endogenous inhibitors (such as TIMPs and α2-macroglobulin), proteolysis, or internalization and recycling [19]. In addition, other proteins with the ability to inhibit MMPs have been described, including the reversion-inducing cysteine-rich protein with Kazal motifs (RECK), tissue factor pathway inhibitor-2 (TFPI-2), and the pro-collagen C-terminal proteinase enhancer (PCPE). Nevertheless, TIMPs are the most potent endogenous inhibitors of MMPs and therefore considered key regulators in the physiological regulation of MMP activity. Four TIMPs have been identified within vertebrates, TIMP-1, -2, -3, and -4, which exhibit diverse inhibitory actions toward different MMP family members [22]. For example, TIMP-1 has a poor inhibitory effect on MMP-9, -14, -15, -16, and -24. TIMPs also harbor the ability to inhibit members of both the ADAM and ADAMTS family of proteinases [22]. TIMP expression is tissue specific and, similarly to MMPs, is finely regulated during development and remodeling. Most of their inhibitory capacity has been ascribed to the N-Terminal domain since it is able to form, when isolated, a stable native molecule with an inhibitory effect on MMPs [23]. TIMPs are normally secreted proteins, however they can localize to the cell membrane associated with membrane protein, including several MT-MMPs. Regulation of the equilibrium between MMPs and TIMPs is essential in homeostasis. Alterations in this balance can trigger patho-physiological conditions associated with atypical ECM turnover of the matrix and/or dysregulation of processes involved in wound healing, remodeling and inflammation. Cardiovascular disease, cancer, arthritis, and neurological disorders are all examples of pathologies where an imbalance between MMPs and TIMPs is apparent [23].

5 MMPs and Atherogenesis

5.1 Early Stage: Pathological Intimal Thickening

Atherogenesis is a multi-step sequence of events that leads to atherosclerotic plaque formation on the luminal side of major arteries. In humans, it is a process that develops and evolves over several decades, beginning with early lesions that can occur during childhood. The development of atherosclerotic lesions is dependent on multiple risk factors which can be genetic or modifiable in nature, including hypercholesterolemia, smoking, high blood pressure, sedentary lifestyle, and diabetes [24]. In man, the first event that generally occurs branch points within major arteries, is the formation of an early lesion, commonly termed pathological intimal

thickening (although sometimes referred to as fatty streaks) [25]. These early lesions are characterized by the accumulation of lipid-laden macrophages (also called foam-cell macrophages due to their appearance under the microscope), within a preexisting smooth muscle and ECM-rich intima. Raised levels of low density lipoprotein (LDL) within the blood stream, alongside alterations in shear stress, the presence of free radicals such as reactive oxygen species (ROS), or exposure to infection-related pathogens, can result endothelial damage. A damaged endothelium is subject to inflammatory activation that triggers expression of adhesion molecules (including vascular cell adhesion molecule-1; VCAM-1) that mediate leukocyte recruitment. Such adhesion molecules facilitate a transient contact, allowing leukocyte rolling at the luminal surface of the vessel wall. After firm adhesion to the endothelium, monocytes and lymphocytes transmigrate, penetrating into the *tunica intima* (the innermost layer of the artery) driven by a chemoattractant gradient, through molecules including monocyte chemoattractant protein-1 (MCP-1). Monocyte recruitment is considered a fundamental process during early lesion formation and atherosclerosis onset. In order to invade the arterial wall, monocytes are required to degrade the physical barrier represented by the ECM, therefore it is essential that they possess potent protease activity. Human monocytes constitutively express several MMPs and TIMPs including MMP-8, MMP-12, MMP-19, TIMP-1, and TIMP-2. Whereas upon adhesion and in response to inflammatory stimuli they can be activated and subsequently upregulate the expression of MMP-1, MMP-3, MMP-10, and MMP-14 via the stimulation of MAP kinase and NF-κB transcription factors [26]. Specifically, MMP-14 expression and activity is necessary for monocyte endothelial transmigration and invasion this process can be blocked by MMP-14 inhibition either by a neutralizing antibody, recombinant TIMP-2 or gene silencing in vitro [27–30]. Moreover, MMP14 inhibition of activated circulating monocytes by a neutralizing antibody, retards monocyte recruitment into existing atherosclerotic lesions in mouse model of atherosclerosis [30]. Once within the intima monocytes differentiate into macrophages in response to several stimuli, in particular the Colony Stimulating Factors (CSFs), which concomitantly drives the expression of scavenger receptors, growth factors, cytokines giving rise to a survival impulse [31]. Accordingly, recently recruited monocytes at sites within the artery where lipoproteins have accumulated and after their differentiation into macrophages, begin to internalize the modified lipoproteins from the surrounding areas, through their cell-surface scavenger receptors. This process results in transformation of macrophages into foam-cells macrophages (FCMs) [32]. Macrophages also interact with T-cells which are also recruited to developing plaques, inducing an array of immune and inflammatory responses including the expression of adhesion molecules, MMPs, cytokines, apoptotic mediators, and pro-thrombotic activities, which collectively drive an inflammatory amplification loop and therefore promoting atherosclerotic plaque progression [33]. Intra-plaque macrophages and foam-cells express a diverse range of MMPs and TIMPs [34]. In particular, it has been observed that MMP-7, MMP-9, and TIMP-3 expression is induced during macrophage differentiation in vitro [26], whereas MMP-1, MMP-3, and MMP-12 expression can be induced in macrophages

in response to inflammatory mediators and cytokines [34]. However, within atherosclerotic plaques, most macrophages are lipid-laden and therefore characterized as foam cell macrophages, therefore the accumulation of lipid within macrophages may exert the most dominant role on MMP and TIMP regulation. Indeed, immunohistochemistry (a valuable method for studying atherosclerotic plaque composition alongside macrophages and foam-cell macrophages in situ), has revealed the expression of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-11, MMP-12, MMP-13, MMP-14, and MMP-16 in foam-cell macrophages within lesions (Table 1). Moreover, the detection of matrix proteolysis (as assessed by in situ zymography) [35, 36] alongside the presence of cleaved collagen fibres at the corresponding sites [13], suggest that at least some of the MMPs expressed within these regions are in an active form. Recent direct evidence has demonstrated that MMP-1, MMP-3, and MMP-14 are over-expressed in foam-cell macrophages isolated from human plaques [37, 38] and that in vivo generated foam-cell macrophages from cholesterol-fed rabbits display heightened expression of MMP-1, MMP-3, MMP-12, and MMP-14, when compared to nonlipid-laden macrophages [39]. Additionally, increased expression and activity of MMP-14 in a sub-population of rabbit foam cell macrophages was associated with a concomitant loss of TIMP-3 expression, resulting in their increased invasiveness, proteolytic activity, and susceptibility to undergo apoptosis [40]. Therefore, the presence of foam-cell macrophage-derived MMPs within the atherosclerotic lesions may direct disease progression and predict future clinical outcome.

5.2 MMPs and Atherosclerotic Plaque Progression

One of the principal processes that determines the progression of a pathological intimal thickening toward the development of a mature atherosclerotic plaque is the formation of a fibrous cap which overlies a recently formed lipid core. The fibrous cap originates following the organized migration of vascular smooth muscle cells (VSMCs) from the *tunica media* (the middle layer of the artery that lies between the tunica intima on the inside and the tunica externa on the outside) toward the arterial lumen, alongside the continual growth of VSMCs already resident within the intimal thickening. The VSMCs overlying the lipid core proliferate and produce fibrous ECM components, such as collagen and fibronectin, providing a structural barrier that separates the thrombogenic lipid core from the blood stream, providing strength and hemodynamic stability to the developing lesion. In addition to the production of MMPs and TIMPs, macrophages within the plaque secrete numerous cytokines and mediators such as platelet-derived growth factor (PDGF), heparin-binding epidermal growth factor (EGF), and insulin-like growth factor (IGF) that facilitate the mobilization and recruitment of VSMCs [41]. In order to expedite their migration, VSMCs need to release themselves from their cell–cell and cell–matrix interactions which act as physical barriers—dysregulated MMP activity directs this process. Studies investigating the role of MMP activity on VSMC migration have focused their attention principally on MMP-2, MMP-9, and

MMP-14, presumably due to these MMPs harboring the ability to degrade the basement membrane protein collagen type IV [16]. MMP-2 has been shown to augment VSMC migration across basement membrane proteins in vitro [42, 43], and MMP-9 overexpression can also promote the migratory capacity of isolated VSMCs [44]. A comprehensive study revealed MMP-14 is critical during VSMC migration, facilitating VSMCs to first degrade and then infiltrate 3-D collagen barriers, including the arterial wall [45]. These findings have been substantiated through subsequent in vivo studies utilizing genetically modified mice lacking either MMP-2, MMP-9, or MMP-14 which all reported attenuated VSMC migration. MMP-3 has also been shown to promote VSMC migration, predominantly through the activation of MMP-9 [46]. A role for the collagenase MMP-13 in VSMC migration has been documented, induced through an Akt-ERK dependent pathway [47]. Additionally, MMPs can contribute to VSMC migration by cleavage of nonmatrix substrates. For instance, MMP-14 can cleave and shed from the cell membrane CD44 (a cell surface hyaluronan receptor), promoting increased cell motility [48]. Conversely, intact CD44 can serve as a docking station for secreted MMP-7 and MMP-9 on the VSMC membrane, localizing their proteolytic activity to the cell surface and potentially facilitating cell migration [49, 50]. MMP activity has also been linked to VSMC proliferation. Similarly to migration, proliferation requires the removal of cell–cell and cell–matrix interactions, which otherwise exert an inhibitory effect on cell division. Cadherins are a family of adhesion proteins involved in cell–cell contact regulation of proliferation, and have recently been identified as new substrates of MMP activity [51]. Cadherins also serve as membrane receptors for cell signaling transduction and their cleavage by MMPs can modulate β-catenin nuclear translocation (a member of Wnt/wingless signaling pathway), known to activate the transcription of several pro-proliferative genes [51]. Indeed, MMP-7 and MMP-12 can induce N-cadherin cleavage/shedding, and through β-catenin signaling, promote VSMC proliferation [52]. Taken together, MMP-directed VSMC growth and migration participates in fibrous cap formation and therefore plays a prominent role in atherosclerotic plaque formation—but is considered beneficial as it protects the developing plaque from instability.

5.3 MMPs and Unstable Plaque Development and Rupture

During atherosclerotic plaque progression, foam-cell macrophages undergo cell death via apoptosis or necrosis. Macrophage and foam-cell death promotes the establishment and expansion of an extracellular lipid-rich core, which is highly thrombogenic and harbors the potential to destabilise advanced atherosclerotic plaques. Unsurprisingly, a role for MMPs has been suggested in macrophage and foam-cell apoptosis [53]. For example, macrophage and foam-cell susceptibility to undergo apoptosis can be retarded by inhibition of MMP-12 or MMP-14 activity, through use of a selective inhibitor or a neutralizing antibody, respectively [40, 54]. Accordingly, TIMP-2 and TIMP-3 can both reduce foam-cell macrophage apoptosis, in part through inhibition of MMP-14-dependent N-Cadherin cleavage [30,

40, 55]. Furthermore, loss of TIMP-2 in vivo increases the number of apoptotic macrophages within atherosclerotic plaques of hypercholesteroleamic mice, whilst TIMP-1 depletion had no effect [30]. Therefore, uncontrolled cell death and subsequent lipid core enlargement contributes to plaque progression and is associated with plaque instability and propensity to rupture [56]. As can know be appreciated, the stability of atherosclerotic plaques is determined by its composition, specifically the VSMC and fibrous ECM content (which reflects the thickness and strength of the fibrous cap), together with the macrophage and lipid content (which reveals the size and possible rate of expansion of the lipid core) [57]. The vast majority of acute coronary events originate from atherosclerotic plaque instability, notably the rupture of the fibrous cap and ensuing leakage of the thrombogenic lipid core into the arterial lumen, triggering thrombosis [58]. As such, clinical symptoms, including myocardial infarction or stroke, are often a result of plaque rupture and subsequent thrombus formation, resulting in distal impairment of blood flow or embolization and consequent ischemia. Indeed, fibrous cap disruption leads to the exposure of highly thrombogenic plaque constituents such as tissue factor (TF), lipids or modified collagen fragments. The interaction of these factors with the flowing blood results in thrombus formation by triggering activation of the coagulation cascade [59]. As earlier discussed, mature atherosclerotic plaques are characterized by a soft and highly thrombogenic lipid-rich core and associated macrophages infiltration, which is encapsulated by a VSMC and ECM-rich fibrous cap that provides structural integrity [56]. However, atherosclerotic lesions are heterogeneous in nature and can vary in fibrous cap thickness and lipid-core size; different combinations of these two variables results in different plaque phenotypes and susceptibility to rupture, with diverse clinical outcome. Pathological studies of human coronary artery atherosclerotic plaques permit histological discrimination between stable and unstable (also defined as vulnerable or rupture-prone) atherosclerotic plaques [25, 56]. Characteristically, stable plaques constitute of a thick fibrous cap, particularly enriched with VSMCs and collagen, and a small lipid core with reduced macrophage accumulation. Plaques with thick caps (and nonstenotic) are generally clinically silent. However, unstable plaques typically present with a large lipid-core and a thin fibrous cap [5] and are characterized by a high number of macrophages plus other inflammatory cell types; and are commonly referred to as thin-cap fibro-atheromas (TCFAs). Histological and in vivo animal studies have demonstrated that inflammation (T-cells and macrophages) not only promotes atherosclerotic plaque formation, but also contributes to plaque destabilization [5]. Foam-cell macrophages produce several pro-inflammatory cytokines such as IFNγ, which in addition to mediating inflammatory responses, can also inhibit VSMC collagen synthesis [60]. As discussed earlier, within atherosclerotic plaques, macrophages are a major source of proteolytic enzymes, especially MMPs, alongside a plethora of inflammatory mediators in plaques, and are therefore considered to play a fundamental in ECM degradation (i.e., collagen and elastin) and subsequent fibrous cap weakening [5]. There is also evidence that macrophage-dependent MMP activity can promote fibrous cap thinning through potentiating VSMC death. For example, MMP activity may detrimentally affect VSMC survival by disrupting

cell–matrix interactions and therefore attenuating matrix-dependent survival signals [16]. The cleavage of death signal molecules and their receptors from the cell surface can trigger apoptosis through autocrine and paracrine processes. A number of MMPs including MMP-7 are able to generate the pro-apoptotic factor TNFα through proteolytic cleavage of pro-TNFα [16]. In addition, MMP-7 can cleave Fas ligand (FasL) to its pro-apoptotic soluble form (sFasL) [61]. Interestingly, MMP-7, TNFα, and FasL all co-localize in human atherosclerotic plaques, suggesting this apoptotic triptych may contribute to formation and expansion of the lipid-rich core [62]. The lateral aspects of an atherosclerotic plaque (commonly termed the shoulder regions) are the sites considered most prone to rupture, and reside between the lipid-rich core and the thinnest part of the fibrous cap. These areas are characterized by accumulations of macrophages and particularly foam-cell macrophages, alongside notable neovascularization [63]. Pathological studies of human atherosclerotic plaques have revealed that macrophages, VSMCs, lymphocytes, and endothelial cells within the rupture-prone shoulder regions express MMP-1, MMP-3, and MMP-9 [64]. MMP-2, MMP-7, MMP-11, MMP-12, MMP-13, MMP-14, and MMP-16 levels are also elevated at the shoulder regions of unstable plaques [8, 10, 12–15], where increased MMP activity and substrate cleavage has also been documented [7, 11, 13, 36]. These findings suggest that MMP expression and activity is strongly associated atherosclerotic plaque progression, highlighting them as therapeutic targets and predictors of clinical outcome in patients with advanced atherosclerotic disease.

6 MMPs as Therapeutic Target for Atherosclerosis

Animal models of atherosclerosis have been widely utilized to investigate the pathogenesis of plaque formation, progression, and instability with the objective of identifying novel therapeutic targets to prevent the clinical manifestations associated with atherosclerosis. Rabbits have been used in multiple studies as several strains spontaneously develop atherosclerotic plaques when fed a high-fat diet. However, most atherosclerosis in vivo studies are conducted in mouse models, despite the fact that wild-type mice atherosclerosis-resistant even after prolonged periods of high fat feeding. The two most commonly used mouse models of atherosclerosis are genetically modified where a key gene of the cholesterol transport pathway has been deleted; these genes are Apolipoprotein E (Apoe) or LDL receptor (Ldlr), thus rendering them hypercholesteroleamic [65–67]. These mice develop atherosclerotic lesions throughout the arterial tree including similar sites to plaque formation in man, even when fed on a normal diet [68]. However, on consumption of a high-fat diet, atherogenesis is significantly accelerated in either Apoe or Ldlr deficient mice, although the hypercholesterolemia is more marked in the Apoe deficient animals. There are also striking similarities in lesion development and progression between both models and humans, as early lesions closely resemble fatty streaks whilst longer periods of high-fat feeding produce complex

advanced lesions [68, 69]. A multitude of studies have been conducted in Apoe deficient mice (and to a lesser degree Ldlr knockout mice) to investigate the roles of MMPs in atherosclerosis. Such studies have utilized genetically modified mice which have global or cell specific knockout or over-expression of a single MMP/TIMP, or treated with a potential therapeutic agent that targets select or all MMPs. These studies have aided the elucidation of potential pathogenic roles of multiple MMPs and TIMPs in atherosclerosis plaque progression and stability (summarized in Table 2).

Table 2 Effect of MMP modulation on atherosclerotic plaque development and stability in animal models

Modulation	Model (species)	Site	Size	VSMCs	Mø	References
MMP-1 Tg	Apoe KO (Ms)	Aorta and root	↓	↔	↔	[70]
MMP-2 KO	Apoe KO (Ms)	Aorta and root	↓	↓	↔	[74]
MMP-3 KO	Apoe KO (Ms)	Aorta, BCA	↑/↑	↓/ND	↓/↔	[76, 77]
MMP-7 KO	Apoe KO (Ms)	BCA	↔	↑	↔	[77]
MMP-8 KO	Apoe KO (Ms)	Aorta	↓	↔	↓	[80]
MMP-9 KO	Apoe KO (Ms)	Aorta, BCA	↓/↑	ND/↓	↓/↑	[77, 78]
MMP-9 Tg	Apoe KO (Ms)	Arch, collar	↔	↔	↔	[71, 72]
MMP-12 KO	Apoe KO (Ms)	Aorta, BCA	↔/↓	↔/↑	↔/↓	[77, 78]
MMP-12 Tg	kbt:JW (Rb)	Aorta	↑	↑	↑	[73]
MMP-13 KO	Apoe KO (Ms)	Root	↔	↔	↔	[81]
MMP-14 KO	Ldlr KO (Ms)	Root	↔	↔	↔	[82]
Non selective MMP inhibitor	Ldlr or Apoe KO (Ms)	Aorta, BCA	↔	↔	↔	[87–89]
MMP-12 inhibitor	Apoe KO (Ms)	Aorta, BCA and root	↓	↑	↓	[54]
MMP-13 inhibitor	Apoe KO (Ms)	Carotid	↔	↔	↔	[92]
miR-24 inhibitor (*MMP-14 over-expression*)	Apoe KO (Ms)	BCA	↑	↔	↑	[84]
TIMP-1 KO	Apoe KO (Ms)	Aorta and root	↔/↓	↔/ND	↔/↑	[30, 83]
TIMP-2 KO	ApoE KO (Ms)	BCA	↔	↓	↑	[30]
TIMP-3 KO	ApoE KO (Ms)	Aorta and root	↓	ND	↓	[85]
TIMP-1 RAd	Apoe KO (Ms)	BCA and root	↓/↔	ND/↔	↓/↔	[55, 86]
TIMP-2 RAd	Apoe KO (Ms)	BCA	↓	↑	↓	[55]
miR-712 inhibitor (*TIMP-3 over-expression*)	Apoe KO (Ms)	Carotid, aorta and aortic arch	↓	ND	↓	[103]

Results of in vivo animal studies evaluating the effects of modulating matrix metalloproteinases (MMP) or tissue inhibitors of MMPs (TIMP) on atherosclerotic plaque size and cellular composition, using transgenic (Tg) or adenoviral (Rad) over-expression, gene knockout (KO), pharmacological inhibitors of MMPs, or microRNA (miR) inhibitors.
VSMC Vascular smooth muscle cell, *Mø* macrophage, *BCA* brachiocephalic artery, *root* aortic root, (↓) decreased, (↑) increased, (↔) no change, and *ND* not determined

6.1 Overexpression Studies

Dissimilar to humans, mice do not constitutively express MMP-1. However, when human MMP-1 was over-expressed exclusively in macrophages of Apoe deficient mice, an unexpected reduction in plaque size, and collagen content was observed [70]. In contrast, macrophage-specific over-expression of pro-MMP-9 did not affect atherosclerotic plaques [71]. However, in a collar-induced carotid artery model of atherosclerosis in Apoe deficient mice, local over-expression of pro-MMP-9 promoted intra-plaque hemorrhage [72]. Furthermore, the transplantation of transduced stem cells permitting the over-expression of an active form of MMP-9, increased plaque progression [71]. Similarly, macrophage-specific over-expression of active of MMP-12 in transgenic rabbits, augmented plaque size and markers of inflammation [73], suggesting that MMP-9 and MMP-12 activation may promote atherosclerosis progression (summarized in Table 2).

6.2 Knockout Studies

A number of studies have been conducted in Apoe knockout mice which are also deficiency for a single MMP or TIMP to elucidate the roles for a selected MMP/TIMP in atherosclerotic plaque formation. Interestingly, these studies have revealed that MMPs exert protective and detrimental effects on atherosclerosis. For instance, Mmp2 knockout mice exhibits a reduction in plaque size, attributed in part to a reduction in VSMC content and implying that plaque stability is compromised in the absence of MMP-2 [74], as MMP-2 is necessary for VSMC migration and intimal formation in vivo [75]. Equally, although Mmp3 deletion resulted in larger aortic and brachiocephalic plaques, a reduction in VSMC number was observed, associated with an increased number of buried fibrous layers (a surrogate marker of plaque instability), suggesting that MMP-3 may promotes plaque stability through promoting VSMC accumulation [76, 77]. Indeed, after carotid ligation, Mmp3 knockout mice shows decreased VSMC migration and associated neo-intimal formation [46]. Likewise Mmp9 deficient mice develop larger plaques with an increased number of buried fibrous layers, and a concomitant reduction in VSMC content [77]. Taken together with findings from an arterial injury model demonstrating MMP-9 promotes VSMC migration and concomitant neo-intimal formation [46], these studies support a beneficial role for MMP-9 in promoting plaque stability through favouring VSMC accumulation. However in another study assessing aortic plaques in mmp9 KO studies, revealed no change in plaque area and a reduced number of lesions, although they also suggested that plaque VSMC number was lowered in Mmp9 deficient animals [78].

In contrast, an increase in VSMC content was reported within the brachiocephalic plaques of mmp7 knockout mice [77], in agreement with a pro-apoptotic role attributed to MMP-7 on VSMCs [79], and indicating a deleterious role for this MMP in atherosclerosis. Mmp8 deficient mice show reduced plaque size and macrophage number but increased collagen content, suggesting MMP-8 promotes

plaque progression [80]. Several lines of evidence have strongly indicated a detrimental role for MMP-12 in plaque progression and instability. Mmp12 deficiency results in smaller brachiocephalic artery plaques, with a reduced number of macrophages and buried fibrous layers [77] and diminished indicators of elastin degradation [78]. Moreover, the ratio between macrophages and VSMCs within the plaques of Mmp12 knockout mice are favourably increased toward VSMCs, in part due to reduced monocyte/macrophage invasion and apoptosis [54], suggesting MMP-12 promotes plaque instability. Further studies in a rabbit model of atherosclerosis have confirmed a detrimental role for MMP-12 in atherosclerosis [73]. Collectively these findings strongly imply that MMP-12 promotes plaque progression and instability. Whilst exerting moderate effects on plaque size, macrophage and VSMC content, mice with either global deletion of MMP-13 or macrophage-specific loss of MMP-14 exhibit a marked increase in plaque fibrillar collagen content, indicating significant roles for these two MMPs in collagen degradation and consequently plaque destabilization [81, 82].

Consequently, these studies imply that some MMPs, such as MMP-2, -3, and -9, exert a protective effect on atherosclerotic plaque progression by promoting VSMC growth and consequent fibrous cap formation. Contrastingly, other MMPs including MMP-7, -8, -12, -13, and -14, may promote plaque instability via increased inflammation, matrix degradation and apoptosis, therefore increasing the propensity of plaque rupture (summarized in Table 2).

6.3 Inhibitor Studies

Although Timp1 deficient mice had larger aortic atherosclerotic lesions with enhanced MMP activity, accompanied with heightened macrophage and lipid content [83], Timp2 knockout mice display a more unstable plaque phenotype than their Timp1 deficient counterparts [30]. These plaques were characterized by increased necrotic core size, buried fibrous layers, macrophage number, and macrophages undergoing apoptosis and proliferation; they also presented reduced collagen and VSMC content, indicative of reduced stability [84]. Equally, Timp3 deficiency in Apoe knockout mice increased lesion size within the aorta and at the aortic root, associated with heightened macrophage accumulation [85]. As therefore expected, systemic over-expression of TIMP-1 or TIMP-2, via adenovirus-mediated gene transfer, reduced lesion development and plaque progression in Apoe knockout mice [55, 86]. Additionally, gene transfer long term over-expression of TIMP-2, but not TIMP-1, arrested progression of established plaques at least in part by constraining monocyte/macrophage invasion and their susceptibility to apoptosis [55]. These findings lend robust support for MMP inhibition as a therapeutic strategy to prevent plaque progression and destabilization. Accordingly, there have been numerous endeavours by academia and industry to develop and deploy synthetic inhibitors of MMPs. Nevertheless, broad spectrum inhibitors containing zinc-chelating groups (such as thiol or hydroxamate groups, or tetracycline derivates) have given inconsistent results. Administration of hydroxamic acid-based,

nonselective MMPs inhibitors to either Lldr knockout or Apoe KO deficient mice revealed no beneficial effects on plaque development or progression [87, 88]. Likewise, doxycycline (a commonly used antibiotic with known nonspecific MMP inhibitory ability) failed to prevent atherosclerosis development in Apoe deficient mice [89]. Furthermore, two independent, randomized, double-blind, and placebo controlled clinical trials involving treatment with of patients with symptomatic coronary and carotid artery disease with doxycycline, did not favourably influence plaque composition or clinical outcome [90, 91]. In contrast, use of a highly selective MMP-12 inhibitor, RXP470.1, arrested plaque progression and improved stability in Apoe deficient mice with preexisting atherosclerosis [54]. In response to MMP-12 inhibition, lesions exhibited reduced lipid core expansion and macrophage apoptosis, increased VSMC to macrophage ratio, decreased plaque calcification, and attenuated elastin degradation [54]. These results, together with a reduction of buried fibrous layers, reflected those observed previously in Mmp12/Apoe double knockout mice [77]. Similarly, a second study where a highly specific MMP-13 inhibitor was deployed, revealed intra-plaque collagenolytic activity was reduced and associated with preservation of fibrillar collagen content within plaques [92], mirroring the effects also witnessed in Mmp13 deficient mice [81]. Taken together, considering broad spectrum MMP inhibition failed to exert any striking benefits on atherosclerosis in either clinical or animal studies, whilst selective MMP inhibition was beneficial in mice, support the tenet that individual MMPs (and therefore possibly TIMPs) play divergent roles in disease development and progression. Consequently, these proof-of-principle studies in mice provide an incentive to translate selective MMP inhibitor treatment into human atherosclerotic patients (summarized in Table 2).

6.4 microRNA Regulation of MMPs

microRNAs (miRs) are small noncoding RNA molecules of approximately 22 nucleotides in length which have the ability to post-transcriptionally regulate gene expression. They are transcribed by polymerase II in the nucleus and are initially produced as primary miRs (pri-miRs). These pri-miRs are processed to miR precursors (pre-miRs) by RNAse III Drosha before they can be exported to the cytoplasm where they are eventually processed into mature and biologically functional miRs through the action of another RNAse III named Dicer. Mature miRs are able to target and bind the 3′ untranslated regions (3′-UTR) of messenger RNA (mRNA) and modulate their expression. It has been predicted that miRs may modulate up to 90% of mammalian genes and therefore play fundamental roles in regulating cellular function [93]. Numerous studies have recently investigated that ability of miRs to regulate MMP expression. For instance, the 3′UTR region of MMP-1 is targeted and regulated by miR-526 [94], which could have potential implications for collagenolysis in plaques. MMP-2 is a direct target of miR-29b, and consequently miR-29b over-expression can inhibit VSMC migration and proliferation and subsequent neo-intimal formation [95]. MMP-3 has been

identified and validated as a putative target of miR-93, as such miR-93 over-expression in human nucleus pulposus cells promoted collagen accumulation [96]. In osteocarcinoma, miR-539 plays a key role in inhibiting osteosarcoma cell invasion and migration through regulating MMP-8 expression in osteosarcoma cells [97]. Direct targeting of MMP-9 by miR-204 can suppress trophoblast-like cell invasion, contributing to the development of pre-eclampsia [98]. Furthermore, MMP-9 expression may also be indirectly regulated by miR-497 via direct targeting of MEK1 in endothelial cells, in response to the anti-hyperlipidaemia drug probucol [99]. Another study conducted in chondrocytes revealed miR-320 was able to directly target and down-regulate MMP-13 expression during chondrogenesis, and vice versa during inflammatory osteoarthritis [100]. Numerous microRNAs have been identified and predicted to target and regulate the expression of MMP-14. miR181a-5p has been shown to downregulate MMP-14 expression by direct targeting of its 3′UTR, reducing cancer cell invasion, and angiogenesis [101]. Similarly, miR-9 can inhibit neuroblastoma cell invasion, metastasis, and angiogenesis by targeting of MMP-14 mRNA [102]. With regard to atherosclerosis, MMP-14 protein expression can be directly modulated by miR-24 in macrophages in response to GM-CSF, influencing the invasive capacity of macrophages [84]. Consequently, administration of a locked nucleic acid (LNA)-miR-24 inhibitor significantly exacerbated preexisting atherosclerosis in Apoe deficient mice, through increasing lesion size, macrophage content, and MMP-14 expression [84]. Moreover, miR-24 expression correlate with more stable coronary plaques in humans, suggesting a protective role of miR-24 in atherosclerosis, presumably through decreased MMP-14 activity [84]. Finally, miR-712 is induced in response to shear stress in endothelial cells of Apoe deficient mice, and through targeting of TIMP-3, exerts a detrimental effect on atherosclerosis via promotion of endothelial inflammation [103]. Collectively, these findings suggest modulation of microRNA may serve as a valuable tool for regulating MMP and TIMP expression in atherosclerosis, highlighting these important and powerful molecules as significant targets for medical intervention.

7 Conclusions

Through studies conducted in isolated cells and animal models, alongside human pathological and clinical findings, MMPs have been established to play a fundamental role in cardiovascular diseases, especially the development, progression, and rupture of atherosclerotic plaques. Seminal studies utilizing animal models that permit genetic modulation of individual MMPs or TIMPs has allowed the identification of specific roles select MMPs exert on all vascular cell types, and the ensuing significance to atherosclerosis. Collectively, this large body of work has demonstrated that modulation of MMP expression/activity can halt and even reverse atherosclerosis, whilst disappointingly broad-spectrum MMP inhibition does not replicate these effects, presumably due perturbation of both beneficial and

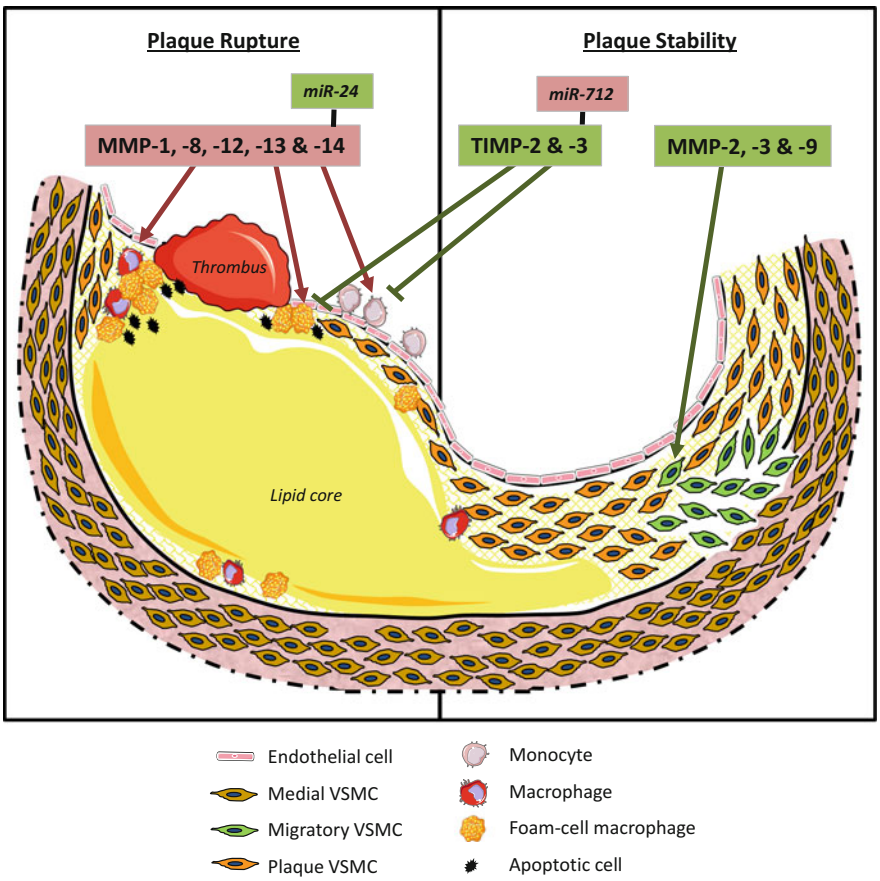

Fig. 2 Divergent roles of MMPs in atherosclerotic plaque progression and stability. Hypothetical model of the potential beneficial and deleterious roles of MMPs and TIMPs during atherosclerotic plaque progression and rupture. Matrix metalloproteinase (MMP)-2, -3, and -9 can facilitate vascular smooth muscle cell (VSMC) migration from the media into the developing atherosclerotic plaque where they participate in fibrous cap formation and maintenance, thus promoting plaque stability. In opposition, MMP-1, MMP-8, MMP-12, MMP-13, and MMP-14 can degrade extracellular matrix proteins present in the fibrous cap whilst also encouraging the recruitment and accumulation of monocytes and macrophages, and their subsequent susceptibility to apoptosis as foam cells—which collectively enhance lipid core expansion, thrombogenicity of the plaque, and thinning of the fibrous cap. Consequently, the stability of the plaque is compromised and vulnerable to plaque rupture and ensuing thrombus formation. More recently, microRNA (miR) have been identified which can regulate MMP and TIMP expression/activity, exerting direct effects on plaque progression

detrimental MMPs (summarized in Fig. 2). Therefore, it is acknowledged and necessary to generate and deploy inhibitors which harbor restricted specificity towards selected MMPs, including MMP-12 and MMP-13, to facilitate transition to man—particularly in the context of atherosclerotic plaque stabilization.

References

1. Virmani R, Burke AP, Farb A et al (2006) Pathology of the vulnerable plaque. J Am Coll Cardiol 47(8):C13–C18
2. Libby P (2012) Inflammation in Atherosclerosis. Arterioscler Thromb Vasc Biol 32 (9):2045–2051
3. Davies MJ (1996) Stability and instability: two faces of coronary atherosclerosis—the Paul Dudley White lecture 1995. Circulation 94(8):2013–2020
4. Hansson GK (2005) Mechanisms of disease—inflammation, atherosclerosis, and coronary artery disease. New Engl J Med 352(16):1685–1695
5. Newby AC (2007) Metalloproteinases and vulnerable atherosclerotic plaques. Trends Cardiovasc Med 17(8):253–258
6. Dollery CM, Libby P (2006) Atherosclerosis and proteinase activation. Cardiovasc Res 69 (3):625–635
7. Galis ZS, Sukhova GK, Lark MW et al (1994) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 94:2493–2503
8. Li Z, Li L, Zielke HR et al (1996) Increased expression of 72-kd type IV collagenase (MMP-2) in human aortic atherosclerotic lesions. Am J Pathol 148:121–128
9. Henney AM, Wakeley PR, Davies MJ et al (1991) Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. Proc Natl Acad Sci 88:8154–8158
10. Halpert I, Sires UI, Roby JD et al (1996) Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. Proc Natl Acad Sci USA 93(18):9748–9753
11. Herman MP, Sukhova GK, Libby P et al (2001) Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling. Circulation 104:1899–1904
12. Schönbeck U, Mach F, Sukhova GK et al (1999) Expression of stromelysin-3 in atherosclerotic lesions: regulation via CD40-CD40 ligand signaling in vitro and in vivo. J Exp Med 189:843–853
13. Sukhova GK, Schonbeck U, Rabkin E et al (1999) Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. Circulation 99:2503–2509
14. Rajavashisth TB, Xu X-P, Jovinge S et al (1999) Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques. Circ Res 99:3101–3109
15. Uzui H, Harpf A, Liu M et al (2002) Increased expression of membrane type 3-matrix metalloproteinase in human atherosclerotic plaque. Role of activated macrophages and inflammatory cytokines. Circ Res 106:3024–3030
16. Johnson JL (2007) Matrix metalloproteinases: influence on smooth muscle cells and atherosclerotic plaque stability. Expert Rev Cardiovasc Ther 5(2):265–282
17. Newby AC, Johnson JL (2005) Genetic strategies to elucidate the roles of matrix metalloproteinases in atherosclerotic plaque growth and stability. Circ Res 97(10):958–960
18. Murphy G (2010) Fell-Muir lecture: metalloproteinases: from demolition squad to master regulators. Int J Exp Path 91(4):303–313
19. Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69(3):562–573
20. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases. Circ Res 93:827–839
21. Itoh Y, Seiki M (2006) MT1-MMP: a potent modifier of pericellular microenvironment. J Cell Physiol 206(1):1–8
22. Baker AH, Edwards DR, Murphy G (2002) Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 115:3719–3727

23. Brew K, Nagase H (2010) The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. BBA-Mol Cell Res 1803(1):55–71
24. Golledge J, Norman PE (2010) Atherosclerosis and abdominal aortic aneurysm: cause, response, or common risk factors? Arterioscler Thromb Vasc Biol 30(6):1075–1077
25. Virmani R, Kolodgie FD, Burke AP et al (2000) Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 20(5):1262–1275
26. Reel B, Sala-Newby GB, Huang WC et al (2011) Diverse patterns of cyclooxygenase-independent metalloproteinase gene regulation in human monocytes. Br J Pharmacol 163 (8):1679–1690
27. Matias-Roman S, Galvez BG, Genis L et al (2005) Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium. Blood 105(10):3956–3964
28. May AE, Schmidt R, Bulbul O et al (2005) Plasminogen and matrix metalloproteinase activation by enzymatically modified low density lipoproteins in monocytes and smooth muscle cells. Thromb Haemostasis 93(4):710–715
29. Sithu SD, English WR, Olson P et al (2007) Membrane-type 1-matrix metalloproteinase regulates intracellular adhesion molecule-1 (ICAM-1)-mediated monocyte transmigration. J Biol Chem 282(34):25010–25019
30. Di Gregoli K, George SJ, Jackson CL et al (2016) Differential effects of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 on atherosclerosis and monocyte/macrophage invasion. Cardiovasc Res 109(2):318–330
31. Di Gregoli K, Johnson JL (2012) Role of colony-stimulating factors in atherosclerosis. Curr Opin Lipidol 23(5):412–421
32. Libby P, Ridker PM, Hansson GK (2009) Inflammation in atherosclerosis: from pathophysiology to practice. J Am Coll Cardiol 54(23):2129–2138
33. Schonbeck U, Libby P (2001) CD40 signaling and plaque instability. Circ Res 89:1092–1103
34. Newby AC (2012) Matrix metalloproteinase inhibition therapy for vascular diseases. Vasc Pharmacol 56(5–6):232–244
35. Galis ZS, Sukhova GK, Libby P (1995) Microscopic localisation of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. Faseb J 9:974–980
36. Johnson JL, Jackson CL, Angelini GD et al (1998) Activation of matrix-degrading metalloproteinases by mast cell proteases in atherosclerotic plaques. Arterioscler Thromb Vasc Biol 18:1707–1715
37. Monaco C, Gregan SM, Navin TJ et al (2009) Toll-like receptor-2 mediates inflammation and matrix degradation in human atherosclerosis. Circulation 120(24):2462–2469
38. Johnson JL, Jenkins NP, Huang W-C et al (2014) Relationship of MMP-14 and TIMP-3 expression with macrophage activation and human atherosclerotic plaque vulnerability. Mediat Inflamm 2014(ID276457):17
39. Thomas AC, Sala-Newby GB, Ismail Y et al (2007) Genomics of foam cells and nonfoamy macrophages from rabbits identifies arginase-1 as a differential regulator of nitric oxide production. Arterioscler Thromb Vasc Biol 27:571–577
40. Johnson JL, Sala-Newby GB, Ismail Y et al (2008) Low tissue inhibitor of metalloproteinases 3 and high matrix metalloproteinase 14 levels defines a subpopulation of highly invasive foam-cell macrophages. Arterioscler Thromb Vasc Biol 28(9):1647–1653
41. Libby P, Ridker PM, Maseri A (2002) Inflammation and atherosclerosis. Circulation 105:1135–1143
42. Pauly RR, Passaniti A, Bilato C et al (1994) Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. Circ Res 75:41–54

43. Haque NS, Fallon JT, Pan JJ et al (2004) Chemokine receptor-8 (CCR8) mediates human vascular smooth muscle cell chemotaxis and metalloproteinase-2 secretion. Blood 103 (4):1296–1304
44. Mason DP, Kenagy RD, Hasenstab D et al (1999) Matrix metalloproteinase-9 overexpression enhances vascular smooth muscle migration and alters remodeling in the injured rat carotid artery. Circ Res 85:1179–1185
45. Filippov S, Koenig GC, Chun T-H et al (2005) MT1-matrix metalloproteinase directs arterial wall invasion and neointima formation by vascular smooth muscle cells. J Exp Med 5:663–671
46. Johnson JL, Dwivedi A, Somerville M et al (2011) Matrix metalloproteinase (MMP)-3 activates MMP-9 mediated vascular smooth muscle cell migration and neointima formation in mice. Arterioscler Thromb Vasc Biol 31(9):e35–e44
47. Yang SW, Lim L, Ju S et al (2015) Effects of matrix metalloproteinase 13 on vascular smooth muscle cells migration via Akt–ERK dependent pathway. Tissue Cell 47(1):115–121
48. Kajita M, Itoh Y, Chiba T et al (2001) Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J Cell Biol 153(5):893–904
49. Yu WH, Woessner JF, McNeish JD et al (2002) CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Gene Dev 16(3):307–323
50. Johnson C, Galis ZS (2004) Matrix metalloproteinase-2 and -9 differentially regulate smooth muscle cell migration and cell-mediated collagen organization. Arterioscler Thromb Vasc Biol 24(1):54–60
51. George SJ, Beeching CA (2006) Cadherin: catenin complex: a novel regulator of vascular smooth muscle cell behaviour. Atherosclerosis 188(1):1–11
52. Dwivedi A, Slater SC, George SJ (2009) MMP-9 and -12 cause N-cadherin shedding and thereby {beta}-catenin signalling and vascular smooth muscle cell proliferation. Cardiovasc Res 81(1):178–186
53. Haider N, Hartung D, Fujimoto S et al (2009) Dual molecular imaging for targeting metalloproteinase activity and apoptosis in atherosclerosis: molecular imaging facilitates understanding of pathogenesis. J Nucl Cardiol 16(5):753–762
54. Johnson JL, Devel L, Czarny B et al (2011) A selective matrix metalloproteinase-12 inhibitor retards atherosclerotic plaque development in apolipoprotein E-knockout mice. Arterioscler Thromb Vasc Biol 31(3):528–535
55. Johnson JL, Baker AH, Oka K et al (2006) Suppression of atherosclerotic plaque progression and instability by tissue inhibitor of metalloproteinase-2: involvement of macrophage migration and apoptosis. Circulation 113(20):2435–2444
56. Davies MJ (2000) The pathophysiology of acute coronary syndromes. Heart 83(3):361–366
57. Davies MJ (2001) Going from immutable to mutable atherosclerotic plaques. Am J Cardiol 88(4A):2F–9F
58. Falk E, Nakano M, Bentzon JF et al (2013) Update on acute coronary syndromes: the pathologists' view. Eur Heart J 34(10):719–728
59. Davì G, Patrono C (2007) Platelet activation and atherothrombosis. New Engl J Med 357 (24):2482–2494
60. Amento EP, Ehsani N, Palmer H et al (1991) Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 11(5):1223–1230
61. Powell WC, Fingleton B, Wilson CL et al (1999) The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol 9(24):1441–1447
62. Geng Y-J, Libby P (2002) Progression of atheroma: a struggle between death and procreation. Arterioscler Thromb Vasc Biol 22:1370–1380
63. de Boer OJ, van der Wal AC, Becker AE (2000) Atherosclerosis, inflammation, and infection. J Pathol 190:237–243

64. Newby AC (2005) Dual role of matrix metalloproteinases (matrixins) in neointima formation and atherosclerotic plaque rupture. Physiol Rev 85(1):1–31
65. Plump AS, Smith JD, Hayek T et al (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 71:343–353
66. Nakashima Y, Plump AS, Raines EW et al (1994) ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb Vasc Biol 14 (1):133–140
67. Ishibashi S, Brown MS, Goldstein JL et al (1993) Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 92(2):883–893
68. Jackson CL, Bennett MR, Biessen EAB et al (2007) Assessment of unstable atherosclerosis in mice. Arterioscler Thromb Vasc Biol 27:714–720
69. Van der Laan PA, Reardon CA, Getz GS (2004) Site specificity of atherosclerosis. Arterioscler Thromb Vasc Biol 24:12–22
70. Lemaître V, O'Byrne TK, Borczuk AC et al (2001) ApoE knockout mice expressing human matrix metalloproteinase-1 in macrophages have less advanced atherosclerosis. J Clin Invest 107(10):1227–1234
71. Gough PJ, Gomez IG, Wille PT et al (2006) Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. J Clin Invest 116(1):59–69
72. de Nooijer R, Verkleij CJ, von der Thuesen JH et al (2006) Lesional overexpression of matrix metalloproteinase-9 promotes intraplaque hemorrhage in advanced lesions, but not at earlier stages of atherogenesis. Arterioscler Thromb Vasc Biol 26:340–346
73. Liang J, Liu E, Yu Y et al (2006) Macrophage metalloelastase accelerates the progression of atherosclerosis in transgenic rabbits. Circulation 113:1993–2001
74. Kuzuya M, Nakamura K, Sasaki T et al (2006) Effect of MMP-2 deficiency on atherosclerotic lesion formation in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 26(5):1120–1125
75. Kuzuya M, Kanda S, Sasaki T et al (2003) Deficiency of gelatinase a suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia. Circulation 108 (11):1375–1381
76. Silence J, Lupu F, Collen D et al (2001) Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. Arterioscler Thromb Vasc Biol 21:1440–1445
77. Johnson JL, George SJ, Newby AC et al (2005) Divergent effects of matrix metalloproteinases -3, -7, -9 and -12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. Proc Natl Acad Sci USA 102(43):15575–15580
78. Luttun A, Lutgens E, Manderveld A et al (2004) Loss of matrix metalloproteinase-9 or matrix metalloproteinase-12 protects apolipoprotein E-deficient mice against atherosclerotic media destruction but differentially affects plaque growth. Circulation 109(11):1408–1414
79. Williams H, Johnson JL, Jackson CL et al (2010) MMP-7 mediates cleavage of N-cadherin and promotes smooth muscle cell apoptosis. Cardiovasc Res 87(1):137–146
80. Laxton RC, Hu Y, Duchene J et al (2009) A role of matrix metalloproteinase-8 in atherosclerosis. Circ Res 105(9):921–929
81. Deguchi JO, Aikawa E, Libby P et al (2005) Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques. Circulation 112(17):2708–2715
82. Schneider F, Sukhova GK, Aikawa M et al (2008) Matrix metalloproteinase-14 deficiency in bone marrow-derived cells promotes collagen accumulation in mouse atherosclerotic plaques. Circulation 117(7):931–939
83. Silence J, Collen D, Lijnen HR (2002) Reduced atherosclerotic plaque but enhanced aneurysm formation in mice with inactivation of the tissue inhibitor of metalloproteinase-1 (TIMP-1) gene. Circ Res 90:897–903

84. Di Gregoli K, Jenkins N, Salter R et al (2014) MicroRNA-24 regulates macrophage behavior and retards atherosclerosis. Arterioscler Thromb Vasc Biol 34(9):1990–2000
85. Stöhr R, Cavalera M, Menini S et al (2014) Loss of TIMP3 exacerbates atherosclerosis in ApoE null mice. Atherosclerosis 235(2):438–443
86. Rouis M, Adamy C, Duverger N et al (1999) Adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-1 reduces atherosclerotic lesions in apolipoprotein E-deficient mice. Circulation 100:533–540
87. Prescott MF, Sawyer WK, Von Linden-Reed J et al (1999) Effect of matrix metalloproteinase inhibition on progression of atherosclerosis and aneurysm in LDL receptor-deficient mice overexpressing MMP-3, MMP-12, and MMP-13 and on restenosis in rats after balloon injury. Ann NY Acad Sci 878:179–190
88. Johnson JL, Fritsche-Danielson R, Behrendt M et al (2006) Effect of broad-spectrum matrix metalloproteinase inhibition on atherosclerotic plaque stability. Cardiovasc Res 71(3):586–595
89. Manning MW, Cassis LA, Daugherty A (2003) Differential effects of doxycycline, a broad-spectrum matrix metalloproteinase inhibitor, on angiotensin II-induced atherosclerosis and abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 23:483–488
90. Brown DL, Desai KK, Vakili BA et al (2004) Clinical and biochemical results of the metalloproteinase inhibition with subantimicrobial doses of doxycycline to prevent acute coronary syndromes (MIDAS) pilot trial. Arterioscler Thromb Vasc Biol 24:733–738
91. Axisa B, Loftus IM, Naylor AR et al (2002) Prospective, randomized, double-blind trial investigating the effect of doxycycline on matrix metalloproteinase expression within atherosclerotic carotid plaques. Stroke 33:2858–2864
92. Quillard T, Tesmenitsky Y, Croce K et al (2011) Selective inhibition of matrix metalloproteinase-13 increases collagen content of established mouse atherosclerosis. Arterioscler Thromb Vasc Biol 31(11):2464–2472
93. Ha M, Kim VN (2014) Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15 (8):509–524
94. Kim K-H, Jung J-Y, Son ED et al (2015) miR-526b targets 3[prime] UTR of MMP1 mRNA. Exp Mol Med 47:e178
95. Lee J, Lim S, Song B-W et al (2015) MicroRNA-29b inhibits migration and proliferation of vascular smooth muscle cells in neointimal formation. J Cell Biochem 116(4):598–608
96. Jing W, Jiang W (2015) MicroRNA-93 regulates collagen loss by targeting MMP3 in human nucleus pulposus cells. Cell Prolif 48(3):284–292
97. Jin H, Wang W (2015) MicroRNA-539 suppresses osteosarcoma cell invasion and migration in vitro and targeting matrix metallopeptidase-8. Int J Clin Exp Pathol 8(7):8075–8082
98. Yu Y, Wang L, Liu T et al (2015) MicroRNA-204 suppresses trophoblast-like cell invasion by targeting matrix metalloproteinase-9. Biochem Biophys Res Commun 463(3):285–291
99. Wang Y-Y, Li H, Wang X-H et al (2016) Probucol inhibits MMP-9 expression through regulating miR-497 in HUVECs and apoE knockout mice. Thromb Res 140:51–58
100. Meng F, Zhang Z, Chen W et al (2016) MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1β-induced chondrocyte responses. OARS 24(5):932–941
101. Li Y, Kuscu C, Banach A et al (2015) miR-181a-5p inhibits cancer cell migration and angiogenesis via downregulation of matrix metalloproteinase-14. Cancer Res 75(13):2674–2685
102. Zhang H, Qi M, Li S et al (2012) microRNA-9 targets matrix metalloproteinase 14 to inhibit invasion, metastasis, and angiogenesis of neuroblastoma cells. Mol Cancer Ther 11(7):1454–1466
103. Son DJ, Kumar S, Takabe W et al (2013) The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis. Nat Commun 4

Protease Cathepsins in Cardiomyopathy: From Mechanism to Intervention

21

Rui Guo and Sreejayan Nair

Abstract

Cardiovascular disease is the leading cause of death in the United States. Risk factors that contribute to the heart disease and cardiac dysfunction include diabetes, obesity, hypertension, high blood cholesterol, smoking, alcohol abuse, sedentary life style, unhealthy diet, family history, and aging. Whereas obesity and uncontrolled hypertension can lead to hypertrophic cardiomyopathy; chronic alcohol consumption and diabetes can cause dilated cardiomyopathy, both of which can eventually result in an impaired cardiac function and heart failure. Cathepsins are lysosomal proteases that are capable of degrading proteins. Studies have shown that cathepsins, particularly those that belong to the cysteine protease family exhibit an important role in the development of cardiomyopathy and heart failure, probably by regulating cardiac remodeling. In diabetic cardiomyopathy, cathepsin K, the most potent cathepsin in terms of its collagenolytic and elastolytic properties, regulates calcineurin/NFAT transcriptional signaling critical for cardiac remodeling. Under obese conditions, inhibition of cathepsin K results in cardioprotection. Cathepsins also exhibit potential effects on epigenetics associated with alcoholic cardiomyopathy. Therefore, targeting cathepsins may represent a novel therapeutic strategy for the prevention and/or treatment of cardiovascular diseases.

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1 Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in the United States [1]. Diabetes, obesity, and alcoholism are among the major risk factors that contribute to heart diseases in the modern society. According to the statistics from Centers for Disease Control and Prevention (CDC), in the year 2012, more than 29 million people or 9.3% of the U.S. population was diabetic. Diabetic subjects are at higher risk for heart attack, congestive heart failure, and atherosclerotic disease all of which can lead to diabetic cardiomyopathy and heart failure [2]. Alcohol abuse is another notable problem worldwide. Chronic alcohol consumption can result in alcoholic cardiomyopathy, characterized by thinned and enlarged heart (dilated cardiomyopathy), disruption of myofibrillary architecture, and cardiac contractile anomalies [3]. Current therapeutic options for dilated cardiomyopathy, such as diabetic cardiomyopathy and alcoholic cardiomyopathy, are limited. Moreover, obesity is an emerging health problem worldwide and is an independent risk factor for developing cardiovascular diseases. Obesity-associated myocardial damage is characterized by cardiac hypertrophy and contractile dysfunction, which is referred to as hypertrophic cardiomyopathy. The numerous preclinical and clinical studies aimed at preventing and/or treating cardiac disorders have not made any dent on the staggering numbers of cardiomyopathies, warranting newer pharmacological strategies to address this problem. This review aims at a brief discussion of the mechanisms leading to the progression of cardiomyopathy under diabetic, obesity, and alcoholic conditions, and addresses the possibility of employing cathepsins as novel targets for prevention and/or treatment of cardiomyopathy.

2 Biological Properties and Functions of Protease Cathepsins

Cathepsins are proteolytic enzymes that are involved in lysosomal protein degradation, which plays a vital role in physiological and pathological processes in living organisms [4, 5]. Dysregulation of cathepsins have been shown to correlate with numbers of diseases such as arthritis [6, 7], cancer [8, 9], autoimmune disease [10], stroke [11], neurodegenerative diseases [12, 13], gastrointestinal diseases [14–16], cardiovascular diseases [17], diabetes, and obesity [18, 19]. The proteolytic property of cathepsins rely on their broad specificities, thus the cleavage sites are different among different cathepsins. Most cathepsins are endopeptidases that catalyze the cleavage of nonterminal amino acids or break peptide bonds within the

target sequences. A few of them are carboxy- or amino-peptidases that cleave the peptide bond at only carboxy- or amino-terminal residues. A main physiological role of cathepsins is protein turnover in the lysosome [5]. Generally, cathepsins are contained and activated within the acidic pH of the lysosomes, sparing the cytosol and membrane of the cells from proteolysis. However, impaired lysosomal integrity leads to leakage of cathepsins to the cytosol and eventually outside of the cell resulting in degradation of cellular components or proteins in the extracellular matrix (ECM) [20].

Cathepsin B, C, F, H, K, L, O, S, V, W, X, and Z, are cysteine proteases with cysteine residue in their catalytic site. Histidine residue in the active site can also assist in the hydrolysis of target peptide bonds on the substrate, as evidenced in cathepsin B His197 or His199, and cathepsin H His166. Other cathepsins are serine protease (cathepsin G), aspartyl proteases (cathepsins D and E), and exopeptidase (cathepsin A). The knowledge of the catalytic sites of different cathepsins is of great importance in developing specific inhibitors for these proteases [5, 21]. Cathepsin K is by far the most potent mammalian cysteine protease [5]. It hydrolyzes various synthetic substrates such as Z-Gly-pro-Arg-MCA, Z-Arg-Phe-AMC, Z-Arg-Arg-AMC, and Bz-Val-Lys-Lys-Arg-AMC [22, 23]. In contrast to the cathepsins B, L, and S, cathepsin K is predominantly present in osteoclasts, and has strong elastase and collagenase properties for the degradation of bone collagen, indicating a special role in bone resorption. Therefore, cathepsin K has been implicated in the pathophysiology of osteoporosis and arthritis [24, 25].

3 Cathepsins in Cardiomyopathies and Heart Failure

Cardiomyopathy, literally "heart muscle disease," is a chronic and sometimes progressive disease of the myocardium (heart muscle) that is abnormally enlarged, thickened, and/or stiffened. The most common case of cardiomyopathy is dilated cardiomyopathy, and posteriorly hypertrophic cardiomyopathy. The weakened heart muscle in these conditions is unable to pump blood to the rest of the body. Cardiomyopathy is caused by a range of risk factors including heredity, coronary heart disease (e.g., atherosclerosis), amyloidosis, diabetes, obesity, long-term alcoholism, endocrine diseases, sarcoidosis, hypertension, and certain drugs (e.g., doxorubicin). All these can lead to peripheral edema, irregular heartbeat, a heart valve problem, heart failure, or other complications.

Cathepsins are ubiquitously expressed in various tissues and play important roles in cardiovascular diseases [17, 26]. Alterations of both extra and intracellular proteolytic activities are invariably observed in heart failure and have been linked to hypertrophic cardiomyopathy, dilated cardiomyopathy, hypertensive cardiomyopathy, ischemic cardiomyopathy, and diabetic cardiomyopathy [27]. Cathepsins B, L, and S are capable of regulating autophagy [28–30], ECM turnover, antigen presentation, neuropeptide and hormone processing, inflammatory response, and apoptosis [10, 26]. Previous studies have shown that the expression and activity of

cathepsin, B, D, K, and S were elevated in atherosclerotic plaque and in the hypertrophic and failing heart in both human and animal models [7, 54, 55]. Cathepsin S and G also displayed detrimental effects by altering ECM degradation, and causing cardiac remodeling [31, 32]. On the contrary, knockout of cathepsin L in mice resulted in dilated cardiomyopathy, whereas over expression of cathepsin L displayed decreased inflammation, fibrosis, and cardiac hypertrophy, probably through AKT/GSK3 beta pathway [33]. These studies strongly suggest a pivotal role for cathepsins in cardiac remodeling and heart failure and attribute protective and detrimental roles for these cysteine proteases. Despite the growing number of recent studies on the role of cathepsins in cardiovascular disease, the cellular and molecular mechanisms by which cathepsin K regulates cardiac dysfunction in the setting of cardiomyopathy and heart failure are yet to be explored. Growing evidence suggests that the expression and activity of cathepsin K are elevated in both clinical and experimental models of neointimal lesions, atherosclerosis, coronary artery disease, hypertrophy, and heart failure [34–37]. Our recent studies have suggested that cathepsin K protein levels were markedly upregulated in human hearts of end-stage dilated cardiomyopathy, and deletion of *ctsk* gene protected against cardiac anomalies induced by pressure overload or high-fat diet (HFD) feeding in mice [38, 39]. We also found that *ctsk* knockout exhibited an overall improvement in systemic glucose utilization [39], which was consistent with the evidence that cathepsin K displayed a negative effect on glucose and lipid metabolism, and inhibition of cathepsin K attenuated body weight gain, elevated serum glucose, and insulin levels in obese mice [19, 40, 41]. Cathepsin K may therefore represent a potential target for prevention or treatment of cardiac hypertrophy and heart failure.

4 Cathepsin K and Calcineurin/NFAT Signaling in Diabetic Cardiomyopathy

Myopathic state of the heart in diabetic subjects is manifested as left ventricular dilation, impaired left ventricular contractility, reduced ejection fraction and cardiac output, cardiac compensatory hypertrophy, and enhanced risk of stroke and hypertension, eventually leading to maladaptation and heart failure [42]. Micro/macrovascular complications also contribute to the cardiac anomalies associated with diabetes [43, 44]. However, the explicit mechanisms underlying the disease are still controversial as the pathogenesis of diabetic cardiomyopathy is multifactorial. Myocardial contractile dysfunction can be attributed to structural changes in the heart as a result of atherosclerosis and hypertension. Recent evidence suggests that diabetes affects cardiac structure and function in the absence of coronary artery disease, valvular disease, or high blood pressure [45]. The general triggering mechanisms behind the complicacy of diabetic cardiomyopathy include metabolic disturbances, altered cellular insulin signaling, small vessel diseases, and myocardial fibrosis which mainly associated with the stimulation of renin–angiotensin–aldosterone system (RAAS) and increased cytokines. Additionally, cardiac autonomic neuropathy,

autophagy, and epigenetics may also contribute to the pathogenesis of diabetic cardiomyopathy [46, 47]. Studies have shown that cathepsin D accelerates cardiac muscle degradation that occurs in the late stage of diabetic cardiomyopathy by triggering autophagy [48, 49]. Impairment of cathepsin L by hyperglycemia has been suggested as a cause of poor neovascularization and regeneration capacity of ischemic tissues in diabetics [50].

Hyperinsulinemia under diabetic conditions contributes to cardiac hypertrophy and remodeling, which can be explained, at least in part, to the inactivation of glycogen synthases kinase-3β (GSK-3β), a well-recognized antagonist of the calcineurin, which in turn inhibits nuclear transcription governing the hypertrophic process via the nuclear factor of activated T cells (NFAT) [51, 52]. It has been demonstrated that a transgene encoding a constitutively active form of calcineurin was sufficient to induce cardiac hypertrophy that progressed to dilated cardiomyopathy, heart failure, and sudden death in transgenic mice [53]. Suppression of calcineurin activity or NFAT transcription inhibits brain natriuretic peptide (BNP) induction and cardiac hypertrophy [52, 54], indicating a potential therapy strategy targeting on calcineurin/NFAT signaling.

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine-protein phosphatase ubiquitously expressed in eukaryotic cells, and involves in a number of cellular processes including Ca^{2+} dependent signaling pathways. In skeletal muscle, calcineurin can modulate fiber type-specific gene expression which is dependent on Ca^{2+} signaling and contractile activity [55, 56]. Indeed, calcineurin has been shown to influence Ca^{2+} fluxes by modulating the activities of L-type Ca^{2+} channel [57], ryanodine receptor (RyR)/Ca^{2+}-release channels [58, 59], sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA 2a) [60] and the inositol 1,4,5-triphosphate receptor [61] in the heart. It has been known that Ca^{2+}-mediated signal transduction is essential for cardiac remodeling and hypertrophy process, and the disturbance of Ca^{2+} homeostasis leads to contractile dysfunction of the cardiomyocyte [62]. Such alterations of Ca^{2+} signals could play a role in the pathophysiology of heart failure. Furthermore, decreased activity of cardiac L-type Ca^{2+} channel induces hypertrophy and heart failure through activation of calcineurin/NFAT signaling in mice [63].

Activation of calcineurin can dephosphorylate the regulatory domains of NFATs within the cytoplasm, and the translocation of dephosphorylated NFATs regulates gene expression in the nucleus [51]. Studies by Fiedlerm [52] and Gao [64] suggest that L-type Ca^{2+}-channel current can also cause NFAT activation. There are four calcineurin-regulated NFAT transcriptional factors, NFATc1-c4, each of which is present in the myocardium [65]. To date, NFATc3, and NFATc4 (NFAT3) have been shown as two main downstream targets of calcineurin for the initiation of hypertrophic response [66]. NFATc4 can in turn stimulate the transcription of pro-hypertrophic genes MEF2 and GATA4, thereby promoting pathological hypertrophy [67]. In addition, NFATc1 has been shown critical for endocardial valve remodeling, coronary vessels, and fibrous matrix formation in the maturing heart, and serves as an essential effector of receptor activator of NFκB ligand (RANKL) signaling, which in turn regulates cathepsin K expression [68, 69]. Research also indicated that in the cardiomyocytes, NFATs may interact with NFκB/p65 and induce

the nuclear translocation of NFκB, and genetic deletion of calcineurin/NFATs displays compromised NFκB transcriptional activation, which predisposes pressure overload-induced cardiac hypertrophy. On the other hand, full transcriptional activation of NFATs requires intact NFκB signaling and p65 transcriptional activity [70]. NFATs and NFκB have also been associated with apoptosis [71, 72].

Both cathepsin K and calcineurin/NFATs signaling pathway have been implicated as critical regulators of cardiomyocyte hypertrophy. It can be postulated that these two previously deemed independent signaling pathways may actually crosstalk with each other, based on the following suppositions: First, increased cathepsins K activity can lead to dysregulated glucose metabolism and increase in glucotoxicity, which can trigger calcineurin/NFATs signaling. Evidence suggests that both hyperinsulinemia and hyperglycemia can trigger calcineurin-NFATs pathway [16, 25, 78, 79]. In our studies, mice rendered diabetic by streptozotocin injection exhibited elevated levels of cardiac cathepsin K, whereas similarly treated cathepsin K knockout mice exhibited attenuation in fasting blood glucose levels and reduced cardiac calcineurin A expression. In addition, diabetic mice exhibited ventricular dilation and cardiac dysfunction that was markedly alleviated by cathepsin K deletion. Since insulin levels were decreased in mice subjected to streptozotocin treatment, it is likely that hyperglycemia and glucotoxicity as a consequence of streptozotocin challenge is the possible trigger for dilated cardiomyopathy and cardiac dysfunction, via the upregulation of cathepsin K. Second, higher level of cathepsin K may induce cardiac anomalies by dysregulation of calcium homeostasis which triggers calcineurin activation. Our studies have demonstrated that cathepsin K knockout dramatically reversed diabetes-induced reduction in SERCA2 and phosphorylation levels of phospholamban at Ser16 and Thr17, as well as attenuated diabetes-induced elevation of intracellular calcium concentration. Taken together, this would suggest that cathepsin K is an upstream signal for regulating Ca^{2+} flux, which contributes to calcineurin stimulation. It is likely that cathepsin K, by virtue of its protease function, cleaves calcineurin to its active form resulting in the activation of NFATs, and subsequently triggers diabetes-induced cardiac anomalies and cardiomyopathy. This may be akin to calpain, a Ca^{2+}-dependent cysteine protease that has been shown to directly cleave calcineurin into its active form both in vitro and in vivo [20]. Future work still needs to be done and to explore the specific cleavage sites of cathepsin K and the structural basis for activation of calcineurin.

5 Role of Cathepsin K in Obesity Cardiomyopathy and Cardiac Dysfunction

Obesity is an independent risk factor for the pathogenesis of cardiovascular diseases such as arteriosclerosis, coronary heart disease, hypertension, cardiomyopathy, and heart failure [73, 74]. Obesity increases the risk for high blood pressure, metabolic syndrome, and abnormal energy metabolism such as glucose intolerance,

dyslipidemia, and insulin resistance, all of which contribute to cardiac anomalies. In addition to genetic predisposition, both clinical and experimental evidence suggests a pivotal role of obesity in cardiac hypertrophy and myocardial dysfunction. Accumulating human studies also confirm that obese people are prone to heart failure [74, 75].

A number of molecular mechanisms including the alteration in cardiac substrate utilization, inflammation, oxidative stress, mitochondrial injury, apoptosis, disrupt of extracellular matrix, fibrosis, endoplasmic reticulum stress, leptin resistance, endothelial dysfunction, lipotoxicity, and impaired Ca^{2+} homeostasis have been speculated as causes for obesity-induced cardiac dysfunction [76–80]. It is speculated that both calcium-dependent CaMKK and calcineurin might participate in obesity-associated cardiac hypertrophy and cardiomyopathy [51, 81, 82]. Similar to diabetic cardiomyopathy, calcineurin may also play an essential role in transducing hypertrophic signals in obese individuals, partially by activating NFAT transcription factors, the signaling of which may involve cathepsin K.

Cathepsin B, L, and K have been shown to be positively associated with lipotoxicity, and cathepsin K negatively regulates lipid metabolism. According to Chiara and co-workers, mRNA levels of *ctsk*, as well as Mitf and TFE3, two transcription factors involved in *ctsk* induction in osteoclasts, were dramatically higher in white adipose tissue (WAT) of obese mice, compared to their wild-type littermates. Interestingly, mRNAs were attenuated in mice undergoing weight loss. *Ctsk* gene expression has been positively correlated with body mass index [41]. In human studies both cathepsin K protein and *ctsk* mRNA expression were elevated in the WAT of overweight/obese patients, supporting the notion that *ctsk* is a novel and reliable marker of adiposity [83]. Studies from our lab showed that *ctsk* knockout significantly attenuated HFD-induced obesity and cardiac dysfunction evidenced as cardiac hypertrophy, cardiomyocyte contractile dysfunction, impaired intracellular Ca^{2+} handling, and apoptosis [39].

Obesity is characterized by defective fat storage, increase in intracellular lipid accumulation, and dyslipidemia. Xiao and co-workers found that *ctsk* is involved in the pathogenesis of obesity by promoting adipocyte differentiation in both human and in cultured cells. Expression and activity of cathepsin K gradually elevates concomitant with the differentiation of 3T3-L1 pre-adipocytes into mature adipocyte [83]. Similar to matrix metalloproteinase (MMP)-2, -3, and -9, cathepsin K, as a cysteine protease, has ability to degrade certain components of the ECM, which contribute to the ECM remodeling and adipocyte differentiation, likely via regulation of peroxisome proliferator-activated receptors (PPAR) and/or CCAAT/enhancer-binding proteins (CEBPB) [84, 85]. In addition, osteonectin that modulates cell adhesion, differentiation, and angiogenesis can be cleaved by cathepsin K in the WAT, resulting in enhanced matrix plasticity, and facilitating adipose remodeling and angiogenesis. Funicello and co-workers [40] found that the rate of lipolysis in adipocytes together with CPT-1 activity were increased in both young and HFD-fed $ctsk^{-/-}$ mice compared to wild-type mice, suggesting an increased release and/or utilization of free fatty acid (FFA) by down-regulating cathepsin K. Furthermore, plasma levels of leptin and triglyceride were significantly lower in adult $ctsk^{-/-}$ mice. The authors concluded that

the absence of *ctsk* is associated with increased energy expenditure, which might be due to increased activation of brown adipose tissue (BAT) for thermogenesis. Studies from Podgorski and co-workers indicated an involvement of cathepsin K in the regulation of adiponectin, an adipokine with anti-inflammatory and anti-angiogenic properties, which is dramatically decreased in obesity [86].

Platt and co-authors revealed that cathepsin K expression is regulated by shear stress in cultured mouse aortic endothelial cells (MAECs) and is elevated in endothelium in human atherosclerosis. Elastase and gelatinase activity was also increased in MAECs exposed to shear stress, which was attenuated by knocking down *ctsk* with siRNA, suggesting that cathepsin K is a shear-sensitive protease [87]. Their study also showed a positive correlation between the cathepsin K expression in endothelium and the integrity of the elastic lamina. These findings suggest that cathepsin K may function as an ECM protease and is involved in arterial wall remodeling and atherosclerosis. Indeed, cathepsin K levels have been positively correlated to plaque volume, and blood levels of cathepsin K have been suggested as independent predictor of coronary artery disease [36].

Sustained obesity can lead to type 2 diabetes and dampened insulin signaling has been observed in the heart from HFD-fed mice. Knockout of cathepsin K improved cardiac function and insulin signaling and reduced apoptosis in obese mice [39]. Yang and co-workers have shown that inhibition of cathepsin K reduced serum glucose and insulin levels by degrading fibronectin [19]. Collectively, these studies suggest that cathepsin K plays may play a role in regulating insulin signaling and preventing apoptosis of the heart of obese mice.

In addition to the above mechanisms, cathepsin K inhibition prevents cardiac hypertrophy by alleviating cardiac remodeling, both in vivo and in vitro studies. Indeed, the stimulation of the mTOR and Erk signaling pathways both of which were induced in the hypertrophic heart was blunted by *ctsk* deletion [38]. HFD can induce not only increased body weight, but also increased heart weight, left ventricular wall thickness, excessive epicardial fat and fatty infiltration of the myocardium, as well as increased total blood volume and cardiac output, all of which contribute to cardiac dysfunction, cardiomyopathy and heart failure. Under these conditions, inhibition of cathepsin K protects against the development of obesity-associated cardiomyopathy via mitigating cardiac remodeling. Furthermore, cathepsin K inhibition by virtue of its beneficial effects on the vasculature can also attenuate obesity-induced hypertension [38]. Furthermore, studies form our lab demonstrated that inhibition of cathepsin K suppresses oxidative stress in the mouse heart and in cultured H9c2 cells.

6 Cathepsins, Alcoholic Cardiomyopathy, and Epigenetics

Alcohol abuse is a serious medical and social problem. Excessive or chronic alcohol intake can lead to alcoholic cardiomyopathy, a disorder of the heart muscle characterized by compensatory cardiac hypertrophy, left ventricular dilation,

impaired left ventricular contractility, reduced ejection fraction and cardiac output accompanied with myocardial fibrosis, cardiomyocyte apoptosis, and mitochondrial impairment. Cardiac remodeling and compensatory cardiac hypertrophy can eventually result in maladaptation and heart failure [88, 89]. However, the explicit mechanisms underlying the disease are yet unclear. A number of mechanisms including direct toxicity of ethanol, indirect toxicity through its metabolites [acetaldehyde and fatty acid ethyl esters (FAEEs)], oxidative stress and impaired autophagy may be involved in alcoholic complications. Acetaldehyde, the primary intermediate in the metabolism of ethanol, is an essential candidate toxin in developing alcoholic cardiomyopathy through hypertrophic responses, interruption of myocardial protein synthesis (as a result of adduct formation) and impairment of mitochondrial integrity [90]. Meanwhile, free radicals produced during ethanol metabolism and FAEEs are also important triggers for alcoholic heart diseases [91]. Additionally, racial and gender differences, genetic variation in certain myocardial proteins, genetic polymorphism of alcohol metabolizing enzymes, epigenetics, and alterations in the levels of microRNA levels may also contribute to the development of alcoholic cardiomyopathy [92].

Epigenetics is a science studying heritable change in the genome, which affects gene expression without any change in the DNA sequence. Potential epigenetic mechanisms include DNA methylation, histone modification, and RNA-based mechanisms such as microRNAs (miRNAs) and long non-coding RNAs (ncRNAs), leading to either transcriptional suppression or activation of the genes independently of the DNA genome sequence [93, 94]. Ethanol can induce epigenetic alterations in different immune cell types including granulocytes, macrophages, and T-lymphocytes which promote inflammation [95]. Epigenetic is emerging as a hot research topic and is a potential target for primary prevention or treating cardiovascular diseases. Epigenetic factors such as methylation and acetylation of histones have been shown to be correlated with enhanced expression of small non-protein-coding ribonucleic acids [92]. Preliminary studies have found that DNA methylation, histone modifications, and RNA-based mechanisms may have an association with the development of cardiac hypertrophy and heart failure. A recent study showed a significant difference in plasma microRNA profile between patients with alcoholic cardiomyopathy and a healthy population [96]. Our previous studies have suggested that ethanol feeding increased the levels of Beclin1 and triggered the formation of autophagosomes in cardiomyocytes, which results in myocardial contractile dysfunction through autophagy. Meanwhile, the expression of miR-30a, a target of Beclin1 was reduced in cardiomyocytes [90]. However, no active epigenetic agents or drugs targeting histone methylation and/or acetylation have actually reached clinical trials for cardiovascular diseases [93].

Generally, epigenetic alterations can be reversed via therapeutic approaches including but not limited in DNA methyltransferase (DNMT) inhibitors, histone deacetylase (HDAC) inhibitors, histone acetyltransferase (HAT) inhibitors, miRNA therapeutics and commonly used medicines like statins [97]. For example, polyphenols and folic acid may be as decent candidates to reduce lipid and ROS levels by regulating DNA methylation and histone modification. Resveratrol, a

DNMT inhibitor, can modulate sirtuin 1, MAP38 kinase, NF-κB, AP-1, eNOS and inflammatory cytokines. Moreover, the HAT inhibitor curcumin is a polyphenol and actually modulates various epigenetic factors such as HDAC, HAT, DNMT, and miRNAs [98]. Curcumin as an antioxidant may influence both acetylation and deacetylation by regulating oxidative stress. Trichostatin A, as a HDAC inhibitor, can also play a pivotal role in the prevention of cardiac performance and alleviate myocardial remodeling through stimulating endogenous cardiac regeneration [93]. Besides, the role of miRNAs in drug exploration as genetic targets has also been investigated. MicroRNAs are a class of short non-coding RNAs that target specific mRNAs thereby inducing degradation or translational inhibition during various physiological or pathological processes. Evidence indicated that miRNAs are involved in the actions of ethanol and play a key role in regulating the progression of cardiomyopathy. Ethanol can cause some miRNAs upregulated and others downregulated simultaneously [94]. For instance, upregulation of miR-212 by alcohol can lead to activation of fetal gene program and heart failure. Therefore, miR-212 can be a potential therapeutic target to protect the heart from chronic alcoholism. According to Jing and colleagues, nine differentially expressed miRNAs including miR-506, miR-1285, miR-512-3P, miR-138, miR-485-5P, miR-4262, miR-548c-3P, miR-548a-5P, and miR-K12-1 may be involved in the development of alcoholic cardiomyopathy. Particularly, miR-138, may be considered as a novel biomarker for the early diagnosis and treatment of human alcoholic cardiomyopathy [96]. Additionally, miR-340 was also regarded as a novel therapeutic target for the heart failure progression by restricting cardiac remodeling [99].

Cathepsin L has been shown to cleave histone H3 that is generated in vivo during mouse embryonic stem cell differentiation. In addition, it was demonstrated that an endogenous osteogenic growth peptide (OGP) that is identical to the histone H4 is responsible for bone regeneration [100]. These findings indicate that the proteolysis of H3 tail may associate with mammalian differentiation and a proliferative effect. Our study found that ethanol treatment increased osteoprotegerin (OPG) level in H9c2 myoblast, which was restored by pharmacological inhibitor of cathepsin K. OPG could increase OGP levels and was recently considered as a hypertrophic marker in patients with cardiomyopathy and heart failure, therefore we postulate that histone H3 and/or H4 may be regulated by cathepsins thereby regulating cardiac remodeling in alcoholic cardiomyopathy. Bulynko and co-workers speculated that cathepsin L-linked phenotypes such as defective skin and bone cell differentiation and dilated cardiomyopathy may result from cathepsin L deficiency-induced epigenetic heterochromatin changes in histone H3(K9) and the histone H2A.Z [101]. It has been suggested that *ctsk*, as a potential cardiovascular target gene, may be regulated by miR-107 that is upregulated in experimental models of heart failure, and gene of cathepsin S (*ctss*) has consensus binding sites for repressed miRNAs [102, 103]. MiR-212 may interact with cathepsin G in connection with collagen deposition [104].

7 Conclusion

Determination of the specific signaling pathways in the development of heart failure is essential for the discovery of novel therapeutic strategies. Cathepsins, especially cysteine cathepsins, are closely associated with cardiac remodeling and contribute to cardiomyopathy and heart failure. Cathepsins may also serve as diagnostic tools or biomarkers for heart failure. Cathepsin inhibitors have already been investigated in clinical trials for a variety of disease conditions such as osteoporosis and rheumatoid arthritis. These agents should be evaluated for their efficacy in preventing and/or treating cardiovascular diseases particularly the progression of heart failure. Inhibition of cathepsins may also alter genetic and epigenetic changes. Further studies are necessary to understand the broader implications and role of cathepsins to successfully target these proteases to treat or control heart disease.

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References

1. Minino AM, Murphy SL, Xu J, Kochanek KD (2011) Deaths: final data for 2008. National vital statistics reports: from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System 59:1–126
2. (1999) Diabetes mellitus: a major risk factor for cardiovascular disease. A joint editorial statement by the American Diabetes Association; The National Heart, Lung, and Blood Institute; The Juvenile Diabetes Foundation International; The National Institute of Diabetes and Digestive and Kidney Diseases; and The American Heart Association. Circulation 100:1132–1133
3. Patel VB, Why HJ, Richardson PJ, Preedy VR (1997) The effects of alcohol on the heart. Adverse Drug React Toxicol Rev 16:15–43
4. Stoka V, Turk B, Turk V (2005) Lysosomal cysteine proteases: structural features and their role in apoptosis. IUBMB Life 57:347–353
5. Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, Turk D (2012) Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochim Biophys Acta 1824:68–88
6. Pozgan U, Caglic D, Rozman B, Nagase H, Turk V, Turk B (2010) Expression and activity profiling of selected cysteine cathepsins and matrix metalloproteinases in synovial fluids from patients with rheumatoid arthritis and osteoarthritis. Biol Chem 391:571–579
7. Jones BA, Riegsecker S, Rahman A, Beamer M, Aboualaiwi W, Khuder SA, Ahmed S (2013) Role of ADAM-17, p38 MAPK, cathepsins, and the proteasome pathway in the synthesis and shedding of fractalkine/CX(3) CL1 in rheumatoid arthritis. Arthritis Rheum 65:2814–2825
8. Jedeszko C, Sloane BF (2004) Cysteine cathepsins in human cancer. Biol Chem 385:1017–1027
9. Turk V, Kos J, Turk B (2004) Cysteine cathepsins (proteases)–on the main stage of cancer? Cancer Cell 5:409–410
10. Reiser J, Adair B, Reinheckel T (2010) Specialized roles for cysteine cathepsins in health and disease. J Clin Invest 120:3421–3431

11. Lee B, Clarke D, Al Ahmad A, Kahle M, Parham C, Auckland L, Shaw C, Fidanboylu M, Orr AW, Ogunshola O, Fertala A, Thomas SA, Bix GJ (2011) Perlecan domain V is neuroprotective and proangiogenic following ischemic stroke in rodents. J Clin Invest 121:3005–3023
12. Sayad A, Noruzinia M, Zamani M, Harirchian MH, Kazemnejad A (2014) Association study of Cathepsin D gene polymorphism in Iranian patients with sporadic late-onset Alzheimer's disease. Dement Geriatr Cogn Disord 37:257–264
13. Paz YMCA, Garcia-Cardenas JM, Lopez-Cortes A, Salazar C, Serrano M, Leone PE (2015) Positive Association of the Cathepsin D Ala224Val Gene Polymorphism With the Risk of Alzheimer's Disease. Am J Med Sci 350:296–301
14. Roberts LR, Adjei PN, Gores GJ (1999) Cathepsins as effector proteases in hepatocyte apoptosis. Cell Biochem Biophys 30:71–88
15. Kang JM, Bahk YY, Cho PY, Hong SJ, Kim TS, Sohn WM, Na BK (2010) A family of cathepsin F cysteine proteases of Clonorchis sinensis is the major secreted proteins that are expressed in the intestine of the parasite. Mol Biochem Parasitol 170:7–16
16. Vreemann A, Qu H, Mayer K, Andersen LB, Stefana MI, Wehner S, Lysson M, Farcas AM, Peters C, Reinheckel T, Kalff J, Brix K (2009) Cathepsin B release from rodent intestine mucosa due to mechanical injury results in extracellular matrix damage in early post-traumatic phases. Biol Chem 390:481–492
17. Qin Y, Shi GP (2011) Cysteinyl cathepsins and mast cell proteases in the pathogenesis and therapeutics of cardiovascular diseases. Pharmacol Ther 131:338–350
18. Kumar VRS, Darisipudi MN, Steiger S, Devarapu SK, Tato M, Kukarni OP, Mulay SR, Thomasova D, Popper B, Demleitner J, Zuchtriegel G, Reichel C, Cohen CD, Lindenmeyer MT, Liapis H, Moll S, Reid E, Stitt AW, Schott B, Gruner S, Haap W, Ebeling M, Hartmann G, Anders HJ (2015) Cathepsin S cleavage of protease-activated receptor-2 on endothelial cells promotes microvascular diabetes complications. J Am Soc Nephrol
19. Yang M, Sun J, Zhang T, Liu J, Zhang J, Shi MA, Darakhshan F, Guerre-Millo M, Clement K, Gelb BD, Dolgnov G, Shi GP (2008) Deficiency and inhibition of cathepsin K reduce body weight gain and increase glucose metabolism in mice. Arterioscler Thromb Vasc Biol 28:2202–2208
20. Turk B, Stoka V (2007) Protease signalling in cell death: caspases versus cysteine cathepsins. FEBS Lett 581:2761–2767
21. Roberts R (2005) Lysosomal cysteine proteases: structure, function and inhibition of cathepsins. Drug News Perspect 18:605–614
22. Lotinun S, Kiviranta R, Matsubara T, Alzate JA, Neff L, Luth A, Koskivirta I, Kleuser B, Vacher J, Vuorio E, Horne WC, Baron R (2013) Osteoclast-specific cathepsin K deletion stimulates S1P-dependent bone formation. J Clin Invest 123:666–681
23. Chapman HA, Riese RJ, Shi GP (1997) Emerging roles for cysteine proteases in human biology. Annu Rev Physiol 59:63–88
24. Aguda AH, Panwar P, Du X, Nguyen NT, Brayer GD, Bromme D (2014) Structural basis of collagen fiber degradation by cathepsin K. Proc Natl Acad Sci USA 111:17474–17479
25. Salminen-Mankonen HJ, Morko J, Vuorio E (2007) Role of cathepsin K in normal joints and in the development of arthritis. Curr Drug Targets 8:315–323
26. Lutgens SP, Cleutjens KB, Daemen MJ, Heeneman S (2007) Cathepsin cysteine proteases in cardiovascular disease. FASEB J 21:3029–3041
27. Muller AL, Freed D, Hryshko L, Dhalla NS (2012) Implications of protease activation in cardiac dysfunction and development of genetic cardiomyopathy in hamsters. Can J Physiol Pharmacol 90:995–1004
28. Goldspink DF, Lewis SE, Kelly FJ (1985) Protein turnover and cathepsin B activity in several individual tissues of foetal and senescent rats. Comp Biochem Physiol B 82:849–853
29. Hsu KF, Wu CL, Huang SC, Wu CM, Hsiao JR, Yo YT, Chen YH, Shiau AL, Chou CY (2009) Cathepsin L mediates resveratrol-induced autophagy and apoptotic cell death in cervical cancer cells. Autophagy 5:451–460

30. Huang CC, Chen KL, Cheung CH, Chang JY (2013) Autophagy induced by cathepsin S inhibition induces early ROS production, oxidative DNA damage, and cell death via xanthine oxidase. Free Radic Biol Med 65:1473–1486
31. D'Amico A, Ragusa R, Caruso R, Prescimone T, Nonini S, Cabiati M, Del Ry S, Trivella MG, Giannessi D, Caselli C (2014) Uncovering the cathepsin system in heart failure patients submitted to left ventricular assist device (LVAD) implantation. J Transl Med 12:350
32. Svystonyuk DA, Ngu JM, Mewhort HE, Lipon BD, Teng G, Guzzardi DG, Malik G, Belke DD, Fedak PW (2015) Fibroblast growth factor-2 regulates human cardiac myofibroblast-mediated extracellular matrix remodeling. J Transl Med 13:147
33. Tang Q, Cai J, Shen D, Bian Z, Yan L, Wang YX, Lan J, Zhuang GQ, Ma WZ, Wang W (2009) Lysosomal cysteine peptidase cathepsin L protects against cardiac hypertrophy through blocking AKT/GSK3beta signaling. J Mol Med (Berl) 87:249–260
34. Hofnagel O, Robenek H (2009) Cathepsin K: boon or bale for atherosclerotic plaque stability? Cardiovasc Res 81:242–243
35. Hu L, Cheng XW, Song H, Inoue A, Jiang H, Li X, Shi GP, Kozawa E, Okumura K, Kuzuya M (2014) Cathepsin K activity controls injury-related vascular repair in mice. Hypertension 63:607–615
36. Cheng XW, Kikuchi R, Ishii H, Yoshikawa D, Hu L, Takahashi R, Shibata R, Ikeda N, Kuzuya M, Okumura K, Murohara T (2013) Circulating cathepsin K as a potential novel biomarker of coronary artery disease. Atherosclerosis 228:211–216
37. Zhao G, Li Y, Cui L, Li X, Jin Z, Han X, Fang E, Gao Y, Zhou D, Jiang H, Jin X, Piao G, Li X, Yang G, Jin J, Zhu E, Piao M, Piao L, Yuan K, Lei Y, Ding D, Jin C, Nan Y, Cheng X (2015) Increased circulating cathepsin K in patients with chronic heart failure. PLoS ONE 10:e0136093
38. Hua Y, Xu X, Shi GP, Chicco AJ, Ren J, Nair S (2013) Cathepsin K knockout alleviates pressure overload-induced cardiac hypertrophy. Hypertension 61:1184–1192
39. Hua Y, Zhang Y, Dolence J, Shi GP, Ren J, Nair S (2013) Cathepsin K knockout mitigates high-fat diet-induced cardiac hypertrophy and contractile dysfunction. Diabetes 62:498–509
40. Funicello M, Novelli M, Ragni M, Vottari T, Cocuzza C, Soriano-Lopez J, Chiellini C, Boschi F, Marzola P, Masiello P, Saftig P, Santini F, St-Jacques R, Desmarais S, Morin N, Mancini J, Percival MD, Pinchera A, Maffei M (2007) Cathepsin K null mice show reduced adiposity during the rapid accumulation of fat stores. PLoS ONE 2:e683
41. Chiellini C, Costa M, Novelli SE, Amri EZ, Benzi L, Bertacca A, Cohen P, Del Prato S, Friedman JM, Maffei M (2003) Identification of cathepsin K as a novel marker of adiposity in white adipose tissue. J Cell Physiol 195:309–321
42. Fein FS (1990) Diabetic cardiomyopathy. Diabetes Care 13:1169–1179
43. Gore MO, McGuire DK (2009) Cardiovascular disease and type 2 diabetes mellitus: regulating glucose and regulating drugs. Curr Cardiol Rep 11:258–263
44. Norby FL, Aberle NS 2nd, Kajstura J, Anversa P, Ren J (2004) Transgenic overexpression of insulin-like growth factor I prevents streptozotocin-induced cardiac contractile dysfunction and beta-adrenergic response in ventricular myocytes. J Endocrinol 180:175–182
45. Rubler S, Dlugash J, Yuceoglu YZ, Kumral T, Branwood AW, Grishman A (1972) New type of cardiomyopathy associated with diabetic glomerulosclerosis. Am J Cardiol 30:595–602
46. Bugger H, Abel ED (2014) Molecular mechanisms of diabetic cardiomyopathy. Diabetologia 57:660–671
47. Fang ZY, Prins JB, Marwick TH (2004) Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications. Endocr Rev 25:543–567
48. Kuo TH, Giacomelli F, Wiener J (1984) Lysosomal and nonlysosomal proteolytic activities in experimental diabetic cardiomyopathy. Exp Mol Pathol 40:280–287
49. Kanamori H, Takemura G, Goto K, Tsujimoto A, Mikami A, Ogino A, Watanabe T, Morishita K, Okada H, Kawasaki M, Seishima M, Minatoguchi S (2015) Autophagic

adaptations in diabetic cardiomyopathy differ between type 1 and type 2 diabetes. Autophagy 11:1146–1160
50. Cheng XW, Shi GP, Kuzuya M, Sasaki T, Okumura K, Murohara T (2012) Role for cysteine protease cathepsins in heart disease: focus on biology and mechanisms with clinical implication. Circulation 125:1551–1562
51. Molkentin JD (2004) Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs. Cardiovasc Res 63:467–475
52. Fiedler B, Lohmann SM, Smolenski A, Linnemuller S, Pieske B, Schroder F, Molkentin JD, Drexler H, Wollert KC (2002) Inhibition of calcineurin-NFAT hypertrophy signaling by cGMP-dependent protein kinase type I in cardiac myocytes. Proc Natl Acad Sci USA 99:11363–11368
53. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN (1998) A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93:215–228
54. Sussman MA, Lim HW, Gude N, Taigen T, Olson EN, Robbins J, Colbert MC, Gualberto A, Wieczorek DF, Molkentin JD (1998) Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science 281:1690–1693
55. Schulz RA, Yutzey KE (2004) Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. Dev Biol 266:1–16
56. Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, Williams RS (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev 12:2499–2509
57. Matthes J, Jager A, Handrock R, Groner F, Mehlhorn U, Schwinger RH, Varadi G, Schwartz A, Herzig S (2004) Ca^{2+}-dependent modulation of single human cardiac L-type calcium channels by the calcineurin inhibitor cyclosporine. J Mol Cell Cardiol 36:241–255
58. Bandyopadhyay A, Shin DW, Ahn JO, Kim DH (2000) Calcineurin regulates ryanodine receptor/Ca(2+)-release channels in rat heart. Biochem J 352(Pt 1):61–70
59. Xiao RP, Valdivia HH, Bogdanov K, Valdivia C, Lakatta EG, Cheng H (1997) The immunophilin FK506-binding protein modulates Ca^{2+} release channel closure in rat heart. J Physiol 500(Pt 2):343–354
60. Munch G, Bolck B, Karczewski P, Schwinger RH (2002) Evidence for calcineurin-mediated regulation of SERCA 2a activity in human myocardium. J Mol Cell Cardiol 34:321–334
61. Cameron AM, Steiner JP, Roskams AJ, Ali SM, Ronnett GV, Snyder SH (1995) Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. Cell 83:463–472
62. Berridge MJ (2006) Remodelling Ca^{2+} signalling systems and cardiac hypertrophy. Biochem Soc Trans 34:228–231
63. Goonasekera SA, Hammer K, Auger-Messier M, Bodi I, Chen X, Zhang H, Reiken S, Elrod JW, Correll RN, York AJ, Sargent MA, Hofmann F, Moosmang S, Marks AR, Houser SR, Bers DM, Molkentin JD (2012) Decreased cardiac L-type Ca(2)(+) channel activity induces hypertrophy and heart failure in mice. J Clin Invest 122:280–290
64. Gao H, Wang F, Wang W, Makarewich CA, Zhang H, Kubo H, Berretta RM, Barr LA, Molkentin JD, Houser SR (2012) Ca(2+) influx through L-type Ca(2+) channels and transient receptor potential channels activates pathological hypertrophy signaling. J Mol Cell Cardiol 53:657–667
65. Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF, Molkentin JD (2002) Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. Mol Cell Biol 22:7603–7613
66. Olson EN, Molkentin JD (1999) Prevention of cardiac hypertrophy by calcineurin inhibition: hope or hype? Circ Res 84:623–632
67. Akazawa H, Komuro I (2003) Roles of cardiac transcription factors in cardiac hypertrophy. Circ Res 92:1079–1088

68. Combs MD, Braitsch CM, Lange AW, James JF, Yutzey KE (2011) NFATC1 promotes epicardium-derived cell invasion into myocardium. Development 138:1747–1757
69. Lange AW, Yutzey KE (2006) NFATc1 expression in the developing heart valves is responsive to the RANKL pathway and is required for endocardial expression of cathepsin K. Dev Biol 292:407–417
70. Liu Q, Chen Y, Auger-Messier M, Molkentin JD (2012) Interaction between NFkappaB and NFAT coordinates cardiac hypertrophy and pathological remodeling. Circ Res 110:1077–1086
71. Serfling E, Berberich-Siebelt F, Avots A, Chuvpilo S, Klein-Hessling S, Jha MK, Kondo E, Pagel P, Schulze-Luehrmann J, Palmetshofer A (2004) NFAT and NF-kappaB factors-the distant relatives. Int J Biochem Cell Biol 36:1166–1170
72. Pu WT, Ma Q, Izumo S (2003) NFAT transcription factors are critical survival factors that inhibit cardiomyocyte apoptosis during phenylephrine stimulation in vitro. Circ Res 92:725–731
73. Sa FG, Lima-Leopoldo AP, Jacobsen BB, Ferron AJ, Estevam WM, Campos DH, Castardeli E, Cunha MR, Cicogna AC, Leopoldo AS (2015) Obesity resistance promotes mild contractile dysfunction associated with intracellular Ca^{2+} handling. Arq Bras Cardiol 105:588–596
74. Kenchaiah S, Evans JC, Levy D, Wilson PW, Benjamin EJ, Larson MG, Kannel WB, Vasan RS (2002) Obesity and the risk of heart failure. N Engl J Med 347:305–313
75. Abel ED, Litwin SE, Sweeney G (2008) Cardiac remodeling in obesity. Physiol Rev 88:389–419
76. Guo R, Zhang Y, Turdi S, Ren J (2013) Adiponectin knockout accentuates high fat diet-induced obesity and cardiac dysfunction: role of autophagy. Biochim Biophys Acta 1832:1136–1148
77. Van Gaal LF, Mertens IL, De Block CE (2006) Mechanisms linking obesity with cardiovascular disease. Nature 444:875–880
78. Ren J, Zhu BH, Relling DP, Esberg LB, Ceylan-Isik AF (2008) High-fat diet-induced obesity leads to resistance to leptin-induced cardiomyocyte contractile response. Obesity (Silver Spring) 16:2417–2423
79. Abel ED (2011) Obesity stresses cardiac mitochondria even when you are young. J Am Coll Cardiol 57:586–589
80. Zaman AK, Fujii S, Sawa H, Goto D, Ishimori N, Watano K, Kaneko T, Furumoto T, Sugawara T, Sakuma I, Kitabatake A, Sobel BE (2001) Angiotensin-converting enzyme inhibition attenuates hypofibrinolysis and reduces cardiac perivascular fibrosis in genetically obese diabetic mice. Circulation 103:3123–3128
81. Despa S, Margulies KB, Chen L, Knowlton AA, Havel PJ, Taegtmeyer H, Bers DM, Despa F (2012) Hyperamylinemia contributes to cardiac dysfunction in obesity and diabetes: a study in humans and rats. Circ Res 110:598–608
82. Zhou X, Zhang Q, Zhao T, Bai X, Yuan W, Wu Y, Liu D, Li S, Ju J, Chege Gitau S, Chu W, Xu C, Lu Y (2014) Cisapride protects against cardiac hypertrophy via inhibiting the up-regulation of calcineurin and NFATc-3. Eur J Pharmacol 735:202–210
83. Xiao Y, Junfeng H, Tianhong L, Lu W, Shulin C, Yu Z, Xiaohua L, Weixia J, Sheng Z, Yanyun G, Guo L, Min L (2006) Cathepsin K in adipocyte differentiation and its potential role in the pathogenesis of obesity. The Journal of clinical endocrinology and metabolism 91:4520–4527
84. Sang QX (1998) Complex role of matrix metalloproteinases in angiogenesis. Cell Res 8:171–177
85. Bouloumie A, Sengenes C, Portolan G, Galitzky J, Lafontan M (2001) Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. Diabetes 50:2080–2086
86. Podgorski I, Linebaugh BE, Sloane BF (2007) Cathepsin K in the bone microenvironment: link between obesity and prostate cancer? Biochem Soc Trans 35:701–703

87. Platt MO, Ankeny RF, Shi GP, Weiss D, Vega JD, Taylor WR, Jo H (2007) Expression of cathepsin K is regulated by shear stress in cultured endothelial cells and is increased in endothelium in human atherosclerosis. Am J Physiol Heart Circ Physiol 292:H1479–H1486
88. Guzzo-Merello G, Cobo-Marcos M, Gallego-Delgado M, Garcia-Pavia P (2014) Alcoholic cardiomyopathy. World J Cardiol 6:771–781
89. George A, Figueredo VM (2011) Alcoholic cardiomyopathy: a review. J Card Fail 17:844–849
90. Guo R, Hu N, Kandadi MR, Ren J (2012) Facilitated ethanol metabolism promotes cardiomyocyte contractile dysfunction through autophagy in murine hearts. Autophagy 8:593–608
91. Guo R, Ren J (2010) Alcohol and acetaldehyde in public health: from marvel to menace. International journal of environmental research and public health 7:1285–1301
92. Piano MR, Phillips SA (2014) Alcoholic cardiomyopathy: pathophysiologic insights. Cardiovasc Toxicol 14:291–308
93. Voelter-Mahlknecht S (2016) Epigenetic associations in relation to cardiovascular prevention and therapeutics. Clinical epigenetics 8:4
94. Rodino-Klapac LR (2013) MicroRNA based treatment of cardiomyopathy: not all dystrophies are created equal. Journal of the American Heart Association 2:e000384
95. Curtis BJ, Zahs A, Kovacs EJ (2013) Epigenetic targets for reversing immune defects caused by alcohol exposure. Alcohol Res Curr Rev 35:97–113
96. Jing L, Jin C, Lu Y, Huo P, Zhou L, Wang Y, Tian Y (2015) Investigation of microRNA expression profiles associated with human alcoholic cardiomyopathy. Cardiology 130:223–233
97. Whayne TF (2015) Epigenetics in the development, modification, and prevention of cardiovascular disease. Mol Biol Rep 42:765–776
98. Reuter S, Gupta SC, Park B, Goel A, Aggarwal BB (2011) Epigenetic changes induced by curcumin and other natural compounds. Genes & nutr 6:93–108
99. Zhu X, Wang H, Liu F, Chen L, Luo W, Su P, Li W, Yu L, Yang X, Cai J (2013) Identification of micro-RNA networks in end-stage heart failure because of dilated cardiomyopathy. J Cell Mol Med 17:1173–1187
100. Bab I, Gazit D, Chorev M, Muhlrad A, Shteyer A, Greenberg Z, Namdar M, Kahn A (1992) Histone H4-related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic activity. EMBO J 11:1867–1873
101. Bulynko YA, Hsing LC, Mason RW, Tremethick DJ, Grigoryev SA (2006) Cathepsin L stabilizes the histone modification landscape on the Y chromosome and pericentromeric heterochromatin. Mol Cell Biol 26:4172–4184
102. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J (2007) MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation 116:258–267
103. Condorelli G, Latronico MV, Dorn GW 2nd (2010) microRNAs in heart disease: putative novel therapeutic targets? Eur Heart J 31:649–658
104. Ucar A, Vafaizadeh V, Jarry H, Fiedler J, Klemmt PA, Thum T, Groner B, Chowdhury K (2010) miR-212 and miR-132 are required for epithelial stromal interactions necessary for mouse mammary gland development. Nat Genet 42:1101–1108

Cysteine-Dependent Aspartate-Specific Proteases in Coronary Artery Disease

22

Gundapaneni Kishore Kumar and Hanumanth Surekha Rani

Abstract

The incidence of coronary artery disease has been the major cause of death all over the globe. Apoptosis an ordered and coordinated cellular process has now been perceived in various vascular diseases including coronary artery disease. It is initiated by atherosclerotic risk factors and is regulated by local and systemic factors and contributes to growth, development, and rupture of plaque. Cysteine-dependent aspartate proteases mediate the both extrinsic and intrinsic apoptotic pathways. Caspases are evolutionarily conserved, intracellular endo-proteolytic enzymes involved in pleiotropic functions like apoptosis, regulation of innate immune system, cell proliferation, cytokine release. Altered caspase activity promotes the various pathological conditions including coronary artery disease. Inhibition of caspases and apoptosis guarantees to be an exceptionally vital focus for coronary artery disease.

Keywords

Coronary artery disease · Proteases · Caspases · Apoptosis · Plaque rupture

1 Introduction

The incidence of coronary artery disease has been the major cause of death all over the globe. It is a chronic multifocal, lipid and immuno inflammatory disease reducing the sufficient blood and nutrient supply to heart leading to heart attack.

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The interplay between genetic and environmental risk factors causes the development of coronary artery disease [1].

Atherosclerosis, principal cause of coronary artery disease develops through the disruption of endothelial function and accumulation of lipoproteins in the intima of the coronary arteries. Elevated concentration of low density lipoprotein (LDL) permeates into disrupted endothelium and modifies into oxidized LDL which is engulfed by macrophages leading to formation of foam cells and a fatty streak, the earliest hallmark of atherosclerosis [2]. Smooth muscle cells are then recruited and undergo proliferation at the site of foamy cells resulting in the initiation of plaque. The plaque is occupied by extra cellular matrix such as collagen and proteoglycans, secreted components of smooth muscle cells. The fatty streak converts into the fibrous plaque and the lesions begins to encroach the lumen of the vessel and disrupts the blood supply to the heart [2].

Apoptosis is a prototypic mode of programmed cell death involved in vital processes such as cell turnover, embryonic development, senescence, and immunity. It has now been perceived in various vascular diseases including coronary atherosclerosis. The occurrence of apoptosis depends on different physiological and pathological conditions of the cell [3].

Apoptosis, a genetically regulated process involves in the pathophysiological progression of atherosclerosis. It is initiated by atherosclerotic risk factors and has been observed in all cells of the atherosclerotic plaque. It also contributes to the growth and development of atherosclerotic plaque. Apoptosis of smooth muscle cells destabilizes the fibrous cap and causes plaque rupture and thrombosis. The effects of apoptosis on plaque depend on various factors such as the cells that are involved, stage of plaque, and region of in plaque [2–5].

The main hallmarks of apoptosis include condensation of chromatin, fragmentation of DNA, membrane blebbing, shrinkage of cell. Membrane-bound apoptotic bodies, processed organelles are removed by macrophages by phagocytosis. The impaired phagocytosis mechanism of foam cells in plaque causes defective efferocytosis and enhances the development of atherosclerosis [3, 6].

2 Biochemical Changes in Apoptosis

The main biochemical changes during apoptosis include breakdown of DNA, changes in membrane structure and phagocytosis. Activation of Caspases ("c" refers to a cysteine protease, the "aspase" refers the cleavage of aspartic acid) is the unique characteristic feature of apoptosis [7].

3 Proteases

Proteases involved in protein catabolism generate amino acids by hydrolyzing the peptide bonds. Intracellular proteases degrade damaged or excess peptides within a cell and are essential for the transport of peptides to their active site. Extracellular

proteases involves in the digestion, activates the blood-clotting system, the complement system, and the fibrinolytic system of the body.

The functions of proteases include regulation of interactions between proteins, production of new bioactive molecules, generation, transformation, and multiplication of molecular signals and processing of cellular information along with various other known functions such as replication, transcription, necrosis, apoptosis, etc. [8]. The size of the proteases ranges from 20 kDa to 0.7–6 MDa. Proteases exhibit different specificity toward the substrates. Proteases like angiotensin-converting enzymes have an exquisite specificity and most of the proteases show the non-specificity toward the substrate while that of proteinase K protease targets on multiple substrates. Altered proteolytic systems have been reported in various pathophysiological conditions including cardiovascular disease [8].

4 Classifications of Proteases

Proteases are enzymes that can hydrolytically break down the enzymes, proteins, and polypeptides.

I. Based on the point of attack used by the proteases, they are classified as either exoproteases or endoproteases. Exoproteases remove a single amino acid from the end (either the N- or C-terminus) of the peptide chain and are subdivided into N-terminal or C-terminal exoproteases. Hydrolytic splitting in the middle of the chain is catalyzed by endoproteases.
II. Depending on the mechanism of catalysis, proteases are classified into six distinct classes, aspartic, glutamic, metalloproteases, cysteine, serine, threonine proteases.
III. On the basis of amino acid sequence, proteases are further grouped into following families.

1. Metalloproteases
2. Serine proteases
3. Cysteine proteases
4. Threonine proteases
5. Aspartic proteases.

Metalloproteases, serine proteases are the most abundant proteases, with 194 and 176 members, respectively, followed by 150 cysteine proteases, whereas threonine and aspartic proteases contain only 28 and 21 members, respectively [7]. Cysteine proteases, i.e., caspase enzymes participates in both death receptor and mitochondrial apoptotic pathways as initiators and executors [8].

5 Cysteine-Dependent Aspartate Proteases

Caspases are generated constantly and exists as inactive proenzymes. Caspases are found in the cytoplasm of cells as inactive monomeric zymogenic precursors known as procaspases. Cell death stimulants activate the procaspases leading to dimerization and formation of mature caspases, which become proteolytically active heterotetramers with two small and two large subunits [9]. These activated caspases are associated with various functions such as tissue differentiation, cleavage of various apoptotic, inflammatory substrates, DNA damage, aging, etc. [9].

6 Discovery and Nomenclature of Caspases

Robert Horvitz H was the pioneer, found the role of caspases in apoptosis and along with Junying Yuan discovered that Ced-3 gene of C.elegans encodes cysteine protease. Cysteine protease enzyme is necessary for apoptosis and has similarities with mammalian interleukin-1-beta converting enzyme (ICE), which is also known as caspase 1. Caspase 1 is the only known enzyme in 1990s, later on other mammalian caspases were subsequently identified and numbered in the order in which they were identified [10, 11].

7 Caspases: Structure and Classification

Caspases are 30–50 kDa zymogens synthesized as inactive proteases. Each caspase consists of three parts including an amine (N) terminal (prodomain), an active site which is located within the large subunit (p20), and a small subunit (p10) (C-terminal) with similar sequences and specificities. These three regions are separated from each other through a cleavage site of aspartic acid. Proteolytic cleavage activates the zymogens and separates the large and small subunits and removes the prodomain and could be recognized as a tetrameric heterodimer. Thus, an active caspase encompasses two subunits of large and small with an active site within the large one [12].

Regarding to amino acid sequences, cysteine proteases can be classified into three classes:

(i) **Initiator or apical caspases**

Initiator caspases are low in abundance and initiates the cell death by receiving pro-death signal and proteolytically activates the effector caspases. For e.g., caspase-2, caspase-8, caspase-9, caspase-10 [13].

(ii) **Executioner or effector caspases**

These caspases are activated by initiators and executes the cell death by proteolysis of essential proteins for life. These caspases also cleave the other protein substrates within the cell, to trigger the apoptosis. For e.g., caspase-3, caspase-6, caspase-7 [13].

(iii) **Inflammation mediators**

During the inflammation process inflammation mediators regulate the cytokine maturation and activated mediators induces apoptosis [12]. For e.g., caspases-1, caspase-4, caspase-5, caspase-12, caspase-14.

8 Caspase Cascade

Regulation of caspases occurs at a posttranslational level as follows.

Cytotoxic T lymphocytes and NK cells releases Granzyme B which activates the caspase-3 and -7.

- Fas, TRAIL, and TNF receptors are considered as death receptors, activates caspase-8 and -10.
- The cytochrome c and Bcl-2 family regulates the apoptosome which activates caspase-9 leading to the execution of apoptotic events.

9 Pleiotropic Functions of Caspases

Caspases are evolutionarily conserved proteases hydrolyze the substrates with aspartic acid and generate active form of caspases from procaspases.

Several studies demonstrated that caspases have pleiotropic functions like apoptosis, regulation of innate immune system, cell proliferation, cytokine release, stem cell self-renewal, and differentiation [8, 14]. Caspases also involves in other cell death types such as pyroptosis, necroptosis, autophagy (antiautophagic (caspase-2, -3, -6, -7, -8, -10) proautophagic (caspase-3 and caspase-9), and mitotic catastrophe. Functions of various caspase genes are shown in Table 1.

Table 1 Cysteine-dependent aspartate proteases (caspases) genes and their functions

S. No.	Caspase gene	Chromosome location	Exons	Functions
1	Caspase 1	11q23	11	Innate immune system, inflammatory apoptosis (pyroptosis)
2	Caspase 2	7q34-q35	13	Apoptosis initiator
3	Caspase 3	4q34	8	Apoptosis executor, activated through both extrinsic and intrinsic pathways
4	Caspase 4	11q22.2-q22.3	10	Inflammation, activator for immature ILs
5	Caspase 5	11q22.2-q22.3	10	Inflammation, activator for immature ILs
6	Caspase 6	4q25	8	Activator of caspase 8
7	Caspase 7	10q25	12	Contributes to apoptosis by influencing on granzyme
8	Caspase 8	2q33-q34	16	Fas-Fas L-apoptosis initiator
9	Caspase 9	1p36.21	14	Apoptosis initiator (mitochondrial-mediated pathway), essential component for activation of apoptosis executioner, activates caspases-3 and 7 which are effective players
10	Caspase 10	2q33-q34	13	Cleaves Bid, activate the mitochondrial pathway
11	Caspase 11	7p22	26	Similarity with Caspase 4, 5 (inflammation)
12	Caspase 12	11q22.3	9	ER stress-induced cell death
13	Caspase 14	19p13.1	7	Least evolved gene, found in keratinocytes, skin barrier
14	Caspase 16	16p13.3	11	Pseudogene

10 Caspase-Mediated Apoptosis

Caspases cleave the proteins responsible for the apoptotic pathways, thereby promoting or inhibiting further reactions. The capase-8, caspase-9, caspase-12, caspase-7, caspase-3 and receptors such as TNF-alpha, FasL, Toll-Like Receptors (TLRs), Death Receptor (DR), etc., participate in the caspase-mediated apoptosis.

(i) **Phases of Apoptosis**

The apoptosis can be divided into:

(a) **Initiation or signaling phase**

Initiation phase of apoptosis may occur following pro-apoptotic stimuli and attachment of relevant promoting molecules to cell surface receptors and subsequent recruitment of death domain proteins promotes the apoptosis [15].

(b) **Control and effector phase**

This phase is dependent on the sequential activation of caspases mediating the transduction and execution of the apoptosis [15].

(c) **The structural alterations phase**

The structural alterations phase occurs due to activation of executor caspases, resulting in the cleavage of lamin, fragmentation, and degradation of genomic DNA leading to an irreversible loss of cell viability [15].

(d) **Recognition and removal phase**

During recognition and removal phase, dead cells and apoptotic bodies are removed as a result of phagocytosis by macrophage cells [15].

11 Apoptotic Pathways

Apoptosis is triggered either by extrinsic or intrinsic pathways involving different caspases but finally these two pathways usually converge on a common effector caspase, such as caspase-3.

(i) **The extrinsic/death receptor apoptosis pathway**

The binding of extra cellular ligand to its specific death receptor triggers the extrinsic apoptotic pathway, resulting in the recruitment of procaspase 8 protein which further activate the downstream effector caspases (caspase-3, caspase-6, caspase-7) to execute the final morphological and biochemical events of apoptosis [16–18]. These effector caspases accelerate the various events targeting the degradation of proteins, DNA fragmentation, cell shrinkage, etc. [19].

(ii) **Intrinsic/mitochondrial apoptosis pathway**

Factors such as deprivation of growth factors, DNA damage, hypoxia, concentration of Ca^{2+} ions, oxidants trigger the intrinsic or mitochondrial apoptotic pathway. The changes in the electrochemical gradient of the inner membrane of the mitochondria are regulated by the Bcl-2 family of proteins resulting in the activation of caspases (caspase 9 and downstream caspases) leading to release of cytochrome c into the cytosol [17, 20]. Bcl-2 family of proteins can be either proapoptotic or antiapoptotic and also determines the commitment of the cell to apoptosis [20].

12 Caspases in Pathogenesis of Human Diseases

Apoptosis eliminates the intracellular components and maintains the homeostasis between cell proliferation and cell death. Activation of initiator and executioner caspases promotes the degradation of important cellular proteins and also activates other enzymes. Altered caspase activation promotes the various pathological

conditions such as cancer, autoimmune diseases, sepsis, immunodeficiency, neurodegenerative, and cardiovascular disorders [17, 21].

13 Apoptosis as Therapeutic Target for Coronary Artery Disease

Apoptosis involves in growth, development, and rupture of atherosclerotic plaque in coronary artery disease and contributes to disease progression and poor prognosis of coronary artery disease. The complexity of the apoptotic pathways is not well defined and the importance of apoptosis in different cell types during the various stages of CAD and its clinical complications are limited. Therefore treatment strategies might be directed towards the stimulants and mechanisms contributing to apoptosis in coronary artery disease [22].

14 Conclusions

Cysteine-dependent aspartate proteases have a significant role in the apoptotic events of coronary artery disease. Several atherosclerotic risk factors induce the apoptosis and all cell types of plaque are involved in the progression of apoptosis resulting in plaque rupture. Inhibition of caspases and apoptosis guarantees to be an exceptionally vital focus for restorative intercession for coronary artery disease. Nonetheless, more work is important to comprehend the molecular mechanisms and the significance of apoptosis in coronary artery disease.

References

1. Ghassibe SM, Platt DE, Youhanna S et al (2012) Genetic and environmental influences on total plasma homocysteine and its role in coronary artery disease risk. Atherosclerosis 222 (1):180–186
2. Badimon L, Vilahur G (2014) Thrombosis formation on atherosclerotic lesions and plaque rupture. J Intern Med 276:618–632
3. Szymanowski A (2015) Detection of apoptosis in patients with coronary artery disease. Printed in Sweden by LiU-Tryck, Linköping, Sweden, ISBN 978-91-7519-029-7. ISSN 0345-0082
4. Kumar GK, Kumar GR, Spurthi KM et al (2016) Polymorphisms of extrinsic death receptor apoptotic genes (FAS−670 G>A, FASL−844 T>C) in coronary artery disease. Apoptosis 21:558–565
5. Jacob FB, Fumiyuki O, Renu V et al (2014) Mechanisms of plaque formation and rupture. Circ Res 114:1852–1866
6. Van Vré EA, Ait-Oufella H, Tedgui A et al (2012) Apoptotic cell death and efferocytosis in atherosclerosis. Arterioscler Thromb Vasc Biol 32:887–893

7. Wong RSY (2011) Apoptosis in cancer: from pathogenesis to treatment. Wong J Exp Clin Cancer Res 30:87
8. Carlos LO, Judith SB (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283(45):30433–30437
9. Şehnaz B, Füsün Ö, Selda G et al (2016) A matter of regeneration and repair: Caspases as the key molecules. Turk J Biol 40:333–352
10. Yuan J, Shaham S, Ledoux S et al (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75(4):641–652
11. Emad SA, David J et al (1996) Human ICE/CED-3 protease nomenclature. Cell 87:171
12. Shalini S, Dorstyn L, Dawar S et al (2015) Old, new and emerging functions of Caspases. Cell Death Differ 22:526–539
13. Amanda BP, Christopher DF, Sally K (2013) Cellular mechanisms controlling caspase activation and function. Cold Spring Harb Perspect Biol 5:a008672
14. Lamkanfi M, Festjens N, Declercq W et al (2007) Caspases in cell survival, proliferation and differentiation. Cell Death Differ 14:44–55
15. Paul SB, Janet DP (2010) Apoptosis: understanding programmed cell death for the CRNA. AANA J 78(3):237–245
16. Stoneman VEA, Bennett MR (2004) Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci 107:343–354
17. David RM, Thorsten B, Tak WM (2013) Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 5:a008656
18. Matthew B, Luis RM, Rebeka LDG et al (2013) Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol 14:32
19. Giuseppa P, Daniela T, Claudia C et al (2016) Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. AGING 8:4
20. Baig S, Seevasant I, Mohamad J et al (2016) Potential of apoptotic pathway-targeted cancer therapeutic research: where do we stand? Cell Death and Disease 7:e2058. doi:10.1038/cddis.2015.275
21. Li J, Yuan J (2008) Caspases in apoptosis and beyond. Oncogene 27:6194–6206
22. HoKim N, Kan PM (2010) Apoptosis in cardiovascular diseases: mechanism and clinical implications. Korean Circ J 40(7):299–305

Association of Matrix Metalloproteinases with CVD: Functional Aspects

23

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Abstract

Matrix metalloproteinases (MMPs) are the members of the family of proteolytic enzymes that have an imperative role in several physiologic and pathologic processes. MMPs mediate changes in extracellular matrix and target growth factor-binding proteins, cell–cell adhesion molecules, and other structural extracellular matrix proteins besides others. Along with their tissue inhibitors, they play an apparent role, ranging from vascular remodeling to neogenesis; extracellular matrix degradation to plaque formation; and from plaque rupture to heart failure. Hence, MMPs regulate a plethora of biological processes and are regulated in vivo by their endogenous inhibitors, e.g., tissue inhibitors of metalloproteinases (TIMPs). Keeping in view the plenty of evidence available in the literature, MMPs can be envisaged as central players in cardiovascular disorders such as heart failure, atherosclerosis, platelet aggregation, stroke, cardiomyopathy, peripheral vascular disease, hypertensive heart disease, and

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aortic aneurysms. Thus, MMPs can be viewed as a most versatile potential targets for therapeutic intervention in such disorders. This chapter highlights the functional role of MMPs in various cardiovascular disease conditions.

Keywords
Cardiovascular disease · Atherosclerosis · Matrix metalloproteinases · Stroke · Tissue inhibitors of metalloproteinases

1 Introduction

Proteinases, either extracellular or intracellular are needed for various developmental and biological processes. The competence to degrade a protein by an enzyme is crucial for a cell to interact correctly with immediate surroundings, either to maintain a controlled physiological process or to disrupt the normal milieu of the cell. The degrading substrates could range from structural to functional proteins. Any unwarranted alteration in protein structure or function may have a deleterious effect on normal physiology, and an abnormal physiology may culminate into a disease or a syndrome. Among these surging cumulative diseases, is a complex set of heart dysfunctions caused due to the degradation of extracellular matrix (ECM). The massive spectrum of cardiovascular diseases triggers the onset of cardiovascular complications from heart failure to acute myocardial infarction (MI) or sudden death, branding it as a major cause of mortality worldwide. Due to the complexity and heterogeneity of cardiovascular disease (CVD), this ailment takes more lives annually than any other disease. Global statistics show that CVD is the foremost cause of death worldwide, representing 31% of all global deaths [1, 2].

The cardiac tissue and cellular framework play an important role in the proper functioning of the heart and vessels. Even a mild change in the cellular framework has been linked to the pathogenesis of several cardiovascular conditions, like peripheral arterial disease, atherosclerosis, and heart failure [3, 4]. A number of molecules have been characterized that have the tendency to alter the cardiac cellular framework and initiate cardiovascular dysfunctions, which are considered as the hallmarks of CVD. Counted in the list are collagen, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) [5]. Of these analytes, MMPs are the proteases that have provided an exciting functional insight into the CVD pathology, prognosis, and management. MMPs play an essential role in the ECM remodeling in both normal and pathological conditions of degradative disorders [6, 7]. Though MMPs monitor cell behavior closely and regulate numerous biologic processes like morphogenesis, angiogenesis, or wound healing [8], our focus in this chapter is to discuss the functional aspects of proteases in the context of CVD perspective. In the present chapter, we highlight the role of these MMPs, their functional and therapeutic aspects related to various categories of CVD pathology.

2 What Are MMPs?

Way back in 1992, MMPs were discovered in an effort to find how the metamorphosing tadpole of a frog lost its tail. Further studies prompted scientists to postulate that, they are omnipresent from the simplest bacteria to the fungi and to the humans [9]. MMPs are a family of Zn^{2+}- and Ca^{2+}-dependent endopeptidases, that degrade several ECM proteins like elastins, gelatin, collagens, matrix glycoproteins, and proteoglycans.

MMPs are secreted as zymogens by a variety of connective tissue and cells like monocytes, endothelial cells, smooth muscle cells, and macrophages or inflammatory T cells. A pro-peptide domain of MMP is connected by a cysteine bond, which when hydrolyzed, frees the pro-peptide domain and exposes the enzymatic site on the parent MMP molecule [10]. Proteolytic activity of MMPs is kept in check under normal physiological conditions and is mainly controlled by zymogen activation, transcription, and by the active forms of their tissue inhibitors known as TIMPs. MMPs are constitutively expressed in low concentrations during normal physiological conditions, however, if this homeostasis is dysregulated, they invoke various pathological responses as is seen in many cardiovascular disease conditions like atherosclerosis, angina, or congestive heart failure besides others [11]. Apart from structural ECM components, MMP substrates also include a cluster of receptors and ligands such as growth factors, cytokines, chemokines, and adhesion proteins that modify cellular migration, adhesion, and activation. MMPs, therefore, have a strong impact on cardiac makeover through various mechanisms [8, 12]. MMPs also play a dominant role in neogenesis or organ development and subsequent tissue remodeling in inflammation and injury. Their role is progressively appreciated in understanding the process of plaque formation and subsequent rupture or in the development of cardiomyopathies, hence MMPs are considered as reliable indicators with regard to CVD diagnosis, prognosis, and management.

2.1 MMP Classification, Structure, and Function

At present, 25 vertebrate MMPs and, at least, 26 human MMPs are known [13]. Additionally, few nonvertebrate MMPs have also been identified that regulate cell differentiation and development process [14, 15]. On the basis of their substrate specificity, these MMPs are categorized into collagenases, gelatinases, stromelysins, and matrilysins (Table 1). Another subtype of MMPs is known as membrane-type MMPs (MT-MMPs) which are membrane-associated proteases and contain additionally a transmembrane and an intracellular domain with a membrane linker domain [16].

A typical MMP consists of four discrete domains of variable amino acid lengths, which are N-terminal, hemopexin (Hpx) or C-terminal domain, a linker peptide or hinge region, and a catalytic domain (Fig. 1). Though there are few exceptions to this general structure, some MMPs may lack one or more domains, for example, MMP-7, MMP-23, and MMP-26 are deficient in the hinge and Hpx domain [17]. These domains may be responsible for the interaction with TIMPs or entail different

Table 1 Matrix metalloproteinases members and their common substrates, biological functions, cellular localization, and an established role in cardiovascular disease

MMP class	Enzyme	Substrate	Cellular localization	Biological effect	Associated CVD pathology	References
MMP-1	Collagenase-1	Collagens I, II, III, VII, X, gelatin	Fibroblasts, interstitial tissue, platelets	Keratinocyte migration and reepithelialization, vasoconstrictor generator, anti-inflammatory	AA, atherosclerosis, platelet aggregation, and MI	[63, 78, 81]
MMP-2	Gelatinase-A	Collagens I, IV, V, VII, X, XI, gelatin, fibronectin, laminin, elastin, proteoglycans	SMCs, VSMCs, platelets	Cell migration and differentiation, collagen affinity, vasoconstrictor, increased bioavailability of TGF-β	Atherosclerosis, AA, arterial restenosis, critical limb, ischemia, heart failure	[52, 70, 71]
MMP-3	Stromelysin-1	Fibronectin, laminin, collagen II, III, IV, IX, X, and XI, MMP-7, -8, -13	Platelets, SMCs, fibroblasts	Generation of angiostatin-like fragment, increase collagen affinity	Atherosclerosis, AA, BBB disruption, stroke, ischemia	[94, 95]
MMP-7	Matrilysin-1	Type I, II, IV, and V gelatins, fibronectin, and proteoglycan, plasminogen	Uterus, cancer cells	Adipocyte differentiation, enhanced collagen affinity, cell aggregation and cell invasion, vasoconstriction and cell growth	Atherosclerosis	[113, 114]
MMP-9	Gelatinase-B	Collagens III, IV, V, VII, X, elastin, vitronectin, gelatin	Monocytes, SMCs, VSMCs, platelets	Generation of angiostatin-like fragment, enhance collagen affinity, cell migration, Bioavailability of TGF-β	Angiogenesis, AA, ischemia, stroke, arterial restenosis, MI, platelet aggregation	[31, 77, 90]
MMP-10	Stromelysin-2	Collagens III, laminin, casein, MMP-1, -8, fibronectin	Uterus, ECs	Cell migration, tissue remodeling, embryonic development	Atherothrombosis, vascular remodeling	[115]
MMP-11	Stromelysin-3	Collagens IV, gelatin, fibronectin, laminin, IGFBP-1	Fibroblasts	Cell proliferation, increase bioavailability of IGF-1	Thrombosis	[116]
MMP-12	Macrophage elastase	Collagen IV, elastin, fibronectin, plasminogen	Microphages	Generation of angiostatin-like fragment	AA, emphysema	[117]

(continued)

Table 1 (continued)

MMP class	Enzyme	Substrate	Cellular localization	Biological effect	Associated CVD pathology	References
MMP-13	Collagenase-3	Collagen I, II, III, IV, XIV, gelatin, fibronectin, osteonectin	SMCs	Anti-inflammatory, enhance collagen affinity	Rheumatoid heart disease, cardiomyopathy	[118]
MMP-14 -15 -16 -17 -24 -25	Membrane type-1 -2 -3 -4 -5 -6	Collagens I, II, III, gelatin, fibronectin, vitronectin, proteoglycans, activate pro-MMP-2 and pro-MMP-13	Fibroblasts, SMCs, platelets, leukocytes	Angiogenesis, apoptosis, activates pro-MMP-2	Atherosclerosis, platelet aggregation, neo-vascularization, tissue remodeling	[119, 120]

AA Aortic aneurysm; *SMC* smooth muscle cell; *VSMC* vascular smooth muscle cell; *MI* myocardial infarction; *TGF-β* transforming growth factor beta; *BBG* blood–brain barrier; *IGF-1* insulin-like growth factor 1

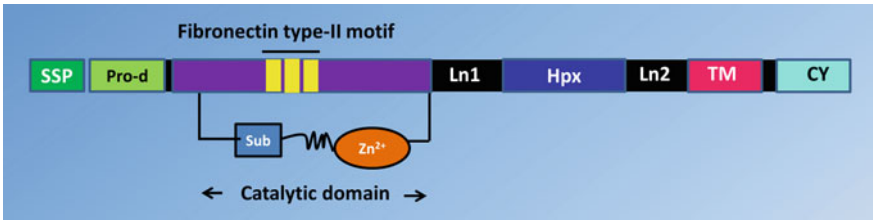

Fig. 1 Domain arrangement and structure of MMP. All the MMP family members share the common signaling sequence peptide, propeptide domain, catalytic and hemopexin-like domains. *SSP* Signal sequence peptide; *Pro-d* pro-domain; *Sub* substrate; *Cat.domain* catalytic domain; *Ln1* linker-1; *Hpx* hemopexin; *Ln2* linker-2; *TM* transmembrane domain; *CY* cytoplasmic tail

functions like substrate recognition, regulation and mediation [18]. The zinc-binding motif HEXXHXXGXXH and PRCGXPD motif of the catalytic domain and the pro-peptide domain, respectively, are the most common structural units among the MMPs. The catalytic zinc ion is coordinated by three histidines of zinc-binding motif and cysteine of the pro-peptide. This Cys-Zn^{2+} coordination complex keeps pro-MMPs inactive by averting a water molecule vital for catalysis from binding to the zinc atom [19]. A highly conserved domain "Met-turn" is also found in the catalytic domain next to the zinc-binding motif, which forms the structural base around the whole catalytic domain [20]. However, the MT-MMP structure is rather different than MMPs and the former contains an extra trans-membrane domain that anchors them to the cell surface [21]. It has been also observed that MMP catalytic domains share a noticeable sequence similarity, ranging from 33% between MMP-21 and -23 to a maximum of 86% between MMP-3 and -10 [22], concluding that the overall topology of the enzyme active-site is extremely conserved among the MMPs.

Although, MMPs enjoy a modest way to interfere in cardiovascular dysfunction and angiogenesis, they also inherit competence to; intervene in the mechanisms of cell behavior like cell shape and movement, growth and differentiation or survival; an affinity to cleave several circulating and pericellular proteins, which empowers them to control cell behavior in copious ways. These mechanisms include the potential activation or inactivation of the cell surface receptors; or inactivation of autocrine or paracrine signaling molecules; or alteration of cell–cell interactions [8]. MMP-mediated ECM degradation disrupts the structural barriers of the cell and such degradation processes allow the cellular invasion to take place. As ECM is not just an inert cellular framework; MMPs affect cell behavior via ECM dysregulation and by sequestering signaling cascade molecules, like growth factors and their binding proteins, or by acting as suitable ligands for cell adhesion receptors, that transduce the signal to the cell interior [23].

3 MMPs and Cardiovascular Diseases

As discussed from time to time, MMPs not only take part in biological processes, such as cell growth and migration, morphogenesis, inflammation, angiogenesis, apoptosis, etc., but also participate in pathological remodeling like CVD or tumor growth [24–27]. Oxidative stress, which is involved in the cardiovascular disease, stimulates endothelial cells, smooth muscle cells, and fibroblasts for MMP production and activation [28]. It is evident now that MMPs play a significant role in any process in which matrix turnover and tissue repair are involved; hence, they have an impending role in various facets of vascular biology, from atherosclerosis to acute MI (Fig. 2). In the following section, we will describe thoroughly the functional role of MMPs in various cardiovascular pathological conditions.

3.1 MMPs and Atherosclerosis

The atherosclerotic plaque comprises a lipid core, fibrous cap and a mixture of inflammatory cells, like lipid-laden macrophages. The fibrous cap is normally built up of ECM surrounded by the smooth muscle cells. The components of the ECM, like collagen and elastin, play a decisive role in the fibrous cap annihilation and creation process [29]. Under normal physiological conditions the annihilation and creation processes, also known as a remodeling process, are balanced and the

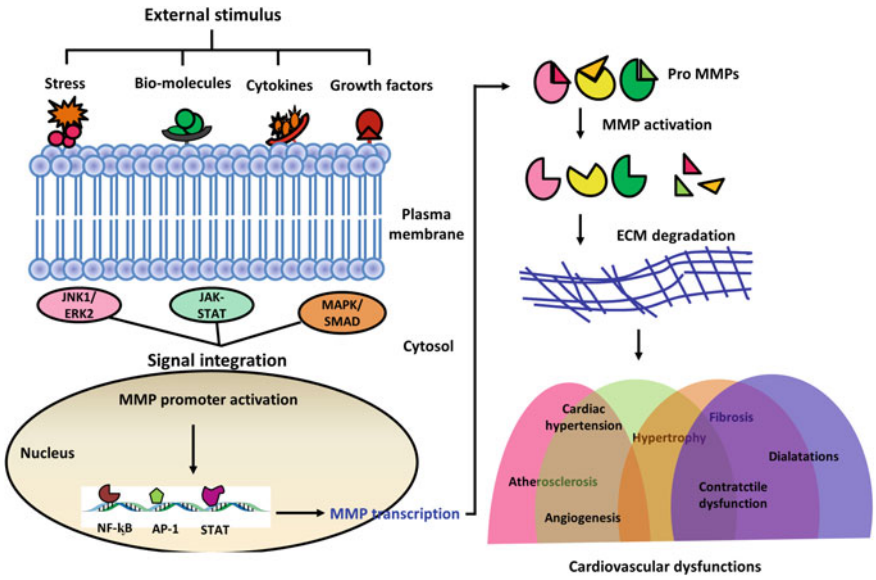

Fig. 2 Mechanism and role of matrix metalloproteinases (MMPs) in various cardiovascular disease conditions

plaque protects the lumen of the vessel to come in contact with the underlying thrombogenic or atherogenic material [30]. However, under hypoxic conditions, which initiate the pro-inflammatory process, the remodeling process can be shifted in favor of resorption of the matrix, leading to a possibly weak fibrous cap and consequent erosion and rupture of the plaque. Previous animal and human studies have documented the presence of co-localization of MMPs (MMP-9 and -3) in the progressing edges of the atherosclerotic plaques [31]. While other MMPs (like MMP-1, -2, -3, -7, -8, -10, -11, -12, and -13) have also been assessed, MMP-9 has been the most studied MMP in the context of atherosclerosis pathology. The increasing presence of the MMPs with inflammatory cells like T-lymphocytes and macrophages at the plaque rupture sites or where extensive remodeling is taking place, have been previously extensively investigated [6].

Interestingly, the atherosclerotic plaque is also embedded with TIMPs, the natural inhibitors of MMPs [32]. However, it has been observed that at certain sites where augmented TIMPs are present, MMPs are associated with vascular calcification [31]. This implicates that vascular calcification is nature's own way of modulating the vascular remodeling and it is possible that TIMPs may overtake the function of MMPs. In nutshell, under normal conditions a balance exists between MMPs and TIMPs, however, if this balance is disturbed by any incitement, the TIMP and MMP activity ratio ceases, favoring an increase in atherosclerotic plaque formation. Further, MMPs once locally activated by inflammatory signals such as free radicals, hypoxia, or by any external stimuli, lead to the degradation of the basic components of ECM like collagen and elastin into free peptide fragments that permit the plaque to rupture. Studies, like those of Luttun et al. [33] have shown that MMPs, particularly MMP-9 is a front runner and plays a vital role in plaque growth and remodeling in the presence of enhanced cholesterol [33]. This study was carried out in transgenic knockout mice deficient in MMP-9, mated with the atherogenic apolipoprotein-E knockout mice. Their study proved that despite excess cholesterol feeding, these mice displayed much less atherosclerotic burden [33].

The MMP activation can also lead to mitogenic signal or growth factor production at the plaque site. The change in the matrix scaffold along with the mitogenic signals induces smooth muscle cell migration into the intima of the vessel and there, these cells transform into macrophage-like structures [34]. Consequently, the activation of MMPs sets up a malicious cycle of the progressive transformation of cells contributing to the atherosclerotic plaque growth and potential rupture [35]. The resultant forces of remodeling with increased inflammatory changes further augment the expression, production, and activation of local MMPs, creating a feedback amplification loop.

Conclusively, it can be summarized that an external stimulus like oxidized low-density lipoprotein (LDL) from the plaque microenvironment results in the elevated transcription of many MMPs while TIMP expression is often depressed in comparison to MMPs. Pro-peptide activation is further enhanced by oxidative stress and foam cell-derived signals. Although growth factors encourage vascular smooth muscle cells to secrete TIMPs, the overall equilibrium between MMP-TIMP balance at the plaque milieu encourages superfluous ECM proteolysis. This is further

aggravated by increased foam cell formation that degrades TIMPs in the extracellular environment, resulting in reduced inhibition of MMPs by TIMPs (Fig. 2).

3.2 MMPs and Heart Failure

The MMP activity and levels have been shown to be augmented in MI and in the development of heart failure [36]. Additionally, during myocardial infarction, plasma MMP levels increase rapidly within hours of the infarction, following the activation of resident macrophages, cytokines, and inflammatory cells [37]. However, as the vascularization process continues, the MMP concentrations decline, but the second upsurge of activation is followed, which is related to ventricular dilation and progression toward heart failure [38]. These observations were also reported earlier in postmortem samples of the patients following a significant MI [36]. In this study, the lytic bands were found to be increased, as evident on the zymographic electrophoretic gels in the myocardial extracts of MI patients, reflective of increased MMP activity particularly of MMP-2 and -9. The levels of these two MMPs are shown to be increased in pericardial and myocardial samples after performing emergent cardiac surgeries of the MI patients [39, 40].

Following MI, a significant contribution of local inflammatory cytokines is attributed toward MMP production and regulation. In a study carried out in an animal model of myocardial infarction, the authors demonstrated that the elevated level of cytokines in the myocardium are directly linked to the augmented production of local MMPs [41], which leads to collagen dissolution that can cause acute myocardial rupture. However, if the process prolongs without myocardial rupture, the heart becomes considerably dilated, promoting reduced heart function and pitiable survival rate. In another animal model study, Spinale et al. [42] reported a time-dependent surge in myocardial MMP levels that accompanies the progression of left ventricular (LV) dysfunction and dilation. These variations in myocardial MMP levels were also accompanied by changes in the ECM structure. These observations suggest that the generation of myocardial MMPs is an early event in the development of LV remodeling and dysfunction. In a rat model of hypertensive heart failure, myocardial MMP levels were shown to increase during the change from compensated LV hypertrophy to the decompensated dilated phenotype [43]. Thus, these animal model studies demonstrate a time-dependent relationship between augmented MMP levels and expression, the remodeling process, and thereby progression to heart failure.

Among the complications which lead to heart failure, diastolic dysfunction is an important contributor toward clinical heart failure [44]. Aberrations in the structure, function, and composition of the ECM have been established to subsidize myocardial compliance and, in turn, impacts the normal diastolic function [45, 46]. Clinical evidence suggests that diminished myocardial MMP activity can facilitate collagen build-up in developing hypertrophy [47–49]. Mujumdar et al. [47] and Li et al. [49] postulated that a time-dependent myocardial MMP activation occurs with the growth of pressure-overload hypertrophy and suppresses the remodeling

process. This hypothesis was corroborated by the findings of Nagatomo et al. [50] where a time-dependent variation in myocardial MMP levels was demonstrated after an acute and lengthy pressure-overload stimulus. These findings suggest that myocardial MMP activity rises with a stimulus that, in turn, changes the architecture of extracellular fibrillar and myocyte support, which is the structural foundation of cardiac hypertrophy.

3.3 MMPs and Cardiomyopathy

Cardiomyopathy is an intrinsic cardiac muscle disease, which can occur due to a genetic disorder, infection, or metabolic dysfunction. One of the common cardiomyopathic disease states that are considered as a major contributor toward the CVD, is known as dilated cardiomyopathy (DCM) [51]. This disease is characterized by significant LV chamber dilatation and severe systolic dysfunction. Since DCM is considered as an advanced CVD condition, this often culminates in severe heart failure and hemodynamic collapse [52].

An easy access to myocardial samples from DCM patients has allowed for an extensive study of the MMP/TIMP system. Additionally, the use of several animal models has paved the way to study changes in myocardial MMPs during the development of DCM [53–55]. These human and animal model studies facilitated significant understanding into the variations in the myocardial MMP system and the relation to the remodeling process [56]. The appearance of MMPs such as MMP-13 and MT1-MMP with DCM clearly indicated that this disease contained the diverse collection of proteolytic substrates and hence may have significantly subsidized to hostile LV remodeling. Rouet-Benzineb et al. [57] demonstrated that MMP-2 and -9 were augmented in DCM and found that MMP-2 levels were actually increased within the myocardial interstitium and cardiomyocytes. Moreover, these investigators came up with the evidence that MMP-2 may destroy contractile proteins such as myosin [58, 59]. Thus, augmented MMP levels within the myocardium of DCM patients may have various deleterious consequences like matrix degradation or proteolytic activation of biologically active signaling molecules and thus, directly affect the structure and function of contractile proteins like myosin. While the earlier studies uniformly established an increase in specific MMP types with DCM, previous studies have reported augmented myocardial TIMP levels [60, 61]. In DCM patients, though the intracellular signaling events and posttranscriptional/-translational processes that enhance the levels of certain MMPs coupled with the decrease in TIMP levels remain to be established, the extracellular outcome of the matrix protein imbalance and of their tissue inhibitors is expected to be significant. Specifically, amplified levels of pro-MMPs within the interstitium would rather deliver a larger pool of MMPs for eventual activation and conversely reduced TIMP levels would favor extended proteolytic activity of MMPs within the interstitium. Conclusively, changes in MMP/TIMP levels would in turn favor myocardial matrix and remodeling, a structural hallmark of DCM [62].

One of the disadvantages of the clinical DCM studies is that the MMP myocardial profiles are determined at the time of transplantation and, therefore, these levels are reflective of end-stage heart failure. Therefore, older studies could not openly address the cause-effect association between LV myocardial matrix remodeling and changes in the MMP/TIMP system. However, few clinical studies have been accomplished in which comparative MMP/TIMP levels were determined in DCM subjects during LV assist device placement, and then reexamined after following several months when the device was detached [63, 64]. For example, the study of Klotz et al. [63] reported that the MMP-1/TIMP-1 ratio was near normal in DCM patients when followed after several months of LV mechanical assisted support. Thus, the data of these clinical findings provide a basis for the mechanistic association between changes in relative MMP/TIMP levels and matrix remodeling in patients with DCM.

3.4 MMPs and Peripheral Vascular Disease

Peripheral vascular disease (PVD) is a chronic circulatory disorder that affects the peripheral blood vessels which supply blood to the arms, legs, and organs located below the stomach. It affects a substantial fraction of the population worldwide. It is projected that around 20% of the adult population older than 55 years have PVD with a linked strong surge in cardiovascular morbidity and mortality [65]. The clinical manifestation of PVD is sporadic claudication, with leg pain at rest and upon exercise. Ulcers and gangrene develop in the later stage of the disease, known as critical limb ischemia. It has been usually observed that PVD patients are completely asymptomatic, whereas others display symptoms mimicking musculoskeletal diseases. Several observations are briefly mentioned below which show that MMPs play a pivotal role in PVD.

3.4.1 Angiogenesis

A series of adaptive changes like angiogenesis and arteriogenesis are observed in response to arterial stenosis and reduced blood flow, aimed at maintaining blood flow to the ischemic tissue. Endothelial cell activation-assisted capillary growth culminates into the proteolysis of the basement membrane, that permits cell proliferation and growth of fresh capillaries [66]. The degradation of the capillary basement membrane is accomplished by MMPs, hence, may modulate angiogenesis by multiple mechanisms. The ECM degradation permits the collapse of the vascular basement membrane, a prerequisite for the dissemination of endothelial cells in the subendothelial matrix and for the formation of a new vascular lumen [66]. Few members of the MMP family like, MMP-1, -2, and -9 have been associated in the angiogenic response to ischemia. MMP-9 appears to play a dual role in the regulation of endothelial cell proliferation; either it promotes the process or inhibits it by angiostatin activation. Angiostatin is produced by the hydrolysis of specific peptide bonds in plasminogen, mediated by several MMPs like MMP-2, -3, -7, -9, and -12 [67].

3.4.2 Post-angioplasty Restenosis

Post-revascularization restenosis is one of the main limitations of the endovascular treatment in PVD. The revascularization episode is the result of two discrete processes: intimal hyperplasia and constructive remodeling. The endothelial damage prompted by angioplasty leads to an enlarged deposition of ECM in the vessel wall, resulting in constrictive remodeling with the reduction of the arterial diameter. As demonstrated by Jenkins et al. [68] in an isolated animal model study, this remodeling infers an upregulation of MMPs. A recent study using MMP activated specific "in-labeled" tracer reported MMP activation in the common carotid artery, 2–4 weeks post-injury in an apolipoprotein $E(^{-/-})$ mice [69].

Intimal hyperplasia is another condition that may hinder in endovascular management of arterial stenoses in PVD. Whether MMPs have any functional role in intimal hyperplasia, was established by the observation that pretreatment with MMP inhibitors, like tetracyclines or batimastat, diminishes neointima formation. Though the exact role of MMPs in intimal hyperplasia is uncertain, an earlier study carried out in knockout mice does suggest that MMP-2 and -9 are intimately involved in this process [70].

3.4.3 Arteriogenesis

The growth of preexisting arteriolar connections into the collateral arteries in retort to the compression of a major axial artery is known as arteriogenesis. During arteriogenesis, normal vessels dilate to form natural bypasses, paving a way for an improved blood flow to the limb [66]. MMPs along with other growth factors and cytokines, stimulate a remodeling cascade that alters small vessels into arterioles proficient of a markedly increased blood flow. MMP-2 and -9 levels are shown to be ~tenfold higher in the adventitia of coronary vessels as compared to the normal coronaries, endorsing the important role of MMPs in arteriogenesis [71]. Arteriogenesis is prominently seen in skeletal muscle of patients with critical limb ischemia, with condensing of the basement membrane of capillaries. This phenomenon is observed early in PVD and is also noticed in patients with recurrent claudication. MMPs are shown to play a pivotal role in the thickening of capillary basement membranes, thus promoting arteriogenesis in PVD subjects.

3.4.4 Arterial Calcification

Arterial calcification, earlier considered to be a degenerative process is a multifaceted and controlled phenomenon [72]. Recent prospective studies endorse a strong prognostic value of arterial calcifications for adverse cardiovascular events. Unambiguously with PVD, arterial calcifications in the mid-thigh femoral artery are connected with an increase in the risk of amputation [73]. MMPs play an active role in the generation of arterial calcifications via elastin degradation, which favors calcium deposition. The elastin degradation leads to the specific cascade process that secretes chemokines, which recruit other inflammatory cells. In fact, different animal model studies demonstrated that in mice where MMP-2 and -9 were deleted, remain resistant to $CaCl_2$-mediated aortic calcification [74, 75].

3.5 MMPs and Aortic Aneurysm

The vascular remodeling process that leads to the formation of an aortic aneurysm (AA) involves inflammation, damage of elastin fibers, and increased collagen in the aortic wall. The structural alterations are complemented by a widespread infiltration of B- and T-lymphocytes, plasma cells, macrophages, cytokines, and increased local concentrations of MMPs. In particular, an imbalance in MMP-2 and -9, and their respective tissue inhibitors, which accounts for degradation of elastin and collagen, is accountable for the fading and dilatation of the aortic wall [32]. Few studies endorse the fact that MMP activity is increased in AA specimens, in particular MMP-2, than cells derived from non-aneurysm tissues [32, 76]. Interestingly, in one of the studies, augmented levels of MMP-2 were detected in vascular tissue distant from the aneurysm site, but not in the healthy controls, demonstrating that the AA exhibits a universal predisposition toward enhanced ECM proteolysis [76]. MMP-9 seems to be the major metalloproteinase expressed in AAs, as evidence in the literature demonstrates much higher levels of MMP-9 than other MMPs studied. Studies have shown that not only are the aortic wall levels of MMPs augmented in patients with AA, but plasma levels of MMP-2 and -9 are also increased, inferring that circulating MMP levels could be considered as biologically applicable markers of connective tissue metabolism in patients suffering from AA [77].

3.6 MMPs and Platelet Aggregation

Numerous MMPs have been identified in platelets, including MMP-1, -2, -3, -9, and MT1-MMP [78]. Platelet aggregation is positively moderated by the release of MMP-2 from the platelets, whereas MMP-9 stabilizes the pro-aggregatory effect of MMP-2 [79]. MMP-2 augments the pro-aggregatory effects of agonists on diverse receptors, delineating the fact that the proteinase facilitates platelet initiation at the level of second messenger system. It is an established fact that MMP-2 primes platelet activation by facilitating phosphatidylinositol 3-kinase activation system [80]. Additionally, as demonstrated in the above-mentioned study, MT1-MMP at the platelet surface contributes to the activation of MMP-2 by the formation of a complex MT1-MMP/TIMP-2/MMP-2 [79].

Apart from MMP-2, MMP-1 is also shown to positively moderate platelet activation by causing accumulation of tyrosine-phosphorylated proteins in platelets, thus initiating the transfer of β3-integrins to cell contact sites [81]. Platelets though impart functional kinetics in hemostasis and thrombosis, they equally contribute to the inflammation and atherogenesis process, via inflammatory mediators [82]. A specific and important role in the inflammatory and pro-atherogenic activity of platelets appears to be played by the CD40/CD40L pathway [83] and it is remarkable to note that the release of sCD40L from platelets is partly reliant on MMP production [84].

3.7 MMPs and Stroke

Stroke is caused by disruption of the blood supply to the brain either by blockage (ischemic stroke) or by the rupture of a blood vessel (hemorrhagic stroke). This pathological condition takes 6 million lives every year, is one of the major contributors to CVD after coronary heart disease which alone accounts for 7 million deaths every year [85]. The pathophysiology and biochemistry of stroke are multifaceted. The earliest vascular events in stroke rapidly lead to energy loss, which eventually initiates a wide and complicatedly linked cascade of neuronal death pathways.

MMPs play an essential role in stroke by crumbling ECM substrates that are indispensable for customary signaling and homeostasis within the neurovascular unit. Stroke onset culminates into uninhibited MMP activity, which facilitates abnormal proteolysis thus leading to blood–brain barrier leakage and ultimately cell death [86, 87]. Two earlier independent studies of Clark et al. [88] and Anthony et al. [89] published in 1997, confirmed for the first time that MMP-9 and -2 levels are elevated in the ischemic human brain. Rosell et al. [90] revealed the presence of high MMP-9 levels not only in the infarcted tissue but also in the peri-infarct areas, suggesting the dominant role of MMPs in the infarct development. MMP-9 levels appear to rise within infarcts after hemorrhagic conversion, correlating with improved neutrophil infiltration surrounding the affected capillaries leading to blood–brain barrier breakage and further capillary damage [91]. These human studies corroborate with the animal model data showing basal lamina injury and loss of collagen type IV, which can be treated with hypothermic therapy that decreases the enzymatic action of MMP-2 and -9 in ischemia-reperfusion rat models [92, 93]. Though, the role of MMP-2 and -9 in stroke is the most discussed topic in the literature, other MMP members, although least studied, may play a significant role as well. For example, MMP-3 can be stimulated after ischemia-reperfusion in rat brain, triggering the cleavage of the cerebral matrix [94]. Similarly, MMP-3 knockout mice display less degradation of tight junctions proteins like claudin-5 and laminin-alpha-1 with condensed neutrophil infiltration as compared with wild-type animals [95].

Although evidence in the literature strongly implicates the role of MMPs in the stroke, the cellular foundation basis of this protease rests to be fully distinct. In an advent of stroke, MMPs are synthesized by local brain cells or peripheral cells that migrate to the damaged brain from the blood stream through the neuroinflammatory response. Increased MMP levels from brain endothelial cells or neurons facilitate neurovascular loss and prolong infarct volumes [96, 97]. Consequently, both brain and blood MMP-9 responses may possibly contribute to the progression of neuronal death, inflammatory infiltration, and neurovascular damage. Future studies in this direction may help to evoke MMP inhibition strategies in stroke.

3.8 MMPs and Hypertensive Heart Disease

Hypertensive heart disease (HHD) is the clinical manifestation in patients that clinically have both diastolic and systolic heart dysfunction. LV hypertrophy and cardiac fibrosis, which are caused by the modifications in the local and systemic neurohormonal environment are the two key facets of heart failure secondary to HHD. As mentioned earlier, every abnormal cardiovascular event is culminated by ECM degradation and the initiation of HHD is no more different. The fibrotic state in HHD develops due to the imbalance between MMPs and their inhibitors, which modify the ECM environment and hence, weaken cardiomyocyte function [98].

Cardiomyocyte and ECM protein synthesis is the predominant process during the onset of HHD and scant data is available regarding the role of MMPs during the early stages of this disease. However, clinical evidence suggests a bigger role of MMP proteases like MMP-1, -2, -8, -9, and -13 in the transaction of this heart dysfunction from compensating state to clinically apparent heart failure, where systolic dysfunction is implicated as the main cause of heart failure in HHD. Further, deterioration of systolic function may trigger the MMP protein activation via the cytokines, causing ECM mutilation, perivascular fibrosis and scarring in the myocardium [99]. These observations suggest that an imbalance in the MMP/TIMP ratio might cause LV dilation and condensed ejection fraction in systolic heart failure. MMPs, either membrane attached or secreted, once exceed the activity level of TIMPs, contribute to the fibrotic process via the ECM degradation by cleaving the α-chains of type I and type II collagens.

Various human and animal model study data has provided a valuable insight into the mechanistic role of MMP proteases in different stages of HHD. The inferences of these studies have given a better understanding of the functional aspects of MMPs in HHD. Laviades et al. [100] have shown that in hypertensive patients with left ventricular hypertrophy (LVH), augmented levels of circulating TIMP-1 are observed, whereas circulating MMP-1 levels and collagen type I telopeptide are reduced when compared with hypertensive patients without LVH. A study conducted in 2006 by Ahmed and his colleagues [101] revealed that patients with normal LV structure and function but with hypertension had normal plasma MMP/TIMP ratio. In contrast, patients with hypertension and LVH had augmented levels of MMP-9 but reduced levels of MMP-2 and -13. Based on these observations, the authors concluded that diminished ECM degradation, a hallmark of cardiovascular dysfunction, was linked with LVH and diastolic dysfunction. Likewise, in an animal model study carried out by Iwanaga et al. [102] in hypertensive Dahl salt-sensitive rats, MMP-2 expression levels were shown to increase along with TIMP-1 and TIMP-2 as LVH progressed. Similarly, in an another isolated study, mice deficient either in MMP-9 or plasminogen activator, were conjointly protected from cardiac fibrosis and LV dysfunction following pressure overload [103]. These human and animal study data further confirm that dysregulation of MMP/TIMP ratio and altered activity of MMPs bear a deleterious effect on the development of HHD.

4 MMPs as Potential Targets in Cardiovascular Disorders

As mentioned in earlier sections of this chapter, enhanced activity of MMPs has been implicated in several physiological and pathological processes such as angiogenesis, morphogenesis, chronic inflammation, autoimmune, neurodegenerative and cardiovascular diseases, chronic obstructive pulmonary disease, cancer, arthritis, etc. The activities of MMPs are kept in check under physiological conditions at the level of transcription and inhibition by endogenous TIMPs. An imbalance in the MMP/TIMP activity ratio is the underlying determining factor in the pathophysiology of vascular diseases associated with tissue remodeling.

In the context of cardiovascular diseases, MMPs have been targeted in several preclinical studies in animal models. Use of synthetic inhibitors in experimental animal models is shown to significantly inhibit aortic calcification, neointima formation, ischemia-reperfusion injury, left ventricular remodeling, and protect against the development of hypertension, heart failure, and atherosclerosis.

The TIMPS are natural endogenous tissue inhibitors of MMPs and are shown to regulate MMP activity at various levels. TIMPs have unique functions and specificity that has been evaluated in experimental animal models in the form of gene-based therapies and provides novel insights into the pathophysiology of such disorders. Using a viral or transgenic approach Zavadzkas et al. [104] demonstrated for the first time, which targeted overexpression of TIMP-4 modified post-MI remodeling in mice. TIMP-2 levels were found to be inversely correlated with infarct size and severity as well as LV dilation. TIMPs have been successfully used in animal models to target neointimal hyperplasia progression and prevent vein graft failure and in-stent restenosis. Though, phase I trials to evaluate the safety of these therapies have been successful with less adverse effects, the efficacy of TIMP therapy has not been confirmed in Phase 2 studies. TIMP-3 gene transfection was associated with reduced left ventricular dilation and recovery of systolic function after MI in a rat model of post-MI, TIMP-3 transfection enhanced the effects of transplanted vascular smooth muscle cells (VSMCs) by inhibiting MMP-2 and MMP-9 and inflammation thus leading to improved myocardial remodeling [105]. Tian et al. [105] reported that cell-based gene transfer of TIMP-3 augmented MMP inhibition and preserved ventricular morphometry and cardiac function in a rat model of MI knockouts. Several other MMP inhibitors including TIMPs, synthetic small molecular weight MMP inhibitors (MMPis), tetracyclines, and inhibitory antibodies have all been tested in experimental animal models of vascular disease till date but only doxycycline which is FDA approved has been extensively evaluated in patients. The TIPTOP (Early Short-term Doxycycline Therapy In Patients with Acute Myocardial Infarction and Left Ventricular Dysfunction to Prevent The Ominous Progression to Adverse Remodelling) trial in primary percutaneous intervention (pPCI)-treated patients demonstrated that doxycycline therapy when used early and for a short term, reduced LV dilation and both infarct size and severity and reduced LV dysfunction and a first ST-elevation myocardial infarction (STEMI) in these patients [106].

Mostly MMP-9 and -2 have been targeted, but the inhibitors have also been considered to target MMP-12 and -13, and for other novel MMPs such as MMP-8, -10, -14, -19, -25, and -28. Several drugs like statins, calcium channel blockers, beta blockers, type II receptor antagonists, angiotensin converting enzyme inhibitors, cholesterol absorption inhibitors, COX-inhibitors have also been shown to inhibit MMP activity in various clinical trials. Polyphenols and flavonoids from natural resources have also been reported to act as potent inhibitors of MMP activity and therefore exert cardioprotective effects [107, 108]. Though many experimental and clinical trials of MMP inhibitors did not demonstrate significant benefits, some trials showed promising results.

Recent studies have reported that MMPs like MMP-2, -9, and -13 are also potential targets of histone deacetylase inhibitors that attenuate biomarkers of cardiovascular risk and inflammation [109]. A study by Lin et al. [110] demonstrated that siRNA increases myocardial contractility and protects against simulated ischemic-reperfusion injury by inhibition of MMP-2 expression. High-mobility group box 1 (HMGB1) is an important mediator of the inflammatory response. HMGB1 knockdown was shown to alleviate cardiac fibrosis and remodeling in diabetic cardiomyopathy in streptozotocin-induced diabetic mice [111]. Recently, in a porcine model of myocardial infarction, Purcell et al. [112] reported that a targeted delivery of polysaccharide-based hydrogels/TIMP-3 construct to areas of MMP overexpression following MI significantly reduced MMP activity and attenuated left ventricular remodeling, thereby demonstrating a localized on demand inhibitor.

The use of several synthetic small molecule inhibitors in experimental animal models, though successful, MMP inhibitors in clinical trials have met with a mixed rate of success and failure. MMP inhibitors have not been translated into clinical application in humans due to adverse side effects following systemic administration of the drugs. The clinical trials have failed due to the problems of lack of selectivity, specificity, and bioavailability of MMP inhibitors. The primary goal in the design of MMP inhibitors is their selectivity to eliminate unwanted side effects to improve their efficacy. The MMP inhibitors with broad specificity may influence the functions of different MMPs in terms of both matrix degradation and tissue repair, Therefore, MMP inhibitors with more restricted specificity are being developed. New genetic and pharmacological tools are now available that may help design disease-specific MMP inhibitors with fewer adverse effects from their use in the therapy of degradative disorders. The MMPs form a part of a diverse and complex proteolytic system involved in performing vital functions in the body. Recent advances in medicinal chemistry may help design future strategies to develop specific MMP inhibitors targeting specific MMPs to minimize their side effects and to enhance their selectivity, bioavailability, and potency.

5 Conclusion

MMPs are the enzymes that degrade substrates ranging from a variety of structural and functional proteins and thus enable the cell to maintain a physiological process or to disrupt the normal milieu of the cell. The present review highlights the crucial functional aspects of MMPs in cardiovascular disorders, highlighting them as key central players involved in remodeling and restructuring functions in vascular biology. Together with their well-documented function in cardiovascular diseases, MMPs have been viewed as potential drug targets and a great interest has been garnered in developing MMP inhibitors to treat them. The effects of MMP inhibition have been well explored in vitro and in preclinical studies in various experimental animal models. The results of the various clinical trials where MMP inhibitors have been used have yielded disappointing and mixed results, therefore translating these results into clinical practice have been unsuccessful. Thus, well-planned and carefully designed preclinical and clinical trials are needed using MMP inhibitors imbibing safety standards, that display therapeutic promise to prevent the development and progression of CVD.

References

1. Roger VL, Go AS, Lloyd-Jones DM et al (2011) Heart disease and stroke statistics—2011 update: a report from the American Heart Association. Circulation 123:e18–e209
2. Horwich TB, Fonarow GC (2010) Glucose, obesity, metabolic syndrome, and diabetes relevance to incidence of heart failure. J Am Coll Cardiol 55:283–293
3. Newby AC (2005) Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol Rev 85:1–31
4. Spinale FG (2002) Matrix metalloproteinases: regulation and dysregulation in the failing heart. Circ Res 90:520–530
5. Velagaleti RS, Gona P, Larson MG et al (2010) Multimarker approach for the prediction of heart failure incidence in the community. Circulation 122:1700–1706
6. Galis ZS, Sukhova GK, Lark MW, Libby P (1994) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 94:2493–2503
7. Cleutjens JP, Kandala JC, Guarda E et al (1995) Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 27:1281–1292
8. Mark D, Sternlicht Zena W (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516
9. Borkakoti N (2000) Structural studies of matrix metalloproteinases. J Mol Med 78:261–268
10. Moore CS, Crocker SJ (2012) An alternate perspective on the roles of TIMPs and MMPs in pathology. Am J Pathol 180:12–16
11. Woessner JF (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5:2145–2154
12. Lindsey ML, Zamilpa R (2012) Temporal and spatial expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases following myocardial infarction. Cardiovasc Ther 30:31–41
13. Kleiner DE, Stetler-Stevenson WG (1994) Matrix metalloproteinases and metastasis. Cancer Chemother Pharmacol 43:S42–S51

14. Lepage T, Gache C (1990) Early expression of a collagenase-like hatching enzyme gene in the sea urchin embryo. EMBO J 9:3003
15. Wang T, Aoki T, Iwata K et al (2000) One-step sandwich enzyme immunoassay using monoclonal antibodies for detection of human enamelysin (MMP-20). Eur J Oral Sci 108:530–537
16. Overall CM, López-Otín C (2002) Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer 2:657–672
17. Hideaki N, Robert V, Gillian M (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69:562–573
18. Murphy G, Nguyen Q, Cockett MI et al (1994) Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant. J Biol Chem 269:6632–6636
19. Bode W, Gomis-Rüth FX, Stocker W (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett 331:134–140
20. Stöcker W, Grams F, Baumann U et al (1995) The metzincins—topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. Protein Sci 4:823–840
21. Jed F, Shahriar M (2006) Recent advances in MMP inhibitor design. Cancer Metastasis Rev 25:115–136
22. Bertini I, Calderone V, Fragai M et al (2003) X-ray structures of binary and ternary enzyme-product-inhibitor complexes of matrix metalloproteinases. Angew Chem 115:2777–2780
23. Streuli C (1999) Extracellular matrix remodelling and cellular differentiation. Curr Opin Cell Biol 11:634–640
24. Lu P, Takai K, Weaver VM, Werb Z (2011) Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol 3:a005058
25. Cox TR, Erler JT (2011) Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Models Mech 4:165–178
26. Kar S, Subbaram S, Carrico PM, Melendez JA (2010) Redox-control of matrix metalloproteinase-1: a critical link between free radicals, matrix remodeling and degenerative disease. Respir Physiol Neurobiol 174:299–306
27. Rajagopalan S, Meng XP, Ramasamy S et al (1996) Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. J Clin Invest 98:2572–2579
28. Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52–67
29. Blanco-Colio LM, Martín-Ventura JL, Vivanco F et al (2006) Biology of atherosclerotic plaques: what we are learning from proteomic analysis. Cardiovasc Res 72:18–29
30. Döring Y, Noels H, Weber C (2012) The use of high-throughput technologies to investigate vascular inflammation and atherosclerosis. Arterioscler Thromb Vasc Biol 32:182–195
31. Orbe J, Fernandez L, Rodriguez JA et al (2003) Different expression of MMPs/TIMP-1 in human atherosclerotic lesions. Relation to plaque features and vascular bed. Atherosclerosis 170:269–276
32. Knox JB, Sukhova GK, Whittemore AD, Libby P (1997) Evidence for altered balance between matrix metalloproteinases and their inhibitors in human aortic diseases. Circulation 95:205–212
33. Luttun A, Lutgens E, Manderveld A et al (2004) Loss of matrix metalloproteinase-9 or matrix metalloproteinase-12 protects apolipoprotein E-deficient mice against atherosclerotic media destruction but differentially affects plaque growth. Circulation 109:1408–1414
34. Konstantino Y, Nguyen TT, Wolk R et al (2009) Potential implications of matrix metalloproteinase-9 in assessment and treatment of coronary artery disease. Biomarkers 14:118–129

35. Lee JK, Zaidi SH, Liu P et al (1998) A serine elastase inhibitor reduces inflammation and fibrosis and preserves cardiac function after experimentally-induced murine myocarditis. Nat Med 4:1383–1391
36. Tyagi SC, Campbell SE, Reddy HK et al (1996) Matrix metalloproteinase activity expression in infarcted, noninfarcted and dilated cardiomyopathic human hearts. Mol Cell Biochem 155:13–21
37. Tyagi SC, Ratajska A, Weber KT (1993) Myocardial matrix metalloproteinase(s): localization and activation. Mol Cell Biochem 126:49–59
38. Sun M, Dawood F, Wen WH et al (2004) Excessive tumor necrosis factor activation after infarction contributes to susceptibility of myocardial rupture and left ventricular dysfunction. Circulation 110:3221–3228
39. Kameda K, Matsunaga T, Abe N et al (2006) Increased pericardial fluid level of matrix metalloproteinase-9 activity in patients with acute myocardial infarction: possible role in the development of cardiac rupture. Circ J 70:673–678
40. Lalu MM, Pasini E, Schulze CJ et al (2005) Ischemia-reperfusion injury activates matrix metalloproteinases in the human heart. Eur Heart J 26:27–35
41. Sun M, Opavsky MA, Stewart DJ et al (2003) Temporal response and localization of integrins beta1 and beta 3 in the heart after myocardial infarction: regulation by cytokines. Circulation 107:1046–1052
42. Spinale FG, Coker ML, Krombach SR et al (1999) Matrix metalloproteinase inhibition during the development of congestive heart failure: effects on left ventricular dimensions and function. Circ Res 85:364–376
43. Peterson JT, Hallak H, Johnson L et al (2001) Matrix metalloproteinase inhibition attenuates left ventricular remodeling and dysfunction in a rat model of progressive heart failure. Circulation 103:2303–2309
44. Little WC, Applegate RJ (1993) Congestive heart failure: systolic and diastolic function. J Cardiothorac Vasc Anesth 7:2–5
45. Kato S, Spinale FG, Tanaka R et al (1995) Inhibition of collagen cross-linking: effects on fibrillar collagen and left ventricular diastolic function. Am J Physiol 269:H863–H868
46. Woodiwiss AJ, Tsotetsi OJ, Sprott S et al (2001) Reduction in myocardial collagen cross-linking parallels left ventricular dilation in rat models of systolic chamber dysfunction. Circulation 103:155–160
47. Mujumdar VS, Tyagi SC (1999) Temporal regulation of extracellular matrix components in transition from compensatory hypertrophy to decompensatory heart failure. J Hypertens 17:261–270
48. Seccia TM, Bettini E, Vulpis V et al (1999) Extracellular matrix gene expression in the left ventricular tissue of spontaneously hypertensive rats. Blood Press 8:57–64
49. Li H, Simon H, Bocan TMA, Peterson JT (2000) MMP/TIMP expression in spontaneously hypertensive heart failure rats: the effect of ACE- and MMP-inhibition. Cardiovasc Res 46:298–306
50. Nagatomo Y, Carabello BA, Coker ML et al (2000) Differential effects of pressure or volume overload on myocardial MMP levels and inhibitory control. Am J Physiol 278: H151–H161
51. Harvey PA, Leinwand LA (2011) Cellular mechanisms of cardiomyopathy. J Cell Biol 194:355–365
52. Elliott P, Andersson B, Arbustini E et al (2008) Classification of the cardiomyopathies: a position statement from the European society of cardiology working group on myocardial and pericardial diseases. Eur Heart J 29:270–276
53. Gunja-Smith Z, Morales AR, Romanelli R, Woessner JF Jr (1996) Remodeling of human myocardial collagen in idiopathic dilated cardiomyopathy. Role of metalloproteinases and pyridinoline cross-links. Am J Pathol 148:1639–1648
54. He KL, Dickstein M, Sabbah HN et al (2004) Mechanisms of heart failure with well preserved ejection fraction in dogs following limited coronary microembolization. Cardiovasc Res 64:72–83

55. Herman MP, Sukhova GK, Libby P et al (2001) Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling. Circulation 104:1899–1904
56. Spinale FG, Coker ML, Heung LJ et al (2000) A matrix metalloproteinase induction/activation system exists in the human left ventricular myocardium and is upregulated in heart failure. Circulation 102:1944–1949
57. Rouet-Benzineb P, Buhler JM, Dreyfus P et al (1999) Altered balance between matrix gelatinases (MMP-2 and MMP-9) and their tissue inhibitors in human dilated cardiomyopathy: potential role of MMP-9 in myosin-heavy chain degradation. Eur J Heart Fail 1:337–352
58. Sawicki G, Leon H, Sawicka J et al (2005) Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. Circulation 112:544–552
59. Wang W, Schulze CJ, Suarez-Pinzon WL et al (2002) Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. Circulation 106:1543–1549
60. Li YY, Feldman AM, Sun Y, McTiernan CF (1998) Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. Circulation 98:1728–1734
61. Li YY, Feng Y, McTiernan CF et al (2001) Down regulation of matrix metalloproteinases and reduction in collagen damage in the failing human heart after support with left ventricular assist devices. Circulation 104:1147–1152
62. George SJ (2000) Therapeutic potential of matrix metalloproteinase inhibitors in atherosclerosis. Expert Opin Investig Drugs 9:993–1007
63. Klotz S, Foronjy RF, Dickstein ML et al (2005) Mechanical unloading during left ventricular assist device support increases left ventricular collagen cross-linking and myocardial stiffness. Circulation 112:364–374
64. Felkin LE, Birks EJ, George R et al (2006) A quantitative gene expression profile of matrix metalloproteinases (MMPS) and their inhibitors (TIMPS) in the myocardium of patients with deteriorating heart failure requiring left ventricular assist device support. J Heart Lung Transplant 25:1413–1419
65. Fowkes FG, Rudan D, Rudan I et al (2013) Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. Lancet 382:1329–1340
66. Hobeika MJ (2007) Matrix metalloproteinases in peripheral vascular disease. J Vasc Surg 45:849–857
67. Sang QX (1998) Complex role of matrix metalloproteinases in angiogenesis. Cell Res 8:171–177
68. Jenkins GM, Crow MT, Bilato C et al (1998) Increased expression of membrane-type matrix metalloproteinase and preferential localization of matrix metalloproteinase-2 to the neointima of balloon-injured rat carotid arteries. Circulation 97:82–90
69. Zang J, Nie L, Razavian M et al (2008) Molecular imaging of activated matrix metalloproteinases in vascular remodeling. Circulation 118:1953–1960
70. Lijnen HR, Soloway P, Collen D (1999) Tissue inhibitor of matrix metalloproteinase-1 impairs arterial neointima formation after vascular injury in mice. Circ Res 85:1186–1191
71. Cai WJ, Koltai S, Kocsis E et al (2003) Remodeling of the adventitia during coronary arteriogenesis. Am J Physiol Heart Circ Physiol 284:H31–H40
72. Guzman JR (2007) Clinical, cellular, and molecular aspects of arterial calcification. J Vasc Surg 45:A57–A63
73. Lehto S, Rönnemaa T, Pyörälä K et al (1996) Risk factors predicting lower extremity amputations in patients with NIDDM. Diabetes Care 19:607–612
74. Basalyga DM, Simionescu DT, Xiong W et al (2004) Elastin degradation and calcification in an abdominal aorta injury model. Circulation 110:3480–3487
75. Qin X, Corriere MA, Matrisian LM et al (2006) Matrix metalloproteinase inhibition attenuates aortic calcification. Arterioscler Thromb Vasc Biol 26:1510–1516

76. Goodall S, Crowther M, Hemingway DM et al (2001) Ubiquitous elevation of matrix metalloproteinase-2 expression in the vasculature of patients with abdominal aneurysms. Circulation 104:304–309
77. Urbonavicius S, Urbonaviciene G, Honorè B et al (2008) Potential circulating biomarkers for abdominal aortic aneurysm expansion and rupture—a systematic review. Eur J Vasc Endovasc Surg 36:273–280
78. Santos-Martínez MJ, Medina C, Jurasz P et al (2008) Role of metalloproteinases in platelet function. Thromb Res 121:535–542
79. Sawicki G, Salas E, Murat J et al (1997) Release of gelatinase A during platelet activation mediates aggregation. Nature 386:616–619
80. Falcinelli E, Guglielmini G, Torti M et al (2005) Intraplatelet signaling mechanisms of the priming effect of matrix metalloproteinase-2 on platelet aggregation. J Thromb Haemost 3:2526–2535
81. Galt SW, Lindemann S, Allen L et al (2002) Outside-in signals delivered by matrix metalloproteinase-1 regulate platelet function. Circ Res 90:1093–1099
82. Pitchford SC, Momi S, Baglioni S et al (2008) Allergen induces the migration of platelets to lung tissue in allergic asthma. Am J Respir Crit Care Med 177:604–612
83. Santilli F, Basili S, Ferroni P et al (2007) CD40/CD40L system and vascular disease. Intern Emerg Med 2:256–268
84. Furman MI, Krueger LA, Linden MD et al (2004) Release of soluble CD40L from platelets is regulated by glycoprotein IIb/IIIa and actin polymerization. J Am Coll Cardiol 43:2319–2325
85. Lo EH, Dalkara T, Moskowitz MA (2003) Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 4:399–415
86. Lo EH, Wang X, Cuzner ML (2002) Extracellular proteolysis in brain injury and inflammation: role for plasminogen activators and matrix metalloproteinases. J Neurosci Res 69:1–9
87. Rosenberg GA, Navratil M, Barone F, Feuerstein G (1996) Proteolytic cascade enzymes increase in focal cerebral ischemia in rat. J Cereb Blood Flow Metab 16:360–366
88. Clark AW, Krekoski CA, Bou S et al (1997) Increased gelatinase A (MMP-2) and gelatinase B (MMP-9) activities in human brain after focal ischemia. Neurosci Lett 238:53–56
89. Anthony DC, Ferguson B, Matyzak MK et al (1997) Differential matrix metalloproteinase expression in cases of multiple sclerosis and stroke. Neuropathol Appl Neurobiol 23:406–415
90. Rosell A, Ortega-Aznar A, Alvarez-Sabin J et al (2006) Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke. Stroke 37:1399–1406
91. Rosell A, Cuadrado E, Ortega-Aznar A et al (2008) MMP-9-positive neutrophil infiltration is associated to BBB breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. Stroke 39:1121–1126
92. Hamann GF, Burggraf D, Martens HK et al (2004) Mild to moderate hypothermia prevents microvascular basal lamina antigen loss in experimental focal cerebral ischemia. Stroke 35:764–769
93. Hamann GF, Liebetrau M, Martens H et al (2002) Microvascular basal lamina injury after experimental focal cerebral ischemia and reperfusion in the rat. J Cereb Blood Flow Metab 22:526–533
94. Sole S, Petegnief V, Gorina R et al (2004) Activation of matrix metalloproteinase-3 and agrin cleavage in cerebral ischemia/reperfusion. J Neuropathol Exp Neurolx 63:338–349
95. Gurney KJ, Estrada EY, Rosenberg GA (2006) Blood-brain barrier disruption by stromelysin-1 facilitates neutrophil infiltration in neuroinflammation. Neurobiol Dis 23:87–96
96. Maier CM, Hsieh L, Yu F et al (2004) Matrix metalloproteinase-9 and myeloperoxidase expression: quantitative analysis by antigen immunohistochemistry in a model of transient focal cerebral ischemia. Stroke 35:1169–1174

97. Harris AK, Ergul A, Kozak A et al (2005) Effect of neutrophil depletion on gelatinase expression, edema formation and hemorrhagic transformation after focal ischemic stroke. BMC Neurosci 6:49
98. Berk BC, Fujiwara K, Lehoux S (2007) ECM remodeling in hypertensive heart disease. J Clin Invest 117:568–575
99. Lopez B, Gonzalez A, Querejeta R et al (2006) Alterations in the pattern of collagen deposition may contribute to the deterioration of systolic function in hypertensive patients with heart failure. J Am Coll Cardiol 48:89–96
100. Laviades C, Varo N, Fernández J et al (1998) Abnormalities of the extracellular degradation of collagen type I in essential hypertension. Circulation 98:535–540
101. Ahmed SH, Clark LL, Pennington WR et al (2006) Matrix metalloproteinases/tissue inhibitors of metalloproteinases: relationship between changes in proteolytic determinants of matrix composition and structural, functional, and clinical manifestations of hypertensive heart disease. Circulation 113:2089–2096
102. Iwanaga Y, Aoyama T, Kihara Y et al (2002) Excessive activation of matrix metalloproteinases coincides with left ventricular remodeling during transition from hypertrophy to heart failure in hypertensive rats. J Am Coll Cardiol 39:1384–1391
103. Heymans S, Lupu F, Terclavers S et al (2005) Loss or inhibition of uPA or MMP-9 attenuates LV remodeling and dysfunction after acute pressure overload in mice. Am J Pathol 166:15–25
104. Zavadzkas JA, Stroud RE, Bouges S et al (2004) Targeted overexpression of tissue inhibitor of matrix metalloproteinase-4 modifies post-myocardial infarction remodeling in mice. Circ Res 114:1435–1445
105. Tian H, Huang ML, Liu KY et al (2012) Inhibiting matrix metalloproteinase by cell-based timp-3 gene transfer effectively treats acute and chronic ischemic cardiomyopathy. Cell Transplant 21:1039–1053
106. Cerisano G, Buonamici P, Valenti R et al (2014) Early short-term doxycycline therapy in patients with acute myocardial infarction and left ventricular dysfunction to prevent the ominous progression to adverse remodelling: the TIPTOP trial. Eur Heart J 35:184–191
107. Zhai X, Chi J, Tang W et al (2014) Yellow wine polyphenolic compounds inhibit matrix metalloproteinase-2, -9 expression and improve atherosclerotic plaque in LDL-receptor-knockout mice. Biochem Pharmacol 90:7–15
108. Sang QX, Jin Y, Newcomer RG et al (2004) Matrix metalloproteinase inhibitors as prospective agents for the prevention and treatment of cardiovascular and neoplastic diseases. Circulation 110:II180–II186
109. Høgh Kølbæk Kjær AS, Brinkmann CR, Dinarello CA et al (2015) The histone deacetylase inhibitor panobinostat lowers biomarkers of cardiovascular risk and inflammation in HIV patients. AIDS 29:1195–1200
110. Lin HB, Cadete VJ, Sra B et al (2014) Inhibition of MMP-2 expression with siRNA increases baseline cardiomyocyte contractility and protects against simulated ischemic reperfusion injury. Biomed Res Int 2014:810371
111. Wang WK, Wang B, Lu QH et al (2014) Inhibition of high-mobility group box 1 improves myocardial fibrosis and dysfunction in diabetic cardiomyopathy. Int J Cardiol 172:202–212
112. Purcell BP, Lobb D, Charati MB et al (2014) Injectable and bioresponsive hydrogels for on-demand matrix metalloproteinase inhibition. Nat Mater 13:653–661
113. Yokoyama Y, Grünebach F, Schmidt SM et al (2008) Matrilysin (MMP-7) is a novel broadly expressed tumor antigen recognized by antigen-specific T cells. Clin Cancer Res 14:5503–5511
114. Hao L, Du M, Lopez-Campistrous A, Fernandez-Patron C (2004) Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor-receptor pathway. Circ Res 94:68–76

115. Rodriguez JA, Orbe J, Martinez de Lizarrondo S et al (2008) Metalloproteinases and atherothrombosis: MMP-10 mediates vascular remodeling promoted by inflammatory stimuli. Front Biosci 13:2916–2921
116. Anglard P, Melot T, Guerin E et al (1995) Structure and promoter characterization of the human stromelysin-3 gene. J Biol Chem 270:20337–20344
117. Curci JA, Liao S, Huffman MD et al (1998) Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms. J Clin Invest 102:1900–1910
118. Johansson N, Ahonen M, Kähäri VM (2000) Matrix metalloproteinases in tumor invasion. Cell Mol Life Sci 57:5–15
119. Nagase H, Woessner JF (1999) Matrix metalloproteinases. J Biol Chem 274:21491–21494
120. Takino T, Sato H, Shinagawa A, Seiki M (1995) Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) gene from a human placenta cDNA library. MT-MMPs form a unique membrane-type subclass in the MMP family. J Biol Chem 270:23013–23020

microRNAs-Mediated MMPs Regulation: Novel Mechanism for Cardiovascular Diseases

24

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Abstract

Cardiac remodeling and its role in disease progression is a multimechanistic and complex process. Disruption of normal extracellular matrix homeostasis is the most important event responsible for cardiac remodeling, altering heart structure and function. Therefore, targeting extracellular matrix remodeling enzymes such as matrix metalloproteinases has received much interest in terms of developing novel therapeutics strategies. Recent findings of microRNAs (endogenous, non-coding, ~22 nucleotide small RNA) in the cardiac tissue as dynamic modifiers of disease pathogenesis have provided glimpses of undiscovered regulatory mechanisms underlying cardiovascular diseases. The implication of several microRNAs in targeting extracellular matrix components (microRNA-29: collagen, fibrillin, elastin) and matrix metalloproteinases (microRNA-21: MMP-2; microRNA-320: MMP-9) has been well documented. The combined strategy of manipulating extracellular matrix remodeling through targeting matrix metalloproteinases by microRNAs has produced encouraging results in preclinical studies. This chapter reviews the potential of microRNA as

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therapeutics tool for cardiovascular diseases through direct and indirect interactions with the matrix metalloproteinases.

Keywords
Cardiovascular diseases · Extracellular matrix · Matrix metalloproteinases · microRNAs

1 Introduction

Cardiovascular diseases (CVD) are the leading cause of human morbidity and mortality worldwide. Adverse myocardial remodeling in response to hemodynamic load and cardiac stress in association with the hormonal response is a significant cause of CVD. In almost all CVD, an increase in cardiac fibroblasts number (due to the transformation of bone marrow (BM)-derived monocytes, BM progenitors, and fibrocytes) and extracellular matrix (ECM) deposition occurs [1]. The ECM forms a milieu surrounding cells that reciprocally influences many aspects of normal cell behavior, including proliferation, adhesion, migration, and survival; thereby assuring the harmonic structure and function of the heart. To do so, ECM undergoes dynamic remodeling regulated by a number of ECM synthesizing as well as degrading enzymes such as matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). MMPs are zinc-dependent endopeptidase that plays a fundamental role in remodeling of the ECM under both normal and pathological conditions. The proteolytic activity of MMPs is strictly controlled by tissue inhibitors of metalloproteinases (TIMPs) [2, 3]. Considerable evidence implicates that the expression level of MMPs deregulates in cardiovascular diseases such as hypertrophy, atherosclerosis, leading to myocardial infarction and heart failure. Nearby, 60 MMP inhibitors have been pursued as clinical candidates targeting multiple disorders such as cardiovascular diseases, arthritis, and cancer. Since, the actual molecular mechanisms underlying their role in onset and progression of disease are still uncertain, clinical developments of most of the drugs have been discontinued due to nontargeted approach. The aim of this chapter is to review the involvement of a novel class of small noncoding RNAs (microRNA) known to acts as fine-tuner of the protein expression, in the regulation of MMPs and their use as a potential therapeutic tool in CVD.

2 Extracellular Matrix Remodeling in Cardiovascular Diseases

Every cell influences their surroundings and gets influenced from the same. In the human body, fibroblasts are present in every tissue bridging the voids and mediating the connections between other cell types. In myocardium, although cardiac

myocytes constitute nearly three-fourth of normal myocardial tissue volume, cardiac fibroblast accounts for more than two-third of cell population [2]. Fibroblast is cells of mesenchymal origin, predominantly responsible for synthesizing and secreting a diverse group of proteins and nonprotein components which constitute a dynamic, noncellular, and three-dimensional support structure called the ECM. Nearly 300 proteins which constitute the ECM are fibrillar, non-fibrillar as well as glycosylated in nature. They include 43 subunits of collagens, 36 glycosylated proteins, and nearly 200 glycoproteins complex. The nonprotein component includes water, minerals, and polysaccharides such as glycosaminoglycans [3]. Depending upon their origin, composition, location, and interactions among different cellular components (epithelial-endothelial elements, fibroblast, myocytes, and adipocyte), ECM forms different specialized structures unique to each tissue. In the cardiac matrix, collagen (types I, III, IV, V, and VI), glycoproteins (fibronectin, laminins, periostin, fibromodulin, and vitronectin), proteoglycans (versican, lumican, and biglycan), and glycosaminoglycans (hyaluronic acid and dermatan sulfate) are the major constituents. The components such as collagen IV, laminin, nidogen, and heparan sulfate synthesized by epithelial and endothelial cells forms the basement membrane. Whereas, the fibrillar collagens, fibronectin, and proteoglycans primarily synthesized by stromal cells (fibroblasts, pericytes) forms the interstitial matrix. Combined together in a systematic manner, the ECM component regulates the cardiac cells behavior biochemically, biophysically, and biomechanically. The biochemical regulations enable the cells to interact with their surroundings through direct and indirect signaling. Whereas, biophysical and biomechanical regulation is the support provided for tissue architecture and integrity which further decides cell proliferation, differentiation, migration, polarity, adhesion, and survival. These dynamic interactions between ECM and intracellular events are mediated by a family of transmembrane receptors known as integrins, discoidin domain receptors, and syndecans [4]. The substitution of even a single amino acid in any ECM component can alter the cell structure as well as its behavior. Therefore, under normal development and physiology, ECM modifying enzymes maintains a precise equilibrium between ECM synthesis and degradation. This equilibrium insures harmonic structure and function of every tissue including myocardium. However, if unconstrained, it leads to accumulation of ECM resulting in fibrosis or disruption of ECM network altering heart structure and function. As discussed above, the arterial wall consists of collagen types I and III, macrophages, and smooth muscle cells. Precursors released during biosynthesis of collagen type I and III such as carboxy-terminal or the amino-terminal propeptides (PICP, PINP, PIIICP, PIIINP) as well as during degradation such as carboxy-terminal or the amino-terminal telopeptide for type I and III collagen (CITP, NITP, CIIITP, PIIINP) have been studied as serological marker of ECM turnover in cardiac patients. A reduced serum level of CITP as well as increased level of CITP, PICP, and TIMP1 has been observed in hypertensive patients' suggesting both elevated synthesis and degradation occurs during high blood pressure [5–7]. Similarly, elevated levels of PIIINP and PICP (fibrosis) have been reported in patients with congestive heart failure and acute myocardial infarction (MI) [8]. These studies

vividly illustrate that dysregulation in ECM rebuilding and remodeling contributes to several pathological conditions as severe as heart failure and MI. Majorly, ECM remodeling is mediated by families of metalloproteinases viz., matrix metalloproteinase (MMPs), meprins, a disintegrin and metalloproteinase (ADAMs), and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) [9, 10]. In addition, serine, cysteine, heparanases, sulphatases, and cathepsin proteases also degrade ECM resulting in the release of growth factors and cytokines. Alterations in the level of proteases may lead to different types of heart diseases. Therefore, regulating MMPs may prevent the onset or progression of disease.

3 Matrix Metalloproteinases-Mediated Cardiac Remodeling

MMPs are the major class of proteases involved in ECM remodeling, therefore; playing a key role in biological (morphogenesis, angiogenesis, and wound healing) and pathological (tumor growth, arthritis, tissue ulceration, and cardiovascular disease) processes [11]. In 1962, a study on collagen remodeling during tadpole tail metamorphosis leads to the discovery of MMPs [12]. Since then, 25 vertebrate MMPs and 23 human homologues have been identified. Although human carries 24 MMP genes, chromosome 1 possesses two identical genes for MMP-23 making a total 23 MMP proteins [13]. On the basis of substrate specificity, domain organization and sequence similarity they are classified into four groups namely: (a) archetypal MMPs that includes collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10), and other archetypal MMPs (MMP-12, -19, -20, -27), (b) matrilysins (MMP-7, -26), (c) gelatinases (MMP-2, 9), and (d) furin-activatable MMPs such as secreted MMPs (MMP-11, -21, and -28), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25), and type II transmembrane MMPs (MMP-23A and -23B). Generally, MMPs possesses four domains structure: a $\sim$80-residue pro-peptide at the amino terminus, a $\sim$165-residue catalytic domain with zinc localized at the active site, a $\sim$200-residue hemopexin-like carboxyl-terminal domain linked to the catalytic domain by 15–65 residue linker region and a transmembrane domain. MMP-7 and MMP-26 lacks hemopexin domain whereas, MMP-23 has unique cysteine-rich, proline-rich, and IL-1 type II receptor-like domains instead of a hemopexin domain. Due to the interaction of a cysteine residue of the pro-peptide domain with the zinc ion of the catalytic site, the MMPs are proteolytically inactive [14]. The proteolytic activity of MMPs is strictly controlled and activated at three different levels: (a) transcriptional and posttranscriptional level (by several cytokines (IL-1, TNF-α), growth factors (TGF-β), glucocorticoids, interferons), (b) zymogen activation (by other already activated MMPs, several serine proteinases or auto-proteolysis), and (c) by tissue inhibitors of metalloproteinases (TIMPs) [15, 16]. These regulatory measures insure the presence of MMPs in precise amount, at the right time and precise location, and in appropriate state (activated or inactive). However, cardiac or hemodynamic load

(hypertension), hyperhomocysteinemia, myocarditis, and RAAS-mediated cardiac stress alters the level of MMPs and their regulators leading to morphological, structural, and functional changes of the heart, referred to as cardiac remodeling. Cardiac remodeling includes degradation or deposition of extracellular matrix (ECM), myocardiocytes hypertrophy, abnormal angiogenesis, coronary collateralization, alterations in receptor signaling cascade, apoptosis, abnormal differentiation, and survival of cardiac stem cell, differential expressions of miRNAs and epigenetic modifications.

4 Matric Metalloproteinases and Cardiovascular Diseases

In heart, the function of MMPs extends well beyond ECM degradation, such as, stimulation of multiple biological effects through liberating and mobilizing growth factors, cytokines [17]. Since all cardiac remodeling and myocardial architecture is associated with a change in the ECM composition; MMPs and their tissue inhibitors (TIMPs) are involved in the pathogenesis of a wide spectrum of cardiovascular disorders including atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction, and aortic aneurysm. Out of 23 MMPs identified in human, MMP-1, -2, -3, -7, -8, -9, -12, -13, -14, and TIMPs-1, -2, -3, -4 have been evaluated to respond to cardiac tissue repair stimuli [18]. The studies on patients tissue extracts with dilated cardiomyopathy revealed a decrease in MMP-1,-12, and increase in MMP-3 level [19, 20]. Similarly, post-MI studies on murine detected an increased level of MMP-8, -9, -13, and -14 [21–23]. Out of 23 MMPs, MMP-9 is surfaced as a leading candidate for having direct effects on cardiac remodeling. Its level increases post-MI, its inhibition improves post-MI outcomes and patients with the highest MMP-9 levels at baseline showed the greatest cardiovascular mortality [24]. A study on rat MI model also demonstrated time-dependent increase in relative TIMP-1 and TIMP-2 mRNA levels and decrease in TIMP-4 level [25]. Later, several clinical studies have utilized plasma samples to profile TIMP levels [26, 27].

These observations have led to the recognition of MMPs and TIMPs as potential therapeutic targets through the development of pharmacological reagents. Previous studies revealed that MMP inhibition have reduced the accumulation of myocardial ECM (fibrosis), LV dilation as well as preserved cardiac function and geometry in ischemic cardiomyopathy [25]. A number of pharmacological MMP inhibitors such as trandolapril and ramipril studied in vitro as well as in rat model have found to control MMP level but at the expense of induction or reduction of other MMPs and TIMPS [28–30]. Lately, though peptides were found to be selective inhibitors of MMP-9 and MMP-2, their clinical usage is highly questionable because of their susceptibility to proteolysis [31]. In addition, clinical trials of other MMP inhibitors like PG-116800 failed to reduce cardiac remodeling or improve clinical outcomes after MI [32]. Therefore, novel molecular therapies have been investigated to more effectively treat or prevent CVDs. RNA-based epigenetics mechanisms including those controlled by small noncoding micro-RNAs (miRNAs) plays a pivotal role in

the regulation of cardiovascular diseases [33]. As a result, miRNAs have emerged as potential diagnostic and therapeutic tool for cardiovascular disease.

5 Small Non-coding RNAs with Big Potential

The existence of miRNAs came into picture nearly two decades ago (1993) when Victor Ambros, Rosalind Lee, and Rhonda Feinbaum discovered that lin-4, a gene involved in development of *C. elegans* larvae does not code for protein but instead codes a pair of small RNAs [34]. These small RNAs are highly conserved, endogenously expressed, and noncoding RNAs that negatively modulate gene expression by either promoting the degradation of mRNA or downregulating the protein production by translational repression. They are transcribed from either intergenic, intronic, or polycistronic regions of the genome previously thought to be the junk region ($\sim$97%). Since their discovery, more than 2000 human miRNAs have been cataloged in mirBase (miRNAs database) (http://www.mirbase.org) predicted to regulate expressions of at least 30% of all human protein-encoding genes. The biogenesis of miRNAs is a complex process where every step is subjected to tight molecular regulation (Fig. 1). It begins within nucleus by transcription of miRNAs genes by RNA polymerase II into $\sim$2000 nucleotides primary transcripts (pri-miRNAs) that can encode one or more miRNAs. A combination of Drosha (an RNase III enzyme) and Pasha also known as DGCR8 (an RNA-binding protein) cleaves primary miRNAs into precursor miRNAs (60–100 nucleotides) which gets transported to cytoplasm in a Ran/GTP/Exportin-5-dependent manner. Once in the cytoplasm, another cleaving enzyme Dicer (RNase III) modifies the precursor miRNAs and generates double-stranded miRNA duplexes of 19–23 nucleotides containing functional guide strand and a passenger strand. The duplex miRNA gets incorporated into RNA-induced silencing complex (RISC) by associating with Argonaute proteins (Ago). The passenger strand gets degraded while the guide strands remains intact and binds the 3′ untranslated region (UTR) of target messenger RNA (mRNA). Pairing across all 22 nucleotides rarely occurs in mammalian cells instead, the seed region of the miRNA (nucleotides 2–7 or 8) appears most important for targeting mRNAs. If the base pair at 3′ UTR matches perfectly, the mRNA gets cleaved and degraded by Ago2-RISC complex. Otherwise, it causes downregulating of protein production through translational repression [35, 36]. miRNAs not only target single gene but often functionally related genes networks thus maintain optimal amount of cellular proteins and hence play a crucial role in the regulation of biological functions. In human cardiac tissue, miRNAs have been identified at all stages of development (for example, miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, miR-208, and the let-7 family) [37]. Our previous study has characterized several known and novel miRNAs in chicken cardiac tissue at all stages of development. Using next-generation sequencing, we identified a total of 1056 microRNAs, out of which 353 were known (for example, let-7, miR-140, miR-181, miR-30,

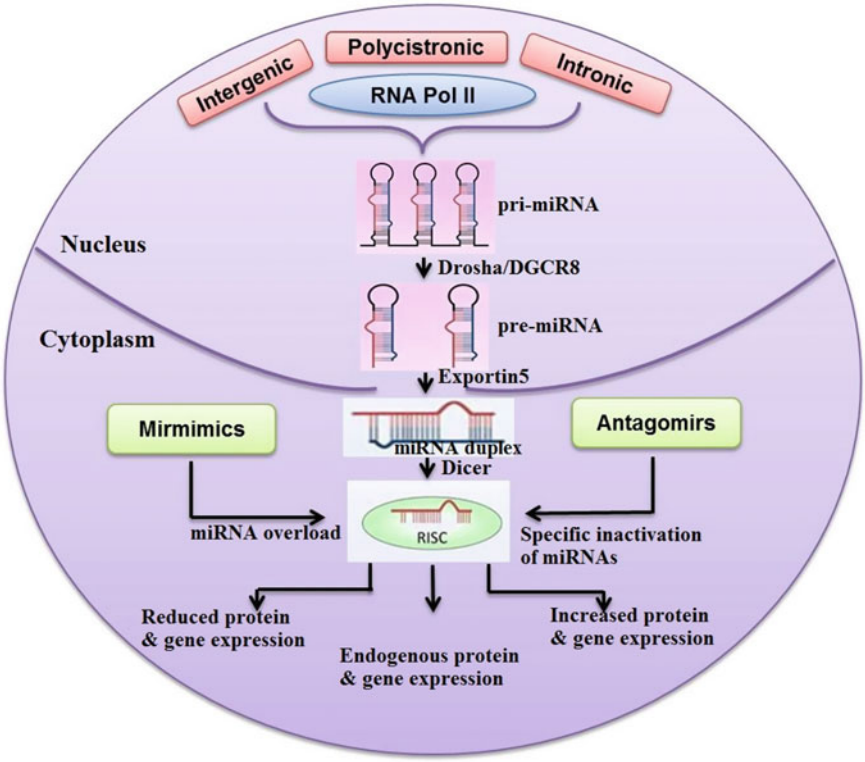

Fig. 1 Schematic representation of miRNA biogenesis as well as therapeutics. Inside nucleus, either of the intergenic, polycistronic or intronic regions of DNA gets transcribed by RNA Polymerase II (RNA Pol II) into nearly 2 kb long primary transcripts (pri-miRNAs). Drosha/DGCR8 cleaves pri-miRNAs into precursor miRNAs (pre-miRNA) of 60–100 bp which gets transported to cytoplasm by exportin 5. In cytoplasm, Dicer modifies pre-miRNA into double-stranded miRNA duplexes. One of the strand (guide strand) activates the RNA-induced silencing complex (RISC) which in turn causes translational repression as well as mRNA cleavage. mirmimics (*left*) activates RISC and reduce protein and gene expression while, antagomirs (*right*) reduce RISC activation resulting in translational repression/mRNA cleavage and in the end increase protein and gene expression

miR-205, miR-103, and miR-22) while 703 were novel miRNAs [38]. These finding implicate that the miRNAs are highly expressed in non-diseased cardiac tissue from embryogenesis to adult life and play a key role in both normal cardiac maintenance and disease. The role of miRNAs in cardiac remodeling of the adult heart was further established with Dicer deletion through the use of a tamoxifen-inducible Cre recombinase in the postnatal murine myocardium. In juvenile, it lead to premature death within 1 week due to atrial enlargement and mild ventricular remodeling whereas, in adult, it induced myocyte hypertrophy, fibrosis, biventricular enlargement, and induction of fetal transcription [39]. These phenotypes were consistent with the defects during heart development observed in zebrafish embryos devoid of Dicer

function [40]. Later, another study came up with the similar outcome but by deletion of the DiGeorge syndrome critical region gene 8 (DGCR8 instead of dicer) using muscle creatine kinase-Cre mice and a conditional floxed allele of the DGCR8 [41]. MicroRNA expression profiling studies revealed that, the 18 most strongly expressed miRNAs and miRNA families account for >90% of all miRNA expressed in the adult mouse heart. Surprisingly, most of them are differentially expressed during cardiac disease, indicating the importance of miRNAs as modifiers of gene expression programs in cardiovascular disease. However, not all differentially expressed miRNAs are implicated in the cardiac disease. For example, miR-214, which is most highly upregulated among the 24 miRNAs studied, failed to induce cardiac phenotypes in transgenic mice in ischemic cardiomyopathy, dilated cardiomyopathy, and aortic stenosis, [42]. Whereas, ablation of miR-133 via double knockout resulted in aberrant proliferation and apoptosis of myocytes, cardiac defects, suggesting their therapeutic application in heart disease [43].

6 Role of microRNAs in Cardiovascular Diseases

miRNAs are highly stable in blood circulation due to their incorporation into microvesicles, exosomes, their association with RNA-binding proteins, and their adherence to circulating lipoproteins. These protective mechanisms enable miRNAs to fulfill biological functions outside the cell and serve as potential prognostic or diagnostic biomarkers in CVD pathologies [44]. Because of their nucleic acid nature they can be detected with high sensitivity and specificity using various molecular techniques such as microarrays and quantitative real-time PCR. Previous studies have shown a number of miRNAs to be unregulated in plasma of cardiovascular disease patients (Fig. 2). miR-208b and miR-499 were highly elevated (1600-fold and 100-fold respectively) in plasma from acute myocardial infarction (AMI) patients as compared with patients presenting with atypical chest pain without AMI [45]. Similarly, elevated miR-208a was detected in plasma of ~90% AMI patients whereas; it was undetectable in patients with non-AMI coronary heart disease or patients with other cardiovascular diseases [46]. However, unlike miR-208a which is specifically expressed in the heart, not all miRNAs are tissue specific. For example, miR-208b and miR-499 are also expressed in skeletal muscle therefore confounding the interpretation of miRNAs as diagnostic biomarkers in cardiac diseases [47]. Although recent advances in prognostics and/or diagnostics have increased the survival rate of patients with AMI, no significant change in mortality rates in patients who survive the initial AMI is observed. In a recent cohort study of 7733 AMI patients, 71% of AMI survival developed heart failure within 5 years [48]. Therefore, an expanded understanding of the function of miRNAs in gene regulatory networks engaged in MMP mediated cardiac remodeling is looked upon as a novel therapeutic strategy for CVD.

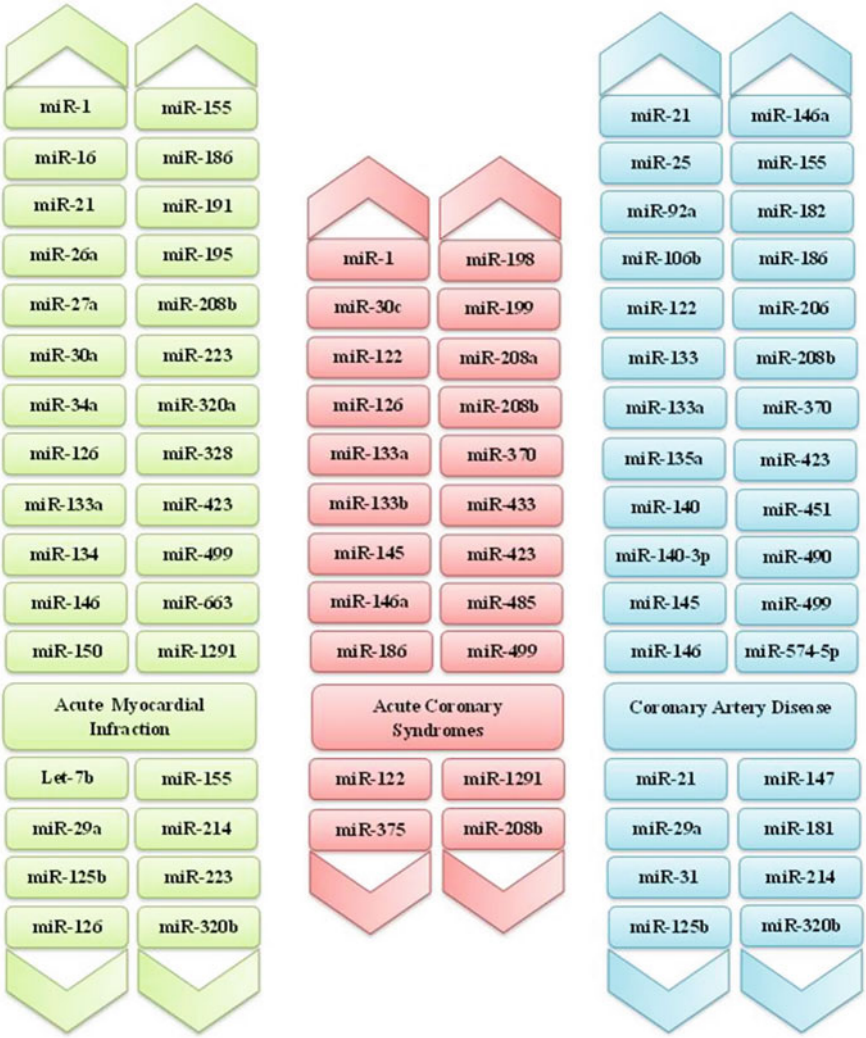

Fig. 2 Overview of upregulated and downregulated miRNAs in acute myocardial infarction (AMI), acute coronary syndromes (ACS) and coronary artery disease (CAD). Information is extracted from multiple studies included in references section [37, 42, 78, 80, 81]

7 microRNAs-Mediated Matrix Metalloproteinases Regulation

Recent studies have predicted/validated multiple gene targets of several miRNAs implicated in the pathophysiology of cardiovascular diseases [49] (Fig. 3). However, a major deficiency in the available literature on miRNA alterations occurring

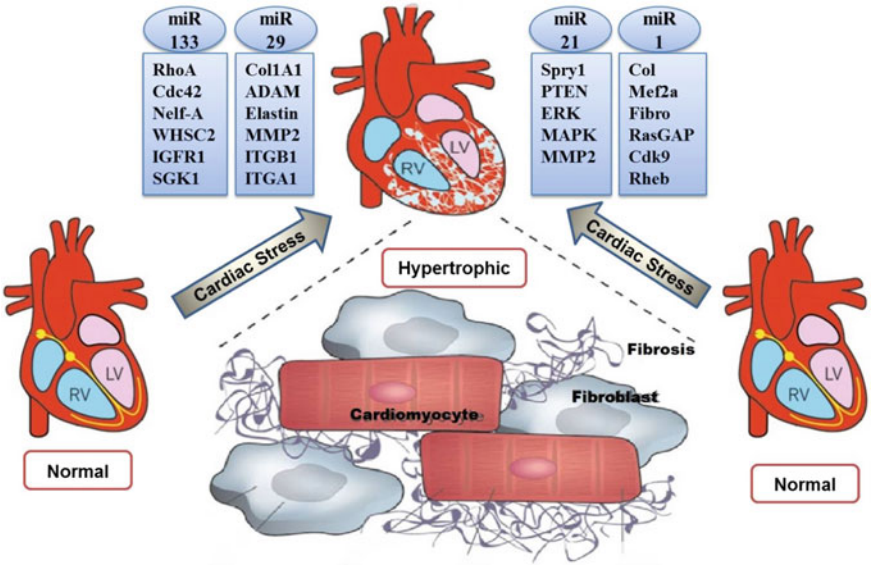

Fig. 3 Schematic representation of miRNAs, involved in development of cardiac hypertrophy and myocardial fibrosis. Cardiac stress leads to the dysregulation of miRNAs (few of them are represented: miR133, miR29, miR21 and miR21) as well as their target genes/proteins (few of them are represented). *Rho A* Ras homolog gene family, member A, *Cdc42* Cell division control protein 42, *Nelf-A* Negative elongation factor A, *WHSC2* Wolf–Hirschhorn syndrome candidate 2, *IGFR1* Insulin-like growth factor receptor 1, *SGK1* Serum-and glucose- regulated kinase, *Col1A1* Collagen type 1 α1, *ADAM* A disintegrin and metalloproteinase, *MMP-2* Matrix metalloprotease-2, *ITGB1* Integrin beta-1, *ITGA1* Integrin Alpha 1, *Spry1* Sprouty RTK Signaling Antagonist 1, *PTEN* Phosphatase and tensin homolog, *ERK* Extracellular signal–regulated kinases, *MAPK* Mitogen-activated protein kinases, *Col* Collagen, *Mef2a* Myocyte Enhancer Factor 2A, *Fibro* Fibronectin, *Ras-GAP* GTPase-Activating Protein, *Cdk9* Cyclin-dependent kinase 9, *Rheb* Ras homolog enriched in brain

during cardiovascular diseases is that very few studies have assessed miRNA/MMP interactions. Since all the MMPs have not been explored to the same intensity, especially in the context of miRNA/MMP interactions as it relates to onset and progression of cardiovascular diseases, this part of discussion is skewed in favor of those miRNAs/MMPs and ECM components where the literature is more abundant. Roy et al. published the first report showing that miR-21 regulates MMP-2 expression in cardiac fibroblasts in response to myocardial ischaemia–reperfusion via PTEN (phosphatase and tensin homologue) pathway [50]. Later, Chen et al. found that miR-29b epigenetically regulates MMP-2/MMP-9 genes expression in the context of atherosclerosis. They observed that oxidized low-density lipoprotein (oxLDL) reduces methylation (DNA methyltransferases) of the MMP-2/MMP-9 genes via upregulation of miRNA-29b leading to the increase MMP-2/MMP-9 gene expression which is known to have a profound influence on cardiovascular diseases [51]. The member of miR-29 family suppresses the expression of several ECM proteins and has been predicted to downregulate during myocardial infarction.

Abonnenc et al. confirmed the role of miR29 in ECM remodeling through pre-miR-29b and anti-miR-29b transfection in murine cardiac fibroblast (CF) [52]. Based on CF secretome data, they concluded that pre-miR-29b treatment reduced the collagen and MMP-2 secretion while anti-miR-29b did not stimulate their secretion [52, 53]. Lately, Chen et al. examined miRNA changes in different heart-cell compartments using congestive heart-failure experimental model. They observed that with decrease expression of miR-29b, the expression of MMP-2 did not change significantly in fibroblasts of left atrial. However, with increases expression miR-21 in left ventricular fibroblasts, the expression of MMP-2 did change [54]. These studies clearly suggest that aberrant posttranscriptional regulation of matrix metalloproteinases (MMPs) by miRNA is an important factor in cardiovascular diseases. Considering, the possibility of exploiting small noncoding RNAs as therapeutic tool for cardiovascular pathology, few studies used exogenous small interfering RNA (siRNA) which follow the same mechanism as miRNA to targets MMP-2 and -9. As described previously, MMP-2 is known to involve in various vascular complications including plaque destabilization and restenosis. Due to its constitutive expression by vascular smooth muscle cells, MMP-2 can facilitate vascular SMCs migration through hydrolysis of numerous extracellular matrix components leading to atherosclerosis. Transfection of vascular smooth muscle cells with MMP-2 siRNA has significantly decreased MMP-2 gene expression, resulting in the suppression of cell migration [55]. Similarly, atherosclerotic plaque stability has been obtained through silencing of MMP-9 gene using MMP-9 siRNA in apolipoprotein E (ApoE)-/- mice, suggesting that miRNAs and/or siRNAs represents potential pharmacological tool to target MMPs and their associated proteins [56].

8 microRNA Research and Therapeutics

Despite tremendous efforts in the traditional approach of drug design involving enzymes, cell surface receptors, increasing number of mortality and morbidity due to cardiovascular disease poses a great challenge to the therapeutic strategy adopted until now. Because of their specificity to their targets in a particular cellular pathway, miRNAs are rapidly becoming the most promising pharmacological tool to diagnose and treat cardiovascular disease. Therefore, it is necessary to understand methods, material, and tools required to resolve complex findings of miRNA research. miRNAs represent only about 0.01% of total cell RNA requiring extremely precise and sensitive instruments often with additional purification and enrichment steps. For visualization of individual pre-miRNA or mature miRNA expression in cells or tissues, quantitative real-time PCR (qRT-PCR), northern blotting, and in situ hybridization (ISH) in combination with immunohistochemistry are widely used. Northern blotting and ISH are very simple yet sensitive techniques but consumes relatively more time and sample since they do not involve miRNA amplification. qRT-PCR represents a balance of cost, precision, and sample size

using modified way of reverse transcribing individual miRNA from a pool of miRNAs present in isolated total mRNAs. For screening of genome-wide miRNA expression, high-throughput molecular biology techniques such as miRNA microarray, deep sequencing are followed by validation with qRT-PCR. While miRNA microarray and qRT-PCR are two of the most common methods for evaluating known miRs, there are still contrasting reports on the correlation between microarray and qRT-PCR [57, 58]. To study novel miRNAs whose complement has not been ascertained, a more sensitive technique for small RNA sequences, which provides absolute abundance values of miRNAs is next-generation sequencing. It is known that a specific miRNA can regulate the expression of multiple genes (targets) while the expression of individual gene can be regulated by multiple miRNAs [59]. Therefore, for a comprehensive understanding of miRNA function and potential therapeutic use in heart disease, identification and validation of miRNA targets is of fundamental importance. There are several *in silico* target prediction programs such as TargetScan, PicTar, MiRanda/mirSVR, miRBase/MicroCosm, RNA22, and PITA. These databases use common feature like complementarity between the 3′ UTR of the target mRNA and the 5′-seed of the miRNA, conservation among species, the presence of several miRNA target sites and pattern recognition [60–62]. The predicted target needs to be further validated which can be achieved using biochemical methods, proteomic/transcriptome analysis, and RISCome analysis. Biochemical methods include qRT-PCR, western blotting, reporter assays, hybrid PCR, cytoplasm/nucleus ratio of mRNA, affinity purification, biotin-tagged miRNAs, and labeled microRNA pull-down assay (LAMP) [63–68]. Proteome analysis includes stable isotype labeling with amino acids in cell culture (SILCA) along with mass spectrometry to identify miRNA–mRNA interactions [69, 70]. Lastly, RISCome analysis includes target identification by sequencing of RNA-induced silencing complex and Argonaute. Other novel high-throughput assays based on immunoprecipitation are RIP-Chip (RNA-Binding Protein Immunoprecipitation-Microarray Profiling), PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation), and HITS-CLIP (High-throughput Sequencing of RNAs isolated by cross-linking immunoprecipitation [46, 71–76]. Not all these methods have been applied for cardiovascular research yet, but since miRNA research is a growing field, these methods could be adapted because of their accurate and quantitative estimation of miRNA profiles and expression.

The general strategy of therapy for any disease is to reverse the pathological changes that resulted due to disease. Therefore, those MMPs and miRNAs whose overexpression is responsible for cardiovascular disease should be suppressed and vice versa. The suppression of overexpressed MMPs/miRNAs can be achieved by miRNA mimic technology (miR-mimic) which is an approach for gene silencing by generating artificial double-stranded miRNA-like RNA fragments. These RNA fragments mimic endogenous miRNAs and bind specifically to its target mRNA activating the RNA-induced silencing complex (RISC), downregulating specific mRNAs and thus induce gene suppression [77]. On the contrary, the expression of MMPs can be induced by antimiRs (cholesterol-conjugated, 2-O-methyl–modified

antimiRs, called antagomiRs), which are a class of chemically engineered oligonucleotides specifically silencing single endogenous miRNA (Fig. 1). They competitively inhibit specific miRNA by binding to the target mature miRNA and lead to a reduced activation of RISC and consequently to an upregulation of specific mRNA and gene expression [78]. As mentioned above, few studies have demonstrated the value of mimic in downregulating the expression of MMP-9 in mouse model of atherosclerosis as well as in vitro. Besides, to promote miRNAs as a viable therapeutic target there are several difficulties to overcome such as, difference in predicted and actual physiological targets, potential off-target effects of antimiRs, and global effect on ECM of other organs. Further studies are required to elucidate the exact methods by which miRNAs are able to repress translation and initiate mRNA degradation of selected targets. The recent success of the first human clinical trial of a miravirsen (SPC3649) therapeutic for suppression of hepatitis C virus (HCV) replication has raised possibilities for the use miRNAs as a therapeutic target in CVD and clinical trials are eagerly awaited [79].

9 Conclusion

Recent studies have established a cause-and-effect relationship between the induction and activation of MMPs and cardiovascular diseases raising great expectations for MMPs as promising target. Also, miRNAs targeting MMPs and/or their regulators, as directly or indirectly discussed above are sufficient to draw a plausible inference that miRNA regulation of MMPs expression is an important mechanism for causing CVD. Since the activity of MMPs relies upon several complex interconnected molecular interactions, many clinical trials of MMP inhibitors failed to improve clinical outcomes. miRNA-based therapeutics under preclinical trials have shown promising results in numerous animal models of CVD such as cardiac hypertrophy, fibrosis, and MI. Till now, the role of less than 12 MMPs have been investigated post-myocardial infraction and very few MMPs have been predicted to be the targets of miRNAs. Therefore, extensive studies analyzing the complex interactions between specific miRNAs and MMPs in context with CVD will definitely bridge this gap and provide novel opportunities for diagnosis and therapy of CVD.

References

1. Krenning G, Zeisberg EM, Kalluri R (2010) The origin of fibroblasts and mechanism of cardiac fibrosis. J Cell Physiol 225:631–637
2. Vliegen HW, van der Laarse A, Cornelisse CJ, Eulderink F (1991) Myocardial changes in pressure overload-induced left ventricular hypertrophy. A study on tissue composition, polyploidization and multinucleation. Eur Heart J 12:488–494

3. Naba A, Clauser KR, Hoersch S et al (2012) The Matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. Mol Cell Proteomics 11:M111.014647–M111.014647
4. De Haas HJ, Arbustini E, Fuster V et al (2014) Molecular imaging of the cardiac extracellular matrix. Circ Res 114:903–915
5. Zannad F, Rossignol P, Iraqi W (2010) Extracellular matrix fibrotic markers in heart failure. Heart Fail Rev 15:319–329
6. Laviades C, Varo N, Fernandez J et al (1998) Abnormalities of the extracellular degradation of collagen type I in essential hypertension. Circulation 98:535–540
7. Lindsay MM, Maxwell P, Dunn FG (2002) TIMP-1: a marker of left ventricular diastolic dysfunction and fibrosis in hypertension. Hypertension 40:136–141
8. López B, Querejeta R, Varo N et al (2001) Usefulness of serum carboxy-terminal propeptide of procollagen type I in assessment of the cardioreparative ability of antihypertensive treatment in hypertensive patients. Circulation 104:286–291
9. Zannad F, Alla F, Dousset B, Perez A, Pitt B (2000) Limitation of excessive extracellular matrix turnover may contribute to survival benefit of spironolactone therapy in patients with congestive heart failure: insights from the randomized aldactone evaluation study (RALES). Rales Investigators Circ 02:2700–2706
10. Lin YH, Ho YL, Wang TD et al (2006) The relation of amino-terminal propeptide of type III procollagen and severity of coronary artery disease in patients without myocardial infarction or hibernation. Clin Biochem 39:861–866
11. Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516
12. Gross J, Lapiere CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci USA 48:1014–1022
13. Park HI, Ni J, Gerkema FE et al (2000) Identification and characterization of human endometase (matrix metalloproteinase-26) from endometrial tumor. J Biol Chem 275:20540–20544
14. Auld DS (2004) Structural zinc sites. In: Messerschmidt A, Bode W, Cygler M (eds) The handbook of metalloproteins, vol 3. Wiley, Chichester, pp 403–415
15. Nagase H, Woessner JF (1999) Matrix metalloproteinases. J Biol Chem 274:21491–21494
16. Fini ME, Cook JR, Mohan R (1998) Brinckerhoff, CE. Regulation of matrix metalloproteinase gene expression. In: Parks WC, Mecham RP (eds) Matrix metalloproteinases. Academic Press, New York, pp 299–356
17. Mittal B, Mishra A, Srivastava A et al (2014) Matrix metalloproteinases in coronary artery disease. Adv Clin Chem 64:1–72
18. Rodríguez D, Morrison CJ, Overall CM (2010) Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta Mol Cell Res 1803:39–54
19. Zamilpa R, Lindsey ML (2010) Extracellular matrix turnover and signaling during cardiac remodeling following MI: causes and consequences. J Mol Cell Cardiol 48:558–563
20. Thomas CV, Coker ML, Zellner JL et al (1998) Increased matrix metalloproteinase activity and selective upregulation in LV myocardium from patients with end-stage dilated cardiomyopathy. Circulation 97:1708–1715
21. Lindsey ML, Goshorn DK, Squires CE et al (2005) Age-dependent changes in myocardial matrix metalloproteinase/tissue inhibitor of metalloproteinase profiles and fibroblast function. Cardiovasc Res 66:410–419
22. Deten A, Volz HC, Holzl A et al (2003) Effect of propranolol on cardiac cytokine expression after myocardial infarction in rats. Mol Cell Biochem 251:127–137
23. Greene J, Wang M, Liu YE et al (1996) Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. J Biol Chem 271:30375–30380
24. Blankenberg S, Rupprecht HJ, Poirier O et al (2003) Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation 107:1579–1585

25. Peterson JT, Li H, Dillon L, Bryant JW (2000) Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat. Cardiovasc Res 46:307–315
26. Kai H, Ikeda H, Yasukawa H et al (1998) Peripheral blood levels of matrix metalloproteases-2 and -9 are elevated in patients with acute coronary syndromes. J Am Coll Cardiol 32:368–372
27. Wagner DR, Delagardelle C, Ernens I et al (2006) Matrix metalloproteinase-9 is a marker of heart failure after acute myocardial infarction. J Card Fail 12:66–72
28. Jayasankar V, Woo YJ, Bish LT et al (2004) Inhibition of matrix metalloproteinase activity by TIMP-1 gene transfer effectively treats ischemic cardiomyopathy. Circulation 110(11)
29. Lindsey ML, Zamilpa R (2012) Temporal and spatial expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases following myocardial infarction. Cardiovasc Ther 30:31–41
30. Yamamoto D, Takai S, Jin D et al (2007) Molecular mechanism of imidapril for cardiovascular protection via inhibition of MMP-9. J Mol Cell Cardiol 43:670–676
31. Seeland U, Kouchi I, Zolk O et al (2002) Effect of ramipril and furosemide treatment on interstitial remodeling in post-infarction heart failure rat hearts. J Mol Cell Cardiol 34: 151–163
32. Hudson MP, Armstrong PW, Ruzyllo W et al (2006) Effects of selective matrix metalloproteinase inhibitor (PG-116800) to prevent ventricular remodeling after myocardial infarction: results of the PREMIER (prevention of myocardial infarction early remodeling) trial. J Am Coll Cardiol 48:15–20
33. Tyagi AC, Sen U, Mishra KP (2011) Synergy of microrna and stem cell: a novel therapeutic approach for diabetes mellitus and cardiovascular diseases. Curr Diabetes Rev 7:367–376
34. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854
35. Davison TS, Johnson CD, Andruss BF (2006) Analyzing micro-RNA expression using microarrays. Methods Enzymol 411:14–34
36. Zeng Y, Cullen BR (2003) Sequence requirements for micro RNA processing and function in human cells. RNA 9:112–123
37. Thum T, Galuppo P, Wolf C et al (2007) microRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation 116:258–267
38. Rustagi Y, Jaiswal HK, Rawal K et al (2015) Comparative characterization of cardiac development specific microRNAs: fetal regulators for future. PLoS ONE 10:10
39. Da Costa Martins PA, Bourajjaj M, Gladka M et al (2008) Conditional dicer gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling. Circulation 118:1567–1576
40. Giraldez AJ, Cinalli RM, Glasner ME et al (2005) microRNAs regulate brain morphogenesis in zebrafish. TL-308. Science 308:833–838
41. Rao PK, Toyama Y, Chiang HR et al (2009) Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res 105:585–594
42. van Rooij E, Sutherland LB, Liu N et al (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci USA 103:18255–18260
43. Carè A, Catalucci D, Felicetti F et al (2007) MicroRNA-133 controls cardiac hypertrophy. Nat Med 13:613–618
44. Gilad S, Meiri E, Yogev Y et al (2008) Serum microRNAs are promising novel biomarkers. PLoS ONE 3:9
45. Corsten MF, Dennert R, Jochems S et al (2010) Circulating microRNA-208b and microRNA-499 reflect myocardial damage in cardiovascular disease. Circ Cardiovasc Genet 3:499–506
46. Wang G-K, Zhu J-Q, Zhang J-T et al (2010) Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. Eur Heart J 31:659–666
47. van Rooij E, Quiat D, Johnson BA et al (2009) A family of micrornas encoded by myosin genes governs myosin expression and muscle performance. Dev Cell 17:662–673

48. Ezekowitz JA, Kaul P, Bakal JA et al (2009) Declining in-hospital mortality and increasing heart failure incidence in elderly patients with first myocardial infarction. J Am Coll Cardiol 53:13–20
49. Neppl RL, Wang DZ (2014) The myriad essential roles of microRNAs in cardiovascular homeostasis and disease. Genes Dis 1:18–39
50. Roy S, Khanna S, Hussain SR et al (2009) MicroRNA expression in response to murine myocardial infarction: MiR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 82:21–29
51. Chen K-C, Wang Y-S, Hu C-Y et al (2011) OxLDL up-regulates microRNA-29b, leading to epigenetic modifications of MMP-2/MMP-9 genes: a novel mechanism for cardiovascular diseases. FASEB J 25:1718–1728
52. Abonnenc M, Nabeebaccus AA, Mayr U et al (2013) Extracellular matrix secretion by cardiac fibroblasts: role of MicroRNA-29b and MicroRNA-30c. Circ Res 113:1138–1147
53. van Rooij E, Olson EN (2009) Searching for miR-acles in cardiac fibrosis. Circ Res 104: 138–140
54. Chen Y, Wakili R, Xiao J et al (2014) Detailed characterization of microRNA changes in a canine heart failure model: relationship to arrhythmogenic structural remodeling. J Mol Cell Cardiol 1–12
55. Kim D, Lee D, Jang YL et al (2012) Facial amphipathic deoxycholic acid-modified polyethyleneimine for efficient MMP-2 siRNA delivery in vascular smooth muscle cells. Eur J Pharm Biopharm 81:14–23
56. Jin Z, Xiong Q, Jia F et al (2015) Investigation of RNA interference suppression of matrix metalloproteinase-9 in mouse model of atherosclerosis. Int J Exp Med 8:5272–5278
57. Ach RA, Wang H, Curry B (2008) Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods. BMC Biotechnol 8:69
58. Chen Y, Gelfond JAL, McManus LM, Shireman PK (2009) Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. BMC Genom 10:407
59. Romaine SPR, Tomaszewski M, Condorelli G, Samani NJ (2015) microRNAs in cardiovascular disease: an introduction for clinicians. Heart 101:921–928
60. Witkos TM, Koscianska E, Krzyzosiak WJ (2011) Practical aspects of microRNA target prediction. Curr Mol Med 11:93–109
61. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20
62. Krek A, Grün D, Poy MN et al (2005) Combinatorial microRNA target predictions. Nat Genet 37:495–500
63. Thum T, Catalucci D, Bauersachs J (2008) microRNAs: novel regulators in cardiac development and disease. Cardiovasc Res 79:562–570
64. Huang Y, Qi Y, Ruan Q et al (2011) A rapid method to screen putative mRNA targets of any known microRNA. Virol J 8:8
65. Li J, Xia W, Huang B et al (2010) A strategy to rapidly identify the functional targets of microRNAs by combining bioinformatics and mRNA cytoplasmic/nucleic ratios in culture cells. FEBS Lett 584:3198–3202
66. Orom UA, Lund AH (2007) Isolation of microRNA targets using biotinylated synthetic microRNAs. Methods 43:162–165
67. Vo NK, Dalton RP, Liu N et al (2010) Affinity purification of microRNA-133a with the cardiac transcription factor, Hand2. Proc Natl Acad Sci USA 107:19231–19236
68. Hsu RJ, Tsai HJ (2011) Performing the labeled microRNA pull-down (LAMP) assay system: an experimental approach for high-throughput identification of microRNA-target mRNAs. Methods Mol Biol 764:241–247
69. Baek D, Villén J, Shin C et al (2008) The impact of microRNAs on protein output. Nature 455:64–71

70. Yang Y, Chaerkady R, Kandasamy K et al (2010) Identifying targets of miR-143 using a SILAC-based proteomic approach. Mol BioSyst 6:1873–1882
71. Fleissner F, Jazbutyte V, Fiedler J et al (2010) Short communication: asymmetric dimethylarginine impairs angiogenic progenitor cell function in patients with coronary artery disease through a MicroRNA-21-dependent mechanism. Circ Res 107:138–143
72. Matkovich SJ, Van Booven DJ, Eschenbacher WH, Dorn GW (2011) RISC RNA sequencing for context-specific identification of in vivo microRNA targets. Circ Res 108:18–26
73. Tan LP, Seinen E, Duns G et al (2009) A high throughput experimental approach to identify miRNA targets in human cells. Nucleic Acids Res 37:20
74. Wang W-X, Wilfred BR, Hu Y et al (2010) Anti-Argonaute RIP-Chip shows that miRNA transfections alter global patterns of mRNA recruitment to microribonucleoprotein complexes. RNA 16:394–404
75. Hafner M, Landthaler M, Burger L et al (2010) Transcriptome-wide identification of RNA-binding protein and MicroRNA target sites by PAR-CLIP. Cell 141:129–141
76. Chi SW, Zang JB, Mele A, Darnell RB (2009) Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. Nature 460:479–486
77. van Rooij E, Sutherland LB, Thatcher JE et al (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105:13027–13032
78. Stenvang J, Petri A, Lindow M et al (2012) Inhibition of microRNA function by antimiR oligonucleotides. Silence 3:1
79. Lanford RE, Hildebrandt-Eriksen ES, Petri A et al (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327:198–201
80. Thum T, Gross C, Fiedler J et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 456:980–984
81. van Rooij E, Sutherland LB, Qi X et al (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. Science 316:575–579