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Naranjan S. Dhalla
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Role of Proteases in Cellular Dysfunction

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Role of Proteases in Cellular Dysfunction

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This book is dedicated to
Prof. Ranbir C. Sobti, Vice Chancellor, Babasaheb Bhimrao Ambedkar University, Lucknow, India, for his untiring efforts to promote excellence in science throughout the Indian subcontinent. Prof. Sobti served the Punjab University with great distinction, first as Professor and Head and then as Vice Chancellor. In view of his outstanding record as scientist in the field of Biotechnology as well as high commitment for developing scientific culture and promoting young investigators, he was elected President of the 101st Indian Science Congress. Dr. Sobti was bestowed the great honor "Padmashree" by the Government of India.

Preface

This book entitled "Role of Proteases in Cellular Dysfunctions" is the second book on Proteases for the Series "Advances in Biochemistry in Health and Disease." It is now well known that proteases are found everywhere, in viruses and bacteria as well as in all human, animal, and plant cells, and play a role in a variety of biological functions ranging from digestion, fertilization, and development to senescence and death. Under physiological conditions the ability of proteases is regulated by endogenous inhibitors. However, when the activity of proteases is not regulated appropriately, disease processes can result in, as seen in Alzheimer's disease, cancer metastasis and tumor progression, inflammation, and atherosclerosis. Thus it is evident that there is an absolute need for a tighter control of proteolytic activities in different cells and tissues.

For scientists working on proteases, it is important to consider the components that are intimately involved in the activation/inhibition of proteases. Additionally, proteases regulate a plethora of cell functions via interactions with other enzymes/proteins in cells. Efficacy of protease inhibitors to treat diseases has also been recognized and is currently an important field of research. This book is the result of the commendable effort by a large group of world leading experts to provide excellent, comprehensive, and up-to-date reviews on the subjects in the field. It contains 23 chapters contributed by experts in their respective fields and elucidates the crucial role of proteases in biological processes, including how proteolytic function and regulation can be combined for new strategies for the development of therapeutic interventions.

The book consists of three parts in specified topics based on current literatures for a better understanding for the readers with respect to their subject-wise interests. The first section of this book covers a brief idea about the neuronal disorders and the involvement of proteases such as calpains, caspases, and matrix metalloproteases (MMPs). The second section covers the deadly disease, cancer, and its relation to ubiquitin–proteasomal system, MMPs, and serine proteases. The last section is about the role of proteases such as calpains, MMPs, and serine protease as well as urokinase-type plasminogen activator receptor (uPAR) in causing cardiovascular

defects. The readers will find these chapters both stimulating and interesting. We hope that this book will prove useful for graduate students and also researchers, who have an interest in cellular proteolytic events.

As editors of this book, we are greatly indebted to the authors for the time and effort they spent in making the book as an advancement of knowledge in this field. We would also like to thank Prof. Dilip Kumar Mohanta, Vice Chancellor, University of Kalyani, West Bengal, India, and Prof. Bimal K Ray, University of Missouri, Columbia, Missouri, USA, for their encouragement. The time and efforts of Dr. Vijayan Elimban and Mrs. Eva Little (St. Boniface Hospital Research) during the editing of this book are highly appreciated. We are also grateful to Ms. Melanie Tucker and Ms. Rita Beck (Springer, New York) for their understanding as well as patience for the preparation of this book.

Winnipeg, MB, Canada — Naranjan S. Dhalla
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Part I
Proteases and Neural Disorders

Chapter 1
Role of Calpain in Immunobiology of Neurodegenerative Diseases

Nicole Trager, Azizul Haque, Swapan K. Ray, Arabinda Das, and Naren L. Banik

Abstract Calpain is a Ca^{2+}-dependent protease that significantly contributes to the pathogenesis of demyelinative and neurodegenerative diseases and injuries of the central nervous system (CNS). Studies from our laboratory and other laboratories clearly indicate that calpain plays crucial roles in pro-inflammatory immune responses to perpetuate inflammation in multiple sclerosis (MS), which is a demyelinative and neurodegenerative disease of the CNS, and experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Calpain mediates its pro-inflammatory roles with activation of nuclear factor-kappa B (NF-κB), promotion of synthesis of cytokines and chemokines, maintenance of Th1/Th2 imbalances, and reactive astrogliosis and microgliosis in MS and EAE. Besides, calpain is

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known to take part in promoting activation of caspases for neurodegeneration in a wide range of diseases and injuries of the CNS. Recently, there is a great interest in developing water-soluble and cell-permeable small molecule inhibitors of calpain for treatment of MS and other neurodegenerative diseases. Although exciting results are being reported showing efficacy of experimental calpain inhibitors in preclinical models, these inhibitors have not yet been successfully used for treatment of MS and other neurodegenerative diseases in humans.

Keywords Calpain • Neurodegeneration • Neurodegenerative diseases • Inflammation • Calpain inhibitors • Multiple sclerosis

1 Introduction

Often, diseases are caused by a specific cellular dysfunction with a common underlying pathway. Neurodegenerative diseases have been a recent topic of this type of investigation. Uncovering a common underlying pathway that results in neuronal damage can lead to understanding of several neurological diseases and development of therapeutics as well. A number of laboratories have been shedding light on one cellular protease, calpain, which can be a common variable in the development and progression of neurodegenerative diseases.

It has been shown that upregulation of calpain and deregulation of calcium homeostasis participate in a variety of pathological processes of the diseases such as multiple sclerosis (MS), Parkinson's disease (PD), stroke, spinal cord injury (SCI), traumatic brain injury (TBI), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), muscular dystrophy (MD), and cataract formation. Calcium ions are intracellular messengers involved in the regulation of important cellular functions, including synaptic activity, membrane excitability, exocytosis, and enzyme activation. Neurons do possess mechanisms to regulate intracellular calcium levels, but it is also known that a dramatic increase in the cytoplasmatic levels of calcium is the first indicator of a trigger for neuronal death [1–3]. Thus, calpain, a calcium ion-dependent protease, could be activated under these conditions and be a significant contributor in neuronal injury. While calpain can be directly involved in neuronal injury through intracellular calcium ion deregulation, it is becoming clear that calpain also acts on the immune system along with how it interacts with nervous system and the development and advancement of neurodegenerative disorders. Many of the neurodegenerative disorders also result from an inflammation or calpain-initiated immune components, which activate inflammatory arms of several immune pathways in the host. These inflammatory events are often implicated in the development and continuation of various neurodegenerative disorders including MS. Researchers are now uncovering how calpain affects the cells of the immune system during these disorders and are suggesting a common pro-inflammation pathway.

2 Calpain Isoforms and Functions

In 1964, calpain was first found in the central nervous system (CNS) [4]. Calpain is an intracellular protease that is present in the cytoplasm as an inactive form [5, 6]. Research has revealed 14 large subunit members and at least one small subunit member of the mammalian calpain family [7]. Calpains 1, 2, 4, 5, 7, and 10 [8–13] are ubiquitously expressed, whereas others are tissue specific: calpain 3 (skeletal muscle) [10], calpain 6 (placenta) [14], calpain 8 (smooth muscle) [15], calpain 9 (stomach) [15], calpain 11 (testis) [16, 17], calpain 12 (skin after birth) [18], and calpain 13 (testis and lung) [16]. There are two dominant isoforms of calpain, micro-calpain (μ-calpain or calpain 1) and milli-calpain (m-calpain or calpain 2) [19–21]. This nomenclature stems from the concentration of Ca^{2+} that is needed for activation of μ-calpain or m-calpain (2–80 mM and 0.2–0.8 mM Ca^{2+}, respectively). The catalytic subunits of calpain found in the CNS include isoforms 1, 2, 3, 5, and 10 [5, 6]. The typical calpain isoform consists of an 80 kDa catalytic subunit and a 30 kDa regulatory subunit. The regulatory subunit possesses a hydrophobic, glycine-rich domain for membrane association. Each subunit contains an EF-hand domain, a characteristic of the most Ca^{2+}-binding proteins [6].

The specific physiological function of calpain is still a topic of continuing research, but calpain is clearly involved in numerous intracellular signaling substrates. After the activation of calpain by Ca^{2+}, it is able to interact with a large number of substrates including cytoskeletal proteins, growth factor receptors, mitochondria, actin-binding proteins, tubulin, microtubule-associated proteins (MAP2, tau), and neurofilaments [21, 22]. Calpain was first thought to be involved in just necrosis, but it is currently known to be involved in apoptosis, cell mobility, cell cycle progression, cell fusion, and many other important processes [23]. These calpain activities are modulated by interactions with calpastatin, which a specific endogenous protein inhibitor of calpain [24, 25].

3 Calpain Upregulation in Neurodegenerative Diseases

Neurodegenerative disorders such as AD, PD, HD, MS, and ALS manifest in various stages of adulthood and involve the dysfunction and ultimate death of neurons in the CNS. While the etiology underlying each disorder varies, the pathological mechanisms, at least in part, converge on impaired intracellular calcium homeostasis, leading to activation of calpain. Calpain has been implicated in tissue degeneration in CNS trauma [26, 27], cerebral ischemia [28], MD [29, 30], PD [31], AD [32, 33], EAE, MS [34, 35], ALS [36], and HD [37]. Increased activity of calpain has also been found in the cerebrospinal fluid (CSF) of MS patients [38].

Accordingly, calpain inhibition has proven to be neuroprotective [39, 40]. Most of the aforementioned studies suggest a direct involvement of calpain in

Table 1.1 Calpain in pathogenesis of neurodegenerative diseases

Neurodegenerative disease	Hypothesized calpain involvement	Ref #
CNS trauma	Neurofilament protein degradation, cleavage of membrane, and cytoskeletal proteins leading to cell death	[26, 27]
Cerebral ischemia	Calpain inhibition shows neuroprotective effect, suggesting calpain's involvement in neuron death	[28]
Muscular dystrophy	Calpain is increased in muscular dystrophic mice and is correlated with loss of important enzymes	[29, 30]
Parkinson's disease	High levels of activation disrupt the structural and functional integrity of the spinal cord	[31]
Alzheimer's disease	High levels of activation correlate with abnormal proteolysis underlying the accumulation of plaques	[32, 33]
Multiple sclerosis	Direct cleavage of myelin and activation of inflammatory T cells	[34, 35]
ALS	Calpain activates caspase cascade to motor neuron death	[36]
Huntington's disease	Inhibits NMDA receptor trafficking and function	[37]

neurodegeneration, but there is also evidence that calpain plays a role in many other cellular events that lead to immune activation and release of inflammatory mediators involved in neurodegeneration (Table 1.1).

4 Calpain and Immunobiology of MS

MS is a demyelinating disease associated with neurodegeneration in the central nervous system (CNS). MS is thought to result from an attack on myelin proteins by autoreactive T helper (Th) cells, thus making it also an autoimmune disorder. Calpain has been shown to be involved in T cell activation and migration [41–44]; and since calpain expression has been shown to be increased in infiltrating inflammatory cells in MS tissue [34] and in EAE spinal cord [45], calpain is thought to play a key role in the migration of T cells into the CNS. In MS, Th1 and Th17 cells play crucial roles in the pathogenesis of the disease. Th2 and T regulatory (Treg) responses have been shown to be pivotal in controlling MS. Researchers have shown calpain to be involved in the deregulation of Th1-/Th2-type responses in MS patients and EAE animals [45–47]. In addition, calpain activity and expression are increased in reactive endogenous glial cells (astrocytes, microglia) in MS [34]. Calpain has also been shown to be involved in different signaling pathways leading to apoptosis and necrosis [27, 48], suggesting that calpain plays a crucial role in cell death in this demyelinating disease as well as other neurodegenerative diseases.

Signal transducers and activators of transcription (STATs) mediate many cytokine-mediated responses, including those seen in autoimmune diseases [49, 50]. Various STAT molecules are substrates of calpain [51]; however, depending on whether these signaling molecules are involved in pro-inflammatory or anti-inflammatory cytokine

production, calpain differentially alters their activity. STAT4 protein, which is critical for the induction of the inflammatory Th1 immune response, is activated by IL-12 and induces the transcription of IFN-γ [52, 53]. Similarly, STAT3 is needed for inflammatory Th17 immune response. In contrast, anti-inflammatory Th2 immune response requires STAT6 protein and is activated by IL-4 [54]. Calpain acts as a negative regulator by degrading STAT6 by a Ca^{2+}-dependent mechanism [47, 51, 55], suggesting that calpain plays a role in signaling events leading to Th1-/Th2-type responses through modulation of STAT6 or other transcription factors. Interestingly, IL-4 inhibits activation of osteoclasts, a bone disorder often seen in MS patients, in vitro through inhibition of NF-κB and JNK activation in a STAT6-dependent manner [56]. It has also been reported that STAT6 is cleaved by calpains and in vivo treatment with calpain inhibitors prevented loss of STAT6 protein [47]. Our laboratory has been working on a hypothesis that activation of STAT6 and inhibition of NF-κB and JNK pathways by calpain inhibition may lead to amelioration of inflammatory processes in MS. Since STAT1, STAT4, and T-bet are important for Th1 subtype activation and proliferation, and since STAT6 and GATA3 are important for Th2 subtype activation, it seems plausible that controlling these signals may result in inflammatory/anti-inflammatory balance(s) in MS patients.

Cytokines are key regulators of immune responses, and blocking their actions has become an important modality in treating inflammatory disorders in MS. Pro-inflammatory Th1 cells produce interleukin-2 (IL-2), interferon-gamma (IFN-γ), IL-12, and tumor necrosis factor-alpha (TNF-α), and Th17 cells produce IL-17A, IL-21, and IL-22. Anti-inflammatory Th2 cells produce IL-4, IL-5, IL-13, and low levels of IL-10 and, in some cases, transforming growth factor-beta (TGF-β), while T regulatory (Treg) cells produce higher levels of IL-10 and TGF-β [57]. Researchers have shown that Th1 cytokines are upregulated, while Th2 cytokines are downregulated in MS [58]. In MS patient, blood calpain inhibition has been shown to help restore this Th1/Th2 balance by decreasing Th1 and increasing Th2 cell markers [59]. Similarly, studies have shown that IL-17 mRNA levels are reduced in MS patient's PBMCs following treatment with calpain inhibitors [59]. A recent study in EAE, the mouse model of MS, shows that the Th2/Treg cytokines IL-4 and IL-10 are significantly increased in animals treated with calpain inhibitors [60], suggesting a role for calpain in disease severity. These emerging data in the field are significant in that they strongly support the hypothesis that blocking calpain may shift Th profiles away from the inflammatory Th1/Th17 profile associated with MS relapse and toward a protective Th2/Treg profile associated with MS remission. A further understanding of the mechanisms of T cell deregulation in MS and calpain's contribution to these pathways is necessary.

T cell activation requires a co-stimulatory response, which lead to production of cytokines, depending on the local environment of the cell. The primary signal is given by the interaction of the T cell receptor (TCR) on the surface of the T cell with the major histocompatibility class II protein (MHCII) upon the surface of antigen-presenting cells (APC) [61]. A major co-stimulatory signal is given by the interaction of T cell surface CD28 molecule with APC cell surface molecules B7-1 (CD80) or B7-2 (CD86) [62]. The result of proper TCR/MHCII and co-stimulation can

result in significant proliferation of T cells [62, 63]. Researchers have shown that a rise in Ca^{2+} levels, liberation of diacylglycerol (DAG) [64, 65], and co-stimulation through CD28 are responsible for the activation of a number of signal transduction pathways that eventually lead to the synthesis of cytokines through activation of transcription factors, which include nuclear factor-kappa B (NF-κB) [45, 66] and nuclear factor of activated T cells (NFAT) [67, 68]. NF-κB has been shown to promote synthesis of IL-2 and other inflammatory factors after calpain-mediated activation, indicating that calpain may play a role in NF-κB-driven gene expression. Interestingly, researchers have also shown that increases in Ca^{2+} lead to the stimulation of calcineurin, which dephosphorylates NFAT at several sites, allowing translocation of NFAT to the nucleus [69, 70]. While there might be a significant link between calpain and NFAT in MS, no studies on NFAT and MS have been reported, and it remains an area that needs further investigation. However, it remains a possibility that calpain is involved in T cell activation and deregulation of Th subpopulations through NFAT and STAT signaling pathways, and calpain inhibition may decrease these inflammatory events in MS.

Migration of immune cells into the CNS and demyelination are hallmarks of MS pathology [71]. One key cytokine involved in chemotaxis and migration of immune cells is IL-8. IL-8 and IL-8 receptors (CXC1, CXC2) have been shown to be upregulated in MS brain tissue [72], implicating this chemokine as a major player in migration of immune cells in MS. It is known that Th1 cells differentially express chemokines receptors CCR5, CXCR3, CXCR6, and CX3CR1, and they migrate to sites of Th1 inflammation where the ligands for these chemokine receptors are upregulated [73–75]. Th2 cells are known to differentially express chemokine receptors CCR3, CCR4, and CCR8 and migrate to sites of inflammation where the ligands for these receptors are expressed [73–75]. Migration of Th1 and Th2 cells may be differentially regulated by STAT6, which particularly regulates Th2 cell trafficking. STAT1 can also regulate the recruitment of Th1 cells through the induction of chemokine ligands RANTES, CXCL9, CXCL10, CXCL11, and CXCL16 [73–75]. Higher levels of chemokine ligands for CCR5 and CXCR3 have also been found in inflammation, suggesting that the inhibition of chemokine recruitment may help block migration of inflammatory T cells [73–77]. It has been shown that inhibition of calpain reduces T cell migration. In specific, CCL2 action migration is slowed or stopped upon treatment with calpain inhibitor [44]. These studies show that calpain has a direct involvement not only in the activation of possible disease causing T cells but also in their active migration to the CNS.

Chemokine receptors are distinct to certain cell types and significantly influence immune responses in inflammation. It has been reported that CXCR3, CCR5, and CCR7 receptors influence Th1 [47, 51], and CCR3, CCR4, and CCR8 receptors influence Th2 [78–81] profiles. In this scenario, chemotaxis appears to be selective. For instance, Th1 cells selectively migrate in response to IFN-α-inducing protein (IP-10), which binds to CXCR3, and macrophage inflammatory proteins (MIPs), which are ligands for CCR5 [81, 82]. Interestingly, recent studies involving T cells from STAT6−/− Balb/c mice suggest that STAT6 may differentially regulate expression of Th1/Th2 chemokine receptors [83]. As mentioned before, STAT6 is a

Table 1.2 Calpain's effects on the immune system

Immune target	Function	Calpain activation	Calpain inhibition	Ref #
STAT 6	Transcriptional factor of Th2 Cells	Directly cleaves	Results in more	[51]
IL-2	T cell activation cytokine	Leads to synthesis of cytokine	Results in less	[41]
CD25	T cell activation cytokine	Leads to synthesis of cytokine	Results in less	[41]
CCR2	T cell pro-migration chemokine	Increased migration toward CCL2	Results in less migration toward CCL2	[42, 44]
IFN-γ	Th1 inflammatory cytokine	Leads to synthesis of cytokine	Results in less	[46]
IkB alpha	Inhibitor of NFκB, marker of unactivated cells	Degrades	Results in more	[45]
IL-12	Th1 inflammatory cytokine	Leads to synthesis of cytokines	Results in less	[59]
IL-17	Th17 inflammatory cytokine	Leads to synthesis of cytokines	Results in less	[59]
IL-23	Th17 inflammatory cytokine	Leads to synthesis of cytokines	Results in less	[59]
IDO	Immune inhibitory	Degrades	Results in more	[59]

substrate of calpain, and thus, it can be construed that calpain's actions on STAT6 can be directly involved in T cell chemotaxis.

Another avenue of calpain influence on the immune system regulation is through the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is known to degrade the essential amino acid tryptophan and other downstream metabolites that suppress effector T cell function and favor the differentiation of regulatory T cells [59, 84, 85]. IDO is also significantly expressed in a variety of immune cell types, including APCs, and is associated with many aspects of immunopathology. These findings suggest that IDO is a possible molecular target for therapeutic intervention of inflammatory autoimmune responses particularly by calpain inhibition. Recent studies showed that calpain inhibition elevated IDO gene expression in MS PBMCs, which was markedly decreased upon calpain activation [59]. All of these findings were summarized in Table 1.2.

5 Calpain as a Therapeutic Target in MS

Calpain obviously plays a strong role directly and indirectly in the progression and retention of inflammatory T cell responses, which in turn lead to neurodegenerative pathology in the CNS. Calpain has also been shown to play crucial roles in lymphocyte activation, migration, cell death, cell cycle progression, cell fusion, and many other important processes in the host.

Accumulative evidence suggested that calpain inhibition could be a viable treatment avenue in inflammatory autoimmune and neurodegenerative diseases. Currently there are no calpain inhibitors approved for clinical use in neurodegenerative diseases, but some are being researched in the animal models of these diseases,

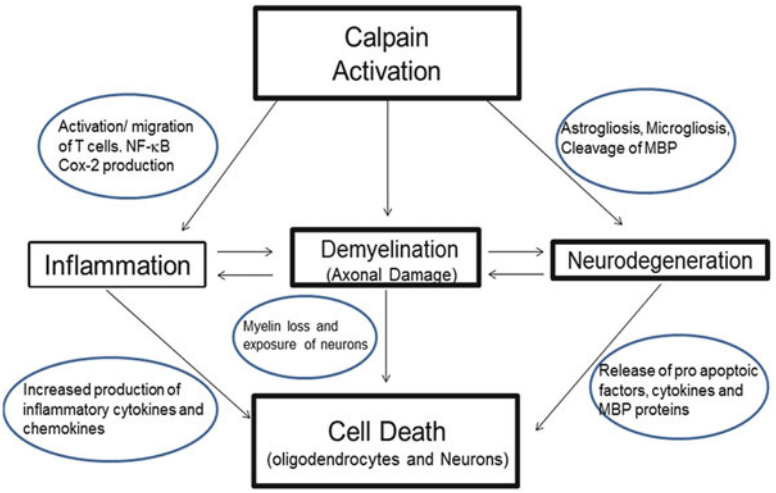

Fig. 1.1 A schematic representation of calpain's role in inflammation, demethylation, and cell death

specifically MS [45, 60, 86]. For similar mechanistic reasons, calpain inhibition therapy has also been a topic of interest in PD, ALS, and AD [2, 31, 32, 36, 87]. Currently, there are new orally available calpain inhibitors that may possibly be used in future clinical trials for treating MS and other neurodegenerative diseases [88]. The following diagram shows potential roles of calpain in inflammation, demyelination, and neuronal/glial cell death (Fig. 1.1). Obviously, successful inhibition of calpain could significantly inhibit inflammation, demyelination, neurodegeneration, and thus pathogenesis in MS and other diseases of the CNS. Calpain so far seems to be an intractable target. Development of water-soluble and cell-permeable calpain inhibitor is not as easy as previously thought. However, remarkable progress has been reported in this area, and all these endeavors are expected to produce clinically useful calpain inhibitors for treatment of demyelinative and neurodegenerative diseases in humans in the near future.

6 Conclusions

Neurodegenerative diseases are incurable, complex, and not completely understood. In MS, the role of the immune system in the pathobiology of the disease is well explored, but not completely understood. Calpain's role in MS and other neurodegenerative disease processes is an avenue of continuing research. But it is clear that calpain has a vital role in the progression and stabilization of inflammation involved in neurodegeneration in MS. The relationship is as complex as the chicken and the egg, which comes first? And do you need both to have both? Based on recent

progress, it is clear that calpain is both involved in activation and migration of immune cells, which then leads to neurodegeneration and activation of the immune system, creating a vicious cycle in progression of disease. This type of pattern has been hypothesized in other related neurodegenerative diseases such as PD and AD, but it is not well documented. Overall, calpain is a pathogenic protease that can be targeted but not without consequences. As with many treatments, a smoking gun is unlikely, but calpain inhibition is a promising target to help inhibit both the inflammatory and the neurodegenerative arms of multiple syndromes.

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Chapter 2
Calpain Interactions with the Protein Phosphatase Calcineurin in Neurodegeneration

Christopher M. Norris

Abstract Dysregulation of intracellular Ca^{2+} is a major cause of neurologic dysfunction and likely plays an important role in the pathophysiology of numerous acute and chronic neurodegenerative conditions. The Ca^{2+}-dependent protease, calpain, and the Ca^{2+}/calmodulin (Ca^{2+}/CaM)-dependent protein phosphatase, calcineurin, are primary effectors of multiple deleterious functions arising from altered Ca^{2+} handling. Increasing evidence suggests that the calpain-dependent, irreversible conversion of calcineurin to a constitutively active phosphatase occurs in intact cellular systems as a result of injury and disease. In this chapter, a brief overview of calpain and calcineurin functions in nervous tissue is given, followed by a more in-depth discussion of calpain/calcineurin interactions in vitro and in vivo. Particular emphasis is placed on recent studies that have identified calpain proteolysis of calcineurin as a key step in neurodegeneration associated with acute neurologic insults as well as chronic terminal diseases, like Alzheimer's.

Keywords Protease • Phosphatase • Calcium • Ischemia • Alzheimer's • Neurodegeneration • Dementia

1 Introduction

The calcium ion (Ca^{2+}) is a ubiquitous messenger involved in countless, diverse cellular functions. In biological systems, Ca^{2+} leads a dual existence of sorts. On the one hand, Ca^{2+} is essential for life. In the nervous system, the release of neurotransmitters, remodeling of growth cones and dendritic spines in response to extracellular

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stimuli, activation and termination of transcriptional programs at the proper stages of development, and many, many other cellular functions depend critically on Ca^{2+}. On the other hand, Ca^{2+} is also commonly the prelude to cellular degeneration and death. Studies in the early mid-1980s suggested that neuronal Ca^{2+} regulation is disrupted during normal aging, leading to deleterious changes in neuronal excitability and plasticity [1–4]. Around the same time, cytosolic Ca^{2+} overload was demonstrated to be one of the primary mechanisms of neuronal death following excitotoxic insults [5–7]. These findings led to the hypothesis that Ca^{2+} dysregulation is a general mechanism for neurologic dysfunction and/or neurodegeneration associated with aging, stroke, acute brain injury, and progressive neurodegenerative diseases [1–3, 8–13]. Today, the Ca^{2+} hypothesis remains viable but has evolved in important ways to emphasize selective changes in discrete Ca^{2+} signaling mechanisms in different cell types and/or in different disorders (e.g., see [14–23]).

Of the numerous Ca^{2+}-sensitive proteins and enzymes, the protease calpain and the phosphatase calcineurin have emerged as two of the most common effectors of Ca^{2+}-induced dysfunction and degeneration. Interestingly, calpains and calcineurin are present in many of the same subcellular domains and exhibit similarly high levels of activity following many of the same types of insults. Comparable changes in the expression/activity of calpains and calcineurin have also been observed in several distinct neurodegenerative diseases and/or conditions, while pharmacologic and genetic inhibitors of these enzymes ameliorate deleterious changes in common biomarkers. Taken together, the evidence suggests that calpain/calcineurin interactions may be a fundamental neurodegenerative mechanism and an opportune target for future therapeutic strategies. The purpose of this chapter is to provide a brief review of calpains and calcineurin and their roles in neurologic dysfunction, with particular emphasis placed on calcineurin signaling and the ramifications of calcineurin proteolysis in human neurodegenerative disease. Outstanding comprehensive reviews of the biochemistry and regulation of each of these Ca^{2+}-dependent enzymes (as well as historical backgrounds) can be found here [24–27] (for calpain) and here [28–30] (for calcineurin).

2 Calpain

2.1 Calpain Structure

Calpains are a family of intracellular, nonlysosomal cysteine proteases that belong to the papain superfamily of proteases. Calpains are regulatory proteases found in most mammalian species and show varying degrees of sensitivity to fluctuating Ca^{2+} concentrations. Calpain proteins are heterodimers consisting of a large (~80 kDa) catalytic subunit and a smaller (28 kDa) regulatory subunit derived from different genes (Fig. 2.1). The catalytic subunit is made up of four distinct domains (I–IV), including a regulatory Ca^{2+} binding domain (IV) containing five EF-hand motifs; a

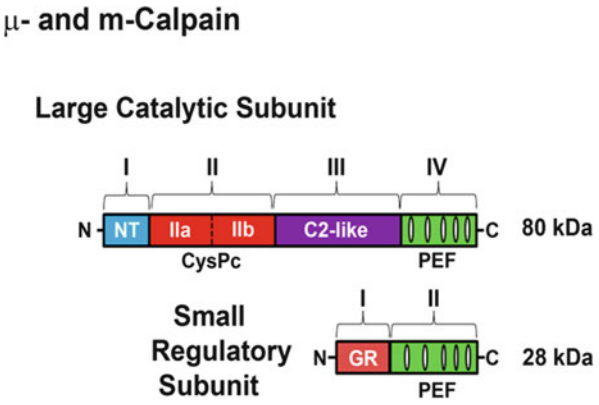

Fig. 2.1 **μ**- and m-calpain subunits. Schematic illustration of the structure of the typical calpain (i.e., **μ**- and m-calpain) catalytic and regulatory subunit. *NT* N-terminus region, *CysPC* calpain-like cysteine protease core region, *PEF* pentameric EF-hand domain, *GR* glycine-rich domain. See text for a description of these subunits and their domains

"C2-like" domain (III) that includes Ca^{2+} and phospholipid binding sites; a catalytic domain (II) that consists of at least two Ca^{2+} binding sites along with two proteolytic core subdomains (IIa and IIb) that come together upon Ca^{2+} binding to form a functional cysteine protease core domain (CysPc); and an N-terminal domain (I) that may be autolyzed upon activation of the holoenzyme. Though relatively poorly understood, the regulatory subunit appears to remain associated with the catalytic subunit during activation [31] (contrary to earlier findings, e.g., see [32]) and is essential for maintaining the stability of the catalytic subunit in vivo. The regulatory subunit contains two domains (I and II): a glycine-rich domain (I) believed to regulate calpain interactions with membranes and/or membrane-related proteins and a Ca^{2+} binding domain (II) that includes five EF-hand motifs.

In humans, there are more than a dozen calpain (or calpain-like) catalytic subunit genes (termed *CAPN1*, *CAPN2*, *CAPN3*, …), while there are at least two distinct regulatory subunit genes (*CAPNS1* and *CAPNS2*). These genes are expressed in most cell types and/or tissues, though some genes can show tissue-type specific expression. The best characterized calpain holoenzymes consist of CAPN1 or CAPN2 and are commonly referred to as μ- and m-calpains, respectively (or calpains 1 and 2). CAPN1/μ-calpain is activated by micromolar concentrations of Ca^{2+}in vitro, while CAPN2/m-calpain is activated when Ca^{2+} is in the millimolar range. In addition to Ca^{2+}, the catalytic activity of calpain is also held in check by endogenous proteins called calpastatins. These proteins very specifically suppress the activity of μ- and m-calpains and are the only known endogenous proteins to serve this function. Unlike the calpain catalytic and regulatory subunits, there is only one human gene for calpastatin (*CAST*), though splicing variations can give rise to many distinct protein products [25, 27].

While Ca^{2+} is clearly the critical activating factor for calpains—binding to EF-hand motifs on both subunits as well as to multiple other binding sites within the catalytic and C2-like domains—the precise biochemical mechanisms/interactions that couple Ca^{2+} binding to increased protease activity have remained surprisingly elusive. One of the central issues is that concentrations of Ca^{2+} required for calpain activation in vitro seem too high to be physiologically relevant, since cytosolic Ca^{2+}

concentrations are not likely to rise into the high micromolar range. This has led to much speculation that an additional biochemical event—such as autolysis, subunit dissociation, and/or the binding of some other cofactor—is necessary for calpain activation [24].Of these possibilities, Ca^{2+}-dependent autolysis of the N-terminal region of the large calpain subunit has been widely accepted as an essential step in calpain activation. Early studies showed that autolysis reduces the Ca^{2+} concentration for activation of both μ and m-calpain in vitro [25]. However, many later studies have shown that autolysis is not necessary for activation in vivo, though it may still play an important regulatory function (for in-depth discussions, see [24, 25, 27]). Conversely, it has been suggested (i.e., [24]) that Ca^{2+} elevations in cellular microdomains (e.g., in postsynaptic spines and/or immediately adjacent to Ca^{2+} channels) may indeed be high enough to meet calpain activation requirements without the need of autolysis. In this case, calpain activation would only be brief due to the rapid drop in Ca^{2+} concentration in these microdomains and/or due to inhibition by calpastatins. Prolonged calpain activation would therefore only occur under pathologic conditions in which Ca^{2+} levels are chronically elevated and/or calpastatin function/expression is downregulated [24].

2.2 Calpain Functions in Nervous Tissue

Calpains are highly expressed in nervous tissue and have long been recognized for their important roles in modulating cellular structure and function. Numerous substrates for calpains have been identified and include cytoskeletal proteins, membrane receptors, ion channels, protein kinases, protein phosphatases (as discussed later), other proteases, and many other protein targets. Consequently, calpains are believed to take part in numerous and diverse signaling cascades. For a comprehensive list of calpain substrates and description of calpain functions, see [25, 27]. One of the earliest proposed functions of calpain in nervous system was the rapid, activity-dependent degradation of cytoskeletal proteins, such as spectrin, leading to the structural reorganization of dendritic spines and other neuronal processes [33–37]. Subsequently, calpain was also shown to target key glutamatergic receptors [38–42], as well as the proteins that modulate glutamate receptor expression/function including membrane-anchoring proteins [43–45] and protein kinases and phosphatases [46–48]. Calpain-mediated cleavage of protein kinases, such as protein kinase C, and phosphatases, such as CN, results in high levels of kinase/phosphatase activity that can persist long after the restoration of basal Ca^{2+} levels [46, 48]. The reorganization of the dendritic cytoskeleton, along with the generation of so-called memory molecules by calpain, may be critical to the expression and maintenance of long-term synaptic potentiation (LTP) and other forms of synaptic plasticity involved in neurodevelopment and cognition.

In addition to these beneficial functions, calpains also mediate numerous deleterious functions and are commonly implicated in neurodegenerative processes

associated with severe Ca^{2+} dysregulation [26]. High levels of calpain expression/activity (or downregulation of calpastatins) are consistently found in primary neural cultures exposed to ischemia/hypoxia, glutamate/kainate, amyloid-β peptides (Aβ), and numerous other neurotoxic insults (e.g., [36, 48–51]). In intact animal models, elevated forms of activated calpain (both μ and m) have been reported in the brain within hours following injury due to carotid artery occlusion [52, 53], glutamate/kainate insult [48, 54], controlled cortical impact [55], or fluid percussion [56, 57]. In many of these same studies, calpain inhibitors exhibited strong neuroprotective and/or nootropic properties. Aberrant calpain activation also appears to be an excellent biomarker for chronic, progressive neurodegenerative disorders including Alzheimer's disease (AD) [50, 58, 59], Parkinson's disease [60], multiple sclerosis [61], and glaucoma [62, 63], to name a few. Moreover, similar to acute injury models, inhibition of calpains using pharmacologic or genetic approaches generally ameliorates functional and pathologic changes in cell culture and/or animal models of these disorders [50, 64–69].

The cellular mechanisms for calpain-mediated neurotoxicity can be difficult to pin down and may vary considerably depending on the brain region/cell type investigated or on the nature of the injury or disease state. One of the difficulties is that calpain interacts with so many different target proteins linked to cell death and degeneration. Indeed, distinct proapoptotic factors including caspase-3, BAX, apoptosis-inducing factor, and several others are directly targeted by calpains and have been proposed to mediate the deleterious actions of calpains in nervous tissue [52, 70–74]. Another complicating issue is that calpains interact extensively with other Ca^{2+} signaling mechanisms, many of which play a critical role in regulating Ca^{2+} homeostasis. For instance, several kinds of Ca^{2+} channels and pumps responsible for shuttling Ca^{2+} from the cytosol to the extracellular space, or into intracellular stores, are degraded by calpains leading to elevated cytosolic Ca^{2+} levels [75–77]. Calpains also appear to be involved in the cleavage of the pore-forming subunit of the L-type voltage-sensitive Ca^{2+} channel to a smaller, higher-conductance channel [78]. Each, or all of these changes, would be expected to exacerbate Ca^{2+} dysregulation, promoting further calpain activation and/or hyperactivation of other Ca^{2+}-dependent enzymes.

Herein lies an additional complication: Does hyperactivation of other Ca^{2+}-dependent enzymes following injury arise simply from increased Ca^{2+} binding or from direct calpain-mediated proteolysis? High activity levels resulting from increased Ca^{2+} binding is perhaps less troublesome because an ebb in cytosolic Ca^{2+} levels would be expected to result in a corresponding decrease in enzyme activity. Calpain-dependent activation, on the other hand, would appear to be a far greater threat to the cell because the resulting proteolytic enzyme fragments are generally uncoupled from their normal regulatory mechanisms and prone to dangerously high and enduring activity levels. The Ca^{2+}-/calmodulin-dependent protein phosphatase, calcineurin, is one potential target of calpain. The following sections will discuss the structure and function of calcineurin, with particular emphasis on recent studies that have uncovered important calpain/calcineurin interactions in neurodegeneration and disease.

3 Calcineurin

3.1 Calcineurin Structure

Calcineurin, or protein phosphatase 3 (PPP3, formerly protein phosphatase 2b), is a nearly ubiquitously expressed serine/threonine protein phosphatase and the only known phosphatase to exhibit direct regulation by Ca^{2+}/CaM. Calcineurin is typically found in intact cells as a heterodimer (see Fig. 2.2a) consisting of a catalytic subunit (CN A or PPP3C, ~61 kDa) and a smaller regulatory subunit (CN B or

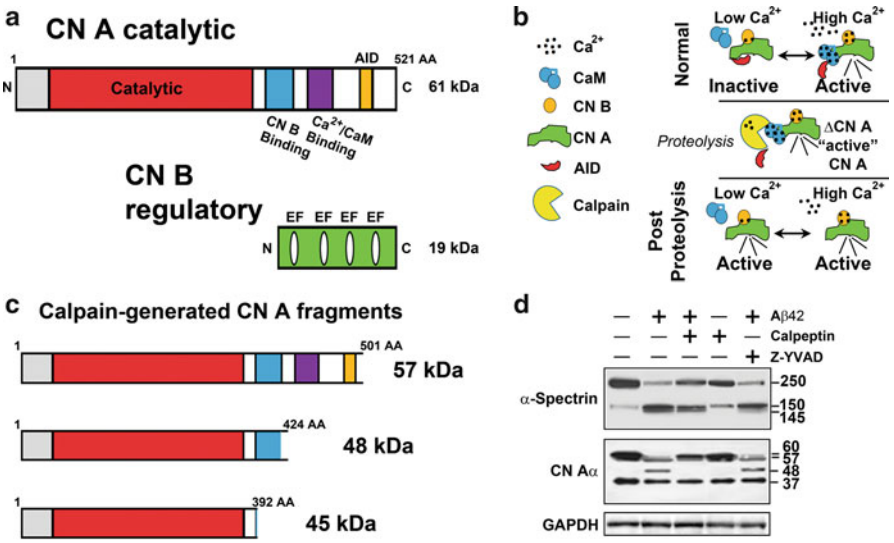

Fig. 2.2 Calcineurin and its regulation by calpain proteolysis. (**a**) Schematic illustration of the structure of the CN A catalytic and CN B regulatory subunits. See text for a description of these subunits and their domains. (**b**) Cartoon illustration of the regulation of CN A activity by Ca^{2+}/CaM, the CN A AID, and calpain proteolysis. Under normal conditions when intracellular Ca^{2+} levels are low, CN A activity is held in check by the CN A AID. When Ca^{2+} levels rise, Ca^{2+}/CaM binds to the CN A subunit displacing the AID from the catalytic domain, resulting in high levels of phosphatase activity. Under abnormal conditions, like severe Ca^{2+} dysregulation, the protease calpain cleaves CN A at several locations near the C terminus, thus removing the AID. Without the AID, the CN A catalytic domain is no longer occluded resulting in high levels of phosphatase activity, even after the local Ca^{2+} concentration falls to basal levels. (**c**) Schematic illustration of the 57, 48, and 45 kDa CN A fragments generated by calpain-dependent cleavage, as demonstrated by Wu et al. [48]. (**d**) Western blot showing CN A**α** proteolysis to 57 and 48 kDa fragments in primary hippocampal neural cultures 24 h after addition of neurotoxic amyloid-**β** peptides (A**β**). The lower 37 kDa band in the CN A**α** blot was not sensitive to local Ca^{2+} levels nor to the addition of calpain, suggesting it represents a nonspecific band, a calpain-insensitive fragment, or an alternative splice variant. Proteolytic breakdown of the calpain substrate, a-spectrin, occurred in parallel with CN A proteolysis. Blockade of calpain activity with calpeptin prevented CN A**α** proteolysis to 57 and 48 kDa fragments. However, a specific caspase 1 inhibitor (Z-YVAD-FMK) was without effect. Blot shown in panel **d** was from Mohmmad Abdul et al. [50] and used with permission

PPP3R, ~19 kDa). The catalytic subunit contains the catalytic core region, CN B binding domain, a Ca^{2+}/calmodulin binding domain, and an autoinhibitory domain (AID) near the C-terminus that lies over the cleft of the catalytic domain and precludes substrate binding when cytosolic Ca^{2+} levels are low [79]. The regulatory CN B subunit is a calmodulin-like Ca^{2+} binding protein with four EF-hand motifs, two of which show very high affinity for Ca^{2+} and are likely occupied at normal resting Ca^{2+} levels (<10 nM). Ca^{2+} binding to CN B is thought to increase the physical association between CN A and CN B and appears to promote low levels of catalytic activity [80]. Early in vitro experiments suggested that the physical association between CN A and B could only be disrupted under supraphysiologic conditions, such as protein denaturation [80]. However, recent investigations on intact primary neurons indicate an increased physical association between catalytic and regulatory subunits in response to neurotoxic stimuli [81], suggesting the possibility that these subunits are not always bound to one another in vivo. These observations are consistent with other work showing that the CN B subunit can associate with and regulate specific target proteins in a CN A-independent manner [82–85]. Additional distinct roles of the CN B subunit in neurologic function are largely unknown but will likely be forthcoming in the next few years.

There are two major CN A isoforms expressed in brain (CN Aαor PPP3CA and CN Aβ or PPP3CB), of which, the CN Aα isoform is the most abundant [86]. A "testis"-specific CN A isoform (CN Aγ or PPP3CC) is also expressed in nervous tissue but at comparatively much lower levels. At least two regulatory CN B isoforms (CN Bα and CN Bβ or PPP3R1 and PPP3R2) have been characterized. CN Bα exhibits similar expression patterns as CN Aα and CN Aβ, while CN Bβ is testes specific [30]. Although isoform-specific differences in tissue distribution and cellular function have been well characterized outside of the brain, much less is known about the expressional/functional differences of CN Aα and CN Aβ inside the brain. However, as discussed in a later section, studies from our lab have shown that CN Aα is the isoform that exhibits the most striking changes and is most susceptible to calpain-mediated proteolysis during the progression of Alzheimer's disease (AD) [14, 50, 87].

3.2 Calcineurin Function in Nervous Tissue

Calcineurin is perhaps best known and characterized in T and B lymphocytes where it coordinates transcriptional programs involved in lymphocyte activation, cytokine production, and lymphocyte anergy [88, 89]. However, calcineurin is most abundantly expressed in brain, especially in regions like the hippocampus [90], which is important for learning and memory and highly susceptible to age-related neurodegenerative disease [91, 92]. In fact, calcineurin was originally named for its high abundance in nervous tissue and its critical dependence on Ca^{2+} [93]. In healthy brain tissue, calcineurin is primarily enriched in neurons [86, 94] where it is highly expressed in dendrites and postsynaptic spines. Glial cells, in contrast, appear to express very low levels of calcineurin under normal conditions [94]. However, after injury, or during aging and age-related neurodegenerative disease, activated glial

cells (especially astrocytes) can label very intensely for the presence of calcineurin [87, 95–97]. As discussed below, the major functions of calcineurin are likely very different in neurons and glia.

While not nearly as promiscuous as other related serine/threonine phosphatases (e.g., protein phosphatase 1 and 2a), calcineurin nevertheless acts on a broad range of substrates; many of which are directly involved in the structural and functional regulation of synapses. In neurons, calcineurin has long been known to dephosphorylate a host of cytoskeletal proteins involved in the dynamic modulation of dendritic spines, including MAP2b and cofilin [98–100]. Calcineurin has also been shown to modulate (i.e., reduce) glutamate receptor activity and/or surface expression via direct dephosphorylation of glutamate receptor subunits [101, 102] and/or through indirect activation of protein phosphatase 1 or other accessory proteins [103, 104]. Through these interactions, neuronal calcineurin is widely believed to play an essential role in mediating long-term synaptic depression (LTD) [105].

In addition to its close functional association with the cytoskeleton, calcineurin is also one of the primary mechanisms for coupling fluctuations in cytosolic Ca^{2+} to changes in gene expression. In neurons, calcineurin-dependent dephosphorylation of transcription factors, such as the cyclic AMP response element binding protein, is widely believed to underlie long-term reductions in key synaptic proteins involved in activity-dependent plasticity and cognitive function [106, 107]. Among the numerous transcription factors that exhibit sensitivity to calcineurin, perhaps none are as closely associated with calcineurin or are more important to overall calcineurin signaling than *n*uclear *f*actor of *a*ctivated *T* cells (NFATs). These transcriptions typically reside in the cytosol in a heavily phosphorylated state when the cell is at rest and Ca^{2+} levels are low. However, with cellular activation and elevated Ca^{2+}, NFATs are bound tightly by calcineurin and dephosphorylated. This event leads to the transport of NFATs into the nucleus, where they remain until they are re-phosphorylated by a variety of "NFAT kinases" and transported back to the cytosol.

NFATs are clearly best known for their role in coupling calcineurin activation in lymphocytes to the transcriptional induction of numerous cytokines and immune/inflammatory mediators [108]. While much less is known about NFAT functions in neural cells, the existing data suggest these calcineurin-dependent factors play unique roles in different cell types and are likely key players in neurologic dysfunction and disease [14]. In neurons, activation of NFATs leads to the upregulation of proteins involved in Ca^{2+} signaling and homeostasis including inositol type 3 receptors [109, 110]. In glial cells, NFATs play a critical role in the induction of immune/inflammatory signaling factors, including a number of cytokines [111–113]. These functions are similar to that observed in T and B lymphocytes, as well as other peripheral immune/inflammatory cells [108]. Interestingly, glial-specific excitatory amino acid transporters (EAATs), particularly Glt-1/EAAT2 (i.e., the major glutamate transporter in the brain), also show high sensitivity to calcineurin/NFAT activity [87, 113]. However, unlike many cytokine factors, Glt-1/EAAT2 appears to be downregulated by calcineurin/NFAT in response to inflammatory and/or neurotoxic insults. Thus, in addition to its immune/inflammatory functions, the glial calcineurin/NFAT pathway also appears to be critical for regulating glutamate homeostasis.

Similar to calpain, calcineurin is often a "usual suspect" when it comes to neurodegeneration associated with Ca^{2+} dysregulation (e.g., see [114]). Through its actions on cytoskeletal proteins and glutamate receptors, neuronal calcineurin has been shown to mediate dendritic spine retraction and/or impaired synaptic function in response to a variety of injurious stimuli (e.g., see [115–118]). Aberrant calcineurin activity has also been linked to cell death cascades through the direct dephosphorylation of proapoptotic factors, such as BAD [119, 120], or through the transcriptional induction of other proapoptotic proteins such as the Fas ligand (FasL) [121]. In glial cells, calcineurin activity induces the expression of numerous proinflammatory mediators [97, 111–113, 122] and promotes glutamate dysregulation and excitotoxicity through activation of NFAT transcription factors [87, 113]. Finally, as alluded to above, the transcriptional and posttranslational modulation of Ca^{2+} channels and pumps by calcineurin may be a key mechanism for promoting and maintaining neuronal Ca^{2+} dysregulation in aging and age-related neurodegenerative diseases [109, 123–125].

Consistent with these observations, elevated calcineurin activity/signaling is often observed following acute injury to nervous tissue [126, 127] or during CNS aging [128] and/or disease [58, 87, 118, 129, 130]. In aged animals and transgenic animal models of AD, increased calcineurin activity/expression is linked to synaptic dysfunction [131, 132], dendritic spine irregularities [133, 134], elevated neuroinflammation [97, 135, 136], and cognitive decline [128, 129]. In human brain tissue, calcineurin/NFAT signaling is elevated during the emergence of clinical symptoms associated with AD [50, 87] and continues to increase with the progression of amyloid pathology and dementia [87]. Suppression of calcineurin activity using commercially available immunosuppressants, or through genetic manipulations, provides strong neuroprotection in many experimental models of acute injury [137, 138]. Glial activation and neuroinflammation in animal models of AD or stroke are also blunted by calcineurin inhibitors (or NFAT inhibitors) [132, 135, 136], as are numerous other biomarkers including synaptic dysfunction/degeneration [132–134, 139], amyloid pathology [132, 140], and cognitive impairment[129, 132, 141, 142]. However, despite all this evidence implicating a causative role of calcineurin in neurodegeneration, it deserves noting that other studies have shown that calcineurin can also activate cell survival pathways in neurons [143] and help resolve harmful neuroinflammatory signaling in glial cells under certain conditions [112, 144]. The precise conditions and cellular mechanisms that transform calcineurin from cellular protector to killer remain unclear and will require further investigation.

3.3 Mechanisms for Calcineurin Regulation

Clearly, changes in the activation state of calcineurin can spell the difference between optimal physiologic function and neurodegeneration. Consequently, multiple mechanisms are available for keeping calcineurin activity in check and/or for directing calcineurin to its proper substrates [29]. Anchoring proteins, such as

A-kinase anchoring proteins (AKAPs), FK-506 binding protein 12 (FKBP12), and postsynaptic density 95 (PSD-95), can help sequester calcineurin to the membrane, thus limiting the access of calcineurin to cytosolic substrates. Usually, calcineurin is anchored along with other protein kinases to provide rapid and dynamic regulation over nearby membrane channels and pumps [145]. Moreover, membrane anchoring of calcineurin close juxtaposition to ligand and/or voltage-gated Ca^{2+} ionophores (e.g., NMDA receptors and L-type Ca^{2+} channels) allows calcineurin to respond rapidly to, and/or provide feedback regulation over, Ca^{2+} influx. In addition to sequestration mechanisms, calcineurin activity is also directly modulated by a handful of endogenous proteins, the most widely studied of which are cabins (*ca*lcineurin *bin*ding) and RCANs ((*R*egulator of *Ca*lc*in*eurin) *D*own *S*yndrome *C*ritical *R*egion) [146]. These modulating proteins have gone by several different names (i.e., cabins–cains; RCANs–MCIPs and DSCRs) depending on the species investigated. Cabins and RCANs are highly expressed in brain and exhibit distribution patterns similar to calcineurin. While both proteins can bind to and inhibit calcineurin activity in vitro and in vivo (especially when inhibitors are overexpressed), RCANs may also facilitate calcineurin activity at physiologic levels, depending on the presence of other accessory proteins, as well as the phosphorylation state of RCAN [147, 148]. Finally, similar to calpains, calcineurin shows high redox sensitivity. Oxidation of the Fe^{2+}–Zn^{2+} binuclear center in the calcineurin A catalytic domain, due to elevated superoxide and peroxide levels, is typically associated with reduced calcineurin activity, and a number of antioxidants have been shown to preserve calcineurin function [149].

3.4 The Importance of the Calcineurin AID

Among the numerous mechanisms for calcineurin regulation, none are more important than Ca^{2+}/calmodulin and the calcineurin AID (Fig. 2.2a). Indeed, the interaction between these mechanisms is what permits discrete and high-fidelity coupling of calcineurin activity to local Ca^{2+} gradients [150]. Calcineurin is exquisitely sensitive to Ca^{2+} and has a Kd to Ca^{2+}-saturated CaM in the picomolar range (28–100 pM) [151]. This value is far lower than that for other CaM-regulated enzymes, including the CaM kinases [152]. When Ca^{2+} is very low, calcineurin phosphatase activity is allosterically blocked by the AID (Fig. 2.2) [150]. Binding of Ca^{2+} to the four EF-hand motifs of the CN B subunit during elevations in cellular Ca^{2+} triggers a conformational change in the CN A subunit, exposing the Ca^{2+}/CaM-binding site. Though Ca^{2+}/CN B can, by itself, stimulate low levels of phosphatase activity and modulate the affinity of CN A for substrate phosphoproteins [80], it is the exposure of the calmodulin binding domain and subsequent binding of Ca^{2+}/CaM that fully unleashes catalytic activity. Indeed, this binding event physically displaces the AID from the CN A catalytic core region [153], where it remains fully accessible to phosphosubstrates for as long as Ca^{2+}/CaM is bound. Subsequently, when Ca^{2+} levels in the cell fall, allosteric inhibition of the catalytic domain by the AID is rapidly

restored as Ca^{2+}/calmodulin dissociates from calcineurin. Without the AID, calcineurin loses much of its sensitivity to Ca^{2+} and, as discussed below, becomes a highly disruptive constitutively active phosphatase (Fig. 2.1) [48].

3.5 Early In Vitro Evidence for Proteolysis of the Calcineurin AID

It has been known since the early to mid-1980s that calcineurin is susceptible to proteolysis in vitro. Early studies showed that exposure of the CN A/CN B holoenzyme to trypsin or chymotrypsin produced an enzyme complex containing the CN B subunit and a truncated ~40–46 kDa CN A subunit [154–156]. This proteolized CN A fragment retained physical interactions with the CN B subunit, but was incapable of binding Ca^{2+}/CaM, and did not require CaM for high enzymatic activity. Application of trypsin/chymotrypsin to Ca^{2+}/CaM-bound CN A resulted in slower rates of proteolysis with the additional appearance of 57, 55, and 54 kDa CN A fragments, suggesting that Ca^{2+}/CaM binding offers some degree of protection from proteolysis. Interestingly, these proteolytic fragments also retained the capacity to bind to Ca^{2+}/CaM. In subsequent studies, CN A was shown to undergo proteolysis in vitro by exposure to Ca^{2+} and calpain [157, 158]. Similar to earlier work, calpain protein was applied in vitro at different Ca^{2+} concentrations, in the presence or absence of CaM. Again, proteolysis of CN A did not affect interactions with the regulatory CN B subunit but did produce high levels of phosphatase activity independent of Ca^{2+}/CaM. However, unlike earlier studies with trypsin, the presence of Ca^{2+}/calmodulin did not protect calcineurin from calpain and instead hastened the rate of proteolytic cleavage. Under these conditions, calpain exposure produced CN A fragments of 55 and 48 kDa, which retained some capacity to bind to and/or respond to Ca^{2+}/CaM [157]. These results suggest that calpain-mediated proteolysis greatly reduces but does not fully eliminate the responsiveness of CN A to Ca^{2+}. In contrast to CN A, the regulatory CN B subunit does not appear to be vulnerable to proteolysis.

3.6 Effects of Overexpressing Truncated CN A in Intact Cell Systems

The proteolysis studies discussed above provided the first evidence that calcineurin activity is held in check by an AID located near the CaM binding domain in the C-terminus of the CN A subunit. Later work confirmed the existence of a C-terminus AID; demonstrated that the AID physically obscures the CN A catalytic core when Ca^{2+}/CaM is absent; and showed that the binding of Ca^{2+}/CaM to CN A displaces the AID from the catalytic region. Studies on calcineurin proteolysis also led to the development of cDNA clones that encode an ~48 kDa C-terminus truncated CN A fragment (ΔCN) that retains CN B binding properties but exhibits high levels of

activity in the absence of Ca^{2+}/CaM. The use of ΔCN, combined with newly developing gene delivery techniques, provided a convenient way to produce elevated calcineurin signaling without stimulating key cellular receptors and/or indiscriminately raising intracellular Ca^{2+} levels, which, in turn, greatly increased our understanding of calcineurin's role(s) in cellular physiology. A consistent theme to emerge from ΔCN overexpression studies is that unchecked calcineurin activity, whether in peripheral tissues or in brain, leads to severe cellular dysfunction and/or death. In primary neuron cultures, ΔCN has been shown to induce numerous detrimental outcomes including postsynaptic spine retraction, dendritic atrophy, and/or apoptosis [118, 119, 159]. In astrocytes, ΔCN was found to trigger cellular hypertrophy and induce numerous genes involved in immune/inflammatory signaling [97]. In intact rodents, forebrain expression of ΔCN caused deficits in LTP and spatial memory [160–162]. Interestingly, these alterations are very similar to those observed in animal models of aging, injury, and or neurodegenerative disease in which endogenous calcineurin activity is aberrantly high.

4 Calpain Proteolysis of Calcineurin in Intact Nervous Tissue

Given the early evidence showing that calcineurin is highly susceptible to calpain-mediated proteolysis in vitro, it is somewhat surprising that the first demonstrations of calcineurin proteolysis in intact cellular systems were not provided until relatively recently [48]. It's possible that the presence of proteolyzed calcineurin in neurologic disease and other disorders escaped detection because the majority of commercially available calcineurin antibodies target the CN A carboxy-terminus which is, of course, missing in smaller proteolyzed calcineurin fragments. Regardless, in the early to mid-2000s, proteolysis of calcineurin was shown to occur in both heart and neural tissue under pathologic conditions [48, 163, 164]. Western blots of CN A (using a primary antibody targeting amino acid residues 264–283) performed on primary neuronal cultures exposed to an excitotoxic glutamate insult revealed at least three truncated CN A products in conjunction with an elevation in Ca^{2+}/CaM-independent calcineurin activity [48]. In the same study, a similar banding pattern for CN A was observed in Western blots of whole hippocampal lysates from mice treated with a kainic acid insult. However, when nervous tissue was treated with distinct calpain inhibitors prior to the administration of glutamate/kainite, CN A appeared as a single 60 kDa band. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ProTOF/MS), it was shown that calpains induce cleavage of the CN A subunit in vitro at amino acid residues 392, 424, and 501 resulting in cleavage products of 45, 48, and 57 kDa, respectively (see Fig. 2.2c). Note that these bands corresponded very closely to the CN A truncation products observed in cell cultures following excitotoxic injury. These results demonstrated that the majority of the AID remains intact in the 57 kDa CN A fragment but is excluded from the 45 and 48 kDa fragments. In addition to lacking the AID, the 45 kDa fragment (but not the 48 kDa fragment) is also devoid of

the Ca^{2+}/CaM binding domain. The work by Wu et al. [48] not only showed that calcineurin undergoes calpain-mediated proteolysis in intact cellular systems but that it also likely plays a significant role in driving pathologic outcomes. Consistent with this study, investigations across multiple laboratories have discovered calpain-dependent proteolysis in several distinct neurologic injuries and disease states including Alzheimer's disease, ischemia, and glaucoma. The major findings of these studies are highlighted in Table 2.1 and discussed further below.

4.1 Calcineurin Proteolysis in Alzheimer's Disease

Alzheimer's disease (AD) is a devastating and terminal neurodegenerative disorder leading to profound cognitive deficits, personality alterations, and the eventual loss of most all daily life skills. The pathologic hallmarks of AD are extracellular Aβ plaques, intracellular neurofibrillary tangles, and extensive neuronal degeneration and neuronal death [165]. There is also ample evidence implicating neuroinflammation [166, 167], Ca^{2+} dysregulation [16, 20], and excitotoxic mechanisms [168, 169] in the pathophysiology of the disorder and, by corollary, calpain, and calcineurin signaling pathways, as well.

In 2005, it was demonstrated that changes in calpain and calcineurin during AD are extensively and directly intertwined, providing a novel mechanism for AD-related neurologic dysfunction and degeneration [58]. In this study by Liu et al., levels of the 57 kDa CN A truncation product were detected at significantly higher levels in medial temporal cortex of human subjects with severe AD pathology, compared to age-matched, non-demented control subjects. Higher levels of the 57 kDa fragment were directly correlated with levels of the 76 kDa active calpain fragment and, importantly, corresponded to greater calcineurin phosphatase activity. Furthermore, levels of proteolyzed calcineurin showed a direct positive correlation with neurofibrillary tangle load, suggesting that calpain/calcineurin interactions play an important role in disease pathology. Consistent with this observation, a later study from this group showed that calcineurin proteolysis in human neocortical regions coincided with elevated phospho-tau levels [170]. These results are particularly intriguing because tau hyperphosphorylation in AD was previously suggested to result from a decrease, rather than an increase, in calcineurin activity. It therefore appears that the relationship between calcineurin phosphatase activity and tau pathology may be different, or perhaps more complicated, than originally proposed.

Subsequent studies have provided further evidence that calcineurin is proteolyzed and activated to a greater degree during the progression of AD, though there are some discrepancies among the reports. In 2010, Wu et al. [118] observed increased expression of calcineurin proteolytic fragments in human AD cortical tissue, but unlike the Liu et al. study [58], the calcineurin truncation product associated with AD had a molecular weight of 48 kDa and was detected primarily in nuclear fractions. Increased nuclear localization of the 48 kDa fragment corresponded to increased nuclear levels of the NFAT3 isoform. Similar observations were observed in primary neuronal cultures from transgenic amyloidogenic mice.

Table 2.1 Studies showing calpain proteolysis of calcineurin in intact CNS tissue

CNS insult/disease state [with reference]	Source of CNS tissue	CN proteolytic fragment generated	Evidence for calpain involvement	Comments
Alzheimer's disease [58]	Human medial temporal cortex	CN A: 57 kDa	Levels of CN A proteolysis were positively correlated activated calpain levels within the same subjects; blockade of calpain activity in vitro inhibited CN A proteolysis as well as Ca^{2+}/CaM-independent calcineurin activity	First demonstration that increased calcineurin activity in Alzheimer's disease is linked to calpain-dependent proteolysis
Alzheimer's disease [118]	Human cortex	CN A: 45 kDa	na	Levels of the CN A 45 kDa fragment were elevated in the nucleus of AD subjects along with NFAT3
Mild cognitive impairment (MCI) [50]	Human hippocampus	CN Aα: 57, 48 kDa	Levels of the 48 kDa CN A fragment were positively correlated with levels of activated μ-calpain within the same subjects	First demonstration that CN A proteolysis is elevated at the early clinical stages of AD; no changes in proteolysis of the CN Aβ isoform were observed in MCI subjects
Elevated amyloid peptide levels/ Alzheimer's disease [50]	Primary neural cultures (neuron and astrocyte) from embryonic rat neocortex	CN Aα: ~48 kDa	Pharmacologic blockade of calpains with calpeptin prevented CN A proteolysis and reduced calcineurin-dependent NFAT activity over a 24-h period	Blockade of either calpain or calcineurin activity reduced Aβ-mediated neuronal degeneration and limited the proteolysis of non-synaptic NR2B receptor subunits; blockade of caspase I activity had no effect on CN A proteolysis
Elevated amyloid peptide levels/ Alzheimer's disease [172]	Human neuroblastoma SH-SY5Y cells	CN A 57 kDa	Generation of CN proteolytic fragment was coincident with increased levels of activated μ-calpain	Aβ-mediated elevations in activated calpain and CN A proteolysis was reduced by blockade of acetylcholinesterase

Alzheimer's disease [170]	Human medial temporal and frontal cortices	CN A: 57 kDa	Levels of CN A proteolysis increased in parallel with activated calpain; blockade of calpains in vitro inhibited CN A proteolysis	Elevated CN proteolysis and activity in human brain were associated with increased, rather than decreased, tau phosphorylation
Glutamate/kainate excitotoxicity [48]	Primary neuronal cultures from embryonic rat pups; intact mouse hippocampus	CN Aα: 57, 48, and 45 kDa	Generation of CN A fragments was coincident with increased levels of activated calpain spectrin breakdown; inhibition of calpain activity prevented CN A proteolysis and reduced the Ca^{2+}/CaM-independent activity of calcineurin	First direct evidence of calpain-dependent CN A proteolysis by calpain in intact cellular system; first evidence that CN proteolytic fragments mediate pathologic changes in nervous tissue
Carotid artery occlusion/ischemia [174]	Mouse whole brain and hippocampus	CN Aα: ~48 kDa	Generation of CN A proteolytic fragment and Ca^{2+}/CaM-independent activity was coincident with increased spectrin breakdown	CN A proteolysis and activation was associated with the increased nuclear localization of NFAT4 in hippocampal pyramidal neurons
Carotid artery occlusion/hypoxia/ischemia [173]	Rat whole brain	CN Aα: 54, 48, and 46 kDa	Generation of CN A proteolytic fragments was coincident with increased calpain protein levels and increased spectrin proteolysis	CN A proteolysis was coincident with elevated GFAP levels suggesting a possible relationship between CN A proteolysis and astrocyte activation
Intraocular pressure/glaucoma [178]	Mice/rats; retinal ganglion cells (RCG)	CN Aα: ~45 kDa	na	CN A proteolysis was associated with increased dephosphorylation of BAD; blockade of calcineurin activity with FK-506 increased phospho-BAD levels and reduced RGC apoptosis
Intraocular pressure/glaucoma [62]	Rats; RCG	CN Aα: ~50 kDa	Generation of CN proteolytic fragment was coincident with increased levels of activated calpain and increased spectrin proteolysis	Work from this study suggested that proteolysis observed by this group on a similar model of glaucoma [178] was also due to calpain

Moreover, forced overexpression of the 48 kDa calcineurin fragment in wild-type neuron cultures recapitulated dendritic dystrophy and spine loss typically observed with elevated amyloid levels. Whether the 48 kDa calcineurin fragment found in human AD tissue resulted from increased calpain-mediated proteolysis was not investigated in this study.

A year later, Mohmmad Abdul et al. [50] reported an increase in the expression of the 48 kDa CN A product in the hippocampus of human subjects diagnosed with mild cognitive impairment (MCI): a putative transition state between normal age-related cognitive decline and AD-related dementia [171]. Generation of the 48 kDa fragment showed a direct positive correlation with levels of the activated calpain 1 fragment, suggesting an increased interaction between calpain and calcineurin during the early clinical stages of AD. While both the CN Aα and CN Aβ isoforms each exhibited signs of proteolysis in human brain tissue, significant differences between subject categories were only observed for the CN Aα isoform. Consistent with an earlier report [172], proteolytic conversion of full-length CN Aα to the 48 kDa fragment as assessed by Mohmmad Abdul et al. [50] was also observed in primary rat hippocampal cultures 24 h after treatment with cytotoxic amyloid peptides (also see Fig. 2.2d). This proteolysis was associated with increased NFAT transcriptional activity, elevated proteolysis of the NR2B isoform of the NMDA receptor, and increased neuronal degeneration. Blockade of calpain activity significantly attenuated each of these effects, while inhibition of caspase 1 was largely ineffective, suggesting selective involvement of calpains in calcineurin proteolysis.

Interestingly, unlike the Wu et al. [118] report, the 48 kDa CN A fragment reported by Mohmmad Abdul et al. [50] was localized to cytosolic, rather than nuclear, fractions. The reason for this discrepancy is unclear but may be due in large measure to disease severity. Work on cardiomyocytes suggests that maintenance of truncated calcineurin in the nucleus may be more disruptive to cellular structure and function than cytosolic calcineurin [164]. It's possible that the nuclear localization of calcineurin AD brain results from a more toxic stage of Ca^{2+} dysregulation. If true, nuclear localization of proteolyzed calcineurin may reflect a critical transition state between MCI and AD-related dementia.

4.2 Brain Ischemia/Hypoxia

Brain ischemia resulting from stroke or other vascular accidents can cause irreversible neuronal damage and/or death due to excitotoxicity and/or other deleterious processes. Recent studies on several rodent species subjected to ischemic insults (i.e., carotid artery occlusion) discovered the appearance of truncated CN A products in damaged brain tissue, in conjunction with elevations in activated calpain and/or with the breakdown of spectrin, a major calpain substrate [121, 173, 174]. In one report by Shioda et al. [174], proteolysis of calcineurin to a 48 kDa fragment occurred within hours of the ischemic insult and was associated with an increase in Ca^{2+}/CaM-independent phosphatase activity along with an increase in the nuclear localization of NFAT4 in hippocampal CA1 pyramidal neurons. A follow-up study from this group suggested that proteolytic activation of calcineurin after ischemia underlies

delayed neuronal death in the hippocampus [121]. Neuronal loss was hypothesized to occur via the nuclear translocation of NFAT4 and forkhead, followed by the transcriptional induction of the proapoptotic factor, FasL. Indeed, each of the events in this pathway was prevented in ischemic animals treated with the calcineurin inhibitor, FK-506. In the Rosenkranz et al. study, a proteomics approach was used to identify modified proteins following perinatal hypoxic–ischemic brain damage in rats [173]. CN A was among the proteins significantly upregulated after ischemia. In addition to elevated levels of full-length calcineurin, several CN A truncation products were also observed including 54, 48, and 46 kDa fragments. The appearance of these smaller calcineurin products were associated with reduced phosphorylation of the calcineurin substrate, DARP32, suggestive of elevated calcineurin activity.

4.3 Glaucoma

Glaucoma is one of the leading causes of blindness and involves the progressive death of retinal ganglion cells (RGC), followed by the degeneration of optic nerve fibers. Increased intraocular pressure (IOP), which leads to RGC apoptosis and optic nerve degeneration in experimental models (e.g., see [175, 176]), is widely believed to be a primary cause of glaucoma [177]. In 2005, a study by Huang et al. [178] reported on the progressive accumulation of a 45 kDa CN A fragment in rat retina during treatments that increase IOP. A similar calcineurin proteolytic fragment was also observed in retinal cell lysates harvested from transgenic mice that spontaneously develop increased IOP and other glaucoma-like symptoms. The appearance of the 45 kDa CN A fragment coincided with a reduction in the phosphorylation state of the proapoptotic factor BAD and an increase in the mitochondrial release of cytochrome C. Consistent with previous reports linking calcineurin to mitochondrial dysfunction and apoptosis [119, 159], pretreatment of rats with the calcineurin inhibitor FK-506 suppressed the dephosphorylation of BAD, reduced cytochrome C release, and ameliorated RGC death and degeneration. Using MALDI-ProTOF/MS to identify cleavage sites in CN A, a follow-up study from the same research group suggested that IOP-related calcineurin proteolysis is most likely attributable to the activation of calpains, rather than other proteases, such as caspases [62].

5 Unresolved Issues and Future Avenues of Research

5.1 Calpain/Calcineurin Interactions in Brain: Role of Different Isoforms and the Contribution of Different Cell Types

There are multiple isoforms of calpain and at least two major CN A isoforms expressed in brain. As alluded to above, we know relatively little about isoform-specific differences in calcineurin, in terms of function and distribution in the brain. However, our previous work on human AD tissue indicates that disease-related

changes in both the subcellular localization and proteolysis of calcineurin are far more prominent for the CN Aα isoform [50, 87]. It's possible that the different CN A isoforms show different patterns of co-localization with calpains or interact with different affinities to the calpains, among other possibilities. One complicating factor in resolving this issue is that we don't really know which cell types exhibit calcineurin proteolysis. Most investigations of calpain/calcineurin interactions have dealt with neurodegenerative processes in neurons (discussed above and see Fig. 2.3). However, in recent years it has become increasingly clear that calcineurin also appears at high levels in activated glial cells, especially astrocytes [97], where it likely contributes to increased neuroinflammation during aging, injury, and disease. Calpains, too, are found in activated astrocytes and microglia with certain types of injury [179–182], and there is evidence that several astrocyte-enriched proteins, including the glial fibrillary acidic protein (GFAP) and vimentin, are targets of calpain-mediated proteolysis [183, 184].

Early work in spinal cord tissue further showed that calpain inhibitors attenuated several markers of gliosis following acute injury [185], suggesting that calpains may help drive astrocyte activation. If so, neuroinflammatory signaling in glial cells may be yet another disease-related process in which calpains and calcineurin are common mechanisms (Fig. 2.3). This possibility raises an interesting dilemma: i.e., are calpain and calcineurin inhibitors neuroprotective because they suppress harmful neuroinflammatory cascades? Or, do calpain and calcineurin inhibitors reduce neuroinflammation by stemming neurodegeneration and/or apoptosis? The extent to which calpain-mediated proteolysis contributes to calcineurin signaling in glial cells is not presently known and difficult to assess in intact tissue with available research tools. Although N-terminus antibodies to the CN A subunit detect the appearance of full-length and truncated forms of CN A in Western blots, these antibodies do not make a distinction between full-length and truncated forms in immunohistochemistry applications. Thus, until a primary antibody is generated that selectively identifies CN A proteolytic products (i.e., recognized proteolyzed but not full-length CN A), it will be very difficult to determine where (i.e., which cell type) calcineurin is actually proteolyzed in heterogeneous tissues, such as brain.

5.2 Are There More Effective Ways to Selectively Target Calpain/Calcineurin Interactions for Therapeutic Purposes?

The evidence to date suggests that calpain/calcineurin interactions are possibly an important upstream mechanism of numerous deleterious changes associated with a variety of neurodegenerative diseases. A critical question is this: can the physical interaction between calpains and calcineurin be exploited for the development of new treatment strategies? Separately, calpain and calcineurin inhibitors have shown neuroprotective properties in numerous disease models. However, the use of these inhibitors in the clinic is fraught with many difficulties. For instance, commercially available calcineurin inhibitors are notorious for their numerous adverse effects,

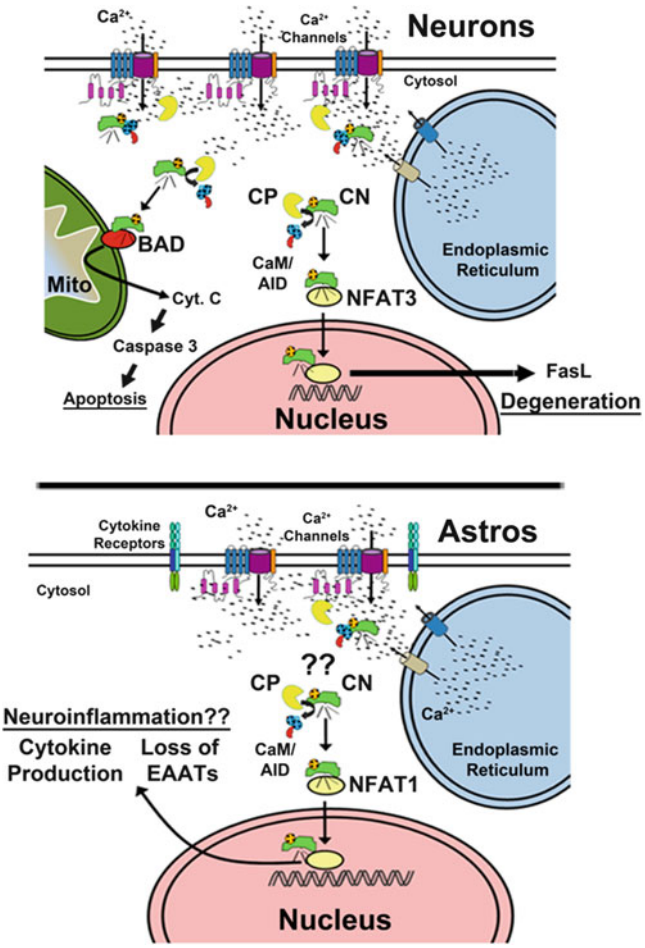

Fig. 2.3 Calpain/calcineurin interactions in neurons and astrocytes. Cartoons showing putative functions/outcomes of calpain/calcineurin interactions in neurons (*top panel*) and astrocytes (*bottom panel*). Based on the literature discussed, calpain-mediated proteolysis of calcineurin in neurons is strongly linked to neurodegenerative processes. Dephosphorylation of BAD leads to its translocation to the mitochondrial membrane and the subsequent release of cytochrome C, followed by caspase activation and apoptosis (e.g., see [119, 178]), while activation of NFAT3 and 4 isoforms triggers the transcriptional induction of the FasL proapoptotic factor and/or hastens the degeneration of dendrites and synapses (e.g., see [118, 121]). Though astrocytic calpain/calcineurin interactions (*bottom panel*) have yet to be investigated extensively, both appear at high levels in activated astrocytes as a result of injury. In astrocytes, calpain-dependent proteolysis of calcineurin could lead to extensive activation of the NFAT1 isoform followed by the upregulation of numerous cytokines involved in neuroinflammation, as well as the downregulation of EAATs resulting in excitotoxicity (e.g., see [87, 97, 111–113]). Note that calpain/calcineurin interactions in either cell type could lead to deleterious processes common to many neurologic disorders. *CP* calpain, *CN* CN, *Cyt. C* cytochrome C, *Mito.* mitochondria, *CaM* calmodulin, *AID* autoinhibitory domain

many of which are potentiated in elderly populations, who also show greatest susceptibility to neurodegenerative disease [186]. The relatively high level of toxicity associated with these drugs is very likely due to their poor specificity. Indeed, the most commonly used calcineurin inhibitors (cyclosporine and tacrolimus) are well known to bind to an inhibit immunophilins [30], which participate in many calcineurin-independent signaling cascades [187]. In addition, calcineurin is ubiquitously expressed and has pleiotropic functions all of which are suppressed by calcineurin inhibitors. Based on the toxic responses to these drugs, it seems clear that many calcineurin and immunophilin-dependent signaling pathways are critical for cell function and viability and should be left unperturbed.

The ideal drug or treatment would prevent calpain from proteolyzing CN A into a constitutively active fragment but would not interfere with the normal activation of calpains and calcineurin or interfere with the interactions of these enzymes with other substrates. This would require extensive investigation into the molecular mechanisms through which calpains recognize, bind to, and proteolyze the CN A subunit. Is there something unique about the primary sequence of CN A that makes it a good substrate for calpain? Is there a way that we could pharmacologically modify the CN A subunit to permit normal Ca^{2+}/CaM binding but exclude binding of calpain? In this regard it may be instructive to consider strategies that have been used successfully to disrupt calcineurin interactions with NFATs for the purpose of developing safer and more effective immunosuppressive agents. Calcineurin interacts with NFATs, in part, by binding to a specific amino acid substrate, PxIxIT, located upstream from the NFAT DNA binding domain. In the late 1990s, Rao and colleagues developed a peptide (i.e., MAGPHPVIVITGPHEE or VIVIT) based on the PxIxIT sequence in an attempt to disrupt calcineurin/NFAT interactions [188]. VIVIT was shown to prevent NFAT activation as effectively as commercial calcineurin inhibitors but did not inhibit calcineurin catalytic activity, per se, in vitro. Since this report, VIVIT has been used by many labs as an alternative to calcineurin inhibitors for the study of diverse processes in numerous and distinct cell types. Proof of principle studies on intact animal models has also shown the potential of VIVIT as a prophylactic in allogenic tissue transplants [189] and as a neuroprotectant in AD-like amyloid pathology [132, 133]. Development of similar peptide or chemical-based reagents to selectively prevent calpain/calcineurin interactions could have a similar impact on therapeutic strategies for treating neurodegenerative disease. At the least, reagents of this type would rapidly advance our understanding of the specific functional consequences of calpain/calcineurin interactions and would therefore have great value to basic research.

6 Conclusions

Calpain and calcineurin are fascinating enzymes and important effectors of Ca^{2+}-mediated neurotoxicity. Recent work has shown us that calpain and calcineurin are not merely regulators of their own discrete signaling pathways but interact extensively. This interaction could prove to be a key step in the transition from normal

cellular function to pathological function. Extensive work will be necessary to determine not only when, but where, calpain/calcineurin interactions occur. Moreover, a greater understanding of the molecular basis of this interaction could lead to more specific and effective therapies for a variety of neurodegenerative disorders and diseases.

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Chapter 3
Involvement of Caspases in the Pathophysiology of Neurodegeneration and Stroke

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Abstract A family of proteases known as caspases is a key element in the proteolytic machinery involved in apoptosis or programmed cell death. Apart from their involvement in cell death, caspases are also associated with the developmental process and other normal functions of adult organisms. Caspases are named such because they constitute a family of cysteine proteases which always cleave an Asp residue in their substrates. Stroke results from a rapid malfunctioning of the brain due to lack of blood supply and is a major health threat producing mortality and morbidity. Majority of strokes are ischemic (80 % of all strokes) and the rest are hemorrhagic. Both forms of divergent cell death mechanisms, necrosis, and apoptosis are observed at different spatial region of ischemic attack. Involvement of multiple caspases in stroke has been documented with caspases 1, 3, 8, 9, and 11 playing major roles. Many neurodegenerative diseases result from loss of functional neurons

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from the brain through enhanced death of neurons. Neurodegenerative diseases are usually late onset and progressive. Among the most common neurodegenerative disorders in aging populations worldwide, Alzheimer's disease (AD) and Parkinson's disease (PD) definitely warrant mentioning. Abnormal protein deposits in specific regions of the brain give rise to both these diseases triggering reactive oxygen species formation and mitochondrial dysfunction. Caspases are activated by these changes resulting in loss of neurons through cell death. In this review we provide a brief overview of the involvement of caspases in diseases associated with the impairment of brain function.

Keywords Stroke • Parkinson's disease • Alzheimer's disease • Caspase • Apoptosis

1 Introduction

Knowledge of two fundamental types of cell death, apoptosis and necrosis, has existed since long ago [1]. Cells are known to die in two different ways: traumatic death through accidental injury or exposure to cytotoxic chemicals, resulting in swelling, bursting, and flooding out the internal components [2, 3]. But cells may also undergo a controlled death termed "apoptosis" or "programmed cell death." Apoptosis, a word coined in the nineteenth century [4] and described in Kerr et al. [5], refers to a specific form of programmed cell death. In the face of an apoptotic trigger, the major cellular regulatory systems will collapse and the internal cellular machinery will be taken apart. The small pieces of the disintegrating apoptotic cells will finally be consumed by neighboring cells. Apoptosis progresses through plasma membrane asymmetry, condensation of nucleus and internucleosomal cleavage of DNA [6]. Finally, apoptotic cell forms "apoptotic bodies" which is rapidly consumed by phagocytes without causing any localized inflammation [7]. Apoptosis, or programmed cell death, plays a central role in the development and homeostasis of all multicellular organisms, for the proteolytic cleavage of a broad spectrum of cellular targets, leading ultimately to cell death. Alterations in apoptotic pathways have been linked to numerous human pathologies such as cancer and neurodegenerative disorders [8].

A proteolytic system of the apoptotic machinery involves a family of proteases known as caspases as the core component [9, 10]. Caspases are essential in cells for apoptosis in development and almost every stage of adult life. The critical involvement of a cysteine protease *Ced-3* in apoptosis was first discovered in the nematode *Caenorhabditis elegans* [11]. Since then, ample evidence has demonstrated that the mechanism of apoptosis is evolutionarily conserved. Caspases are a family of cysteine proteases which always cleave an Asp residue in their substrates, and thus they are named "caspase": c, cysteine protease and "asp," strong aspartate preference [12]. In *C. elegans* cell death, the most important elements are the two genes Ced-3 and Ced-9. Homology of these genes was found with vertebrate counterparts: the caspase family and the Bcl-2 family [11, 13]. This knowledge facilitated the entry of apoptosis into the mainstream of modern biology. Thus, the importance of caspases began with the discovery that the *C. elegans Ced-3* gene encodes a

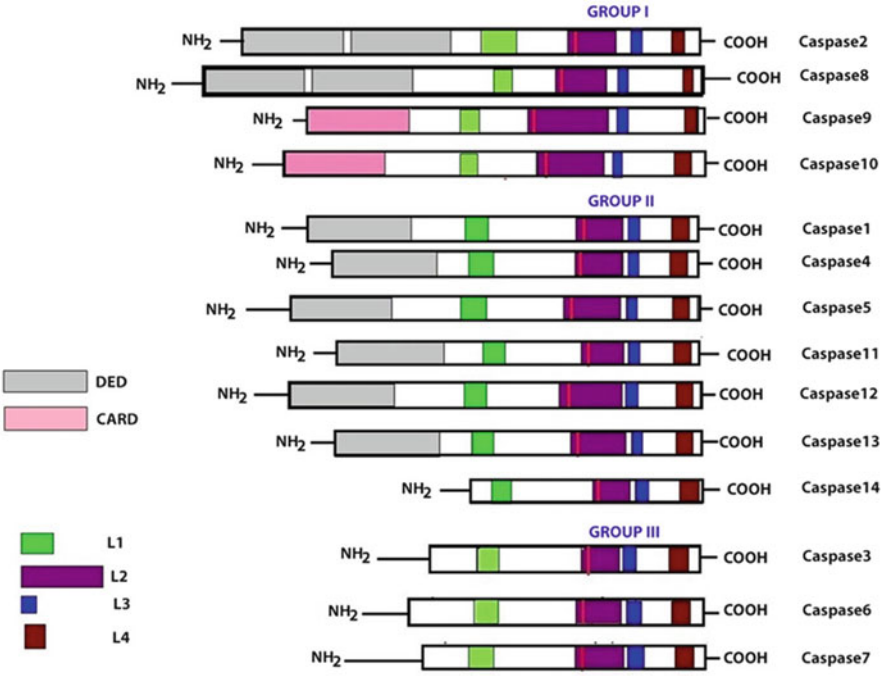

Fig. 3.1 The caspase family. Three major groups of caspases are presented diagrammatically according to functional segments. The CARD, DED, and the four surface loops (L1,L2,L3,L4) that shape the catalytic groove are indicated. The catalytic residue Cys is shown as a red line at the beginning of loop L2. Group I: Apoptosis initiator caspases; Group II: Inflammatory caspases; Group III: Apoptosis effector caspases

homologue of the independently identified human interleukin-1β-processing enzyme (ICE) [14] involved in inflammation. The caspases work on a specific sequence in their target proteins, typically cleaving next to aspartate amino acids. Expression of either *Ced-3* or ICE in mammalian cells induced cell death. ICE became the first member (*caspase 1*) of a family of proteases dependent on a cysteine nucleophile to cleave motifs possessing aspartic acid (aspase) [15]. Identification of at least 14 distinct mammalian caspases has taken place; their orthologs were found to be present in species as diverse as the nematodes to the dipteran and the lepidopteran (Fig. 3.1). Even though the first mammalian caspase, caspase 1 or ICE, was identified as a key mediator of inflammatory response, 8 of the 14 caspases were described to possess apoptotic functions. Of the 14 members of the caspase family, seven are classified [16] as cytokine-processing enzymes comprising caspase 1 [17], caspase 4 [18], caspase 5 [19], caspase 11, caspase 12, caspase13, and caspase 14 [20], and the other seven are classified as apoptosis-related enzymes comprising caspase 2 [21], caspase 3, caspase 6, caspase 7, caspase 8 [22], caspase 9 [23], and caspase 10. The caspases 2, 8, 9, and 10 trigger apoptosis and are known as upstream or initiator caspases; they activate the executioner or downstream caspases comprising caspases 3, 6, and 7 which ultimately execute apoptotic cell death [12, 24, 25].

Caspases carry out inflammatory, death-inducing, and normal developmental functions. Many disease processes are accompanied by activation of many caspases. Here we plan to concentrate on the vital roles played by caspases in the brain which are associated with ischemic stroke and neurodegenerative disorders such as PD and AD.

2 Structural Aspects of Caspases

Caspase proteins are formed as inactive zymogens containing N-terminal prodomains. Key regulatory proteins such as Rb (retinoblastoma protein) are cleaved suppressing cell-cycle progression, and nucleic acid synthesis is inhibited through damage to polymerases. Cleavage of the major enzymes required for homeostasis and repair results in the destruction of these vital mechanisms. Cytoplasmic actin and nuclear lamins are specifically dismantled. Fragmentation and condensation of the genetic material takes place through activation of the DNA fragmentation factor (DFF). The lipids in the cell membrane are subtly changed, signaling to neighboring cells that the apoptotic process inside the cell is on. Crystallographic studies revealed that the active caspase is a tetramer of two heterodimers, thus containing two active sites. Upstream initiator caspases are capable of autocatalytic activation and generally have a long prodomain. Downstream effector caspases need initiator caspases for their activation by cleavage. A well-designed amino acid library scan identified an optima of four amino acid motif situated N-terminal to the aspartic acid cleavage site for each caspase, which helps define substrate specificity as well as specific peptide inhibitors for caspases [26]. In General, caspases are specific proteases recognizing at least four contiguous amino acids, named P4, P3, P2, and P1, and are known to cleave after the C-terminal residue (P1), usually an Asp. Although the P1 residue was thought to be exclusively Asp, recent studies indicate that some caspases can also cleave after Glu [27].

Caspases are zymogens; structurally all procaspases contain a highly homologous signature motif protease domain, distinctive of the family of caspases. The protease domain is divided into two subunits, large subunit of about 20 kDa, p20, and a small subunit of about 10 kDa, p10 [20, 28]. In a number of procaspases, the p20 and p10 subunits are separated by a small linker sequence. This is followed by N-terminal prodomain of variable length. Caspases are usually divided into three major groups based upon length, structure, and function of the prodomain. Group I (inflammatory caspases) are the caspases with long prodomains (over 100 amino acids); group II are initiator of apoptosis caspases, while group III are effector caspases with a short prodomain of 20–30 amino acids.

The long prodomains contain distinct structural motifs which belong to “death domain” (DD) superfamily. DDs are 80–100 residue long motifs involved in the transduction of the apoptotic signal. This superfamily consists of the “death effector domain” (DED) and the “caspase recruitment domain” (CARD). A novel motif termed as the “death-inducing domain” (DID) was also recently identified. Procaspases 8 and 10 each contain two tandem copies of DEDs, whereas the CARD

domain is found in caspases 1, 2, 4, 5, and 9 and caspase 3 [29]. Each of these motifs interacts with other proteins by homotypic/homophilic interactions and has important roles in procaspase activation. DD superfamily members are identified by structural similarity that includes six or seven antiparallel, amphipathic α-helices. Structural likeness of all recruitment domains is indicative of a common evolutionary origin [30]. But DD and CARD contacts occur through electrostatic interactions, whereas DED connections are made by hydrophobic interactions [31]. Cleavage of a procaspase at the specific Asp-X bonds results in the formation of the mature caspase, which comprises the heterotetramer p20–p10 and causes release of the prodomain. Studies on active caspases 1 and 3 suggest release of large and small subunits from the procaspase through proteolytic processing and requiring both subunits for the protease activity. X-ray structures determined for mature caspases 1, 2, 3, 7, 8, and 9 indicated that the overall structural design of all caspases is similar and revealed that the mature caspase is a heterotetramer with two adjacent small subunits surrounded by two large subunits [20]. In the caspase heterotetramer, the two heterodimers align in a head-to-tail fashion. Correspondingly, two active sites are positioned at the opposite ends of the molecule making up four active sites in the heterotetramer. The active center comprises amino acid residues from both subunits. The activation of procaspase involves the proteolysis of inter-domain linker and subsequently of the prodomain [10]. Caspase activation may proceed through auto-activation via oligomerization [32], death receptor, or mitochondrial pathway-induced transactivation and proteinase-evoked proteolysis (granzyme B, cathepsin G, calpains, and apoptotic serine proteinase (p24)) [23, 29].

3 Pathways Which Influence/Activate Caspase Cascade

Four pathways to caspase activation during apoptosis have been established as follows: (a) mitochondria-mediated pathway, (b) death receptor-mediated pathway, (c) granzyme B-mediated pathway, and (4) the endoplasmic reticulum (ER)-mediated pathway.

3.1 Mitochondria-Mediated Pathway

Chemotherapeutic agents, UV radiations, oxidative stress, and growth factor withdrawal appear to mediate apoptosis via mitochondrial pathway. In apoptosis mitochondria undergoes two major changes: first, the outer mitochondrial membranes become permeable to proteins, resulting in the release of proteins normally found in the space between the inner and outer membranes (include cytochrome c and apoptosis-inducing factor); and secondly, the transmembrane potential of inner mitochondrial membrane is reduced [33, 34]. The release of cytochrome c forms a heptameric wheel-like caspase-activating apoptosome. The components of apoptosome (Cyto C, Apaf-1, dATP, and procaspase 9) lead to the formation of a situation

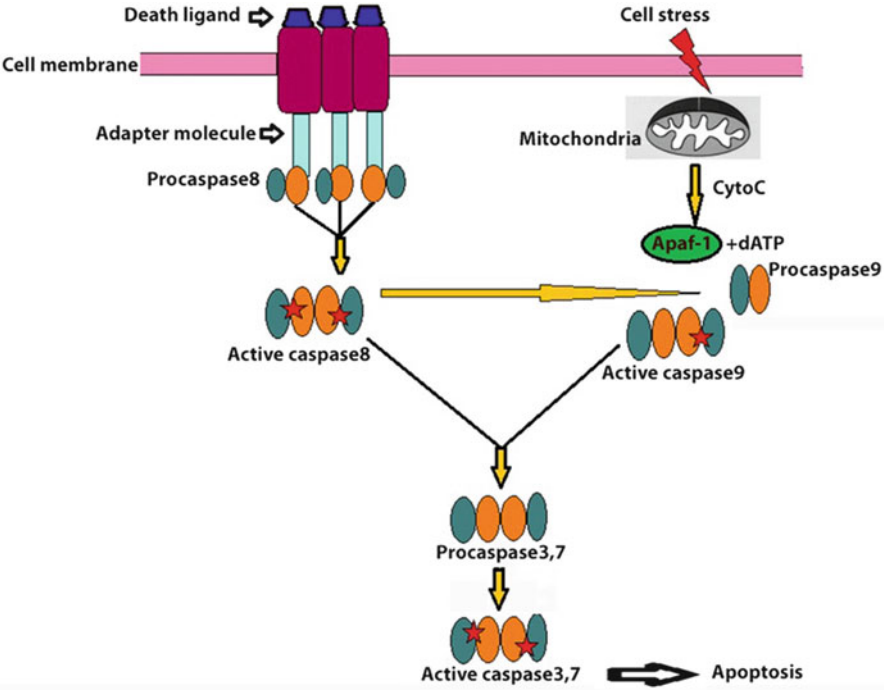

Fig. 3.2 Schematic overview of caspase signaling in two pathways of apoptosis. Engagement of either the extrinsic or the intrinsic death pathways leads to the activation of the initiator caspases by dimerization at multiprotein complexes. In the extrinsic pathway, the DISC is the site of activation for caspase 8 and interaction with its ligands, death receptors recruit adaptor protein, and initiator caspases. The active sites are represented by *red stars*. Active initiator caspase activate the effector caspase to induce apoptosis. Stimulation of the intrinsic death signals stimulate mitochondria directly resulting in the release of cytochrome c that bind to an adaptor protein Apaf-1 and recruit initiator caspase 9. Active caspase 9 activates effector caspases to induce apoptosis. Caspase 9 is shown as having one active site as seen in its crystal structure. However, the number of active sites in vivo is unknown. Following activation, the initiator caspases then cleave and activate the effector caspases 3 and 7 to induce apoptosis

for processing and activation of caspase 9. Caspase 9 has a CARD in the N-terminus, which is the key site to associate with Apaf-1 and cytochrome c [20, 35]. Activation of caspase 9, in turn, cleaves effector caspases, e.g., caspases 3, 6, and 7 [36] (Fig. 3.2). The effector caspases cleave the target proteins resulting in a disciplined and controlled way to cell death. In this pathway of apoptosis, caspases 3 and 9 are the most important as their activities influence the process of apoptosis as well as the type of cell death. Deregulation of this type of apoptotic process can lead to many diseases and pathological disorders including cancer, autoimmunity, and neurodegeneration. Therefore, caspases, the major executioners of cell death, could be used as potential targets for the development of therapeutic approaches.

3.2 Death Receptor-Mediated Pathway

The involvement of membrane receptors is required in death receptor pathway. Members of tumor necrosis factor receptor (TNFR) superfamily of which TNFR and Fas are best characterized and share a distinct death domain (DD) within their cytoplasmic tails [37]. After their association with corresponding DD-containing ligands, oligomerization, and/or a conformational change in the receptor occur. Then recruitment of an adaptor molecule, Fas-associated death domain (FADD) or TNF receptor-associated death domain (TNFADD), and procaspase 8 (probably procaspase 10 also) form the death-inducing signaling complex (DISC) [38, 39]. TNFR2, a TNF receptor-related member without a death domain, engages only TNF receptor-associated factor-2 (TRAF2) and consequently do not have an effect on the TRADD-dependent apoptotic pathway [38]. At the DISC, procaspase 8 is processed and caspase 8 ensures the direct activation of caspase 3 [20]. Thus, the mitochondrial pathway and the death pathway converge on caspase 3. Caspase 3 then can cleave a series of proteins such as PARP, a DNA repair enzyme, nuclear lamins, gelsolin, and fodrin [20]. The two apoptotic pathways can be linked through Bid (usually found in the cytosol and is cleaved by caspase 8 to form a truncated protein, tBid), a proapoptotic BH3-only member of the Bcl-2 family of proteins [40]. tBid translocates to the mitochondria where tBid activates Bax, initiating the release of cytochrome c and mitochondrial dysfunction [37]. A cross talk between the receptor and mitochondria-mediated pathways can amplify caspase activation necessary for apoptosis. In fact, more recent studies focus on multiple signal transduction cascades that trigger cells to undergo apoptosis (Fig. 3.2).

3.3 ER-Mediated Pathway

If cells are exposed to endoplasmic reticulum (ER) stress, caused by agents such as tunicamycin (a special inhibitor of N-glycosylation in the ER) and brefeldin A (an inhibitor of ER/Golgi transport), misfolded or unfolded proteins (as ER is the site of assembly of polypeptide chains) accumulate in the ER lumen [41]. Thus, ER is another center of regulation for cell death [42]. In this pathway, caspase 12, specifically localized on the cytoplasmic (outer) side of the ER, is thought to be an initiator caspase and plays a very important role [43]. Like most other members of the caspase family, caspase 12 needs separation of the prodomain for activation. Several likely molecular mechanisms for the processing of caspase 12 have been proposed. Caspase 12 is initially processed at the N-terminal region by the protease calpain, which is activated by ER stress. Caspase 12 is an inactive enzyme in humans, and it has also a caspase recruitment domain, like caspase 9; so it may be possible that like caspase 9, caspase 12 is also activated by its association with an Apaf-1-like protein [44, 45]. A Bcl-2 protein targeted to the ER, Bcl-2/cb5, inhibits ER-stress-mediated cytochrome c release [46]. Thus, the release of cytochrome c has a critical role in ER-stress-mediated apoptotic pathway.

3.4 Granzyme B-Mediated Pathway

The two major populations of cytotoxic lymphocytes, natural killer cells and cytotoxic T lymphocytes (CTLs), have an essential role in the clearance of virally infected cells and tumor cells. The central mechanisms of cytotoxicity used by these cells are the stimulation of the Fas death pathway and granule exocytosis [47]. In granule exocytosis, two proteins are required: perforin (pore-forming protein facilitates the delivery of other granule components into target cells) and granzyme B (serine proteinase with substrate specificity similar to caspase family of apoptotic cysteine proteinase). The architecture of the substrate-binding site of granzyme B is designed to accommodate and cleave hexapeptides [48]. Cell death can be induced by granzyme B through two complementary pathways: a cytosolic pathway which includes activation of proapoptotic caspases and a nuclear pathway possibly comprising a cell-cycle-regulating protein and/or Cdc2 kinase activation. They play an important role in human pathologies such as transplant rejection, viral immunity, and tumor immune surveillance. Releasing from the CTL, granzyme B binds to receptor and is endocytosed but remains arrested in endocytic vesicles until released by perforin. In the cytosol, the granzyme B targets caspase 3 and triggers the caspase cascade which culminates in apoptosis. However, granzyme B initiates caspase processing, but it cannot fully process procaspase 3. The full activation may involve other proapoptotic mediators such as cytochrome c, Smac/Diablo, and Htra2/omi, which are released from mitochondria. Bcl-2 overexpression inhibits the release of these proapoptotic molecules from the mitochondria, bringing about cell survival even in the presence of partial procaspase processing by granzyme B [49]. Other reports have demonstrated that in the absence of caspases, granzyme B can still initiate mitochondrial events via the cleavage of Bid [48].

4 Caspases and Neurodegenerative Diseases

Neurodegenerative diseases result from loss of functional neurons from the brain. Many neurodegenerative diseases are late onset and progress as the patient ages. In many aging populations across the globe, incidence of Alzheimer's disease and Parkinson's disease is becoming increasingly prevalent [50, 51]. Apart from the aging process, no common causative factors may be common to the diseases. However, subcellular changes documented in the brains affected by the two diseases bear many similarities (Fig. 3.3). Abnormal protein aggregates are observed in the brains affected by both these diseases [52].

4.1 Parkinson's Disease

Both motor and cognitive dysfunctions are salient features of Parkinson's disease (PD) [53]. Progressive loss of motor function resulting in resting tremor, abnormal

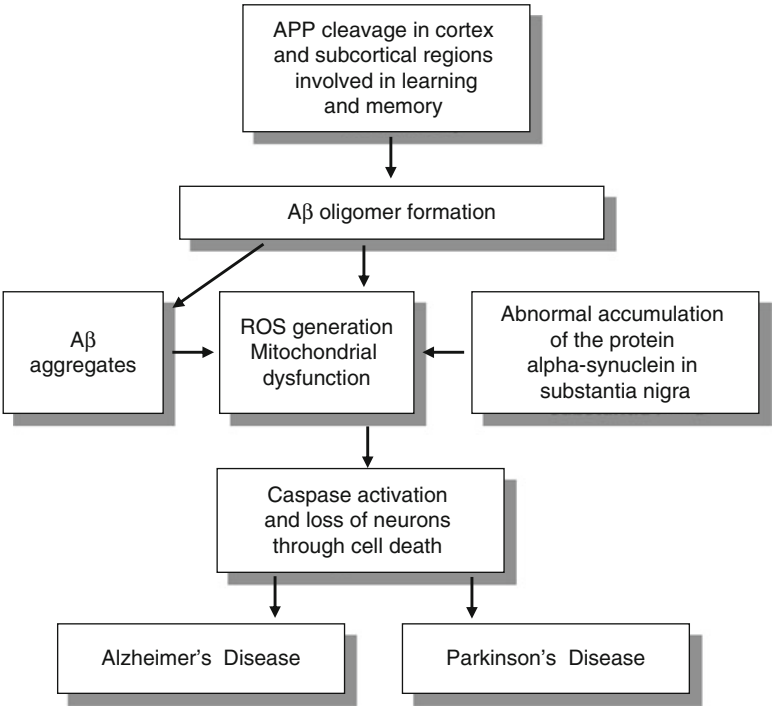

Fig. 3.3 General overview of the involvement of caspases in the etiology of neurodegenerative disorders Alzheimer's disease and Parkinson's disease

gait, rigidity, and dementia is often observed in Parkinson patients. Both neurotoxic substances and genetic mutations may be the cause of disease initiation. The disease is characterized by progressive loss of dopaminergic neurons in the substantia nigra. α-synuclein is a protein which accumulates in these neurons, and protein aggregation triggers oxidative stress and mitochondrial malfunction. Loss of dopaminergic neurons through cell death is an important element in Parkinson's disease pathology. α-synuclein is thought to be a key player in the cytotoxicity as expression of the mutant forms of the protein, or overexpression of the normal gene may result in cell death [53–57]. Cell death is triggered by α-synuclein accumulation in the presence of dopamine in human neuronal cells revealing the reason for selectivity of the toxic effects for dopaminergic neurons [57]. Accumulation of protein aggregates which includes α-synuclein triggers apoptosis mediated by reactive oxygen species [55]. Experimental Parkinson's disease-like symptoms were produced in animals through administration of neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) [55, 58, 59]. It was found that under these experimental conditions, significant activation of caspases, namely, caspases 8, 9, and 3 occurred [55, 59, 60]. Similar results were obtained when human neuronal cell lines were treated with these neurotoxins [61]. Increasing the expression of mutant α-synuclein in PC 12 cells resulted in induction of mitochondrial cell death pathways corroborated by mitochondrial cytochrome c release and heightened

activity of caspases 9 and 3. Inhibitors of caspases 9 and 3 partially reversed the cytotoxicity [62]. Furthermore, endoplasmic reticulum (ER) stress was also detected to be present as expression of mutant α-synuclein triggered the activation of caspase 12, and specific inhibitors of these caspases resulted in a complete inhibition of cell death [63]. Presence of activated caspase 3 was demonstrated in the substantia nigra of PD patients [64, 65]. Researchers have reported that in 1-methyle-4-phenyl-1,2,3,6-tetrahmydropyridine (MPTP)-induced Parkinsonian syndrome, gene disruption of caspase 3 protected mice from PD-like symptoms and decreased tyrosine hydroxylase expression in the nigra-striatum [59]. Inherited forms of PD result from mutations in certain genes among which mutation in LRRK2 is very common. Primary neuronal cells expressing mutant LRRK2 activate caspase 8 through increased activity of the cell death receptor pathway [66].

4.2 Alzheimer's Disease

Among the neurodegenerative disorders affecting the elderly population, Alzheimer's disease (AD) is most common. The disease progresses by causing gradual memory loss and cognitive impairment [67]. Postmortem examination of brains of AD patients revealed the presence of beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFT) in neurons of brain regions responsible for learning and memory [67, 68]. Aβ aggregates predominantly comprised Aβ plaques. Aβ is produced in neurons through the actions of enzyme β and γ secretase on amyloid precursor protein (APP). Two predominant forms of Aβ are produced through action of γ secretase. The shorter form with 40 amino acids residues is known to be less toxic, and a longer form with 42 amino acids is more toxic and forms oligomers resulting in Aβ aggregates [51, 67, 69]. NFTs are composed of the protein tau. In a normal brain, tau is associated with microtubules and is involved in axonal transport [70].

In AD tau is hyperphosphorylated and loses its normal function and produces NFTs. Hyperphosphorylation of tau is preceded by Aβ oligomer formation [51]. Oxidative stress and mitochondrial dysfunction have been associated with both Aβ oligomer formation and tau pathology in AD.

Considerable evidence exists for potentially linking caspase activation and Aβ and NFT formation. However, studies on animal models directly confirmed this hypothesis. LaFerla and colleagues developed triple transgenic mice [71], termed 3×Tg-AD mice expressing mutant PS1 (presenilin1 a gene affected by familial AD), APP, and tau. A progressive development of both plaques and tangles with plaques taking place prior to tangle formation was observed in these mice. Rohn and his colleagues [72] also generated mice which overexpressed antiapoptotic protein Bcl-2, termed as 3×Tg-AD/Bcl-2. Overexpression of Bcl-2 in the neurons of 3×Tg-AD mice blocked the caspase activation and the cleavage of tau leading to its accumulation within neurons. That caspase cleavage of tau is an important step towards NFT

formation was indicated by the absence of fibrillary tangle formation in 3×Tg-AD mice overexpressing Bcl-2 even in the presence of high protein levels of tau. Caspase 8 and consequently caspase 3 activation through death receptor signaling were linked to Aβ toxicity in neuronal cell lines [73]. The exposure of primary cortical neurons to Aβ of 42 amino acids resulted in a loss of mitochondrial membrane potential, increase in ROS levels, and activation of caspase 9 and caspase 3 [74]. Induction of the extrinsic apoptotic pathway resulting in the activation of caspase 8 and 9 through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptors DR4 and DR5 by Aβ oligomers has also been documented in human microvascular cerebral endothelial cells [75]. In neuronal LAN5 cells, Aβ oligomers were shown to activate mainly caspase 9, but insoluble aggregates activated mainly caspase 8 suggesting that they are unable to activate an internal pathway. The authors suggest that the aggregates mimic the extracellular plaques and activate a death receptor activating caspase 8, whereas the diffusible oligomers may cross the neuronal membrane triggering the intrinsic apoptotic pathway [76].

A crucial role for caspase 6 [77] in axonal degeneration has been described in a neurodegenerative disorder like AD. Induction of the extrinsic apoptotic pathway takes place through activation of death receptor 6 (DR6) by N-terminal APP fragment (NAPP) fragment cleaved by β-secretase. Activation of DR6 leads to an increase in caspase 6 activity with consequent axonal degeneration which is an important feature of AD.

The studies discussed above suggest a clear indication that caspases are involved in the etiology associated with AD and PD. Therefore, a question whether targeted inhibition of this class of proteases will offer an effective approach to treat this disease may be raised. To get the answer to this question, it may be important to look for pharmacological agents which target caspases. The pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone (Z-VAD-fmk) was used on the transgenic mouse model of amyotrophic lateral sclerosis (ALS) decreasing mortality and delaying the time of onset [78]. Z-VAD has been investigated for the treatment of many neurodegenerative disorders like ALS, Huntington's disease, PD, and also for acute neurological trauma like ischemia [78–80]. Unfortunately, clinical development of Z-VAD was discontinued, and a number of other molecules have been developed for clinical application. Quinolyl-valyl-*O*-methylaspartyl-[-2, 6-difluorophenoxy]-methyl ketone (Q-VD-OPh) is a more recent, broad-spectrum caspase inhibitor that showed therapeutic potential [81]. Q-VD-OPh appears to be much better than Z-VAD in many respects including greater potency, selectivity, stability, and cell permeability [81, 82]. Also, Q-VD-OPh may cause lesser toxic side effects than Z-VAD even at very high doses. Above all, Q-VD-OPh may be able to cross the blood–brain barrier. Since this is always the deciding factor when developing a drug for brain disorders, satisfying this criterion may establish Q-VD-OPh in the treatment of these diseases. Treatment with Q-VD-OPh in animal models of PD, Huntington's disease and stroke confirms its role as a neuroprotective agent [81–83]. Using animal models of AD, caspase inhibitors like Q-VD-OPh could be tested for their efficacy in preventing or halting the damage brought about by AD.

5 Caspases and Stroke

A rapid malfunctioning of the brain due to lack of blood supply is generally termed as stroke. Worldwide stroke is one of the leading threats of death. According to the causative, stroke is classified broadly into two categories, ischemic and hemorrhagic. Eighty percent of strokes are ischemic and the remainders are hemorrhagic [84]. Even these subclasses can cross talk as it has been found that hemorrhages develop inside the ischemic region as well as pre-hemorrhages can be the cause of ischemic attacks [85]. The term ischemia means reduction in blood supply to the particular organ. For brain, it is called cerebral ischemia, which may be focal or global in nature. Numerous factors including atherosclerosis, thrombosis, embolism, occlusion, hypoglycemia, and hypertension contribute to the development of ischemic stroke [86–90].

Ischemic stroke initiates a number of events including neuronal injury, oxidative damage, disruption of ionic homeostasis, altered microvascular permeability, and inflammation either temporally or spatially. The brain does not store glucose so the effects due to ischemia initiate very rapidly, but it may continue hours after ischemic onset. Due to ischemic attack, decrease in blood flow at the core reaches below the threshold value for neuronal viability that causes complete energy depletion at the center and increasing energy gradient from center to outer periphery. This peripheral region is called the penumbra. Due to absolute energy depletion, neurons at attacked region become electrically silent and the ultimate outcome of it is neuronal death. Necrosis and apoptosis, both mechanistically divergent cell deaths, are observed at different spatial region of ischemic attack. Due to massive energy depletion, the center of the attacked region experiences necrotic cell death [91, 92]. Whereas predominant apoptotic cell death is observed at penumbra [92, 93]. Brain cells face death insult not only at the time of ischemia but also during reperfusion, a sudden return of blood flow to the oxygen-starved energetically compromised tissue, and the efficacy is as severe as ischemia [94].

Caspases are the most active participants of the apoptotic process. After extensive work on caspases since 20 years, the role of caspase in cerebral ischemia and brain death has been understood more deeply. So a new therapeutic direction of stroke opened up targeting these caspases. The role of multiple caspases in stroke is documented in the literature, but the task of caspases 1, 3, 8, 9, and 11 is more predominant. Most of the studies have been performed in rodent model. Extensive study in human model is still needed to understand the cerebral ischemia in human more closely. According to the modes of action, caspases may be categorized in two classes: a) inflammatory and b) apoptotic. Previously it was considered that inflammatory pathways and death pathways are independent. But recent studies strongly established the fact that released inflammatory cytokine can disrupt the blood–brain barrier and can be one of the main components for causing cerebral edema, which is considered as the obligatory factor for ischemic attack. Status and role of different caspases in stroke are discussed below.

5.1 Caspase 1 and Caspase 11

Caspase 1 and caspase 11 are reported as inflammatory response inducers. The information that during ischemic stroke in rodent, cytokine IL1-β levels get elevated and less cell death occurs after ischemic attack in IL1-β null mice strongly supports the contribution of this cytokine in ischemic damage [95]. Caspase 1 proteolytically cleaves proinflammatory IL1-β to inflammatory IL1-β [96]. Overexpression of dominant negative form of caspase 1 prevents maturation of proinflammatory IL1-β [96]. Recent study in genetically caspase 11 knockout mouse showed prevention against stroke [97] and lipopolysaccharide-mediated immune response [98]. Caspase 1 and caspase 11 levels get elevated after ischemic attack [99, 100]. Neuroprotection against ischemic damage of genetically knockout caspase 1 or caspase 11 mouse further establishes the role of caspase 1 and caspase 11 in ischemic damage [99–103].

5.2 Caspase 2

Some studies showed increase in caspase 2 expression during ischemia [100], but no such direct evidence has been found that suggest an importance of caspase 2 in ischemia. Even caspase 2 knockout mice are not protected from transient or global ischemia [104]. It should be noted in this context that caspase 2 knockout mouse showed increase of caspase 9 and Diablo/Smac in neurons, and therefore caspase 2 may play an indirect effect in ischemic injury [105].

5.3 Caspase 3

The most studied member in the caspase family is caspase 3. Increase in both transcriptional and translational levels for caspase 3 and also activation of caspase 3 are reported after ischemic challenge [100, 106, 107]. Moreover, a mouse lacking caspase 3 showed less ischemic infarcts strongly establishing the involvement of caspase 3 in ischemic injury [108].

5.4 Caspase 6 and Caspase 7

Effector caspases, caspase 6 and caspase 7, are poorly expressed in the brain cell. Elevation of mRNA and protein of caspase 6 and mRNA of caspase 7 is observed after ischemic injury [100, 109]. Due to lack of extensive studies, the role of these effector caspases in ischemic injury is not conclusive.

5.5 Caspase 8

Increase in transcription, translation, and activation is observed for caspase 8 after ischemic stress [100, 109]. Knockout of caspase 8 is lethal for the mice, so no data is available for genetically knocked out mouse [110]. Overall data suggest that caspase 8 has a definite role in ischemic death pathways.

5.6 Caspase 9

Caspase 9 levels remain unaltered after ischemia [100]. But many groups reported enhanced mitochondrial release of cytochrome c to cytosol and thus increase in apoptosome complex after ischemic insult [111]. Therefore, the role of caspase 9 is not conclusive. Even knocking out caspase 9 is lethal so experiments could not be performed in adult caspase 9 knockout mouse.

5.7 Caspase Inhibitors, a New Target for Stroke Therapy

Till now there are no such remedies for progressive neuronal death shortly after a stroke. Tissue plasminogen activator (tPA), which degrades the blood clot, is the only FDA-approved drug for ischemic stroke. Even tPA has large limitations mainly due to short treatment window and medical contradictions, like hypertension [112]. Since cell death is the primary concern after ischemic attack and caspase is the key regulator of this cell death, targeting caspase may be the new approach to reduce the neuronal death and thus prevent ischemic injury. Death receptor blockage, genetic manipulation, or use of catalytic inhibitor can be adopted to fight against the neuronal cell death. Introduction of antibody against death receptors after 30 min of stroke reduced the neural injury in rodent model [113]. As mentioned earlier, expression levels change in different caspases after ischemic insult, and genetic knockout of caspase 1, 3, 6, and 11 gives neuroprotection against stroke. Therefore, genetic alterations caused by knockdown of the caspases may be a logical approach. Recently caspase 3 siRNA-loaded carbon nanotubes has been introduced into cortical motors in rodent model, which gave protection against stroke to the region where it was introduced [114]. Short peptides, based on optimal substrate motif, have been examined as catalytic inhibitors of caspases in rodent model to manipulate the catalytic activity of caspase. Such inhibitors Z-VAD (benzyloxycarbonyl-Val-Ala-Asp) and putative caspase 3 inhibitor DEVD showed neuroprotection against stroke [115]. But due to the high doses required for inhibition and lack of specificity, these peptides can never be tested in clinical trial. Incorporation of the natural protein inhibitors (inhibitors of apoptotic proteins, IAP) against caspase can be a strategy to overcome neural cell death. The only disadvantage is the cellular impermeability of

these proteins. Recently a new approach has been adopted to make these proteins permeable by anchoring some carrier molecules. Penetratin 1 is anchored with the XBIR3 domain of X-linked inhibitor proteins (XIAP) and administered in stroke [116]. Results show promising inhibition of intrinsic apoptotic pathway and neuroprotection in rodent model. The biggest challenge to prepare small molecule drugs against stroke is crossing the blood–brain barrier (BBB). Recently intranasal delivery of drugs has been used to achieve to some extent success to reach CNS bypassing BBB [117]. Although new approaches are coming up, more investigations are needed to attain a potent therapy for stroke.

6 Conclusions

We have explored the involvement of the apoptotic process, more specifically the major perpetrator, the caspase family enzymes, in the sudden damage to the central nervous system caused by stroke or a more chronic insult by the degenerative disorders of the nervous system. We have presented different viewpoints about the efficacy of targeting caspases as means to block cell loss in the brain. Our review also attempts to shed light on the importance of looking seriously for suitable caspase inhibitors which will find clinical applications. Although the use of caspase inhibitors in clinical therapies is still a phenomenon of the future, recent research summarized here indicates that this approach may hold some promise for development of therapeutic strategies.

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Chapter 4
Matrix Metalloproteinases in Ischemia–Reperfusion Injury in Brain: Antioxidants as Rescuer

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Abstract Cerebral ischemia–reperfusion (CIR) injury exerts a potential threat on neuronal cell survival. Cerebral ischemia, a type of stroke, ensues due to occlusion of oxygen in common carotid arteries and blockage of nutrients in brain tissues. It is the most common and lethal neurological disorder especially in the aged individuals. When neuronal cells become deprived of sufficient oxygen because of low blood flow rate following ischemic stroke, a cascade of events occurs, leading to cell death by toxicity and oxidative stress. Oxidative stress appears to be an important role in CIR injury wherein a large amount of reactive oxygen species generated by the mitochondria provokes the release of cytochrome c and other apoptotic proteins, leading to defective gene expression and subsequent cell death. Under CIR, natural defense mechanism fails to protect neurons from oxidative damage. Matrix metalloproteinases (MMPs), mainly MMP-2 and MMP-9, are elevated after cerebral ischemia which are involved in accelerating matrix degradation, disrupting the blood–brain barrier (BBB), and increasing the neuronal infarct size. Some compounds (flavonoids, antioxidants, MMP inhibitors) show the potency as neuroprotectant against CIR. Question arises how to reduce the cytotoxicity of the compounds and overcome the BBB permeability? Over the past few years, different vesicular formulations, especially liposome and nanocapsule, have received attention as effective modality in enhancing therapeutic concentration while rescuing CIR. This chapter is focused on the mechanism of MMPs' action during CIR injury and to delineate the effect of MMP inhibitors and antioxidants with their different formulations in modulating MMP activity.

Keywords Cerebral ischemia–reperfusion • Oxidative stress • Reactive oxygen species • MMP • BBB • Antioxidant • Vesicular formulation

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1 Introduction

CIR is a medical emergency that can cause permanent neurological damage, complications, and even death. Risk factors for CIR include elderliness, high blood pressure, transient ischemic attack (TIA), diabetes, high cholesterol, tobacco smoking, and arterial fibrillation [1, 2]. CIR, which is one of the major leading cause of death worldwide [3, 4], occurs due to oxygen occlusion and obstruction of blood flow or cerebrovascular hemorrhage in brain arteries. Reperfusion injury, opposite to cerebral ischemia, occurs when blood supply returns to the tissue after a period of lack of oxygen during ischemia. The absence of blood flow reduces supply of oxygen and nutrients during the ischemic period while after the restoration of circulation creates a condition of inflammation and oxidative damage via induction of oxidative stress rather than restoration of normal function. Oxidative stress occurs very early after the ischemia–reperfusion injury. Oxidative damage to BBB usually leads to vascular leakage and rupture in ischemic brain tissue [5]. Arachidonic acid released from brain phospholipids during CIR is a major source of free radical generation which in turn mediates BBB disruption and brain edema [6, 7]. White blood cells when carried to the previously ischemic area by the reflowing blood release a host of inflammatory factors such as interleukins and free radicals and give rise to an inflammatory response which is partially responsible for the reperfusion tissue damage. White blood cells may also bind to the endothelium of small capillaries, obstructing them and leading to more severe damage in ischemic condition [6]. On the onset of ischemia restored blood flow reintroduces oxygen within cells and it damages cellular proteins, DNA, and the plasma membrane by causing oxidative stress that, in turn, causes apoptosis.

In prolonged cerebral ischemia (60 min or more), hypoxanthine is formed as breakdown product of ATP metabolism. The enzyme xanthine oxidase acts on molecular oxygen and produce superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet}$). Xanthine oxidase (XO) also produces uric acid which may act both as prooxidant and as scavenger of ROS (H_2O_2, $O_2^{\bullet -}$, $OH^{\bullet}$). Excessive nitric oxide (NO) produced during reperfusion insult reacts with superoxide radical to produce peroxynitrite ($ONOO^{\bullet}$) that attack the cell membrane lipids, proteins, and glycosaminoglycans causing further tissue damage. Reperfusion injury thus also cause hyperkalemia [8]. CIR causes significant elevation in infarct size, XO activity, $O_2^{\bullet -}$ production, lipid peroxidation, and significant depletion of endogenous antioxidants (i.e., reduced glutathione (GSH), superoxide dismutase (SOD), and total tissue sulfhydryl (T-SH) groups systems) in brain and often leads to impairment in short-term memory and motor coordination.

Brain is the most susceptible organ to the ischemic damage and subsequent reperfusion injury [7, 8]. Superoxide dismutase (SOD), catalase, glutathione peroxidases (GSHPx), and vitamin C and E levels in neuronal tissue become insufficient to combat the ROS production after cerebral ischemia–reperfusion [9, 10]. Thus, oxidative damage contributes significantly to neuronal cell death during ischemia–reperfusion injury. So, free radicals are important pathophysiological mediators of cellular

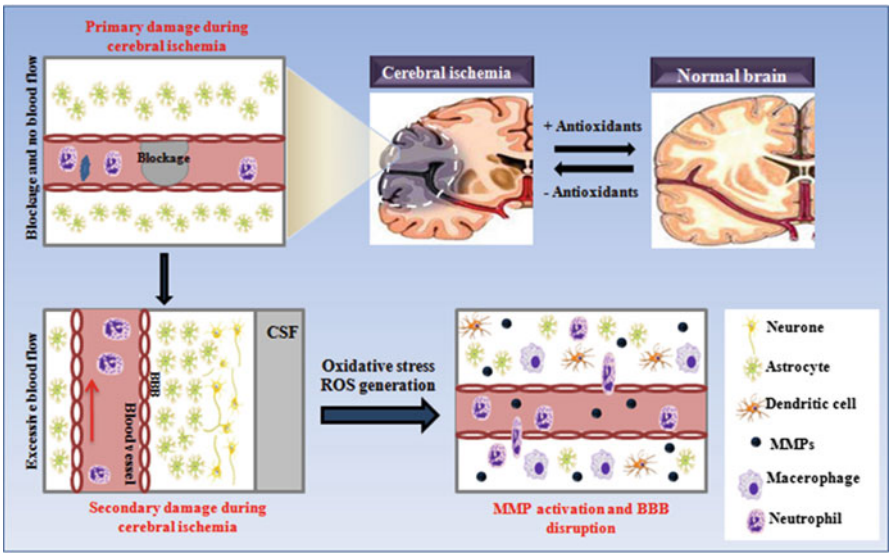

Fig. 4.1 Reactive oxygen species are generated as a consequence of ischemia–reperfusion injury and antioxidants or neuroprotectants reduce oxidative stress by modulating MMPs. Antioxidants reversibly act through interaction among neurons, astrocytes, microglia, and cerebral blood vessels. MMPs attack the basal lamina of endothelial cells and disturb the BBB integrity, leading to infiltration of inflammatory cells (e.g., neutrophils, macrophages)

injury in CIR [11]. These ROS are normally produced in very low amounts as mitochondrial metabolites by activated microglia and endothelial cells [12]. A number of events predispose of the brain to ischemia–reperfusion insult by ROS formation (Fig. 4.1). After reperfusion, a number of events predispose of the brain set off a cascade of biochemical and molecular sequelae due to the production of ROS [13]. Among the ROS, $O^{\bullet -2}$ is believed to be directly toxic to neurons, leading to central nervous system (CNS) damage [14]. Additionally, total sulfhydryl (T-SH) oxidation mediated by these free radicals released during ischemia–reperfusion also participates in xanthine dehydrogenase (XDH)-XO reversible oxidative conversion. Both mechanisms occur due to a conformational change in flavin adenine dinucleotide (FAD) binding domain of the xanthine dehydrogenase [15].

The endothelial cells forming the BBB in the brain are highly specialized structure to restrict important parts of the brain, restricts the entry of foreign particle into the extracellular environment of the brain due to the endothelial tight junction present thereat, but during CIR, parenchymal bleeding is responsible for the leakiness of BBB. Astrocytic end feet encircling endothelial cells were thought to aid in the maintenance of BBB, but recent research indicates that the brain microvessel endothelial cells (BMEC) render important morphological characteristics such as the presence of tight junctions between the cells, the absence of fenestrations, and a diminished pinocytic activity. In acute cerebral ischemia, fine-tuned chemokine responses lead to recruitment of T cells, macrophages, and mast cells into the brain tissue. After CIR, different cytokines such as tumor necrosis factor (TNF-α) and

interleukin-1 (IL-1) and cell adhesion molecules are found to be activated and upregulated [16]. The lymphocytes infiltrate the BBB-releasing proteases, particularly MMP-2 and MMP-9 which help in BBB breakdown. On release of other chemoattractants, polymorphonuclear neutrophils and monocytes enter the brain parenchyma and impose massive oxidative stress on the reperfused tissue, thus resulting in structural and functional change of BBB [17].

Generally, thrombolysis promotes matrix degradation in the ischemic brain parenchyma by MMP-9 activation [17], which imposes oxidative stress via upregulation of inducible proinflammatory enzymes such as nitric oxide synthase (NOS) [18]. It also induces vascular disturbances by downregulating endothelial NOS (eNOS) [19, 20]. As a consequence, neuronal injury occurs in a caspase-8-dependent pathway which is regulated by activated protein C [17, 21]. Thrombolytic therapy or tissue plasminogen activator (tPA) therapy of acute CIR increases both reperfusion damage and hemorrhage risk. The fact that the half-life of tPA itself is short (8–12 min) [22] and exemplifies the profound influence of tPA on acute ischemic injury. Although, till date, the common effectors propagating the actions of tPA remain unknown.

Oxidative insults during CIR result in damage in vulnerable regions of the brain which causes other neurodegenerative diseases. Posttranscriptional regulation is involved in modulating antioxidant enzyme activities during aging. Furthermore, the rate of mitochondrial generation of the $O_2^{\bullet -}$ increases gradually with aging. The increase of oxidative potential and loss of proper antioxidant defense appear to be crucially involved in aging of the brain [23]. So, by introducing biological antioxidants, attempts are made to protect those cells from ROS-mediated damage in CIR [24–29]. In cerebral ischemia and reperfusion injury, antioxidant compounds such as vitamins C and E, β-carotene, and glutathione are tested for their efficacy to protect neuron from oxidative attack, but endogenous antioxidant therapy has not been found to be an effective approach to counter this disease condition [30–33]. Most in vivo and clinical studies on the effects of lipid soluble antioxidant supplementation in combating neurological diseases and aging have focused on vitamin E [34]. It is reported that high doses of vitamin E result in its elevated plasma levels, but fails to increase Vitamin E levels in cerebrospinal fluid (CSF). In spite of therapeutic inconvenience, conjugated antioxidants have been applied against cerebral reperfusion injury [28, 29, 34]. Hence, it is necessary to replenish this exhausted antioxidant population by introduction of large amount of exogenous antioxidants as drug which will act as neuroprotectant to counter the CIR-mediated oxidative attack [35, 36]. This article is focused on the contribution of antioxidant by inhibiting the molecular pathway of apoptosis after CIR and also attenuating the inhibitory activity.

2 Cerebral Ischemia–Reperfusion

CIR, a type of cerebral attack or stroke, ensues when blood flow to the brain gets obstructed resulting in occlusion of oxygen and deprivation of nutrients. Cerebral stroke are of two major types: (1) ischemic stroke and (2) hemorrhagic stroke.

The *cerebral ischemic stroke* occurs due to the blood clot formation which prevents entry of blood into the brain. As a consequence, brain cells suffer from the lack of nutrients and oxygen eventually leading to their death. There are four reasons behind ischemic stroke occurrence, namely, (a) thrombosis (obstruction of blood vessel by local blood clot) [5], (b) embolism (obstruction due to an embolus formed elsewhere in the body) [2], (c) systemic hypoperfusion (general decrease in blood supply, e.g., in shock) [37], and (d) venous thrombosis [38]. Depending on where the blood clot forms, cerebral ischemic stroke can be divided into two subtypes, *thrombotic stroke* where the clot forms inside the cerebral blood vessels and *embolic stroke* where the clot forms elsewhere in the body but travels toward the brain until they become lodged in a narrow artery causing a blockage. Generally, 87 % of the cerebral strokes are of ischemic type and the remaining are hemorrhagic. *Cerebral hemorrhagic stroke*, on the other hand, results from rupture of blood vessel or an abnormal vascular structure. Hemorrhagic stroke occurs due to high blood pressure or aneurism when a weakened portion of the blood vessel balloons out, ruptures, and causes bleeding in the brain. Besides ischemic and hemorrhagic stroke, TIA is also known as pre-cerebral stroke that is regarded as a warning sign of an impending major cerebral stroke. This is a minor cerebral stroke which is also usually caused by blood clot but generally does no significant damage. TIA produces a temporary stroke and then subsides. CIR disables the function of the affected area which eventually leads to an inability to move limbs on one side of the body, inability to understand or formulate speech, and an inability to see one side of the visual field [2], a condition also known as paralysis.

Following CIR, the affected brain tissue ceases to function when it suffers an oxygen-deprived condition for more than 60–90 s and up to 3 h. After ischemia, blood flow burst in during reperfusion that result in tissue death or infarction (for this reason fibrinolytic or antepsin are given only until 3 h since the onset of stroke). Reperfusion injury is caused by return of blood flow resulting in progression of vasogenic edema, hemorrhagic transformation, and an increase in stroke volume. Following cerebral ischemia, the injured area of brain tissue (ischemic penumbra [39]) becomes low in energy due to oxygen and glucose deficiency, and as a consequence energy-dependent processes (such as ion pumping) necessary for cell survival fail. Under such condition, anaerobic metabolism occurs in the brain. Unfortunately, this kind of metabolism produces less ATP but releases lactic acid as by-product which is an irritant that can potentially destroy cells by disrupting the normal acid–base balance in the brain. One of the major causes of neuronal injury is the release of the excitatory neurotransmitter glutamate. The concentration of glutamate outside the cells of the nervous system is normally kept low levels by the so-called uptake carriers, which are powered by the concentration gradients of ions (mainly Na^+) across the cell membrane. However, CIR disrupts this ion gradient homeostasis causing reversal of glutamate transporters direction and thus releasing glutamate into the extracellular space. Glutamate acts on receptors on nerve cells (especially NMDA receptors), producing an influx of calcium which activates enzymes that digest the cellular proteins, lipids, and nuclear materials. Calcium influx also triggers several reactions leading to the activation of lipases and

endonucleases, resulting in the failure of mitochondria which leads further towards energy depletion and cell death by apoptosis [14]. Ca^{2+} activates influx of acid-sensing ion channels (ASICs), thus mitochondrial membrane truncated bid interacts with apoptotic proteins (Bad and Bax) releasing cytochrome *c* (Cyt*c*) or apoptosis-inducing factor (AIF) into cytosol. Cyt*c* then binds with apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to form an "apoptosome," which activates caspase-9 and subsequently caspase-3. Activated caspase-3 cleaves nDNA repair enzymes, such as poly(ADP-ribose) polymerase (PARP), which leads to nDNA damage and apoptosis. Two distinct phases of neuronal cell death, apoptosis or programmed cell death and necrosis may occur following CIR. Although cellular injury mechanisms are different, they coexist within injured brain (neurons in the core being necrotic and in the penumbra being apoptotic) and is related to duration and severity of ischemic insult.

Cerebral ischemia leads to the release of IL-1 and TNF-α by ischemic neurons, and glia generate adhesion molecules (selectin, integrin, intercellular adhesion molecules) in the cerebral vasculature which results in the breakdown of BBB and edema formation. This is also disrupted by enhanced secretion of IL-1 and proteases while TNF-α plays a dual role in the ischemic brain.

Many studies have shown that IL-1β, TNF-α, FGF, and EGF are associated with MMP-9 [40–43] through the MEK1-Erk, P38, and PI3K-Akt signaling pathways in monocytes and neutrophils under CIR [44–47]. MMP-9 promoter sequence contains AP-1 binding site and NF-κB binding that promotes the transcription of MMP-9 expression [48, 49]. Another study of using MMP-9 and chimeric knockouts lacking MMP-9 either in leukocytes or in resident brain cells has shown that MMP-9 is to be the key player in promoting leukocyte recruitment and subsequent BBB breakdown and neuronal injury [50, 51]. The elevated expression of MMP-2 and MMP-9 is severely responsible for BBB breakdown after cerebral ischemia [52–54]. Reports shown that while MMP-9 responses dominate the acute phase of CIR, MMP-2 elevations, on the other hand, seem to occur in the delayed phases [53]. MMP-9 involvement in infarct growth has been elucidated by studies showing its upregulated presence in infarct tissue as well as in the peri-infarct areas [55, 56]. When the integrity of BBB is lost, inflammatory cells can infiltrate the brain, which are otherwise inhibited by tight junctions in BBB endothelial cells, causing hemorrhage, vasogenic edema, and neuronal cell death. Studies have shown Pro-MMP-9 levels in plasma at 24 h to be related with final infarct volumes [57], and its level along with that of activated MMP-9 levels in brain homogenates has progressively increased after permanent middle cerebral artery occlusion. MMP-9, followed by laminin, was reported to be the most powerful and only predictor of the infarct volume and size [58, 59], suggesting MMP-9 as a potential therapeutic target for the treatment of stroke. High plasma levels of MMP-9 are also shown to be independently associated with hemorrhagic transformation in acute ischemic stroke [60] and the baseline MMP-9 level was the only factor independently associated with late hemorrhagic infarction [61]. In addition to MMP-2 and MMP-9, MMP-3 has been found to be a contributing factor in cleavage of the cerebral matrix agrin and subsequent BBB opening after CIR and during neuroinflammation [62, 63]. CIR

also induces oxidative stress which is implicated in tissue injury and death via oxidation of lipids, proteins, and nucleic acids in ischemic tissues or through indirect redox signaling pathways [64–66]. During reoxygenation by spontaneous or thrombolytic reperfusion following ischemia, oxygen serves as a substrate for pathophysiologically relevant enzymatic oxidation reactions in the cytosolic as well as subcellular organelles to produce $O_2^{\bullet-}$ and H_2O_2 [67]. At the period of reperfusion, the endogenous oxidative defenses (namely, SOD, GSHPx, and catalase) fail. Small molecular antioxidants like GSH, ascorbic acid, and α-tocopherol are perturbed and fail in subsequent replenishment of antioxidants. NOS, cyclooxygenases (COX), XDH, XO and NADPH oxidase, myeloperoxidase, and monoamine oxidase are few of the prooxidant enzymes which are known to participate in oxidative stress-induced injury in cerebral ischemia. Among the three isoforms of NOS existent in the CNS parenchyma, neuronal NOS (nNOS) and endothelial NOS (eNOS) act in a Ca^{2+}-dependent mode to induce cell death by *N*-methyl-D-aspartate (NMDA) receptor activation [68], whereas induced NOS (iNOS) acts in a Ca^{2+}-independent manner. Several in vitro and in vivo studies and in vivo ischemia studies have shown that while NO produced by nNOS and iNOS has been associated to ischemic brain damage [69], while NO produced by eNOS, on the other hand, neuroprotection by increasing the cerebral blood flow in the penumbra [70] through vasodilative activities. Indeed, eNOS is reported to be upregulated in the cerebral cortex and hippocampus after transient global cerebral ischemia and reperfusion in indomethacin-sensitive mechanisms [71], whereas induction of iNOS and the formation of 3-nitrotyrosine under oxygen–glucose deprivation are found to kill cerebral endothelial cells by apoptosis [72]. Under cerebral ischemic condition, iNOS-induced NO has been found to enhance COX-2 activity which is also evident from the presence of iNOS-positive neutrophils in close proximity to COX-2-positive neurons [73].

In severe cases of cerebral ischemia, direct mitochondrial swelling and damage occur which causes the inhibition of ATP synthesis and increased ROS production and thus directly causing necrotic cell death. In addition to usual signaling pathway, superoxides can directly regulate cytochrome c-dependent apoptosis. Some reports have shown that overexpression of CuZnSOD reduced the levels of activated caspase-3 [74] and caspase-9 [75] after transient focal cerebral ischemia.

APE/Ref-1, a constitutive multifunctional protein that is also known as a redox factor for redox-regulation-sensitive AP-1 transcription factors [76], is found to be involved in DNA-base excision repair of oxygen radical-induced AP site [77]. Downregulation of APE expression is associated with apoptosis [78] possibly by incomplete repair of AP sites which lead to mutagenesis and genetic instability. The early rapid reduction of apurinic/apyrimidinic endonuclease (APE), which appears to be sensitive to oxidative stress, has been reported in vulnerable hippocampal CA1 neurons after transient global cerebral ischemia [79, 80], which is also supported by the fact that overexpression of CuZnSOD prevents the early reduction of APE in the ischemic brain [81]. In addition to AP-1, many other transcription factors like NF-κB, HIF-1, SP-1, and EIK-1 are known to be redox sensitive [82]. NF-κB is particularly known for its role in regulation of the redox state of a cell [83]. Oxidative stress-mediated activation of NF-κB during reperfusion is detrimental to the

ischemic brain. But due to the involvement of NF-κB in various other cellular functions, its particular role in ischemic brain can be illusive. While overexpression of SOD1 in mice reduces the activation of NF-κB thus leading to reduction of infarction after transient focal cerebral ischemia, on the other side, p50 (a component NF-κB dimer) knockout mice exhibit increased vulnerability of hippocampal neurons to excitotoxic injury [84]. The highly inducible iNOS gene which contains a NF-κB-responsive element at the 5′ region [85] may suggest the importance of oxidative stress-induced NF-κB signaling after cerebral ischemia and reperfusion. Apart from iNOS, the downstream inducible genes also include COX-2, MMP-9, intercellular adhesion molecules (ICAM-1), HOX genes, and cytokines, which are known to be involved in neuronal injury and blood–brain barrier compromise and inflammatory response after cerebral ischemia. MMP-9 promotor sequence contains AP-1 and NF-κB binding site that promotes the transcription of MMP-9. The role of NF-κB activation in regulating these inducible genes is also supported by the finding that overexpression of CuZnSOD in SOD1 mice reduces the expression of MMP-9 and COX-2 after brain injury in CIR [52, 86–88].

3 Antioxidants and Their Formulation in Arresting CIR Injury

The production of ROS is the major detrimental factor in reperfusion injury after CIR [89–91], and endogenous antioxidant enzymes are believed to be the major mechanism behind detoxifying the effects by ROS. Antioxidants have been the focus of interest for the last several years for developing therapeutic agents in attenuating CIR injury and other neurodegenerative disorders.

SOD, an endogenous antioxidant, catalyzes the dismutation of superoxide free radicals and plays an important role in the defense against free radical-induced damage in reperfusion and helps in reducing the infarct size after CIR. Three isoforms of SODs, copper/zinc SOD (CuZnSOD, SOD1), manganese SOD (MnSOD, SOD2), and extracellular SOD (ECSOD, SOD3), are major antioxidant enzymes. CuZnSOD is a major cytosolic enzyme that is constitutively expressed in mammalian cells and has been involved in various CNS disorders. MnSOD is a mitochondrial antioxidant enzyme, whereas ECSOD is an isoform that is localized in extracellular space, cerebrospinal fluid, and cerebral vessels [92–94]. These three SODs dismutate O_2 and form H_2O_2, which is then scavenged by peroxisomal catalase or GSHPx at the expense of GSH which is also an endogenous antioxidant and is regenerated from oxidized GSH by GSH reductase in the presence of NADPH.

CuZnSOD dismutates $O_2^{\bullet-}$ to H_2O_2; overexpression of SOD1 in the presence of developmentally low activities of the catalytic enzymes GSHPx and catalase leads to an increased production of H_2O_2 and may explain the increased brain injury observed after hypoxia–ischemia in neonatal SOD1 transgenic mice.

CuZnSOD has been extensively used in experimental studies on CIR. It is very difficult to use this enzyme for the treatment of cerebral ischemia by systemic

administration because of its short half-life (6 min) and it fails to pass the BBB. However, it has been shown that modified enzyme with an increased half-life, such as polyethylene glycol-conjugated CuZnSOD, is used successfully to reduce infarction volume in rats with CIR. Liposome-entrapped CuZnSOD also has an increased half-life of up to 4.2 h, BBB permeability, and cellular uptake and is reported to be protective against cerebral ischemia and traumatic brain injury [95, 96].

Overexpression of CuZnSOD (SOD1) in transgenic mice resulted in a reduction of infarction volume, edema formation, and better neurological outcomes after transient CIR [97]. In contrast, target disruption of SOD1 in mutant mice resulted in a marked exacerbation of cerebral infarction and edema formation after transient middle cerebral artery (MCA) occlusion [98]. These results suggest that the constitutively expressed CuZnSOD has a potential role in reducing CIR. Overexpression of MnSOD (SOD2) has also been reported to show neuronal protection against oxidative stress after transient CIR as well as ECSOD (SOD3). SOD1 dismutates superoxide to H_2O_2; overexpression of this enzyme in the presence of developmentally low activities of the catalytic enzymes GSHPx and catalase leads to an increased production of H_2O_2 and may explain the increased brain injury observed after hypoxia–ischemia in neonatal SOD1 transgenic mice.

We all know that ROS are directly as well as indirectly involved in signaling cellular pathway in neuronal damage after CIR. During reperfusion, these endogenous antioxidants act as a defensive agent and likely to be disturbed as a result of overproduction of ROS by cytosolic and mitochondrial system, thus fails to replenish damage in ischemic brain tissue.

The causative role of ROS in ischemic brain injury has not yet been determined. The recent development and availability of transgenic and knockout mutant rodents that either overexpress or are deficient in antioxidant genes have provided powerful tools for the molecular and cellular mechanisms of signaling pathways, direct oxidative damage, or both that are involved in ischemic brain injury. Endogenous antioxidants are not enough to combat with oxidative load, and for this reason, sufficient exogenous antioxidants are needed.

Vitamin E, a potent chain breaking lipid soluble antioxidant, reacts with lipid peroxyl radicals and terminates the peroxidative chain reaction and reduces oxidative damage. Reduction of serum vitamin E levels after CIR is directly related to the more production of free radicals. Reduction of vitamin C, a water-soluble antioxidant in the human body, is also directly related to the more production of ROS after reperfusion injury in CIR. It may be due to the exhaustion of antioxidant in the neutralization of free radicals which are formed largely after CIR. Uric acid, the most abundant endogenous antioxidant, may protect against oxidative modification of endothelial enzyme and preserves the ability of endothelium to mediate vascular dilatation.

Quercetin is an important flavonoidal antioxidant which can be found in fairly large amounts in fruits, vegetable oils, red wine, and tea [97, 99, 100]. Owing to its polyphenolic hydroxyl groups, quercetin exhibits its antioxidant property and is effective against neurodegenerative diseases [101–104]. To fight against the oxidative attack produced during cerebral stroke, the present-day challenge is to isolate nontoxic antioxidants of herbal origin that can be selectively targeted to brain.

Ginkgo biloba is the best-selling herbal product in the world which is also a good antioxidant. It is an extract from the green leaves of the Ginkgo tree which is native to Asia but is also grown worldwide. The active ingredients in the extract are the Ginkgo flavone glycoside, bilobalide, and terpene lactones including ginkgolides A, B, and C. Ginkgo biloba extract has been shown to produce neuroprotective effects in different in vivo and in vitro models [105]. Ginkgo biloba has been reported as a potent component to protect neurons from both necrotic and apoptotic death in cerebral ischemia model [106]. Flavonoids, e.g., ginkgo biloba and quercetin, have long been considered as great antioxidants for their extraordinary free radical scavenging properties that are especially effective against cerebral ischemia-induced lipid peroxidation and subsequent cell death.

Cytidine 5′ diphosphocholine (CDP-choline), a neuroprotective drug used in cerebral stroke [107] has shown beneficial effects in a variety of neurodegenerative brain injury cases because of its possible protective effect on cell membrane integrity [108]. But CDP-choline therapy is not an effective approach in combating CIR-induced neurodegeneration. CDP-choline is known to get hydrolyzed into and accumulate as cytidine and choline when administered orally or i.v. [109]. The uptake of cytidine and choline into the brain from the circulation is dependent on transporters located at the BBB. Some antioxidant compounds which are hydrophilic and of size less than 50 nm can cross the BBB easily and can possibly prevent CIR damage easily. For this reason, different vesicular formulations, namely, liposome and nanoencapsulation, are necessary to carry the therapeutic compounds to their target in the brain. Liposomes are well-accepted forms of drug carriers not only because of their biocompatible nature but also because of the negative biological response they elicit as a drug carrier in the living system [110]. Although it is unlikely that large size liposomes (~100 nm) are able to cross the BBB [111], liposomes less than 100 nm are known as effective drug carriers to the CNS, especially during CIR [112]. Again, receptor-mediated carriers are also often used. The mannose receptor is a transmembrane glycoprotein which is expressed in brain cells, particularly on the macrophages and microglia [113], and facilitate endocytosis of any mannosylated moieties binding to liposomes. Mannose receptor expression is noticed in perivascular, meningeal, and choroid plexus macrophages in normal, diseased, and inflamed brain [114]. Thus, because of the presence of these mannose receptors, mannosylated liposomes are effective in site-specific drug delivery to cerebral tissue [115, 116]. To reduce the size (less than 50 nm) extruder is used for liposomal drug preparation. Liposome encapsulated compounds are found efficient in delivering its antioxidant components to the ischemic brain [117].

Nanoencapsulation, on the other hand, has been well accepted in present age as a potent drug delivery mechanism because of its nontoxic, biodegradability, nonimmunogenic, and sustained drug-releasing ability in biological system. The important technological advantages of nanoencapsulation also include high stability in biological system, high carrier capacity, high feasibility of incorporation of both hydrophilic and hydrophobic substances, and feasibility of different routes of administration including oral application. These properties of nanocapsules enable improvement of drug bioavailability and reduction of losing frequency [100, 118, 119].

4 Effect of MMPs in ECM Remodeling After Cerebral Ischemia–Reperfusion

The *extracellular matrix* (ECM) plays a crucial role in maintaining tissue architecture and organ homeostasis. It provides the basic scaffold, which is necessary for the organization of cells into tissues. ECM also regulates various cellular functions by acting as a reservoir of myriad growth factors and cytokines [120, 121]. The ECM is composed of a variety of noncellular, biological macromolecules such as proteins, glycoproteins, proteoglycans, and polysaccharides [122, 123]. The specialized components of the ECM structure such as the basement membrane, which separates endothelium from stroma, comprise of type IV collagen; laminin; fibronectin; linker proteins such as nidogen and entactin, which connect collagens with other protein components; and various growth factors and proteases [124]. Another specialized structure known as interstitial matrix, which is primarily made up of stromal cells, is rich in fibrillar collagens, proteoglycans, and various glycoproteins such as tenascin-C and fibronectin (Fig. 4.2) that contribute to its high charge and hydration state and, most importantly, to the tensile strength of tissues [125].

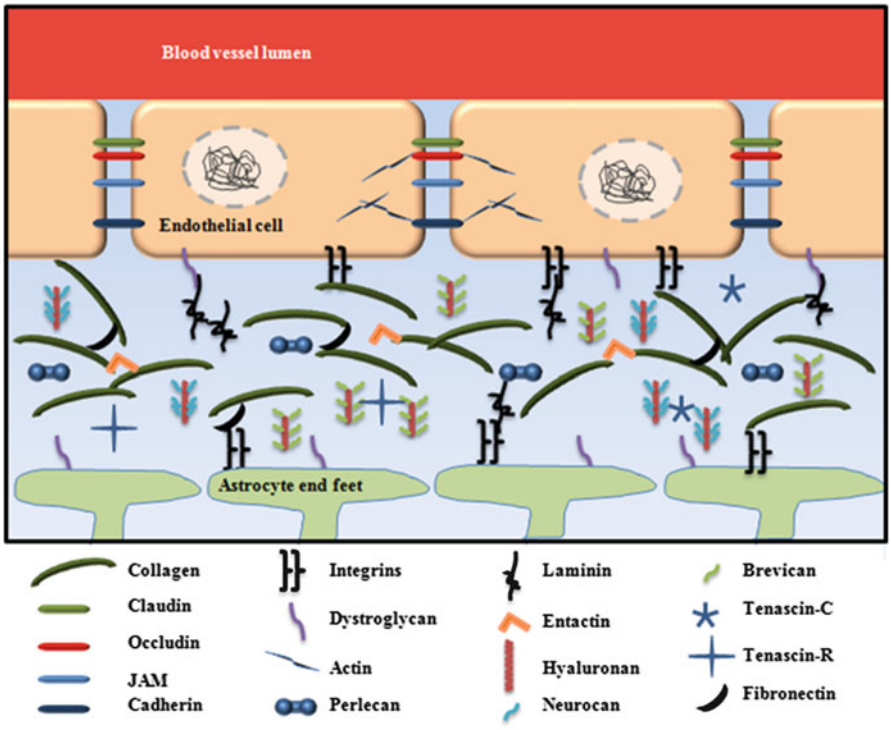

Fig. 4.2 BBB is formed by inter-endothelial cell tight junctions and adherens junctions. The extracellular matrix (ECM) of the basement membrane, pericytes, and astrocytic end feet supports the BBB architectural integrity. Laminin, collagen, fibronectin, perlecan, integrin, and dystroglycan are the main components of ECM. Endothelial cells and astrocytes contribute to the permeability and stability of the BBB in the brain

ECM remodeling is mainly evident during development and maturation. In some cases while extensive tissue remodeling is a part of normal physiology (e.g., bone, mammary gland, adult hippocampus during memory formation, etc.) for some tissue sites, it can also be a consequence of some pathological and repair processes (e.g., neuronal death caused by metabolic perturbations, inflammation, wound healing, oncogenesis, etc.). MMPs play pivotal roles in both cell–cell and cell–ECM interactions by proteolytically degrading or activating cell surface and ECM proteins, which in turn influence cell proliferation, differentiation, migration, and, ultimately, survival as well. Cells under physiological conditions employ a variety of strategies to regulate these MMPs at different levels, i.e., at transcriptional, localization and trafficking, activation of zymogen forms, and inhibition of active forms by its respective endogenous inhibitors (TIMPs). TIMPs are found to regulate MMP activities during tissue or ECM remodeling, which is supported by the fact that a particular TIMP deficiency has shown to have similar phenotype to that of integrin mutants [126]. During remodeling of adult tissues, respective development-associated molecules are upregulated or are reexpressed which otherwise would have been downregulated. Such examples of molecules of the CNS include tenascin-C, an oligomeric glycoprotein [127]; neurocan, a chondroitin sulfate proteoglycan [128]; and the polysaccharide hyaluronan [129]. While tenascin-C and hyaluronan are more permissive to cellular migrations and complex remodeling events [130, 131], on the other hand, neurocan, which connects both tenascin-C and hyaluronan, is somewhat inhibitory for tissue plasticity [132, 133]. Neurocan, apart from its moderate yet constitutive activity in adult rat brain, is generally proteolytically processed by MMP-2 which separates the hyaluronan-binding domain from the tenascin-binding part of the molecule [53, 128].

During maturation of the CNS, characteristic changes in the composition of the molecules of the extracellular matrix occur which, in many cases, typically involve replacement of proteins with members of the same protein family and thus permitting the maintenance of the overall structural organization [134–136]. As, for example, neurocan, versican V1 splice variant, tenascin-C, and cartilage link protein are the typical molecules abundant in developing rodent brain while their homologues brevican, shorter versican V2 splice variant, and tenascin-R are major molecules present particularly in the adult rodent brain [137–139]. Based on their abundance, close temporal expression, and avid physical interactions, brevican and tenascin-R are considered as a representative molecular pair of mature brain matrix molecules, while neurocan and tenascin-C, on the other side, are regarded as representatives of developing juvenile brain matrix [140, 141].

Tenascin-C has also been implicated in the induction and activation of MMPs [120, 127]. Two serine-type proteolytic systems, the plasminogen activator/plasmin system and neuropsin, and their substrates, laminin and fibronectin, respectively, play vital roles in remodeling [142]. The neuroprotective effect of plasminogen activator or plasminogen deficiency was attributed to the maintenance of interstitial depositions of laminin-10 [143]. ADAMTS1 and ADAMTS4 are involved in the proteolytic processing of aggrecan, brevican, and the versican V1 and V2 variant that are the prominent constituents of the adult brain ECM and also play a part in the

pathological matrix remodeling processes [144–146]. ADAMTS1 and ADAMTS4 recognize a defined, centrally localized cleavage site and thus produce an N-terminal hyaluronan-binding glycoprotein fragment and a C-terminal proteoglycan fragment as cleavage products [147]. It has also been reported that tenascin-C mRNA and MMP-9 are significantly upregulated under such disease conditions [148, 149]. In the brain, the extracellular space volume fraction condenses from about 40 % at early developmental stages to about 20 % in adulthood [150] which becomes occupied by the ECM [151]. Hyaluronan, the primary space-filling molecule of the ECM, can aggregate with the lectican family chondroitin sulfate proteoglycans (versican, neurocan, and brevican) to form lampbrush-like superstructures [152–154]. The lecticans have a terminal globular domains that are separated by an elongated, highly glycosylated core region. The link proteins that are able to bind to both the other components consist of two hyaluronan-binding link modules identical to the hyaluronan-binding N-terminal globular domains of the lecticans and a module of the immunoglobulin type. The C-terminal globular ends of the lecticans have been found to bind to glycolipids and various glycoproteins [153, 155]. For example, fibulin-2, a dimeric glycoprotein, is reported to interact with the C-terminal domain of versican, thus playing a role in cross-linking two versican molecules [156].

Oligomeric glycoproteins of the tenascin family, namely, tenascin-R and tenascin-C and recently added member tenascin-N, are found to be especially suitable for cross-linking hyaluronan aggregates in the CNS. Interactions of these molecules with the C-terminal domains of lecticans have been found to be highly promiscuous and particularly specific. For example, tenascin-C can specifically and avidly interact with C-terminal domain of neurocan, but not as such with that of brevican, while tenascin-R shows its preference towards the C-terminal domain of brevican [157–159].

5 Role of MMPs and Their Inhibitors in CIR Injury

CIR injury can result in the loss of structural integrity of brain tissue and blood vessels, partly through the release of MMPs, which are zinc-dependent endopeptidases that break down collagen, hyaluronic acid, and other elements of connective tissue. Other proteases also contribute to this process. The loss of vascular structural integrity results in a breakdown of the protective BBB that contributes to cerebral edema, which can cause secondary progression of the brain injury [160]. The MMP activity is often inhibited by various specific and nonspecific inhibitors. These nonspecific inhibitors include 2-macroglobulin, 1-antiprotease, and BB-94. Among the specific inhibitors, the most important physiological inhibitors are the tissue inhibitors of metalloproteinases or TIMPs. TIMPs are the coding proteins of multigene family, and their expression is tightly regulated during development and tissue remodeling. A total of four types of TIMPs (Table 4.1) have been found in vertebrates. They form the high-affinity complexes with activated MMPs at the molar ratio of 1:1 and inhibit the degradation of ECM by blocking the MMP catalytic domain [161].

Table 4.1 Characteristic features of different TIMPs

Characteristics	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Molecular weight	28	21	24/27	22
Localization	Soluble	Soluble or cell surface	ECM	Soluble or cell surface
Interacting MMPs	Pro-MMP-9	Pro-MMP-2	Pro-MMP-2/-9	Pro-MMP-2

TIMP-1 inhibits the activity of most MMPs except for MT1-MMP and MMP-2 while TIMP-2 inhibits majority of MMPs except MMP-9. Again, while TIMP-3 inhibits MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13, TIMP-4, on the other hand, inhibits MMP-1, MMP-3, MMP-7, and MMP-9. Apart from its inhibiting activities, TIMP-2 can form complexes with MT1-MMP in the cell membrane to activate MMP-2 activity [162, 163]. Corresponding to MMPs, TIMPs play a negative role in the regulation of the ECM metabolism by preventing MMP activation and inhibiting their functions and thus affecting the extent of protein breakdown duration of injury [164]. An imbalance in MMP and TIMP concentration often leads to an overall increase in MMP activity, thus playing a crucial role in excessive matrix degradation under pathological conditions [54]. Apart from their MMP-specific inhibitory activities, TIMP-1 and TIMP-2 are multifunctional proteins with different biological activities. TIMP-1 and TIMP-2 have been shown to have a growth factor-like activity and also to inhibit the angiogenesis while TIMP-3 is reported to be associated with apoptosis [165].

TIMPs inhibit the MMPs activity in two ways. In zymogen activation step, TIMP-2 and TIMP-1 are reported to form stable complex with pro-MMP-2 and pro-MMP-9, respectively. Again in the stage of activated MMPs, both TIMP-1 and TIMP-2 can directly form a tight complex with the activated MMP-9 and MMP-2, respectively, to inhibit their activity [166].

MMP-2 and MMP-9 have been found in CNS particularly in perivascular cells, brain vascular endothelial cells, astrocytes, and microglias under normal physiological conditions. Microglias in activated state have also been found to secrete MMP-9. Human hippocampal pyramidal cells have also been reported to synthesize MMP-9. Several reports suggested MMP-9 expression in the developing mouse embryo brain, suggesting that MMP-9 is related to neurodevelopment. On the other hand, MMP-2 also is often associated with regeneration of axons. Along with their normal physiological roles, MMPs are also believed to play an important role in various pathologic processes of CNS diseases such as in CIR injury [167].

It is reported that the MMP-2 and MMP-9 play a key role in the process of injury and the formation of atherosclerotic lesions, which are one of the major risk factors for developing cerebral ischemia. The overexpression of gelatinases (MMP-2 and MMP-9) by the endothelial cells covered on the plaque can dissolve collagen and significantly alter the plaque composition that leads to the relative increase of the lipid content and thus increases plaque instability. As a result, the plaque cap thins, splits, and eventually leads to cerebral ischemia [168, 169]. It is found that while MMP-1, MMP-8, and MMP-12 are important in maintaining atherosclerotic plaque stability [170], the rupture of atherosclerotic plaque depends on the proportion and

activity of MMP-9 [171]. Under normal condition, cerebral vascular endothelial cells express small basal amount, if any, of MMP-9, while focal increase of MMP-9 activity is regarded as an early warning of acute plaque rupture [172]. Under ischemic and reperfusion condition, brain tissue produces MMP and some other active proteases that lead to the rapid and significant degradation of microvessels [173]. These MMP, especially MMP-2 and MMP-9, activity increases and ultimately leads to cerebral microvascular and BBB permeability, thus resulting in BBB damage, inflammatory cell invasion, and cerebral edema [52, 54, 164, 174]. MMP-9 plays a proinflammatory role in ischemic brain tissue by helping neutrophil and other leukocytes migrate from the blood into the ischemic tissue, subsequently causing BBB damage by proteolyzing microvascular basement membrane and eventually leading to neurological damage [50].

Studies have demonstrated that increased MMP- 9 acts on the tight junctions and basement membrane of the BBB endothelial cells which leads to eventual BBB damage, increase permeability, and vasogenic brain edema with the worst being the occurrence of herniation [175]. MMP-2 and MMP-9 expressed by macrophages may contribute to their exudation into the ischemic lesions and thereby promote wound healing after focal stroke [54]. Activated MMP-9 are also found to act on the cell apoptosis cascade in the damaged area after transient cerebral ischemia [176]. MMP-9 can also cause damage by degrading the myelin basic protein of brain white matter after ischemic brain. It is reported that like MMP-2 and MMP-9, MMP-1 not only had a direct proteolytic role by digesting collagen types I, II, and III but also played a pivotal part in the MMP (importantly MMP-2 and MMP-9) activation cascade [58].

Studies have shown that exogenous inhibitors could reduce the ischemic and reperfusion injury by reducing the burden of increased MMP activity after CIR [54, 164]. Thus, MMP inhibitors may be looked upon as a new potential treatment modality for combating CIR injury [177]. MMP inhibitors are indeed found to reduce the incidence of acute plaque rupture by reducing MMP-9 activity. Cerebral ischemia and subsequent reperfusion cause the increased proportion of TIMPs which combine with their corresponding MMPs and inhibits their function to stabilize the ECM and thus significantly reduces BBB damage and brain edema after ischemia. The initial BBB breakage during CIR injury was linked to increased MMP-2 level while later opening was correlated with increased MMP-9 activity [55, 164, 178, 179].

6 Avenues in the Future: Targeting MMPs for Therapy in CIR Injury

In the normal individual, cerebral vascular endothelial cells do not or negligibly express MMP-9. But after the induction of cerebral ischemia-reperfusion, brain tissue can produce some active MMPs and consequently lead to the degradation of microvessels [172], increase in cerebral microvascular permeability, invasion of inflammatory cells, and cerebral edema [52, 54, 164, 174]. The activity of MMP-9

is normally increased in the early stages of stroke and proinflammatory response, while the activity of MMP-2 act in the repair phases of stroke. It has been shown that MMP-9 is expressed in neutrophil 1 week after infarction while the macrophage expresses matrilysin and MMP-2 1 week later. The level of MMP-2 is higher in the previous history of cerebral ischemia [179]. Thus, MMP-9 plays an important role in secondary brain damage while MMP-2 is involved in tissue repair and regeneration.

Application of MMP-9 monoclonal antibody significantly reduces the infarction volume in a rat model of ischemia [54]. It is reported that matrix metalloproteinase inhibitors and MMP-neutralizing antibodies can reduce the vasogenic brain edema and infarction volume [55]. In addition, MMP inhibitors are also effective in preventing atherosclerosis and ischemic brain damage. So, reducing the activity of MMP-9 is one of the important targets for the therapy of CIR.

The balance between MMP and TIMP is very much crucial in any given normal or pathophysiological condition. TIMP-1 and TIMP-2 play important roles in endogenous repair particularly during angiogenesis, reestablishment of cerebral blood flow, and regulation of the neurogenic response. Increase in TIMP-1 and TIMP-2 level is found to be concurrent with the decreased MMP-9 and MMP-2 levels, respectively, which further illustrates the importance of their respective proportions in the system [164]. The use of commercially available synthetic inhibitors of MMPs like BB-94 and BB-1101 further confirms the relation between BBB opening and brain edema following CIR and MMPs, as their application was found to reduce or even prevent both the consequences of CIR [54, 164, 174]. Hence, MMP inhibitors can well be employed for cerebrovascular disease treatment [177]. MMP inhibitors are found to combine with the divalent cation in vitro and inhibit the leukocyte-associated MMP function, thus reducing reperfusion injury. In support, MMP inhibitors and MMP-specific antibodies can reduce the vasogenic cerebral edema and infarction volume [55]. Urokinase or rt-PA in conjugation with the synthetic MMP inhibitors (BB-94, BB-1101, doxycycline, etc.) can well be used in thrombolysis treatment which will reduce the extent of hemorrhage after thrombolysis and the thrombolytic time window can be extended [178, 180]. The reason behind the fact working efficiency being the fact that MMP inhibitors' early application, before subsequent rt-PA, or urokinase treatment will result in inhibition of MMP-1, MMP-2, MMP-3, and MMP-9 (which are the key players of BBB damage), thus maintaining BBB and blood vessel integrity and increasing the overall safety of thrombolytic therapy [174, 179].

It has been demonstrated that the novel roles for MMPs and TIMPs in the regulation of neuronal cell death and apoptosis are through the MMP modulation of excitotoxicity, anoikis, death receptor activation, and neurotrophic factor bioavailability. MMPs and TIMPs provide therapeutic targets to abrogate neuronal death and can continue for weeks to months within the penumbra surrounding the necrotic core of the infarct. MMP inhibition is considered to be a therapeutic target, so when, where, and how it provide either detrimental or beneficial effects during the injury and repair phases during injury? MMP inhibitors are excellent candidates for therapeutics if administered selectively at the appropriate time points. MMP-9 has been suggested to be a promising therapeutic agent because its activity rises after CIR and either genetic or pharmacological inhibition of MMP-9 shows protection against CIR [148].

7 Conclusion

In CIR, MMPs play an important role by degrading the ECM and destroying the BBB which leads to brain edema, secondary brain injury, and ultimately cerebral infarction. MMP inhibitors are found to be effective in preventing the ischemic brain damage. Thus, MMPs may be regarded as a new potential target for CIR therapy and MMP inhibitors can be used as therapeutic agent in cerebrovascular diseases. In addition, vesiculated compound that attenuate MMP activity may also be looked upon as a next generation therapeutic neuroprotectant in combating the CIR injury. Thus, nanoformulation using biocompatible and biodegradable compound can be an attractive and effective means in designing a novel drug delivery mode for treatment of CIR.

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Chapter 5
The Role of Matrix Metalloproteinases in Neurovascular Injury

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Abstract Cell–matrix homeostasis is vital in the CNS. In a large number of CNS disorders, abnormal activation of extracellular proteases may disrupt neuronal function by degrading neurovascular matrix integrity. This chapter surveys the role of a key family of extracellular proteases, the matrix metalloproteinases (MMPs), in stroke and brain injury. Blood–brain barrier (BBB) leakage and brain edema is a critical part of stroke pathophysiology. A large body of data in both experimental models as well as clinical patient populations suggests that MMPs may disrupt BBB permeability and interfere with cell–cell signaling between neuronal, glial, and vascular compartments. Hence, ongoing efforts are underway to validate MMPs as potential therapeutic targets as well as biomarkers in stroke. Because BBB perturbations may also occur in neurodegeneration, MMPs and associated neurovascular mechanisms may also be potential targets in a broader range of CNS disorders.

Keywords Matrix metalloproteinase • Edema • Hemorrhage • Blood–brain barrier • Stroke • Trauma

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1 Introduction

The concept of the neurovascular unit emphasizes that central nervous system (CNS) function requires cross talk between all cell types in neuronal, glial, and vascular compartments. In order for these cell–cell signals to operate, intercellular matrix integrity is vital. Hence, any disorders that abnormally activate extracellular proteolysis may disrupt matrix homeostasis, interfere with normal cell–cell and cell–matrix signaling, and further amplify disease pathophysiology. In this regard, enzymes from the family of matrix metalloproteinases (MMPs) have been shown to play significant roles in a wide spectrum of CNS disorders including stroke, brain trauma, and neurodegeneration. This chapter will survey the role of MMPs in mediating neurovascular injury in the context of stroke.

2 The Blood–Brain Barrier and Edema

From a clinical standpoint, disruption of neurovascular matrix most clearly manifests as damage to the blood–brain barrier (BBB). The BBB comprises a critical interface between the CNS and the rest of the body. Its primary function is to help regulate the microenvironment of the CNS. To do so, the BBB functions both as a true barrier and a dynamically controlled influx/efflux transport system for handling a wide of metabolic and signaling factors.

Historically, the BBB was thought to be solely mediated by tight junctions between cerebral endothelial cells [1, 2]. However, recent data now suggest that the system is much more complicated. Astrocytes and pericytes are now known to be vital contributors in barrier function as well [3, 4]. Additionally, the BBB is now also considered as part of the "neurovascular unit"—a concept that emphasizes the importance of cell–cell signaling between all cell types residing in neuronal, glial, and vascular compartments [5–7]. Hence, BBB leakage in CNS disorders can be thought of as merely one manifestation of neurovascular unit dysfunction [8, 9].

In the context of brain injury and disease, BBB leakage and neurovascular unit dysfunction is most clearly observed clinically as brain edema. Classically, brain edema has been defined as either cytotoxic (swelling and intracellular accumulation of water in astrocytes and neurons) or vasogenic (accumulation of water in extracellular space) in origin [10, 11]. In acutely ill patients, brain edema most commonly leads to central and systemic complications due to elevations in intracranial pressure, regardless of whether the edema is "cytotoxic" or "vasogenic." Indeed, it is now accepted that these theoretical concepts underlie a more complicated reality where BBB dysfunction and leakage comprise a mix of extracellular and intracellular responses in multiple cell types.

The spatial and temporal evolution of BBB leakage and edema is also complicated, depending on the type of injury or disease. There are many mechanisms at play, including loosening of tight junctions, alterations in transporters, alterations in

pinocytosis, degradation of matrix lamina, etc. In acute stroke, biphasic patterns of BBB permeability have been described with openings and closings taking place during ischemia–reperfusion [12]. The BBB is not an inert static barrier, intact or breached. But rather, BBB permeability and transport are dynamically regulated—with various degrees of opening and closing depending on the nature of molecular factors or size of tracers involved.

Ultimately, the BBB is an interface where all components of the neurovascular unit come together. Connecting all these elements together is the neurovascular matrix with substrates including collagen, laminin, fibronectin, etc., all vulnerable to MMP degradation. Hence, overactivation of MMPs after stroke or brain injury will disrupt the neurovascular matrix, interfere with cell–cell signaling, and induce a wide spectrum of neurological dysfunction. Due to the limited scope of this chapter, the reader is referred to many excellent reviews on MMPs, the BBB, and brain for more in-depth dissections of these important topics [13–17].

3 Matrix Metalloproteinases and the Neurovascular Unit

When stroke or brain injury occurs, MMPs become dysregulated and further amplify damage to the neurovascular matrix. MMPs comprise a large family of extracellular zinc endopeptidases [14]. But in the context of stroke, the largest amount of data may exist for the gelatinases MMP-2 and MMP-9. In animal models of focal and global cerebral ischemia, MMPs are upregulated, and treatment with MMP inhibitors prevent neuronal cell death, decrease infarction, and improve outcomes [18–20]. Knockout mice that lack MMP-9 show significantly reduced brain cell death after cerebral ischemia or traumatic brain injury [21–24]. Conversely, transgenic mice that overexpress tissue inhibitors of metalloproteinase (TIMP) have better outcomes [25].

Mechanistically, the data in animal models fit well with the premise that high levels of MMPs can damage neurovascular matrix and cause BBB injury, edema, and hemorrhage [13]. Degradation of various basal lamina and tight junction proteins has been correlated with BBB leakage and blockade of MMPs reduce edema [21, 23]. Matrix proteolysis and BBB disruption was reduced in knockout mice lacking MMP-9 [23]. MMP activation and BBB leakage also appears to coincide with the generation of free radicals. And as neurovascular injury continues to evolve, recruitment of cytokines and vascular adhesion molecules add onto the accumulating tissue damage and may even further amplify MMPs and inflammation [26].

Beyond vascular leakage per se, MMP-mediated proteolysis of neurovascular matrix may also interfere with homeostatic signals between different cell types in the neurovascular unit. Resting matrix signaling via integrins is vital for normal cell function. Disruption of extracellular matrix by MMPs can induce anoikis in neurons and cerebral endothelial cells [27, 28]. In animal models, degradation of matrix correlates with cell death [29]. In a nonhuman primate model of focal cerebral ischemia, areas where matrix antigens are lost correspond to growing regions of collapsing penumbra and dying cores [30]. The importance of these matrix signals

is further confirmed in fibronectin knockout mice in which neuronal apoptosis and brain damage are amplified after cerebral ischemia [31].

From a molecular perspective, matrix coupling may also help sustain trophic coupling between cells of the neurovascular unit. For example, cerebral endothelial cells may be a rich source of trophic factors such as FGF and BDNF, and this type of vascular neuroprotection is a critical defense against multiple insults such as hypoxia, oxidative stress, and perhaps even amyloid-beta [32, 33]. In white matter, an analogous oligovascular unit may exist. Cerebral endothelial cells and astrocytes also may produce trophic factors that sustain and protect oligodendrocyte precursor cells against injury [34, 35]. By interfering with these vital interactions between multiple cell types, MMP-mediated disruption of matrix–trophic coupling in the gray and white matter may significantly contribute to stroke and trauma pathophysiology.

4 Biphasic Properties of Matrix Metalloproteinases and Brain Plasticity

MMPs play a deleterious role during acute stroke by augmenting BBB disruption, edema, hemorrhage, and brain injury. However, emerging data now suggest that MMPs may play biphasic roles. During the acute stages of stroke, MMPs are deleterious. But during delayed phases of stroke recovery, MMPs may play surprisingly beneficial roles [36, 37]. In part, this duality of MMP phenotype may be related to its original physiologic roles in normal development of brain morphology [38]. In developing brain, these proteases modify extracellular matrix to allow newborn cells to migrate and neurites and axons to extend and connect. Additionally, MMPs may also facilitate the actions of other signaling molecules. For example, MMP-9 may be an "angiogenic switch" by processing and releasing bioactive VEGF to promote vascular growth and/or remodeling [39]. MMP-9 has also been implicated in associative learning in the hippocampus. The broad-spectrum MMP inhibitor FN-439 interferes with long-term potentiation [40]. MMP-9 knockout mice display deficits in learning and memory [41].

During stroke recovery, the brain attempts to remodel. MMPs may be recruited as part of this endogenous recovery process. So blocking MMPs at the wrong place or wrong time may worsen outcomes. Following focal cerebral ischemia in mice, endogenous neurogenesis is amplified in the subventricular zone and newborn neuroblasts are diverted from their original rostral migratory stream toward the damaged brain [42]. This process requires MMPs, and delayed blockade of MMPs disrupts neuroblast migration [43]. At 2 weeks after focal strokes in rats, a secondary upregulation of MMPs in peri-infarct cortex can be detected in astrocytes and endothelial cells [44]. Late blockade of MMPs in this model system is damaging since MMPs appear to mediate VEGF processing, compensatory angiogenesis, and stroke recovery [44]. Hence MMP inhibitors can sometimes lead to beneficial reductions of acute edema, while resulting in impaired long-term recovery [45]. Similar biphasic properties of MMPs may also exist in spinal cord injury, where

MMP-2 is increased together with reactive gliosis [46]. But genetic deletion of MMP-2 exacerbated white matter damage and decreased motor recovery [47]. Of course, not all MMP-mediated plasticity is guaranteed to be beneficial. How MMPs augment normal or abnormal rewiring in recovering brains after stroke or trauma remains to be fully elucidated.

5 Matrix Metalloproteinases in Clinical Stroke

Because MMPs can degrade the neurovascular matrix and interfere with cell–cell signaling between neuronal, glial, and vascular cells, they have been proposed as potential biomarkers in clinical stroke and brain injury. Plasma levels of MMP-9 are elevated during acute stages of both ischemic and hemorrhagic stroke and appear to be correlated with poor neurological outcomes [48, 49]. In animal models of embolic stroke, tPA amplifies MMP-9 [50]. Emerging clinical data may be consistent with the experimental literature. Patients with higher plasma levels of MMP-9 may be more susceptible to hemorrhagic transformation following tPA thrombolysis for acute ischemic strokes [51, 52].

In addition to serving as positive protein signals in plasma, MMP responses have also been detected in genetic and brain compartments of stroke patients. After ischemia or brain injury, circulating blood cells show rapid alterations in gene expression. In particular, responses in MMP-9 genes are highly conserved [53, 54]. In the brain parenchyma itself, MMP-9 positive astrocytes colocalize with peri-hematoma edema [55]. After ischemic strokes, MMP-9 positive neutrophils appear to coincide with local disruptions in microvessels [56, 57]. Taken together, these signals are broadly consistent with data and mechanisms derived from experimental models.

Beyond their utility as biomarkers, MMPs have also been pursued as potential therapeutic targets. In animal models of focal cerebral ischemia, MMP inhibitors reduce infarct volumes when administered early during acute ischemic strokes. Consistent with its proposed mechanisms, MMP inhibitors appear to be especially effective in terms of reducing brain edema and hemorrhage. One aspect of this pathophysiology with particular clinical relevance may be the relationship between MMPs and tPA thrombolysis. tPA is known to bind several lipoprotein receptors in cerebral endothelium that can upregulate MMP-9 [58]. Therefore, it is possible that some of the hemorrhagic transformation complications seen in tPA-thrombolysis patients may be caused by an inadvertent increase in MMPs [51, 52]. An obvious question is whether clinically acceptable compounds can be used as MMP inhibitors in stroke thrombolysis. In this regard, minocycline has been proposed as a potential “re-purposed drug” to target MMP-9 [59]. In experimental clot-based models of focal stroke in hypertensive rats, minocycline plus tPA as combination stroke therapy seems to suppress MMP-9, decreases hemorrhagic transformation, and widens the therapeutic time window for safe and effective reperfusion [60]. Based in part on these experimental data, clinical trials have been started [61]. Initial findings are promising as minocycline appeared to dampen plasma MMP-9

biomarker levels, as hypothesized [62]. Nevertheless, some caution might be warranted. As discussed earlier, MMP blockade may interfere with endogenous recovery after brain injury, and long-term use of minocycline worsened outcomes in an ALS clinical trial [63].

Although the majority of clinical MMP data has been collected in ischemic strokes, recent efforts extend the role of these proteases to hemorrhagic strokes as well. MMPs are upregulated in subarachnoid hemorrhage patients, and blood and CSF levels of MMP-9 may track vasospasm and clinical outcomes [64]. Mechanistically, MMPs contribute to early brain injury and may also process gelsolin that can further amplify neuroinflammation [65]. In experimental models of subarachnoid hemorrhage, MMP inhibition improves outcomes [66]. Whether these targets work clinically remains to be determined.

6 Neurovascular Abnormalities and Neurodegeneration

The data implicating MMPs in neurovascular matrix injury appear to be strongest in stroke and brain trauma. However, accumulating data now suggest that similar mechanisms may operate in other CNS disorders. MMP-2 may be upregulated in the brain tissue of human patients with Parkinson's disease [67]. Blockade of MMPs can decrease cell death, neuroinflammation, and functional impairment in some experimental animal models of Parkinson's disease [68]. MMPs are also induced by and can process amyloid [69, 70]. In Alzheimer's disease and cerebral amyloid angiopathy, abnormal activation of MMPs may contribute to the BBB pathophysiology and neurodegeneration [71].

BBB leakage is commonly associated with edema in stroke and brain trauma. But BBB disturbances may also be important in other CNS disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, ALS, multiple sclerosis, etc. [16]. Furthermore, BBB dysfunction is not always a binary "open or shut" phenomenon. There may be gradations in BBB permeability that reflect subtle perturbations in cell–cell signaling within the entire neurovascular unit. Therefore, MMP-mediated perturbations in neurovascular matrix integrity may impact a wide spectrum of neurodegenerative disorders.

7 Conclusions

The extracellular matrix within the neurovascular unit comprises a critical locus where cell–cell signaling operates. Damage to the neurovascular matrix disrupts function in the entire neurovascular unit. In this chapter, we briefly surveyed the roles of MMPs as mediators of neurovascular injury. In the context of stroke, a large body of preclinical data and accumulating clinical findings support a role of MMPs both as biomarker and as potential target. However, some caution may also be

warranted since MMPs can play biphasic roles after brain injury—deleterious in the acute phase but potentially beneficial in delayed remodeling and recovery. Further dissection of the complex actions of MMPs in neurovascular function is warranted.

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Chapter 6
The Role of Proteases in Embryonic Neural Crest Cells

Avi Bar, Irit Shoval, Efrat Monsonego-Ornan, and Dalit Sela-Donenfeld

Abstract As the embryo develops, multiple cellular events of division, differentiation, migration, and invasion occur. Cells are formed at specific locations and migrate along different axes to various destinations, by acquiring diverse types of molecular machineries and processes. One such process is the epithelial-to-mesenchymal transition (EMT), in which epithelial cells with highly ordered shapes and contacts transform into mesenchyme in order to start migration. Consequently, these separated cells react to intracellular and extracellular signals to travel through different microenvironments along stereotypical, long-distance migratory routes to their precise homing targets. Different types of proteases are necessary to execute such complex events. One excellent system to evaluate cell movements during embryonic development is the population of neural crest cells. These unique cells are initially formed as part of the neural epithelium, but then they undergo a dramatic EMT after which they extensively migrate and differentiate into various fates including craniofacial skeleton, skin pigments, and peripheral nerves. In this review, we will discuss the central roles of proteases, mainly the family of matrix metalloproteases, in facilitating neural crest cell migration, and propose an integrative model to suggest the orchestrated action of two such proteases in these developmental events.

Keywords Matrix metalloproteinases • Neural crest cells • Extracellular matrix • Epithelial-to-mesenchymal transition • Tissue inhibitor of MMPs • A disintegrin and metalloproteinases • Cell migration • Embryo

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1 Introduction

Neural crest cells (NCC) comprise a discrete embryonic cell population, vertebrate's specific, which arises from the dorsal margins of the neural tube. This multipotent population of cells responds to an array of signals, including cellular communication between NCC themselves, as well as secreted factors from neighboring tissues, and migrates via known and defined routes in the embryo, where they differentiate in the anterior–posterior axis to a vast number of derivates. These include most of the sensory neurons, ganglia, and Schwann cells of the peripheral nervous system, as well as the pia and arachnoid matters that cover the brain and spinal cord; the majority of bone and cartilage tissues of the head and face, such as the frontal, nasal, premaxillary, maxillary, mandibular and temporal bones, Meckel's cartilage, and tooth dentin; and connective tissues in the cardiac system such as the septa of the heart outflow tract and cushion cells of the heart valves. NCC also develop into all skin melanocytes and yield the secreting cells of endocrine tissues such as the thymus, thyroid, and adrenal glands [1–8].

The development of NCC, which begins approximately at week 3 in humans and embryonic day 8 (E8) in mice [9, 10], involves many steps from early induction up to final differentiation and relies on accurate genetic plan, specific factors, and expression pattern: (1) while born at the lateral edges of the neural plate, NCC acquire a unique specification program that distinguishes them from their neighboring neuroepithelial cells; (2) after the closure of the neural tube, they localize to the dorsal-most part of the neural tube and face a dramatic epithelial-to-mesenchymal transition (EMT) that enables them to separate and to begin to migrate extensively throughout the embryo in a well-controlled process; (3) they are required to stop migrating, home, and differentiate in various embryonic tissues to form their astonishing range of cell types [2, 11–14]. Given the complex ontogeny and multipotency of NCC, it is not surprising that numerous birth defects are associated with NCC. Abnormal NCC development has been shown to be responsible for the majority of craniofacial congenital defects as well as to pathologies in skin pigmentation, innervations, heart, and secretion glands. Few examples include cleft palate, craniosynostosis, neurofibromatosis, dysautonomia, Hirschsprung's disease, and persistent truncus arteriosus. Many types of syndromes including DiGeorge, craniofrontonasal, velocardiofacial, Treacher Collins, and Waardenburg are also associated with NCC defects. Intriguingly, although the migration and differentiation of NCC are highly regulated, several of the most aggressive tumors, including melanoma, neuroblastoma, and glioblastoma, are of NCC origin [11, 15–20].

2 NCC and the Extracellular Matrix

The inductive signals for NCC formation include bone morphogenetic proteins (BMPs), Wnts, fibroblast growth factors (FGFs), and retinoic acid (RA), which are also highly crucial for many other processes in embryonic development.

These signals set off an array of cascades which lead to NCC survival, segregation, and delamination from the dorsal neural tube [1, 3, 21–24]. One central finding showed that BMP4, along with its secreted inhibitor noggin, are active in the dorsal neural tube in a dynamic pattern to regulate emigration of neural crest progenitors from the neuroepithelium. The balance between these two factors was shown to be at highly importance for this process which involves an intimate crosstalk between the embryonic paraxial mesoderm and neural primordium [22, 23]. Another recent study elucidated the role of FGF and RA in regulating the timing of NCC migration. FGF was found to prevent NCC EMT (through the MAP kinase pathway), whereas RA signaling triggers EMT, and the balance between the two signaling factors determines the right timing for noggin and Wnt expression, which are required for NCC delamination [24]. These and many other studies indicated an interface between different signaling molecules and pathways, in the complex process of NCC EMT and migration.

The processes of EMT and migration of NCC involve dramatic changes in cell behavior and extracellular matrix (ECM). These includes modifications in cell–matrix interactions, such as the breakdown of the basal lamina of the neural tube, switches in the expression of cell–cell and cell–matrix adhesion molecules, changes in cytoskeleton assembly, development of motility, and the expression of guidance molecules [2, 3, 11, 16, 25, 26]. Taking into consideration these dramatic changes in ECM properties and cell interactions, the composition and remodeling of the ECM should play a central role in the acquiring of cell motility. The ECM is composed of a complex mixture of insoluble molecules including collagens, glycoproteins, and proteoglycans. In addition, to provide a solid-state support for cells, it also acts as a reservoir for embedded cytokines and growth factors, as well as by harboring cryptic information within the molecules that makes up the ECM network itself. Receptors at the cell surface provide signals for cells to sense their microenvironment and react to stimuli. Therefore, the state of macromolecules within the ECM and its interaction with neighboring cells are of critical importance [27, 28].

Proteolysis is a major process leading to changes in the ECM by affecting the adherence of cells to the ECM, the release of bioactive fragments, the sequestering of growth factors and cytokines, as well as the shedding of receptors on the cell surface [28]. Previous studies described that the ECM plays an active role in the migration of NCC by regulating the migration itself, as well as by providing directional cues via permissive and nonpermissive molecules, such as fibronectin, several laminin and collagen isoforms, and aggrecan [29–31]. For instance, ECM molecules which hold opposing effects on NCC migration are thrombospondin-1 and F-spondin. These two extracellular proteins participate in many cell-to-cell and cell-to-matrix communications and were shown to be implicated in neural cell adhesion and neurite extension [32, 33]. Thrombospondin-1 and F-spondin promote and inhibit NCC migration, respectively, and hence mediate directional migration of NCC into the rostral half of the somite, rather than to its caudal half [34, 35].

Detachment of cell–cell contacts is a critical event to allow EMT and separation of NCC to single mesenchymal cells. Cadherins are a large family of calcium-dependent, cell–cell adhesion molecules. Members of this family are widespread

and the alternation in their expression patterns is highly important for NCC emigration. Prior to migration, NCC transform from the epithelial form, which expresses type I cadherins (such as E-cadherin and N-cadherin), to mesenchymal form, which expresses type II cadherins (such as cadherin-5, cadherin-7, and cadherin-11). This shift in the distribution of cadherins is critical for the NCC to migrate [36–41]. Type II cadherin 6b is also required to be downregulated in NCC upon migration from the dorsal neural tube and was shown to control the timing for NCC delamination in embryos [42–44]. Finally, laminins are a family of glycoproteins which are major component of basement membranes (basal laminae). Laminin has a principal role in allowing NCC migration [29–31, 45, 46], and its absence results in NCC migration abnormalities [42]. Laminin role in NCC is coupled with integrin receptors, to mediate the adhesion and migration of the cells, as functional knockout of integrins using antisense oligonucleotides results in reduced attachment of NCC to laminin in vitro and reduced migration in vivo [47].

3 Matrix Metalloproteinases in the ECM

Given the pivotal role of the ECM in NCC delamination and migration, both as a surrounding and supporting matrix, and as the provider of directional cues, it is highly plausible to speculate involvement of matrix remodeling proteases in these processes. A significant family of proteases that acts in the ECM is the matrix metalloproteinases (MMPs). At present, at least 28 family members have been reported [48]. Each MMP has distinct but often overlapping substrate specificity, and together they can cleave numerous extracellular substrates, including virtually all ECM and basal-lamina proteins [49]. The MMPs are grouped to four subfamilies according to their domain structure and substrate specificity: collagenases, gelatinases, stromelysins, and the membrane-type MMPs (MT-MMPs), which are collagenases that contain a transmembrane domain and are hence anchored to the cell membrane. All MMPs have an N-terminal signal sequence that enables their extracellular secretion. The N-terminal pre-domain is followed by a propeptide that maintains enzyme latency (pro-MMP) until the pro-domain is removed. Next is the catalytic domain that contains the conserved zinc-binding motif, since MMPs depend on metal ions for their catalytic activity. Following are a specific cleavage site and a binding site. A secondary substrate-binding site is located outside the active site itself [50–52]. Most MMPs have a hemopexin-like domain that is involved in tissue inhibitor of MMPs (TIMP) binding, substrate binding, and some other less understood proteolytic activity. In addition to their ECM substrates, MMPs also cleave cell-surface molecules and other pericellular nonmatrix proteins, thereby capable to regulate cell behavior in several ways [53]. MMPs influence diverse physiologic and pathologic processes, including tissue morphogenesis and growth, wound repair, inflammatory diseases, and cancer [49, 54]. For example, gelatinases A (MMP2) and B (MMP9) were shown to be overexpressed in a variety of malignant tumors, where their overexpression and activity are often associated with tumor aggressiveness and poor prognosis, thus making gelatinases as cancer biomarker

[55]. Both gelatinases, as well as membrane-bound collagenase MMP14 (MT1-MMP), were also reported to directly regulate angiogenesis [56]. Intriguingly, in addition to their secretion and localization at the membrane or the ECM, MMPs were also reported to be expressed in the cell nucleus in different tissues, suggesting their ability to cleave also nuclear matrix proteins. The existence of nuclear matrix has been studied and revealed functions in DNA replication, transcription, and RNA splicing, indicating possible role of MMPs in such processes [57–61]. For example, among MMPs localized to the cell nucleus are the gelatinases in cultured reactive astrocytes [62] and the stromelysin MMP3 (stromelysin-1), which is not only localized to the nucleus but possesses a transcription factor-like function [63]. Moreover, the ECM itself was suggested to affect gene expression [64], which strengthens the role of MMPs in gene expression regulation. Yet, much is unknown regarding such possible intracellular roles of MMPs in embryos and adults.

As multiple MMPs exist within an organism, each with its own profile of expression, accumulation, activation, inhibition, and clearance, as well as its own, sometimes broad, range of preferred substrates, multiple control levels of MMP activity are required. In vitro evidences have shown that MMP expression is regulated by several cytokines and growth factors, including interleukins, FGF, and transforming growth factor-β (TGF-β). Many of these stimuli induce the expression/activation of c-*fos*/c-*jun*, which bind to activator protein-1 sites within several MMP promoters [49, 50, 65, 66]. In addition to being differentially regulated at the level of transcription, the activation of pro-MMP into an active protease is stepwise and can be achieved by protease cleavage of the pro-domain. Since some MMPs were found to activate other members of the family, a specific hierarchy among these proteins is crucial to be maintained [49, 57, 66].

MMP activity is also controlled by endogenous inhibitors. Tissue inhibitor of metalloproteinases (TIMP) is a family of four members termed TIMP1–4. The overall shape of the TIMP molecules is like a wedge, which slots into the active-site cleft of an MMP in a substrate-like manner, thus preventing MMP activity. Among the TIMPs, TIMP3 is also capable of inhibiting other types of proteases such as a disintegrin and metalloproteinases (ADAMs) and aggrecanases, whereas TIMP2 holds inhibition ability but also assists in the activation of pro-MMP2, in a context-dependent manner [66]. According to the importance of TIMPs in the MMP's activity balance, they hold diverse biological function, including cell differentiation, signaling, cytoskeleton changes (leading to growth and migration), invasion, angiogenesis, and apoptosis. Like MMPs, TIMPs are also present in the nucleus, which further strengthens the possibility for transcription influence by TIMPs themselves, as well as by MMPs [57, 58, 66, 67].

4 The Role of MMPs in NCC Migration

Given the importance of the ECM in NCC motility, and the fundamental role of MMPs in remodeling the ECM, effects of MMPs in promoting the detachment of NCC from the neural tube and facilitating their migration in the embryo are anticipated.

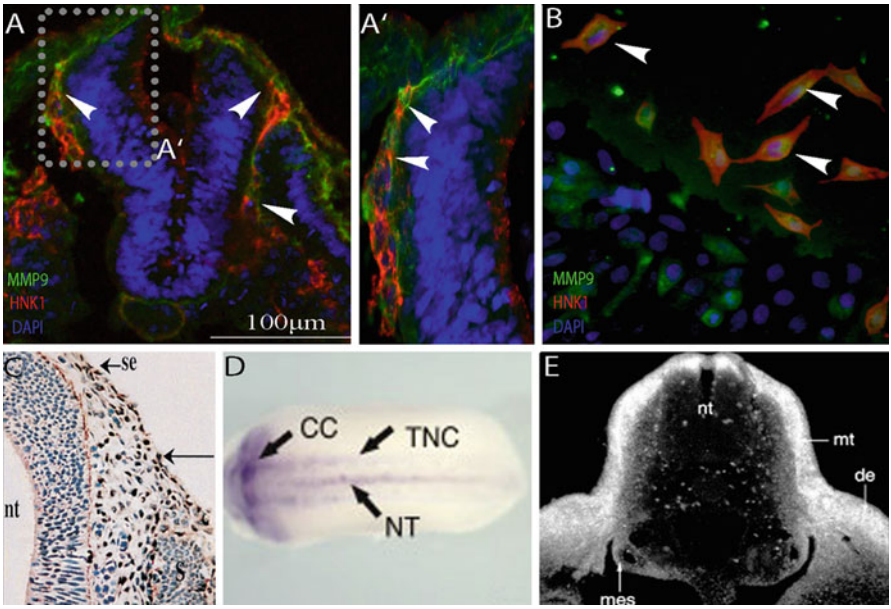

Fig. 6.1 The expression of MMPs and ADAMs in neural crest cells. (**a**, **b**) Immunofluorescence labeling of neural crest cells (NCC) using MMP9 (*green*) and HNK1 (*red*) antibodies. (**a**) A transverse section from the trunk level of an embryo of 25 somites. (**b**) An ex vivo explant of NCC obtained from the hindbrain of 6–8 somite-staged embryo. *Blue* staining (DAPI) represents cell nuclei. *White arrowheads* indicate MMP9- and HNK1-positive NCC. Panel **a′** is an enlargement of the *boxed areas* in panel **a**. Reproduced with permission, Developmental Biology, Monsonego-Ornan et al., 2012 [70]. (**c**) Immunohistochemistry on a cross section of an E9.5 mouse embryo demonstrating expression of MMP-8 (*arrow*) in NCC migrating in the dorsal lateral pathway under the surface ectoderm and in the ventromedial pathway between the neural tube and a somite. Adapted with permission, Matrix Biology, Giambernardi et al., 2001, [71]. (**d**) In situ hybridization on stage 22 *Xenopus laevis* embryo using MMP14 antisense riboprobe. Streams of migratory NCC express MMP14 are evident (*arrow*). Reproduced with permission, Developmental Dynamics, Harrison et al., 2004, [72]. (**e**) In situ hybridization on a transverse section obtained from the trunk level of day 4 chick embryo, using ADAM 10 antisense riboprobe. Expression persists in the dorsal neural tube, dermis, myotome, and mesonephros. Adapted with permission, Developmental Biology, Hall and Erickson, 2003, [137]. Abbreviations: *se* surface ectoderm, *nt* neural tube, *s* somite, *CC* cephalic neural crest, *TNC* trunk neural crest, *de* dermis, *mt* myotome, and *mes* mesonephros

A first support for MMP's role in NCC can be supplied by their expression patterns in several models. For instance, MMP2 was demonstrated in chick NCC when they undergo EMT at the dorsal neural tube [68] and later in cardiac NCC when they migrate into the heart [69]. Moreover, we have recently discovered that the other member of the gelatinases subfamily, MMP9, is expressed in emigrating NCC of avian embryos along the entire cranial-trunk axis of the dorsal neural tube, as well as in motile NCC and in their migration routes in the surrounding mesoderm [70] (Fig. 6.1a, b). The secreted collagenase, MMP8, was found in mice embryonic NCC and in their melanocyte derivatives in adults [71] (Fig. 6.1c). MMP14 was reported

to be expressed in cranial and trunk NCC in frog (*Xenopus laevis*) (Fig. 6.1d) [72] and chick embryos (Roth L., Blum M., and Sela-Donenfeld D, unpublished results). At later stages of NCC development, when they differentiate into craniofacial skeletal derivatives, multiple types of MMPs, such as MMP3 and gelatinases MMP2&9, were also demonstrated in many craniofacial tissues [73–77].

Whether the expression of MMPs in NCC is associated with their activity to promote their development in mammalians is an important question. One support for the function of MMPs in cranial NCC development in humans originated from the discovery of mutations in the MMP2 gene. Individuals with these mutations manifest a disorder involving characteristic facial features such as a sclerotic cranial sutures, narrow nasal bridge, and bulbous nose. As these tissues largely arise from NCC, such defects may be attributed to their impaired development. Yet, other pathologies connected to ECM degradation were also found in these patients, in NCC-unrelated tissues, such as in the body skeleton [78–80]. MMP2-null mice also displayed defects in the development of both cranial and long bones [81, 82], although the phenotype was less severe than the human syndrome. Furthermore, mice mutated for the other gelatinase, MMP9, showed no defects in a known NCC derivate, although exhibiting impaired angiogenesis in the growth plate of adults [83]. On the other hand, an association between craniofacial impairments and MMP9 polymorphism was suggested in humans [84]. Along this line, a different set of experiments found that general MMP inhibition altered Meckel's cartilage development in mice [85, 86] and that MMP2 acts in the leading processes of cranial NCC when they migrate from the first branchial arch to the nasal primordia, as well as in ECM remodeling during periodontitis and in postnatal development of cranial sutures [77, 87, 88]. Since MMP2 and MMP9 are the only existing gelatinases, a possible compensation between these MMPs may explain the weak or no effect in NCC in the single knockout mice versus their effects on NCC in more restricted experimental systems. Hence, while the abovementioned studies suggest an involvement of gelatinases in the ontogeny of mammalian NCC, future studies are required to examine their activity during earlier embryonic stages as well as to assess for their compensatory actions.

Further support for the function of MMPs in mammalian cranial NCC arises from the analysis of MMP14 null mice, which displayed some cranial features similar to those found in the MMP2 human mutation [89]. Since MMP14 was shown to activate MMP2 in vivo in lung and skin tissues [90], it is possible that a deficiency in either of these enzymes led to the common pathology in craniofacial NCC development. Moreover, mice deficient for both membrane-type MMPs, MMP14 and MMP16, were found to be born with multiple developmental defects in collagen-rich tissues, including a severe dysfunction in palatal shelf formation, and to die shortly after birth. This phenotype indicates for the critical role of MT-MMPs in the development of craniofacial NCC derivatives in utero. Whether these non-secreted MMPs act directly on NCC and their ECM or act by activating other soluble MMPs remains unclear [91, 92]. Finally, an association between a polymorphism in MMP3 and cleft lip/palate has been observed [93]. As MMP3 was also shown to be expressed during palatal morphogenesis [73, 94], it is possible that this protease also plays a role in the development of cranial NCC derivatives. Taken together,

while multiple evidences suggest an involvement of MMPs in the correct forming of the cranium and face in mammalians, their possible activity during early stages of NCC development awaits further research.

Additional work examining the direct role of MMPs in murine NCC was carried out in the enteric nervous system. This system is formed by extensive migration of cervical and lumbar NCC into the gut, where they differentiate into autonomic neurons responsible for the gut peristaltic movements [95, 96]. MMP2 activity was reported to be important for the migration of the enteric NCC in mice embryos and for the formation of the neural networks within the developing gut [97]. In this study, Ret^{TGM+} mice, which express GFP in all enteric NCC, were utilized. When explants of mid-plus hindgut from E11.5 mice were cultured in the presence of a general gelatinase (MMP2/MMP9) inhibitor, the migration distance of enteric NCC was reduced. To verify which of the gelatinases is central to this process, gelatinase activity was assessed by gelatin zymography assay in gut samples, revealing MMP2 but not MMP9 activity to be essential for enteric NCC migration in mammalian. Yet, as no gut-related disorder was reported in MMP2-null mice [81, 82], this research further supports the hypothesis of compensation between different MMPs in the genetic mice mutants.

The direct role of MMPs in executing the EMT and migration of NCC in young embryos was more thoroughly investigated in other embryonic models. In the chick embryo, application of a synthetic MMP inhibitor into the cell-free space, adjacent to the pre-migratory cardiac NCC at the second somite level, inhibited their ability to migrate [98]. Furthermore, concordantly to showing MMP2 localization in pre-migratory avian NCC, Duong and Erickson [68] utilized tools to inhibit MMP2 in vivo and ex vivo using pharmacological inhibitors and electroporation of morpholino-antisense oligonucleotide sequence against MMP2 that prevents its translation. These treatments prevented NCC from undergoing EMT and detaching from the dorsal neural tube. Yet, prevention of MMP2 activity in NCC that were already commenced in migration did not prevent their motility suggesting that MMP2 is involved in promoting EMT but not motility of NCC. However, another study claimed that MMP2 plays a slightly later role in the avian embryonic NCC which is restricted to cardiac NCC movements [69].

In *Xenopus laevis* embryos, both MMP2 and MMP14 were suggested to play an essential and specific role in melanophore migration [99]. This study was conducted using a small molecule compound (NSC 84093) that selectively inhibited the migration of melanophores. As MMP2 and MMP14 were shown to be expressed in these NCC derivatives, this study suggested that NSC 84093 acts as a putative MMP2/14 inhibitor. These findings were confirmed using specific MMP2 and MMP14 antisense morpholinos, which led to a loss of the dorsal and lateral stripes of melanophores in the developing embryo. Notably, a more dramatic effect was gained in the MMP14 morphants compared to MMP2 morphants, in agreement with the phenotypes gained in the similar knocked-out mice [81, 82, 89]. As MMP2 zymogen was previously found to be activated by MMP14 in vivo [90], such a result may support the hypothesis of MMP14 acting to promote NCC migration upstream to MMP2, in addition to possible other MMP2-unrelated mechanisms.

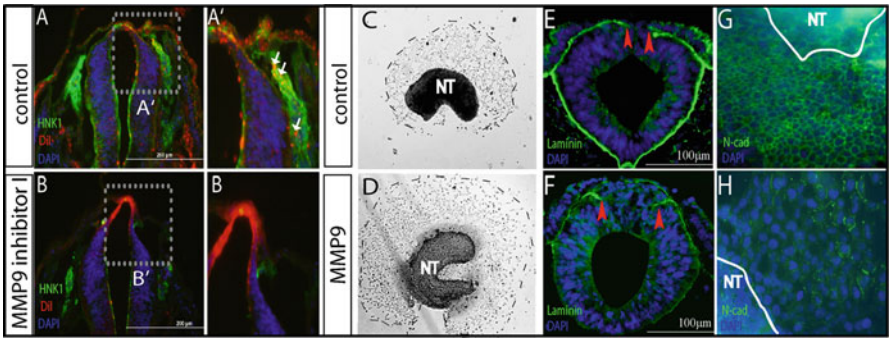

Fig. 6.2 MMP9 is required for neural crest cell migration in avian embryos. (**a**, **b**) Chick embryos of 12 somite-stage were injected into the neural tube with control (**a**) or MMP9 inhibitor I (**b**) solutions, both containing the membrane fluorophore CM-DiI. The following day embryos were harvested, transverse frozen sections were obtained from the hindbrain axial level, and immunofluorescence was conducted. Panels **a′** and **b′** are enlargement of the *boxed areas* in panels **a** and **b**. *Red* represents CM-DiI labeled cells, *green* represents migratory NCC which express HNK1, and *blue* (DAPI) represents nuclear staining. *White arrows* in **a** mark DiI- and HNK1-positive NCC, which are missing in **b**. (**c**, **d**): *Bright field* images of isolated neural tubes obtained from the caudal trunk levels of 16 somite embryos grown for 16 h in control conditioned media (**c**) or in MMP9-containing media (**d**). *Dashed circles* represent borders of NCC migration area, which significantly enlarges upon MMP9 addition. (**e**, **f**) Chick embryos of 12 somite stage were injected into the neural tube with control media (**e**) or MMP9-enriched media (**f**). Embryos were harvested 6 h later and forwarded to transverse sectioning followed by immunofluorescence using laminin antibody. *Green* represents laminin and *blue* (DAPI) represents nuclear staining. Gaps in laminin (*red arrows*) are wider in the basement membrane around the neural tube treated with excess MMP9 (**f**), compared to control (**e**). (**g**, **h**) Isolated neural tubes were obtained from the hindbrain level of 6–8 somite-old embryos and allowed to grow for 16 h to allow NCC migration. The next day, explants were added with control media (**g**) or MMP9-containing media (**h**) for 30 min and analyzed using anti-N-cadherin (N-cad) antibody. Typical N-cad staining is observed in the cell membranes of early migrating NCC (*green*) of control explants (**g**). Excess MMP9 abolished N-cad staining in NCC surface (**h**). *Blue* (DAPI) represents nuclear staining, and *white lines* represent borders of neural tubes. Abbreviation: *NT* neural tube. Adapted with permission, Developmental Biology, Monsonego-Ornan et al., 2012, [70]

We have recently discovered a new player that participates in the delamination and migration of avian NCC, MMP9 [70]. MMP9 was found to be expressed in pre-migratory and migratory NCC along the entire rostral-caudal axis of the embryo as well as along NCC migratory pathways in the nearby mesoderm (Fig. 6.1a). Inhibition assays using a specific pharmacological inhibitor against MMP9 [100] in the head and trunk regions of the embryo showed the requirement of active MMP9 in the promotion of both EMT and migration of NCC, which were significantly diminished in this experiment (Fig. 6.2a). Molecular knockdown experiments were also carried out by electroporating an antisense morpholino against MMP9 into the pre-migratory NCC, which resulted in a similar prevention of NCC migration in the embryo, without perturbing their viability or earlier specification. The reciprocal affect of excess MMP9, either via adding MMP9-enriched media to ex vivo explants or by overexpression MMP9 cDNA into the neural tube in vivo, resulted in accelerated EMT and

migration of NCC, compared to controls (Fig. 6.2b). This effect also appeared in younger areas of the neural tube where typically NCC are not yet engaged in migration, implicating for the sufficient application of MMP9 to trigger the separation and migration of NCC. This impact of MMP9 was associated with loss of epithelial morphology and enhanced transition toward mesenchymal phenotype of NCC, together with a dramatic reduction of the neuroepithelial cell marker N-cadherin, in the treated embryos. Analysis of the mechanism by which MMP9 executed the migratory phase of NCC revealed its function in breaking N-cadherin and the integrity of laminin at the basement membrane around the dorsal neural tube to enable the cell emigration (Fig. 6.2c). Together, these findings, illustrated in Fig. 6.4, revealed for the first time the prominent role of MMP9 in the EMT and migration of NCC in the avian model, which is mediated through its digestive activity on ECM components.

Since MMPs are highly homologous between vertebrates and seem to be involved in NCC migration and differentiation in several species, it is logical to expect a well-established, evolutionary conservation of their activities. However, in contrast to avian, amphibian, and mammalians, in zebrafish embryos much less is known about MMPs' involvement in NCC. Nevertheless, several reports described disruptions in zebrafish craniofacial morphogenesis upon knockdown of MMP2, MMP9, MMP13, and MMP14 [101, 102]. Further indirect support for the involvement of MMPs in zebrafish cranial NCC was provided in a study where elevation of glucocorticoids increased the embryonic expression of MMP2, MMP9, and MMP13, which was linked to changes in craniofacial morphogenesis [102, 103]. Craniofacial abnormalities were also induced by gasoline oxygenates along with decreased MMP2 and MMP9 transcript level in zebrafish embryos [104]. Finally, as cell invasion factors and remodeling of the ECM were suggested to be required for NCC migration in the zebrafish [105–107], it is highly plausible that MMPs play a key role in this process.

Given MMP roles in early NCC stages and in the subsequent development of their derivates, it is expected that TIMPs may also be involved in NCC ontogeny, where they can play a role in either inhibition and/or facilitating MMP activity. TIMP2 was shown to be expressed and specifically involved in regulation of early migrating cardiac NCC in the chick by facilitating MMP2 activation [108]. TIMP2, along with TIMP3, was elucidated to possess an important, but unique, role in early cardiac development in chick. TIMP2 was predominantly associated with continued cardiac cushion development, and TIMP3 was predominantly associated with myocardial remodeling [109]. Furthermore, TIMP1 and TIMP2 mRNA transcripts were localized to the ossifying areas along the outer edges of mice Meckel's cartilage, indicating a potential role in forming this structure [85, 86]. TIMP3 affects head and axial tissue formation, where overexpression during frog embryogenesis leads to defects in the head and in the dorsal axis [110]. Altogether, these data implicate for the significant effects TIMPs hold on NCC, both in early NCC migration and in later NCC derivatives.

A non-desired outcome of NCC is the developing of cancer, such as neuroblastoma, which is a common pediatric solid tumor derived from primordial NCC. MMPs are pivotal for the progression of many types of cancer, where their expression is tightly correlated with tumor aggressiveness and poor prognosis [56, 111]. Concomitantly, MMP2 and MMP9 were found to be expressed in neuroblastoma [112], where MMP2 activity in these cells was found to be strongly associated with

elevated MMP14 levels, advanced stages of the tumor, and poor clinical outcome in these patients [113]. Furthermore, TIMP3 was reported to successfully restrict the NCC-derived neuroblastoma growth in mice [114]. MMP2 and MMP9 were also reported to exhibit reduced expression and activity upon treating NCC-derived human tumor cell lines with the anticancer drug valproic acid (VPA), whereas TIMP1 expression was increased following exposure to VPA [115]. These reports elucidate not only the involvement of MMPs in cancer tumors of NCC origin but also the role of TIMPs in these processes and the importance of the delicate balance between MMPs and TIMPs in normal and malignant tissues.

Another type of cancer which originates from the melanocyte derivative of NCC in the skin is melanoma. This highly invasive tumor exploits many similarities to the well-controlled program of embryonic NCC migration, including the expression of MMP2 and additional EMT and cell migration genes, such as *Myb* [116]. A recent study which compared between the transcriptomes of normal NCC and aggressive melanoma revealed a significant upregulation of MMP2 expression in the cancer cells compared to embryonic NCC [117]. Hence, unraveling the regulatory mechanisms that govern the controlled expression of MMPs in naive embryonic NCC would be significant for better understanding of these differences as well as for developing innovative treatments for the nonregulated expression of MMPs in cancer.

In addition to MMPs, several other intrinsic factors, such as members of the Twist and Snail families of transcription regulators, control NCC development and tumor progression. Since both processes share multiple cellular characteristics, any information on the regulation of EMT and cell motility via a crosstalk between MMPs and intracellular factors may be beneficial for developmental biology and cancer research [25, 27, 36, 49, 56, 118]. Indeed, additionally to their well-established ability of MMPs to cleave ECM components, several studies have shown an intracellular function of MMPs in the expression of the transcriptional repressor factor *Snail*, which is an important pro-tumorigenic factor in many cells. Snail acts by repressing the expression of E-cadherin during several EMT processes, including in NCC, leading to the separation and spreading of cells [119–124]. In mouse mammary epithelial cells, treatment with MMP3 led to the expression of an alternatively spliced form of the small GTPase protein Rac1, which in turn stimulated the expression of *Snail*. Although the data was not presented, the authors imply that similar effect was mediated by MMP9, but not MMP2 [124]. Therefore, while we have recently discovered that MMP9 is capable to cleave E-cadherin and laminin in NCC to promote their separation [70], it awaits to be discovered whether, in addition to its direct proteolytic actions on ECM substrates, it also modulates migration of NCC in the embryo's expression regulation of central genes such as *Snail*.

5 The ADAM Family of Metalloproteinases in NCC

In addition to MMPs, the large family of a disintegrin and metalloproteinases (ADAMs) has also been shown to participate in promoting NCC development [125]. So far, 40 family members have been identified in the mammalian genome [126, 127]

and also in other species [128]. The ADAMs are type I membrane-anchored metalloproteinases with an extracellular domain and disintegrin and cysteine-rich domains [128]. Their substrates include cell adhesion molecules, ECM proteins, growth factors, and cytokines, as well as receptors and ligands of signaling pathways [129]. The ADAMs are widely expressed in embryos and adults and play fundamental roles during different developmental processes by their ability to regulate cell–cell and cell–matrix interaction upon cleaving the extracellular domains of cell-surface proteins and the ECM. Through these functions, ADAMs modulate differentiation, migration, and even transcriptional activation in different cells [130].

Different studies have investigated the proteolytic ability of ADAMs to shed cell-surface proteins that can either activate (i.e., Notch or epidermal growth factor (EGF) ligands) or inactivate cleaved proteins (i.e., ephrin, cadherins), leading to different extracellular and intracellular outcomes [129]. Apparently, the ectodomain shedding by ADAMs is the rate-limiting step before further cleavage events within the plasma membrane. This process, termed regulated intermembrane proteolysis (RIP), is best known for Notch proteolysis [131], in which ADAMs cleavage is followed by further cleavage by the γ-secretase complex, generating an active intracellular Notch domain that transduces signaling events into the cell nuclei.

Another major group of ADAM substrates are the cadherin family of adhesion molecules. For instance, ADAMs were found to control the cleavage of N-cadherin in mouse and chick embryos [132–135] and of cadherin-11 in *Xenopus laevis* embryos [136]. As these cadherins are crucial players in NCC development and migration, the participation of ADAMs in these processes was assumed. Indeed, ADAMs were shown to be localized in several embryonic models to NCC and surrounding tissues. For example, ADAM10 is expressed in dorsal neural tube of avian embryos and in emigrating NCC in vitro [137] (Fig. 6.1e), as well as in colocalization with N-cadherin in the neural tube of E9.5 mouse embryos [134]. Other ADAMs, such as ADAM12 and ADAM13, were found in later NCC derivatives in chicks, such as in craniofacial structures, as well as in the mesoderm which is tightly contacted with migratory NCC [138, 139]. Concomitantly, in *Xenopus laevis*, ADAM13 and ADAM19 were both demonstrated in the somitic mesoderm, the neural tube, and cranial NCC [140, 141]. Together, these expression data fit well with the possibility of ADAMs playing roles in the modulation of NCC migration.

Determination of ADAM's function in NCC development using mice knocked-out for ADAMs is not yet possible, since most mutants die early in development due to deficits in multiple cellular pathways. For example, ADAM10-deficient mice die at E9.5 and show multiple Notch-related defects of the developing central nervous system, somites, and cardiovascular systems [142]. Moreover, these mutants are much smaller than WT mice, probably due to abnormal high accumulation of cadherins, compared to WTs [133]. Yet, these mice were used to develop ADAM10-deficient fibroblast cell lines and to demonstrate the ability of ADAM10 to cleave cadherins, leading to reduction in cell–cell adhesion and promotion of cell migration and separation in vitro. Experiments using these fibroblasts also showed that the accumulation of cadherins at the fibroblast cell membranes sequestered β-catenin, leading to prevention of its translocation to the nucleus and alterations in intracellular signaling [133, 134].

Concomitantly with these findings, ADAM10 was demonstrated to promote NCC EMT in the trunk of avian embryos, via its cleavage of N-cadherin in pre-migratory NCC [135]. In its typical cell-surface localization, N-cadherin negatively regulates NCC delamination by maintaining their epithelial cell–cell contacts in the dorsal neural tube, whereas its downregulation enables them to undergo EMT [43, 135] (Fig. 6.3a, b). N-cadherin was reported to be cleaved extracellularly by ADAM10 in HEK293 cells and in primary cultured cells from the cerebral cortex of fetal mice [143], generating a C-terminal fragment, termed CTF1, which was further processed by γ-secretase to form the soluble intracellular CTF2 fragment, which is in turn involved in the regulation of gene expression in multiple different systems [134, 144, 145]. In the context of NCC, Shoval et al. revealed that upstream control of BMP signaling in triggering NCC delamination [22, 23] is mediated by its ability to promote N-cadherin degradation through ADAM10 activity. The ability of BMP to trigger NCC EMT was blocked by the addition of GI254023X, a specific ADAM10 inhibitor, which preserved N-cadherin in its intact structure (Fig. 6.3d–g). As such, the persistent N-cadherin actively antagonized β-catenin-dependent signaling induced by BMP/Wnt and consequently prevented NCC migration. Conversely, the CTF2 end-product of N-cadherin cleavage by ADAM10 was found to rescue the inhibitory effect of GI254023X and to actively promote NCC migration by enhancing β-catenin-dependent signaling in the nucleus (Fig. 6.3c, h, i). Thus, ADAM10 cleavage of N-cadherin was suggested to play a key role in NCC emigration by converting the negative modulation of N-cadherin into a stimulatory intracellular signal, downstream of BMP [135], as illustrated in Fig. 6.4.

Other members of the ADAM family were also found to participate in cranial NCC development in frog embryos. Knockdown of ADAM19 in *Xenopus laevis* caused reduction in the expression of NCC markers, perturbation in the migration of cranial NCC, and impairment in somite organization [146]. ADAM13 was also suggested to be involved in NCC de-adhesion and migration as well as in myoblast differentiation in this species [140]. Interestingly, in *Xenopus tropicalis*, ADAM13 was found to act earlier in the induction of cranial NCC. Cleavage of Ephrin B1/B2 by ADAM13 in neural-progenitor cells was found to be required for prevention of canonical Wnt signaling, which led to appropriate levels of *Snail2* expression and to NCC induction [147]. Furthermore, ADAM9 and ADAM13 were found essential for cranial NCC development in frogs upon cleavage of cadherin-11 [136]. At variance from N-cadherin, cadherin-11 is expressed in migrating NCC and necessary for their mobility. The extracellular domain of cadherin-11, which is the end-product of ADAM cleavage, was found to stimulate NCC migration. Moreover, this extracellular fragment could also rescue the migration of cranial NCC that was inhibited by knocking-down ADAM13, via a non-cell autonomous mechanism. For instance, the cleaved cadherin-11 was shown not only to directly reduce cell adhesiveness but also to bind to full-length cadherin-11, thus reducing cadherin-11 cell–cell interactions and facilitating motility. Notably, ADAM13 function was recently suggested to be required in the 3-dimentional context of the embryo, as inhibition of cranial NCC migration upon ADAM13 depletion could only occur in vivo [148]. This study suggested that ADAM13 is involved in cleaving fibronectin and/or other ECM proteins, resulting in the opening or widening of the dense 3-dimentional

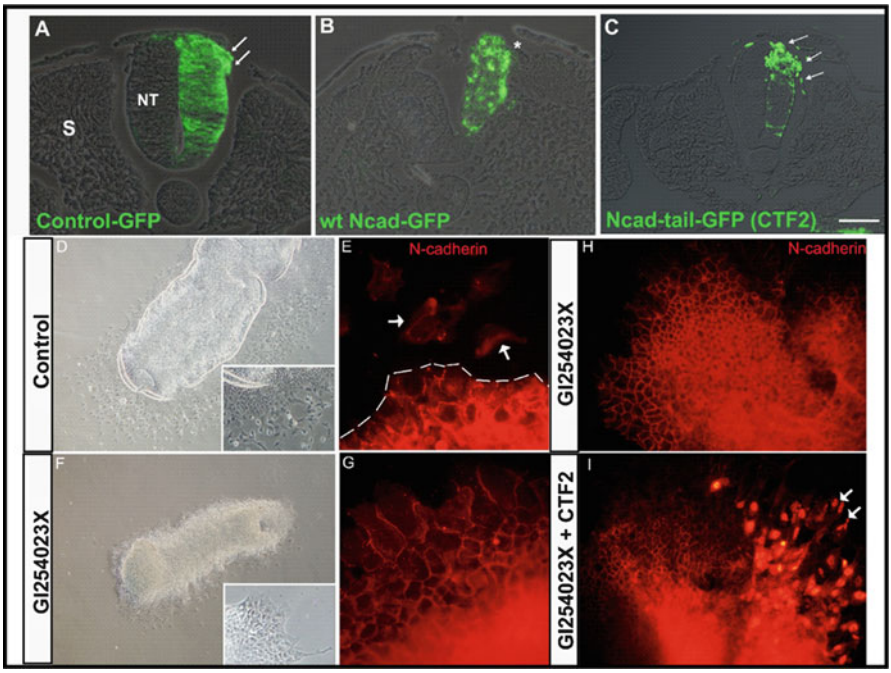

Fig. 6.3 N-cadherin cleavage by Adam10 is required for the generation of EMT in neural crest cells. (**a**–**c**) Transverse sections of chick embryos that were unilaterally electroporated with different plasmids into the trunk level of the neural tube 20 h earlier. (**a**) Control GFP plasmid shows typical early delaminating NCC (*arrows*) opposite to a dissociating somite. (**b**) Full-length N-cadherin-GFP-expressing plasmid inhibits NCC delamination opposite to an already dissociated somite (*asterisk*). (**c**) The N-cadherin tail CTF2-GFP plasmid stimulates an enhanced NCC migration (*arrows*). (**d**–**g**) *Bright field* (**d**, **f**) and immunofluorescent images (**e**, **g**) of explanted neural-tube primordia obtained 18 h after explantation. (**e**, **g**) High magnification of N-cadherin-stained NCC explants. (**d**, **e**) Control-untreated explant shows migration of NCC with reduced N-cadherin levels (*arrows*). The *dotted line* in (**e**) marks the border between the epithelial-N-cadherin-expressing NCC and migrating mesenchyme NCC. (**f**, **g**) Treatment with GI254023X, an ADAM10-specific inhibitor, blocks NCC delamination by maintaining full-length membrane-bound N-cadherin. (**h**, **i**) Electroporation of neural tubes with the N-cadherin tail CTF2, the end-product of ADAM10 cleavage, followed by their explantation in the presence of GI254023X. Explants were stained with antibodies directed to the intracellular domain of N-cadherin that also react with transfected CTF2 which is evident in the cell nuclei. (**h**) Control explants in the presence of ADAM10 inhibitor show that cells retain membrane-bound N-cadherin immunoreactivity and are adhered to each other. (**i**) Explants treated with CTF2 and ADAM10 inhibitor show that NCC expressing CTF2 in their nucleus have emigrated from the neural-tube, lack membrane immunostaining, and are detached from each other (*arrows*). *Scale bar*: 44 **μ**m for **a**, **b**; 62 **μ**m for **c**; 200 **μ**m for **d**, **f**; 20 **μ**m for **e**, **g**; 72 **μ**m for **h**, **i**. Abbreviations: *NT* neural tube, *S* somite. Adapted with permission, Development, Shoval et al., 2007, [135]

ECM that surrounds the migrating cranial NCC. Yet, the mechanism of ADAM13 function is not fully understood, since, unlike the end-product of N-cadherin, the cleavage of cadherin-11 by ADAM13 does not modulate β-catenin [136]. Interestingly, ADAM13 itself was also found to be cleaved by γ-secretase, leading

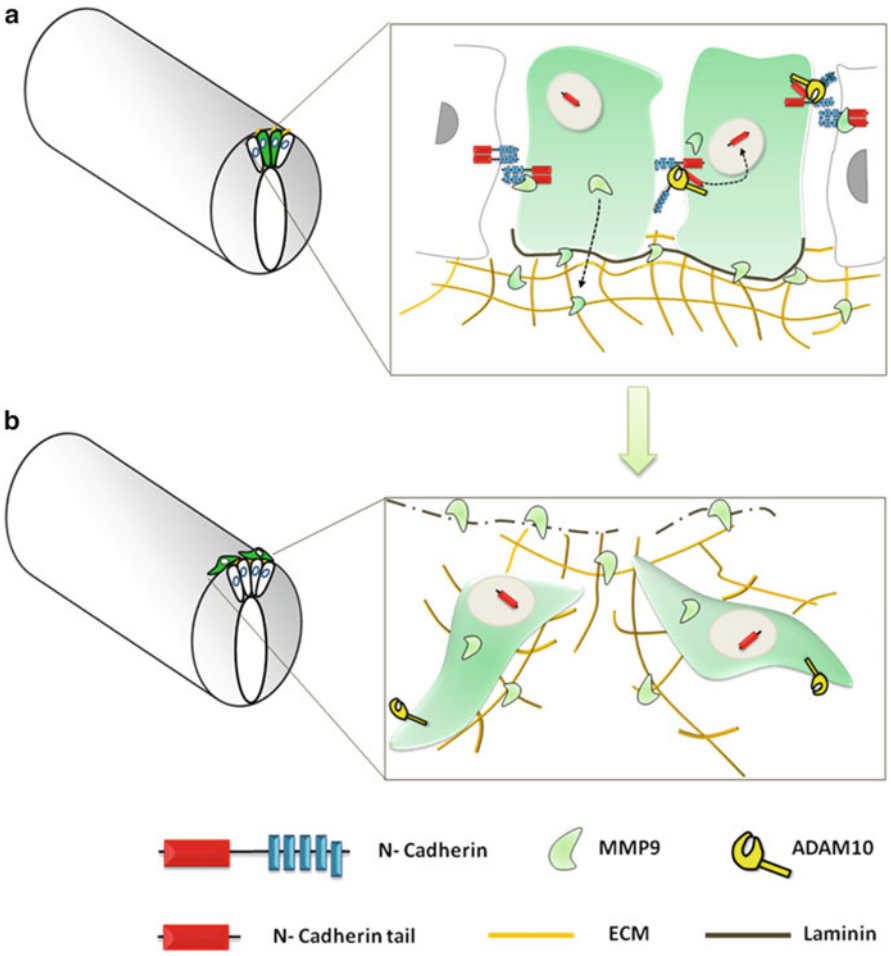

Fig. 6.4 A schematic illustration of the proposed activities of MMP9 and ADAM10 in executing the detachment of neural crest cells from the neural tube. (**a**) Prior to migration, neural crest cells (NCC, *green*) compose the dorsal-most part of the neural tube epithelia, where they retain cell–cell and cell–matrix contacts. In order to undergo the epithelial-to-mesenchymal transition (EMT) and engage in migration, secreted MMP9 (*green*) cleaves N-cadherin molecules (*blue*), basement membrane proteins (laminin, *black*), and ECM components (*dark yellow*) around and adjacent to NCC. ADAM10 (*yellow*) is anchored to NCC membranes, where it cleaves N-cadherin (*blue*). As a result of the cleavage, soluble intracellular CTF2 fragment (*red*) is generated and enters NCC nucleus. (**b**) Following these proteolytic activities, NCC are now separated from each without N-cadherin contacts, the basement membrane/surrounding ECM is loosen, and they display a mesenchymal morphology that enables their migration

to its cytoplasmic domain to be translocated into the nucleus and to activate transcription of genes essential for NCC development. One such gene, Calpain8, was found to retain the migration capacity of cranial NCC in embryos lacking the ADAM13 cytoplasmic domain. Another putative downstream candidate of

ADAM13 in promoting NCC migration is MMP13, as its expression was enhanced by ADAM13 cytoplasmic domain in NCC [149], raising the attractive hypothesis of a crosstalk between different types of proteases in enabling NCC migration.

Altogether, multiple studies in different model organisms have found ADAM family members to be critically involved in many aspects and stages of NCC development through extracellular and intracellular mechanisms. Moreover, different ADAM members were shown to compensate for the loss of a certain ADAM protein in mediating NCC migration, indicating for a conserved role of ADAMs during evolution [149]. Future studies will be required to further elucidate upstream regulators and downstream target genes of ADAMs in normal and perturbed NCC development. Developing of conditional knockout mice is also necessary to unravel the role of ADAMs in mammalian NCC. Finally, a regulatory interaction between MMPs and ADAMs in facilitating NCC migration requires further investigation.

6 Other Types of Proteases in NCC Migration

In addition to the central roles of MMPs and ADAMs in promoting NCC EMT and migration, some other proteases were also shown to be involved in these processes, as well as in the development of later NCC derivatives. Plasminogen activator (PA) is an inducible serine protease found in a variety of embryonic tissues. It was shown to be produced by cephalic NCC during their onset of migration and their colonization in the developing head and neck [150], indicating for its possible role in promoting these processes. Furthermore, an additional secreted serine protease, urokinase-type plasminogen activator (uPA), was found to regulate cranial NCC migration in vitro, via catalysis of plasminogen to plasmin. In this study, addition of plasminogen/plasmin to NCC enhanced their migration, which was prevented by blocking uPA activity using anti-catalytic uPA antibody. This shows that plasminogen plays a role in NCC migration, which is mediated by uPA activity [151]. Not surprisingly, uPA was also found to be involved in the activation of several MMP family proteins [152]. uPA from cultured hepatic stellate cells (HSC) significantly induced the activity of MMP2 and MMP9 in cirrhotic tissues. Similarly, transfection of a vector encoding human uPA into HSC which are not producing uPA resulted in overactivation of MMP3, MMP2, and MMP9 [153]. Furthermore, uPA, along with MMP9, was reported to activate pro-MMP13 in the joint fluid of patients suffering from osteoarthritis and rheumatoid arthritis [154]. Plasmin was also reported to activate MMP3 and MMP9 [155]. While these studies suggest a regulatory network between MMPs and serine proteases, it is not known whether these two players also interact in NCC.

Finally, dipeptidyl peptidase IV (DPPIV) is a cell-surface serine protease, which is expressed in normal, noncancerous neuronal NCC derivatives in adults, where it was shown to decrease the gelatinolytic activity of MMP9. However, its expression is greatly decreased in cells derived from neuroblastoma. Interestedly, restoration of DPPIV expression in neuroblastoma cells suppressed their tumorigenic potential,

indicating for the regulatory role of this serine protease in preventing cell invasiveness, possibly through the modulation of MMP9. Whether this protease plays an earlier regulatory role in embryonic NCC migration is not known [156].

7 Conclusions

MMPs and their inhibitors (TIMPs), as well as other proteases such as ADAMs and serine proteases, hold great influence on controlling NCC EMT, migration, and tissue formation on multiple levels, stages, and species. Alternation in the expression and activity of these proteases lead to impaired NCC development and to related malformations and disorders. The pivotal extracellular, cell surface, and intracellular roles of MMPs and ADAMs (as illustrated for MMP9 and ADAM10 in Fig. 6.4), the regulatory cascade that modulate their expression and activity, and the possible crosstalk between themselves and other proteins in the unique system of embryonic NCC are only starting to be understood and await future research to be fully unfolded. Furthermore, the similarity in MMPs involvement in metastatic cell invasion and in the well-controlled process of embryonic NCC migration, together with the fact that some of the highly aggressive tumors are derived from NCC progeny, strengthens the need to fully characterize and compare the role and regulation of proteases in these processes. Finally, new knowledge about MMPs and other proteases in NCC will enable the better understanding and reducing congenital malformations, cancer, and other NCC-related disorders.

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Part II
Proteases and Cancer

Chapter 7
Proteases and Cancer Development

Shudong Zhu and Zhoufang Li

Abstract Dysfunction of proteases is observed in many cancers. Signaling and functional roles of both intracellular proteases and extracellular proteases in the development of cancer are discussed in this chapter. As mitochondrial proteases, HtrA2/Omi regulates inhibitors of apoptosis proteins, while Lon protease degrades misfolded proteins and maintains the stability of the mitochondrial genome. Caspases are closely interconnected with mitochondria in apoptosis and serve as the major executors of the apoptosis machinery. Cathepsin proteases have multiple substrates including growth factors and extracellular matrix proteins. Matrix metalloproteinases trigger the release of growth and angiogenic factors and modulate extracellular matrix molecules. Changes of these proteases affect various aspects of cancer development, including transformation, apoptosis, invasion, and metastasis of cancer cells. Targeting these proteases is becoming an important approach to cancer treatment.

Keywords Protease • Cancer • Mitochondria • Caspase • Cathepsin • Matrix metalloproteinase • Apoptosis • Invasion • Metastasis

1 Introduction

Proteases are responsible for proteolysis, one of the most fundamental posttranslational regulatory systems. They are involved in various physiological processes. Dysfunction of proteases is observed in many cancers. Here we will discuss both

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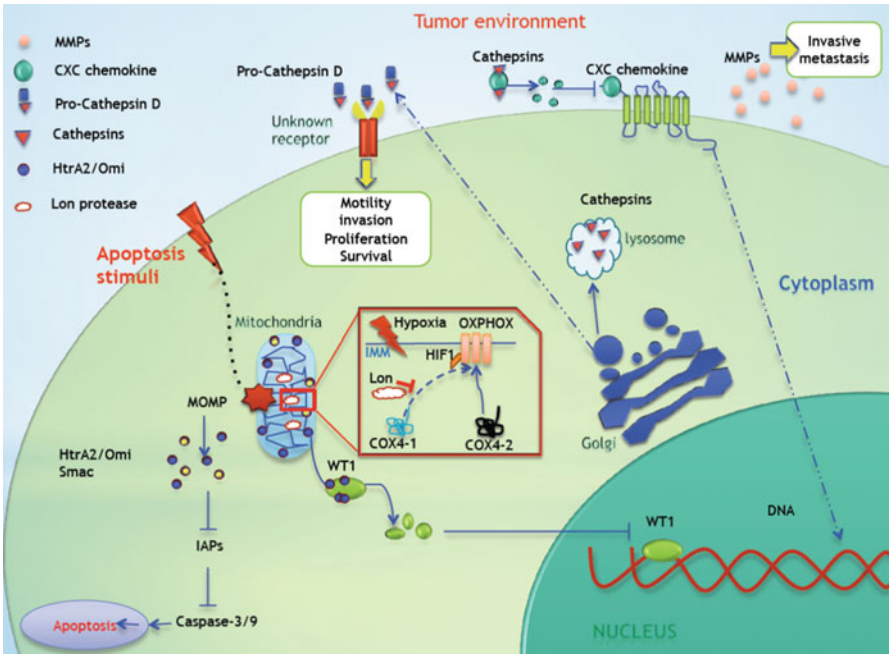

Fig. 7.1 Network of intracellular and extracellular proteases in cancer development. *MMP* matrix metalloproteinases, *HtrA2/Omi* high temperature requirement protein A2, *HIF* hypoxia-inducible factor, *COX* cytochrome C oxidase, *MOMP* major outer membrane protein, *IAP* inhibitors of apoptosis proteins, *WT1* Wilms' tumor suppressor protein

intracellular proteases and extracellular proteases in cancer development, including mitochondrial proteases, lysosome proteases, cytosolic proteases, and matrix metalloproteinases (MMPs). Figure 7.1 shows the network of the proteases in cancer development (Fig. 7.1).

2 Mitochondrial Proteases in Cancers

Mitochondria are essential for the survival and proliferation of normal and cancer cells. Besides their role as an energy factory for maintaining metabolism and proliferation of cells, they are also part of the apoptosis machinery. Dysfunction of mitochondria may lead to the development of various cancers.

Mitochondrial proteases play crucial roles in cancer development directly or indirectly. Some of them strictly control the quality of proteins in organelles by degrading misfolded and non-assembled polypeptides. Others are key factors in the apoptosis pathway. The roles of two mitochondrial proteases, including high temperature requirement protein A2 (HtrA2/Omi) and Lon protease, in cancers are summarized below.

2.1 HtrA2/Omi

HtrA2/Omi is a serine protease in the mitochondrial intermembrane space. It is a crucial regulator to maintain mitochondrial homeostasis, facilitating cell survival. However, HtrA2 acts as a proapoptotic factor under stress. HtrA2/Omi is widely expressed in a variety of cancer cell lines, including gastric cancer [1], ovarian cancer [2], prostate cancer [3, 4], and lymphoma [5].

HtrA2/Omi is associated with cancer development mainly because of its role in apoptosis. It promotes apoptosis in human cells in a caspase-dependent manner, as well as in a caspase-independent manner, via its proteolytic activity. In response to apoptotic signals activated by cisplatin [6] and staurosporine [7], HtrA2/Omi undergoes proteolytic processing, releasing the mature form of the protease from the mitochondria into the cytoplasm, where its exposed N-terminal Ala–Val–Pro–Ser motif mediates interaction with the BIR domains of its substrates, inhibitors of apoptosis proteins (IAPs), including the X-linked inhibitors of apoptosis (XIAP), cellular inhibitor of apoptosis protein-1 (cIAP1) [8], and cIAP2. Binding of HtrA2 with IAPs leads to the degradation of IAPs and activates caspase-3-dependent apoptosis pathway [7, 9, 10]. Hence, upregulation of Omi/HtrA2 sensitizes cells to apoptosis. On the contrary, decreased expression of HtrA2 increases the resistance of multiple cell lines against apoptotic stimuli, reduces cell death, and was observed in various human cancer cells [2, 9].

Besides the interaction of HtrA2 with IAPs and being involved in caspase pathways, other mechanisms of HtrA2 action have also been reported. For example, HtrA2 controls cell proliferation through WARTS kinase [11]. HtrA2 can also elicit its protease activity by cleaving the oncogenic Wilms' tumor suppressor protein WT1 at multiple sites under apoptotic stimuli. This leads to WT1's removal from gene promoter regions and enhances apoptosis [12]. Knockdown of HtrA2 prevents its proteolysis activity on WT1 [13]. HtrA2 also binds to receptors, such as integrin alpha 7 (ITGA7), and triggers cell death in cancers [4]. The activity of HtrA2 is directly regulated by Akt, providing a mechanism by which Akt induces cell survival at the post-mitochondrial level [14].

Abundant work has been done to examine the precise mode of action and the importance of HtrA2 in apoptosis in mammalian cells through biochemical, structural, and genetic studies. While the N-terminal Ala–Val–Pro–Se motif is crucial for binding with the IAPs, both the N-terminal alanine and the catalytic serine residue control the proapoptotic activity of mature HtrA2 protein. Ablation in either of the sites reduces the ability, whereas inhibition of both sites completely blocks the proapoptotic activity of HtrA2/Omi [10].

2.2 Lon Protease

Lon protease is an ATP-dependent serine peptidase. It plays vital roles in the degradation of misfolded and damaged proteins and maintains the stability of the mitochondrial genome. Overexpression of Lon is associated with tumor transformation

[15, 16]. Downregulation of Lon leads to massive apoptosis, disrupts mitochondrial structure, and causes cell death [17].

Lon protease maintains the respiratory system in tumors through one of its substrate, COX4-1 [18]. Cytochrome C oxidase (COX) is a terminal enzyme in the respiratory electron transport chain of mitochondria. There are two isoforms of COX4. COX4-1 is expressed under aerobic condition, whereas COX4-2 plays its major role under hypoxia. Rapid proliferation of cells may lead to hypoxia, which is lethal in normal cells. However, in malignant cells, the hypoxia induces Lon protease and COX4-2 expression through hypoxia-inducible factor-1 (HIF-1). COX4-1 is degraded by the Lon protease, whereas the level of COX4-2 is elevated to maintain the energy supply under hypoxia to promote tumor cell survival.

Lon protease also maintains the stability of the mitochondrial genome through its chaperon activity. Alteration of mitochondrial DNA is reported in cancer cells [19]. Mitochondrial DNA is in close proximity to the respiratory chain and is susceptible to high levels of reactive oxygen species (ROS) [20]. The D loop region controls replication and transcription. This three-stand segment in the mitochondrial genome where Lon binds contains many hot spots of mutation in cancer cells. However, despite our current level of knowledge, we still don't fully understand the precise mechanism by which Lon protease contributes to cancer development yet.

Biochemical and structural studies have revealed that Lon consists of three major functional segments. The N-terminal domain (N domain) is responsible for interaction with protein substrates. Blockage or mutation in N domain decreases its expression, reducing its proteolytic activity and even life cycle in *Podospora anserina* [21]. The second part is related to ATP binding and hydrolysis. ATP stimulates the proteolysis activity of Lon, whereas ADP inhibits this process [22]. The P domain carries the catalytic binding sites [23]. However, the P domain itself doesn't show proteolytic activity on protein substrates, but only on small peptides [24].

Recently, several drugs targeting Lon protease such as the obtusilactone A and sesamin have been shown to induce apoptosis in human lung cancer cells [25]. Synthetic triterpenoid CDDO and its derivatives also selectively inhibit Lon and cause the cell death in lymphoma [26].

3 Caspases

Evasion of apoptosis is one of the hallmarks of tumor development. Caspases are closely interconnected with mitochondria regarding their roles in apoptosis and serve as the major executors of the apoptosis machinery. Of the 12 caspases so far identified, 7 are involved in apoptotic pathways. CASP9, CASP2, CASP8, and CASP10 are categorized as initiator caspases which are recruited to the apoptosome when death stimulus occurs. The initiator caspases contain death-fold motifs, such as caspase recruitment domain (CARD, as in caspase 2 and caspase 9) or death effector domain (DED, as in caspase 8 and 10) at the prodomains. These initiator

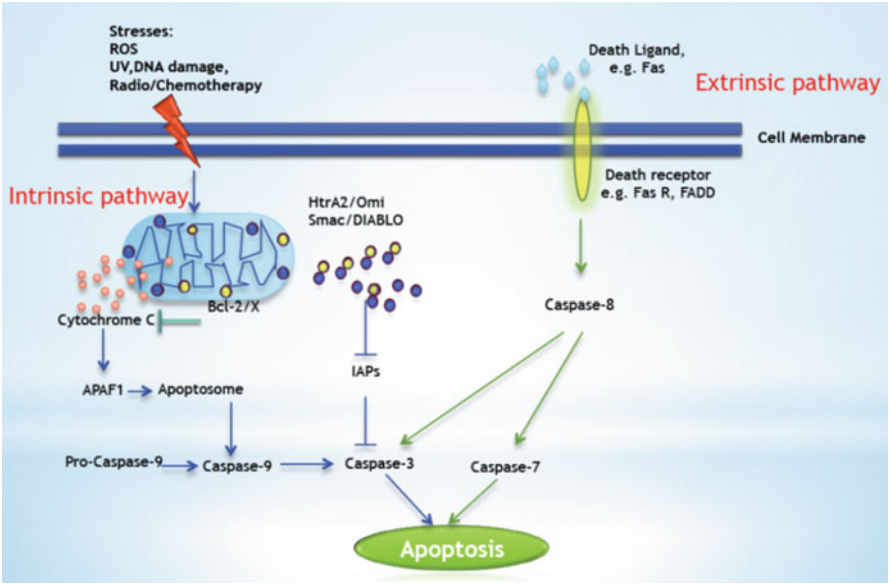

Fig. 7.2 Intrinsic and extrinsic apoptosis pathways. *HtrA2/Omi* high temperature requirement protein A2, *IAP* inhibitors of apoptosis proteins, *ROS* reactive oxygen species, *COX* cytochrome C oxidase, *Apaf-1* apoptosis-activating factor 1, *SMAC/Diablo* second mitochondrial activator of caspases/direct IAP-binding protein with low p*I*, *FADD* Fas-associated protein with death domain, *UV* ultraviolet

caspases further cleave the inactive pro-forms of members of another caspases family, the effector caspases, including CASP3, CASP6, and CASP7, which cleave protein substrates within cells and trigger the apoptotic process. Both groups of caspases play vital roles in apoptosis.

There are two main apoptosis pathways. One is the extrinsic receptor mediated and the other is intrinsic mitochondrial pathways. The extrinsic pathway is initiated by extracellular ligands through oligomerization of cell surface receptor and assembly of death-inducing signaling complex (DISC), whereas the intrinsic involves the participation of mitochondrial proteins such as Bcl-2 family and assembly of apoptosome (Fig. 7.2). Cells utilize an advanced machinery to get rid of genetic or biochemically abnormal cells through apoptosis. Repression of apoptosis, by either antiapoptotic proteins or mutation of the apoptotic executors (the caspases), can lead to cancers (Table 7.1) [27–31, 36, 44]. In particular, caspase 8 is associated with advanced and invasive cancers. In addition, recent evidence suggests that caspases per se could act as tumor suppressors. For instance, knockdown of caspase 2 not only promotes growth of mouse embryonic fibroblasts (MEFs) but also aggressively accelerates tumor formation [32]. Here, we will take initiator caspase 8 and effector caspase 3 as examples to illustrate the mechanisms by which caspases are involved in cancers.

Table 7.1 Alteration of caspase genes in various cancers

Cancers	Caspases	References
Breast	Caspase 3, 8, 10	[44]
Colorectal	Caspase 7, 8, 9	[28]
Gastric	Caspase 2, 6, 8	[30]
Head and neck	Caspase 8	[28]
Hepatocellular	Caspase 8	[29]
Lung	Caspase 8, 10	[31]
Neuroblastoma	Caspase 8	[36]

3.1 Caspase 8

Caspase 8 belongs to the family of cysteine proteases and is one of the initiator caspases in the extrinsic apoptosis pathway [33]. The activation of caspase 8 depends on the DISC in mammalian cells [34].

Several mechanisms are involved in caspase 8-induced cancer development. Caspase 8 plays a central role in the ligand-induced apoptosis of tumor cells. For example, the extracellular ligands of cytotoxic T lymphocytes such as TNF family death ligands (TRAIL, Fas/CD95 ligand, and TNF-a) interact with the corresponding death receptors (TRAIL receptors, Fas/CD95, and TNFR1), which then recruit adaptor proteins such as FADD in the cytosol, followed by recruiting pro-caspases 8. Caspase 8 is generated, and the active caspase 8 is capable of cleaving and activating downstream caspase 3 and other proteases. The cell death machinery is triggered and the tumor cells die [35].

In addition, genetic mutation or alteration in the caspase 8 gene or its regulatory sequence is associated with tumor formation. Silencing of caspase 8 caused by gene deletion or promoter hypermethylation has been identified in pediatric tumors and corresponding cell lines [36]. A six-nucleotide deletion in the promoter region of caspases 8 reduced the expression level of caspase 8 protease and was associated with squamous cell carcinoma of the head and neck cancer [37]. Other variants of caspase 8 such as caspase 8 long (caspase-8L) have also been identified in tumors. Caspase 8L is a splice variant produced by the insertion of a 136bp sequence between exon 8 and exon 9. This insertion leads to a premature stop codon, generating a truncated caspase 8 with only the DED domains but lacking the C-terminal proteolytic domain [38, 39]. This truncated, inactive caspase 8 can still be recruited to the DISC complex due to its retention of DED domains [40, 41]. As a result, caspase 8L acts as a dominant negative to the wild-type caspase 8 and therefore prevents cell death [42, 43].

Moreover, caspase 8 may also have multiple effects on tumor development. Caspase 8 can suppress oncogenic transformation, independent of its role in apoptosis. Loss of caspase 8 expression is often associated with amplification of the MYCN oncogene and increased expression of the corresponding protein. However, it is not clear if these two genetic alterations are functionally linked or if they just fit into a two-hit model providing a permissive environment for tumor growth.

3.2 Caspase 3 (Effector Caspase)

Caspase 3 belongs to the effector caspase family. It is both required in extrinsic and intrinsic apoptotic pathways. Signals from caspase 8 (extrinsic) or from the complex of caspase 9, apoptosis-activating factor 1 (Apaf-1), ATP, and cytochrome c (intrinsic) cleave the procaspase 3 and activate caspase 3 and then further cleave and activate caspases 6 and 7. Effector caspases target a broad spectrum of cellular proteins, ultimately leading to cell death. The proteolytic activity of mature caspases 9 and 3 is inhibited by inhibitor of apoptosis proteins (IAPs). In turn, IAPs are inactivated and caspase activity restored by regulatory proteins such as SMAC/Diablo (second mitochondri-derived activator of caspases/direct IAP-binding protein with low p*I*) or HtrA2/Omi, which are released from the mitochondria (Fig. 7.2).

Disturbances of caspase 3 have been reported in several types of cancers. In a screen of primary breast tumor samples for caspase 3 levels using PCR, northern blot, and western blot, approximately 75 % of the tumors as well as morphologically normal peritumoral tissue samples were found to lack caspase 3 transcripts and caspase 3 protein expression [44]. Interestingly, upregulation of caspase 3 has also been reported in clinical breast tumor samples, using immunohistochemistry [45, 46]. This discrepancy is likely due to the difference in the methods of analysis.

Structural and biomedical studies reveal that several mechanisms are involved in cancer development in association with caspase 3. For example, the different observations in the previous reports may be due to the different forms of caspase 3 being detected in cancer. CASP-3 gene can undergo alternative splicing, giving rise to two forms, the wild-type caspase 3 and a short-form caspase 3s which has antiapoptotic function [47]. Co-expression of two caspase 3 isoforms has been detected in diverse tumor cell lines, as well as in breast carcinomas, where the ratio of expression levels has been used as a prognostic marker to guide the use of cyclophosphamide-containing chemotherapy in patients [48]. At present, the exact role of caspase 3 in tumor formation/progression and tumor sensitivity to treatment is still unclear.

Inhibiting apoptosis is one of the important aspects of cancer; therefore finding ways to reverse this inhibition, and thus activate caspases, is important in cancer therapies and in the developing novel strategies in treating cancers. For instance, inactivation of caspase 8 has been shown to cause resistance to current treatment approaches; thus restoration of caspase 8 represents a promising therapeutic way to treat human cancers. Regulators of the caspase family are also promising pharmacological tools for treating of cancers.

Recently, a report from *oncogene* shows that caspase 3 activation in dying tumors cells in patients undergoing chemo- and radiotherapeutic regimens can not only induce cell death but also proteolytically activates a cytosolic Ca^{2+}-independent phospholipase A2 (iPLA2) (Fig. 7.3). iPLA2 then adopts the plasma membrane lipid as a substrate and produces arachidonic acid (AA) and releases soluble lipid messengers, notably prostaglandin E2 (PGE2), in a cascade of enzymatic reactions. The PGE2 stabilizes EP2 protein (a G-protein-coupled receptor expressed in the surface of tumor cells), which promotes tumor cell proliferation possibly through

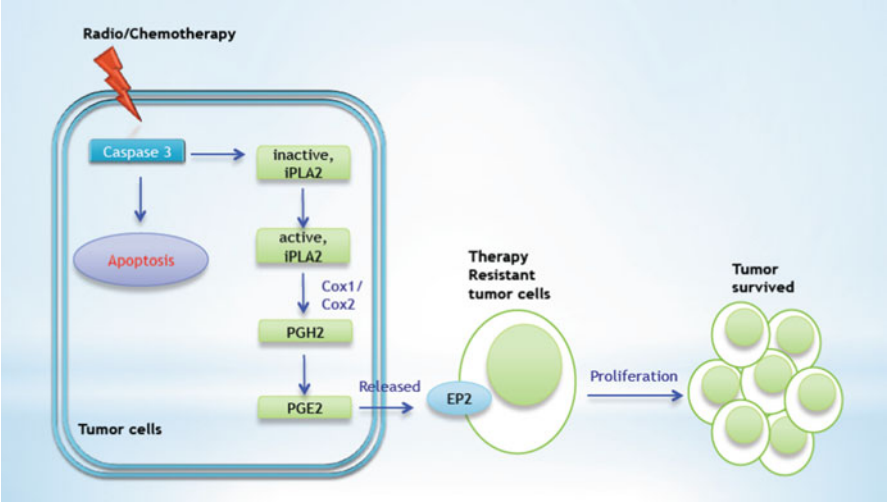

Fig. 7.3 Caspase 3-mediated stimulation of tumor cell repopulation during cancer therapy. *COX* cytochrome C oxidase, *iPLA2* Ca^{2+}-independent phospholipase A2, *PGH2* prostaglandin H2, *PGE2* prostaglandin E2, *EP2* E-prostanoid receptor 2

the WNT-β-catenin signaling pathway [49]. The report suggests that therapies that block the activation of caspase 3 alone may result in significant clinical and therapeutic failure.

4 Cathepsin Proteases

Cathepsin proteases are lysosomal peptidases ubiquitously expressed in animals. Most of them are activated and take effect in lysosomes with only few exceptions such as Cathepsin K, which takes effect in the extracellular matrix. Elevated Cathepsin proteases have been reported in several types of cancers including breast cancer [50–52], prostate cancer [53], colon cancer [54], and lung cancer [55]. Two representative Cathepsin proteases, the aspartyl Cathepsin D and the cysteine Cathepsin B, and their roles in cancers are summarized below.

4.1 Cathepsin D

Cathepsin D is a lysosomal aspartyl protease, a dimer of disulfide-linked heavy and light chains. Mutations in the Cathepsin D gene are involved in the pathogenesis of breast cancer and other diseases. Overexpression of Cathepsin D has been observed in breast cancer and prostate cancer and is associated with poor prognosis [56–59].

Cathepsin D has been implicated in activating growth factors, such as bFGF, which is known to be able to promote cancer cell growth and angiogenesis [60]. More direct evidence supports such roles of Cathepsin D: While overexpression of Cathepsin D was found to increase proliferation by reducing the cellular production of unknown secreted growth inhibitors [61], Cathepsin D also promotes cancer cell invasion and metastasis [62]. Interestingly, mutated Cathepsin D devoid of catalytic activity did not lose its mitogenic activity in cancer and endothelial and fibroblastic cells. Studies in estrogen receptor-positive breast cancer cell lines revealed that this housekeeping enzyme is highly regulated by estrogens and certain growth factors (i.e., IGF1, EGF). The regulation of Cathepsin D mRNA levels by estrogen is mainly due to increased initiation of transcription [63]. In addition, cancer cells and stromal cells overexpress pro-Cathepsin D [64]. The elevated pro-Cathepsin D in the extracellular matrix (ECM) of tumors also suggests an extracellular mode of action of Cathepsin D. The acidic environment of tumors facilitates the maturation of the pro-Cathepsin D and triggers downstream signaling pathways, either through some unknown cell surface receptor or to promote fibroblast outgrowth via a paracrine loop. This overexpressed and hypersecreted pro-Cathepsin D also stimulates motility and invasion of fibroblasts, or cancer cell angiogenesis, and results in enhanced tumor–host homeostasis. However, the mechanism is not yet clear.

Cathepsin D can also attenuate the immune response in mouse tumor models through its proteolytic activity, by degrading a series of chemokines including MIP-1a, MIP-1b, and SLC [65]. MIP-1a and MIP-1b recruit immature dendritic cells (DCs) to the tumor tissues, and then these immature DCs mature and express SLC receptor CCR7, which initiates an antitumor immune response at sites of the primary tumor [66, 67]. The cleavage of SLC by Cathepsin D interferes with the migration of antigen-loaded mature DCs to secondary lymphoid organs and thus attenuates the antitumor effect of the chemokines [68].

Researchers have tested major types of tumors for a correlation between Cathepsin D expression and cancer progression stages and the clinical outcome. Meta-analysis has established a correlation between high levels of Cathepsin D and poor disease-free survival in node-negative breast cancer patients [69]. Cathepsin D could also serve as a potential prognostic marker for lung cancer [55].

4.2 Cathepsin B

Cathepsin B belongs to the large family of cysteine Cathepsin proteases. Cysteine cathepsin proteases play important roles in cancer development. The Cathepsin B, as a major member in this family, is involved in several cancers, including breast cancer and melanoma [70, 71]. Cathepsin B is upregulated at the levels of mRNA, protein, and activity in these human cancers. For example, in inflammatory breast cancer (IBC), high levels of Cathepsin B are detected in the caveolar membrane microdomains. There is also a significant positive correlation between the expression of Cathepsin B and the number of positive metastatic lymph nodes in IBC.

Table 7.2 Cathepsins and inhibitors

Cathepsins	Cancers	Inhibitors
Cathepsin B	Melanoma	CA-074
	Breast cancer	
Cathepsin D		Pepstatin A
Cathepsin K	Breast cancer, bone metastasis	MK-0882
	Bone metastasis	SB-553484
Cathepsin L		SC-364671
Cathepsin S	Rheumatoid arthritis	RWJ-445380

Cathepsin B is proposed to be a prognostic marker for lymph node metastasis, and elevated Cathepsin B activity correlates to poor therapy outcome [71].

Cathepsin B expression is increased in tumor cells, particularly at the invasive edges with abundant stromal fibroblasts and inflammatory cells. Cathepsin B enhances the tumor proteolysis. It degrades extracellular matrix proteins, such as laminin and collagen IV, and activates the precursor form of urokinase plasminogen activator (uPA). Alternatively, it binds to the annexin II heterotetramer (AIIt) at the caveolar region on the tumor surface, with the AIIt binding site for Cathepsin B differing from that for plasminogen/plasmin or tissue plasminogen activator (tPA). Activation of Cathepsin B on the cell surface by AIIt leads to the regulation of downstream proteolytic cascades. In addition, AIIt also interacts with extracellular matrix proteins, such as collagen I and tenascin-C, forming a structural link between the tumor cell surface and the extracellular matrix. One assumption is that the complexes formed by Cathepsin B, AIIt, tPA, and tenascin-C facilitate the activation of precursors of proteases and initiation of proteolytic cascades, hence promoting the tumor cell detachment, invasion, and motility [72]. Increased secretion of pro-Cathepsin B has also been observed in tumors. The intracellular trafficking and location of Cathepsin B are also modulated in cancer cells [73].

Since Cathepsin B protease has recently emerged as an important member of proteolytic enzymes in cancer progression and invasion, it is not surprising that Cathepsin B inhibitors have been proposed as anticancer agents. Up to now, numbers of inhibitors of Cathepsin B have been identified either as endogenously expressed molecules, such as cystatins, or as chemically synthesized agents, such as CA-074. CA-074 significantly reduces the percentage of invading cells in melanoma [70] and limits bone metastasis in breast cancer [74]. Inhibitors for other cysteine Cathepsins such as K, L, and S (the CLIK series) have also been developed and tested in vivo [75–78]. In summary, Cathepsins in general have emerged as promising pharmacological targets for cancer therapy. A lot of synthetic Cathepsin inhibitors are under different stage of clinical trials (Table 7.2).

5 MMPs

MMPs are a family of zinc-dependent endopeptidases that facilitate cancer development by triggering the release of growth and angiogenic factors and by modulating extracellular matrix molecules. MMPs are present in the extracellular matrix in an

inactive form, and once activated, MMPs are further regulated by three major types of endogenous inhibitors, α2-macroglobulin, reversion-inducing cysteine-rich protein with Kazal motifs (RECK) [79], and tissue inhibitors of metalloproteinases (TIMPs) (reviewed in [80]). The bioactivity of TIMPs is further regulated posttranslationally by processes such as inactivation by serine protease cleavage [81]. So far, 23 MMPs are identified in vertebrates and categorized into several classes. Among them, MMP9 is one of the most studied protease that is actively involved in cancer development. Several other MMPs are also discussed briefly.

5.1 MMP9

The evidence for MMP9 contributing to neoplastic progression was from mouse model studies, where eliminating MMP9 significantly reduced the incidence of pancreatic islet carcinomas [82] and cervical carcinogenesis [83], while reconstitution of MMP9 restored cellular programs for the neoplastic progression and tumor development [84, 85]. The infiltrated leukocytes in these cancers are the major source of MMP9.

Several mechanisms are involved in MMP9-associated tumor progression. MMP9 alters the stromal microenvironment by mediating liberation of ECM-sequestered growth-promoting factors, such as basic fibroblast growth factor (FGF-2), or proteolytic cleavage of growth factor latent precursors, such as transforming growth factor (TGF-a) [86]. MMP9 is also found to be an active regulator of tumor angiogenesis: Vascular endothelial growth factor (VEGF) is a key factor in neovessel formation. However, VEGF requires both high MMP9 and the VEGF receptor (VEGFR1) to activate the angiogenic program. Low expression levels of VEGFR1 fail to induce subsequent metastatic cell growth in the presence of MMP9, while increases of VEGFR1 combined with a population of endothelial cells capable of inducing expression of MMP9 induce significant metastasis. It is now believed that activated MMP9 releases matrix-sequestered VEGFa to interact with its receptors, to be able to stimulate efficient vascular remodeling and angiogenesis necessary for metastatic cell growth and survival [82, 87]. Taken together, MMP9 not only induces a microenvironment favorable for primary tumor growth but is also crucial for the metastasis and survival of these metastatic cells [88, 89].

5.2 Other MMPs

Several other MMPs have been shown to enhance tumor progression. MMP1 increases susceptibility of chemical skin carcinogenesis [90]; MMP14, MMP2, or MMP3/stromelysin-1 promotes mammary carcinogenesis [91], while reduction of MMP11 attenuates chemically induced skin cancer [92]. Among them, MMP14 and MMP2 could release cryptic fragments of laminin-5 gamma 2 chain domain, which binds to the EGF receptor on tumor cells, thus activating downstream signaling

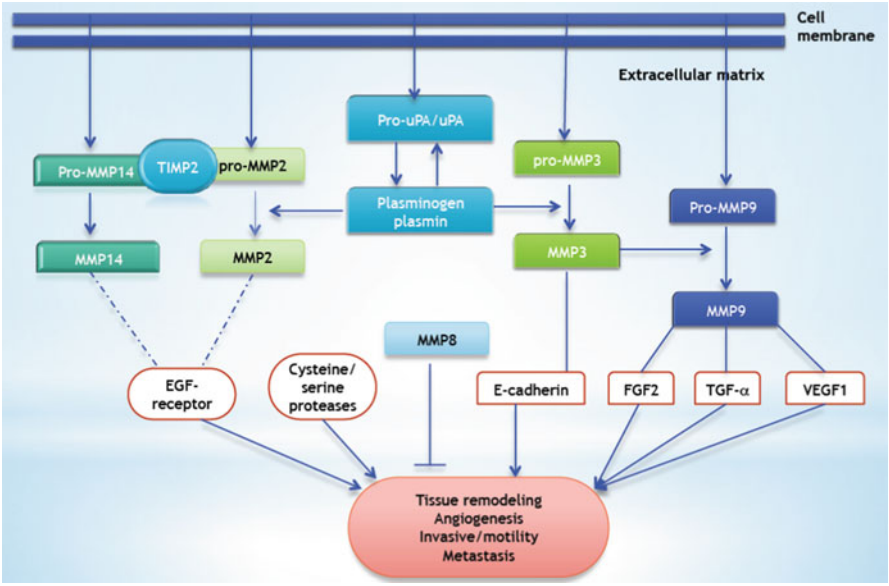

Fig. 7.4 MMPs and cancer progression. *MMP* matrix metalloproteinases, *uPA* urokinase plasminogen activator, *EGF* endothelial growth factor, *FGF-2* basic fibroblast growth factor, *TGF-α* transforming growth factor alpha, *VEGF* vascular endothelial growth factor

events that lead to tumor cell motility [93, 94]. Others such as MMP3 target the adhesion molecule E-cadherin which will trigger progressive phenotypic conversion of normal epithelial cells into the invasive mesenchymal phenotype, as observed in mammary epithelial cells [95]. Most of these MMPs are capable of altering cell–cell and cell–matrix interactions. While most MMPs promote tumor progression, however, some MMPs also exhibit antitumor functions. For instance, MMP8 (collagenase 2) reduces skin tumor susceptibility [96].

The mechanisms by which MMPs contribute to the malignant transformation and cancer metastasis are illustrated in Fig. 7.4. Due to the active involvement of MMPs in tumor progression, they have been selected as potential targets for anticancer therapy. Quite a lot of MMP inhibitors have been identified; however, the results of clinical evaluation in cancer patients of these MMP inhibitors have not been as promising as initially expected. The reason is not clear. In the case of MMP9, although the MMP9 inhibitor attenuated or blocked its activity, as a feedback control, several other proteases in parallel have been upregulated in compensation for the loss of the MMP9 proteolytic activity. Therefore, therapies that target a series of proteases or a protease pathway rather than an individual protease may have better outcomes.

6 Conclusions

Proteases including mitochondrial proteases, lysosome proteases, cytosolic proteases, and matrix metalloproteinases play important roles in the development of various cancers. Changes of these proteases affect various aspects of cancer development, including transformation, apoptosis, invasion, and metastasis of cancer cells. More details of the signaling pathways of the proteases are starting to be revealed. Targeting these proteases shows promise in cancer treatment.

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Chapter 8
Matrix Metalloproteinase and Its Inhibitors in Cancer Progression

Ajay K. Chaudhary, Anita H. Nadkarni, Shruti Pandya, and Kanjaksha Ghosh

Abstract Matrix metalloproteases (MMPs) are a family of zinc-dependent endopeptidases that participates in the degradation of various components of the extracellular matrix (ECM) and basement membrane. The main functions of MMPs are in wound healing, embryogenesis, angiogenesis, invasions, and tumor cell metastasis. MMPs are involved in solid and hematological malignancy through modification of cell growth, activation of cancer cells, and modulation of immune functions. Several polymorphisms of different MMPs and their expression levels have been well documented in different types of solid cancer. These polymorphic variations were found to be associated with angiogenesis, cancer progression, invasion, and metastasis. There is paucity of data available in the field of hematological malignancies. Hence, the field of matrix biology of hematological malignancies is an area of active exploration. Last 20 years, intensive drug discovery programs are carried out in many clinical trials of matrix metalloproteinase inhibitors (MMPIs) for cancer therapy. Number of MMP inhibitors (MMPIs) have been devolved for the cancer treatment. However, their efficacy and action have not been confirmed, and more data is required for better conclusions. For discovery of drug target motive, the regulatory mechanisms of MMPs and its inhibitors may provide several new avenues for the development of therapeutic intervention for the patient care.

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1 Introduction

Matrix metalloproteinases (MMPs) are the family of zinc-dependent endopeptidases that degrades the various components of the extracellular matrix (ECM) and basement membrane, which are normally found in the space between cells. This space is known as extracellular matrix (ECM) space, and presence of proteins in this space is known as extracellular matrix proteins (ECMP). It contains zinc or calcium ions. Therefore, it is known as a family of zinc-dependent endopeptidases. Nowadays more than 24 types of human MMPs have been discovered. It is highly involved in wound healing, angiogenesis, and tumor cell metastasis.

The ECM plays an important role in the networking of molecules for the supporting and dividing tissues. ECM space consists of structural proteins (collagen and elastin) and specialized proteins (fironecting and laminin). The main composition of the ECM is related to the uniqueness of the specific tissues, so that each tissue seems to have slightly different types of ECM. For example, ECM of the bone marrow is mainly made up of collagen types I, II, and IV–VI, fibronectin, vitronectin, laminin, and proteoglycans [1]. Another form of specialized ECM is the basement membrane. The basement membrane is thin flexible mat that separates epithelial cells from the underlying stroma. It provides a first barrier against cell invasion, and therefore, the basement membrane is implicated in angiogenesis, tumor growth, and metastasis [2].

1.1 History, Structure, and Classification of the MMPs

Jerome Gross and Charles Lapiere first described MMPs in 1962, who observed enzymatic activity in tadpole tail metamorphosis [3]. Latter purified MMPs are isolated from human skin by Eisen in 1968 [4]. The MMPs have a common domain structure. The domain structure of MMPs includes the signal peptide domain, which guides the enzyme into the rough endoplasmic reticulum (RER) during synthesis. The propeptide domain sustains the latency of these enzymes until it is removed or disrupted, the catalytic domain, which houses the highly conserved Zn^{2+} binding region and is responsible for enzyme activity. The hemopexin domain determines the substrate specificity of MMPs, and a small hinge region helps the hemopexin for the presentation of the substrate to the active core of the catalytic domain (Fig. 8.1). Based on the substrate specificity, MMPs have been divided into distinct subclasses such as collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysins (MMP-7, MMP-26), and other MMPs.

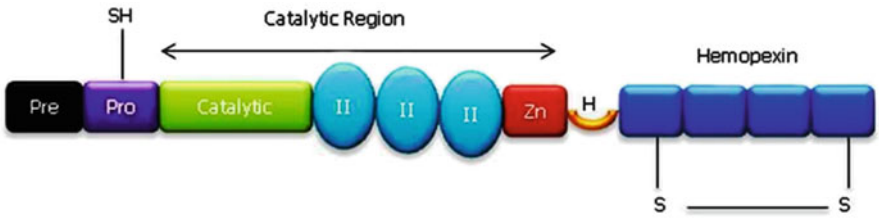

Fig. 8.1 Basic domain structure of the gelatinases, modified from Visse and Nagase (2003) [5]. The general structure of the MMPs consists of a signal peptide (pre-), a propeptide domain (pro-), a catalytic domain with a highly conserved zinc-binding site, and a hemopexin-like domain linked to the catalytic domain by a hinge region (Pre, signal sequence; Pro, propeptide with zinc-ligating thiol (SH) groups; II, collagen-binding fibronectin type II inserts; Zn, zinc-binding site; H, hinge region; hemopexin, this domain contains four repeats with the first and last linked by disulfide bond)

1.2 Brief View of Collagenases, Gelatinases, Stromelysins, and Matrilysins

Collagens are the main protein in connective tissues of the mammals. It is one of the long, fibrous structural proteins called collagen fibers, and it plays an important role in a major component of the ECM. Collagen supports most tissues and gives shape to the cell structure and strengthens to the blood vessels which involved in the development of tissue morphology. In addition to fibrillar collagens, collagenases can cleave several other matrix and non-matrix proteins including growth factors, and this way it regulates cell growth and survival. There are approximately 34 genes associated with collagen formation.

Collagenases are important proteolytic tools for ECM remodeling during organ development and tissue regeneration and also play an important role in many pathological conditions including tumor progression and metastasis. Synthesis of collagenases is regulated by extracellular signals via cellular signal transduction pathways at transcriptional and posttranscriptional level. Collagenases are synthesized as inactive pro-forms, and once it is activated, their activity is inhibited by specific tissue inhibitors of metalloproteinases known as TIMPs. Collagen-related disorder commonly occurs due to the genetic defects or nutritional deficiencies that affect the posttranslational modification, secretion, biosynthesis, or other processes concerned in the production of normal collagen.

Gelatinase is a proteolytic enzyme that allows a living organism to hydrolyze gelatin into its sub-compounds (polypeptides, peptides, and amino acids) that can cross the cell membrane. Gelatinase family includes MMP-2 and MMP-9 gene. The chromosomal locations of MMP-2 and MMP-9 genes are at chromosome number 16 (16q) and 20 (20q), respectively (Fig. 8.2a, b). It is characterized by gelatinases A and B, respectively. Gelatinase A (MMP-2) and gelatinase B (MMP-9) are two closely related members of the MMP gene family that efficiently degrade denatured collagens [6]. The size of gelatinase A is 72 kDa and gelatinase B is 92 kDa, which are capable of breaking down the ECM. MMP-2 and MMP-9 are key molecules which participate in inflammation, autoimmunity, and cancer progression.

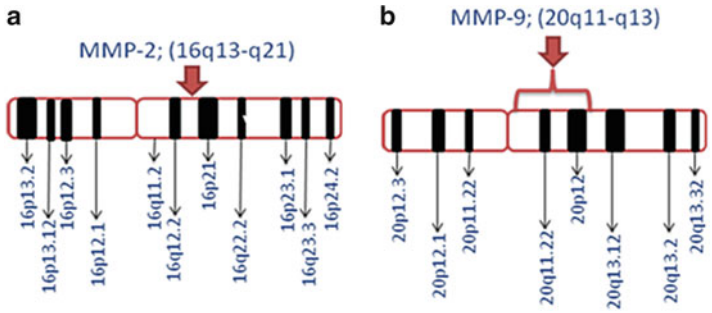

Fig. 8.2 Chromosomal location of MMP-2 and MMP-9 gene. (**a**) Chromosomal location of MMP-2 is 16q13–q21. (The MMP2 gene is located on the long (q) arm of chromosome 16 between positions 13 and 21.) More precisely, the MMP2 gene is located from base pair 55,513,080 to base pair 55,540,585 on chromosome 16. (**b**) Chromosomal location of MMP-9 is 20q11.2–q13.1. (The MMP9 gene is located on the long (q) arm of chromosome 20 between positions 11 and 13.) More precisely, the MMP9 gene is located from base pair 44,637,546 to base pair 44,645,199 on chromosome 20

Stromelysin includes MMP-3, MMP-10, and MMP-11 gene type. It has a similar domain arrangement as that of the collagenase, except they don't cleaved interstitial collagens. MMP-3 and MMP-10 digest a number of ECM molecules and participate in proMMP activation. On the other hand, MMP-11 has very weak activity toward ECM molecules but cleaves serpins more readily [7]. Overexpression of MMP-10 promotes tumor development, and this indicates that MMP-10 induction is an important pathway which is activated in tumor cells by inflammatory cytokines response [6]. The MMP-3 gene has been mapped on the long arm of chromosome 11q22.3, and the level of expression of this gene can be influenced by single-nucleotide polymorphisms (SNPs) in the promoter region of their respective gene [8] in many solid cancers. Matrilysins (MMP-7 and MMP-26) lack a hemopexin domain. MMP-7 is synthesized by epithelial cells. Besides ECM components, it processes cell-surface molecules such as Fas-ligand, pro-tumor necrosis factor-α, and E-cadherin.

2 Regulation of Matrix Metalloproteinase

2.1 Transcriptional Regulation of MMPs

MMPs are highly regulated proteins. Its regulation is carried out at three levels; the first level of regulation is transcriptional regulation, second is activation of latent MMPs, and third is inhibition of MMPs [9]. In the process of transcriptional regulation, MMPs have the fundamental ability to degrade all forms of matrix components, and they are very precisely regulated at the transcriptional level. Most of the MMPs are transcribed, whereas only some are constitutively expressed. Tumor growth factor beta (TGF-β) superfamily are suggested to be associated with healing and the

expression of MMPs genes is transcriptionally induced by oncogenic transformation, cytokines, as well as growth factors—including interleukins, interferons, EGF, KGF, NGF, VEGF, PDGF, TNF-α, and TGF-β [10].

The regulation of different MMPs also occurs at the protein level. MMPs are secreted as latent enzymes, and this process can be achieved by activators and inhibitors. The expression of MMPs is primarily regulated at the level of transcription, and their proteolytic activity requires zymogen activation. Many stimuli increase the expression of c-fos and c-jun proto-oncogene products, and these activate the activator protein-1 (AP-1) at proximal promoter regions of several MMPs such as MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13 types. Several oncogenes and viruses induce MMP expression in malignant cell lines [11].

All MMPs are synthesized in the form of latent MMPs (zymogen). They are secreted as proenzymes and require extracellular activation. When disturbances occur in physiological and biological activity of the cell environment due to the effect of xenobiotics and many environmental toxicants, then the activation is controlled by latent proenzymes and inhibitors of the matrix metalloproteinases, known as TIMPs, which are secreted by the MMP-producing cells. For this motive different types of MMP inhibitors such as TIMP-1, TIMP-2, TIMP-3, and TIMP-4 are produced to maintain the physiological activity of the cells.

2.2 Activation and Inhibition of MMPs

MMPs are normally synthesized as latent proenzymes. This can be achieved by several proteolytic enzymatic activities, including serine proteinases together with other MMPs. In vivo activation of MMP involves tissue and plasma proteinases and bacterial proteinases activity together with oxidative stress. MMPs are usually activated at the cell surface, for example, activation of MMP-2 by a MMP-2/TIMP-2/MT1-MMP complex. Several MMPs may also be activated intracellularly by furin or related proprotein convertases [12]. MMP activity can be controlled by inhibition in two ways: nonspecific endogenous inhibitors such as *a*2-macroglobulin and by specific tissue inhibitors of MMPs known as TIMPs. Currently, four TIMPs (TIMP 1–4) are known to be expressed in vertebrates.

3 Role of Matrix Metalloproteinases in Cancer

3.1 Progression of Cancer, Tumor Angiogenesis, and Metastasis

The stromal fibroblasts are the primary source of MMPs in the most malignant tumors. The infiltrations of inflammatory cells are a prominent feature of many progressive tumors. An inflammatory cell produces cytokines, which enhance the expression of MMPs by tumor and stromal stimulation of cancer cell–platelet

interactions. Stromal fibroblasts take part in the inhibition of many MMP functions, as well as the proliferation of immune responsible cells, such as T cell and natural killer (NK) cells.

Almost all tissues develop a vascular network that provides cells with nutrients and oxygen and enables them to eliminate metabolic wastes. It is well known that angiogenesis performs a critical role in the development of many types of cancer. Smaller solid tumor (<2 mm^3) cannot vascularize. Beyond the critical volume of 2 cubic millimeters (>2 mm^3), oxygen and nutrients have difficulty diffusing into the center of the tumor cell, so it causes the state of cellular hypoxia that marks the initial onset of tumor angiogenesis. New blood-vessel development is an important process in tumor progression. It favors the transition from hyperplasia to neoplasia, i.e., the passage from a state of cellular multiplication to a state of uncontrolled proliferation, which is the characteristic of tumor cells. The endothelial cells that form the blood vessels respond angiogenically by differentiating and secreting MMP, which digest the blood-vessel walls to escape and migrate toward the site of the angiogenic stimuli.

The normal regulation of angiogenesis is governed by a fine balance between factors that induce the formation of blood vessels and those that inhibit the angiogenic process. When this balance is destroyed, it usually results in pathological angiogenesis which causes increased blood-vessel formation in diseases. More than 20 endogenous positive regulators have been described, including growth factors, matrix metalloproteinases, cytokines, and integrins (i.e., transmembrane receptors) [13]. Growth factors such as vascular endothelial growth factor (VEGF), transforming growth factors (TGF-beta), fibroblast growth factors (FGF), and epidermal growth factor (EGF) have shown to induce the division in cultured endothelial cells indicating a direct action of growth factor on these cells [14].

The tumor cells time to time induce the growth-stimulating signals to the neighboring cells. The release of the extracellular proteins from the cell surface that involves metalloprotease-directed proteolysis is referred to as ectodomain shedding. MMPs participate in cell-surface proteolysis, leading to the release of several cell-surface growth regulators. As MMPs degrade proteins in ECM, their primary function was considered to be the remodeling of ECM. However, MMPs also act on the non-matrix substrates (e.g., chemokines, growth factors, growth factor receptors, adhesion molecules, and apoptotic mediators) that give the rapid and critical cellular responses required for tumor growth and progression.

3.2 Detection of MMPs in Solid Hematological Malignancies

The released or secreted MMPs can be detected by the zymography analysis. This technique was first described in 1980 by C. Heussen and E. B. Dowdle [15]. It is a very sensitive technique. This is semiquantitative, which can be used to determine the MMP levels [16]. The expression of MMPs can be analyzed by substrate zymography. This method is based on the degradation of preferential substrate of MMPs. Using this technique, one can determine whether the MMP is in active or latent

form. To localize MMPs in tissue sections, in situ zymography can be performed. TIMPs can be detected by reverse zymography, which is based on their ability to inhibit MMPs. The techniques are the same except that the substrate differs depending on the type of MMPs or TIMPs to be detected. In zymography, the proteins are separated by electrophoresis under denaturing sodium dodecyl sulfate (SDS), non-reducing conditions. The separation occurs in a polyacrylamide gel containing a specific substrate that is copolymerized with the acrylamide.

During electrophoresis, the SDS causes the MMPs to denature and become inactive. After electrophoresis, the gel is incubated in an appropriate activation buffer. During this incubation, the concentrated, renatured MMPs in the gel will digest the substrate. After incubation, the gel is stained with Coomassie® Blue, and the MMPs are detected as clear bands against a blue background of undegraded substrate [17]. The clear bands in the gel can be quantified by densitometry [18].

Gelatin zymography is used for the detection of the gelatinases, MMP-2, and MMP-9. Casein zymography is used for the detection of stromelysins, MMP-1, MMP-7, MMP-11, MMP-12, and MMP-13. Collagen zymography is used for the detection of MMP-1 and MMP-13. Therefore, zymography is a valuable tool used for MMP research.

3.3 Status of MMPs in Solid and Haematological Malignancies

The status of MMPs and its inhibitors in solid malignancies have been well documented. Many studies have documented association of different polymorphic associations with cancers such as MMP-9 (-1562 C/T) in lung cancer [19], ovarian cancer [20], colorectal carcinoma [21], oral squamous cell carcinoma (OSCC) [22], oral submucous fibrosis [23], head and neck cancer [24], gastric carcinoma [25], and oral cancer [26]. Above studies suggested that individuals carrying T allele were more susceptible. On the other hand in hepatitis C cases, the frequency of C alleles was higher in cirrhosis patients as compared to chronic hepatitis patients [27]. It has been reported that oral submucous fibrosis (OSMF) and head and neck squamous cell carcinoma (HNSCC) are most prevalent in Asian countries, because of the habit of tobacco, smoking, and areca nut chewing [28, 29]. The polymorphic associations of many MMPs susceptible to the disease have been reported in Asians. Chang et al. also reported that areca nut ingredient such as arecoline inhibits the gelatinolytic activity of the many MMPs in the mucosal layer [30].

The normal physiological process of the hematopoiesis, proliferation, differentiation, and migration of the hematopoietic stem cells (HSCs) are regulated by their complex interactions with the surrounding bone marrow (BM) microenvironment, such as marrow stromal cells, cytokines, and extracellular matrix proteins [31]. Under steady-state conditions, most stem cells are maintained in G0 phase of cell cycle by contact with BM stromal cells [32]. Bergers et al. reported that release of cytokines and growth factors from ECM membrane by MMPs can alter the stem cells–stromal cells interaction and promote HSCs migration and differentiation [33].

The main function of MMPs is the degradation of ECM. MMPs participate in the turnover of ECM in the hematopoietic microenvironment, regulating the release of hematopoietic stem cells and mature leucocytes from bone marrow (BM) to peripheral blood (PB). Recently, Chaudhary et al reported that polymorphic association of different MMPs and its expression are involve in the progression of solid and haematological malignancies and also discuss about the drug target therapy [34]. As in solid tumors, MMPs participate in ECM proteolysis and growth factor or cytokine release which are important to leukemia progression. The expression of MMPs, especially gelatinases A and B (MMP-2 and MMP-9), was first described in adult acute or chronic myeloid leukemia (CML) and myelodysplastic syndromes (MDS). Kuittinen et al. studied the expression level of MMP-2 and MMP-9 in the clinical course of acute lymphatic leukemia (ALL) and using immunocytochemical staining. They reported that remarkable difference in the expression level of MMP-2 and MMP-9 between pediatric and adult ALL cases. In adult ALL patients 65 % of the cases showed positive staining in blast cells for MMP-2 and 25 % for MMP-9 [35]. Ries et al. reported that the BM-MNCs produce MMP-9 and TIMP-1 in myeloproliferative malignancies and suggested that MMP-2 can be a potential marker for dissemination in malignancies [36]. Travaglino et al. investigated potential role of MMP-2 and MMP-9 expression in myelodysplastic syndrome (MDS) and compared it with acute myeloid leukemia (AML). They reported high MMP expression in AML blasts and suggested possible involvement of these enzymes in the invasive phenotype of AML [37]. This may provide a useful tool for diagnosis and prognosis and as strategies for targeting MMPs as a new cancer treatment.

4 Role of MMP Inhibitors in Drug-Targeted Therapy

4.1 MMP Inhibitors and Its Clinical Trials

The several approaches have been used to inhibit MMP gene transcription, which is based on targeting extracellular factors, signal transduction pathways, or nuclear factors that activate expression of inhibitor genes [38]. There are a number of matrix metalloproteinase inhibitors (MMPIs) that are currently being tested in all three phases of clinical trials against a variety of human cancers [39].

Peptidomimetic MMPIs are pseudopeptide derivatives that mimic the structure of collagen at the cleavage site of MMPs. These substrate-based MMPIs are usually broad spectrum and block MMP activity by occupying the substrate-binding site and chelating the zinc. The earliest generations of these inhibitors are batimastat (BB-94). It has low water solubility and not orally available. The next generation of hydroxamate-based inhibitors is marimastat (BB-2516) and designed to be orally available. It is commonly associated with musculoskeletal syndrome, probably due to their off target effects on non-MMP metalloproteinases [40].

Another group of MMPIs is also known as chemically modified tetracyclines (CMTs). They do not possess antibiotic activity [41]. CMTs may inhibit MMPs by

binding to the key metal ions, such as zinc and calcium and which are involved in regulation of MMP transcriptional activity [42]. Some of the CMTs which are used as MMPIs include metastat (COL-3), minocycline, and doxycycline.

Several small molecule inhibitors targeted specifically to the ADAM (a disintegrin and metalloproteinase, adamlysins) family of enzymes have also been recently studied [43, 44]. Of these, INCB7839 (Incyte Corporation, Wilmington, DE) is in phase II clinical trials against breast carcinoma and several other solid tumors. Such inhibitors could be useful in targeting tumors dependant on epidermal growth factor receptor (EGFR) signaling either as single agent or in a synergistic manner with currently approved tyrosine kinase inhibitors (TKI) [45].

4.2 Status of the MMPs in Cancer Therapy

Many drugs have been designed as inhibitors of matrix metalloproteinases (MMPIs), but no one has completely reached to the clinical utility level. The one compound (Periostat™ CollaGenex Pharmaceuticals, New York, NY) has approved for clinical use because of its ability to inhibit MMPs. This is used in periodontal inflammation and its formulation is used as low-dose doxycycline. Initial clinical testing of MMPIs was started over 20 years ago. There were problems in the design of the clinical trials of these agents, which certainly contributed to their failure. As MMPs are promising therapeutic targets, an intensive drug discovery program led to many clinical trials of MMP inhibitors (MMPIs) for cancer therapy. However, until very recent reports of success in gastric carcinoma, these trials have largely been disappointing.

5 Conclusions

In conclusion, as in solid malignancies, the role of different types of MMPs and its tissue inhibitors has been well established, while in case of hematological malignancies, no promising study has investigated so far. Even though the effects of altered transcription of all MMPs are unclear, overexpression of MMPs caused by promoter polymorphisms may enhance cancer progression by their role in degradation of the ECM. Thus, the altered expression of MMPs generated through functional SNPs and the consequent effect on tumor microenvironment may contribute to the patient-to-patient variability in different solid and soft cancer susceptibility. Further studies of the in vitro, in vivo, and clinical trials are required to clarify the exact biological roles of MMPs and TIMPs.

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Chapter 9
Ubiquitin–Proteasome System in the Hallmarks of Cancer

Pushpak Bhattacharjee, Minakshi Mazumdar, Deblina Guha, and Gaurisankar Sa

Abstract The paramount role of ubiquitin–protein conjugation for its ability to regulate protein turnover and nonproteolytic signaling functions has been implicated in the regulation of various biological and pathological phenomena. Malignant cells utilize modified ubiquitination to augment or attenuate signaling pathways on the basis of whether the outcome of this signaling is conducive or not for tumor growth and survival. Hence, there lies a necessity for a fresh view at the ubiquitin-dependent mechanisms that play an important role in human oncological diseases. To control ubiquitination-dependent mechanisms of cell transformation within tumor cells themselves, along with increased rate of protein synthesis, translation and protein quality control processes are often required to support the transforming events for their contribution to the mechanisms of tumor progression. Given that ubiquitin metabolism is governed by enzymes—E1, E2, E3, E4, deubiquitinases (DUBs), and the proteasome—the system as a whole is ripe for target and drug discovery in cancer. Recently, the hallmarks of cancer designated by Hanahan and Weinberg in 2011 comprise ten biological capabilities acquired during the multistep development of tumor. Based on these hallmarks, the present review enlightened the role of ubiquitination in every hallmark for rationalizing the complexities of neoplastic disease and also discusses therapeutic implications targeting the ubiquitin–proteasome system as well as synthetic and natural compounds with potent inhibitory effects.

Keywords Apoptosis • Cancer hallmarks • Natural product • Proteasome • Ubiquitin ligase E3

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1 Introduction

Cancer research is an increasingly logical science, in which myriad phenotypic complexities are manifestations of a small set of underlying organizing principles. The circuit diagrams of heterotypic interactions between the multiple distinct cell types that assemble and collaborate to produce different forms and progressively malignant stages of cancer. Based on the signaling circuitry describing the intercommunication between various cells within tumors, in 2000, Hanahan and Weinberg [1] distinguished six hallmarks of cancer comprising biological capabilities acquired during the multistep development of human tumors. To provide further useful conceptual framework for understating far greater detail and clarity, they revisit, refine, and extend the concept of cancer hallmark in 2011 [2] and sought out (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) avoiding immune destruction, (4) enabling replicative immortality, (5) tumor-promoting inflammation, (6) activating invasion and metastasis, (7) inducing angiogenesis, (8) genome instability and mutations, (9) resisting cell death, and (10) deregulating cellular energetics as hallmarks of cancer. In the light of these hallmarks, we aimed to revise the role of ubiquitination in development and progression of cancer in the present review. The Nobel Prize in Chemistry in 2004 was awarded to Aaron Ciechanover, Avram Hershko, and Irwin Rose for the discovery of ubiquitin-mediated protein degradation. Eight years later, it is clear that we have only scratched the surface of the complex mechanisms by which ubiquitination regulates cancer cell signaling and biology. Critical cellular processes are regulated, in part by maintaining the appropriate intracellular levels of proteins, where de novo protein synthesis is a comparatively slow process, but proteins are rapidly degraded at a rate compatible with the control of cell cycle transitions and cell death induction. One of the major pathways for protein degradation is initiated by the addition of multiple 76-amino acid ubiquitin monomers via a three-step process of ubiquitin activation and substrate recognition. Polyubiquitination targets proteins for recognition and processing by the 26S proteasome, a cylindrical organelle that recognizes ubiquitinated proteins, degrades the proteins, and recycles ubiquitin. The critical roles played by ubiquitin-mediated protein turnover in cell cycle regulation makes this process a target for oncogenic mutations. Alterations in ubiquitination and deubiquitination reactions have been directly implicated in the etiology of many malignancies. In general, specific cancers can result from stabilization of oncoproteins or destabilization of tumor suppressor genes. Some of the natural substrates for degradation by the ubiquitin–proteasome system are growth-promoting factors that if not properly removed from the cell can promote cancer.

2 Ubiquitin–Proteasome Dynamics

The ubiquitin–proteasome system (UPS) is comprised of ubiquitin, a three-enzyme ubiquitination complex, the intracellular protein ubiquitination targets, and the proteasome that is the organelle of protein degradation. The ubiquitination machinery is present both in the cytosol and the nucleus. Ubiquitination involves a three-step enzymatic reaction catalyzed by three different types of proteins, termed E1, E2, and

E3 ubiquitin ligases [3]. The hierarchical organization of the ubiquitin–proteasome enzymatic conjugation cascade places a single E1 enzyme at the top of the cascade that activates ubiquitin for all subsequent downstream reactions and interacts with all E2s. At the next stage, there are many E2 ubiquitin-conjugating enzymes (Ubc) that have defined but broad specificity. The last phase of ubiquitination is mediated by the E3 family of ubiquitin ligases, which is largely responsible for target protein specificity. Each E2 interacts with several E3s, and each E3 targets several substrates based on shared recognition motifs. Each E3 can interact with more than one E2, and some substrates can be targeted by more than one E3. E3 ubiquitin ligases include the following two main classes of enzymes: homologous to E6-associated protein (E6-AP) C terminus (HECT) E3 ligases and really interesting new gene (RING) E3 ligases. The RING finger proteins can be categorized into two distinct groups, single- and multi-subunit proteins. Examples of single-subunit RING E3s are the MDM2 oncoprotein, the E3 ligase of P53 [4] and CBL as well as the E3 ligase of receptor tyrosine kinases such as epidermal growth factor receptor and platelet-derived growth factor receptor [5]. Other RING E3s include the Skp1-cullin1-F-box protein family (SCF), which consists of multi-subunit complexes that include a RING finger domain as one of several components. Another subset of UPS is a large subcellular organelle, the proteasome, which is a multi-subunit 26S protein complex, that is the site for ATP-dependent degradation of ubiquitin-tagged proteins [6]. The proteasome is essentially a hollow cylinder-shaped particle that is deployed to different sites in the cytosol or nucleus. The 26S proteasome is composed of two major subunits that can assemble in an ATP-dependent manner [7]. The 20S catalytic component contains multiple proteolytic sites, and the 19S regulatory component contains multiple ATPases and a binding site for ubiquitin concatemers.

Degradation of a protein *via* the ubiquitin–proteasome pathway involves two discrete and successive steps: (1) tagging of the substrate by covalent attachment of multiple ubiquitin molecules and (2) degradation of the tagged protein by the 26S proteasome complex with release of free and reusable ubiquitin. Conjugation of ubiquitin, a highly evolutionarily conserved 76-residue polypeptide, to the protein substrate proceeds via a three-step cascade mechanism (Fig. 9.1). Initially, the ubiquitin-activating enzyme E1 activates ubiquitin in an ATP-requiring reaction to generate a high-energy thiol ester intermediate, E1-S∼ubiquitin. One of several E2 enzymes transfers the activated ubiquitin moiety from E1, via an additional high-energy thiol ester intermediate, E2-S∼ubiquitin, to the substrate that is specifically bound to a member of the ubiquitin–protein ligase family, E3. For the HECT domain E3s, the ubiquitin is transferred once again from the E2 enzyme to an active site Cys residue on the E3, to generate a third high-energy thiol ester intermediate, ubiquitin∼S-E3, before its transfer to the ligase-bound substrate. RING finger-containing E3s catalyze direct transfer of the activated ubiquitin moiety to the E3-bound substrate. E3s catalyze the last step in the conjugation process: covalent attachment of ubiquitin to the substrate. The ubiquitin molecule is generally transferred to a ε-NH_2 group of an internal Lys residue in the substrate to generate a covalent isopeptide bond. In some cases, however, ubiquitin is conjugated to the NH_2-terminal amino group of the substrate. By successively adding activated ubiquitin moieties to internal Lys residues on the previously conjugated ubiquitin molecule, a polyubiquitin chain is synthesized. The other modes of recognitions of

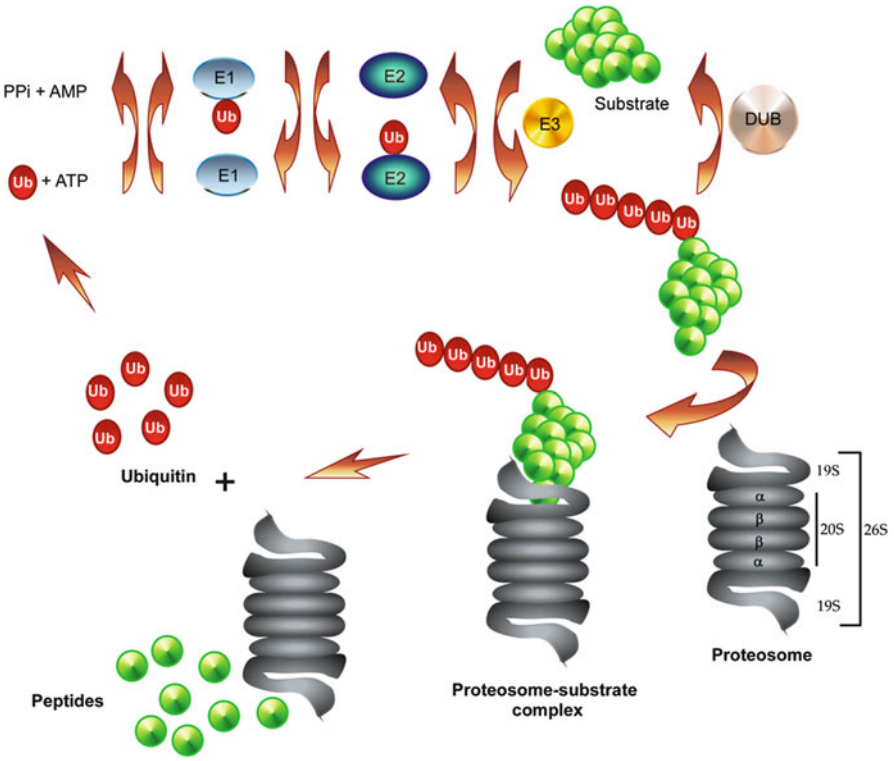

Fig. 9.1 The ubiquitin–proteasome pathway. A ubiquitin-activating enzyme (E1) binds ubiquitin in an adenosine triphosphate (ATP)-dependent step. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2). A ubiquitin ligase (E3) helps transfer ubiquitin to the target substrate. *DUB* deubiquitinating enzymes, *AMP* adenosine monophosphate, *PPi* pyrophosphate, *Ub* ubiquitin

proteolytic substrates by ubiquitin–protein ligases are through peptide-induced allosteric activation, phosphorylation of either substrate or ligase enzyme or both, recognition in *trans* via an ancillary protein, recognition of the selectively abnormal/mutated/un- and misfolded proteins, including also defective ribosomal products, or recognition via hydroxylated proline substrate. The chain is recognized by the downstream 26S proteasome complex. Proteins are degraded in a processive manner by the proteasome; thus, a single protein is hydrolyzed to final products before the next substrate enters [8]. This ordered process contrasts to the activity of cytosolic proteases that cleave proteins once before dissociating from their substrates. Various protein components of the 26S proteasome have differential ability to bind multiubiquitin chains in protein conjugates and may confer an additional degree of specificity in the targeting of proteins for degradation [9]. Cleavage products in the proteasome average six to ten amino acids in length, and eventual hydrolysis to individual amino acids occurs in the cytosol [10]. Evidently, no part of the cell is out of reach of the ubiquitin–proteasome regulatory system. Levels of proteins in the nucleus, cytoplasm, ER lumen, as well as membrane proteins, are all kept in check

by the ubiquitinating enzymes and the proteasome. Even the stability of mRNA can be regulated via proteasome-dependent protein degradation. As an additional outcome of this system, the peptide products of the proteasome are a critical factor in deciding whether a cell will be recognized as infected or tumorigenic by the immune system and destroyed, or recognized as self and spared.

An important step in the ubiquitin pathway involves the release of ubiquitin from its various adducts. Release of ubiquitin plays an essential role in two processes, the first of which is protein degradation. During degradation, it is important to release ubiquitin from Lys residues of end proteolytic products, to disassemble polyubiquitin chains and to "proofread" mistakenly ubiquitinated proteins. The second process is ubiquitin biosynthesis. Ubiquitin is synthesized in a variety of functionally distinct forms. One of them is a linear, head-to-tail polyubiquitin precursor. Release of the free molecules involves specific enzymatic cleavage between the fused residues by deubiquitinating enzymes (DUBs). The last ubiquitin moiety in many of these precursors is encoded with an extra C-terminal residue that has to be removed in order to expose the active C-terminal Gly. In a different precursor, ubiquitin is synthesized as an N-terminal fused extension of two ribosomal proteins and serves as a covalent "chaperone" that targets them to the ribosome. Following their incorporation into the ribosomal complex, ubiquitin is cleaved [11].

Ubiquitin mediates many of its functions by interacting with highly specialized ubiquitin-binding domains (UBDs) in downstream effector proteins. More than 15 UBDs (UBA, UIM, IUIM, UEV, GAT, CUE, PAZ, NZF, GLUE, UBM, UBZ, VHS, etc.) have been discovered so far. The complexity of cellular signaling networks is further increased by modifications with ubiquitin-like (Ubl) proteins, including the small ubiquitin-related modifier (SUMO), neural precursor cell-expressed developmentally downregulated 8 (Nedd8), interferon-stimulated gene 15 (ISG15), FAT10, Atg8, and Atg12 [12], all of which regulate a variety of physiological processes. The versatility of protein ubiquitination as a cellular regulatory mechanism is now well established and appears to be comparable to that of phosphorylation, another well-studied protein modification. Indeed, in many cases, protein phosphorylation and ubiquitination go hand in hand in the regulation of many cellular processes when phosphorylation typically precedes ubiquitination. Recognition of the widespread applicability of these concepts will increasingly affect the development of new means to treat human cancer.

3 Involvement of Ubiquitin on Different Hallmarks of Cancer

3.1 Sustaining Proliferative Signaling

To retain the power of proliferation and survival, cancer cells develop few strategies. One could be the alterations in ubiquitination and degradation of signaling receptors. Such alterations include stabilization of pro-growth/pro-survival receptors as well as

accelerated degradation of those receptors whose downstream signaling is detrimental for cell proliferation and viability. The mechanism behind such alteration includes changes in levels and activities of specific ubiquitin ligases or affinity of substrates to these ligases. Epidermal growth factor receptor (EGFR) is a molecule known to be found on the surface of many cancer cells that can activate signals to promote cell growth and cell division. EGFR ubiquitination prevents its recycling but promotes the degradation of the internalized receptors in lysosome. Such events switch off the signaling initiated by EGF and limit the magnitude and duration of pro-growth and pro-survival responses [13]. Phosphotyrosine within the intracellular domain of EGFR is the site for the recruitment of the c-Cbl (for Casitas B-lineage lymphoma protein) E3 ubiquitin ligase (Fig. 9.2). Upon EGFR activation, Cbl proteins are tyrosine phosphorylated by Src kinases [14]. However, phosphorylation of Cbl proteins is not required for their interaction with EGFR. In addition, phosphorylation of Cbl proteins enhances interaction with another adaptor protein, namely, Cbl-interacting protein of 85 kDa (CIN85). Binding of Cbl to the activated EGFR and recruitment of CIN85 coupled EGFR is then mediated through clathrin-mediated endocytosis [15]. From the early endosome, the growth factor receptor can either be recycled to the plasma membrane or it can be degraded. The decision to recycle or degrade appears to be regulated in part by the E3 activity of Cbl proteins. Ubiquitination of the EGFR increases its trafficking to the multivesicular body and eventually to the lysosome, where it is degraded [16]. Interestingly, interactions of EGFR with an HECT-type ubiquitin ligase Smad-ubiquitination regulatory factor II (Smurf2), which can ubiquitinate, but stabilize EGFR by protecting it from c-Cbl-mediated degradation [17].

Met receptor tyrosine kinase (RTK) another important regulator of many biologic functions in malignant cells, including cell proliferation, also undergoes ubiquitination-dependent regulation [18] by c-Cbl. Mutant Met (Tpr-Met) participates in enhanced downstream signaling and transform mammalian fibroblasts and epithelial cells [19]. The cause behind such effect is the deletion of juxtamembrane tyrosine residue of Met (the recruitment site of c-Cbl) that fails to recruit c-Cbl, thus impaired ubiquitination [20]. Akt, a serine/threonine protein kinase, is a key regulator of cell proliferation and survival and is also known to be dysregulated in human cancers. Constitutive activation of growth factor receptors is found in the majority of glioblastomas and breast, endometrial, and prostate cancers. Tumor necrosis factor receptor-associated factor 6 (TRAF6) was found to be a direct E6 ligase for Akt and is essential for Akt ubiquitination, membrane recruitment, and phosphorylation upon growth factor stimulation. The human cancer-associated Akt mutant displayed an increase in Akt ubiquitination in turn contributing to oncogenic Akt activation [21].

Another family of transcription factor NFκB (nuclear factor-κB) regulates gene expression important for cell proliferation and survival [22]. Constitutive activation of NFκB is linked to its anti-apoptotic role in pre-neoplastic and malignant cells. NFκB activation is regulated by ubiquitination of several signaling proteins of the NFκB pathway [23]. Translocation of NFκB from cytoplasm to nucleus is initiated by its release from the inhibitor IκB. Action of IκB is under the control of IκB kinase (IKK), whose regulatory subunit IKK/SUMO promotes IκB phosphorylation that recruits the E3 ubiquitin ligase SCF-β-transducin repeat-containing proteins (βTRCP) to IκB which in turn promotes Lys48-linked ubiquitination and proteasomal degradation, thereby releasing NFκB [23, 24].

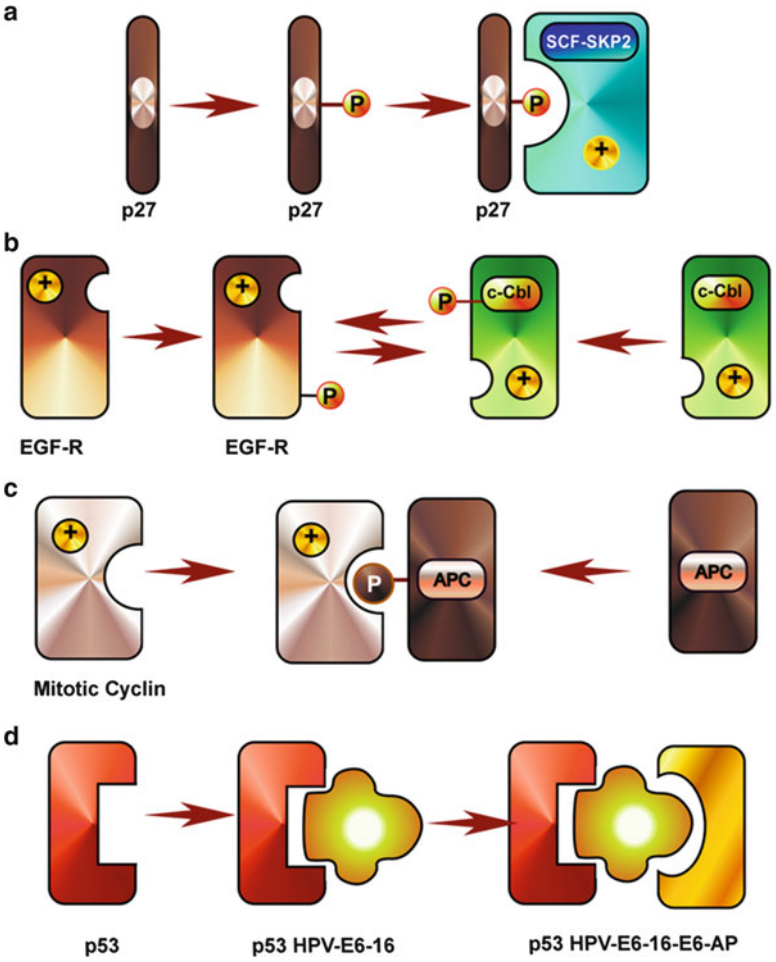

Fig. 9.2 Certain examples of different substrate E3-ligase interactions: (**a**) p27 with SCF-SKP2, (**b**) EGFR with c-Cbl, (**c**) cyclin D1 with APC, and (**d**) p53 with HPV-E6-18 and E6-AP. *SCF-SKP2* S-phase kinase-associated F-box protein 2, *EGFR* epidermal growth factor receptor, *c-Cbl* Casitas B-lineage lymphoma protein, *APC* anaphase-promoting complex, *HPV* human papillomavirus, *E6-AP* homologous to E6-associated protein

3.2 Evading Tumor Suppressors

In addition to the hallmark capability of inducing and sustaining positively acting growth-stimulatory signals, cancer cells must also circumvent powerful programs that negatively regulate cell proliferation; many of these programs depend on the actions of tumor suppressor genes. Dozens of tumor suppressors that operate in various ways to limit cell growth and proliferation have been discovered through their characteristic inactivation in one or another form of animal or human cancer. The mostly known tumor suppressor is the guardian of genome p53, a sequence-specific

DNA-binding transcription factor. The tumor suppressor p53 plays a critical role in maintaining the integrity of genome and preventing tumor development. p53 is activated when cells are subjected to DNA-damaging agents [25, 26], oncogene activation, hypoxia, and lack of growth factors. It has been demonstrated that the level of p53 is mainly regulated at the posttranscriptional stage through ubiquitination-dependent degradation [27, 28] and the ring-containing protein Mdm2, the E3 that ubiquitinates p53 keeps it at a low level in unstimulated cells. Ubiquitination of p53 by Mdm2 also facilitates the export of p53 from nucleus to cytoplasm, where it could not activate transcription of target genes [29]. Under oncogene activation, the expression of Mdm2-binding protein, ARF, is transcribed from an alternate reading frame of the INK4a/ARF locus and is increased; this directly inhibits Mdm2's E3 activity and increases intracellular p53 level [30]. Enhancement of p53 degradation has also been recognized as one of the strategies used by oncoviruses that stimulate cell proliferation for the sake of their own life cycle [31]. Adenoviral E6 oncoprotein, a "high-risk" human papillomavirus (HPV) type, has also been demonstrated to catalyze the ubiquitin-mediated degradation of p53 [32, 33]. E6 is a small protein of about 150 residues composed of two 70-residue zinc-binding domains [34]. E6 interacts with E6-AP, an 850-residue E3 ubiquitin ligase [32]. E6-AP contains an E6-binding site within a central 18-residue stretch comprising the "LxxLL" motif that is found in several other targets of E6 [35]. The core domain of p53 appears to contain an E6-binding site restricted to E6s of high-risk HPVs (Fig. 9.2). E6 binding to this site is E6-AP dependent and is required for p53 ubiquitination and subsequent degradation and its subsequent degradation by the 26S proteasome [36]. Deubiquitination enzyme herpes virus-associated ubiquitin-specific protease (HAUSP) can specifically remove ubiquitin from p53 and stabilize it, even in the presence of Mdm2. Overexpression of HAUSP suppressed colony formation in a p53-dependent manner, presumably through p53-induced growth arrest and/or apoptosis. It is not known yet whether or how HAUSP is regulated by p53-inducing stimuli such as DNA damage and oncogene activation [37].

A normal cell proliferates in response to mitogenic stimulus; in contrast, cancer cells precede following their own tune. The later event occurs when the negative feedback system fails to prevent uncontrolled proliferation. Looking at the detailed picture of a normal cell cycle, it reveals a series of cyclins and cyclin-dependent kinases (CDKs) are involved in regulation of cell cycle. Activities of CDKs are dependent on the level of cyclins in the cell and CKI (CDK inhibitor). These cell cycle regulators remain under the supervision of UPS, which is tightly linked to cancer development as known from the reported evidences. S-phase kinase-associated protein 2 (SKP2), a component of E3 ligase of the SCF complex, targets the negative regulator of cell cycle p27 (Fig. 9.2). p27, a well-known tumor suppressor, is one of the targets for degradation [24, 38]. It is evident that SKP2 expression is inversely correlated with levels of p27 in many cancers and also with the grade of malignancy in certain human tumors. Also, frequent amplification and overexpression of the *SKP2* gene has been observed in lung cancers [39]. Among the cyclins, cyclin E is the cell cycle regulatory protein that increases to a peak at G1-S transition and activates CDK. This commits the cell to the S-phase genomic duplication. Reports show that aberrant accumulation and overabundance of cyclin E leads to

premature S-phase entry, chromosome instability, and tumor formation [40, 41]. In some cases where cyclin E is overexpressed, there exists defect in its ubiquitin-mediated degradation. The gene encoding the F-box protein of SCF complex is mutated in breast and ovarian cancer shows high level of cyclin E [42]. F-box and WD-40 domain protein 7 (FBW7), another component of SCF complex, was biochemically and genetically shown to act as a tumor suppressor. Well-known oncoproteins such as cyclin E, cMyc, JUN, Notch-1, and Notch-4 are the substrates for FBW7 [43]. Mutations in *FBW7* were found in ovarian cancer, breast cancer, lymphoma, and colorectal cancer. Such mutations might therefore result in impaired degradation of the substrates and their subsequent accumulation, which might then contribute to carcinogenesis. Beta-transducin repeat-containing protein (β-TRCP) is a versatile F-box protein of SCF complex that targets several cell cycle regulators EMI1/2 [44], WEE1A [45], and Cdc25A/B [46]. Genetic alteration of β-*TRCP* genes in human cancers has been shown in several studies. Evidences indicate that loss of function of β-TRCP activates the Wnt signaling pathway which is related to human cancer development. Also, increased β-TRCP level is associated with increased β-catenin activation. The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase that plays an essential role in G1 phase and mitosis through the degradation of cell cycle proteins. APC/C also functions as a tumor suppressor and is mutated in more than 70 % of colorectal carcinomas [47]. APC/C, anaphase-promoting complex/cyclosome, promotes polyubiquitination and degradation of mitotic cyclins and securin, which are required for termination of the mitotic cycle and separation of the sister chromatids, respectively [48].

3.3 Avoiding Immune Destruction

Studies indicate that ubiquitination plays potent roles in regulating a variety of signals in both innate and adaptive immune cells. Among them E2 enzymes play a major role in determining the length and linkage type of ubiquitin chains that are formed [49]. For example, the E2 enzyme Ubc13 is required for interleukin-1 (IL-1) and lipopolysaccharide (LPS)-induced MAP kinase activation but appears less important for nuclear factor-κB (NFκB) signaling from these ligands in macrophages and fibroblasts [50]. Ubc13 also appears to be dispensable for tumor necrosis factor (TNF)-induced NFκB signaling. In contrast, Ubc13 is important for T cell receptor (TCR)-induced NFκB signaling in thymocytes [51]. It is possible that other E2 ligases, such as Ubc5, can support K63 ubiquitin-dependent signals, depending on the cell type and stimulus. The selectivity of E2s for certain subsets of E3 enzymes (and hence substrates) and the predilection of E2s to form particular ubiquitin chain linkages combine to render these enzymes important regulators as well as mediators of ubiquitination. Ubiquitin replacement strategy is employed for testing the requirements for specific ubiquitin chain linkage in cells. This strategy was used to show that K63 ubiquitination is required for viral activation of interferon regulatory factor-3 (IRF3) [52]. These studies and others uncovered the physiological importance of K63 ubiquitin chains in immune signaling. In addition, functions

of other non-K48 linkages have been described in immune cells. K33 linkages have recently been described in T cells, where the E3 ubiquitin ligases Cbl-b and ITCH appear to cooperatively promote K33-linked ubiquitination of T cell receptor-ζ (TCRζ). This modification inhibits TCRζ's phosphorylation and association with the tyrosine kinase Zap-70 and thereby restricts TCR signaling [53]. Hence, K33 ubiquitination can disengage TCRζ from TCR signaling without inducing its proteasomal degradation, providing an additional mechanism by which ubiquitination can restrict signals. Utilization of this type of ubiquitin-mediated restriction of signals, rather than proteasomal degradation, allows cells to reutilize TCRζ without spending energy on degrading and resynthesizing this protein. ITCH is an E3 ubiquitin ligase that plays a role in T cell receptor activation and signaling through ubiquitination of multiple proteins including MKK4, a member of the MAP kinase kinase family, which directly phosphorylates and activates the c-Jun NH2-terminal kinases (JNK), in response to cellular stresses and proinflammatory cytokines. ITCH contributes to a ubiquitin-dependent nonproteolytic pathway that regulates inducible Foxp3 expression, a transcription factor necessary for induction of the immunosuppressive functions in regulatory T lymphocytes in cancer [54].

Lineage-specific deletions of ubiquitin-modifying enzymes using LoxP-flanked alleles have recently begun to unveil cell-autonomous functions for these enzymes in mice. Tumor necrosis factor receptor-associated factors (TRAFs) are important signaling adaptors that can mediate signals from TNF superfamily receptors to activate various transcription factors. Mice deficient for TRAF2, TRAF3, or TRAF6 die in utero or perinatally from multiple organ abnormalities, demonstrating their nonoverlapping roles in mouse development [55]. TRAF6, the seminal E3 ligase shown to synthesize nondegradative K63 ubiquitin linkages and activate NFκB signaling, is a good example of the divergent functions that one E3 ligase can play in cell-specific contexts. It was found that lineage-specific deletions of the genes encoding TRAF6, A20, and ACT1 to illustrate the differing roles E3 ubiquitin ligases can play in different cell types. In contrast to TRAF6's roles in supporting NFκB and MAPK signals, mice lacking TRAF6 specifically in T cells develop multiorgan inflammatory disease, indicating that TRAF6 plays a T cell-intrinsic role in preventing spontaneous inflammation. TRAF6-deficient T cells are resistant to both induction of T cell anergy or immunologic tolerance and suppression by regulatory T cells [56]. Act1, originally identified as an NFκB activator, is a positive signaling regulator of IL-17-mediated responses in T cells by recruiting transforming growth factor-associated kinase-1 (TAK1) and TRAF6 [57]. Act1 possesses a U box domain and functions as an E3 ubiquitin ligase that K63 polyubiquinates TRAF6. The ubiquinating activity mediated by the Act1 U box appears to be required for IL-17-dependent signaling [58].

3.4 Enabling Replicative Immortality

DNA damage repair is important for a healthy cell to survive without a mutated or uncorrected genome. As an error made during the replication if continued impacts

on the genomic integrity and may lead to onset of cancer, several proteins sequentially take part in correcting the erroneous DNA. Damage repair occurs at three checkpoints at G1/S, G2/M, and an intra-S checkpoint in cell cycle. These checkpoints are controlled by two essential kinases ATM and ATR. Structurally modified chromatin and double-stranded DNA breaks are the substrates for ATM [22, 59] while ATR is recruited at stalled replication forks [24]. Ubiquitin-mediated regulation is an important feature for checkpoint activation. The checkpoint pathways target the CDK regulators of the cell cycle like cyclins, CDK inhibitors, or Cdc25 (Cdc, cell division cycle) depending upon the stages of cell cycle in which DNA is damaged. Phosphatase Cdc25A is a dual-specificity phosphatase that controls entry into and progression through various phases of the cell cycle and is the substrate for SCFβ-TrCP [40] in response to DNA damage resulting in cell cycle arrest. Another ligase APC/C remains active in M and G1 phases. These target the Cdc20 for its degradation during G1 or associate with CDH1 to form a complex that functions during G2/M phase. Also, APC/C participates in a p53-independent checkpoint response in order to target the degradation of cyclin D1 (Fig. 9.2). Monoubiquitination of the components of Fanconi anemia (FA) DNA repair pathway participates in interstrand cross-links (ICL) during DNA replication. DNA ICL are recognized by a protein complex comprising of Fanconi anemia complementation group M (FANCM), FA-associated protein 24 (FAAP-24), and DNA-binding histone-fold proteins MHF1 and MHF2. This complex recruits the FA core complex. Within the core complex, FANCL interacts with UBE2T, an E2-conjugating enzyme, to ubiquitinate FANCD2 and FANCI [44]. A significant repair pathway gets on following a double-stranded break in DNA. Events following such damage are largely dependent on the multifunctional E3 enzyme breast cancer type-1 susceptibility protein (BRCA1). Recruitment of BRCA1 is mediated by a series of ubiquitination events, initiated by RING finger 8 (RNF8) along with Ubc13. Among the large number of proteins recruited at the breakage site, H2AX is one of them which encodes histone H2A. RNF8 with its interacting partner Ubc13 ubiquitinylates H2AX and H2A at the site of repair foci formation and plays a role in the repair of damaged chromosomes. RNF8 may serve as the master for orchestrating the events associated with damage at the double-stranded break. Hence, depletion in RNF8 level might be a failure in orchestration of the events during the double-stranded break repair [60].

3.5 Tumor-Promoting Inflammation

Recent data have expanded the concept that inflammation is a critical component of tumor progression. Many cancers arise from sites of infection, chronic irritation, and inflammation. Ubiquitination-mediated regulation of the inflammatory suppressor homeodomain-containing transcription factor NKX3.1 was found very significantly reduced in regions of inflammatory atrophy and in preinvasive prostate cancer. Inflammatory cytokines tumor necrosis factor (TNF)-alpha and interleukin-1beta accelerate NKX3.1 protein loss by inducing rapid ubiquitination and proteasomal

degradation. TOPORS, ubiquitously expressed E3 ubiquitin ligase that ubiquitinates p53, has also been shown to ubiquitinate the tumor suppressor NKX3.1 that leads to its proteasomal degradation in prostate cancer cells [61]. In many chronic inflammatory diseases and cancer-related inflammation, ubiquitination plays an eminent role through TNF signaling. TNF receptor 1 (TNFR1) is ubiquitously expressed, whereas TNFR2 is mainly expressed on lymphocytes and endothelial cells. The different outcomes of TNF signaling originate at the apical signaling complex that forms when TNF binds to TNFR1, the TNFR1 signaling complex (TNF-RSC). By integrating recently gained information, it has given insight on the functional importance of the presence of different types of ubiquitination in the TNF-RSC, including linear ubiquitin linkages generated by the linear ubiquitin chain assembly complex (LUBAC), i.e., a Ub ligase (E3) that catalyzes head-to-tail ligation of Ub [62]. LUBAC mediates ubiquitylation of NFκB essential modulator (NEMO) with linear Ub chains, which is required for efficient NFκB activation following TNF stimulation [63]. However, it was recently shown that linear Ub chains can be attached to NEMO and that this is important for NFκB activation [63].

Transforming growth factor-β (TGF-β) is a potent pleiotropic cytokine, that affects the host immune/inflammatory reactions in the tumor microenvironment. TGF-β is known to inhibit cell growth by transcriptional repression of growth-promoting gene, e.g., Myc, but an aberrant TGF-β signaling can promote inflammation during tumor growth. High level of expression of the Smad-ubiquitination regulatory factor II (Smurf2), an E3 ligase that downregulates TGF-β receptor II (TβRII) [64], correlates with poor prognosis in patients with esophageal squamous cell carcinoma [65].

3.6 Activating Invasion and Metastasis

Research into the capability for invasion and metastasis characterized by epithelial mesenchymal transition (EMT) has accelerated dramatically over the past decade as powerful new research tools and refined experimental models as critical regulatory genes have been identified. During cancer-associated EMT, epithelial cancer cells acquire the ability to detach from their initial site, pass through the dismantled basement membrane into adjacent tissues, and metastasize to distant sites. This event is programmed in tune with the downregulation of epithelial cell markers and the upregulation of mesenchymal counterparts. Cells that undergo EMT have a greater capacity to migrate, to invade, and to resist therapies. Many signal transducers and transcription factors involved in EMT are regulated by ubiquitination and the ubiquitin–proteasome system. Zinc finger E box-binding homeobox (ZEB) protein ZEB1, the most apical EMT transcription factor, is a very powerful inducer of EMT via its transcriptional suppression of E-cadherin and zona occludens protein ZO-1 expression. ZEB1 has been found to be upregulated by E3 ligase cullin7/FBXW8 complex (FBXW8 stands for F-box/WD repeat-containing protein 8), resulting in downregulation of E-cadherin and increased invasion of human trophoblastic cell lines.

Another major transcription factor governing EMT, SNAIL is upregulated by NFκB in a posttranslational manner; NFκB inhibits SNAIL phosphorylation by glycogen synthase kinase 3β (GSK3β) and thus prevents subsequent ubiquitination by E3 ligase βTRCP and proteasome degradation [66]. Such fine regulation of SNAIL by NFκB positively contributes to mesenchymal transition. Similarly, NFκB is a critical regulator of basic helix-loop-helix (bHLH) transcription factor TWIST which regulates several hundred metastatic genes. Hence, a feed-forward loop is established in this case, as TWIST activates transcription of kinase AKT2, an NFκB activator [67]. ZEB1 as well as SLUG (also known as SNAIL2) has been found to be upregulated by E3 ligase cullin7/FBXW8 complex, resulting in downregulation of E-cadherin and increased invasion of human trophoblastic cell lines. Another protein, Smad ubiquitination regulatory factor 2 (Smurf2) was originally identified as the Smad E3 ligase that induces the ubiquitination and degradation of Smad1 and Smad2 [68]. Reduction of Smurf2 expression with specific short interfering RNA in metastatic breast cancer cells has been showed to induce cell rounding and reorganization of the actin cytoskeleton, which are associated with a less motile and invasive phenotype [69]. Gp78 is a RING finger E3 ubiquitin ligase identified as the tumor autocrine motility factor receptor mediating tumor invasion and metastasis also remains integral to the endoplasmic reticulum (ER) and involved in ER-associated degradation (ERAD) of diverse substrates [70]. These studies anticipate the progress toward more translational efforts that exploit ubiquitin-related targets for the anticancer therapies.

3.7 Inducing Angiogenesis

The tumor-associated neovasculature, generated by the process of angiogenesis, is governed by countervailing factors that either induce or oppose angiogenesis. The well-known prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Secretion of the above pro-angiogenic factors remains under the transcriptional regulation of hypoxia-inducible factor1α (HIF1α). Recent findings demonstrate a novel role for protein ubiquitination in regulation of cancer-induced angiogenesis. To keep a control over the regulation of angiogenesis-promoting factors, *Von Hippel–Lindau* (*VHL*) gene product acts as an antiangiogenic factor. The *VHL* gene encodes a component of an SCF-like ubiquitin ligase and is mutated in patients suffering from the familial cancer susceptibility, VHL-syndrome that is associated with cancer of the kidney and tumors in the blood vessels of the central nervous system. Mutations in components of the ubiquitination machinery can also cause malignancies. Mutations in *VHL* predispose individuals to a wide range of malignancies, including renal cell carcinoma, pheochromocytoma, cerebellar hemangioblastomas, and retinal angiomas. A hallmark of $VHL^{-/-}$ tumors is a high degree of vascularization that arises from constitutive expression of hypoxia-inducible genes, including the master switch transcription factor HIF1 and VEGF. It has been recently shown that pVHL

is a ubiquitin ligase [40] that is involved in targeting of HIF1 for ubiquitin- and proteasome-mediated degradation [71]. Mutations in VHL prevent the degradation of HIF α-subunits under normoxic conditions and are the cause behind the formation of hypervascular lesions and renal tumors [72]. Deregulated HIF-1α then triggers the transcription of several genes encoding pro-angiogenic growth factors, including VEGF, platelet-derived growth factor (PDGF), and transforming growth factor-α (TGF-α) [73]. As a consequence, these upregulated factors even under normoxic condition trigger the formation of new blood vessels and tumor. Therefore, it can be suggested that identification of molecules that inhibit the angiogenic signaling of VEGF and its receptors will provide new avenues for development of better and more effective agents to combat angiogenesis-associated diseases like cancer.

3.8 Genome Instability and Mutation

Genomic stability is maintained by a system which can detect and resolve the defects by reducing the rate of spontaneous mutation in each cell generation. Rate of mutation often increases in cells acquiring mutant genes which may lead ahead to tumor development. Tumorigenesis-associated mutations in ubiquitin–proteasome pathway can be classified into two groups: (1) those that result from loss of function, mutations in a ubiquitin system enzyme or target substrate that result in stabilization of certain proteins, and (2) those that result from gain of function, abnormal or accelerated degradation of the protein target.

p53, which keeps a check on the genomic integrity of a cell, gets activated upon a genomic insult and causes cell cycle arrest or apoptosis of the cell depending upon the extent of the DNA damage. p53 itself remains under fine regulation by the ubiquitin ligase protein which is necessary for the maintenance of an optimum level of the target protein. Mutant p53 (a single amino acid substitution in DBD) found in 50 % of human cancer [74] fails to transactivate Mdm2 as it normally does and hence accumulation of mutated p53 prevails in tumor. Such inability of mutated p53 which is no more regulated by the negative regulator Mdm2 remains nonfunctional and shows a halt to its tumor suppressive action. Recently it is reported that mutant p53 also undergoes Mdm2-independent degradation where conformationally mutated p53 proteins in cells are specifically recognized by molecular chaperones heat-shock protein90 (Hsp90) and heat-shock cognate70 (Hsc70), which present the substrates to the ubiquitin ligase chaperone-assisted E3 ligase C terminus of Hsc70-interacting protein (CHIP), a U-box-dependent E3 ubiquitin ligase [75]. CHIP is able to degrade both the wild type and the mutated form of p53. Downregulation of CHIP in some cancers has shown reduced degradation of mutant form of p53, hence enhancing its stability. This encourages mutant form of the tumor suppressor to promote various aspects of tumorigenesis, including metastatic spread. Two proteins which remain extensively mutated in serous ovarian cancer are FBW7, a substrate recognition component of an SCF-type E3 ubiquitin ligase, and speckle-type POZ protein (SPOP) another ubiquitin ligase, and are responsible for degradation of other proteins, hence regulating their turnover.

SCF-FBW7 degrades several proto-oncogenes that function in cellular growth and division pathways, including Myc, Notch, and Jun [76]. Even, protein encoded by SPOP, in mutated form has also been reported to be found in prostate cancer. Substrates recruited by SPOP and targeted for ubiquitylation via the CUL-3/SPOP complex include Bmi-1, PIPK II β, and Daxx. These substrates are subsequently degraded by the proteasome. In addition, SPOP itself becomes ubiquitylated by the CUL-3-based ubiquitin ligase and is targeted for proteasomal degradation. Mutations are also reported in genes involved in the ubiquitin-mediated proteolysis pathway. One of them is protein encoded by VHL. Mutations in VHL are known to be associated with the development of clear cell renal cell carcinoma [77]. A better understanding of the ubiquitylation machinery will provide new insights into the regulatory biology of cell cycle transitions and the development of anticancer drugs.

3.9 Resisting Cell Death

The tremendous progress in understanding the ubiquitination process over the last couple of years made it possible to identify ubiquitination substrates and study the role of ubiquitination in regulating the levels and functions of many apoptosis-related proteins. Bcl-2 family proteins are critical regulators of apoptosis. The anti-apoptotic members of the family such as Bcl-2, Bcl-xL, and Bcl-w all contain the conserved Bcl-2 homology (BH) regions BH1, BH2, and BH3, which form a hydrophobic groove that binds to BH3 domain of other family members [78]. The Bcl-2 family has two types of proapoptotic members: the Bax and Bak subfamily and the BH3-only proapoptotic members such as Bid, Bad, Bim/Bod, and PUMA [79]. Studies with gene-targeted cells indicated that the presence of Bax or Bak is required for many forms of apoptosis [80], and each type of cell needs at least one of the anti-apoptotic Bcl-2 family members to survive [81]. Besides being controlled through transcription, phosphorylation, and proteolytic cleavage, it is becoming evident that Bcl-2 family members are regulated by ubiquitination and proteasome degradation systems. The death-promoting multidomain Bcl-2 members Bak and Bax also seem to be controlled by the proteasome. The oncoprotein human papillomavirus E6 can inhibit apoptosis in differentiating keratinocytes, which express high levels of Bak. Interestingly, the ubiquitin ligase E6-AP interacts with Bak, which results in the degradation of Bak in vivo [82]. E6-AP-mediated p53 ubiquitination and subsequent degradation have already been discussed in evading growth suppressor section (Fig. 9.2). Overexpression of Bcl-2-associated athanogene-1 (BAG-1), a protein that harbors ubiquitin domain (a domain that is related to ubiquitin), potentiates the anti-apoptotic effects of Bcl-2 in response to several apoptotic stimuli. Furthermore, BAG-1 interacts with, and suppresses the actions of, the ubiquitin ligase seven in absentia homologue 1A (Siah-1A), which is induced by, and contributes to the proapoptotic functions of, p53 [83].

Many molecules of the extrinsic apoptosis pathway are closely related to the ubiquitination and proteasome system. For example, the intracellular domains of

Fas and type-I TNF receptor bind with small ubiquitin-related modifier-1 (SUMO-1) and Ubc9 [84], suggesting that they are targets of SUMO modification or may function as a scaffold to promote sumoylation. The death receptor-associated protein Daxx can also bind with SUMO-1 and Ubc9 [85] and was conjugated with SUMO-1 [86]. Since the SUMO-1- and Ubc9-binding site is overlapped with the Fas-binding region of Daxx, it is conceivable that SUMO association or sumoylation may affect signal transduction of death receptor. Since we now know that sumoylation modulates many proteins and cellular processes, the mechanisms and physiological significance of the earlier transfection experiments indicating that overexpression of SUMO-1 altered the susceptibility to apoptosis remain to be determined. Another Fas-associated protein, FAF1, contains a ubiquitin-like domain required for the apoptosis induced by its overexpression [87]. c-FLIP contains two death effector domains and acts as a dominant negative inhibitor of death receptor-mediated activation of caspase 8. RING finger protein 34 (RNF34), E3 ubiquitin–protein ligase, inhibits death receptor-mediated apoptosis through ubiquitination/degradation of caspase 8 and caspase 10 [88]. Lack of this protein made cells become highly sensitive to FasL or TNF [89]. The resistance of certain melanoma cells to the death receptor ligand TRAIL has been attributed to the expression of c-FLIP [90]. Further studies indicated that the downregulation of c-FLIP was due to its ubiquitination and subsequent proteasomal degradation. Instead of direct c-$FLIP_L$ phosphorylation, JNK promotes accelerated decay of c-$FLIP_L$ through activation of the ubiquitin ligase ITCH, the first member of the HECT family of E3 ligases [91]. Ubiquitination is a critical process in regulating many signal transduction pathways, transcription factors, and adaptor molecules that have profound effects on the growth and death of cells. For example, suppressors of cytokine signaling (SOCS) are a family of intracellular proteins that contain a center SH2 domain and characteristic carboxyl-terminal SOCS box [92]. They are often induced by cytokines and in turn suppress the Jak-Stat pathways that mediate the signal of cytokines, forming a negative feedback to limit cytokines' biological effects.

3.10 Deregulating Cellular Energetics

Proliferation in cancer cells is accompanied by activation of glycolysis, which occurs even in the presence of a normal oxygen concentration. The purpose and mechanism of this aerobic glycolysis, known as the Warburg effect [93], are still unclear. This has led to the suggestion that aerobic glycolysis may be required for new biomass formation [94]. The glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3 (PFKFB3), and glutaminolysis-regulating enzyme glutaminase 1 (GLS1) are degraded by the E3 ubiquitin ligase APC/C-Cdh1. A decrease in the activity of APC/C-Cdh1 in mid-to-late G1 releases both proteins, thus explaining the simultaneous increase in the utilization of glucose and glutamine during cell proliferation [95]. Glutamine provides energy through the TCA cycle as well as nitrogen, sulfur, and carbon skeletons for growing and

proliferating cancer cells [96]. PFKFB3 is also a substrate at the onset of S-phase for the ubiquitin ligase SCF-β-TRCP, coinciding with a peak in glycolysis in mid-to-late G1 page of cell cycle [95]. E3-ubiquitin ligase, VHL, responsible for HIF-1α protein turnover under normoxic conditions, provided a direct link between glucose metabolism and some forms of cancer. Glucose metabolism was required for glucose-induced monoubiquitination in the cultured glioma cells. Monoubiquitinated histone H2B (uH2B) is a semiquantitative histone marker for glucose. Loss of uH2B occurs specifically in cancer cells of tumor specimens of breast, colon, lung, and additional 23 anatomic sites. In contrast, uH2B levels remain high in stromal tissues or noncancerous cells in the tumor specimens. Taken together this basic information, Urasaki et al. have recently reported that glucose deficiency and loss of uH2B are novel properties of cancer cells in vivo, which may represent important regulatory mechanisms of tumorigenesis [97]. Glucose-dependent insulinotropic polypeptide (GIP) released from duodenum and jejunum into the plasma during a meal binds to its receptor (GIP-R). Interference of this binding results in impairment of insulin secretion and variable degrees of glucose intolerance. In the diabetic range with the glucose levels, the degree of ubiquitination is increased and the speed of ubiquitination is accelerated. GIP-R is partially regulated, by the multivesicular body (MVB) sorting pathway in islets, that it is degraded by ubiquitination and not recycled following prolonged exposure of islets to glucose in the diabetic range and that this explains the profound decrease in GIP-R levels seen after several hours of exposure to elevated glucose levels [98].

Moreover, cancer cells seem to be highly dependent on de novo lipogenesis for their proliferation and survival. The expression and activity of many enzymes involved in fatty acid synthesis, i.e., ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FASN), are upregulated in many types of cancers. Alteration of lipid metabolism is another nearly ubiquitous change in tumor cells. In fact, fatty acid oxidation is a dominant bioenergetic pathway in various cancer cells. Fatty acid synthase (FASN), a key player in the de novo synthetic pathway of long-chain fatty acids, has been shown to contribute to the tumorigenesis in various types of solid tumors. In prostate cancer, the isopeptidase ubiquitin-specific protease-2a (USP2a) has been found to interact with and stabilize FASN protein through removing ubiquitins from FASN. USP2a is androgen regulated and overexpressed in prostate cancer, and its functional inactivation results in decreased FASN protein and enhanced apoptosis [99].

4 Targeting Ubiquitin–Proteasome Pathway in Cancer Therapy

There are multiple enzymes that modulate ubiquitin conjugation and deconjugation. Particularly ubiquitin E3-ligases play a key role in the ubiquitin-mediated proteolytic cascade as anticipated in the different hallmarks of cancer (Fig. 9.3 and Table 9.1). Thus, targeting these enzymes offers a greater degree of specificity and

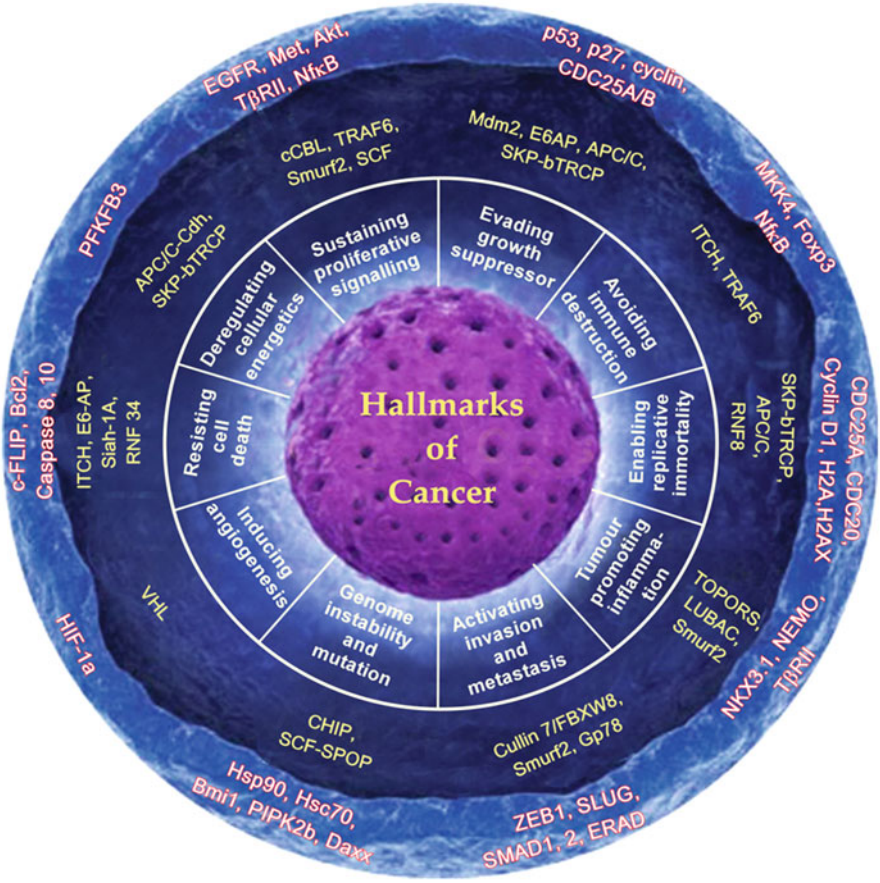

Fig. 9.3 Role of ubiquitination on different hallmarks of cancer. Ubiquitins and their substrate proteins that interfere with each of the acquired capabilities necessary for tumor growth and progression have been highlighted. *EGFR* epidermal growth factor receptor, *TβRII* transforming growth factor-β receptor II, *NFκB* nuclear factor-κB, *c-Cbl* Casitas B-lineage lymphoma protein, *TRAF6* tumor necrosis factor receptor-associated factors 6, *Smurf2* Smad-ubiquitination regulatory factor II, *SCF* Skp1-cullin1-F-box protein, *Cdc* cell division cycle, *E6-AP* homologous to E6-associated protein, *APC/C* anaphase-promoting complex/cyclosome, *βTRCP* β-transducin repeat-containing proteins, *MKK* MAP kinase kinase, *H2A* histone 2A, *H2AX* genes coding for histone H2A, *RNF8* RING finger 8, *NEMO* NFκB essential modulator, *LUBAC* linear ubiquitin chain assembly complex, *FBXW8* F-box/WD repeat-containing protein 8, *ZEB* zinc finger E box-binding homeobox, *ERAD* endoplasmic reticulum-associated degradation, *CHIP* chaperone-assisted E3 ligase C terminus of Hsc70-interacting protein, *SPOP* speckle-type POZ protein, *Hsp* heat-shock protein, *Hsc* heat-shock cognate, *PIPK2β* phosphatidylinositol 5-phosphate 4-kinase type-2 beta protein, *VHL* Von Hippel-Lindau, *HIF1α* hypoxia-inducible factor1**α**, *Siah-1A* seven in absentia homologue 1A, *PFKFB3* 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3, *GLS1* glutaminolysis-regulating enzyme glutaminase 1

Table 9.1 Role of ubiquitin ligase E3, their substrate, and the biological function(s) impacted by dysregulation of the ligase in the different hallmarks of cancer

Hallmarks of cancer	Ubiquitin ligase E3	Substrate	Biological function(s) impacted by dysregulation
Sustaining proliferative signaling	c-Cbl	EGFR, Met	Cell growth [14–20]
	TRAF-6	Akt	Cell survival [21]
	SCF-βTRCP	NFκB	Cell proliferation, survival [23, 24]
Evading growth suppressor	Mdm2	p53	Apoptosis [25–29]
	E6-AP	p53	Apoptosis [32, 36, 37]
	SCF-SKP2	p27	Cell cycle control [24, 38]
	SCF-FBW7	Cyclin E	Cell cycle control [41–43]
	SCF-βTRCP	EMI1/2, WEE1A Cdc25A/B	Cell cycle control [44–46]
	APC/C	Cyclin, securin	Cell cycle control [48]
Avoiding immune destruction	Cbl-b	TCRζ	TCR signaling [53]
	ITCH	MKK4, Foxp3	Immunosuppression by Treg [54]
	TRAF-6	NFκB	T cell anergy [55, 56]
Enabling replicative immortality	SCF-βTRCP	Cdc25A	Cell cycle control [40]
	APC/C	Cdc20, cyclin D1	Cell cycle control [40]
	RNF8	H2A, H2AX	Repair damaged chromosomes [60]
Tumor-promoting inflammation	TOPORS	NKX3.1	Inflammatory suppression [61]
	LUBAC	NEMO	Cell proliferation [63]
	Smurf2	TβRII	Cell growth [64]
Activating invasion and metastasis	Cullin7/FBXW8	ZEB1, SLUG	EMT [68]
	Smurf2	SMAD1, SMAD2	Cell survival and invasion [68]
	Gp78	ERAD	Tumor invasion and metastasis [70]
Inducing angiogenesis	VHL	HIF-1α	Tumor vascularization [71, 72, 77]
Genome instability and mutation	CHIP	Hsp90, Hsc70	Tumor suppression [75]
	SCF-FBW7	Myc, Notch, Jun	Cell cycle control, cell growth [76]
	SCF-SPOP	Bmi1, PIPK2β, Daxx	Apoptosis [76]
Resisting cell death	E6-AP	p53, Bak	Apoptosis [37, 82]
	Siah-1A	Bcl2	Apoptosis [83]
	RNF34	Caspase 8, caspase 10	Apoptosis [88]
	ITCH	c-FLIP	Apoptosis [91]
Deregulating cellular energetics	APC/C-Cdh1	PFKFB3	Glycolysis [94, 95]
	SCF-βTRCP	PFKFB3	Glycolysis [95]
	APC/C-Cdh1	GLS 1	Glutaminolysis [95]

therefore a reduced potential for side effects. Below we discuss the current status of small molecule therapeutics that target ubiquitin conjugation (E1, E2, and E3) and deconjugation (DUBs). The three enzymes sequentially involved in target protein ubiquitylation (E1-activating, E2-conjugating, and E3 ligase enzymes) are currently active targets in anticancer drug discovery [100].

4.1 Targeting by Synthetic Drug

E1 catalyzes the first step in the conjugation of ubiquitin or a ubiquitin-like protein to a target protein. An adenosine sulfamate analogue, MLN4924, inhibits the E1 enzyme responsible for NEDDylation, the covalent addition of a ubiquitin-like protein, NEDD8, to specific target proteins including SCF-Skp2 [101], an E3 ligase linked to cell cycle regulation. In the case of Skp2, NEDDylation results in progrowth activation, and MLN4924 is currently in phase II clinical trial for hematologic cancers. Experimental inhibitors of E1 have also been reported, for example, PYR-41, an irreversible ubiquitin E1 active site binder that enters cells and, while possibly too reactive to be a clinical candidate, is nonetheless useful as a tool compound [102].

Recently a small molecule selective allosteric site inhibitor of the E2 enzyme hCdc34, named CC0651, was reported [103]. Cdc34 ubiquitylates p27, among other target proteins, and inhibition of p27 ubiquitylation and degradation is predicted to prevent tumor cell cycle progression. Thus, compounds such as CC0651 are in preclinical development as potential anticancer agents.

Inhibition of MDM2 was one of many molecular oncology strategies employed in the last 10–15 years to maximize p53 presence and activity in tumors [104], and the two E3 ligase antagonists currently in clinical trial for cancer, RO5045337 (nutlin-3) and JNJ-26854165, are directed at MDM2, specifically, at the regulation of its substrate, p53 [105]. Recently in our laboratory, functional restoration of p53 was achieved by nonsteroidal anti-inflammatory drug celecoxib via multiple molecular mechanisms: (1) inhibition of p53 degradation by suppressing viral oncoprotein E6 expression, (2) promoting p53 transcription by down-modulating Cox-2 and simultaneously retrieving p53 from Cox-2 association, and (3) activation of p53 via ATM-/p38MAPK-mediated phosphorylations at serine-15/serine-46 residues [106]. The nutlin compounds might not progress to clinical trial owing to poor animal efficacy and that E3s in general are perhaps too complex for drug discovery. Disulfiram, another compound, that is an FDA-approved drug for the treatment of alcoholism was also found to have specific activity against zinc fingers and RING finger ubiquitin E3 ligases that play an important role in cancer development. There is ongoing clinical trial of disulfiram with copper gluconate against liver cancer and disulfiram as adjuvant against lung cancer [107].

There are approximately eighty known DUBS, proteases that hydrolyze isopeptide or α-peptide bonds linking ubiquitin to its target protein. Many of these have been validated as targets for cancer and other diseases [108]. In the past 15 years,

tool compounds and/or preclinical development candidate small molecule DUB inhibitors have been reported; these compounds were identified as inhibitors of several DUBs and have a range of reported selectivities with respect to other DUBs and other cysteine proteases [109]. A classic example of the strategy of ablating oncoproteins by inhibiting the DUBs that protect them from ubiquitylation and degradation is exemplified in the search for suitable inhibitors of USP7/HAUSP, one of the first therapeutically relevant DUBs to be described. Although USP7, like all DUBS, deconjugates ubiquitin from several target proteins, inhibition of USP7 promotes the degradation of its primary cellular target, HDM2, resulting in net p53 stabilization and activation [110].

The simple consequence of blocking cellular protein degradation in the proteasome is the accumulation of ubiquitylated proteins of all sorts, which appears upon first consideration to be an intolerable consequence for any cell. The proteasome was thought of as the cell's garbage disposal unit, and its blockade assumed to result in a huge excess of unwanted proteins—a toxic event. On the contrary, however, this global response has been harnessed to provide antitumor activity against multiple myeloma and mantle cell lymphomas in patients, accompanied by manageable toxicity. In 2003 [111], bortezomib, a synthetic proteasome inhibitor developed by Millennium Pharmaceuticals, Inc., which strongly indicates that the proteasome would be a novel target for cancer treatment that binds reversibly to the β5 subunit of the 20S degradation chamber of the proteasome and thereby inhibits it, was approved by the FDA for treatment of relapsed refractory multiple melanoma, following impressive activity in clinical trials [112]. Subsequently, bortezomib was approved for first-line treatment of multiple myeloma and for the treatment of relapsed mantle cell lymphoma [113].

4.2 Targeting Through Natural Product

Two natural E1 inhibitors, panepophenanthrin and himeic acid A, have been isolated from microorganisms. As the first natural E1 inhibitor, panepophenanthrin was isolated from a mushroom strain, *Panus rudis*. This compound inhibits the formation of an E1-ubiquitin thioester intermediate. A culture of the fungus *Aspergillus* sp., isolated from the mussel *Mytilus edulis galloprovincialis*, showed strong E1 inhibitory activity, and bioassay-guided fractionation of the culture afforded himeic acid A as an E1 inhibitor [114]. This compound was found to inhibit the binding of ubiquitin to the ubiquitin-binding site in the E1 enzyme. As himeic acid A cannot inhibit E1-like enzymes for other ubiquitin-like modifiers, at least, SUMO-1 and ISG15 could be a specific inhibitor of the ubiquitin E1 enzyme. Subsequently, a search for inhibitors of Ubc13-Uev1A interaction in natural resources revealed the presence of leucettamol A in the marine sponge *Leucetta aff. microrhaphis* [115].

Girolline was originally isolated as an antitumor compound from a marine sponge, but a phase I clinical study with this compound showed severe side effects in patients and no apparent antitumor activity [116]. Ubistatin was discovered by searching a

chemical library for an inhibitor of destruction box-dependent protein degradation [117]. It was found that ubistatin binds to the ubiquitin chain of ubiquitinated proteins and inhibits ubiquitin-dependent proteolysis. Thus, compounds inhibiting the delivery system for ubiquitinated proteins could serve as novel inhibitors targeting the ubiquitin–proteasome system. Punaglandins isolated from the soft coral *Telesto riisei* are highly functional cyclopentadienone and cyclopentenone prostaglandins chlorinated at the endocyclic α-carbon position and inhibit ubiquitin isopeptidase activity and exhibit antiproliferative effects more potently than J series PGs [118].

Chlorofusin, the first MDM2 antagonist from natural sources, isolated from the culture of a *Fusarium* sp. found to inhibit MDM2-p53 binding and was evaluated with seven chromophore diastereomers of chlorofusin [119]. Since (−)-hexylitaconic acid is unable to inhibit the interaction of p53 with COP1, another E3 of the HECT-type for p53, it can be inferred that hexylitaconic acid binds to HDM2 protein. Several compounds inhibiting members of a family of E3 ligases known as anti-apoptotic proteins (IAPs) have also recently entered clinical trial [120]. These IAPs ubiquitylate proteins that are essential to apoptosis, eliminating their function and, thus, blocking apoptosis. Analogues of Smac, a naturally occurring protein that binds to the IAP, triggering its auto-ubiquitylation, subsume this function and restore apoptotic activity.

Peptide aldehydes, the first proteasome inhibitors [121], are the most widely used as molecular tools for the investigation of various cellular events. Tyropeptin A, isolated from the culture broth of *Kitasatospora* sp., contains an aldehyde moiety that can inhibit the chymotrypsin-like and trypsin-like activities of the proteasomes. Fellutamide B was originally isolated from the marine-derived *Penicillium fellutanum* as a cytotoxic compound [122] and found to induce the release of nerve growth factor (NGF) from fibroblasts and glial-derived cells [123]. Fellutamide B was tested for inhibitory activity against the proteasome and found to potently inhibit the chymotrypsin-like activity along with the trypsin-like and caspase-like activities. Natural products can also serve as an adjuvant chemotherapeutic drug for curing resistant cancer cells. A recent report from our laboratory showed that curcumin pretreatment of drug-resistant cells alleviated SMAR1-mediated p65NFκB activation by proteasomal degradation of its transcriptional repressor IkBα and hence restored doxorubicin sensitivity [27].

5 Conclusion

The ubiquitin–proteasome system controls a wide range of cellular events in the various hallmarks of cancer. Thus, the ubiquitin–proteasome system is emerging as a significant target in anticancer therapies. In preclinical studies, few drug showed antitumor activity against a variety of solid tumors, including breast, gastric, colon, pancreas, and non-small lung cancers. In addition, several natural and synthetic inhibitors targeting the proteasome have evolved. Although the mechanisms and functions of the ubiquitin system have been investigated extensively, a

comprehensive understanding of the complex ubiquitin–proteasome system as well as the development of inhibitors of this system by searching natural sources and chemical libraries and also by chemical synthesis is needed to develop efficient anticancer drugs in the future.

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Chapter 10
Matrix Metalloproteinases in Cancer Metastasis: An Unsolved Mystery

Shravanti Mukherjee, Argha Manna, Minakshi Mazumdar, and Tanya Das

Abstract Tumor progression is a complex, multistage process by which a normal cell undergoes genetic changes that result in phenotypic alterations and acquisition of the ability to spread and colonization to distant sites in the human body. Understanding the molecular mechanisms of metastasis is crucial for developing novel therapeutic strategies to combat metastatic cancers. Early studies established the importance of the extracellular matrix on tumor cell growth and differentiation. With time, the role of the extracellular matrix and matrix metalloproteinases (MMPs), a family of degradative enzymes, in the regulation of tumor invasion, metastasis, and angiogenesis was recognized. Initially, it was believed that the major role of MMPs in metastasis was to facilitate the breakdown of physical barriers to metastasis, thus promoting invasion and entry into and out of blood or lymphatic vessels (intravasation, extravasation). However, recent evidence suggests that MMPs may have a more complex and divergent role in metastasis as well as in cancer stem cell maintenance. In the present review, the role of MMPs and their functional contribution in metastasis have been revisited and discussed. Upcoming approaches target MMPs and their inhibitors, e.g., tissue inhibitors of metalloproteinases (TIMPs), genetically or pharmacologically, suggesting that MMPs are key regulators of growth of tumors, both at primary and metastatic sites. These evidences present MMPs as the important candidates in creating and maintaining an environment that supports the initiation and maintenance of growth of primary and metastatic tumors. Future endeavors to target matrix metalloproteinases would be important in the development of novel therapeutic strategies against metastatic cancers.

Keywords Cancer stem cell • Epithelial to mesenchymal transition • Invasion • Matrix metalloproteinases • Metastasis • Migration • Tissue inhibitors of metalloproteinases

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1 Introduction

Cancer originating from mutations in genes that regulate essential pathways of cell function leading to uncontrolled outgrowth of tissue cells [1] seems to be one of the leading causes of disease and mortality worldwide [2]. The tumors are complex structures of malignant cancer cells embedded in vasculature and surrounded by a dynamic tumor stroma consisting of various nonmalignant cells, such as fibroblasts and myeloid cells. The milieu of the tumor microenvironment is akin to the inflammatory response in a healing wound, which promotes angiogenesis, turnover of the extracellular matrix (ECM), and tumor cell motility. Understanding the molecular mechanisms of this complex interplay between malignant cancer cells and the surrounding nonmalignant stroma represents one of the major challenges in cancer research. However, the past two decades of biomedical research have yielded an enormous amount of information on the molecular events that take place during carcinogenesis and the signaling pathways participating in cancer progression. Our laboratory has also marked a significant contribution to elucidate the key molecular machineries responsible for carcinogenesis along with the therapeutic approaches using dietary polyphenols [3–8].

Metastasis is a cascade of linked sequential steps involving multiple host-tumor interactions [9]. To successfully create a metastatic colony, a cell or group of tumor cells must be able to leave the primary tumor, invade the local host tissue, enter the circulation, arrest at the distant vascular bed, extravasate into the target organ interstitium and parenchyma, and proliferate as a secondary colony. During invasion, tumor cells disobey the social order of organ boundaries and cross into tissues where they do not belong. The mammalian organism is divided into a series of tissue compartments separated by the extracellular matrix unit consisting of the basement membrane and its underlying interstitial stroma [10]. The basal cells of the epithelium or organ parenchymal side of this unit are attached to the basement membrane. On the opposite side, the interstitial stroma contains stromal cells, fibroblasts, and myofibroblasts. During all types of benign tissue remodeling, proliferative disorders, and carcinoma in situ, the cell populations on either side of this connective tissue unit do not intermix. Only during the transition from in situ to invasive carcinoma do tumor cells penetrate the epithelial basement membrane and enter the underlying interstitial stroma to interact with the stromal cells. Thus, a definition of the behavior of the metastatic tumor cell is the tendency to cross tissue compartment boundaries and intermix with opposite cell types [11]. The continuous basement membrane is a dense meshwork of collagen, glycoproteins, and proteoglycans which normally does not contain any pores large enough for passive tumor cell transversal. Consequently, invasion of the basement membrane must be an active process. Once the tumor cells enter the stroma, they gain access to lymphatics and blood vessels for further dissemination.

Interactions of the tumor cell with the basement membrane can be separated into three steps: attachment, matrix dissolution and migration. The first step is binding of the tumor cell to the basement membrane surface-mediated cell-surface receptors

of the integrin [12] and non-integrin [13] variety. Matrix receptors recognize glycoproteins such as laminin, type IV collagen, and fibronectin in the basement membrane. Two to eight hours after attachment, a localized zone of lysis is produced in the basement membrane at the point of tumor cell contact. Tumor cells directly secrete degradative enzymes [14] or induce the host to elaborate proteinases to degrade the matrix and its component adhesion molecules. Matrix lysis takes place in a highly localized region close to the tumor cell surface [15], where the amount of active enzyme outbalances the natural proteinase inhibitors present in the serum, those in the matrix, or that secreted by normal cells in the vicinity. Locomotion is the third step of invasion which propels the tumor cell across the basement membrane and stroma through the zone of matrix proteolysis.

During metastatic dissemination, cancer cells activate a complex molecular machinery to migrate through the surrounding extracellular matrix (ECM) and intravasate into blood or lymphatic vessels [16]. To negotiate barriers to cell migration, cancer cells secrete their own proteolytic enzymes or induce their expression in other cells through the release of cytokines (e.g., endothelial cells, tumor-infiltrating fibroblasts or leukocytes) [17]. In particular, matrix metalloproteinases (MMPs) are considered key players in tumor progression because of their ability to remodel the ECM and cleave/activate membrane-bound and matrix molecules, and cytokines that stimulate cancer cell migration and proliferation [18].

Studies conducted over more than 40 years have revealed mounting evidence supporting that extracellular matrix remodeling proteinases, such as matrix metalloproteinases (MMPs), are the principal mediators of the alterations observed in the microenvironment during cancer progression. MMPs belong to a zinc-dependent family of endopeptidases implicated in a variety of physiological processes, including wound healing, uterine involution and organogenesis, as well as in pathological conditions, such as inflammatory, vascular and auto-immune disorders, and carcinogenesis [19]. MMPs have been considered as potential diagnostic and prognostic biomarkers in many types and stages of cancer [20]. The notion of MMPs as therapeutic targets of cancer was introduced 25 years ago because the metastatic potential of various cancers was correlated with the ability of cancer cells to degrade the basement membrane. Subsequently, a growing number of MMP inhibitors (MMPIs) have been developed and evaluated in several clinical trials. Recent report from our laboratory also suggested that downregulation of MMPs like MMP-2 and MMP-9 is prerequisite for the anti-migratory effect of the flavins in breast cancer cells [21].

2 Matrix Metalloproteinases: What Is So "Mysterious" About These Enzymes?

The matrix metalloproteinases (MMPs), also called matrixins, are a group of genetically distinct but structurally related calcium-dependent zinc-containing endopeptidases that are involved in the degradation and repair of major macromolecular components of extracellular matrix (ECM), connective tissue, and cell-surface-bound

molecules. They are naturally occurring proteolytic enzymes found in most mammals that are secreted especially by mesenchymal cells, macrophages, and polymorphonuclear leukocytes [22]. A large set of experimental data indicated that MMPs play essential roles in the processes of tissue remodeling and repair, morphogenesis, angiogenesis, embryonic development, apoptosis, ovulation, neural development, wound healing, chemotherapy-induced alimentary tract (AT) mucositis, cell adhesion and proliferation as well [23]. Moreover, these enzymes have frequently been detected in human tumor specimens and their production and/or misregulation has been associated with the tumor aggressiveness and poor prognosis [24, 25].

Under normal physiological conditions, the expression and activity of these enzymes are very low and strictly controlled by endogenous specific tissue inhibitors of metalloproteinases (TIMPs). Generally, there are a total of four TIMPs (TIMP-1, -2, -3 and -4) and these four protein inhibitors are able to control the proteolytic activity of all MMPs and mediate the stability of cells. That is, overexpression or high activation of MMPs has been causally linked with the pathological destruction of connective tissue and the ensuing pathological disorders characterized by the breakdown of ECM components or connective tissues [26]. These diseases include cancer, osteoarthritis (OA), rheumatoid arthritis (RA), angiogenesis, chronic periodontitis, pulmonary emphysema, skin ulceration, atherosclerosis, gingivitis, central nervous system disease, type I diabetes, myocarditis and dilated cardiomyopathy, coronary artery disease, multiple sclerosis (MS), congestive heart failure and cardiovascular disease [27]. On the basis of their primary roles in various oncologic events, the MMPs have been a highly active set of targets for the design of therapeutic agents to intervene the MMP-related pathological states, such as carcinogenesis and arthritis [28]. Here, we review the recent advances in our understanding of MMP-driven regulation of the tumor invasion.

2.1 Domain Structures of Matrix Metalloproteinases

Matrix metalloproteinases belong to a family of zinc- and calcium-dependent endopeptidases called metzincin. The endopeptidases belong to the wide metzincin group, in turn constitutes one of several metalloendopeptidase families; according to their structural characteristics, all the metzincins are mainly subdivided into astacins, ADAMs/adamalysins/reprolysins, serralysins, matrix metalloproteinases/matrixins, snapalysins, leishmanolysins, and pappalysins [29]. All the metzincins are mostly multidomain proteins with approximately 130–260-residue globular catalytic domains showing a common core architecture characterized by a long zinc-binding consensus motif, HEXXHXXGXX(H/D), and a methionine-containing Met-turn. Metzincins have been characterized to participate in unspecific protein degradation such as digestion of intake proteins and tissue development, maintenance, and remodeling, but they are also involved in highly specific cleavage events to activate or inactivate themselves or other (pro)enzymes and bioactive peptides [30]. Among these proteinases, the matrix-degrading metalloenzymes are the most common enzymes, mainly named

matrixins or matrix metalloproteinases (MMP). They form a multigenic family of proteolytic calcium-/zinc-dependent enzymes (expressed as 26 distinct proteins), functioning at neutral pH, secreted in their latent form (proenzymes or inactive zymogens or pro-MMPs), and requiring proteolytic activation [31].

2.2 Structural Classification of Matrix Metalloproteinases

MMPs are a family of zinc-dependent endopeptidases first described almost half a century ago [32]. They play a crucial role in various physiological processes including tissue remodeling and organ development [33], in the regulation of inflammatory processes [34], and in diseases such as cancer [35]. The general structural blueprint, shared by 23 MMPs, shows three domains that are common to almost all MMPs - the pro-peptide, the catalytic domain, and the hemopexin-like C-terminal domain that is linked to the catalytic domain via a flexible hinge region. MMPs are initially expressed in an inactive state due to the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site. Only after disruption of this interaction by a mechanism called cysteine switch, which is usually mediated by proteolytic removal of the pro-domain or chemical modification of the cysteine residue, does the enzyme become proteolytically active. The pro-domain contains a consensus sequence and requires proteolytic cleavage by convertases, which, depending on the sequences, occurs intracellularly by furin or extracellularly by other MMPs or serine proteinases such as plasmin [36].

Structurally related members of MMPs can be broadly classified into five subfamilies based on the variation in their primary structure and function, substrate specificity, as well as their cellular sources: collagenase group (MMP-1, -8, -13, -18), gelatinase group (MMP-2, -9), stromelysin group (MMP-3, -10, -11), membrane-type (MT)-MMP group (MMP-14, -15, -16, -17), and a nonclassified group (MMP-7, -12) [37] (Fig. 10.1). This superfamily shares a conserved structural topology comprising a catalytic domain containing three histidines that constitutes the zinc-binding site and a "methionine-turn" motif that lies beneath the active site zinc ion. The ion-binding motif reads HEBXHXHBGBXHZ, where histidine (H), glutamic acid (E), and glycine (G) are invariant, B is a bulky hydrophobic residue, X is a variable residue, and Z is a family-specific amino acid (serine in MMPs). All MMPs have an N-terminal hydrophobic signal sequence, i.e., predomain, which leads their secretion into the extracellular space after the synthesis in the endoplasmic reticulum. Predomain is followed by a 77–87 amino acid-long pro-domain that constitutes the N-terminus of the secreted enzyme and maintains it in its latent form until its removal or disruption. The prodomain keeps the enzyme inactive through a mechanism identified as "cysteine switch" where the unpaired cysteine in the highly conserved "Pro-Arg-Cys-Gly-X-Pro-Asp" sequence forms a bridge with the catalytic zinc, thereby preventing enzymatic activity. The enzyme acquires total proteolytic capacity when the prodomain becomes chemically removed by cleavage [38]. The active site is of great importance: it specifically binds to selective substrates by

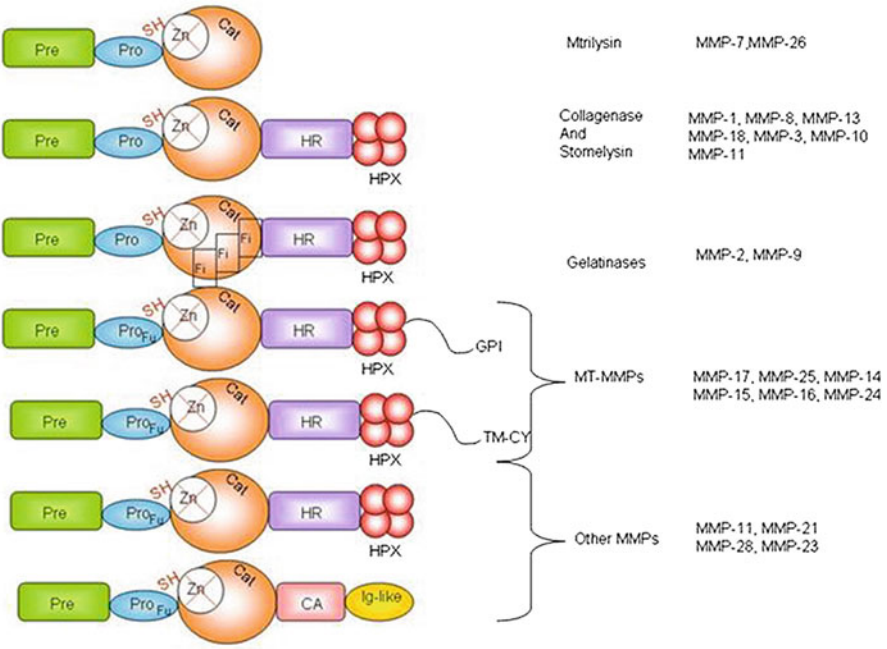

Fig. 10.1 Conserved domain structures of different groups of MMPs. *SH* zinc interacting thiol, *Fi* insert that resemble collagen-binding type II repeats of fibronectin, *Fu* furin cleavage site, *Zn* zinc, *HR* hinge region, *CA* cysteine array, *Cat* catalytic domain, *HPX* hemopexin domain, *GPI* glycosylphosphatidylinositol, *TM* single span transmembrane domain, *CY* short cytoplasmic domain, *MMP* matrix metalloproteinases, *MT-MMPs* membrane-type metalloproteinases, *Ig* immunoglobulin

means of its active site cleft, through specificity subsite pockets that bind amino acids adjacent to the scissile peptide bond, and through secondary substrate binding exosites located outside the active site [39]. These domains represent the minimal structure of MMPs found in MMP-7 (matrilysin) and MMP-26 (endometase/matrylisin-2) which lack any other domain. All the other MMPs have a hinge region varying in length and composition which also influences substrate specificity, and a four-blade structure representing the hemopexin/vitronectin-like domain [40, 41]. Two metalloproteinases, MMP-2 and MMP-9 (also named gelatinase A and B), are further characterized by the presence of three head-to-tail cysteine-rich repeats within the catalytic domain [42]. This structure resembles the collagen-binding type II repeats of fibronectin and is necessary for the binding and cleavaging activities of these MMPs. Not all MMPs are secreted enzymes; membrane-type (MT) MMPs have been identified to contain a single-pass transmembrane domain and a short cytoplasmic C-terminal tail or to be anchored to the cell membrane by a glycosylphosphatidylinositol anchor [43].

Most MMPs are secreted as latent precursors (zymogens) that are proteolytically activated in the extracellular space (Fig. 10.2), with the exception of MMP-11 and

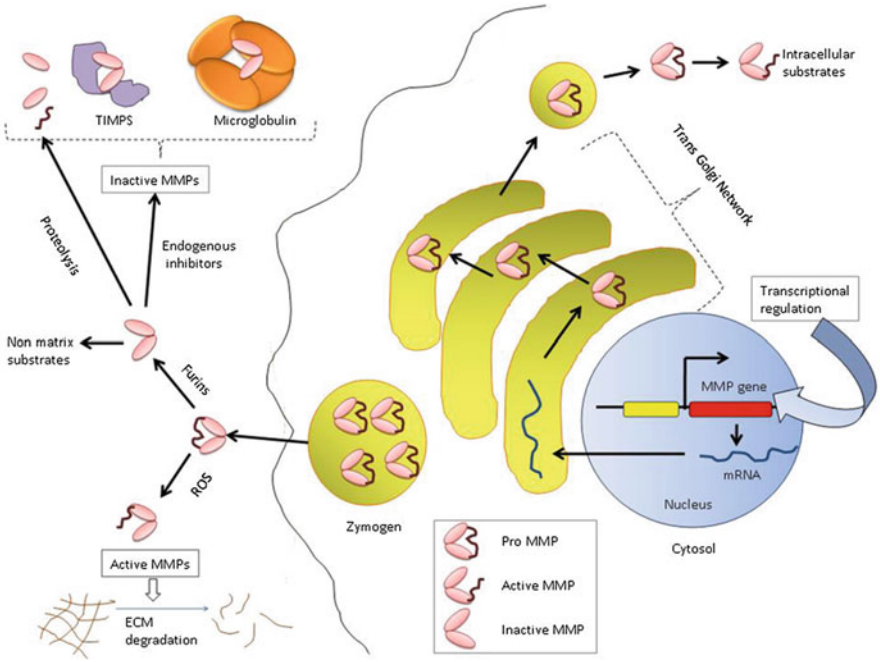

Fig. 10.2 General overview of activation and inactivation mechanism of MMPs within cytoplasm and extracellular space. *TIMP* tissue inhibitor of metalloproteinases, *ECM* extracellular matrix, *ROS* reactive oxygen species, *MMP* matrix metalloproteinases

MT1-MMP, which are activated prior to secretion by Golgi-associated, furin-like proteases. The activity of MMPs in extracellular space is specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind to the highly conserved zinc-binding site of active MMPs at molar equivalence.

3 Regulation of Matrix Metalloproteinases Activity in Tumor Milieu: The Mysterious Interdependence

The complexity of the tumor microenvironment allows a variety of regulatory cascades to determine the functions of the diverse MMPs expressed. Proteolytic activity of MMPs can be regulated at different levels, i.e., gene expression, compartmentalization, conversion from zymogen to active enzyme, and, finally, the presence of specific inhibitors. While judging the patho-physiological relevance of increased expression of proteinases in tumor tissues, it is important to judge whether endogenous inhibitors or activating/converting enzymes are present in the microenviornment. A key step in regulating MMP activity is the conversion of the zymogen into an active proteolytic enzyme (Fig. 10.3). There are several proteinases that mediate

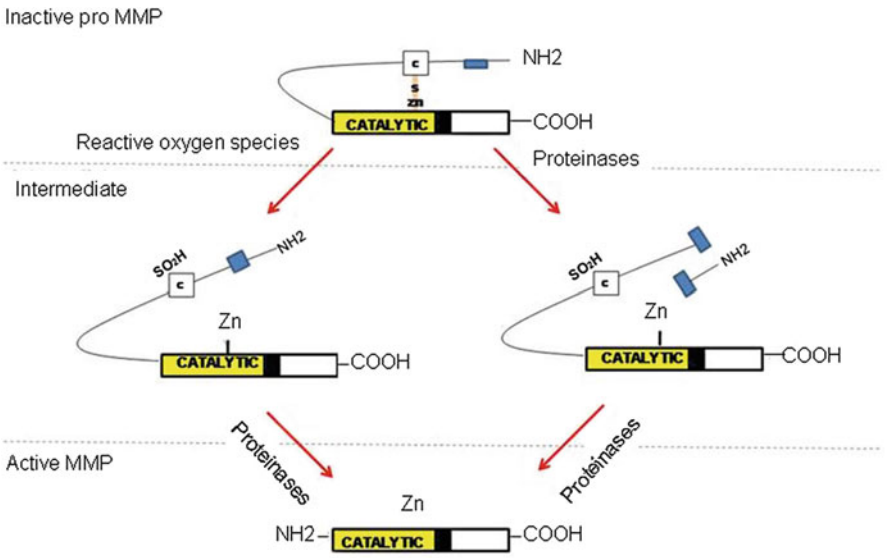

Fig. 10.3 Molecular mechanism of conversion of inactive pro-MMPs into active form of MMPs. *Zn* zinc, *MMP* matrix metalloproteinases, *S* sulfhydryl group

MMP activation, such as plasmin, furin, or active MMPs [44, 45]. The function of MMPs can also be influenced by reactive oxygen species (ROS). The inflammatory response at the tumor site creates large amounts of ROS that are produced by activated neutrophils and macrophages. These oxidants initially activate MMPs via oxidation of the pro-domain cysteine [46] but, eventually, in combination with the enzyme myeloperoxidase contributed by inflammatory cells, inactivate MMPs by modification of amino acids of the catalytic domain by hypochlorous acid [47].

The localization or compartmentalization of MMPs under physiological conditions often dictates their biological function. Several MMPs interact with surface receptors such as integrins or localize to specific areas of the ECM, which potentiates MMP activity by increasing their local concentration and also may interfere with accessibility to endogenous inhibitors [48]. The binding of MMP-2 to integrin αvβ3 via its hemopexin domain is crucial for mesenchymal cell invasive activity [49]. Likewise, high local concentrations of active MMP-14 on the cell membrane of metastatic cancer cells play important roles in cell migration [50]. However, there may also be additional mechanisms to concentrate extracellular proteinases in specific sites in the microenvironment.

Mechanical forces contribute to tumor progression [51], potentially by modulating proteolysis of ECM components. These forces may unwind the conformation of MMP substrate proteins, thus allowing recognition and cleavage by proteinases. For example, the ECM component fibronectin is unfolded by mechanical forces in the ECM of living cells and this unfolded fibronectin then acts as potent MMP substrates [52]. Tumor progression is frequently characterized by increased tissue stiffness, elevated interstitial fluid pressure, and altered blood flow conditions [53].

Thus, it is conceivable that similar mechanisms involving mechanical force are regulatory factors for MMP function in the tumor microenvironment.

The expression and activity of MMPs is regulated at the transcriptional level by cytokines and growth factors and after secretion by endogenous natural inhibitors. The tissue inhibitors of matrix metalloproteinases (TIMPs) provide a negative control of MMP-activity. Four various inhibitors of metalloproteinases characterized so far, are designated as TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Among them, TIMP-1 and TIMP-2 have been characterized most extensively. The TIMPs inhibit active MMPs by forming 1:1 stoichiometric non-covalent complexes with the endopeptidase [54]. TIMP-1 and TIMP-2 are capable of inhibiting the activities of all known MMPs except MT-MMPs, and play a key role in MMP-driven different physiological processes. Moreover, TIMP-1 can also complex with the precursor of MMP-9 [55], whereas TIMP-2 and TIMP-4 can bind to the zymogen form of MMP-2, a 92-kDa type IV procollagenase [56]. TIMP-3 inhibits not only the activity of MMP-1, -2, -3, -9 and -13 [57], but also the activity of MT-MMPs as well as TNF-α converting enzyme. However, the role of TIMPs is not restricted to the inhibition of MMPs. They possess growth-promoting activities for various cell types as well as having antiangiogenic and proapoptotic properties [58].

4 Matrix Metalloproteinases in Cancer Progression: The Mystery Revisited

During development of carcinogenesis, tumor cells participate in several interactions with the tumor microenvironment involving extracellular matrix (ECM), growth factors and cytokines associated with ECM, as well as surrounding cells (endothelial cells, fibroblasts, macrophages, mast cells, neutrophils, pericytes and adipocytes) [59]. Four hallmarks of cancer that include migration, invasion, metastasis and angiogenesis are dependent on the surrounding microenvironment. Critical molecules in these processes are MMPs because they degrade various cell adhesion molecules, thereby modulating cell–cell and cell–ECM interactions.

The emerging view, reflected by several studies, reveals that the expression and role of MMPs and their natural inhibitors, i.e., tissue inhibitor of metalloproteinases (TIMP), is quite diverse during cancer development. The overexpression of MMPs in the tumor microenvironment depends not only on the cancer cells, but also on the neighboring stromal cells, which are induced by the cancer cells in a bidirectional paracrine manner. Cancer cells stimulate host cells (e.g., fibroblasts) and are themselves stimulated by host cell (e.g., neutrophil) to constitute an important source of MMPs through the secretion of interleukins and growth factors and direct signaling through extracellular MMP inducer [60, 61].

Recent studies show that members of the MMP family exert different roles at different stages during cancer progression (Fig. 10.4). In particular, they may promote or inhibit cancer development depending among other factors on the tumor stage, tumor site (primary, metastasis), enzyme localization (tumor cells, stroma), and substrate profile.

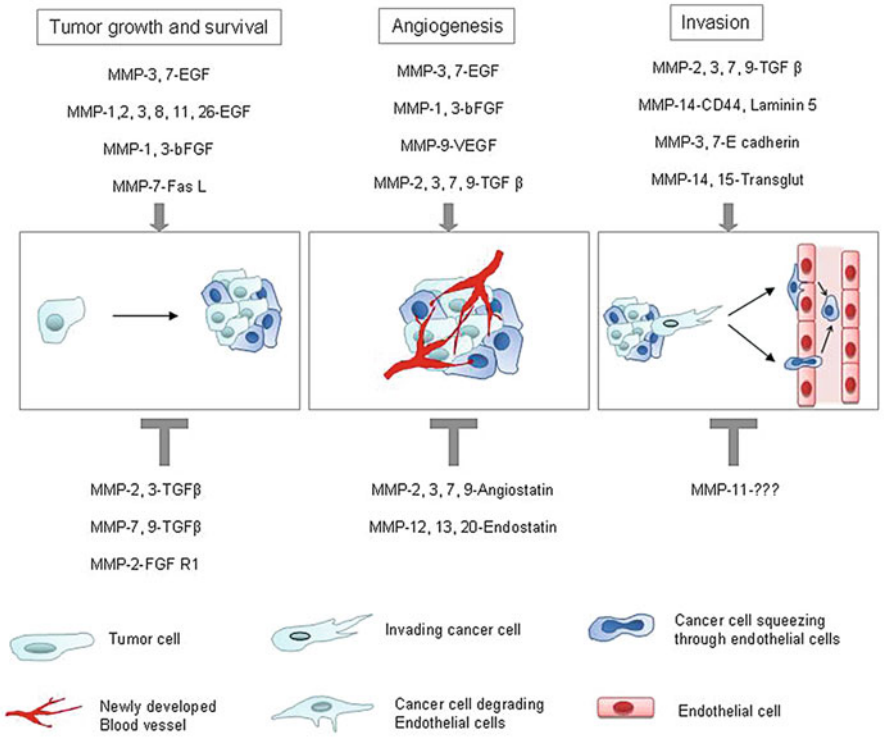

Fig. 10.4 Different stages of metastatic tumor progression (tumor growth and survival, angiogenesis, intravasation and extravasation) are positively and negatively regulated by different MMPs. *EGF* epidermal growth factor, *IGF* insulin-like growth factor, *TGF β* transforming growth factor-beta, *bFGF* basic fibroblast growth factor, *FGF-R1* fibroblast growth factor receptor 1, *MMP* matrix metalloproteinases, *VEGF* vascular endothelial growth factor, *FasL* Fas ligand, *Transglut* transglutaminase

5 Matrix Metalloproteinases in Tumor Metastasis: A Tale of the Mysterious Mediators

The extracellular matrix holds cells together and maintains the three-dimensional structure of the body. It also plays critical roles in cell growth, differentiation, survival and motility. For a tumor cell to metastasize from the primary tumor to other organs, the collagen-rich ECM and basement membrane that are the physical barriers for cell migration must be degraded. The key enzymes responsible for ECM breakdown are MMPs that actively fuel the progression of cancer from localized growth to the invasion of surrounding tissues and the development of distant organ metastasis. Also there is a cooperation between these two components, i.e., ECM and MMPs, enabling the tumor cell to reach its target organ and survive.

However, the classic view that these enzymes simply provide a mechanism for the breakdown of connective tissue barriers has been challenged.

5.1 Matrix Metalloproteinases in Extracellular Matrix Degradation and Distant Metastasis

Although all five major classes (serine, aspartic, cysteine, threonine, and metalloproteinases) are involved in metastasis, a great deal of emphasis has been placed on the type IV collagenases, MMP-2 and MMP-9 [62]. Type IV collagen is a major structural protein in the basement membrane and ECM. A number of studies have linked elevated MMP-2 and MMP-9 levels with an increased metastasis. The conclusions which can be drawn thus far are that the number and the relative levels of MMPs increase with tumor progression.

Several recent studies have been done to try and characterize the phenotypic and enzymatic profiles of more aggressive tumor cell lines. Selection of progressively more invasive human lung carcinoma cells from an established CU cell line revealed that the more invasive cells had a higher expression of MMP-9. These cells had a four- to sixfold increase in invasive activity over the parental and had an increased metastatic potential in vivo. MMP-9 has also been shown to be overexpressed in advanced stage melanoma cells and in breast cancer cell lines. Other tumor models involving MMP-9 in their invasive phenotype include human non-Hodgkin's lymphoma cells and human giant cell tumors [63]. MMP-2 has also been observed to be overexpressed in more aggressive tumor cells. The level of pro-MMP-2 vs. active MMP-2 also plays a role in determining invasive and metastatic capacity of pancreatic tumor cells towards regional lymph node [64–66]. Cell lines displaying an intermediate level of activation were the most invasive while those cells with a high level of activation were the least invasive. This is probably due to the balance required between MMPs and TIMP to create a controlled proteolytic system.

Although the major role of MMPs in metastasis has been inferred from the in vivo and in vitro data presented above to be breakdown of the ECM, recent studies have proposed additional roles for the MMP family. Most of the in vivo and in vitro assays designed to examine the role of MMPs on tumor invasion measure the end results, as in the number of micrometastases formed. The mechanism, however, remains unknown. Intravital video microscopy (IVVM) allows for the observation of the metastatic cascade by following the tumor cell through the microcirculation [67]. The results from these experiments suggest that the destruction of tumor cells in the circulation and during extravasation do not contribute as much as previously thought to the inefficiency of metastasis. Rather, the growth of the individual tumor cell once in the target organ appears to be the rate-limiting step. Tumor cells engineered to overexpress TIMP-1 were shown to extravasate at rates equal to wild-type cells but were unable to form proliferative colonies within the target organ [68]. Although these data suggest that MMPs may play a role in tumor cell growth, the

studies of MMP involvement in ECM degradation and basement membrane invasion still support the core role of MMPs in metastatic invasion.

5.2 Matrix Metalloproteinases in Epithelial to Mesenchymal Transition

Epithelial–mesenchymal transformation (EMT) is a critical step in malignant transformation of epithelial cells into carcinoma [69]. Loss of the homotypic cell–cell adhesion molecule E-cadherin and nuclear translocation of β-catenin are common features of EMT and are associated with the progression of most epithelial cancers [70, 71]. Several MMPs, including MMP-3, MMP-7, and MT1-MMP, cleave E-cadherin releasing a soluble 80 kDa peptide with motility stimulatory activity, which suggest that MMPs could actively contribute to EMT [72]. Interestingly MMPs like MMP-7 and MT1-MMP are also transcriptionally upregulated by β-catenin LEF/TCF complexes [73], suggesting the existence of an MMP-dependent positive feedback mechanism by which E-cadherin degradation by MMPs also results in an increase in MMP expression.

5.3 Matrix Metalloproteinases in Invadopodia Formation

Invadopodia (podosomes) are specialized cell-surface structures that have been identified on transformed malignant cells and are composed of a meshwork of actin ring, microfilaments and metalloproteinases, involved in degradation of underlying matrix. Invadopodia utilize proteases to degrade a variety of immobilized substrates including fibronectin, laminin, type I and IV collagens and other ECM components. Several integral membrane enzymes of different classes have been identified as important functional components of invadopodia [74]. These include the serine proteases, seprase (surface-expressed protease), and dipeptidyl peptidase IV, which must form oligomeric structures for expression of proteolytic activity and also MT-MMP (Fig. 10.5). Plasma membranes shed vesicles containing densely clustered MMP-9 and MMP-2, which might facilitate directional proteolysis of the ECM during cell migration and especially during cancer invasion [75].

5.4 Matrix Metalloproteinases in Cancer Dissemination

A positive correlation between tumor progression and the expression of multiple MMP family members (MMP-1, MMP-2, MMP-7, MMP-9, MMP-11, and MT1-MMP) in tumor tissues has been demonstrated in numerous human and animal studies [76, 77]. On the basis of numerous studies, it was proposed that pharmacologic targeting of MMP activity might provide a mechanism to prevent cancer

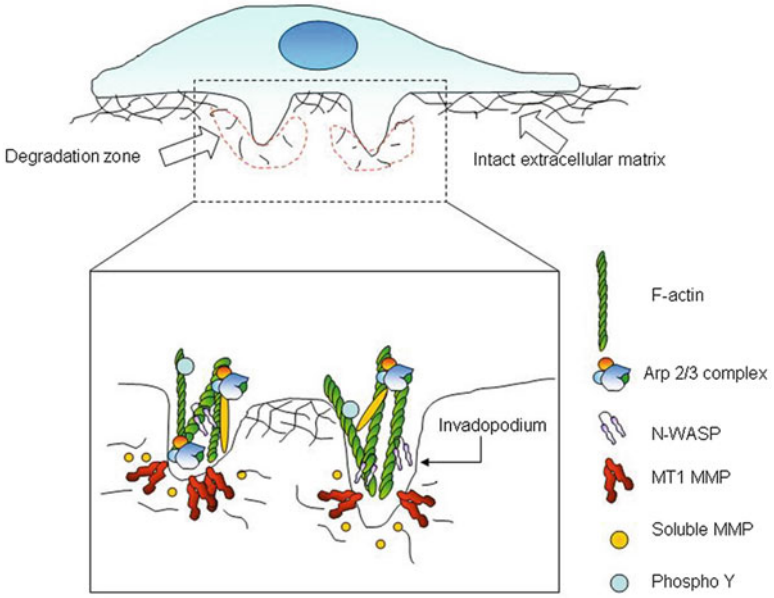

Fig. 10.5 Localization of membrane-bound MMP (MT1-MMP) at invasive bodies of tumor cell (invadopodium). *Arp* actin-related protein, *phospho* phospho tyrosine, *MT1-MMP* membrane-type 1 matrix metalloproteinases, *N-WASP* neuronal Wiskott–Aldrich syndrome protein, *F-actin* filamentous actin

dissemination [78]. Further support for the role of MMPs in cancer dissemination came from the demonstration that TIMPs can interfere with experimental metastasis [79]. However, the role of MMPs and TIMPs in cancer is far more complicated than suggested initially. For example, increased TIMP-1 levels in human cancer tissues have been associated with poor prognoses [80]. It is uncertain whether this reflects the growth-potentiating properties of TIMPs or some other undetermined property of TIMPs [81]. Other experimental studies demonstrated that MMPs act primarily to alter the extracellular environment to allow sustained cancer cell growth in an ectopic site, as opposed to having a specific role of allowing the cells to extravasate from the blood stream [82]. Furthermore, in some experimental tumor systems, increased MMP production did not correlate with increased metastasis [83]. One potential explanation of this finding is that excess proteolysis might degrade matrix signals and receptors, thereby disrupting cell–matrix interactions and inhibiting migration [84].

5.5 *Matrix Metalloproteinases Contribute to Intravasation and Extravasation*

Metastasis is the most devastating event associated with cancer because it heralds an irreversible stage of progression that responds poorly if at all to current therapeutic regimens. Cancer metastasis is a complex, multistage process, which includes cell

detachment from the primary tumor mass, migration through the ECM, degradation of the vascular endothelial basement membrane and penetration into the vascular lumen, survival within the circulation, proliferation on distal vascular endothelia, and finally penetration into a new host tissue microenvironment and establishment of a relationship with the local stroma that is conducive to new tumor colony outgrowth. Several if not all of these steps depend at least in part on MMP activity.

Detachment of cells from the primary tumor mass requires the downregulation of cell–cell adhesion mechanisms. Role of several MMPs in this aspect (especially E-cadherin downregulation), has been discussed before. Migration of tumor cells through the host tissue stroma requires partial degradation of the ECM and coordinated sequential attachment to and detachment from the ECM scaffold. Recent work using two-photon microscopy has provided spectacular real-time evidence that MMP proteolytic activity causes controlled degradation of collagen fibrils that are in contact with the invading tumor cell surface, leaving trail of released cell-surface molecules in the cell's wake [14]. Interestingly, inhibition of MMPs does not result in abrogation of tumor cell migration through the collagen gel but rather transforms the crawling movement associated with collagen fibril cleavage into amoeboid movement that leaves the collagen lattice intact. This model strongly supports the notion that the MMP activity relevant to ECM degradation is associated with the tumor cell surface.

As already discussed, cleavage of ECM components by MMPs generates proteolytic fragments that enhance tumor cell migration. Thus, cleavage of laminin-5 by MMP-2 and -14 results in laminin fragments that trigger migration signals in cells [85], and cleavage of collagen IV discloses cryptic sites that are recognized by integrins and contribute to migration stimuli [86]. MMPs also cleave adhesion receptors responsible for cell–matrix interaction, thereby presumably participating in the detachment of cells from the ECM. The cell-surface hyaluronan receptor and facultative proteoglycan CD44 is cleaved by MMP-14, and its cleavage promotes migration. Expression of CD44 containing a mutation of the proteolytic cleavage site abrogates cell migration on ECM [87].

Intravasation, the process whereby tumor cells penetrate the vascular endothelial wall, has been proposed to be a rate-limiting event in metastasis. Although it is likely that a variety of MMPs may be involved in the degradation of the vascular endothelial basement membrane, MMP-9 has thus far been shown to play a potentially leading role [88].

Survival in the face of the immune response is key for the ability of tumor cells with metastatic potential to establish new colonies. Among the wide range of mechanisms that have been proposed to explain tumor cell evasion of immune surveillance, several are MMP dependent. Tumor cells typically interact with neutrophils, macrophages, cytotoxic T cells (CTLs), and natural killer (NK) cells. T cell proliferation is controlled in large part by the engagement of the interleukin-2 receptor (IL-2R) by its natural ligand IL-2. MMPs, including MMP-9, have been shown to cleave the α-chain of IL-2R [89], resulting in the inhibition of T cell proliferation. MMP-9-mediated activation of latent TGF-β may also contribute to immune suppression, since TGF-β is a potent inhibitor of T cell function [90]. Recent evidence indicates

that MMP-9-mediated shedding of cell-surface ICAM-1 may block the ability of CTLs and NK cells to interact with target cells, thereby reducing the effectiveness of their cytotoxicity. Interestingly, an MMP-11 cleavage product of *α1-proteinase* inhibitor reduces the sensitivity of tumor cells to NK-mediated killing [91].

Recent evidence indicates that MMPs cleave a variety of chemokines in ways that can either enhance or block their function. SDF-1/CXCL12, which is inactivated by several MMPs [92], is a ligand for the CXC chemokine receptor 4 (CXCR4) on leucocytes and breast carcinoma cells. Inhibition of CXCR4 engagement by its ligand using monoclonal antibodies reduces metastasis from breast to lung and lymph nodes in vivo [93].

Extravasation was believed to be a key step in cancer metastasis. However, increasing evidence indicates that extravasation is not a rate-limiting step. This process does not appear to require the proteolytic action of MMPs but results from the mechanical disruption of blood vessels by locally growing tumor cells.

The final step in metastasis is the establishment of tumor colonies at sites distant to that of the origin, which relies on interactions between the tumor cells and host tissue stroma. Invading tumor cells may have their own repertoire of MMPs, but it is becoming increasingly clear that they direct, either by physical contact or in paracrine manner, MMP expression and secretion by stromal cells, including fibroblasts, endothelial cells, and leucocytes [94]. MMPs produced by the stroma augment the release of ECM sequestered growth factors, which may help enhance tumor survival, promote angiogenesis, and contribute to further tumor dissemination. A key question is whether reliance on MMP activity lasts throughout metastatic tumor growth or whether MMP-dependent events serve to initiate colony development, which may then proceed in the absence of further MMP-mediated proteolysis. This is an important consideration for therapeutic strategies targeted toward controlling MMP activity and one that remains to be adequately addressed.

5.6 Matrix Metalloproteinases Help Cancer Cells to Communicate with Distant Organ Cells to Form "Metastatic Niche"

Certain organs such as lung, liver, or bone are the preferential sites for the formation of metastases. MMPs and other proteinases are crucially involved in the formation of receptive environment at distant site, known as "metastatic niche". Soluble factors released from the primary tumor appear to trigger the formation of a metastatic niche that is induced initially by the expression of embryonic-type fibronectin, which is most likely produced by fibroblasts at these sites. This event takes place before disseminated tumor cells are detectable at these distant organs, hence, these authors name this process the formation of a "premetastatic niche" [95]. Increased fibronectin production at these sites allows for the infiltration of VEGFR1-positive, bone marrow-derived progenitor cells, which then establish a metastasis-supporting microenvironment. Interestingly, the production of MMPs, namely MMP-3 and -10,

is upregulated together with the angiogenic modulator angiopoietin-2 in premetastatic lung tissue even before myeloid cells are recruited to these sites [96]. These findings imply an important role of extracellular proteolysis in premetastatic niche generation.

6 Matrix Metalloproteinases and the Risk for Recurrence of Metastasis: The Mysterious Contributors

MMPs can be used as markers to predict tumor recurrence in several cancer types. High preoperative serum levels of MMP-2 or MMP-3 predicts recurrence in patients with advanced urothelial carcinoma [97]. Similarly in ovarian cancer, high expression levels of MMP-2 in tumor cells can predict tumor recurrence [98]. Kuniyasu et al. [99] found that a high ratio of gelatinase expression (MMP-2 or MMP-9) to E-cadherin expression in tumor cells can predict recurrence and death in pancreatic cancer. Similarly, expression of activated MMP-2 is related to regional lymph node and distal metastasis as well as to postresection recurrence of the same tumor [100]. The expression of certain MMPs in primary tumor can predict the risk of metastasis. Expression of MMP-1 is associated with lymph-vascular invasion and lymph node metastasis in stage IB cervical cancer [101] and peritoneal metastasis in gastric cancer [102]. Expression of MMP-2 in tumor cells can indicate increased risk of metastasis in uveal melanoma and in SCC of tongue [103]. Similarly, increased MMP-9 expression by tumor cells in colorectal cancer is associated with advanced Dukes stage and presence of distant metastases [104]. Interestingly, MMP-2 and MMP-9 expression levels are especially high in lung carcinomas and melanomas metastasizing to the spine, suggesting that they contribute to enhanced invasive properties of metastatic spinal tumors [105]. MMP determinations from patient serum have shown predictive value in estimation of metastasis risk. High serum levels of MMP-2 correlate with the presence of metastases in lung cancer or to disease progression in patients with prostate cancer [106], and a high serum MMP-9/E--cadherin ratio can predict metastasis of renal cell carcinoma [107].

7 Matrix Metalloproteinases in Cancer Stem Cell Maintenance: Escalating the Mystery

Matrix metalloproteinases (MMPs) stimulate tumor invasion and metastasis by degrading the extracellular matrix. Here we reveal an unexpected role for Mmp10 (stromelysin 2) in the maintenance and tumorigenicity of mouse lung cancer stem-like cells (CSC). MMP-10 is highly expressed in oncosphere cultures enriched in CSCs and RNAi-mediated knockdown of MMP-10 leads to a loss of stem cell marker gene expression and inhibition of oncosphere growth, clonal expansion, and transformed growth in vitro. Interestingly, clonal expansion of Mmp10-deficient

oncospheres can be restored by addition of exogenous MMP-10 protein to the culture medium, demonstrating a direct role for MMP-10 in the proliferation of these cells [108]. Oncospheres exhibit enhanced tumor-initiating and metastatic activity when injected orthotopically into syngeneic mice, whereas MMP-10 deficient cultures show a severe defect in tumor initiation. Conversely, oncospheres implanted into syngeneic non-transgenic or MMP-10/MMP-2 mice show no significant difference in tumor initiation, growth, or metastasis, demonstrating the importance of MMP-10 produced by cancer cells rather than the tumor microenvironment in lung tumor initiation and maintenance. Analysis of gene expression data from human cancers reveals a strong positive correlation between tumor MMP-10 expression and metastatic behavior in many human tumor types. Thus, MMP-10 is required for maintenance of a highly tumorigenic, cancer-initiating, metastatic stem-like cell population in lung cancer. Our data demonstrate for the first time that MMP-10 is a critical lung cancer stem cell gene product and novel therapeutic target for lung cancer stem cells.

8 Matrix Metalloproteinases as Targets for Anti-metastatic Therapy: Aiming Toward Unraveling the Mystery

The data from model systems, reviewed above, suggest that MMPs are involved in most phases of carcinogenesis from initiation to metastasis. Inhibition of these proteinases might thus lead both to prevention of cancer development and to inhibition of dissemination.

Two main types of MMP inhibitor exist: the TIMPs and low-molecular-weight synthetic inhibitors [109]. Because of their protein nature and multiplicity of actions, it is unlikely that TIMPs will be widely used as anticancer molecules. Because of this, most research in recent years has focused on the synthetic inhibitors. Many of these are peptides and are similar to the cleavage site in collagen [110]. Some of the zinc-binding groups that are currently being investigated in model systems include the hydroxamates, carboxylates, amino carboxylates, and sulfhydryls [111]. Some of these inhibitors (e.g., the hydroxamates) are presently undergoing clinical trials in patients with advanced cancers [112]. We are unaware of any studies so far in human breast cancer, however.

Although MMP inhibitors are currently being evaluated in patients with metastatic cancers, there are still many unanswered questions concerning the use of these compounds. Some of these are as follows. Is it better to use a broad-spectrum or specific matrix metalloproteinases inhibitor? In order to answer this question, it will be necessary to establish which are the MMPs whose involvement in the different phases of cancer progression is critical. If the action of MMP inhibitors is blocking of MMP activity only, these compounds may not induce the type of tumor shrinkage that is seen with the traditionally used cytotoxic agents. Conventional approaches that are used to assess tumor regression may thus not be possible. A novel approach taken to address this issue has been to monitor the rate of rise in levels of serum tumor markers. The use of these tests in phase 2 trials has shown a dose-dependent

decrease in rate of rise after treatment with the MMP inhibitor marimastat [108]. Furthermore, this decreased rate of marker rise appeared to correlate with extended patient survival.

It is reported that MMPs have functional overlap with other proteases, e.g., plasmin, and arrest of invasion will require inhibition of plasmin as well as of the MMPs [113] thereby making the targeting of matrix metalloproteinases in cancer therapy even more challenging.

9 Therapeutically Targeting EMT-Promoting Matrix Metalloproteinases: The Mystery Unfolded

An obvious point for intervention in MMP-induced or -mediated EMT is the catalytic inhibition of MMPs themselves. Unfortunately, clinical trials of first- and second-generation small-molecule MMP-inhibiting drugs in breast cancer and other cancers proved disappointing [114]. A phase III trial of the MMP inhibitor marimastat in patients with metastatic breast cancer found no therapeutic benefit [115], while phase II trials of marimastat and rebimastat in patients with early-stage breast cancer concluded that large adjuvant trials with these agents were not feasible due to musculoskeletal toxicity and failure to achieve therapeutic plasma levels [116]. Many of the problems with the MMP inhibitors tested to date appear to stem in large part from a lack of specificity; the drugs employed simply target too many enzymes. This is a critical problem, because some MMPs appear to protect against tumor progression at certain stages of breast cancer development, and inhibition of these MMPs at the wrong time can lead to increased tumor aggressiveness [117]. For example, high levels of MMP-8 have been shown to suppress breast cancer metastasis [118, 119] significantly. Ribozyme-mediated knockdown of MMP-8 in a nonmetastatic, high MMP-8 breast cancer cell line conferred metastatic competence [120]. Thus, pharmacological inhibition of MMP-8 along with invasion- and metastasis-promoting MMPs would be anticipated to reduce or limit the potential benefit of the therapy.

As another consequence of poor specificity, clinical trials of MMP inhibitors were plagued by the serious side effect of musculoskeletal syndrome (MSS). This dose-limiting toxicity frequently resulted in failure to achieve targeted plasma levels, and in patients withdrawing from treatment. The specific molecular target responsible for these side effects has not been conclusively identified. Remaining candidate mediators of MSS include MT1-MMP, metalloproteinases outside of the MMP and ADAM families [121], or nonprotease metalloproteins. To minimize off-target effects, well-tolerated MMP-directed therapeutics will need to achieve selectivity for the MMP family in preference to other metalloenzymes, as well as the ability to distinguish among MMPs. In the arena of more highly selective small-molecule MMP inhibitors, slow progress is being made. These synthetic compounds typically feature a zinc-chelating group such as hydroxamate derivatized with peptidic or nonpeptidic groups designed to mimic a peptide substrate; they target the MMP active site zinc and substrate binding site [122]. Structure-based design of selective

inhibitors has been hampered by the close structural homology of active sites and overlapping substrate specificities among the MMPs, and by the elastic and flexible nature of the MMP active site, which further complicates computational drug design even when high-resolution crystal structures are available [123, 124]. Current approaches to small-molecule MMP inhibitors include optimization of compounds based on an array of different zinc-binding groups to yield more selective inhibitors toward a variety of MMPs, as well as the development of non-zinc-binding inhibitors that selectively target unique aspects of the MMP-13 active site. A less conventional approach has pursued development of irreversible mechanism-based inhibitors, selective for gelatinases MMP-2 and MMP-9 that covalently modify the catalytic glutamate residue of the MMP active site. In yet another approach, several groups have attempted to exploit the selective substrate binding exosites present on MMP accessory domains to develop selective allosteric inhibitors of MMPs; while a promising concept, this approach has yet to yield highly potent and selective drug leads. Thus, the challenges are clear: while some MMPs facilitate breast cancer development and could potentially be targeted for therapeutic benefit, others are essential for basic physiological processes, interference with which can have serious negative consequences. We now need better understanding of which MMPs to target and when, as well as new generation technologies to target specific matrix metalloproteinases for regression of metastatic cancer.

10 Conclusions

MMPs are associated with multiple human cancers; hence they were early considered as drug targets to treat cancer. The first drug development programs based on the notion of blocking MMP-mediated angiogenesis and metastasis were started about 25 years ago and led to a number of small-molecule metalloproteinases inhibitor (MPI) drugs in phase III clinical trials. The effects of MPIs in these trials turned out to be disappointing as they failed to increase the survival rate of cancer patients. Possible reasons for the failure of MPIs have been extensively discussed previously. Indeed, the clinical studies were suboptimally designed with respect to the stage of cancer, so the question remains whether MPIs might have proven more effective when used in earlier stages of the disease.

Part of the rationale to use MPIs as anticancer drugs was to block interstitial migration of metastatic cancer cells. However, recent analyses have shown that cancer cells can switch to an amoeboid-like protease-independent migration mode by forming actin-rich protrusions and “squeezing” through the ECM. This would render MPIs impotent to inhibit the migratory behavior of metastatic tumor cells. Whether this alternative mode of migration is actually relevant for cancer cell migration, under in vivo conditions, in the presence of a naturally cross-linked collagen matrix, currently remains questionable.

The cytostatic potential attributed to MPIs is certainly in keeping with the numerous studies describing MMP-mediated regulation of cell growth signals, such as the

activation of TGF-β by MMP-2, -9 and -14, the proteolytic release of soluble EGFR ligands, or the degradation of E-cadherin by MMP-3 or -9. Moreover, MMPs interfere with apoptosis induction, especially after chemotherapy, by cleaving Fas ligand from the surface of cancer cells as shown for MMP-7. In the clinical trials, MPIs were administered to patients with advanced cancer, which was most likely too late to exert any beneficial effect on survival.

Interfering with the tumor vasculature is regarded as one of the most promising strategies to inhibit tumor growth and has motivated the development of drugs like Bevacizumab (Avastin, anti-VEGF monoclonal antibody), which has been FDA approved for the treatment of metastatic cancers in combination with chemotherapy. Many studies also support a dominant role of MMP-9 in the angiogenic switch by regulating the bioavailability of VEGF tumors, suggesting a beneficial effect of MPI on tumor angiogenesis. However, in other cancer models, MMP-9 generates ECM fragments like tumstatin, a potent suppressor of tumor vasculature formation, resulting in increased tumor growth in MMP-9-deficient mice. This illustrates that one MMP can have opposing effects in different tumor types and highlights that the use of MPIs has to be carefully considered and evaluated for each specific kind of cancer.

Certainly, the complexity of the mode of action of MMPs has expanded considerably from proteinases that simply degrade the ECM, to specific modulators of angiogenesis as well as fine-tuners of cell signaling pathways and the inflammatory response. One of the major, recent advances in MMP research is the discovery of specific regulatory effects of MMPs on the stromal cells in the tumor microenvironment. MMPs regulate the course of the inflammatory reaction in multiple ways and facilitate the recruitment of inflammatory cells by altering the function of chemokines and the bioavailability of important proinflammatory cytokines. Regarding the link between inflammation and cancer, the interference with MMP-mediated immunoregulatory functions could prove beneficial for cancer patients. For example, given that TNF-α contributes to progression of several sorts of cancer, inhibiting TNF-α activation using MPIs might dampen the inflammatory milieu at the tumor microenvironment.

Effects of MMPs on myeloid cells may well be implicated in the generation of the premetastatic niche. In fact, MMP-2, -3 and -9 have already been shown to contribute to the establishment of metastasis-prone sites at tumor-distant organs. These insights argue for the use of MPIs at early stages of malignant disease prior to the full initiation of tumor-associated inflammation and before the soil have been primed for metastasis in distant organs.

The tumor-suppressing function of these MMPs is probably another reason for the failure of broad-spectrum MPIs as anticancer drugs. The inflammation-suppressing function of MMPs accounts for increased incidence of cancer development in *MMP-8* knockout mice and for the link between MMP-8 loss-of-function mutations and melanoma in humans. Also, MMP-12 delivered by macrophages can suppress the growth of lung metastases, which appears to involve regulation of the tumor vasculature. Apart from that, some MMPs carry out biological functions other than proteolytic, mediated by specific binding to certain target molecules, for instance, via their hemopexin domain. Small-molecule MMP inhibitors as used in clinical trials are certainly ineffective to interfere with a nonproteolytic role of MMPs.

One of the major tasks for the future is the development of active site-directed inhibitors or antibodies that are specific for single MMPs and show little or no cross-reaction with other MMPs. Antibodies could also target functional noncatalytic domains of MMPs. Moreover, MMP activity can be exploited to activate cytotoxic agents such as anthrax toxin to target the tumor vasculature. New activity-based imaging probes specific for MMPs will facilitate monitoring the effect of MPIs on the function of MMPs in vivo. Imaging activity of specific MMPs in vivo will further advance our understanding of the time frame of MMP function during the progression of certain tumors. Like the development of tailor-made therapies and medications based on individual oncogenic pathway signatures in human cancers, expression patterns of MMPs in cancer patients could facilitate a fully rational decision about when and in what combination MPIs and anticancer drugs should be used in the future.

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Chapter 11
A Disintegrin and Metalloproteinase-12 as a New Target for Cancer Treatment

Alpana Ray and Bimal K. Ray

Abstract Metastatic spread of cancer is a leading cause for the loss of life from this disease. In metastatic cascade, cells undergo multifaceted phenotypic transformations that include breakdown of extracellular matrix (ECM) encasing the tumor, cancer cell migration and invasion of surrounding tissues, and relocation of cancer cells in secondary organs. Recently a multitasking protein, A Disintegrin and Metalloproteinase-12 (ADAM-12), has attracted particular interest because of its potential roles in tumor growth and development by facilitating remodeling of extracellular matrix and cell migration that are so essential for cancer growth and metastasis. ADAM-12 is an active metalloproteinase; it regulates release of growth factors and is capable of promoting cell–cell and cell–matrix adhesion and cell signaling as well. Overexpression of ADAM-12 is reported in many types of human cancers. Furthermore, a statistical correlation between the urinary levels of ADAM-12 in breast and bladder cancer patients and cancer progression has been found. These results suggested that ADAM-12 could be used as a diagnostic marker. In addition to cancers, increase of ADAM-12 expression is linked to the pathogenesis of osteoarthritis, cardiac hypertrophy, and Alzheimer's disease, as well as during high-fat diet-induced obesity. This review is meant to provide a broad overview of the regulatory pathways by which ADAM-12 could be expressed to contribute towards tumor development, accelerate tumor progression, and metastasis. A better understanding of the regulation of this multifunctional protein that could well be used as a new therapeutic option.

Keywords ADAM-12 • Gene expression • Breast cancer • Anticancer therapy • Transcription factors • Cellular interactions • Z-DNA • Epigenetic regulation

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1 Introduction

A Disintegrin and Metalloproteinase-12 (ADAM-12), also known as meltrin-α, is a transmembrane glycoprotein and a key member of a large (>33) ADAM family of proteins [1–3]. The name ADAM stands for *a d*isintegrin and *a m*etalloproteinase and represents the two prominent structural domains of the ADAM family of proteins. The disintegrin-like domain of ADAM-12, which is homologous to the disintegrin domain of snake venom metalloproteinases (SVMPs), preferentially binds to the extracellular matrix (ECM) via integrins that are important signal transducers between the intra- and extracellular environments [4]. The metalloproteinase domain of ADAM-12 has considerable similarity with the zinc-binding HEXXHXXGXXH domain of the superfamily of zinc-dependent proteases, known as metzincins that include the matrix metalloproteinases (MMPs), the astacins and serralysins [5]. In addition to these two domains, ADAM-12 contains several other functional domains by which it can perform diverse cellular functions. ADAM-12 is shown to perform proteolytic breakdown of the ECM [6]. ADAM-12 regulates ectodomain shedding of heparin-binding epidermal growth factor (HB-EGF) [7] and cell–cell and cell–matrix contact through interactions with cell surface receptors, such as integrins and syndecans [8, 9]. Recent studies show a strong link between increased expression of ADAM-12 and a variety of pathogenic conditions. In many human cancers, for example, breast, liver, lung, prostate, colon, bladder, gastric, bone, and brain cancers, marked upregulation of ADAM-12 is detected [6, 10–17]. Upregulation of ADAM-12 is observed in cardiac tissues of human patients with hypertrophic obstructive cardiomyopathy [18] and in animal models of hypertension and cardiac hypertrophy [19, 20], in patients with osteoarthritis [21], during the loosening of total hip replacement implants [22] and during the development of high-fat diet-induced obesity [23]. In addition, ADAM-12 has been implicated in Aβ-mediated neurotoxicity in Alzheimer's disease [24].

2 Expression of ADAM-12

Sequence analysis indicates that ADAM-12 protein is highly conserved between several species, including the human, mouse, rat, and bovine [25–28], and the human genome sequence data indicates a single gene for *ADAM-12* which contains 23 exons and spans over 35 kb on chromosome 10q26 [26]. Expression of this gene gives rise to two forms of ADAM-12 mRNAs that are generated via alternative splicing of the primary transcript [26]. The longer form, ADAM-12-L, is a transmembrane-type protein while the shorter form, ADAM-12-S, lacks the transmembrane and cytoplasmic domains present at the C-terminal and thereby is secreted in the cells [26]. However, ADAM-12-S mRNA could not be detected in the mouse [25] or in the bovine [28]. The significance of the two mRNA isoforms of ADAM-12 is far from clear, but the ADAM-12-L is shown to cleave pro-forms of EGF [29], HB-EGF [7], placental leucine aminopeptidase (P-LAP) [30], and Delta-like 1 [31] and the

insulin-like growth factor (IGF)-binding protein-5 (IGFBP-5) [21] proteins. The ADAM-12-S is shown to cleave both IGFBP-3 and IGFBP-5 in vitro [32].

Most adult tissues express ADAM-12 at a barely detectable level except during certain developmental and physiological conditions. For example, ADAM-12 expression is seen to be very high in the placenta [9], in neonatal skeletal muscles [25], in regenerating muscles [33, 34], and in the tissues with chronic wound [35]. ADAM-12 mRNA level declines during in vitro differentiation of myogenic cells but increases markedly during regeneration of muscle in vivo [34]. In addition, ADAM-12 expression is well detectable in the cells of mesenchymal origin, such as osteoblasts [36], chondroblasts [37], and adipoblasts [38]. Highly controlled synthesis of ADAM-12 suggests that a tightly regulatory process is most likely involved in the expression of this gene. This regulatory mechanism is apparently compromised in many pathogenic conditions including cancer leading to increased expression of ADAM-12 in tissues where its expression is otherwise restricted. Undesirable changes in the ADAM-12 expression are most likely associated with the pathogenesis of several diseases including cancer.

3 Structural Components and Functions of ADAM-12

The structural components of ADAM-12 are found to be vital for its crucial role and involvement during cancer progression and metastasis. It is well known that an adequate supply of growth factors, receptors, and enzymes within biological pathways is essential for tumor proliferation, survival, and metastasis. ADAM-12 by virtue of its multiple structural domains can assume multiple hats and thus can act as a protease, an adhesive protein, and can take part in cell–cell interactions and communications. The structural domains of ADAM-12 from the N-terminal end include a prodomain, a metalloproteinase, a disintegrin-like, a cysteine-rich, an epidermal growth factor-like domain, a transmembrane domain, and a cytoplasmic domain. Removal of the prodomain is essential for the activation and secretion of the functional ADAM-12 protease [39, 40]. The metalloproteinase domain of ADAM-12 is shown to cleave various ECM proteins, such as gelatin, type IV collagen, and fibronectin [6] and is instrumental in regulating bioavailability of HB-EGF [7] and IGFBP-3 and IGFBP-5 growth factors [32, 41]. The cell adhesion function of ADAM-12 is contributed by a cysteine-rich domain, which is different from the cell adhesion domains of snake venom metalloproteinases (SVMPs) [9]. In the SVMPs, either an Arg-Gly-Asp (RGD) motif or a XGD (X denotes any amino acid) motif confers the cell adhesion function. The cysteine-rich domain of ADAM-12 also supports in vitro attachment of several tumor cell lines, as well as some non-tumor cells of bone and muscle origins [42, 43], and functions in facilitating cell fusion [25, 26]. The cytoplasmic tail region of mouse ADAM-12 (179 amino acid long) is shown to be involved in a signal transduction for some biological function through the interaction with Src-homology 3 (SH3) containing proteins, such as the SH3 domains of the p85α PI3-kinase [44] and the adaptor protein Grb2 [45].

4 Role of ADAM-12 in Cancer

A marked rise in ADAM-12 protein level is reported in a broad spectrum of human cancers, which include breast, lung, liver, prostate, bladder, gastric, and brain cancers and giant cell tumors of bone [6, 10–17]. In breast [6] and prostate [10] cancers, the increase of ADAM-12 level correlates with cancer progression, suggesting that ADAM-12 could act as a tumor diagnostic marker. The urinary level of ADAM-12 in human bladder cancer patients also is seen to correlate with the stage of bladder cancer, and quite importantly the level of ADAM-12 is seen to decrease following the removal of tumor and increase when the cancer is returned [12]. Although exact mechanisms by which ADAM-12 may promote oncogenesis are still debated, as previously described, ADAM-12 has both protease and adhesion activities, and both functions are likely to be important to development and progression of tumors. Recently, ADAM-12 has been found to partner with receptor of activated C-kinase 1 (RACK1) protein in mediating the PKC-dependent translocation of ADAM-12 to membranes of activated hepatic stellate cells of patients with hepatocellular carcinoma [46]. RACK1 is a scaffolding protein and a homologue of the β-subunit of heteromeric G proteins [47]. In addition, ADAM-12 is shown to regulate Notch-mediated signaling [31].

Studies on transgenic animal models shed further light on the role of ADAM-12 in cancer. Overexpression of ADAM-12-S isoform is shown to increase the rate and aggressiveness of breast tumor formation in the polyoma middle T (PyMT) mouse model of breast cancer [48]. In the transmembrane 4 superfamily 3 (TM4SF3) transgenic mice, upregulation of ADAM-12 was seen during metastasis of esophageal carcinoma, and more importantly, abrogation of ADAM-12 by siRNA significantly suppressed cancer invasion [49]. These findings indicated for a crucial role of ADAM12 in metastatic carcinoma and suggested that ADAM-12 might be a potential target of esophageal carcinoma for antimetastasis therapy [49].

5 Regulation of ADAM-12 Expression in Pathogenic Conditions

Compared to the knowledge gathered on the structure and function of ADAM-12, presently, very little is known about the mechanisms that control the synthesis of ADAM-12 mRNA in adult tissues, during development and under pathogenic conditions. Expression of ADAM-12 appears to be regulated, primarily, at the transcriptional level, and TGF-β is identified as one of the prominent inducers of ADAM-12 expression in a variety of cell types, such as the liver [11], chondrocytes [21], breast cancer cells [50], fibroblasts, chondrosarcoma, and prostate cancer cells [51]. Induction of ADAM-12 by TGF-β is shown to be regulated via both the Smad pathway [51] and non-Smad signaling such as PI3Kinase/p70 (S6) kinase and mitogen-activated protein kinase (MEK) pathways [11, 52]. The search of the promoter elements regulating TGF-β1-mediated induction of human *ADAM-12* gene has

identified a high-affinity NF-κB-binding element between -329 and -318 nucleotides, mutation of which markedly inhibits TGF-β1-mediated increase of human *ADAM-12* expression in MDA-MB-231 breast cancer cells [50]. Furthermore, inhibitors of NF-κB significantly reduced *ADAM-12* transcription [50]. In NIH3T3 fibroblast cells, SnoN, a negative regulator of TGF-β signaling pathway regulates TGF-β1-mediated increase of mouse *ADAM-12* gene expression [51]. The agents controlling epigenetic modification such as inhibitors of histone deacetylases (HDACs) are shown to abrogate TGF-β-mediated induction of ADAM-12 in the C3H10T1/2 pluripotent stem cells [53]. In particular, selective inhibition of HDAC3 was found to be proficient in reducing the effect of TGF-β-induced expression of ADAM-12 [53]. These limited but important studies point towards the complexity of ADAM-12 gene expression. It is noteworthy that HDACs, by catalyzing the removal of acetyl groups from lysine residues in both histone and nonhistone proteins, play an important role in the regulation of gene transcription [54, 55] and tumorigenesis [56]. Several HDAC inhibitors are currently in clinical trials for the treatment of solid tumors [57].

6 Identification of a Negative Transcriptional Regulatory Element in Human *ADAM-12* Gene

By using a series of chimeric reporter constructs consisting of the ADAM-12 promoter, a unique regulatory element in the human *ADAM-12* gene was recently identified [27]. A highly conserved negative regulatory element (NRE) consisting of a stretch of dinucleotide repeat sequences (Fig. 11.1) is found to be present at the 5′-untranslated region (+30/+190) of the human *ADAM-12* gene [27]. The dinucleotide repeat regions, typically, are capable of forming Z-DNA conformation [58], and indeed the dinucleotide repeat sequences in the ADAM-12 NRE were shown to adopt a Z-DNA conformation both in vitro and in vivo [27]. This Z-DNA-forming transcriptional repressor remains inactive in placental cells where ADAM-12 expression is very high. Furthermore, the NRE DNA-binding activity was found to be high in low-ADAM-12 expressing tissues and undetectable in a high-ADAM-12 expressing tissue. Involvement of Z-DNA-mediated regulation of ADAM-12 in human mammary epithelial cells was recently demonstrated [59].

7 Z-DNA and Its Biological Role

Z-DNA is a left-handed double-helical conformation and is distinct from right-handed B-DNA. Chromosomal DNA that can adopt a dramatic zigzag structure, called Z-DNA, was first identified in 1979 by Alex Rich and his group [60]. Z-DNA represents a higher energy state and can either enhance or repress gene expression [27, 61–64]. Z-DNA is most easily formed in alternating purine–pyrimidine sequences, such as $(CG)_n$, $(GT)_n$, $(CA)_n$, or $(TA)_n$ when $n \geq 12$. In addition, some other sequences can also form Z-DNA [58, 65]. Z-DNAs are found

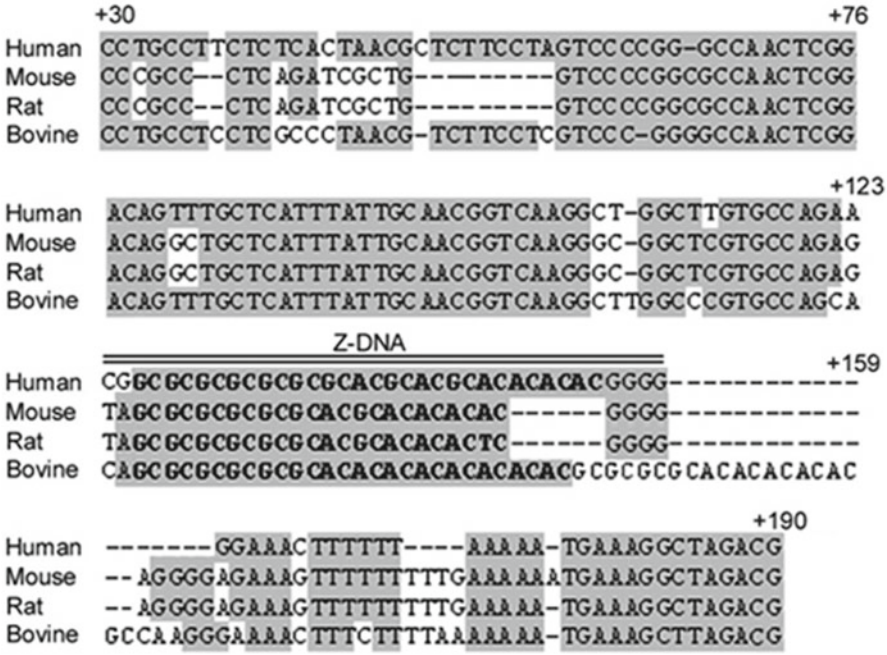

Fig. 11.1 Comparison of the nucleotide sequence of *ADAM-12* gene among different species. This highly conserved domain contains a dinucleotide repeat region that can form Z-DNA

frequently throughout the mammalian genome with a strong bias towards location near the site of transcription initiation but never in any pseudogene [66]. Z-DNA elements can either enhance or repress gene expression [27, 61–64]. Table 11.1 lists several cancer-associated genes that contain putative Z-DNA-forming sequences at the regulatory regions. Increase in the number of (CA) repeats is shown to negatively modulate expression of *EGFR* [67–71], *Cyr61* [72], acetyl-CoA carboxylase (*ACC*) gene [73], heme oxygenase 1 (*HO-1*) [74, 75], type I collagen α2 (*Col1A2*) [76], and matrix metalloproteinase-9 (*MMP-9*) [77–81]. Cyr61 is a cysteine-rich heparin-binding protein, implicated in promoting tumorigenesis; HO-1 is an antioxidant shown to have antiproliferative and apoptotic effect on breast cancer cells. MMP-9 promotes tumor proliferation and metastasis. A Z-DNA-forming $(TG)_n$ repeat is found in the colony-stimulating factor-1 (*CSF-1*) gene [62, 82]. A $(TA)_n$ repeat in the estrogen receptor α (*ER α*) gene [83, 84] and a TCTCT$(TC)_n$ repeat in the *HMGA2* gene [85, 86] have been linked with cancer risk, and a $(TG)_n$ dinucleotide repeat is identified as a candidate DNA marker for breast metastasis [87, 88]. Somatic alteration of polymorphic dinucleotide repeat sequences in two genes of androgen metabolic loci, steroid 5α-reductase (*SRD5A2*) [89] and 3β-hydoxysteroid dehydrogenase type II (*HSD3B2*) [90], is linked with prostate cancer predisposition and higher frequency of prostate cancer risk in African American population. CA repeat polymorphism at *RAD51* and *BRACA2* gene regions are shown to be associated with genetic susceptibility to breast

Table 11.1 Z-DNA elements in cancer-linked genes

Symbol[a]	Z-DNA-forming sequence	Reference
EGFR	$(CA)_{21}$	[67–71]
ERα	$(TA)_{14–24}$	[83, 84]
BRCA2	$(CA)_{17}$	[91]
ADAM-12	$(CG)_6(CACG)_2(CA)_4(CG)$	[27]
CSF-1	$(TG)_4TC(TG)_2TCAG(TG)_{14}$	[62, 82]
Cyr61	$(CA)_{18}$	[72]
ACCA	$(CA)_{28}$	[73]
MMP-9	$(CA)_{18}$	[77–81]
COL1A2	$(CA)_{26}(CG)_7$; $(TG)_{25}$	[76]
HSD3B2	$(TG)_n(TA)_n(CA)_n$	[90]
HMGA2	$TCTC(TC)_n$	[85, 86]
SRD5A2	$(TA)_{9–18}$	[89]
RAD51	$(CA)_{17}$	[91]
HO-1	$(CA)_{18}$	[74, 75]
PRL	$(CA)_{18}$,$(TG)_{65}$	[92]

[a]Symbol represents acronym for different genes which are described in the text

cancer [91]. A 170 bp long $(TG)_n$ and 60 bp long $(CA)_n$ repeat sequences in prolactin gene form Z-DNA and inhibit gene transcription [92]. Prolactin is an invasion suppressor hormone and implicated in the development of mammary glands as well as regulating control of initiation and progression of breast cancer [93].

The role of Z-DNA in transcriptional suppression via binding of specific protein(s) called Z-DNA-binding protein (ZBPs) has been recently demonstrated [27]. More recent investigation has identified MeCP2 and its chaperon partner NF1 as the Z-DNA-interacting epigenetic regulators [59]. Such interaction of MeCP2 and NF1 at Z-DNA element causes suppression of gene expression for the genes carrying the Z-DNA at the promoter region. A model illustrating this molecular event is shown in Fig. 11.2. According to this model, absence or reduction of MeCP2–NF1 interaction with the dinucleotide repeat elements of *ADAM-12* gene in breast cancer cells is a crucial step in the expression of ADAM-12. This unique epigenetic regulatory process is altered in breast cancer cells and could be due to the degradation of MeCP2 in cancer cells or due to a posttranslational modification of the protein.

8 Could ADAM-12 Be a Therapeutic Target?

In the present review the important functions of ADAM-12 and its overexpression in many cancers, as well as in some other pathogenic conditions, are discussed. Given the important roles that ADAM-12 plays in tumor growth and metastasis, there may be significant clinical potentials in exploiting inhibitory molecules that target ADAM-12. In the event of the selection of ADAM-12, next critical decision is whether to target the protein or the mRNA. Traditionally, pharmaceutical industries have preferred targeting the function of the protein and thus focused either on

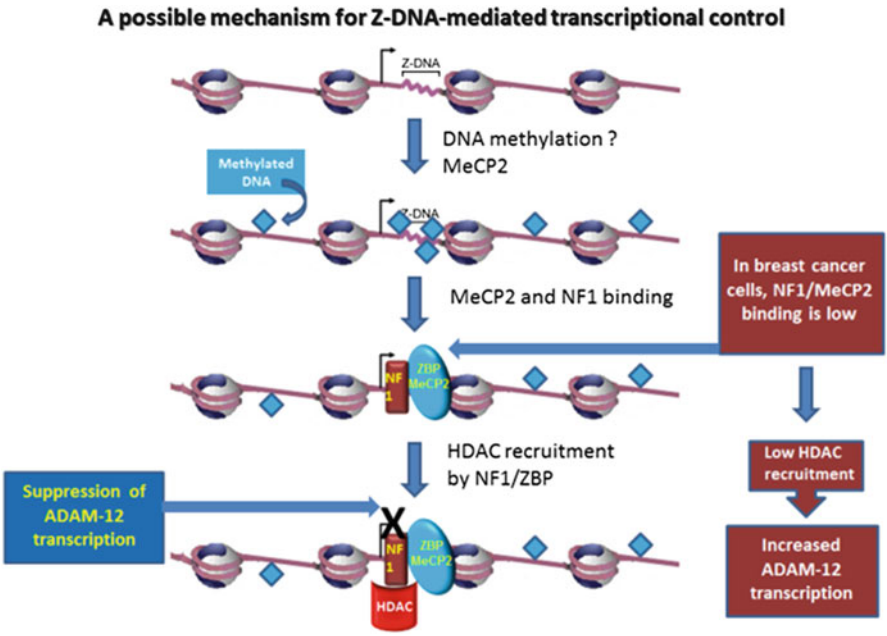

Fig. 11.2 A model depicting possible role Z-DNA and Z-DNA-binding protein (ZBP), MeCP2, and associated factor NF1in regulating ADAM-12 expression in most normal cells. Once bound to the Z-DNA element of *ADAM-12* gene, MeCP2–NF1 complex recruits histone deacetylases (HDAC), and together they suppress transcription of *ADM-12*. Reduced availability of MeCP2 in cancer cells causes low-HDAC recruitment and consequently allows enhanced *ADAM-12* gene transcription

the inhibitory compounds or specific antibodies. But, surprisingly, both scientific community and pharmaceutical industries have ignored the classical negative feedback regulation of a gene's expression by its own product. Blockade of the function of the target protein may very well trigger an urge for the cells to compensate for the loss of the active protein and further induce gene expression to churn out more mRNAs and protein products. In conclusion, whichever path is selected for the inhibition of ADAM-12, interfering with the active protein or gene transcription, mounting evidence points that ADAM-12-based therapeutic approach may prove useful for the treatment of many human cancers and other pathologies as well.

9 Conclusion

9.1 ADAM-12 Plays a Critical Role in Carcinogenesis

- ADAM-12 expression is upregulated in many human cancers.
- ADAM-12 plays vital role in tumor invasion and metastasis.
- ADAM-12 is implicated in several signaling pathways regulating a number of cellular processes such as differentiation, migration, and proliferation.

9.2 ADAM-12 as a Therapeutic Target

- The molecular mechanisms underlying ADAM-12 upregulation provide the potential for defining new therapeutic targets for cancer.
- Other approaches to reduce ADAM-12 expression may include using specific siRNA molecules and antibodies.

9.3 ADAM-12 as a Cancer Diagnostic Marker

- Development of simple tests and diagnostic kits for ADAM-12 to be used as a biomarker of cancer.

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Chapter 12
Proteases and Their Role in Drug Development with an Emphasis in Cancer

Sindhuri Upadrasta and Neeru Saini

Abstract Proteases play a fundamental role in multiple biological and pathological conditions including cancer. They contribute to cancer development and promotion by regulating the activities of growth factors/cytokines and signalling receptors, as well as the composition of the extracellular matrix, thereby suppressing cell death pathways and activating cell survival pathways. With strong evidence of protease involvement in cancer, proteases serve an important role in anticancer drug development. In this review we will first introduce key proteases along with their function in tumorigenesis. Finally we will discuss the key proteases as viable therapeutic targets for anticancer drug development. Further elucidation of the role of proteases in cancer will allow us to design more effective inhibitors and novel protease-based drugs for clinical use.

Keywords Caspases • Cysteine cathepsins • Urokinase-type plasminogen activator • Kallikreins • Matrix metalloproteinases • A disintegrin and metalloproteinases • A disintegrin and metalloproteinase with thrombospondin motifs • Protease-activated prodrugs

1 Introduction

Proteases refer to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. Till recent times, they were essentially considered to be protein degrading enzymes having nonspecific functions in protein catabolism. Recent developments in this field indicate that proteases can cleave specific substrates, thereby having an influence on the varied vital processes and pathological

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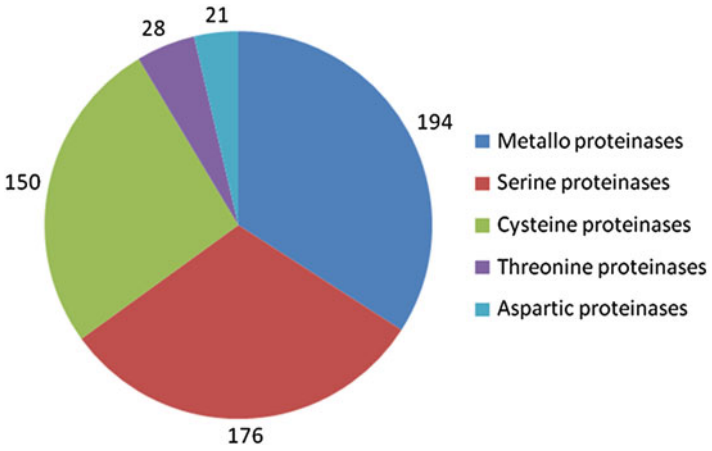

Fig. 12.1 Classification of human degradome. On the basis of the mechanism of catalysis, the human proteases are classified into 5 different classes: metallo, serine, cysteine, threonine, and aspartic proteases

conditions. An insight into the genome of human and other model organisms has revealed the impressive diversity existing in protease functions. There are at least 569 proteases and homologues produced by human cells there by forming a human degradome. These proteases and their homologues are further classified into five classes: 194 metalloproteinases, 176 serine, 150 cysteine, 28 threonine, and 21 aspartic proteases (Fig. 12.1) [1].

The number as well as diversity of these proteases is indicative of their importance in the biological processes. Proteases have been found to regulate the fate, localization, and activity of many proteins, modulate protein–protein interactions, create new bioactive molecules thereby contributing to the processing of cellular information, and generate, transduce, and amplify molecular signals. A direct result of these multiple actions is that proteases influence DNA replication and transcription, cell proliferation and differentiation, adhesion, tissue morphogenesis and remodelling, angiogenesis, stem cell mobilization, autophagy, senescence, necrosis, apoptosis, and evasion of immune system [2]. Therefore, proteases have an influence on cell behavior, survival, and death of all organisms [3]. Alterations in proteolytic systems can lead to multiple pathological conditions such as neurodegenerative disorders, inflammatory and cardiovascular diseases, and cancer.

Given the role of proteases in protein degradation and tissue remodelling, they have been suggested to be involved in cancer invasion and metastasis, which accounts for a majority of lethal outcomes related to cancer [4]. This was first proposed by Fisher in 1946. Following this, many individual proteases were identified to have a role in cellular invasion. Intracellular proteases like cysteine and aspartyl proteases take part in removing damaged or undesirable products and degradation of endocytosed proteins [5]. Other intracellular proteases like cysteine proteases (of caspase family of proteins) and autophagins regulate proteolytic activities which lead to apoptosis and autophagy, respectively [6, 7]. All the intracellular proteases, including

the deubiquitinases, confer protection to the cell via proteolytic cascades, and loss of function mutations in these proteases leads to various human cancers [6, 8]. In contrast to the protective mechanism of intracellular proteases, extracellular proteases take part in facilitating tumorigenesis. Activation of oncogenic transcriptional pathways often leads to over expression of these enzymes in the tumor tissues [9].

The ability of cancer cells to invade normal tissues and cross physiological barriers depends on proteolytic function of these proteins. In order to metastasize, tumor cells must cross the basement membrane which comprises a continuous and dense network of collagen, glycoproteins, and proteoglycans. Recent data suggest that the action of proteases present within and on the surface of cells results in local proteolysis which helps in the movement of the cells from their primary location to a distant location. Metastasis basically involves a sequence of events wherein a cell or group of cancer cells attach to the underlying basement membrane, intravasate into the vasculature, survive in the circulation, arrest at a distant vasculature bed, extravasate into surrounding tissues, and proliferate into a secondary tumor where proteases play a major role [10]. In the pages that follow, you will be introduced to some of the important proteases which have been shown to play key roles at different stages of cancer progression.

2 Caspases

Caspases (cysteine-dependent aspartyl-specific protease) belong to a family of cysteine proteases that mediate proteolytic events indispensable for biological phenomena such as cell death and inflammation. To date, a number of caspases have been identified in various vertebrate and invertebrate species. In humans, 11 caspases including caspase 1 to caspase 10 and caspase 14 have been identified. Several additional caspases, including caspase 11, caspase 12, and caspase 13, have been detected in other mammals such as rodents and the cow *Bos taurus*. To date 14 mammalian caspases have been broadly categorized into initiators (caspases 2, 8, 9, and 10), effectors (caspases 3, 6, and 7), and inflammatory caspases (caspases 1, 4, 5, 11, 12 and 13). Caspase 14 is a unique caspase as it belongs to neither apoptotic caspases nor inflammatory caspases [11]. Recently, caspases 15, 16, 17, and 18 have also been identified as new members of the caspase family in vertebrates, although their function has not yet been identified [12–14]. In addition, fish-specific caspases have been found: caspy, caspy2, and caspase recruitment domain (CARD)-casp8 [15, 16]. Not limited to vertebrates, caspases have been identified in a wide variety of animals such as sponge *Geodia cydonium*, *Hydra vulgaris*, sea anemone *Aiptasia pallida*, nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster*, sea urchin *Strongylocentrotus purpuratus*, and ascidians *Ciona intestinalis* and *Ciona savignyi* [17–25].

Since apoptosis is central to tumorigenesis, the role of caspases has been a subject of great interest. Literature reveals that caspase alterations are not rare in a variety of tumors which might result from mutations, promoter methylation, alternative splicing, and posttranslational modifications. Some of these defects lead to loss of function, but in other cases mutated caspases act as dominant negatives preventing the activation of wild-type protein [26]. The importance of caspase 8 was

brought to light by showing that knockout of caspase 8 leads to embryonic lethality. In contrast, another study pointed out that the deletion or silencing of Caspase 8 gene promotes cell survival and metastasis in neuroblastoma [6, 27]. Somatic mutations leading to inactivation of Caspase 8 gene have also been seen in tumors of head and neck, lung, colorectal, and gastric tumors [28, 29]. Following this, genetic alterations in other caspases have also been observed, and it has been pointed out that Caspase 10 is also frequently mutated in tumors [30, 31]. Presence of mutation in Caspase 8, Caspase 10, and other caspase family members suggests their involvement in progression of malignant tumors.

Literature further reveals that altered caspase function can also be a consequence of modified expression of their specific inhibitors, for example, cFLIPs that competes with caspase 8 for FADD binding, thereby preventing its activation. cFLIPs is often elevated in tumors, while its downregulation has been shown to sensitize tumor cells towards therapy [32]. Among caspase inhibitors an important role is played by inhibitors of apoptosis (IAPs). While initially described as caspase inhibitors, IAPs are now recognized to regulate a multitude of other cellular functions including regulation of the immune response, cell migration, mitosis, and proliferation [33]. Alterations of IAPs are found in a variety of human cancers and are associated with poor prognosis and resistance to therapy. In some cases however, loss of IAPs correlates with tumor progression complicating the issue and suggesting that the role of IAPs has to be carefully evaluated based on cell context.

3 Cysteine Cathepsins

Cathepsins were originally identified as endopeptides that are located in the lysosomes, while recent reports have uncovered nontraditional roles for cathepsins in the extracellular space as well as in the cytosol and nucleus [34, 35]. Cysteine cathepsins specifically are capable of efficiently cleaving a wide variety of substrates and thought to participate in protein turnover. They comprise 11 proteases that show increased expression in tumors and are referred to as clan CA, family C1a: cathepsins B, C (also known as cathepsin J and dipeptidyl-peptidase 1), F, H, K (also known as cathepsin O2), L, O, S, W, V (also known as cathepsin L2), and Z (also known as cathepsin X and cathepsin P) in humans.

Cysteine cathepsins are highly upregulated in a wide variety of cancers by mechanism which includes gene amplification, transcript variation (arises from the use of alternate promoters and alternative splicing), transcriptional regulation, posttranscriptional regulation, and epigenetic regulation to name a few [5]. Presence of diversity in the expression of specific cysteine cathepsins in tumor cells and tumor-associated cells at different times during neoplastic progression indicates that individual enzymes have distinct roles during progression in the various cell types that comprise the tumor microenvironment and in the tumor cells. The pattern of expression often varies with tumor type and the cellular composition of the tumors. Increase in the expression of cysteine cathepsins occurs in premalignant or early lesions, for example, cathepsin B in Barrett's esophagus and stage I esophageal tumors [36, 37],

Table 12.1 Tumor cells and tumor-associated cells that express cysteine cathepsins

Fibroblasts	Cat. B, Cat. C, Cat. K, and Cat. L
Osteoclasts	Cat. B and Cat. K
Neutrophils	Cat. B and Cat. C
Mast cells	Cat. C and Cat. S
Myoepithelial cells	Cat. F, Cat. K, and Cat. L
T-lymphocytes	Cat. C and Cat. W
Endothelial cells	Cat. B, Cat. L, and Cat. S
Tumor associated macrophages	Cat. B, Cat. C, Cat. K, Cat. L, Cat. S, Cat. V, and Cat. X

cathepsin H in node-negative lung tumors [38], and cathepsin S and cathepsin X in high-grade prostatic intraepithelial neoplasias. In addition to these in ductal carcinoma in situ of the breast, there is increased expression of the cysteine cathepsins F, K, and L [39]. Cathepsin S, which is increased in stage IV astrocytomas, is found in both tumor cells and tumor-associated macrophages [40], as has been reported for cathepsin B in colon carcinomas and observed for cathepsin B in transgenic mouse mammary tumors. Moreover, overexpression of Cathepsin B gene has also been found in esophageal carcinoma and transformed rat ovarian cells. Recent studies also show that increased expression of Cathepsin B is associated with breast, lung, gastric, colorectal, and prostate carcinomas, melanomas, gliomas, and osteoclastomas and with low survival rates in patients with colorectal cancer [10]. Cathepsin B can cleave a wide variety of substrates including extracellular matrix proteins, proteinases, as well as proteinase inhibitors. Reports suggest that Cathepsin B is involved in detachment of migrating cancer cells, and inhibitors of cysteine cathepsins can reduce the proteolysis and migration of oral squamous cell carcinoma cells. Inhibitors against intracellular Cathepsin B have been shown to reduce the invasiveness of human melanoma and prostate carcinoma cells [5]. Cysteine cathepsins are also upregulated during HPV16-induced cervical carcinogenesis, further encouraging consideration of this protease family as a therapeutic target in human cancers. In contrast to these studies, deletion of Cathepsin L in HPV16-induced skin carcinogenesis mouse model leads to formation of early onset aggressive tumors. Keratinocytes with homozygous deletion of Cathepsin L show increased proliferation rates, suggesting a tumor suppressor function of Cathepsin L with respect to squamous cell skin carcinoma [41]. Table 12.1 shows the distribution of cysteine cathepsins in tumor and tumor-associated cells that express cysteine cathepsins.

4 Urokinase-Type Plasminogen Activator

Urokinase-type plasminogen activator (uPA) is an extracellular proteolytic enzyme belonging to serine protease family of enzymes. The uPA system comprises urokinase-type plasminogen activator, 2 inhibitors of plasminogen activators, namely, PAI-1 and PAI-2, and the urokinase receptor (uPAR) [42, 43]. The binding of uPA to the cell surface receptor uPAR leads to activation of uPA, and it further cleaves the surface-associated plasminogen into the serine protease called plasmin

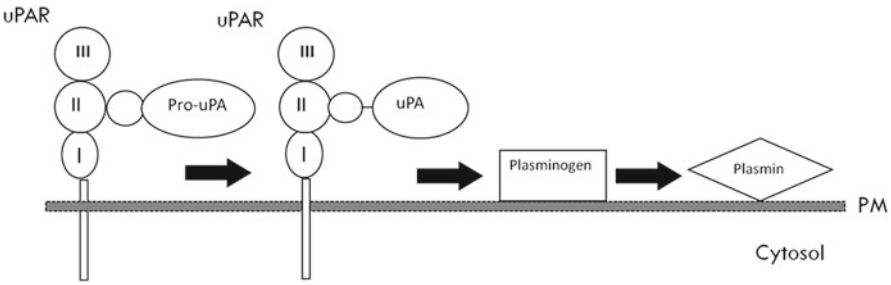

Fig. 12.2 Schematic representation of the PAS system. Given illustration represents the proteolytic cascade which leads to generation of plasmin

which in turn is involved in a number of pathophysiological processes requiring basement membrane (BM) or extracellular matrix (ECM) remodelling, including tumor progression and metastasis (Fig. 12.2) [44, 45]. Plasmin can also activate specific growth factors like FGF2, VEGF, IGF-2, and HGF that stimulate cell proliferation/mitogenesis [46–48]. FGF-2 and VEGF the two well-known stimulators of endothelial cell growth have also been found to play a role in angiogenesis [49].

Preliminary evidences support the role of uPA system in early stages of tumorigenesis. The expression of both uPAR and uPA is significantly upregulated during cancer progression and is primarily confined to the tumor-associated stromal compartment. uPA, plasminogen, and plasmin have also been shown to play roles in cell migration and adhesion [42]. Increased expression of uPAR in virtually all human cancers suggests possible clinical applications as diagnostic marker, predictive tool of survival or clinical response, and as a target for therapy and imaging. In fact, increased expression of uPA/uPAR and PAI-1 in tumors shows strong correlation with metastatic potential and lower rates of patient prognosis [50] and indicates poor survival. uPA/PAI-1 has been designated as a prognostic markers associated with poor disease outcome for early stage breast cancer and has been recommended by the American Society of Clinical Oncology for screening in routine clinical practice [51]. The cleaved forms of uPAR are also prognostic markers and a potential diagnostic, and predictive impact of the different uPAR forms has been reported [52, 53].

Literature further reveals that in comparison to wild-type mice, uPA-deficient mice which have been chemically induced with blue nevi failed to progress to melanomas [54]. In vivo studies have shown that uPA and matrix-metalloproteases (MMPs) work together for the degradation of the ECM thereby facilitating metastasis. Recent data have also provided new insights into the role of uPAR in gastric cancer progression, and in addition to mediating proteolysis, this receptor also appears to mediate cell signalling, proliferation, and survival, and these observations have revealed novel ways to target uPAR. Dual role has also been suggested for uPA in angiogenesis depending on the stage of angiogenesis. In the beginning, uPA helps by degrading the ECM and promoting the proliferation of endothelial cells, while later on it might activate angiostatin (inhibitor of angiogenesis) formation [55].

5 Kallikreins

Kallikrein (Greek synonym for pancreas: kallikreas) was named by the three German scientists H. Kraut, E.K. Frey, and E. Werle, who in 1930 reported that the pancreas is a rich source of this endogenous hypotensive substance. The human kallikrein gene locus spans a region of 2, 61,558 bp on chromosome 19q13.4. It is formed of 15 tandemly localized kallikrein genes with no intervention from other genes and is the largest cluster of serine proteases within the human genome.

Kallikreins are basically serine proteases which are responsible for the coordination of various physiological functions including blood pressure, semen liquefaction, and skin desquamation [56]. The expression of kallikreins has also been found to be altered in hormonally regulated human carcinomas, and there have been reports in the literature that suggest that kallikreins might function as tumor-promoting or tumor-suppressing enzymes on the basis of hormonal balances and tissue type. Prostate-specific antigen (PSA) kallikrein 3 is proposed to promote tumorigenesis by initiation of growth factors and proteolytic degradation of the ECM. Kallikrein 3, the most commonly known kallikrein, is a useful biomarker that aids in the diagnosis, staging, and follow-up of prostate cancer. Apart from KLK3 several other kallikreins, including kallikreins 2 (KLK2) and 11 (KLK11), are also emerging as complementary prostate cancer biomarkers [57, 58]. Kallikrein 4 has also been found to be overexpressed in prostate cancer [59, 60]. Along with these kallikreins, several others have been implicated in the other cancers. For example, KLK5, KLK6, KLK7, KLK10, KLK11, and KLK14 are emerging biomarkers for ovarian cancer [61–63], and kallikrein 1, also known as tissue kallikrein, cleaves kininogen to release the vasoactive kinin peptide, bradykinin, or lysyl bradykinin. Kallikrein 5 is widely expressed but found at high levels in skin, breast, brain, and testis, and its overexpression is an indicator of aggressive ovarian tumors which result in poor patient prognosis [64, 65]. Kallikrein 8 is expressed in the brain and is a novel marker of ovarian and cervical cancer. It is worth mentioning here that upregulation of 12 kallikrein genes has been found in ovarian cancer. KLK3, KLK8, and KLK10 have been shown to have tumor suppressive functions [66, 67]. Owing to their interaction with other serine proteases like uPA and its receptor uPAR, human kallikreins have been implicated in tissue invasion, angiogenesis, and metastasis. Furthermore, KLKs can activate MMPs like collagenase IV and thereby promote tumorigenesis. KLK2 and KLK4 can inactivate PAI-1 and in turn activate the uPA pathway [68]. Controversies still exist on the role of KLK2 in angiogenesis. While KLK2 can activate tumor growth factor β (TGF-β) and promote angiogenesis, it is also known to block FGF 2 and hence inhibit angiogenesis. Furthermore, KLKs can activate MMPs like collagenase IV and thereby promote tumorigenesis. Due to their role as biomarkers, KLKs are used for the screening, diagnosis, prognosis, and monitoring of various cancers, e.g., prostate, ovarian, breast, testicular, and lung; human tissue kallikreins (KLKs) are attracting increased attention these days.

6 Matrix Metalloproteinases

This family consists of 23 zinc-dependent endopeptidases which are expressed during processes involving tissue remodelling like embryonic development, wound healing, uterine and mammary involution, cartilage to bone transition during ossification, and trophoblast invasion into endometrial stoma during placenta development [69–72]. This extracellular remodelling property of MMPs has implications in pathological processes like periodontitis and rheumatoid arthritis. Recent findings further provide evidence that MMPs can modulate the different stages of tumor formation which include tumor growth, invasion, metastasis, and angiogenesis.

MMP-9 has also been found to enhance endothelial cell growth in vitro [73, 74], and MMP-8 when overexpressed in breast cancer cells reduces their metastatic potential. Literature also reveals that MMP-8-deficient mice show increased incidence of skin carcinomas [75]. Furthermore, MMP-12, which is mainly expressed in macrophages, has been shown to reduce tumor growth rates in mice [76]. However, the precise role of MMP-12 in human cancers is not yet clear. This stems from studies supporting its dual role: its expression in colon and hepatocellular carcinomas has been found to have favorable outcomes, while its expression in other tumors correlates with poor prognosis of patients [77–79]. Dual roles have been reported for other MMPs like MMP-3, MMP-9, and MMP-11 which have been associated with tumor progression and in some instances with antitumor effect [1].

MMPs facilitate metastasis and angiogenesis by degrading the physical barriers and allowing increased signalling by signalling molecules like growth factors and cytokines. MMPs mediate cleavage of the ectodomain of VE-cadherins which leads to loss of cell–cell adhesions. In vivo studies show that MMP-7 (matrilysin) is necessary for endothelial cell proliferation, and upregulation of other MMPs like MMP-1 and MMP-2 causes induction of angiogenesis [80]. In comparison to quiescent vessels, angiogenic and tumor blood vessels contain exposed cryptic binding sites for αvβ3 which is brought by cleavage of type IV collagenase by MMPs. This also correlates with increased expression of MMP-2 which binds to αvβ3 and facilitates angiogenesis [81, 82]. Further studies on these enzymes showed that loss of MMP-8 causes abnormalities in inflammatory response induced by carcinogens leading to sustained inflammation thereby generation of a favorable environment for tumor development.

Another zinc-dependent metalloprotease family is the disintegrin and metalloproteinase which include two subgroups: the membrane-bound ADAM and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [83, 84]. This family of proteases bears structural relationships with other MMPs. ADAMs and ADAMTS have been implicated in different stages of cancer progression. ADAMs mediate the shedding of cytokines and growth factors and regulate the fusion of membranes and motility of cells as well as muscle development, fertilization, and cell fate determination [85]. ADAM17 plays a major role in inflammatory processes by facilitating the shedding of TNF-α [86]. ADAM10 and ADAM12 have been found to be overexpressed in a number of carcinoma tissues and cell lines consistent with their ability to regulate adhesion and motility of cells [87]. Reports show that ADAMTS1, the first identified member of ADAMTS family, inhibits angiogenesis and reduces growth of tumor and metastasis [88–90]. Table 12.2 shows role of different MMPs and their implications in tumorigenesis.

Table 12.2 Role of different MMPs and their implications

	Activity	Effect
Cancer cell invasion		
Several MMPs such as MT1-MMP, MMP-2 and MMP-9 Several members of the ADAM family	Proteolytic	Degrade physical barriers
Cancer cell proliferation		
MMP-1, -2, -3, -7, -9, -11, -19, ADAM12	Cleavage of IGF-binding proteins	Proliferation
MMP-3, -7, ADAM17, ADAM10	Shedding of membrane-anchored ligands of EGFR (HB-EGF, TGF-α, and amphiregulin)	
ADAM10	Shedding of E-cadherin	
MMP-9, -2, -14	Activation of TGF-β	
MMP-7 (anchored to CD44)	Shedding of HB-EGF	
Cancer cell apoptosis		
MMP-7, ADAM10	Cleavage of Fas ligand Anti-apoptotic	
ADAM10	Shedding of tumor associated major histocompatibility proteins complex class-I	
Several MMPs and ADAMs	Indirect activation of Akt through activation of EGFR and IGFR	
Tumor angiogenesis and vasculogenesis		
Several MMPs (including MMP-2, -9, MMP-3, -10, -11, MMP-1, -8, -13)	Degradation of COL-IV, perlecan; release of VEGF and bFGF, respectively	Up-regulation of angiogenesis
	Degradation of COL-IV, COL-XVIII, perlecan, generation of tumstatin, endostatin, angiostatin, and endorepellin, respectively	Down-regulation of angiogenesis
Cell adhesion, migration, and epithelial to mesenchymal transition		
MMP-2	Degradation of COL-IV; generation of cryptic peptides	Promote migration
MT1-MMP	Degradation of laminin-5; generation of cryptic peptides	
MMPs	Integrins as substrates	
MMP-2,-3, -9, -13, -14	Over-expression; related to EMT	Induction of EMT; cell migration
ADAM10 MMP-1, -7	Shedding of E-cadherin	
MMP-28	Proteolytic activation of TGF-β	Powerful inducer of EMT; cell migration
Immune surveillance		
MMP-9	Shedding of interleukin-2 receptor-α by T-lymphocytes surface	Suppress T-lymphocyte proliferation
MMP-9, -2, -14	Release of active TGF-b	Suppress T-lymphocyte reaction against cancer cells
MMP-7, -11, -1, -8, -3	Release of a1-proteinase inhibitor	Decrease cancer cell sensitivity to NK cells
MMP-7, -8	Cleavage of a- and b-chemokines or regulation of their mobilization	Affect leukocyte infiltration and migration

7 Proteases as Viable Therapeutic Targets for Anticancer Drug Development

7.1 Caspases as Drug Targets

Cell death inhibition is a very successful strategy that cancer cells employ to combat the immune system and various anticancer therapies. An alteration in the apoptotic signalling pathway is one of the main reasons for tumorigenesis. Hence, components as well as triggers and regulators of this pathway are among the most promising targets for pharmacological interventions with respect to cancer. Currently chemotherapeutic treatments aim to promote cellular toxicity and damage which in turn induces apoptosis either directly or indirectly via caspases. Approaches are being developed that will activate death receptor pathways, synthetically activate caspases, restore the activity of tumor suppressor genes such as p53, and counteract the effects of antiapoptotic factors. Among these approaches, small molecules are in clinical trials against several antiapoptotic players, namely, IAP proteins and the Bcl-2 protein. Cellular IAP proteins regulate expression of antiapoptotic molecules and prevent assembly of proapoptotic protein-signalling complexes. In addition, amplifications, mutations, and chromosomal translocations of IAP genes are associated with various malignancies. Several therapeutic strategies have been designed to target IAP proteins, including a small-molecule approach that is based on mimicking the IAP-binding motif of an endogenous IAP antagonist—the second mitochondrial activator of caspases (Smac). Other strategies involve antisense nucleotides and transcriptional repression. Inhibitors of IAPs like second mitochondria-derived activator of caspase/direct IAP-binding protein with low p*I* (Smac/DIABLO) and heat-inducible serine protease (A2HtrA2) have increased the interest of the pharmacological industry in them. Omi/HtrA2 is a mitochondrial serine protease that is released from mitochondria during apoptosis. It binds to IAP and antagonizes its binding to caspase 9, thereby modulating the caspase activity. Another member of the IAP family is survivin, which plays a role in apoptosis as well as cell cycle regulation. Caspase 3 and cyclin-dependent kinase inhibitor p21 have been found to colocalize with survivin. Regardless of its function as an inhibitor of caspase 3 or as a regulator of cell cycle, downregulation of survivin affects the growth of transformed cells. As many of these processes are often modified in cancer, it is clear how alteration of IAPs can play a role in tumorigenesis. The most important pathway regulated by IAPs that contributes to cancer development is the NF-kB signalling pathway, and XIAP, cIAP1, and cIAP2 have been shown to regulate this pathway and in turn regulate inflammation, immunity, and cell survival. Moreover evidence is there in the literature that IAPs protect from TNF-α killing. In addition, recent findings show a role for IAPs in metastasis also as XIAP/survivin complex has been found to trigger the NF-kB pathway leading to activation of cell motility kinases [91]. This however is still a controversial issue, and other studies show a suppressive effect of IAPs on cell mobility, therefore raising the need for further investigations. Experiments with administration of antisense nucleotides and ribozymes against survivin have also

Table 12.3 Non-inflammatory compounds which are being tested as possible drugs targeting the caspases and their inhibitors

Targeted molecule	Principal compound	Sponsor	Name
Caspases	Peptide based irreversible inhibitors	INSERM	
Caspases	Caspase inhibitors	Merck Frosst	M-920 (L-826, 920)
Caspases	Caspase inhibitors	Vertex Pharmaceuticals	VX-799
Survivin	Antisense oligodeoxy-nucleotides	Isis Pharmaceuticals/ Abbott Laboratories	
Caspase-3	Highly selective inhibitor of caspase-3	Merck Frosst	MF-286, MF-867

Table 12.4 Drugs targeting Bcl2 family proteins

Agents	Target proteins	Sponsor	Stage
Apogossypol	Bcl-2, Bcl-Xl, Mcl-1	Burnham (NCI)	Preclinical
HA-14	Bcl-2	Maybridge Chem	Preclinical
Antimycin A	Bcl-2, Bcl-Xl	University of Washington	Preclinical
BH3Is	Bcl-Xl	Harvard University	Preclinical
Oblimersen sodium	Bcl-2	Genta	Phase III
Gossypol (AT-101)	Bcl-2, Bcl-Xl, Bcl-W, Mcl-1	Ascenta (NCI)	Phase I/Phase II
ABT-737 (ABT-263)	Bcl-2, Bcl-Xl, Bcl-W	Abbott	Phase I
GX15-070	Bcl-2, Bcl-Xl, Bcl-W, Mcl-1	Gemin X	Phase I

been shown to induce apoptosis in various cell lines. This finding has triggered the development of antisense-based strategies which target the expression of survivin. In any case due to their involvement in cancer progression and their ability to suppress apoptosis IAPs have become an attractive therapeutically target, leading to the development of IAP inhibitors, some of which are based on natural inhibitors such as Smac/DIABLO [91–93]. These drugs appear to be able to directly kill cancer cells or at least sensitize them to other killing agents while sparing normal cells. A number of these compounds are currently entering clinical trials; Table 12.3 [94].

Bcl-2 is an antiapoptotic molecule and overexpression of Bcl-2 protein has been reported in many types of cancers, including leukemia, lymphomas, and carcinomas. Bcl-2 has also been associated with chemotherapy resistance in various human cancers. Thus targeted inhibition of Bcl2 can be used as a tool for the treatment of different cancers. Several classes of drugs have been found to regulate gene expression of antiapoptotic Bcl-2 members, and several of these are in different phases of clinical trials. Until recently, most research efforts aimed at developing anticancer tools were focusing on small molecules. Alternative compounds are now being increasingly assessed for their potential anticancer properties, including peptides and their derivatives. Most anticancer peptidic compounds induce apoptosis of tumor cells by modulating the activity of Bcl-2 family members that control the release of death factors from the mitochondria. Some of these peptides have been shown to inhibit the growth of tumors in mouse models. Several agents targeting antiapoptotic Bcl-2 family of proteins are also in preclinical/clinical trials (Table 12.4).

7.2 Cysteine Cathepsins as Drug Targets

An increased cell proliferation rate represents a key aspect of tumor biology, and cysteine cathepsins have been discovered to influence the regulation of cell proliferation by several means. Upregulation of cysteine cathepsins has been reported in many human tumors, including breast, lung, brain, gastrointestinal, prostate, and melanoma. Knockout of specific cathepsins in mice has confirmed that targeting of individual cysteine cathepsins can prove to be a beneficial strategy for cancer treatment. Avascular tumors are severely restricted in their growth potential because of the lack of blood supply, and it is well known that angiogenesis is required for invasive tumor growth and metastasis and constitutes an important point in the control of cancer progression. Therefore, inhibition of angiogenesis is a valuable approach to cancer therapy. There is also increasing evidence that cysteine cathepsins promote invasion and metastasis by remodelling the extracellular matrix (ECM) in the tumor microenvironment. Active cathepsins have been shown to be able to degrade the protein components of basement membranes and the interstitial connective matrix including laminin, fibronectin, elastin, tenascin, and various types of collagen. Following the report by Szpaderska et al. that inhibitors of intracellular cathepsins can reduce the invasiveness of human melanoma and prostate carcinomas, they are being studied for anticancer therapies. Endogenous inhibitors of these enzymes, known as cystatins, are also being used to reduce tumor growth, invasion, metastasis, and angiogenesis. Administration of small molecule inhibitors of cathepsins or increasing the expression of endogenous inhibitors is suggested to be of therapeutic benefit. Presently, only one small molecule inhibitor has been successful which is a broad-spectrum inhibitor and targets the intracellular and extracellular pool of cathepsins. Therapeutic agents that can be activated by subsequent cleaving at the tumor cell surface by cathepsins have also proven to be efficacious. Such therapeutic agents generally contain a pore forming toxins conjugated to cathepsin B cleavable linkers. Prodrugs which can be cleaved by cathepsins are also being developed, for example, prodrugs of doxorubicin [5].

7.3 uPA System as Drug Targets

Breast cancer is one of the most common malignancies and is responsible for many deaths. The plasminogen activation system (PAS) has been found to be frequently upregulated in metastatic breast cancer and also correlates with poor prognosis of patients. Many antimetastatic prophylactic drugs are being developed which target the PAS. Inhibitors of uPA and the interaction of uPA with its cell surface receptor (uPAR) are promising molecules for drug development. Known inhibitors bind to the S1 subsite of uPA which forms a salt bridge with the negatively charged Asp^{189} residue by incorporation of positive charges. Essentially all uPA inhibitors which are being used as antimetastatic drugs retain the crucial

interaction with improvisations in the pharmacokinetics of the positively charged molecules. An example is amiloride that is a potassium-sparring diuretic that has been reported to prevent lung metastasis in a rat adenocarcinoma model. It has also been found to significantly reduce metastasis of MATB rat mammary cancer cells. Amiloride as well as B428, another uPA inhibitor, have showed the potential to reduce the invasive capacity of two breast cancer cell lines, namely, MDA-MB-231 and MDA-MB-436. Other small molecule inhibitors of uPA are tranexamic acid, aprotinin, and leupeptin. Arginine mimetics which bears either an *N*-tri-isopropyl-phenylsulfonamide group or 4-amidinobenzylamine group are also being considered as potent inhibitors. Besides this cyclic peptide antagonists who have the potential to displace the uPA molecules bound to the surface receptors and hence inhibit the tumor cell associated activation of plasminogen and fibrin degradation [50]. The interaction between uPA and its receptor uPAR has also been investigated to find possible strategies of intervention. This has devised the development of linear peptide antagonists which compete with uPA for the binding site/epitope on its receptor.

7.4 MMPs as Drug Targets

Given the positive correlation between tumor aggressiveness and the levels of proteases, these enzymes have become a subject of interest for development of anticancer drugs. MMPs regulate the destruction of the extracellular matrix which facilitates malignant invasion making them a suitable target for drug development. One such strategy involves the use of tissue inhibitor of metalloproteinases (TIMPs) or TIMP fragments as direct inhibitors of MMP activation or activity. Another strategy involves using peptide inhibitors which mimic the amino terminal motif of the MMPs which contain the latent state enzyme. A third strategy involves using synthetic compounds as competitive inhibitors of substrates which bind to the active site of the enzymes. In vivo studies have shown that TIMP-1, TIMP-2, and synthetic substrate inhibitors can be used to block angiogenesis. In vivo studies in animal models provide evidence that TIMP-1 inhibits invasion and metastasis. Simultaneously, TIMP-2 has also been shown to inhibit cell invasiveness in in vitro and in vivo studies. An example of synthetic inhibitors of MMPs is BB94 whose administration to athymic nude mice-bearing fragments of colon cancer inhibited the growth of primary tumor and a reduction in incidence of tumor invasion and spontaneous metastasis. In a separate study it has also been shown to delay growth of B16-BL6 transplanted primary melanoma. Chemically modified tetracycline derivatives have also been shown to inhibit the enzyme activity as well as the synthesis of MMPs by blocking its transcription. These compounds inhibit MMPs by chelating the metal ions like zinc and calcium. A few examples of these compounds are metastat, minocycline, and doxycycline. Doxycycline is currently the only approved drug which is being used against periodontitis, brain tumor, and Kaposi sarcoma [86].

8 Development of Protease-Activated Prodrugs

As mentioned above, proteases are molecules important for the development of anticancer drugs. Besides being target molecules for therapeutic drugs, proteases are also instrumental molecules for development of prodrugs. Prodrugs are derivatives of therapeutic agents which upon chemical or environmental stimuli release the parent drug. They are inactive molecules in their native state and are transition to active drugs upon being acted by a stimulus. Given that proteases lead to selective cleavage of their substrates, they have been considered for development of protease-activated prodrugs (PAP), ensuring an efficient delivery and efficacy of the drug with minimum toxic effects on healthy cells. They are made up of a parent drug conjugated to a polymer or peptide substrate via a cleavable linkage and/or a targeting moiety for specific delivery, which in the case of PAPs should be stable in blood stream till it reaches target protease [95]. Examples are prodrugs made by conjugating single amino acids or dipeptides to cancer drugs like doxorubicin and daunorubicin. For further information please go through reference [95].

9 Conclusions

Proteases are involved in a wide variety of functions like immunological responses, degradation of the articular cartilage matrix, and other pathological processes, playing a major role in both intra- and extracellular protein turnover. Apart from their role in various physiological and pathological processes, proteases have also been found to be involved in tumor growth, invasion, and migration. A number of proteases have been associated with various stages of cancer and also serve as biomarkers which can predict the prognosis of patients. Given their role in tumorigenesis, proteases are being considered as highly relevant targets. Endogenous inhibitors of proteases have been found to be suitable molecules for drug development. Synthetic peptides mimicking the inhibitors of pro-tumorigenic proteases are also being developed. Research is also going on to identify selective inhibitors of proteases rather than broad-spectrum inhibitors. In addition to this, RNA interference can also prove to be instrumental in silencing protease genes which show aberrant expression in tumors. Such therapies can prove to be useful not only in cancer but several pathological states, such as immune disorders, osteoporosis, rheumatoid, and osteoarthritis, where proteases are known to be involved. In addition to being target molecules for different anticancer drugs, proteases are now also being used for the development of protease-activated prodrugs which will ensure the efficient delivery and action of conventional anticancer drugs.

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Part III
Proteases and Cardiovascular Defects

Chapter 13
Role of Matrix Metalloproteinases in Atherosclerosis

Karina Di Gregoli and Jason L. Johnson

Abstract Matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) play a complex role in the pathogenesis of atherosclerosis and plaque instability. Proposed roles for MMPs included matrix degradation but also regulation of the proliferation, migration and apoptosis of monocytes, macrophages and vascular smooth muscle cells. Accordingly, in vitro and in vivo studies have demonstrated that individual MMPs are utilised by distinct cell types to modulate their behaviour. As a result some MMPs clearly promote plaque growth and development of "vulnerable" morphologies in experimental models but others do not. Likewise some MMPs associate with vulnerable atherosclerotic plaque phenotypes in man, whilst others correlate with stable lesions. This premise is underlined by the fact that in both mouse and man, broad-spectrum MMP inhibition fails to exert beneficial effects on atherosclerotic plaque development and stability. Given that MMPs exert such diversity on atherosclerotic lesions, one therapeutic strategy may be to selectively inhibit those MMPs particularly implicated in adverse plaque phenotypes. An example is our recent use of a selective MMP-12 inhibitor in the apolipoprotein E (Apoe) mouse model. The inhibitor retards progression of established plaques, in part by reducing the recruitment of monocytes into plaques but also by preventing apoptosis of foam-cell macrophages and calcification. Consequently, although our understanding of the multifaceted role MMPs play during the development, progression and rupture of atherosclerotic plaques are becoming clearer; the need for selective MMP inhibition is required for the translation of the experimental findings to the clinic.

Keywords Matrix metalloproteinases • Macrophages • Atherosclerosis • Vascular smooth muscle cells • Plaque rupture

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1 Introduction

The formation and progression of atherosclerotic plaques are the main underlying pathology of cardiovascular disease and now recognised as the principal cause of mortality and morbidity in developed countries [1]. Atherosclerosis can be described as a chronic inflammatory, autoimmune-like disease, which progresses in the presence of high plasma lipid levels [2]. It is characterised by the accumulation of lipids (atheroma) and fibrous elements (sclerosis) in the large arteries nourishing the heart (coronary arteries) and the brain (carotid arteries) [1]. The rupture of atherosclerotic plaques can precipitate several clinical events including myocardial infarction, stroke and peripheral vascular disease. Consequently there has been a wealth of research conducted to elucidate the pathophysiology of atherosclerotic plaque formation, progression and rupture. A principal aim has been to delineate appropriate targets to enable the development of new medical therapies aimed at a specific cell type or molecule involved in atherosclerosis. Multiple studies have highlighted matrix metalloproteinases (MMPs) as key mediators of atherosclerotic plaque progression [3]. MMPs are implicated in all stages of plaque development and progression from lesion initiation to plaque rupture [4]. The foremost cause of plaque rupture is thought to be the loss of extracellular matrix (ECM) proteins within the lesions, such as the degradation of collagen and elastin, which generally corresponds with areas of inflammation. These regions are principally characterised by the presence of lipid-laden macrophages, but also T and B cells, mast cells and smaller amounts of other lymphocytes [5]. MMPs play other roles in plaque rupture as well as ECM degradation. For example, MMPs are able to promote the death of vascular smooth muscle cells (VSMC), the major cell type responsible for plaque ECM synthesis [6]. Elevated levels of MMPs such as MMP-1, -2, -7, -8, -9, -11, -12, -13, and -14 have been identified in human atherosclerotic plaques (see Table 13.1). Furthermore, MMP expression is higher in macrophage-rich areas such as the shoulder regions and around the lipid-rich core indicating macrophage-derived MMPs

Table 13.1 MMPs upregulated 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]

Abbreviations: *Mø* macrophage, *VSMC* vascular smooth muscle cell, *EC* endothelial cell

may play a critical role in atherosclerosis progression. Consequently, many studies have focused their attention on the potential of MMP inhibitors as a therapeutic approach to stabilise and eventually enable the regression of atherosclerosis [7].

2 Matrix Metalloproteinases

MMPs (also named the matrixins) are a large family of proteolytic enzymes involved in important processes such as morphogenesis, wound healing, tissue repair and remodelling. They play a central role in pathological events such as cancer progression and atherosclerosis, becoming important therapeutic targets for medical research. The MMPs are multidomain proteins able to degrade components of the ECM. Their activity is tightly regulated by a family of endogenous inhibitors termed the tissue inhibitors of metalloproteinases (TIMPs) that, together with MMPs, maintain an important balance over ECM homeostasis during physiological and pathophysiological conditions. However, non-ECM molecules have also been highlighted as potential MMP substrates, conferring new roles for this family of proteases and their inhibitors in processes such as inflammation, cell migration, differentiation, growth and apoptosis (as reviewed by [8]).

At present 24 human MMP genes have been identified. MMP expression is tissue specific and can be regulated by inflammatory cytokines, growth factors, hormones and physical cell–cell and cell–matrix interactions [8]. They are included in the Metzincin family together with 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 [9].

3 MMPs Structure and Classification

All MMP family members share structural homology. Generally they have:

- *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 cleaved during secretion [8]
- *Pro-peptide*, highly conserved motif responsible for pro-MMP latency [8]
- *Catalytic domain* with a zinc binding site responsible for the endopeptidase activity of MMPs
- *Hinge region* (linker peptide), situated between the catalytic domain and the hemopexin-like domain, that is a determinant for the stabilisation of collagenolytic activity due to the presence of several proline residues
- *Hemopexin-like domain*, located at the C-terminus that has strong sequence similarity to the serum protein hemopexin and with a wide range of roles amongst different MMPs [9]

However, between all the MMPs there are some differences in structure that confer different biological properties. On the basis of their domain organisation and their substrate preference, the MMPs are routinely divided into five different groups: collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysins (MMP-7 and MMP-26) and membrane-type MMPs (MT1-, MT2-, MT3-, MT4-, MT5- and MT6-MMP also known as MMP-14, -15, -16, -17, -24 and -25, respectively).

4 MMP Activation and Inhibition

All the MMPs are produced as zymogens. To have full activation the MMP protein is required to pass an important regulatory step, the cleavage of the pro-domain. The interaction of the pro-domain with the catalytic domain maintains the MMP in an inactive conformation [8]. The activation of the pro-form occurs in a multistep manner, called also "stepwise activation" [8]. In the pro-domain of these enzymes, there is a "proteinase susceptible bait region" that may be a substrate of plasma or bacterial proteinases. The cleavage of this region does not remove the whole pro-peptide domain, but results in destabilisation of the cysteine-Zn^{2+} negative interaction, leaving the MMP in an intermediate form. To gain full activation the *in-trans* activity of other MMPs (in the intermediate or active form) is necessary to remove all the inhibitory interaction [8]. Some MMPs are fully activated intracellularly by furin or other pro-protein convertases and then secreted, or cell surface bound, as active enzyme. The activity of furin-activated MMPs is regulated by endogenous inhibitors (such as α_2-macroglobulin and TIMPs), proteolysis, or internalisation and recycling [8]. Recently, other proteins have been identified with the ability to inhibit MMP activity; the reversion-inducing cysteine-rich protein with Kazal motifs (RECK), tissue factor pathway inhibitor-2 (TFPI-2), and the procollagen C-terminal proteinase enhancer (PCPE) [10]. TIMPs are the most potent endogenous inhibitors of MMPs and therefore believed to play the major role in regulating their activity physiologically. In vertebrates four TIMPs have been identified (TIMP-1, -2, -3, -4), which exhibit different inhibitory efficacy against different members of the MMP family [10]. Their expression is tissue specific and are regulated during development and remodelling [11]. Most of the inhibitory functions of TIMPs are attributed to the N-terminal domain since it is able to form, if isolated, a stable, native molecule that has inhibitory activity against MMPs [11]. TIMPs are secreted proteins but they can also be found on the cell membrane associated with membrane proteins such as MT-MMPs. All the TIMPs can inhibit the activity of all MMPs; however, TIMP-1 has a poor inhibitory effect on MMP-9, -14, -15, -16, and -24. TIMPs also harbour the ability to inhibit members of both the ADAM and ADAMTS family of proteinases [10]. The balance between MMPs and TIMPs is crucial for homeostasis. Any alteration of this balance may result in pathological dysfunctions associated with an aberrant turnover of the matrix. Examples include cardiovascular disease, cancer, arthritis and neurological disorders [11].

5 MMPs and Atherogenesis

5.1 Early Stage: Fatty Streak Formation

Atherogenesis is the process that leads to atherosclerotic plaque formation on the luminal side of an artery. It is a long, multistep sequence that develops and evolves over several decades in man, starting with early lesions that may occur during early adolescence. Atherosclerotic plaque progression can depend on genetic factors and/or some well-recognised risk factors such as hypercholesterolemia, smoking, sedentary lifestyle or diabetes [12]. The first step in atherogenesis is the formation of an early lesion, commonly termed a fatty streak or more recently coined as pathological intimal thickening [13]. These lesions are characterised by the presence of lipid-laden macrophages, also more commonly called foam-cell macrophages, within the arterial intima. Elevated levels of low-density lipoprotein (LDL), shear stress, and presence of free radicals such as reactive oxygen species (ROS) or infection-related pathogens can generate endothelial damage. Damaged endothelium is subject to inflammatory activation that leads to an increased expression of various adhesion molecules that mediate leukocyte recruitment. Adhesion molecules expressed by the endothelium mediate transitory contact and leukocyte rolling on the luminal surface of the vessel wall. Once adherent to the endothelium, monocytes and lymphocytes penetrate into the *tunica intima*, passing through the endothelium, driven by a chemoattractant gradient. Monocyte recruitment can be considered one of the crucial events in atherosclerosis development and early lesion formation. For a monocyte to invade tissue, it must be able to degrade the physical barrier represented by the ECM, and therefore protease activity is strongly required. Human monocytes constitutively express a range of MMPs and TIMPs such as MMP-8, MMP-12, MMP-19, TIMP-1 and TIMP-2. However, upon adhesion and in response to proinflammatory mediators, they upregulate the expression of others MMP such as MMP-1, MMP-3, MMP-10 and MMP-14 through the activation of MAP kinase and NF-κB transcription factors [14]. In particular, MMP-14 expression and activity are required for human monocyte endothelial transmigration and invasion since these events can be retarded by MMP-14 inhibition either by a neutralising antibody or gene silencing in vitro [15–17]. Therefore, MMP expression in monocytes, in response to adhesion stimuli, may be necessary to mediate cell transmigration and subsequent invasion into the arterial wall. Once within the intima monocytes differentiate into macrophages under the influence of numerous stimuli. The most prevalent stimuli are colony-stimulating factors (CSFs), which induce the expression of scavenger receptors, cytokines and growth factors giving rise to a survival incentive [18]. Macrophages expressing scavenger receptors start to internalise modified lipoproteins resulting in their transformation into foam-cell macrophages (FCMs) [19]. Additionally, the interaction between macrophages and T cells mediates a broad variety of immune and inflammatory responses, such as the expression of adhesion molecules, cytokines, prothrombotic activities, MMPs and apoptotic mediators driving an inflammatory amplification loop and therefore

promoting atherosclerotic plaque progression [20]. Within the atherosclerotic lesion, macrophages and foam-cell macrophages can express an extensive range of MMPs and TIMPs [21]. MMP-7, MMP-9 and TIMP-3 are induced during macrophage differentiation in vitro [14], and the expression of other MMPs such as MMP-1, MMP-3 and MMP-12 can be induced in mature macrophages by inflammatory mediators and cytokines [21]. Within the atherosclerotic lesion most macrophages are in reality foam-cell macrophages. Immunohistochemistry is a valuable tool used to study foam-cell macrophages in situ. Using this technique it has been possible to identify the presence 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 13.1). Furthermore, the identification of cleaved collagen in the same sites [22] alongside matrix proteolysis as assessed by in situ zymography [23, 24] also indicates that at least some of these enzymes are also active. Recently, it has been shown that cells from human plaques overexpress MMP-1 and MMP-3 [25] and similarly, in vivo-generated foam-cell macrophages from cholesterol-fed rabbits overexpress MMP-1, MMP-3, MMP-12 and MMP-14 [26, 27]. Moreover, the loss of TIMP-3 expression by a subpopulation of rabbit FCMs increased their invasiveness, proteolytic activity and propensity to undergo apoptosis [27]. Therefore, the presence of foam-cell macrophage-derived active MMPs may indicate atherosclerotic plaque progression and predict future clinical outcomes.

5.2 Mature Atherosclerotic Plaque Formation

Alongside MMPs and TIMPs, macrophages produce cytokines and mediators such as platelet-derived growth factor (PDGF), heparin-binding epidermal growth factor and insulin-like growth factors that mediate the recruitment of VSMC [28] from the *tunica media*. In order to facilitate their migration, VSMC need to release themselves from physical barriers, such as cell–cell and cell–matrix contacts, for which MMP activity may facilitate this process. Studies focussing on the role of MMPs in VSMC migration have directed their attention primarily on MMP-2, MMP-9 and MMP-14 [6]. It has been shown that MMP-2 is required for VSMC migration through the basement membrane in vitro [29, 30] and that MMP-9 overexpression can increase the migratory capacity of isolated VSMC [31]. An elegant study revealed that MMP-14 is crucial for VSMC to degrade and infiltrate 3D collagen barriers including the arterial wall [32]. These findings were then corroborated by subsequent in vivo studies using genetically modified mice lacking either MMP-2, MMP-9 or MMP-14, which all demonstrated attenuated VSMC migration [32–34]. Moreover, MMPs can also drive VSMC migration by processing non-matrix substrates. CD44, a cell surface hyaluronan receptor, can be cleaved and shed from the cell membrane by MMP-14 resulting in increased cell motility [35]. However, it has been pointed out that, conversely, SMCs can also use CD44 as a docking station for secreted MMP-7 and MMP-9, localising their proteolytic activity to the cell surface and thus possibly facilitating migration [36, 37]. Once in the lesion VSMC start to proliferate and synthesise ECM components, such as collagen and fibronectin, leading to the formation

of a fibrous cap. This event fixes the point of mature atherosclerotic lesion formation. VSMCs proliferation has also been linked with MMP expression and activity. As described for migration, also VSMC proliferation requires the loss of cell–cell and cell–matrix connection which otherwise exerts an inhibitory effect on cell division. Furthermore, a more recently identified target of MMP proteolytic activity are a family of adhesion molecules named cadherins. Cadherins are principally involved in cell–cell contact but can also act as cell-signalling receptors, modulating the nuclear translocation of β-catenin, a member of Wnt/wingless signalling pathway known to activate several genes, some of which are involved in cell proliferation [38]. It has been demonstrated that MMPs can cleave cadherins from the VSMC surface, eliciting β-catenin nuclear translocation and leading to cell proliferation [39].

Mature atherosclerotic plaques can progress without impairing lumen capacity due to compensatory vascular remodelling [40]. However, ensuing foam-cell macrophage death, including both necrosis and apoptosis, leads to formation and slow enlargement of an extracellular lipid-rich core, which is highly thrombogenic. A role for MMPs in macrophage/foam-cell macrophage apoptosis has also been described [41]. Indeed, blocking either MMP-12 activity with a selective inhibitor, or reducing MMP-14 proteolytic activity using a neutralising antibody, reduced the susceptibility of foam-cell macrophages to undergo apoptosis [27, 42]. Similarly, an excess of the endogenous inhibitor of MMPs, TIMP-2, reduced foam-cell macrophages apoptosis, although exogenous TIMP-1 had no effect [43]. Ongoing foam-cell macrophage apoptosis and lipid-rich core expansion contributes to plaque progression. The stability of atherosclerotic plaques is due to the composition and thickness of the fibrous cap; the majority of acute coronary events result from rupture of the cap and the subsequent leakage of the thrombogenic lipid core into the arterial lumen [44].

6 MMPs and Atherosclerotic Plaque Progression

Clinical symptoms such as myocardial infarction and stroke are often triggered by the rupture of an atherosclerotic plaque and ensuing thrombus formation, resulting in distal impairment of blood flow or embolisation. As described earlier, the mature atherosclerotic plaque shows a characteristic structure. It presents a soft and highly thrombogenic lipid-rich core with associated foam-cell macrophage infiltration, covered by a VSMC- and ECM-rich fibrous cap that provides the plaque's structural integrity. Fibrous cap disruption results in the exposure of highly thrombogenic plaque constituents like tissue factor (TF), lipids or modified collagen fragments and their interaction with flowing blood, triggering activation of the coagulation cascade, thrombin generation and ensuing thrombus formation [45]. Atherosclerotic plaques are heterogeneous in nature and as such there is high variability in fibrous cap thickness and lipid core size. Different combinations of these two factors can lead to different clinical outcomes. Pathological examination of human atherosclerotic coronary arteries [13, 46] has allowed researchers to distinguish and define plaque phenotypes histologically as stable and unstable (also termed vulnerable or rupture prone). A typical stable plaque presents a thick fibrous cap rich in VSMCs

and collagen with a small lipid-rich core and minimal macrophage infiltration. This kind of lesion usually remains clinically silent in the coronary arteries. On the other hand, a vulnerable plaque typically contains a large lipid-rich core and a thin fibrous cap [3] and is characterised by a higher number of macrophages and other inflammatory cells. Such plaques are commonly referred to as thin-cap fibroatheroma (TCFA). As well as being important in atherosclerotic lesion formation, inflammation (T cells and macrophages) has been proposed to promote plaque destabilisation [3]. Foam-cell macrophages produce proinflammatory cytokines such as IFNγ, which can inhibit VSMC collagen synthesis. Moreover, they produce proteolytic enzymes such as MMPs, and inflammatory mediators capable of activating MMPs, resulting in the degradation of collagen and many other ECM components such as elastin, which can further weaken the fibrous cap [3]. Furthermore, inflammation also induces activation of apoptotic pathways that trigger VSMCs death, additionally contributing to fibrous cap thinning. It has been proposed that MMPs may affect VSMC apoptosis by cleavage of cell–matrix connections, thus attenuating matrix-dependent surviving signals [6]. Moreover, ECM protein degradation due to increased MMP activity may release peptide fragments exhibiting novel cryptic sites that can bind to VSMC surface receptors which induce apoptosis signalling pathways. The processing of death molecules, and their receptors, can trigger apoptosis through both autocrine and paracrine processes. Several MMPs are able to cleave pro-TNF-α exposing the cells to a proapoptotic factor [6]. Fas ligand, together with TNF-α, has been indicated as a target of MMP-7 [47]. Interestingly, MMP-7, TNF-α and Fas ligand are all present in human atherosclerotic plaques [48].

The most fragile sites in the plaque where most of the ruptures take place are at their lateral aspects, termed the shoulder regions. This region is characterised by a high density of macrophages and foam-cell macrophages and the presence of neovascularisation [49]. The expression of MMP-1, MMP-3 and MMP-9 in FCMs, VSMCs, lymphocytes and endothelial cells has been demonstrated in the shoulder regions of human atherosclerotic plaques. Moreover, in situ *zymography* also revealed that MMP activity is increased in the shoulder regions of advanced plaques, compared to undiseased arteries where MMP-2, TIMP-1 and TIMP-2 are also detected [24, 50, 51]. Higher levels of MMP-2, MMP-7, MMP-11, MMP-12, MMP-13, MMP-14 and MMP-16 are expressed in prone-to-rupture regions of unstable plaques and their presence colocalised with cleaved collagen fragments, the main ECM component of the fibrous cap [22, 52–56]. These findings indicate that MMP expression and ensuing activity are crucial in the process of plaque rupture and therefore in the clinical outcomes observed in patients with advanced atherosclerotic disease.

7 MMPs as Therapeutic Targets for Atherosclerosis

Animal models have been largely used to study the pathogenic steps of atherosclerotic plaque formation, progression and rupture, with the aim of identifying potential therapeutic targets to combat plaque advancement. Rabbits when fed a high-fat diet spontaneously develop aortic atherosclerotic plaques, and thus, this model has been

Table 13.2 Effect of MMP modulation on atherosclerotic plaque development and stability using in vivo animal models

Modulation	Model (species)	Site	Size	VSMCs	Mø	References
MMP-1 Tg	Apoe KO (Ms)	Aorta and root	↓	↔	↔	[63]
MMP-2 KO	Apoe KO (Ms)	Aorta and root	↓	↓	↔	[64]
MMP-3 KO	Apoe KO (Ms)	Aorta, BCA	↑/↑	↓/ND	↓/↔	[65, 66]
MMP-7 KO	Apoe KO (Ms)	BCA	↔	↑	↔	[66]
MMP-8 KO	Apoe KO (Ms)	Aorta	↓	↔	↓	[67]
MMP-9 KO	Apoe KO (Ms)	Aorta, BCA	↓/↑	ND/↓	↓/↑	[66, 68]
MMP-9 Tg	Apoe KO (Ms)	Arch, collar	↔	↔	↔	[69, 70]
MMP-12 KO	Apoe KO (Ms)	Aorta, BCA	↔/↓	↔/↑	↔/↓	[66, 68]
MMP-12 Tg	kbt:JW (Rb)	Aorta	↑	↑	↑	[71]
MMP-13 KO	Apoe KO (Ms)	Root	↔	↔	↔	[72]
MMP-14 KO	Ldlr KO (Ms)	Root	↔	↔	↔	[73]
Non-selective MMP inhibitor	Ldlr or Apoe KO (Ms)	Aorta, BCA	↔	↔	↔	[74–76]
MMP-12 inhibitor	Apoe KO (Ms)	Aorta, BCA and root	↓	↑	↓	[50]
MMP-13 inhibitor	Apoe KO (Ms)	Carotid	↔	↔	↔	[77]
TIMP-1 KO	Apoe KO (Ms)	Aorta and root	↔/↓	↔/ND	↔/↑	[78, 79]
TIMP-1 RAd	Apoe KO (Ms)	BCA and root	↓/↔	ND/↔	↓/↔	[51, 80]
TIMP-2 RAd	Apoe KO (Ms)	BCA	↓	↑	↓	[51]

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) overexpression, gene knockout (KO) or pharmacological inhibitors of MMPs
Abbreviations: VSMC, vascular smooth muscle cell; Mø, macrophage; BCA, brachiocephalic artery; root, aortic root; ↓, decreased; ↑, increased; ↔, no change; ND, not determined

used in a number of studies. However, the majority of in vivo atherosclerotic studies have been conducted in mice. Yet contrary to the rabbit, wild-type mice do not spontaneously develop atherosclerotic lesions in response to high-fat diet. Accordingly two mouse models were generated where components of cholesterol transport were genetically deleted; mice with targeted deletion of apolipoprotein E (Apoe) [57, 58] or LDL receptor (Ldlr) [59] spontaneously develop atherosclerotic plaques throughout the arterial tree [60]. The pathogenesis can be greatly accelerated when the mice are fed a high-fat diet, inducing severe hypercholesterolemia and the development of plaque which resemble the human analogues and progress similarly from fatty streaks to complex advanced lesions. Utilising mainly Apoe KO mice (and to a lesser degree Ldlr KO animals), a number of studies have been conducted where these mice are either bred with transgenic or knockout mice or treated with potential therapeutic agents. These studies have elucidated the roles of MMPs and TIMPs in atherosclerotic plaque progression and stability (summarised in Table 13.2).

7.1 Overexpression Studies

Unlike humans, mice do not actively express MMP-1. Nonetheless a study where human MMP-1 was overexpressed selectively in macrophages of Apoe KO mice

showed unexpectedly a reduction in plaque size and collagen content [61]. However, macrophage-specific overexpression of pro-MMP-9 did not influence lesion stability in arterial plaques [62]. Conversely, pro-MMP-9 expression induced intra-plaque haemorrhage in advanced lesions induced in carotid arteries by collar implantation in Apoe KO mice [63]. Moreover, overexpression mutated form of MMP-9, which is fully auto-activated, augmented plaque progression [62]. Similarly, macrophage overexpression of an active form of MMP-12 in rabbits also increased plaque size and increased inflammation [64] suggesting that MMP-9 and MMP-12 activation may promote atherosclerosis progression (summarised in Table 13.2).

7.2 Knockout Studies

MMP knockout studies in Apoe KO mice have indicated both protective and detrimental effects. Mmp2 KO mice presented a reduction in plaque size, although this may have been due to a decrease in VSMC accumulation, suggesting plaque stability may be compromised [65]. Mmp3 deletion resulted in larger aortic and brachiocephalic plaques [66, 67] associated with less medial elastin breaks [66] but lowered VSMC content and concomitant increase in buried fibrous layers (a surrogate marker of plaque instability) [67] implying MMP-3 affords greater stability. In agreement reduced VSMC migration and associated neo-intimal formation were observed in Mmp-3 KO mice [68]. In contrast, an increase in VSMC content was observed within brachiocephalic plaques of Mmp7 KO [67], consistent with a proapoptotic role of MMP-7 on VSMCs [69]. Lesion size and macrophage numbers were reduced in Mmp8 KO mice, suggesting improved stability [70]. Similar to MMP-3, contradictory conclusions have been yielded in Mmp9 KO mice; whereas plaque burden and macrophage number were reduced in one study [71], increased plaque size, macrophage content and buried fibrous layers in another [67]. In agreement with promoting plaque instability, reduced VSMC migration and associated neo-intimal formation were observed in Mmp-9 KO mice [68]. Mmp12 KO reduced brachiocephalic artery lesion area and number of buried fibrous layers [67], whilst also decreasing aortic elastin fragmentation [71]. Additionally, the intra-plaque ratio between VSMCs and macrophages was favourably increased towards VSMC content in implying heightened stability, in part through modulation of monocyte/macrophage invasion and apoptosis [42]. Taken together with the detrimental role of MMP-12 in rabbit atherosclerosis [64], the findings suggest that MMP-12 promotes plaque progression and instability. Deletion of Mmp13 and Mmp14 had negligible effects on plaque macrophage and VSMC composition, but increased fibrillar collagen content, indicating a role in plaque instability [72, 73]. Taken together these findings indicate that some MMPs (such as MMP-2, MMP-3 and MMP-9) may exert a protective role in atherosclerosis by promoting VSMC growth and associated fibrous cap formation and therefore enhance plaque stability. Conversely, other MMPs (including MMP-7, MMP-8, MMP-12, MMP-13 and MMP-14) may promote plaque progression through increased matrix degradation, inflammation and susceptibility to apoptosis and thus participate in triggering of plaque rupture (summarised in Table 13.2).

7.3 Inhibitor Studies

In line with above findings, adenovirus-mediated gene transfer systemic overexpression of two endogenous inhibitors of MMPs, TIMP-1 and TIMP-2 has retarded atherogenesis and plaque progression of early lesions [43, 74]. Moreover, long-term overexpression of TIMP-2, but not TIMP-1, retarded progression of advanced plaques to a certain extent by inhibiting monocyte/macrophage invasion and their susceptibility to apoptosis [43]. These studies thus provide strong support for MMP inhibition as a therapeutic strategy to prevent plaque instability and associated clinical events. Consequently, scientific research has focussed on the development and testing of synthetic inhibitors of MMPs. However the results, with synthetic compounds containing zinc-chelating groups (such as thiol or hydroxamate groups, or tetracycline derivates) have been contradictory. Hydroxamic acid-based, non-selective MMPs inhibitor exhibited no beneficial effects on plaque development or progression in Lldr KO mice [75] or Apoe KO animals [76]. Similarly, the widely used antibiotic doxycycline, that also displays broad-spectrum MMP inhibitory properties, failed to prevent plaque development in Apoe KO mice [77]. Moreover, treatment with doxycycline in two independent, randomised, double-blind and placebo-controlled clinical trials in patients with symptomatic coronary and carotid artery disease failed to have any positive effects on plaque phenotype or clinical outcome [78, 79]. The lack of any clear benefits against atherosclerosis in either animal or clinical studies may be due to the divergent roles MMPs play. Accordingly, selectively targeting MMPs with a clearly detrimental role could be more effective for retarding atherosclerotic plaque progression. Indeed two recent studies have examined the effectiveness of highly specific MMP inhibitors in mouse models of atherosclerosis. Firstly, a highly selective MMP-12 inhibitor, RXP470.1, was administrated to Apoe KO mice with established plaques. The study showed that MMP-12 inhibition blocked plaque progression and improved stability through reduction of lipid core expansion and macrophage apoptosis, increased VSMC to macrophages ratio, decreased plaque calcification and attenuated elastinolysis [42]. All these effects, together with a reduction of buried fibrous layers in plaque mirrored those observed on Mmp12/Apoe double KO mice [67]. A second study utilised a highly specific inhibitor towards MMP-13 [80]. Intra-plaque collagenolytic activity was inhibited and associated with preservation of fibrillar collagen levels in plaques, resembling what the effects previously observed in Mmp13 KO mice [72]. Therefore, these proof-of-principle studies in mice provide motivation to translate selective MMP inhibitor treatment human patients harbouring atherosclerosis (summarised in Table 13.2).

8 Conclusions

A multitude of studies in cells, animal models and humans has firmly established that MMPs play a central role in cardiovascular pathologies, in particular the development, progression and rupture of atherosclerotic plaques. Alongside in vitro

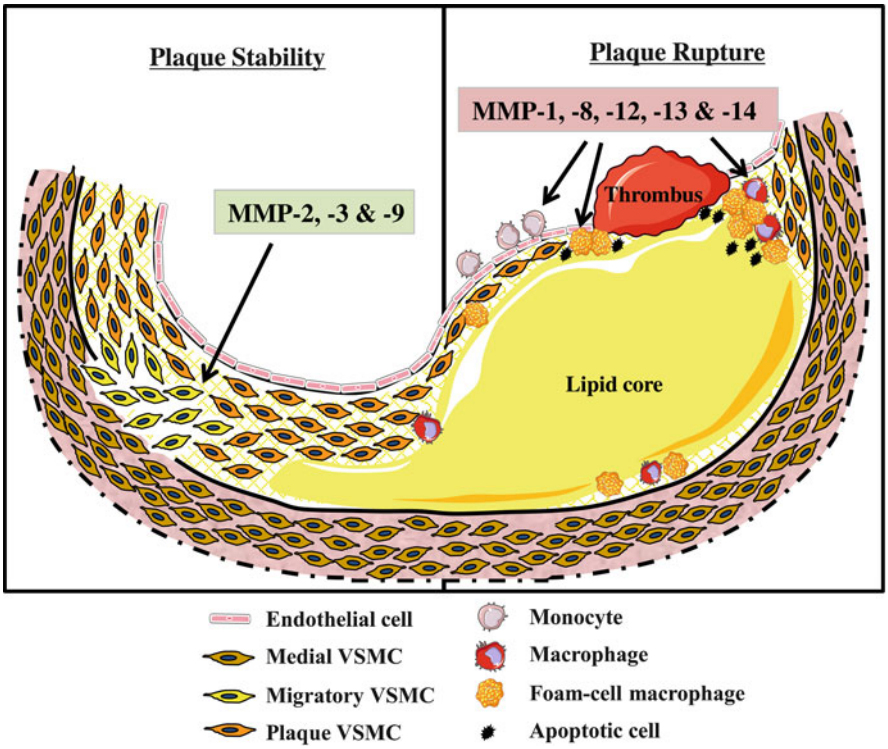

Fig. 13.1 Divergent roles of MMPs in atherosclerotic plaque progression and stability. Hypothetical model of the beneficial and detrimental roles of MMPs during atherosclerotic plaque progression and rupture. Matrix metalloproteinase (MMP)-2, -3 and -9 can facilitate vascular smooth muscle cell (VSMC) migration from the media into the developing atherosclerotic lesion where they participate in fibrous cap formation and promotion of plaque stability. Conversely MMP-1, MMP-8, MMP-12, MMP-13 and MMP-14 can degrade extracellular matrix proteins present in the fibrous cap whilst also promoting the recruitment of monocytes and macrophages and their subsequent susceptibility to apoptosis—together these processes would enhance lipid core expansion and the thrombogenicity of the lesion. Subsequently the stability of the plaque is compromised and vulnerable to plaque rupture and ensuing thrombus formation

studies, the advent of animal models allowing genetic manipulation of individual MMPs and TIMPs has elucidated the functions of MMPs in all vascular cell types and the subsequent consequences for atherosclerosis. Such work has highlighted that modulation of MMP activity can reverse atherosclerosis, but that broad-spectrum MMP inhibition cannot replicate these effects, possibly owing to effects on both beneficial and detrimental MMPs (summarised in Fig. 13.1). Consequently it is now accepted that inhibitors with restricted specificity towards individual MMPs such as MMP-12 and MMP-13 are desirable for translation to man, particularly in the context of atherosclerotic plaque stabilisation.

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Chapter 14
The Importance of the Urokinase-Type Plasminogen Activator and Its Receptor for the Development and Progression of Atherosclerosis

Nicole Paland and Bianca Fuhrman

Abstract Urokinase-type plasminogen activator (uPA) is a multifunctional multi-domain protein that is not only a regulator of fibrinolysis but it is also associated with several acute and chronic pathologic conditions. uPA mediates extracellular matrix (ECM) degradation and plays a pivotal role in cell adhesion, migration, and proliferation during tissue remodeling. On the cell surface, uPA binds with high affinity to its receptor, the uPAR, providing a strictly localized proteolysis of ECM proteins. The uPA/uPAR complex also activates intracellular signaling, thus regulating cellular function. An imbalance in the uPA/uPAR system leads to disorders in tissue structure and function. This book chapter summarizes recent progress in understanding the role and mechanisms of the uPA/uPAR system in the initiation and progression of atherosclerosis.

Keywords Urokinase • Monocytes/macrophages • Smooth muscle cells • Foam cells • Vascular remodeling • Atherogenesis

1 Introduction

Atherosclerosis, the leading cause of death in industrialized countries, is a progressive inflammatory disease [1]. Atherogenesis refers to the development of atheromatous plaques in the inner lining of the arteries [2]. Early atherosclerosis is characterized by the attachment of monocytes to the endothelium of the blood vessel and their infiltration into the subendothelial space in the intima of the vessel.

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In the intima, the monocytes differentiate into macrophages [3]. Mature macrophages accumulate lipids and cholesterol due to the uptake of oxidized low-density lipoprotein (Ox-LDL), thereby developing into foam cells, a process which further contributes to plaque formation and growth [4]. Macrophages in the atheroma may have proinflammatory characteristics of M1 macrophages, which produce high levels of proinflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor (TNFα), or M2 macrophages, which are more prominent producers of anti-inflammatory cytokines IL-1RA, IL-10, and transforming growth factor-β (TGFβ) [5]. Eventually, macrophage-derived foam cells undergo apoptosis. In early atherosclerotic lesions, apoptotic macrophages are rapidly cleared by efferocytosis, resulting in suppression of the proinflammatory response [6]. Overall, these processes result in a reduction in lesion cellularity and size.

Lesion progression involves the recruitment of smooth muscle cells (SMC) from the media into the intima, where they proliferate and form the neointima in a process called "remodeling" and in later stages convert to foam cells [7]. Moreover, SMC produce extracellular matrix (ECM) molecules, which can trap and retain LDL within the artery wall [8]. When bound, LDL can undergo oxidative modification, enhancing thus lipid uptake, foam cell formation, and lesion progression. The ECM can form a fibrous cap that covers and stabilizes the plaque.

In advanced lesions, due to the increased number of macrophages in the atheroma, efferocytosis becomes inefficient, causing secondary necrosis of the cells. This causes the release of lipids and other cell components from the dead cells, which accumulate extracellularly, causing an overkill of debris to be cleared. The buildup of necrotic debris promotes inflammation. Furthermore, there is the ongoing persistent influx of monocytes into the intima, which contribute to increased lesion cellularity. All these processes lead to the formation of a necrotic core, which causes thinning of the fibrous cap. The decrease of SMC, which produce collagen, or the stimulation of metalloproteinases (MMP) release from apoptotic macrophages further destroys the fibrous cap [9] eventually leading to plaque rupture. The ruptured plaque triggers thrombus formation causing lumenal thrombosis and arterial occlusion.

Urokinase-type plasminogen activator (uPA) is an enzyme belonging to the serine proteases family. It resides in the extracellular space and by binding to its receptor, the uPAR, it gets activated mediating plasmin generation [10], thus playing a pivotal role in plasmin-mediated pericellular proteolysis. During the past decades, several other roles of the uPA/uPAR system have emerged that extend beyond its role in fibrinolysis [11], such as cell migration, differentiation, proliferation, apoptosis, and matrix degradation through activation of intracellular signaling [12]. uPA is the crucial protein for neointimal growth and vascular remodeling [13]. The uPA/uPAR system play a role in the pathogenesis of atherosclerosis [14]. There is increasing evidence that cells in the atherosclerotic arterial wall, including macrophages, endothelial cells, and SMC, secrete high levels of uPA in the advanced stages of atherogenesis [15] and express uPAR on the surface [16, 17]. Elevated expression of uPA especially in atherogenic macrophages leads to accelerated atherosclerosis [18–20]. Plasma levels of uPA were elevated in patients with unstable

angina and correlated positively with plaque plus media and external elastic membrane areas, as determined by intravascular ultrasound, suggesting that the urokinase system is associated with signs of plaque instability [21]. On the contrary, another study [22] has shown that uPA is essential for maintaining cellularity and collagen content thus stabilizing the plaque, and lack of uPA increased neointimal size and luminal stenosis but reduced media thickness and the cellularity of lesions in a double knockout apolipoprotein $E^{-/-}/uPA^{-/-}$ mice. Therefore, up to date there is no consensus on the contribution of membrane-bound uPA and uPAR to the development of atherosclerosis.

Generation of transgenic mice with macrophage-targeted overexpression of uPA by Dichek et al. enabled the researchers to establish that uPA has atherogenic activity when it is expressed at elevated levels by macrophages [18]. The level of uPA expression by macrophages was shown to be an important determinant of atherosclerotic lesion growth in the atherosclerotic apolipoprotein E-deficient mice [20]. The atherogenic effects of macrophage-expressed uPA in the atherosclerotic apolipoprotein E-deficient mice were mediated via plasminogen [19]. Moreover, macrophage uPA overexpression caused intraplaque hemorrhage and fibrous cap disruption, features associated with atherosclerotic plaque rupture, via plasminogen activation-dependent activation of MMP(s) other than MMP-9 [23]. Results from macrophage microarray suggested that macrophage uPA overexpression affects cell migration and cell–matrix interactions.

These recent studies in humans and in animal models have suggested that uPA might be involved in the development of atherosclerosis. However, the exact roles of the uPA/uPAR system in atherogenesis are not fully elucidated yet. This book chapter summarizes recent advances on the impact of the uPA/uPAR system in the initiation and progression of atherosclerosis.

2 Structure and Function of the uPA/uPAR System

The uPA/uPAR system is a very important component of the fibrinolytic system. It is composed of the 55 kDa serine protease urokinase-type plasminogen activator (uPA), its cell membrane-associated receptor uPAR, (also defined as CD87), the substrate plasminogen, the serpins plasminogen activator inhibitor 1 (PAI-1), and the plasminogen activator inhibitor 2 (PAI-2) [24]. uPA is produced by diverse cell types, including vascular endothelial cells, SMC, monocytes and macrophages, fibroblasts, epithelial cells, and by malignant tumor cells [25]. It gets secreted as an inactive single-chain 411 amino acids zymogen form (pro-uPA), which consists of three domains: a serine protease domain, a kringle domain, and a growth factor-like domain. Pro-uPA undergoes several posttranslational modifications, such as glycosylation, phosphorylation, and fucosylation. These modifications contribute to the catalytic independent chemotactic and mitogenic activities of uPA. Proteolytic cleavage by plasmin, glandular kallikrein mGK6 or cathepsin B, generates a two-chain molecule held together by a single disulfide bond forming the active enzyme

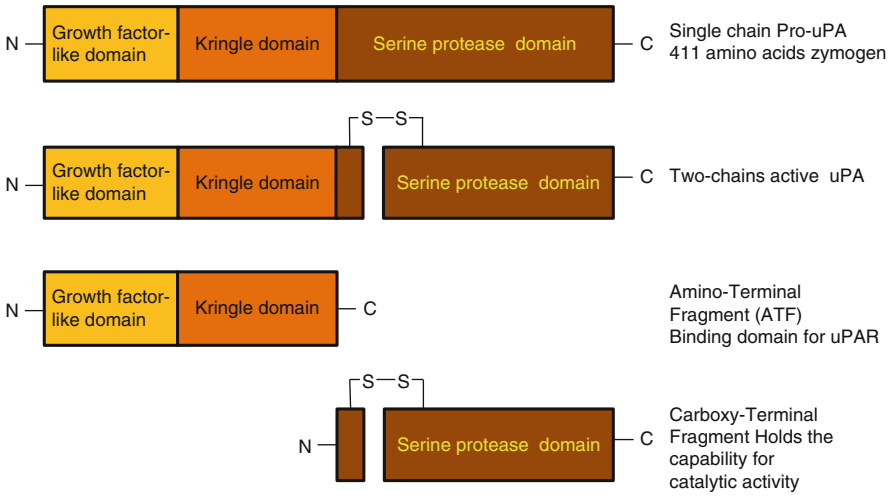

Fig. 14.1 Schematic representation of the structure of uPA. Pro-uPA consists of three domains, a growth factor-like domain, a kringle domain, and a serine protease domain. Catalytic cleavage produces a two-chain active uPA bound by a disulfide bridge. Further cleavage forms the amino-terminal fragment consisting of the growth factor-like domain and the kringle domain, sufficient for binding to the uPA receptor (uPAR). The remaining C-terminal fragment making up the serine protease domain holding the catalytic activity of uPA

uPA. Further cleavage releases the amino-terminal fragment domain (ATF), which comprises the receptor-binding domain consisting of the growth factor-like domain and the kringle domain, which stabilizes the interaction and is sufficient to trigger intracellular signaling through its receptor uPAR. The remaining carboxy-terminal fragment consisting of the serine protease domain holds full capability to transform plasminogen to the active enzyme plasmin independent from receptor binding (Fig. 14.1). Generation of processed uPA from pro-uPA occurs either in the extracellular space but more efficiently when bound to its receptor uPAR.

The uPAR is a highly glycosylated 55–60 kDa single-chain polypeptide bound to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. It lacks a transmembrane domain, which enables high mobility in the plasma membrane, and its location depends on the functional state of the cell. Dimerization of uPAR leads to reclustering of the receptor in lipid rafts and was firstly described to occur in neutrophils [26, 27]. uPAR can bind both the single-chain pro-uPA or the processed two-chains uPA with high affinity. Receptor-bound uPA activates plasminogen more efficiently than the fluid phase enzyme. uPAR is a multifunctional receptor, which not only binds uPA but also interacts with members of the family of Receptor Tyrosine Kinases (RTKs), G-protein-coupled receptors (GPCRs), and integrins [28], thus facilitating cell–matrix interactions or mediating cellular responses, such as cell adhesion, migration, cell signaling, proliferation, and apoptosis, via activating intracellular signaling pathways [29, 30]. The extracellular part of the uPAR can get cleaved and shedded from the cell surface to body fluids, including plasma and

urine. Soluble uPAR (suPAR) has similar extracellular functions as the cell bound receptor. Soluble uPAR is increased in symptomatic carotid and plasma, thereby serving as a marker for the severity of atheroma progression [16].

2.1 Plasmin-Dependent and Plasmin-Independent Effects of uPA

uPA was first described as a serine protease of the fibrinolytic system, thus involved in clot lysis and extracellular matrix degradation. Its main function in proteolysis is the catalysis of the plasminogen conversion to plasmin, which subsequently mediates most of the uPA functions connected to proteolysis in the fibrinolytic system. This includes the downregulation of the local concentration of fibrin, fibronectin, laminin, vitronectin, and proteoglycans [31]. Moreover, the cleavage of inactive metalloproteases leads to the degradation of collagen, which further contributes to extracellular matrix degradation [25]. As a result of ECM degradation, growth factors are released, including basic Fibroblast Growth Factor (bFGF), Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), or Transforming Growth Factor-β (TGF-β). Thus, the role of plasmin extends beyond its function in fibrinolysis and extracellular matrix degeneration to cell proliferation, differentiation, and tissue regeneration. Additionally, uPA can directly activate fibronectin [32], the scatter factor pro-hepatocyte growth factor (HGF) [33], and the macrophage stimulating protein (MSP) [34], which contain cleaving sites that are homologous to plasminogen. Thus, not only plasmin but also uPA can directly control cell proliferation, ECM invasion, and apoptosis inhibition. Many cell signaling effects mediated by uPAR are plasmin independent. Since uPAR lacks an intracellular domain, intracellular signal transduction requires the interaction of uPAR with transmembrane coreceptors. Up to date, 42 proteins were determined to directly interact with uPAR, nine of which are soluble ligands and 33 coreceptors. Interaction of uPAR with the uPAR-associated protein (uPARAP/ENDO180) is implicated in extracellular matrix rearrangements [35] or assisting in chemotaxis under normal and pathological conditions [36]. Cross talk between platelet-derived growth factor receptor-β (PDGFR-β) and uPAR in human vascular SMC leads to PDGF-independent PDGFR-β activation [37]. The activity of the chemokine receptor CXCR4 is regulated by the interaction of uPAR with fMLP receptors (fMLP-Rs) [38]. Transactivation of the epidermal growth factor receptor by uPAR was shown to involve Src kinases and metalloproteases, which contribute to proliferation and enhanced invasiveness of breast cancer cells [39]. Complexes formed with the LR11, a member of the LDL receptor family, lead to enhanced migration of smooth muscle cells of the hyperplastic intima [40]. Signaling impinged by the interaction of uPAR with the integrin MAC1 (αMβ2 integrin) was shown to contribute to the adhesion of macrophages and neutrophils to endothelial cells [41].

Figure 14.2 summarizes both plasmin-dependent and plasmin-independent signaling initiated by the uPA/uPAR system.

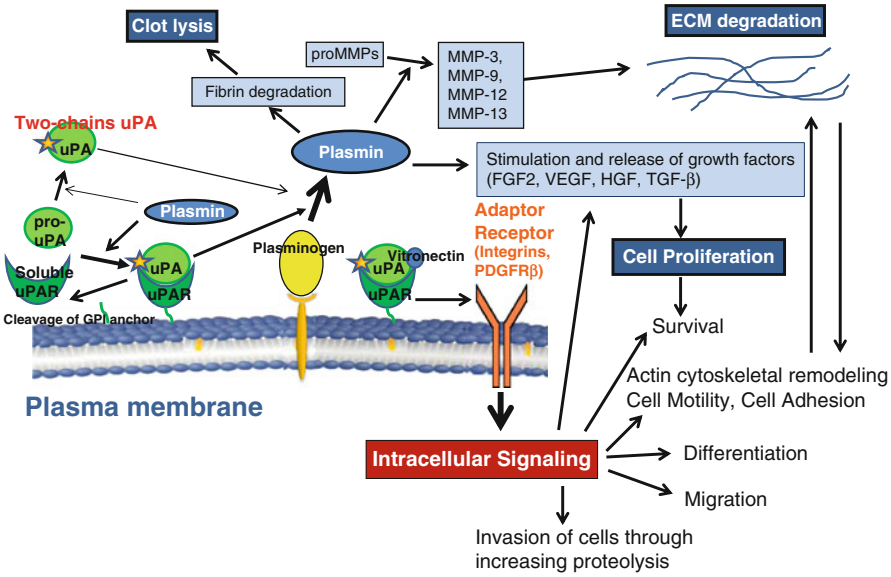

Fig. 14.2 Plasmin-dependent and plasmin-independent effects of uPA. uPA catalyzes the formation of plasmin from plasminogen. Plasmin in turn can activate free or receptor-bound uPA. uPA can also get activated by kallikrein or cathepsin B. Plasmin is involved in the degradation of fibrinogen which leads to clot lysis. It also activates metalloproteinases which are responsible for extracellular matrix degradation. This leads to the stimulation and release of growth factors resulting in advanced cell proliferation and survival. uPAR can also bind to other ligands such as vitronectin or transmembrane receptor molecules, such as integrins or PDGF-b leading to the initiation of intracellular signaling cascades involved in differentiation, cell migration, proliferation, actin remodeling influencing matrix rearrangements, and cell invasion

3 The uPA/uPAR System in the Early Atheroma

Innate immune responses due to subendothelial accumulation of lipoproteins activate the endothelium to secrete chemokines and to express adhesion molecules that attract and bind monocytes [42]. uPAR can facilitate monocyte adhesion by binding to vitronectin [43] and by forming complexes with complement receptor 3 (CR3) (CD11b/CD18), a β2 integrin [44]. Recently, it was shown that uPAR expression regulates monocyte migration and is associated with accelerated atherosclerosis in apoE$^{-/-}$ mice on high-fat diet [17]. This is in accordance with another study showing that uPAR expression is elevated on monocytes from patients with acute myocardial infarction (AMI) [45].

After adhesion of monocytes to the endothelium infiltration of them into the intima of the vessel comprises the next step in atherogenesis. Intravasation of monocytes through the endothelium requires the action of extracellular proteases and remodeling of the extracellular matrix and of the cell–cell contacts in the endothelium. A recent study on the role of uPAR on macrophage infiltration into the

vascular wall was examined in stable murine macrophage (Raw264.7) cell lines expressing high levels of human uPAR, uPA, or both. The human uPAR expressing Raw264.7 cells showed increased adhesion to both human uPA and vitronectin. Raw264.7 cells expressing human uPAR or both human uPAR and uPA, but not uPA alone, were detected in the aortic wall of apoE$^{-/-}$ mice, and no cells were detected in that of age-matched C57BL/6J mice after intravenous infusion of the cells, thus demonstrating that uPAR plays a key role in promoting macrophage infiltration into the arterial wall of apoE$^{-/-}$ mice [46].

Monocyte-to-macrophage differentiation has physiological as well as pathological consequences. The uPA/uPAR system plays a role in monocyte-to-macrophage differentiation. uPA was shown to be necessary and sufficient for the formation of the monocyte/macrophage maturation promoting fragment, mactinin, in vitro and in vivo [47]. uPA could also enhance PMA-induced differentiation of THP-1 monocytes [48]. Further evidence for the implication of uPA/uPAR in monocyte proliferation and differentiation stems from a study, which demonstrated that the plasminogen activator inhibitor type 2 (PAI-2) interferes with an uPA/uPAR-dependent adhesion, proliferation, and differentiation of monocytes [49].

Progression of the development of the early atheroma involves the uptake of modified lipoproteins such as oxidized-LDL (Ox-LDL) and the accumulation inside of the matured macrophages. This leads to foam cell formation which culminates eventually in stress of the endoplasmic reticulum. This increases oxidative stress within the macrophage by the production of reactive oxygen species (ROS) [50]. ROS induces macrophage lipid peroxidation and, subsequently, increases macrophage atherogenicity. These "oxidized macrophages" exhibit an increased capability to oxidize LDL [51] and to accumulate cholesterol [52, 53] which leads to foam cell formation, the hallmark of early atherogenesis. We have shown that monocyte-to-macrophage differentiation both in vitro and in vivo during maturation of mouse peritoneal macrophages is characterized by an increase in cellular oxidative stress [54, 55] followed by an oxidative stress response, as shown by an increased expression of the antioxidant cellular enzyme paraoxonase 2 (PON2) [56]. We have studied the effect of uPA on macrophage oxidative stress and lipid accumulation. Our recent results demonstrated that uPA binding to uPAR increased intracellular oxidative stress in macrophages as a result of NADPH oxidase activation [57]. In response to the increased cellular oxidative stress, uPA upregulated PON2 expression in macrophages via the PI3K/ROS/MEK/SREBP-2 signaling cascade mediated by the PDGFR-β [58]. Furthermore, we could show that uPA downregulates PON1 expression and secretion from hepatocytes, the major source of PON1 generation, by regulating PPARγ activity. PON1 is a major repressor of oxidative stress in the atheroma, thus downregulation of PON1 by uPA can furthermore contribute to oxidative stress [59].

Increased uptake of cholesterol and modified lipoproteins, like oxidized-LDL (Ox-LDL), by macrophages leads to foam cell formation. Monocytes isolated from human blood took up oxidized-LDL (Ox-LDL) at a gradual increasing rate during their differentiation into macrophages, and this process was significantly enhanced in hypercholesterolemic patients [60]. Activation of macrophage uPAR by the soluble form of the LR11, a member of the low-density lipoprotein receptor secreted

from intimal SMC, stimulated lipid uptake via the scavenger receptors and lipid accumulation in macrophages towards foam cell formation [61]. We have recently shown that uPA enhanced macrophage atherogenicity by increasing cellular cholesterol accumulation. This effect was mainly due to the accumulation of unesterified cholesterol. Upon incubation of macrophages with uPA, there was a significant increase in macrophage unesterified cholesterol content, resulting from an increase in cellular cholesterol biosynthesis. This effect was mediated via uPA-induced activation of the transcription factor SREBP-1 through PI3K and MEK signal transduction pathways. Furthermore, we have demonstrated that uPA-induced increase in cholesterol biosynthesis required binding of uPA to uPAR but was independent of its proteolytic activity [62].

Infiltrated monocytes into the intima of the vessel can differentiate into two types of macrophages, to M1 macrophages that promote inflammation or to M2 macrophages that promote inflammation resolution. Thus, at later stages of atherosclerosis an imbalance of the ratio of M1 macrophages and M2 macrophages leads to impaired resolution of the inflammation. Furthermore, the impaired egress of macrophages and the constant influx of monocytes lead to increased lesion cellularity and to the progression of the growth of the plaque [63]. Soluble uPAR was shown to be associated with inflammation in the vulnerable human atherosclerotic plaque [16].

In the atheroma some macrophage-derived foam cells die by apoptosis and are engulfed by phagocytes in a process called efferocytosis. In early stages of atherogenesis apoptosis of macrophages is beneficial due to efficient efferocytosis, preventing formation of post-apoptotic necrosis and fewer proinflammatory cells [42]. This results in the reduction of the lesion cellularity and size.

4 The uPA/uPAR System in the Advanced Atheroma

Atheroma formation involves vascular remodeling. That includes the migration of smooth muscle cells from the media into the intima, their proliferation in response to mediators, such as platelet-derived growth factor, and the production of extracellular matrix (ECM) molecules [8]. ECM can form a fibrous cap that covers and stabilizes the plaque. uPA and uPAR-dependent cell adhesion to the extracellular matrix is an important event in vascular remodeling. uPA exert effects on adhesion, migration, and proliferation of vascular smooth muscle cells (VSMCs) by mechanisms involving both proteolysis and interaction with uPAR and requires the involvement of membrane rafts [64]. In a series of studies carried out by Dumler et al., intracellular signaling processes elicited by uPA in VSMC that culminate in VSMC migration and proliferation were elucidated. The protein kinases activated downstream of uPAR are cell type specific and have been identified as Src, MAPKs, ERK1/2, p38, AKT, PI3-K, the Janus kinases Jak1 and Tyk2, small GTPases and the Rho family, focal adhesion kinase (FAK), and SHP-2 [65–70].

At early stages of the remodeling process, VSMCs have a high migratory potential in the absence of proliferation. Interactions of VSMC with monocytes recruited

at the arterial wall at injured sites resulting in VSMC growth and migration are central to the development of vascular intimal thickening. uPA expressed by monocytes was shown to be a potent chemotactic factor for VSMC in a co-culture model, suggesting that monocyte uPA acts as endogenous activator of VSMC migration, contributing and accelerating vascular remodeling [66]. SMC migration in response to uPA stimulation was shown to be dependent on the kringle domain of uPA and required uPA-activation of p38 MAP-kinase and downstream phosphorylation of non-muscle caldesmon [67] and required the association of uPA with uPAR [68].

Proliferation of migrated VSMCs further contributes to the stabilization of the atherosclerotic plaque. Local increase in uPA in distended injured arteries augmented SMC migration and proliferation leading to thickness of the carotid artery intima and media and early neointima formation, and these effects were attributed to the proteolytic activity of uPA [69]. uPA induced SMC proliferation through ERK1/2 and p38 MAPK-mediated pathways, whereas uPA-induced SMC migration appears to be dependent on ERK1/2 activity alone. Both of these actions are orchestrated via activation of the EGFR [70]. uPA was found to stimulate reactive oxygen species generation in VSMCs via regulation and activation of cellular oxidases, and this effect is essential for uPA-mediated VSMCs proliferation [71, 72]. Furthermore, migrating and proliferating smooth muscle cells (SMCs) along with extracellular matrix proteins form a fibrous cap around the growing plaque resulting in stabilization of the plaque.

At later stages of atherogenesis, macrophage cell death is induced by growth factor deprivation, the presence of cytokines, and oxidized lipids or lipoproteins [73]. This was mainly shown in vitro, but there is an increasing consent that cholesterol accumulation in the foam cells causes stress originating in the endoplasmic reticulum (ER) culminating in Ca^{2+} release from the membranes of the ER and the activation of the C/EBP homologous protein (CHOP), a protein from the Unfolded Protein Response (UPR) [74]. At these later stages of atherogenesis, efferocytosis functions only inefficiently for reasons not yet fully clarified. One reason could be that phagocytes simply become overwhelmed by the high number of dying macrophages and a persistent influx of monocytes from the bloodstream. Another reason might be that advanced lesional macrophage death limits the number of phagocytes or that they are poor substrates for phagocytes [42]. This leads to secondary necrosis promoting the release of lipids from the dead cells that accumulate extracellularly, forming the necrotic core of the plaque.

The involvement of uPA in apoptosis of macrophages is not yet elucidated. We could show recently, that the MM6 mature macrophage cells are prevented from Ox-LDL-induced apoptosis by uPA, by downregulating Bim activation, a direct target protein of CHOP, in an ERK1/2-dependent manner [48]. At these later stages of atherosclerosis, increased apoptosis of macrophages in the plaque leads to plaque rupture, and the lack of insufficient clearance of apoptotic cells by efferocytosis leads to necrotic cell death, which finally leads to plaque instability and plaque rupture.

Plaque rupture includes apoptosis of endothelial cells and of SMC covering the plaque [75, 76], rupture of plaque microvasculature, or other factors that alter plaque

composition or cause thrombosis [77]. Uncleared apoptotic macrophages not only release lipids but also shed plasma membrane microparticles, which can stimulate thrombosis [78]. Additionally, increased proteolytic activity of the plaque leads to extracellular matrix destruction, which further promotes SMC apoptosis [79]. Matrix metalloproteinases (MMP) are the major contributors to proteolytic activity [80]. Macrophage expression of active MMP-9 induced acute plaque disruption in apoE$^{-/-}$ mice, thus linking uPA/uPAR to plaque rupture [81]. Another study in aged apoE$^{-/-}$ mice revealed that overexpression of uPA by plaque macrophages caused increased vascular matrix metalloproteinase activity, and histological features of plaque rupture could be observed [23]. In contrast, an earlier study showed that the lack of uPA promoted atheroma progression and plaque vulnerability in apoE$^{-/-}$ mice [22].

A positive correlation was observed between macrophage infiltration and plaque vulnerability in recently symptomatic carotid atherosclerotic plaques. uPAR was found to be highly expressed in monocyte-derived macrophages and in symptomatic carotid atherosclerotic plaques and co-localized with macrophages predominantly in ruptured plaque segments thus supporting a role for uPAR in plaque rupture [82].

While in vivo studies rather revealed a positive influence of uPA on plaque rupture, in vitro studies on endothelial and vascular smooth muscle cells showed opposing results. One study showed that uPA promoted endothelial cell survival via induction of the X-linked inhibitor of apoptosis protein (XIAP). This function was dependent on the protease activity of uPA and the presence of uPAR [83]. Another ex vivo study with vascular smooth muscle cells derived from PAI-1$^{-/-}$, t-PA$^{-/-}$, or uPA$^{-/-}$ mice showed a correlation between plasmin generation and apoptosis induction in these cells. Plasmin-induced apoptosis was greater in PAI-1$^{-/-}$ mice compared to t-PA$^{-/-}$ or uPA$^{-/-}$ mice stating that plasmin-induced apoptosis of vascular smooth muscle cells in mediated by t-PA or uPA and inhibited by PAI-1, confirming an atherogenic effect of uPA [84].

5 Therapeutic Approaches

Since there is increasing evidence that the uPA/uPAR system is associated with increased atherogenicity, impairment of uPA and/or uPAR functions, or inhibition of their expression may provide a novel therapeutic approach to attenuate atherosclerosis. Targeting uPAR with the aim of disrupting its interaction with uPA in cardiovascular diseases, similarly to its application in cancer disorders [85], might be considered as a possible therapeutic approach in the management of advanced atherosclerosis in order to prevent plaque rupture. In an attempt to inhibit neointima formation, a novel hybrid protein consisting of the tissue inhibitor of metalloproteinase-1 (TIMP-1) linked to the receptor-binding ATF was constructed (TIMP-1. ATF). This hybrid will bind to the uPAR and prevent uPA binding and subsequently inhibit local activation of plasminogen, as well as uPA-mediated intracellular signaling activation. Expression of TIMP-1.ATF by using adenoviral-mediated gene

transfer [86, 87] or a non-viral expression vector [88] reduced neointima formation by inhibiting SMC migration.

Other approaches include the administration of statins. Cerivastatin, an inhibitor of HMG-CoA reductase, inhibited uPA/uPAR expression and MMP-9 secretion by peripheral blood monocytes, which could be an antithrombotic mechanism [89]. Other statins, such as simvastatin and atorvastatin attenuated VCAM-1 and uPAR expression on human endothelial cells [90] further proving an atheroprotective effect of statins.

6 Conclusions

There is increasing evidence that the uPA/uPAR system is involved in the pathogenesis of atherosclerosis. The emphasis on this book chapter was to elucidate the role of the uPA/uPAR for the initiation and progression of atherogenesis. uPA has two well-described physiologic roles: activation of plasminogen and binding to uPAR. Research in this area has provided mechanistic insight into how uPA interaction with uPAR can induce a local proteolytic cascade or cellular signaling activation that are of great importance for both vascular remodeling, macrophage-foam cell formation, and plaque stability. However, as summarized herein, many of the studies use a single-mechanistic approach in a specific model, and thus progression to more pathophysiologically relevant in vitro and animal models is essential. Implication of uPA in macrophage-foam cell formation and SMC migration, proliferation, and vascular remodeling suggest a causal relationship between uPA and vulnerable plaque formation. Understanding the mechanisms responsible for these molecular processes is of fundamental importance for developing novel therapeutic avenues in treatment of atherosclerosis and cardiovascular disorders.

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Chapter 15
Matrix Metalloproteinases and Hypertension

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Abstract The matrix metalloproteinases (MMPs) are known for quite some time to play essential roles in this remodeling process of arteries found in hypertension, as will be discussed here. More recently, evidence indicates that some of them may not only influence hypertension due to their effect in remodeling but possibly by direct mechanisms such as the proteolytic change of vasoactive mediators. The most studied MMP in animal models of hypertension is MMP-2, and many such studies are described here. After a brief description of MMPs, we discuss data on MMPs in hypertension in three sections: findings on arteries, findings on the heart, and on the final section we then briefly discuss some other issues that may indicate that MMPs are related to hypertension in other yet unexplored ways. In this review we also highlight data showing the protective effects of MMP inhibition in arteries, heart, and kidney. MMP inhibition appears to be a relevant target for newer pharmacological intervention in hypertension. Doxycycline is the MMP inhibitor most widely used in cell cultures, animal studies on hypertension, and is a drug approved for commercial use for periodontal disease in the United States. The refinement of MMP assays and the specific delivery of different MMPs may highlight the specific role of each MMP in the different types of hypertension, enabling the use of a rational

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approach for a more specific inhibition of them in hypertension, so that target organs are more protected.

Keywords Metalloproteinases • MMPs • MMP-2 • Hypertension • Doxycycline • Vascular remodeling • Vascular dysfunction • Arteries • Heart • Kidney • Ischemia/reperfusion

1 Introduction

According to the World Health Organization, one in every three adults worldwide has raised blood pressure (BP). High and sustained levels of BP characterize hypertension, a disease that leads to functional and structural changes of target organs (heart, kidney, and blood vessels) [1]. In hypertension we have changes in the mechanisms of BP control [2, 3], increased oxidative stress [4, 5], and in the extracellular matrix [6]. Extracellular matrix changes result from adaptive responses of the body to face increased blood pressure. Some of these responses include cardiac remodeling [7], hypertrophic vascular remodeling that occurs in conductance arteries, and eutrophic remodeling that occurs in resistance arteries [8–10]. The matrix metalloproteinases (MMPs) are calcium- and zinc-dependent proteinases that count as one of the most important classes of proteinases of mammalians, since they are essential for development, remodeling processes, and cell migration. Altogether 24 MMPs have been identified in humans [11].

Traditionally, the MMPs have been subdivided into collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs, according to their substrate specificity, primary structure, and cellular location [12]. There are three collagenases identified so far: collagenase 1 (MMP-1), collagenase 2 (MMP-8), and collagenase 3 (MMP-13). The group of gelatinases is composed by gelatinases A (MMP-2) and B (MMP-9). MMP-3, MMP-10, and MMP-11 are also known as stromelysins 1, 2, and 3, respectively. MMP-7 and MMP-26 belong to the matrilysins group and are the smallest MMPs. There are two types of membrane-type (MT) MMPs, which include four type I transmembrane proteins (MMP-14, MMP-15, MMP-16, and MMP-24) and two glycosylphosphatidylinositol-anchored proteins (MMP-17 and MMP-25). There are seven other MMPs which have not been catalogued in the above subgroups: MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, and MMP-28. MMPs are multidomain proteins. Their domain structure contains a signal peptide, a pro-peptide, a catalytic domain, a hinge region, and, in the majority of cases, a C-terminal domain [12]. The activation of MMPs requires the dissociation of the interaction between the cysteine residue of the pro-peptide domain and the zinc ion of catalytic domain. This cysteine "switch," as it has been called, must be opened for MMP activation [13].

Many agents have been shown to produce the activation, including detergents (sodium dodecyl sulfate, for instance) and chaotropic agents, chemicals (like HOCl and mercurial compounds), and enzymes (trypsin, plasmin). Physical agents can

open up the structure of the MMP and expose the zinc ion. Reagents that react with sulfhydryl groups (NEM, N-Ethylmaleimide; GSSG, glutathione oxidized; HOCl; APMA, amino-phenyl mercuric acetate) will act on two steps: first activating the enzyme (by interacting with the cysteine of the pro-peptide), and thereafter the activation of the enzyme will mostly result in autolysis of the enzyme and its activation. Proteolytic enzymes (trypsin, plasmin) can cleave the pro-peptide (which does not affect the cysteine of the pro-peptide). In a second step, these activated molecules will catalyze their autolysis, and this way the activated MMP will have their pro-peptide permanently removed, being in this way permanently activated [14].

Another possible form of activation involves the action of oxidizing agents, as has been shown in vitro. It has been well characterized for MMP-2 that peroxynitrite (ONOO–) causes the activation of pro-MMP-2 without removing of the pro-peptide, keeping the same molecular weight of the 72 kDa inactive form of MMP-2 [15]. Oxidative stress (ONOO–) and cellular glutathione (GSH) react with the cysteine residue in the conserved domain causing its S-glutathiolation and disrupting its thiolate bond with the catalytic Zn2+ ion, resulting in an active 72 kDa (full-length) MMP-2 [16]. The activation by glutathiolation has also been shown for MMP-1, MMP-8, and MMP-9 [17].

The regulation of MMP activity occurs by tissue inhibitors of metalloproteinases (TIMPs), which are important to prevent excessive degradation of extracellular matrix. Four TIMPs are described: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 [18]. There is a balance between MMPs and TIMPs as a mechanism to prevent excessive degradation of the matrix [19]. In addition to the TIMPs, MMPs are also inhibited by alpha 2-macroglobulin [14].

2 MMP Activity and Vascular Remodeling and Dysfunction

The MMPs, in general, are mostly known as major contributors of the extracellular matrix turnover and tissue remodeling [20]. Increased levels and activity of MMP-2 and MMP-9 were consistently observed in plasma of hypertensive patients and cardiac and vascular tissues of various animal models of hypertension [7, 21–30]. MMP-2 is indeed essentially involved in the long-lasting hypertension-induced maladaptive cardiovascular changes as it contributes to the excessive migration, proliferation, and hypertrophy of cardiac myocytes and vascular smooth muscle cells (VSMCs) to remodel the cardiovascular apparatus [30–33].

In the vasculature, in particular, increased MMP-2 activity may contribute to hypertension-induced increased wall thickness in the conductance arteries (hypertrophic remodeling) [25–27] and reduced lumen diameter, without alterations in the media width, in the resistance arteries (eutrophic remodeling) [34], both resulting in increased media thickness/lumen (M/L) ratio. This latter parameter was shown to be an important marker to predict several hypertension-induced cardiovascular complications. Furthermore, MMP-2 seems to be potentially involved in the maladaptive resynthesis of collagen and elastin in the conductance arterial wall as a result of the

excessive degradation of these extracellular matrix components [25, 26, 35–38]. In fact, treatment of 8 weeks 2K-1C rats with doxycycline significantly diminished hypertension-induced increased collagen and elastin deposition in aortic wall and increased media thickness and MMP-2 activity [25]. Increases in MMP-2 and these arterial alterations are observed at an early phase of renovascular hypertension (2 weeks) and then increase with time (4, 6, and 10 weeks) [36]. Mechanistically, MMP-induced excessive extracellular matrix degradation may contribute to the VSMCs phenotype switch from contractile to synthetic, allowing the cells to migrate, proliferate, and produce new extracellular matrix components in the vascular wall [32, 39–42]. MMP-2 may also trigger the integrin receptors-mediated hypertrophic signaling in VSMCs, thus contributing to hypertension-induced arterial hypertrophy and remodeling [37, 43–45]. Using immunohistochemistry in aortas, our group found that MMP-2 localized particularly to the 2K-1C medial hypertrophied VSMCs and that localization may contribute to its effect as a hypertrophic mediator [26]. As increased MMP-2 activity was also observed near the aortic internal elastic lamina, it might cause fragmentation of fibers and consequently VSMCs migration [46, 47] during hypertension. Doxycycline significantly reduced 2K-1C-hypertension-induced increases in MMP-2 activity in the hypertrophied VSMCs and aortic internal elastic laminas [26]. Furthermore, the levels of MMP-9 and MMP-14 were also increased in the aortas of 2K-1C rats, suggesting that they may also contribute to hypertension-induced arterial hypertrophy [26, 36]. Although MMP-14 is best known to activate MMP-2 through its interaction with TIMP-2, it also regulates VSMCs hypertrophy, migration, and proliferation in animal models of vascular injury [48]. MMP-14 was also localized around the medial hypertrophied VSMCs in the 2K-1C aortas [26], and this localization may contribute to hypertension-induced activation of a hypertrophic and migratory signaling pathway. The increased level and activity of MMP-9 were mostly found in the aortic intima of 2K-1C rat aortas [26], as well as in DOCA-salt hypertensive rat aortas [49]. Due to this restricted localization, MMP-9 levels were determined to be roughly half the MMP-2 levels [26, 49]. However, its presence in the aortic intima layer suggests that it may play an important role in facilitating hypertension-induced neointima formation and endothelial dysfunction [31, 32]. Doxycycline completely prevented those MMP-induced structural changes in the aortas of 2K-1C rats [26].

For the resistance arteries, a recent study performed in cremasteric arterioles exposed to norepinephrine and angiotensin II in vitro showed that increased MMP-2 activity does contribute to the observed hypertension-induced inward eutrophic remodeling, as treatment with the MMP inhibitor GM6001 completely inhibited this change [34]. The inward eutrophic remodeling was recognized by a reduction in the maximal arteriolar passive diameter after exposure to the vasoconstrictor agonists. As increased MMP-2 activity cleaves several proteins of the extracellular matrix, it may lead to the rearrangement of the VSMCs around the media and intima layers, thus contributing to hypertension-induced reduced lumen diameter without alterations in the media width [37]. This remodeling differs from the hypertrophic remodeling as the small arteries have an intrinsic constriction and they are not ordinarily submitted

to the increased vascular wall stress to which large arteries are submitted in hypertension. There are excellent articles in the literature which explain with details the differences between the hypertrophic and eutrophic remodeling [10, 27, 37, 50].

On the other hand, a study using the L-NAME model of hypertension showed that increased MMP-2 activity does participate more in hypertension-induced hypertrophic vascular remodeling than the inward eutrophic remodeling [27]. Although treatment with doxycycline significantly decreased MMP-2 levels and activity in both large and small arteries of L-NAME-treated rats, it prevented only the aortic hypertrophic remodeling [27]. Differences in models of hypertension (in vivo and in vitro), exposure time to the hypertensive stimulus, and MMP inhibitors may significantly contribute to such discrepancies between studies.

The maladaptive changes in the arterial wall mediated by increased MMP activity may lead to significant vascular dysfunction in hypertension. In fact, MMP-mediated excessive proteolysis of collagen and elastin as well as their resynthesis and rearrangement in the vascular wall may cause arterial stiffness, which is associated with arterial rigidity. This, in turn, results in elevated pulse wave velocity (PWV) and pulse pressure as well as increased systolic blood pressure and left ventricular hypertrophy [39, 51]. In addition to collagen (type I and II), MMP also stimulates the resynthesis of fibronectin and proteoglycans, which are all proteins that increase the stiffness of the matrix [52, 53]. Furthermore, MMP-induced excessive proteolysis of arterial elastin in hypertension results in a significant transference of the mechanical load to collagen [54]. Interestingly, the remaining non-degraded and also the resynthesized elastin may be less efficient and stiffer, and thus also contribute to arterial stiffness and fibrosis [54]. A study using a rat model of elastocalcinosis, induced by warfarin and vitamin K, showed that increased MMP-9 activity may mediate arterial stiffness-induced hypertension by causing increased elastin proteolysis, activation of the transforming growth factor β (TGF-β), and calcium deposition in the aortic media [55]. As a result of elastin degradation, soluble elastin peptides may be formed and with TGF-β may stimulate VSMC migration, proliferation and an osteogenic phenotype [55]. Doxycycline significantly reduced MMP-9-induced increase in aortic stiffness, PWV, and calcification [55]. A clinical study performed in patients undergoing isolated systolic hypertension showed that increased plasma levels of MMP-9 were positively associated with aortic and brachial PWV, which suggests that MMP-9 is a mediator of arterial stiffness-induced hypertension [22].

Increased activity of MMP may also contribute to hypertension-induced endothelial dysfunction and impaired vasodilatation in the presence of agonists [25, 56]. Our group showed that treatment of 2K-1C rats with doxycycline completely ameliorated the impaired vasodilator response to acetylcholine in the endothelium [25, 56]. Although the mechanisms involved in MMP-induced endothelial dysfunction still need to be identified, we speculate that it may be a result of MMP-mediated maladaptive structural remodeling in the vasculature. As both MMP-2 and MMP-9 proteolyze the extracellular matrix in the endothelium and media layers in the 2K-1C aortas, they may allow the cells to migrate and relocate, thus impairing the vascular function [31, 32].

Increased expression and activity of MMP-2 was also shown in thoracic aortas of diabetic rats. Those arteries also showed endothelial dysfunction and displayed an improvement of vascular relaxation to acetylcholine when treated with doxycycline [57, 58]. These authors also suggest that increased levels of MMP-2 leads to endothelial dysfunction.

Furthermore, a study showed that MMP-9 *knockout* mice had an increased expression of the endothelial nitric oxide synthase and an improved response of the resistance arteries to acetylcholine [59]. In fact, the acute injection of MMP-9 and/or MMP-7 in the mesentery (via the superior mesenteric artery) of Wistar rats was responsible for a rapid vasoconstriction and reduced diameter of arterioles and venules [60]. Studies also demonstrated that the potential mechanism involved in MMP-7-induced vasoconstriction and hypertrophy in rat mesenteric arteries appears to be dependent on epidermal growth factor receptor activation and mitochondrial ROS production into the VSMC, effects which were blocked by treatment with doxycycline [61, 62].

Several studies also suggest that increased MMP activity may contribute to vascular contractility by cleaving important non-extracellular matrix peptides and cellular receptors. It has been shown that plasmatic MMP activity of spontaneously hypertensive rats may cleave the extracellular domain of the β_2-adrenergic receptor in aortic endothelial cells, and this effect may contribute to MMP-mediated increased arteriolar vasoconstriction [60]. Treatment of these hypertensive rats with the nuclear factor kappa B inhibitor, pyrrolidine dithiocarbamate, also decreased MMP-induced proteolysis of β_2-adrenergic receptor [60], an effect similar to the one of doxycycline. This suggests that nuclear factor kappa B may be an upstream mediator of MMP activation [63]. However, it is unclear what MMP is involved in the of beta-2 receptor cleavage. MMP-2 may increase the vascular resistance in hypertension by cleaving big endothelin-1 (ET-1) to ET-1-32, a stronger vasoconstrictor [64]. A recent study conducted in pregnant rats with reduced uterine blood flow showed that MMP activity may be responsible to enhance the constriction effects of big-ET-1 on the mesenteric vessels, as this effect was inhibited by GM6001 [65]. MMP-2 also cleaves the vasodilator peptide calcitonin gene-related peptide in vitro into degradation products with lower vasodilator action [66]. Adrenomedullin was also shown to be cleaved by MMP-2 in vitro with generation of vasoconstrictor peptides [67].

3 Role of MMPs in the Hypertensive Heart

A time-course study of renovascular hypertension (2K1C) showed that 15–75 days after arterial clipping, increased left ventricular and septum wall thicknesses and cardiomyocyte hypertrophy are observed. These histopathological changes were accompanied with increased levels of ROS and MMP-2 activity and expression [68]. Interestingly 2K-1C rats treated with doxycycline (nonselective MMP inhibitor) reversed cardiac hypertrophy, MMP-2 expression, and activity [7].

Up to now, MMP-2 appears to be the major MMP involved in heart damage. Besides the work cited above, there are some other studies that show that MMP-2 is

increased in the heart that has structural and functional changes resulting from hypertension [7, 68, 69]. Furthermore, the overexpression of MMP-2 in the heart (in transgenic mice) results in ventricular remodeling with myocyte hypertrophy, lysis of myofilaments, destruction of sarcomeres, proliferation of cardiac fibroblasts, and functional loss [70]. After 12–24 weeks of life, these mice that transgenically overexpress MMP-2 in the heart show heart valve degeneration [71]. Furthermore, in the absence of MMP-2 expression (MMP-2–/– mice), the heart is protected from the injury induced by increased ventricular afterload [72]. These results clearly show that MMP-2 alone, in the absence of any other pathological condition, leads to structural changes and loss of cardiac function.

In the setting of ischemia/reperfusion, the transgenic animals that overexpress MMP-2 have larger areas of infarction and a decrease in contractile function in the hearts [73]. It has been neatly shown that in ischemia/reperfusion injury in rats, the expression of MMP-2 is increased, being found in the cytoplasm of cardiomyocytes, where it may be the protease responsible for degradation of structural proteins of the heart, as suggested by results for studies with troponin I [74], myosin light chain 1 [75], α-actinin [76], and titin [77]. Degradation of these structural proteins is likely to result in loss of cardiac function. Again the use of doxycycline and *o*-phenanthroline (MMP inhibitors) attenuated the impaired recovery of mechanical function of the heart in the ischemia/reperfusion [75, 78]. Furthermore MMP-2 is described in the nucleus of cardiac myocytes and is involved in protein degradation in DNA repair poly (ADP-ribose) o polymerase playing a role in apoptotic cell death [79]. To date the MMP-2 is the unique MMP described within the cardiac myocyte, and more recently, a novel intracellular (mitochondrial) isoform of MMP-2 induced by oxidative stress has been identified in cardiac myocytes submitted to oxidative stress conditions [80]. This form of approximate size of 65 kDa may contribute to progressive cardiac dysfunction.

The activity of MMP-9 is also higher in ischemia/reperfusion and heart of mice homozygous (–/–) and heterozygous (+/–) knockout for MMP-9 when exposed period with no flow showed 35.4 % and 17.5 % smaller infarct size, respectively [81].

In hypertension induced by the infusion of angiotensin II, an increased expression of MMP-2 has been observed in acute hypertension (first few weeks), which has decreased when the hypertension was established and cardiac remodeling (hypertrophy and fibrosis) has occurred. In this study, pharmacological intervention targeted at interfering with MMP-2 RNA attenuated hypertension, although it did not prevent heart remodeling. RNA interference with MMP-7 and ADAM-17 (tumor necrosis factor-α converting enzyme) attenuated hypertension and cardiac remodeling [82].

4 Other Aspects of MMPs and Hypertension

Although the focus of this review has not been the description of MMP concentrations in the plasma of hypertensive patients, there are many such studies and some have conflicting results. For instance, while higher plasma MMP-9 levels have been associated with increased arterial stiffness and elevated blood pressure in essential

hypertension [83], other studies showed that plasma MMP-9 levels were lower in hypertensive subjects compared to normotensive controls [84, 85].

Since hypertension is not one sole biological condition, but might be different diseases that have in common the raise in blood pressure, part of the conflict in the levels of MMPs found may actually reflect different underlying diseases, and those might actually be related to different MMPs. There is one interesting study which compared the plasmatic concentrations of different MMPs in controls, hypertensive patients, and hypertensive end-stage renal disease (ESRD) patients [86] and found that MMP-9 was elevated in hypertensive patients versus controls, while in hypertensive ESRD patients, MMP-2 and MMP-10 were elevated compared to both other groups. The finding of those discrete patterns of MMP overexpression in hypertension, with MMP-9 elevated early and MMP-2 and MMP-10 linked to target organ damage, may be important for the scientific knowledge of what is going on regarding damage done by MMPs in hypertension.

While the kidney is one of the target organs, where damage occurs subsequent to hypertension, the kidney has also primary roles in control of blood pressure levels. And it is remarkable that sound biological evidence already exists showing the essential role of MMP-2 in kidney damage, but inhibitors of this proteinase are still not used clinically. Ten years ago it was observed that it was MMP-2's proteolytic activity that was probably responsible for a whole range of pathological changes in the kidney, resulting in kidney failure [87]. Furthermore, when MMP-2 was transgenically expressed solely in the renal proximal tubular epithelium, pathological and functional changes characteristic of human chronic kidney disease were observed, including tubular basement membrane alterations, tubular epithelial–mesenchymal transition, tubular atrophy, fibrosis, and renal failure [88]. Although it is not yet known what the expression of other MMPs would do when expressed in the same way, it is already clear that MMP-2 damages the kidney irreversibly. Together with the information from clinical studies that MMP-2 is found in higher levels in hypertensive patients with ESRD (not reviewed here), there is consistency in the fact that the damaging effects of MMP-2 may be underestimated, and one possible reason is the fact that each model of hypertension has its characteristics and biological similarities with one aspect of clinical hypertension (different models will probably show different levels of MMPs in tissues and plasma), and another reason might be the fact that even today quantification of MMPs is tricky and a universal quantification method of their levels and activity is not available, and thus studies are not comparable.

Along this line, a study by Williams et al. [89] compared the effects of ACE inhibitors with the effects of the association of ACE inhibitors + novel MMP inhibitors (XL081 and XL784) on the development of renal injury in rat models of hypertension (Dahl salt-sensitive) and type 2 diabetic nephropathy. These novel inhibitors tested were highly potent towards MMP-2, MMP-13, and ADAM10, with XL784 also exhibiting high inhibitory potency against MMP-9 and ADAM17. Results markedly reduced proteinuria and even decreased hypertension, suggesting that inhibiting MMP activity is at least as effective as the administration of ACE inhibitor. The authors concluded that chronic treatment with XL784 along

with lowering arterial pressure by blocking the renin–angiotensin system has a great potential for the prevention and possibly reversal of hypertension- or diabetic-induced renal disease [89].

Chronic vascular inflammation has been suggested to be important for the development of essential hypertension, either as a pathogenic or secondary event [90]. C-reactive protein (CRP) and interleukin 6 (IL-6) are chronic inflammation markers highly associated with increased risk of adverse events in cardiovascular diseases [91]. MMP-9 has been also considered a predictor of cardiovascular mortality in patients with coronary artery [92]. MMP-9 expression increases when endothelial cells are exposed to CRP [93]. Interestingly, there are other inflammatory conditions that might be raising MMP levels in the plasma and tissues and in this way contributing to development or worsening of hypertension.

Periodontal disease, also called periodontitis, is a chronic disease of the gums and teeth that is highly prevalent in the adult population, affecting more than half the adults aged 30–90 years of age [94]. Periodontitis is one of those diseases known to raise chronic inflammation markers in plasma, and periodontitis is today clearly linked to cardiovascular diseases [95]. Although many studies exist showing that treatment of periodontal disease leads to decreases of chronic inflammation markers [96], the actual link between hypertension and other cardiovascular diseases to periodontitis remains elusive.

In line with many other studies, as can be read in reviews on this subject [97], patients with chronic periodontitis but no other chronic disease show increased levels of CRP and IL-6 before treatment, as compared to age- and gender-matched controls, and the levels of these two markers decrease 3 months after periodontal therapy [98], while other markers such as CD40 ligand, monocyte chemoattractant protein (MCP)-1, soluble P-selectin (sP-selectin), soluble vascular adhesion molecule (sVCAM)-1, and soluble intercellular adhesion molecule (sICAM)-1 did not show changes [98]. But, maybe more importantly for this discussion, in this cohort MMP-9 and MMP-8 were also increased in the plasma or these apparently healthy patients (except for periodontitis) and decreased after periodontal therapy, which comprises standard scaling and tooth root planning, but no systemic drugs [98]. This change in circulating MMPs after periodontal therapy had not been shown before in patients with periodontitis. Besides total MMP-8 and MMP-9 levels, the total proteolytic activity related to metalloproteinase activity in plasma was also shown to be higher, decreasing 3 months after treatment in the patients [98]. In another experiment that tested the effect of periodontal treatment on inflammation biomarkers and endothelium function, it was found that endothelium function was compromised and could be reversed by periodontal treatment alone [99]. Since MMPs have been shown to be increased in the plasma of periodontal patients, and they decrease after effective periodontal therapy, it is conceivable that the increase in MMPs might be the underlying link between periodontal and cardiovascular disease. And periodontitis might be just one example of one inflammatory condition in which MMPs are increased that may reflect somehow in increases in blood pressure. Other inflammatory conditions known to be related to increased MMP levels in plasma are atherosclerosis [100, 101] and diabetes [102, 103].

Finally, the most widely used inhibitor of MMPs used in the animal studies described in this review is doxycycline, which is in clinical use in the United States since 1998 (it is indicated for periodontitis and its commercial name is Periostat) [104]. The results discussed here regarding organ histology and function, as well as the blood pressure levels, indicate that MMP inhibition is so promising as a possible alternative for the treatment of hypertension, and this supports further investigation of the use of doxycycline or other MMP inhibitors in hypertensive patients.

Furthermore, more specific models/strategies for delivery or expression of MMPs in specific organs have been designed, and can thus be used, what will greatly help identifying which (if any) specific MMP is actually responsible for which effect. We will probably see in the future such strategies used in studies of the different hypertensive models, and those will be the studies that can finally answer which is the most important (if there is only one) MMP in which model of hypertension, and this knowledge will certainly help improve clinical treatments.

5 Conclusions

In conclusion, increased MMP activity plays a significant role in hypertension-induced structural and functional alterations in the cardiovascular system, MMPs inhibition protected from end-organ damage induced by hypertension, and reversed structural and functional alterations in many studies. In this review we discuss data showing the protective effects of MMP inhibition in the arteries, heart, and kidney. MMP inhibition appears to be a relevant target for newer pharmacological intervention in hypertension. Doxycycline is the MMP inhibitor most widely used in animal studies on hypertension so far and is a drug approved for commercial use for periodontal disease in the United States. The refinement of MMP assays and the specific delivery of different MMPs may highlight the specific role of each MMP in the different types of hypertension, enabling the use of a rational approach for a more specific inhibition.

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Chapter 16
Regulation of Proteolysis in Vascular Remodeling

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Abstract The extracellular matrix (ECM) is the scaffolding on which cells adhere and is comprised of fibrous proteins (such as collagens and elastin), proteoglycans, and water. Matrix–cell adhesion provides essential cell survival cues and the matrix sequesters various growth factors that can regulate the behavior of adjacent cells. However, the matrix, and in particular, the basement membrane, presents a confining barrier that limits vascular cell movement. Thus, reorganization of the ECM is an essential step in vascular remodeling processes. ECM proteolysis allows for sprouting angiogenesis and arteriogenesis in response to physiological stimuli, such as increased blood flow or increased metabolic activity. However, unregulated extracellular matrix degradation is associated with vascular diseases such as diabetic retinopathy, tumor angiogenesis, and thrombosis. In this review, we describe the structure and function of the major matrix protease families secreted by endothelial cells: the matrix metalloproteinases (MMPs) and plasminogen activator (PA)/plasmin system. We also discuss the function and regulation of the endogenous protease inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), and the plasminogen activator inhibitors (PAIs). The equilibrium between proteases and inhibitors plays an integral role in establishing the maintenance and structural remodeling of the vasculature. We review the regulation of these proteases and their inhibitors in vascular cells, particularly in response to altered shear stress. The specific involvement of members of the MMP/TIMP system and the PA/plasmin/PAI system proteases in vascular remodeling of capillaries (angiogenesis) and arteries (arteriogenesis) are discussed.

Keywords Angiogenesis • Arteriogenesis • Matrix metalloproteinase • Plasminogen • Extracellular matrix

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1 Introduction

The extracellular matrix (ECM) scaffolding that surrounds blood vessels (the basement membrane and adventitial matrix) largely is composed of proteins such as collagens, laminins, fibronectin, and elastin, which provide structural integrity to the blood vessel wall and promote critical cell-adhesion-based survival signals. The cleavage of ECM proteins is integral for the cell migration and invasion that underlies vascular remodeling. MMPs represent one major class of molecules that degrade the ECM. The serine protease plasmin also plays an important role in the proteolysis of matrix proteins.

2 Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases

2.1 Matrix Metalloproteinases

There are 28 MMPs in vertebrates, all of which share the common structural elements of a pro-domain, a catalytic domain, a hinge region, and a hemopexin domain [1, 2]. The pro-domain of most MMPs contains a conserved motif known as the cysteine switch, which maintains the MMP in its inactive pro-form [2, 3]. The catalytic domain contains the active zinc site where proteolysis occurs [4]. A subset of MMPs, membrane-type MMPs, also has a transmembrane domain. Most MMPs are secreted in their pro-form and are subsequently activated in a two-step process, beginning with proteolytic cleavage of an exposed loop in the pro-domain known as the bait region, which results in partial activation of the MMP. Other active MMPs, or autocatalysis, typically then complete the activation process through removal of the remainder of the pro-domain [5]. MMP-2 is activated through a different mechanism than the other MMPs. Secreted MMP-2 first forms a trimeric complex with MT1-MMP and TIMP-2 on the cell surface. Subsequently, an adjacent MT1-MMP cleaves the bait region of MMP-2, thus activating it [6]. Oxidation and S-nitrosylation also can cause conformational alterations that disrupt the cysteine–zinc interaction that typically stabilizes the pro-form of the MMP [7, 8].

MMP-dependent cleavage of matrix proteins also can generate antiangiogenic factors such as angiostatin and endostatin (cleavage products of plasminogen and collagen XVIII, respectively) [9, 10]. MMP-2, MMP-9, MMP-7, and MMP-12 are capable of cleaving plasminogen and creating angiostatin [9, 11], while MMP-12 creates endostatin [10]. MMP-13 also is thought to produce endostatin in the lungs, which is believed to have a protective effect against melanoma metastasis [12]. It has been hypothesized that MMP-induced angiostatin production results in impaired angiogenesis in diabetes, where high levels of MMPs and low levels of vascular endothelial growth factor (VEGF) are present in the vasculature [13]. High MMP activity, high angiostatin expression, and low vascular density were all correlated in the arteries of diabetic patients [14].

MMPs not only cleave ECM proteins, they also can cleave membrane-bound proteins. For example, MT1-MMP cleaves endoglin bound to the cell surface to create soluble endoglin, which is antiangiogenic [15]. Multiple MMPs are able to cleave Ephrin A1 from the cell membrane, allowing for binding to its receptor and subsequent signal transduction [16]. MMP-1 and MMP-13 cleave and activate protease-activated receptor 1 [17, 18]. These pathways have important implications in tumor progression and angiogenesis.

MMP activity is under rigorous transcriptional and posttranscriptional control [19]. Transcription of MMP genes can be induced by growth factors, cytokines, physical stress, and cell–cell or cell–matrix interactions [19, 20]. The list of transcription factors known to interact with MMP promoters is extensive and includes AP-1, Sp-1, Egr-1, GATA-2, Stat3, and NFκB [21–26]. In endothelial cells, physical stimuli such as 3D collagen type I lattices have been shown to stimulate MMP-2 and MT1-MMP expression [27], via the transcription factors GATA-binding protein 2 (GATA-2) [23] and early growth response-1 (Egr-1) [22], respectively. MMP transcription also is regulated by acetylation and methylation [28, 29].

MMP secretion may be modulated by various signal pathways. We and others showed that growth factor stimulation can induce increased MMP-2 secretion and activation by endothelial cells independent of new protein synthesis [30–32]. Endothelial cells possess cytoplasmic vesicles containing MMP-2 and MMP-9, which act as storage depots that are available for rapid release of the MMPs upon stimulation [33]. Schnaeker et al. also showed that MMP-2 is housed in small cytoplasmic vesicles that appeared to be distributed within the cytoplasm in a "punctate" manner, with a larger concentration apparent within lamellipodia [34]. Many of these MMP-2-positive vesicles were associated with the microtubule network and co-localized with the microtubule motor kinesin [34], consistent with a microtubule-dependent secretion process. Remacle et al. demonstrated that the microtubule cytoskeleton regulates the transport and internalization of MT1-MMP [35]. Cdc42 GTPase also is associated with stimulation of MMP-2 secretion [32].

2.2 Tissue Inhibitor of Metalloproteinases

The tissue inhibitor of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs. There are 4 TIMPs with similar structures. TIMP-2 is expressed constitutively throughout the body, while TIMP-1, TIMP-3, and TIMP-4 exhibit tissue specificity and inducible expression [36, 37]. The TIMP N-terminal domain interacts with the Zn^{2+}-binding pocket of active MMPs in a 1:1 stoichiometric reversible complex, preventing MMP activity [38, 39]. The C-terminal domain of TIMP-1 and TIMP-2 also has the ability to bind to the pro-domains of MMP-9 and MMP-2, respectively, inhibiting the activation of these MMPs [40, 41]. TIMPs 1, 2, 3, and 4 all have been shown to inhibit MMP-2 activity through binding either to the proenzyme or to the active site of MMP-2 preventing its proteolytic activity [42–46].

TIMPs are regulated by transcription, secretion, endocytosis, and extracellular interactions [47]. TIMP-1 transcription is known to be regulated by multiple

transcription factors, including Egr-1, AP-1, Ets-1, Sp-1, NFkB, CRE, and Smad 2/3 [48–53]. Various signaling pathways have been shown to activate TIMP-1 transcription. In fibroblasts, TGF-β activates TIMP-1 transcription through AP-1, and not through its canonical Smad signaling pathway, while in glomerular cells nitric oxide (NO) activates TGF-β1, which increases TIMP-1 via Smad 2/3 [51, 54]. Recently, our lab has shown that TIMP-1 transcription in endothelial cells is increased by short-duration exposure to elevated shear stress via activation of Ets-1 [55]. Continued TIMP-1 transcription after longer periods of elevated shear stress likely involves other signal pathways, since Ets-1 protein expression is depressed after 24 h of shear stress exposure [55].

TIMPs regulate the cellular interaction with the extracellular microenvironment by inhibiting MMP-dependent proteolysis of matrix and cell surface proteins. Typically, this is considered to inhibit the remodeling processes; however, in some cases, the blockade of MMP activity may promote cellular migration and proliferation. For example, MMP-dependent generation of the anti-angiogenic factors angiostatin and endostatin could be reduced in the presence of TIMPs. Though this has not been shown, it follows logically that if TIMPs prevent MMP activation, then MMPs are unable to cleave plasminogen or collagen XIII to produce angiostatin and endostatin.

Distinct from their role as MMP inhibitors, TIMPs also may elicit a variety of signaling events related to cell survival and proliferation by binding to various cell surface proteins. TIMP-1 binds an integrin β1-CD63 complex, integrin αvβ1, and a pro-MMP-9/CD44 complex [56–64]. However, conflicting consequences of these interactions have been reported, as there is evidence that TIMP-1 both promotes and prevents cell proliferation [65–67]. We have observed no effect of recombinant TIMP-1 on promoting microvascular endothelial cell survival signaling (Fig. 16.1). TIMP-2 interaction with integrin α3β1 causes phosphatase activation and subsequent cell cycle arrest in endothelial cells, thus reducing angiogenesis [60]. TIMP-3 binds to VEGF receptor-2 (VEGFR2) and therefore blocks VEGF binding and downstream signaling [59]. TIMP-3 induces apoptosis by preventing cleavage of the TNF-α receptor by the metalloproteinase ADAM-17 [68]. This allows for TNF-α signaling and subsequent cell death. The extent to which MMP-independent roles of TIMPs play in physiological and pathological vascular remodeling processes remains to be established.

3 The Plasminogen Activator/Plasmin System

3.1 Regulation of Plasmin Production

Plasmin cleaves extracellular matrix (ECM) proteins such as laminin, fibronectin [69], and vitronectin [70]. Plasminogen is secreted into the plasma as an inactive zymogen [71] and its subsequent cleavage by urokinase-type plasminogen activator

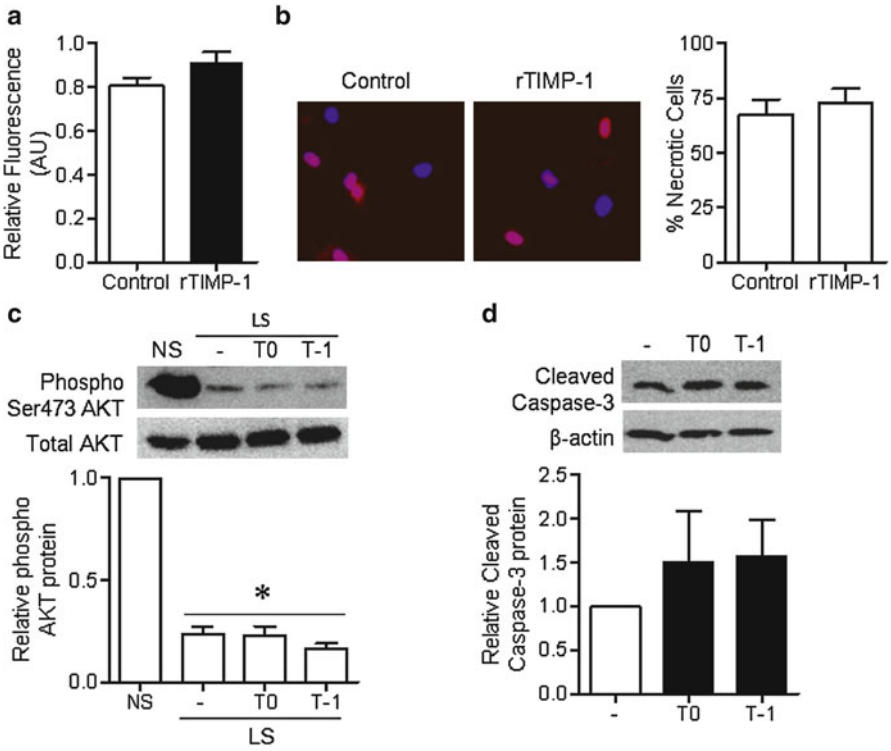

Fig. 16.1 TIMP1 does not promote cell survival in microvascular endothelial cells. In (**a**) and (**b**), rat microvascular endothelial cells were cultured in no-serum media for 48 h, with treatment of recombinant TIMP1 (100 ng/ml). Cell quantification assays (**a**) and cell death assays (**b**), from CyQuant and Biotium, respectively, were performed according to the manufacturer's instructions. In (**b**), necrotic cells are labeled with ethidium homodimer III (*red*) while DAPI (*blue*) demarcates all nuclei. In (**c**), Western blotting for phospho-Ser473 Akt (normalized to Akt) was conducted on microvascular endothelial cells following culture under normal serum (NS) or low serum (LS) conditions for 1 h. In (**d**), Western blotting for cleaved caspase-3 (normalized to **β**-actin) was conducted on endothelial cells treated with mitomycin (M; 10 **μ**g/ml) for 24 h to stimulate apoptosis. In both (**c**) and (**d**), recombinant TIMP-1 was added either at the same time as the low serum/mitomycin treatment (T0) or 1 h prior to treatment ($T-1$). Blots were stripped and re-probed for total AKT or **β**-actin, respectively. Densitometric analyses represent the values obtained from 3 to 6 independent experiments. *$p<0.05$ compared to normal serum (NS) control

(uPA) or tissue-type plasminogen activator (tPA) results in the generation of active plasmin [72]. Within the blood, plasminogen activation is achieved primarily via tPA, while cell-associated plasminogen activation occurs predominantly via uPA [73]. Tight regulation of plasminogen cleavage is achieved by maintaining an equilibrium between uPA and tPA, and their inhibitors, plasminogen activator inhibitor-1 and 2 (PAI-1,2) [74, 75].

uPA itself is secreted in an inactive form that is activated by the cleavage of its pro-domain, through binding to its cell surface receptor, urokinase-plasminogen

activator receptor (uPAR), or via plasmin. The generation of active uPA by plasmin establishes a positive feedback loop that results in increased plasmin levels [76]. Notably, uPA facilitates the activation of plasminogen at localized regions on the cell surface through its interaction with uPAR [77], which is a glycosylphosphatidylinositol-linked membrane protein [78]. During directional cell migration, uPAR is localized to the leading edge of the cell, thereby promoting localized plasmin-dependent ECM degradation [79], similar to the role that MT1-MMP plays in directing matrix proteolysis to the leading edge of migrating cells.

PAI-1 and PAI-2 belong to the serpin family [80]. PAI-1 is synthesized primarily by endothelial cells [81]. PAI-2, although first reported in placental tissue, also is secreted by a number of cell types including endothelial cells [80]. However, PAI-1 is a more efficient inhibitor of both uPA and tPA, as compared to PAI-2 [82], suggesting that it may play a dominant role in controlling plasminogen activation. PAI-1-uPA-uPAR cell surface complexes may undergo rapid internalization via the low-density lipoprotein receptor-related protein [83]. Through promoting the internalization of this complex, PAI-1 serves to regulate cell surface levels of uPAR, which will modulate the process of plasmin generation [84]. PAI-1 also interacts with the matrix protein vitronectin (VN), which stabilizes and extends the half-life of PAI-1 [85, 86].

Plasmin-dependent proteolysis is increased when plasmin/plasminogen is tethered to the cell surface, as this both promotes uPA-dependent plasminogen cleavage and protects plasmin from inactivation [87–90]. A diverse group of over 12 proteins has been shown to act as cell surface proteins for plasminogen and/or plasmin [91]. A novel plasminogen receptor, Plg-R_{KT}, recently was identified and localized on the cell surface in close proximity to uPAR [92], within many migratory cells including macrophages [93].

3.2 Plasmin Functions

Plasmin-dependent cleavage of matrix proteins promotes cellular migration and invasion characteristic of vascular remodeling through loosening basement membrane structural components. In some cases, this is a self-limiting event. For example, cleavage of vitronectin by plasmin reduces the affinity of vitronectin-PAI-1 binding, increasing the PAI-1-uPA interaction and thus reducing plasmin generation [70].

Plasmin cleavage of the ECM also facilitates the release and subsequent activation of matrix-bound proteins such as transforming growth factor β l [94], fibroblast growth factor-2 [95], and hepatic growth factor [96]. The release of FGF-2 from the matrix allows this protein to stimulate endothelial and smooth muscle cell proliferation [96]. Experiments on cultured endothelial cells have demonstrated a positive feedback loop between uPA and FGF-2, as FGF-2 increases endothelial cell production and release of uPA, thereby increasing plasmin levels and facilitating further release of matrix-associated FGF-2 [97]. Plasmin also promotes increased ECM proteolysis by activating a variety of MMPs, including MMP-1, 3, 9, 12, and 13 [98, 99].

3.3 Additional Biological Functions Associated with PAI-1 and uPAR

PAI-1 and uPAR appear to play roles in cell adhesion, migration, and survival that are independent of their regulation of plasminogen activation [100, 101]. Both of these proteins are able to interact with other ECM proteins such as VN and various integrins [100, 101]. PAI-1 binds with high affinity to VN, competitively inhibiting the interaction between uPAR and VN. High levels of PAI-1 thus inhibit uPAR-mediated cell adhesion [102]. PAI-1 bound to VN also interferes with integrin binding to the RGD domain of VN, resulting in reduced integrin-mediated cell adhesion [103].

PAI-1 is reported to affect cellular apoptosis and proliferation. Chen et al. demonstrated that PAI-1 forms a high affinity complex with caspase-3, thereby inhibiting caspase-dependent apoptotic signaling in vascular smooth muscle cells [104]. This also was associated with an increased rate of vascular smooth muscle cell proliferation [105]. Conversely, high extracellular levels of PAI-1 induce endothelial and vascular smooth muscle cell apoptosis, likely through interference with integrin-mediated cell adhesion to VN [106, 107]. We found that recombinant PAI-1 did not induce signaling through Akt or ERK1/2 pathways in microvascular endothelial cells (Fig. 16.2), suggesting that any observed effects of PAI-1 on survival or proliferation likely are mediated indirectly through its effects on modulating uPAR or VN-mediated cell adhesion.

uPA-uPAR signaling, independent of its proteolytic activity, occurs through interactions with vitronectin, integrins, and/or neutrophils as well as via the activation of several downstream pathways including ERK-MAPK pathway [108], FAK-Src [109], and Akt [110].

4 Regulation of Proteases and Protease Inhibitors by Shear Stress

Shear stress signaling is associated with vascular remodeling at the level of the arterial system, where it may promote outward remodeling of the vessel wall [111, 112], and at the level of the capillaries, where it promotes a non-sprouting form of angiogenesis [113, 114]. Not surprisingly, numerous mediators of shear stress signals have been shown to alter the production of proteases and protease inhibitors.

NO inhibits the production of MMP-2 in endothelial cells [115, 116]. In larger vessels, this gives endothelial cells an atheroprotective phenotype, because MMPs weaken atherosclerotic plaques and increase the risk of plaque rupture by degrading the basement membrane [115]. Within capillaries, the reduction in MMP-2 mRNA and protein may help to ensure capillary integrity through minimizing basement membrane degradation.

TGF-β1 is known to be upregulated in response to elevated shear stress [117]. In general, TGF-β1 signaling increases basement membrane production and enhances

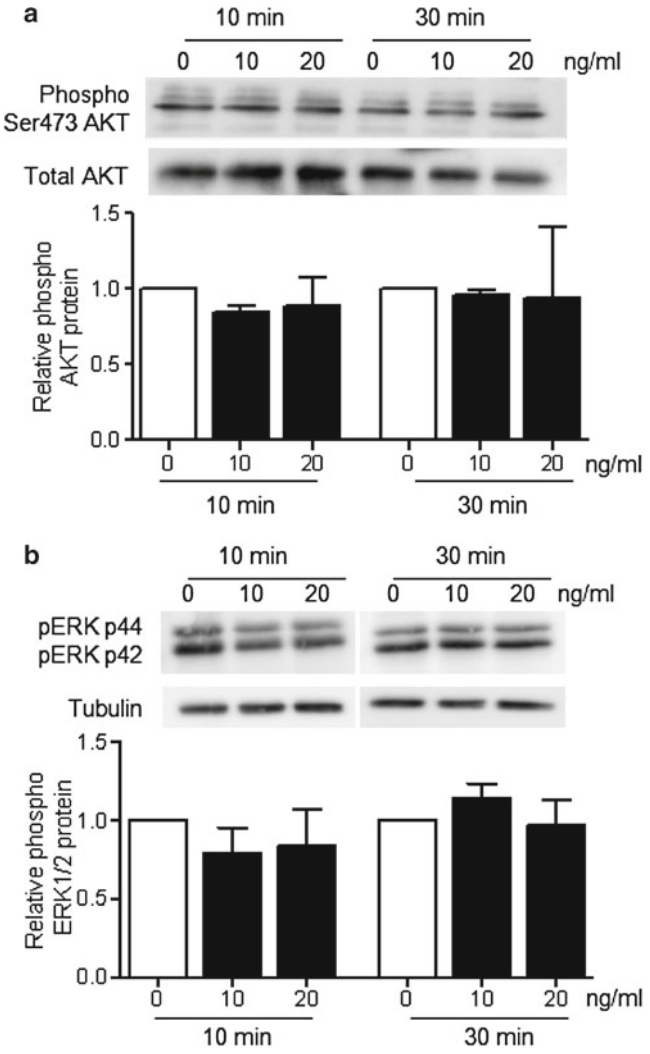

Fig. 16.2 PAI-1 does not activate cell survival or proliferation signal pathways in microvascular endothelial cells. Rat microvascular endothelial cells were treated with recombinant PAI-1 (10 or 20 ng/ml) for 10 or 30 min followed by lysis and Western blotting for phospho-Ser 473 AKT (**a**) or phospho-ERK1/2 (**b**). Blots were stripped and re-probed for total AKT or tubulin, respectively. Densitometric analysis represents the values obtained from 2 independent experiments

endothelial cell barrier function, all of which promote vascular integrity [118–121]. Consistent with these effects, TGF-β1 is a known transcriptional regulator of PAI-1 [122] and TIMP-1 [123–125]. However, the involvement of TGFβ1 in regulating these factors in response to shear stress remains to be elucidated.

In endothelial cells, Sp-1 acts as a coactivator to increase transcription in response to shear stress [126]. Sp-1 is known to regulate transcription of several proteins in

response to shear stress activation, including VEGFR2 [127, 128]. However, Sp-1 represses the transcription of MT1-MMP in endothelial cells subjected to shear stress [128], indicating that it contributes to maintenance of vessel structural integrity.

Another shear-sensitive transcription factor is Ets-1. Ets-1 is a transcription factor that binds to a GGA(A/T) consensus sequence in the promoter region of target genes, termed the ETS domain [129]. Also important to the structure of Ets-1 is its protein-binding domains, which allow for interactions with other transcription factors [130]. These domains include the ETS DNA-binding domain, the N-terminal, the exon VII domain, and the C-terminal transcription activation domain [131]. The transcriptional activity of Ets-1 is regulated by phosphorylation at multiple sites [132]. DNA-binding activity can be both increased and decreased by phosphorylation of Ets-1, depending on the phosphorylation site [132]. Our lab showed that in endothelial cells, Ets-1 mRNA and DNA binding are increased by 2 h of exposure to high shear stress (15 dyne/cm^2) [55]. We found that the transcription of TIMP-1, TIMP-3, and PAI-1 in endothelial cells is increased by short-duration exposure to elevated shear stress, dependent on Ets-1 [55]. Continued transcription of these protease inhibitors after longer periods of elevated shear stress likely involves other signal pathways, since Ets-1 protein expression is depressed after 24 h of shear stress exposure [55]. Interestingly, long-term (24 h) exposure of microvascular endothelial cells to low levels of shear stress (5 dyne/cm^2) also stimulates production of protease inhibitors (TIMP-1 and PAI-1) in the absence of an effect on the production of proteases MMP-2 and uPA (Fig. 16.3). Together, these data imply that the predominant effect of shear stress signaling on endothelial cells is to induce a shift in favor of increased proteolytic inhibition, which may be important in promoting the continued structural integrity of the thin-walled capillaries.

5 Protease and Protease Inhibitor Involvement in Angiogenesis

In sprouting angiogenesis, proteolysis of the basement membrane is necessary for the angiogenic sprout to migrate. It has been recognized for some time that this process requires a critical balance between proteases and their inhibitors to ensure that excessive matrix degradation does not occur [133]. MMPs are recognized to facilitate the pathological processes of tumor angiogenesis and the capillary remodeling associated with diabetic retinopathy [134–139]. Physiologically, production of the proteases MMP-2 and MT1-MMP is upregulated during sprouting angiogenesis in rat skeletal muscle [116, 140, 141]. Increases in MMP-2 also are detected in human skeletal muscle during exercise training [142]. Endothelial MMP-2 and MT1-MMP productions are stimulated by the permeability factors VEGF and histamine, in part via the translocation of the transcriptional coactivator, β-catenin, from the cell membrane to the nucleus [143]. MMP production also is increased in overload-induced angiogenesis, which occurs via activation of ERK and JNK signaling [144]. Cytoskeletal reorganization increases MMP-2 and MT1-MMP

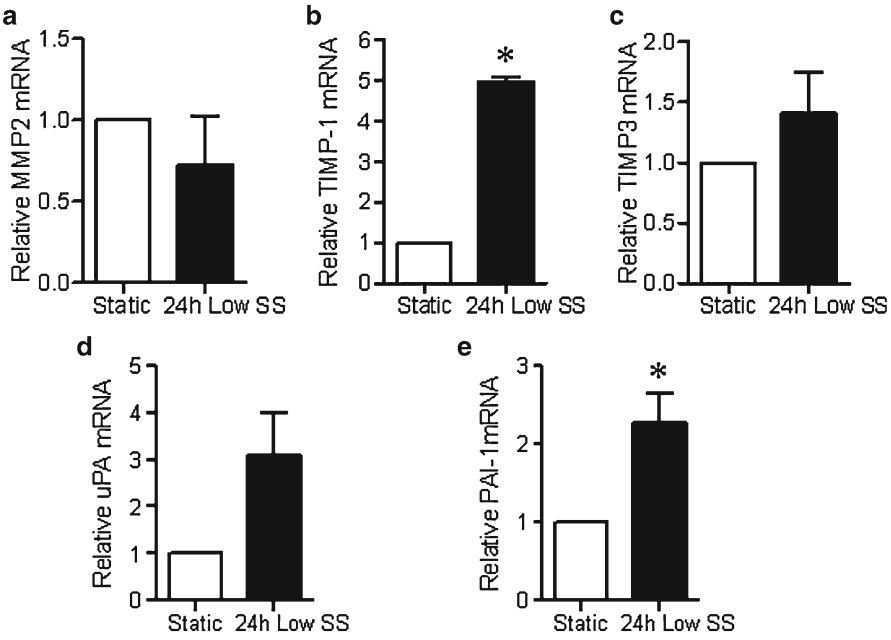

Fig. 16.3 Low shear stress enhances production of proteolytic inhibitors TIMP-1 and PAI-1 in microvascular endothelial cells. Rat microvascular endothelial cells were exposed to static conditions or low laminar shear stress (5 dyne/cm^2; 24 h Low SS) for 24 h. Following cell lysis and reverse transcription, RNA was analyzed by quantitative real-time PCR using TaqMan probes for MMP-2 (**a**), TIMP-1 (**b**), TIMP-3 (**c**), uPA (**d**), and PAI-1 (**e**). In each case, relative changes in transcript levels were calculated, using HPRT-1 as a housekeeping gene. $n = 3–5$ per condition; $^*p < 0.05$ compared to static condition

production, via PI3K and JNK signaling [145]. The oxidative stress associated with high glucose has been shown to promote activation of MMP-2 and MT1-MMP within retinal endothelial cells [137] and the activation of MMP-2 in retinal pericytes [146], thus contributing to excessive vascular remodeling. MT1-MMP localization to sprouting tip cells has been demonstrated, consistent with its significant permissive role in endothelial cell migration [147]. Beyond its role in tip cell sprouting, MT1-MMP also coordinates activities associated with neovessel maturation and stabilization. For example, MT1-MMP within vascular mural cells acts to promote appropriate PDGF receptor β signaling associated with mural cell recruitment and stabilization of vessels [148]. MT1-MMP also regulates lumen formation in vasculogenesis and angiogenesis [149].

Since MMP-2 and MT1-MMP both proteolyze components of the extracellular matrix, the lack of increase in these proteases during shear stress-induced angiogenesis is consistent with the observations that basement membrane degradation and sprouting do not occur during this form of angiogenesis [113]. Our lab showed that MMP-2 production is inhibited by shear stress and that both NO production and p38 MAPK activation contribute to this inhibition [116]. It also is possible that the

activity of MMP-2, in addition to its production, is inhibited by shear stress. This would be another useful regulatory mechanism for the prevention of proteolysis, since inhibiting de novo production of MMP-2 does not prevent preexisting MMP-2 from degrading the extracellular matrix.

Knockout models have shown varying effects of MMPs on angiogenesis. Notably, genetic deficiency or inactivation of individual MMP family members does not result in embryonic lethality due to failed development or expansion of the vasculature, implying that there is functional redundancy of MMPs within the vascular system. MMP-12 knockout mice have decreased pathological angiogenesis, but enhanced physiological angiogenesis in a model of oxygen-induced retinopathy [150]. MMP-9 knockout mice demonstrate impaired angiogenesis in the corneal pocket angiogenesis assay [151]. In a model of diabetic retinopathy, MMP-9 deficient mice show reduced levels of retinal capillary endothelial cell apoptosis and minimized mitochondrial damage [152]. Wound healing-induced angiogenesis is impaired in MMP-13 knockout mice [153]. Conversely, in an epidermal-specific MT1-MMP knockout mouse model, wound healing-induced angiogenesis was sustained for a longer period of time than in wild-type mice, and this was correlated with decreased endostatin expression [154]. Conditioned media from MT1-MMP knockout epithelial cells also enhanced endothelial cell proliferation and migration of wild-type cells [155]. MMP-2 knockout mice show impaired angiogenesis in dystrophic skeletal muscle [156], as well as reduced retinal and tumor angiogenesis [157, 158]. However, combined deletion of MMP-2 and MMP-14 (MT1-MMP) results in perinatal lethality, providing evidence of the critical cooperative actions of these two proteases [159].

TIMPs inhibit angiogenesis using multiple mechanisms. By inhibiting MMP activity, TIMPs prevent angiogenic sprouts from migrating through the extracellular matrix [160]. TIMP-1 secretion enhances soluble VEGFR1 secretion, which is anti-angiogenic [161]. TIMP-2 also is able to inhibit angiogenesis via its interaction with integrin $\alpha 3\beta 1$ which causes phosphatase activation and subsequent cell cycle arrest in endothelial cells [60]. TIMP-2 knockout mice exhibit accelerated angiogenesis compared to wild-type mice [162]. TIMP-3 inhibits VEGF-induced cell migration, preventing collagen invasion and capillary morphogenesis in an in vitro model of angiogenesis [163]. TIMP-3 binds to VEGFR2 and therefore blocks VEGF binding and downstream signaling [59]. However, TIMPs may also be proangiogenic in some circumstances. MMPs have the ability to create potent antiangiogenic factors such as angiostatin and endostatin, which are cleavage products of plasminogen and collagen XVIII, respectively [9, 10]. TIMPs would block the generation of these factors. Also, in luminal splitting, MMP-2 activity is inhibited, so TIMPs may play a positive role in this process [116]. TIMP-3 was shown to regulate lumen formation, which indicates that it plays a role in vessel maturation in angiogenesis [164]. More recently, TIMP-1 has been discovered to play a role in increased cell survival and proliferation, suggesting that the role of TIMP-1 in angiogenesis may be more complicated than originally thought [61, 65, 67]. Additionally, TIMP-1 protein levels were positively correlated with high levels of vascularization in patients with diabetic retinopathy [165]. TIMP-1 also is highly expressed in many types of cancers, and expression

level is correlated with a negative prognosis and metastasis, which requires angiogenesis [166–168]. In this case, TIMP-1 could act to promote angiogenesis.

There is strong evidence demonstrating the importance of the PA/plasmin system (uPA/tPA, plasmin, and PAI-1) in angiogenesis. For example, plasminogen-deficient mice have significant reductions in angiogenesis even with growth factor treatment [169]. tPA is expressed in quiescent endothelial cells, while uPA, uPAR, and PAI-1 are produced predominantly in activated endothelial cells, under the stimulation of VEGF [170, 171]. uPA upregulation correlates temporally with the initiation of endothelial cell migration [172]. A tight regulation of the balance between plasminogen activators and inhibitors is required for angiogenesis to occur properly [173]. VEGF induces the production of both uPA and PAI-1 in microvascular endothelial cells; however, the increase in uPA is greater, which results in a larger ratio of uPA to PAI-1 [174]. This enables increased plasminogen activation and ECM cleavage to occur while a concurrent, albeit smaller, increase in PAI-1 ensures that cells still retain ECM connections in order to avoid apoptosis [175, 176]. Furthermore, the role of PAI-1 in angiogenesis is not solely due to its inhibitory role on uPA/tPA. PAI-1 has been shown to enhance the migratory stage of angiogenesis [175, 176], which likely is due to the interaction of PAI-1 with the ECM protein VN [177]. It has been speculated that PAI-1 interferes with vitronectin (ECM protein) interaction with integrin αvβ3 [178], thereby promoting cell migration. These contrasting roles of PAI-1 in angiogenesis are a function of the independent functions of its carboxy and amino terminals [179].

Research has highlighted the importance of the various components of the plasminogen system in angiogenesis but also indicates that there are substantial differences in the roles of specific proteolytic members across various scenarios involving angiogenesis. In a mouse model of myocardial infarction, a loss of uPA, but not tPA or uPAR, impaired the postinfarction revascularization process [180]. Furthermore, only uPA deficiency significantly reduced plasmin generation [175]. This functional role may be amplified by the capacity of uPA to activate certain MMPs [181], demonstrating the dual importance of this protease in plasmin and MMP-dependent functions. In contrast, the critical involvement of tPA in angiogenesis is less clear. Mawatari et al. exposed human microvascular endothelial cells to a wound migration assay and reported that anti-tPA antibodies resulted in decreased cell migration compared to untreated cells [182]. This notion was further supported by experiments in which capillary sprouts from aortic explants of tPA, uPA, or plasminogen-deficient mice were embedded in either 3D collagen I or Matrigel. Researchers observed a significant reduction in sprouting ability in uPA-deficient mice and an almost complete abolishment in tPA and plasminogen-deficient explants [183]. Significantly, sprouting was compromised to a greater extent when explants were embedded in collagen as compared to Matrigel, indicating that plasmin activity is essential for wound healing angiogenesis in plasmin-nondegradable matrices such as collagen gel [183]. Conversely, Zablocki et al. concluded that tPA does not promote angiogenesis, observing no effect of tPA overexpression on endothelial cell proliferation or migration [184]. Interestingly, researchers reported that PAI-1 deficiency, but not uPA, tPA, or uPAR deficiency, significantly reduced tumor

vascularization in a mouse model [176]. This counterintuitive effect of PAI-1 deficiency highlights the challenges associated with teasing out the functional roles of individual members of these proteolytic pathways and indicates that more work is needed to fully understand the exact roles of each of its components and how to best manipulate them for clinical benefit.

6 Protease and Protease Inhibitor Involvement in Arteriogenesis

Enlargement of collateral vessels, often referred to as arteriogenesis, is a compensatory response that occurs subsequent to occlusion of an adjacent major conduit artery. Collateral artery remodeling can result in a 20-fold increase in arterial diameter [185], which helps to minimize the extent of downstream tissue ischemia [186]. For collateral vessel remodeling to occur, proteolysis in the internal and external elastic lamina as well as the basement membrane of endothelial and smooth muscle cells must occur [187]. Outward remodeling of arteries is associated with a shift in the balance between proteases and their inhibitors, in favor of an increase in ECM proteolysis. This balance must be tightly regulated as excessive proteolysis can result in permanent tissue damage and insufficient proteolysis in impairment of normal tissue turnover and/or remodeling.

Collateral remodeling is thought to occur through a series of stages: endothelial cell activation; recruitment of monocytes; ECM degradation; alterations in smooth muscle cell phenotype, proliferation, and migration; adventitial remodeling; and finally, stabilization and reversion of the differentiated smooth muscle cell phenotype [188]. Endothelial and smooth muscle cells within collateral arteries are activated within 3 days of occlusion of the main conduit vessel, thus beginning the initial steps of arteriogenesis [189]. ECM degradation requires the actions of the plasminogen/plasmin system and MMPs. Plasmin will cleave basement membrane proteins, but also will activate latent TGF-β and release matrix-bound VEGF and bFGF, which in turn will interact with their cell surface receptors to promote cell proliferation and migration [190]. uPA also has been shown to cleave fibronectin and to activate certain MMPs [191]. Basement membrane proteolysis may aid in the transition of smooth muscle cells from a contractile to a proliferative phenotype [192].

In general, a shift in the TIMP-MMP balance in favor of increased proteolysis has been observed in numerous models of outward arterial remodeling, with the expression pattern and involvement of specific MMPs differing moderately in a species and model-specific manner. MMP-2, MT1-MMP, and MMP-9 increased robustly within the wall of rabbit carotid arteries subjected to flow overload [193]. MMP-2 and MT1-MMP, but not MMP-9, were increased in rat mesenteric arteries exposed to flow overload [194]. In a rat model of coronary collateral artery remodeling, MMP-2 and MMP-9 increased robustly, but transiently, while no changes in TIMP-1 or TIMP-2 levels were observed [195]. Increased cleavage of laminin, type IV collagen, and elastin correlated with these changes in protease expression. Similarly, increased

production and activity of MMP-2 and MMP-9 was observed transiently in hind limb muscle following femoral artery ligation. TIMP-1 and TIMP-2 mRNAs also were elevated [196]. Cai et al. demonstrated that activation of fibroblasts within the adventitia is associated with increased expression of several proteolytic factors, predominantly MMP-2 and 9, tPA, and PAI-1, during the late phases of canine coronary collateral artery remodeling [197]. Finally, during the maturation phase, the levels of MMP, PA, and PAI-1 begin to return to basal levels [197].

Several studies provide convincing evidence that MMP activity is required for appropriate collateral artery remodeling. The inhibition of MMP activity prevented the outward luminal expansion while endothelial and medial cell proliferative responses were not impeded [194]. Inhibition of MMP-2 and MMP-9 prevented both the ECM cleavage and substantially reduced the extent of collateral artery remodeling in a coronary collateral model [195]. More specifically honing in on the involvement of MMP-2, Cheng and colleagues demonstrated that MMP-2-deficient mice had a significant reduction in collateral vessel growth following femoral artery ligation, resulting in reduced blood flow recovery compared to WT mice [196]. Notably, MMP-2-deficient mice exhibited a larger increase in MMP-9 activity and mRNA as compared to the WT mice, indicating that MMP-9 was not capable of compensating for the functional role of MMP-2 in arterial remodeling. Increased TIMP-1 mRNA levels also were detected in MMP-2-deficient mice, which may have counterbalanced the increase in MMP-9 activity.

Numerous factors contribute to the upregulation of proteases within the arterial wall during the process of wall remodeling. Early growth response factor 1 (Egr-1) has been labeled the “master switch” as it has the ability to promote the transcription of several growth factors (bFGF, VEGF), cytokines (MCP-1), adhesion molecules (ICAM-1), and proteases (MT1-MMP, uPA) [111, 198, 199]. Increased expression of Egr-1 was detected within the wall of collateral mesenteric arteries, predominantly within the smooth muscle layer, correlating temporally with increased expression of MMP-2 and MT1-MMP [194]. Increased levels of phosphorylated c-jun were increased similarly in the smooth muscle cell nuclei of collateral vessels [194]. c-jun is known to stimulate the production of MMP-2 and MT1-MMP [145], as well as other MMP family members [200, 201]. Reactive oxygen species (ROS) formation is associated with, and required for, the process of coronary collateral artery remodeling [202]. ROS may promote proteolysis in several ways. First, ROS evoke the activation of ERK, JNK, and p38 MAPK, as well as Akt and src kinases [202], all of which may regulate the transcription and secretion of MMPs (as described in Sect. 1). Second, peroxynitrites (ONOO–), which may be produced as a consequence of excessive NO production such as through the activity of macrophage iNOS [203] or via shear stress-dependent activation of p47phox-NADPH oxidase in endothelial cells [204], can directly activate MMP-2 and MMP-9 [205, 206]. Furthermore, uPA has been shown to contribute to neutrophil superoxide production [207] and release [208], indicating uPA to regulate cellular redox state and its associated arterial remodeling [209].

Cues to halt the proteolysis-driven arterial remodeling in part involve a reduction of the primary stimuli, such as reduced shear stress. Furthermore, there is an increased expression of TGF-β1 within collateral vessels undergoing vascular

remodeling [210]. Local infusion of TGF-β1 increases the capacity of collateral vessels in the immediate vicinity to undergo remodeling [210]. Considering that TGF-β1 promotes with production of TIMP-1, 3 and PAI-1, resulting in the restraint of proteolysis (as described in Sect. 3), TGF-β1-induced signaling may act to moderate or shut off the proteolytic activity within the vessel wall.

7 Conclusions

The functional involvement of proteases within the vascular wall is critical for the vascular remodeling that enables blood vessels to undergo structural reorganization in response to an altered hemodynamic environment or in response to metabolic or inflammatory cues emanating from the abluminal side of the vessel. The capacity of cells within the vessel wall to express an extensive repertoire of proteases, including many members of the MMP family and components of the PA/plasmin system, provides multiple mechanisms to initiate the proteolysis of specific targets at localized sites at the surface of endothelial and smooth muscle cells as well as adventitial cells. Studies to date suggest that there is a substantial degree of functional redundancy in the roles of individual proteases, as individual knockouts of MMPs or PA/plasmin members do not abrogate the formation and structural remodeling of the vasculature. It is equally important that the proteolytic actions of these enzymes capacity are limited temporally, so that integrity of the vessel wall is not compromised. Proteolytic inhibitors, namely, TIMPs and PAI-1, contribute to confining the location and the extent of proteolysis and for reestablishing a non-proteolytic environment at the termination of remodeling events. While the physiological regulation of these players ensures an appropriate transition between proteolysis and stabilization within vascular cells, the pathological upregulation of either proteases or inhibitors will disrupt vascular remodeling events, contributing to loss of vascular integrity and/or failure to establish the necessary structural adaptations. Thus, development of strategies for effective manipulation of the proteolytic balance within the vasculature may have potential clinical benefit in a wide range of vascular pathologies.

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Chapter 17
Emerging Role of Genetic Variants of Matrix Metalloproteinases Genes in Left Ventricular Dysfunction

Balraj Mittal, Avshesh Mishra, Anshika Srivastava, and Naveen Garg

Abstract Heart failure (HF) is defined as inability of the heart to pump sufficient blood to meet the body's metabolic demands. Left ventricular (LV) dysfunction with progressive HF is a condition in which the left ventricle of the heart becomes functionally impaired. The impaired ventricular function can be attributed to unfavourable ventricular remodelling. Among the pathways that contribute to remodelling process, matrix metalloproteinases (MMPs) appear to be of particular interest. It has been established that altered MMP activity under pathological conditions leads to a situation favouring proteolysis that results in adverse ventricular remodelling, leading to LV dilatation, loss of contractile function and progressive clinical heart failure. Therefore, the aim of the work described in this chapter is to explore the role of genetic variants of MMP genes [*MMP2* (C-735 T, rs2285053), *MMP7* (A-181G, rs11568818) and *MMP9* (R279Q, rs17576), (P574R, rs2250889), (R668Q, rs17577)] with LV dysfunction in coronary artery disease (CAD) patients. The study included 510 consecutive patients with angiographically confirmed CAD. Among patients with CAD, 162 with reduced left ventricle ejection fraction (LVEF ≤ 45) were categorised as LVD. The *MMP9* R668Q genetic variant was significantly associated with LVD (LVEF ≤ 45) (p-value = 0.007, OR = 3.48), while no significant difference was observed in the distribution of *MMP2* C-735 T, *MMP7* A-181G and *MMP9* R279Q, P574R genetic variants both at genotype and allele levels. Also the frequency of $MMP9_{279R,574P,668Q}$ haplotype comprising *MMP9* R668Q variation was significantly higher in reduced LVEF subjects (p-value = 0.008, OR = 1.83). The study concludes that *MMP9* R668Q polymorphism plays significant

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role in conferring genetic susceptibility to left ventricular dysfunction in coronary artery disease patients.

Keywords Left ventricular dysfunction • Left ventricle ejection fraction (LVEF) • MMP genetic variants • Coronary artery disease (CAD)

1 Introduction

A major challenge in human genetics is elucidating the role of sequence variations in genes that lead to an increased risk of disease. These genetic variants are not necessarily the cause of illness, but markers that will help in improving the diagnosis, therapeutics and risk assessment. A significant portion of current medical research is devoted to the pursuit of genetic variants that can be used to identify disease. Furthermore, the genetic complexity may also be compounded by heterogeneity, where different combinations of gene variants give rise to a similar phenotype. At present, major attention is increasingly focused on elucidating genetic susceptibility to the common multifactorial diseases that clinicians encounter on a daily basis and cardiovascular disorder such as heart failure is one of them [1].

Heart failure (HF) is defined as the inability of the heart to pump sufficient blood to meet the body's metabolic demands. Left ventricular (LV) dysfunction with progressive HF is a multifactorial, complex pathological condition in which the left ventricle of the heart becomes functionally impaired [2]. It is caused by several mechanical, neurohormonal and genetic factors. It has been established that extensive coronary artery disease (CAD) and/or myocardial damage is the leading cause of LV dysfunction [3].

The impaired ventricular function associated with heart failure can be attributed to declines in perfusion and to unfavourable ventricular remodelling. LV remodelling is an important structural indication in patients with progressive LV dysfunction, which results in alterations in left ventricular chamber geometry. Experimental and clinical studies have demonstrated that matrix metalloproteinases (MMPs) play important role in LV remodelling [4–6].

2 Matrix Metalloproteinases and LV Dysfunction Process

MMPs are zinc-dependent endopeptidases with varying substrate specificity and have the capacity to degrade many components of cardiac extracellular matrix (ECM). The cardiac ECM, connective tissue scaffold on which cellular elements are arranged, plays a vital role in the maintenance of myocardial structure and function, particularly that of the left ventricle. Physiological integrity of ECM structure is largely under the control of MMP family of endopeptidases, an activity of which maintains a balance between connective tissue synthesis and degradation. Altered MMP expression and activity under pathological conditions may lead to a situation

Table 17.1 Candidate genes and polymorphisms selected

Genes	Location	Type of polymorphism and rs no.	Minor allele frequency (MAF)	Functional role
MMP2	16q13-q21	C-735 T rs2285053	(0.11)	Decreased promoter activity with T allele
MMP7	11q21-q22	A-181G rs11568818	(0.40)	Altered transcription by influencing the binding of nuclear proteins
MMP9	20q11.2-q13.1 exon 6	R279Q rs17576	(0.55)	Affecting the binding capacity of enzyme with substrate
MMP9	20q11.2-q13.1 exon 10	P574R rs2250889	(0.14)	Affect both substrate and inhibitor binding
MMP9	20q11.2-q13.1 exon 12	R668Q rs17577	(0.28)	Affect both substrate and inhibitor binding

favouring proteolysis. MMPs play a key role in LV geometry and function by maintaining the balance between ECM synthesis and degradation. The ECM provides structural support for myocytes alignment and blood vessels and also coordinates the conversion of myocytes contraction into myocardial force [7].

A plethora of studies have highlighted that changes in the myocardial extracellular matrix contribute to the progressive remodelling process [8]. Among the pathways that contribute to ECM remodelling, the activation MMPs appear to be of particular interest [9]. Promising studies on animal models and expression analysis have shown that altered MMP activities under pathological conditions lead to a situation favouring proteolysis that result in adverse ventricular remodelling, leading to LV dilatation, loss of contractile function and progressive clinical heart failure [7, 10]. Several regulating mechanisms have been explored that may influence the activities of MMPs, such as regulation of transcription, activation of latent MMPs and inhibition of MMP function by tissue inhibitors of metalloproteinases. In fact, there is growing evidence to indicate that natural sequence variations including single-nucleotide polymorphisms (SNPs) in promoters of the *MMP* gene may result in different expression of MMPs in different individuals. Moreover, SNPs in the coding region, especially non-synonymous, may also influence the protein activity and therefore may be associated with LV remodelling and heart failure progression. Therefore, the aim of the work described in this chapter is to explore the role of genetic variants of matrix metalloproteinases genes with LV dysfunction. Based on the pathophysiology of LV dysfunction and the physiological role of MMPs, we have selected five genetic variants of three MMP genes for our study (Table 17.1).

3 Study Design for Elucidating Role of Genetic Variants of MMPs in LV Dysfunction

Here we have designed a case–control association study to elucidate the role of common genetic variants of MMPs in conferring genetic susceptibility to LVD in patients having significant CAD. For this purpose, patients with CAD requiring angioplasty

were enrolled from the cardiology clinic and their clinical profile was evaluated. Based on echocardiography, left ventricular ejection fraction was determined by Simpson's method [11]. The patients were categorised as LVD and non-LVD, based on their left ventricular ejection fraction (LVEF). The CAD patients with LVEF ≤ 45 % were considered as having LVD, while patients with LVEF >45 % were considered as non-LVD patients [12]. In addition to clinical profile, a 5 ml peripheral blood was collected in EDTA vial and stored at −80 °C for DNA extraction.

3.1 The Demographic Profile and the Clinical Characteristics of the LVD and Non-LVD Patients

The demographic profile and the clinical characteristics of the LVD and non-LVD patients are shown in Table 17.2. The mean age (56.24 ± 8.51 vs. 56.00 ± 9.88) and male/female ratio (138/24 vs. 301/47) were not significantly different between the LVD and non-LVD patients. Hypertension and diabetes are commonly observed in CAD patients but were not significantly different among LVD and non-LVD patients. Mean body mass index (BMI) was also comparable between the two subgroups. However, a higher percentage of LVD patients were smokers as compared to non-LVD patients (37 % vs. 21 %, $P<0.001$). The vessel diseases in LVD and non-LVD groups were also comparable. The frequency of STEMI (ST-elevation myocardial infarction), a well-known predictor of LVD, was also significantly different between the LVD and non-LVD patients (73.5 % vs. 37.4 %, $P<0.001$). Furthermore, on analysing the echocardiographic traits, a significant difference was found in mean left ventricle ejection fraction, left ventricle end diastole dimension, left ventricle end systolic dimension, interventricular septum end diastole dimension and left ventricle mass (Table 17.2).

3.2 Influence of MMP Gene Polymorphism Between LVD and Non-LVD Patient

Genomic DNA was isolated from stored EDTA blood by salting-out method [13] and used for genotyping MMP genetic polymorphisms in LVD and non-LVD CAD patients. The polymorphisms were genotyped using the PCR–RFLP method [14, 15]. The frequency distributions of MMP gene polymorphisms were statistically compared between LVD and non-LVD groups by binary logistic regression, using SPSS software version 16.0 (SPSS, Chicago, IL, USA). A two-tailed $p<0.05$ was considered statistically significant. The distribution of *MMP2* C-735 T, *MMP7* A-181G, *MMP9* R279Q, *MMP9* P574R and *MMP9* R668Q genetic variants with LVD is shown in Figs. 17.1, 17.2, 17.3, 17.4 and 17.5, respectively. A significantly higher percentage of CAD patients with *MMP9* 668QQ genotype had reduced

Table 17.2 Demographic and clinical characteristics of LVD and non-LVD patients

Demographic profile	LVD (LVEF ≤ 45)	Non-LVD (LVEF > 45)	*p*-value
Total subjects	**162**	**348**	–
Age[a], years	56.24 ± 8.51	56.00 ± 9.88	0.789
Male sex	138 (85.2 %)	301 (86.5 %)	0.682
Clinical characteristics			
Hypertension	69 (42.2 %)	163 (46.8 %)	0.340
Diabetes	51 (31.5 %)	97 (27.9 %)	0.404
Smoking	60 (37.0 %)	73 (21.0 %)	**<0.001**
BMI[a]	24.09 ± 2.93	24.65 ± 3.29	0.167
ST-elevation myocardial infarction (STEMI)	119 (73.5 %)	130 (37.4 %)	**<0.001**
Myocardial infarction (MI)	139 (85.8 %)	212 (60.9 %)	**<0.001**
Single vessel disease (SVD)	106 (65.4 %)	236 (67.8 %)	–
Double vessel disease (DVD)	36 (22.2 %)	84 (24.1 %)	–
Triple vessel disease (TVD)	20 (12.3 %)	28 (8.0 %)	0.296
Echocardiographic traits[a]			
Left ventricle ejection fraction (LVEF)	37.38 ± 7.47	54.86 ± 5.29	**<0.001**
Left ventricle end diastole dimension (LVEDD), mm	49.07 ± 6.99	44.48 ± 4.58	**<0.001**
Left ventricle end systolic dimension (LVESD), mm	34.42 ± 7.69	28.93 ± 4.87	**<0.001**
Posterior wall end diastole dimension, mm	9.59 ± 1.42	10.07 ± 1.49	**0.027**
Interventricular septum end diastole dimension, mm	9.91 ± 1.45	10.38 ± 1.76	0.051
Left ventricle mass (LV mass), gm	175.39 ± 53.04	158.38 ± 42.76	**0.014**

[a]Values are mean ± SD
Significant values are shown in bold

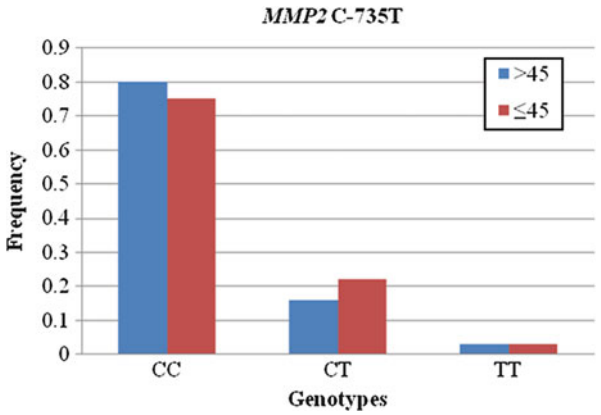

Fig. 17.1 Distribution of *MMP2* C-735 T gene polymorphisms in LVD and non-LVD subjects, as the *blue bar* indicates individuals with LVEF > 45 % (non-LVD), whereas the *red bar* indicates individuals with LVEF ≤ 45 % (LVD). The CC, CT and TT genotypes correspond to wild-type, heterozygote and variant genotypes, respectively. The corresponding *p*-values are: CT; *p*-value = 0.142, OR (95 % CI) = 1.42 (0.89–2.26). TT; *p*-value = 0.656, OR (95 % CI) = 1.29 (0.42–3.92)

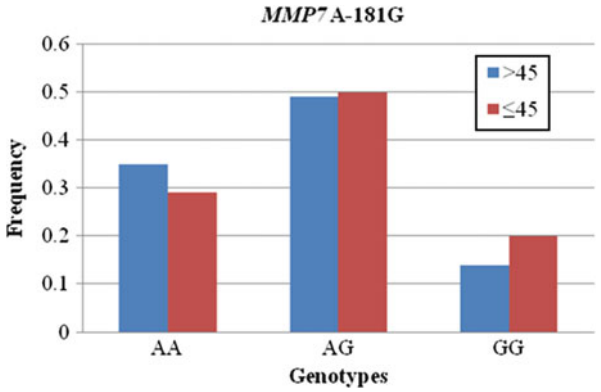

Fig. 17.2 Distribution of *MMP7* A-181G gene polymorphisms in LVD and non-LVD subjects, as the *blue bar* indicates individuals with LVEF >45 % (non-LVD), whereas the *red bar* indicates individuals with LVEF ≤45 % (LVD). The AA, AG and GG genotypes correspond to wild-type, heterozygote and variant genotypes, respectively. The corresponding *p*-values are: AG; *p*-value=0.291, OR (95 % CI)=1.26 (0.82–1.93). GG; *p*-value=0.065, OR (95 % CI)=1.68 (0.97–2.91)

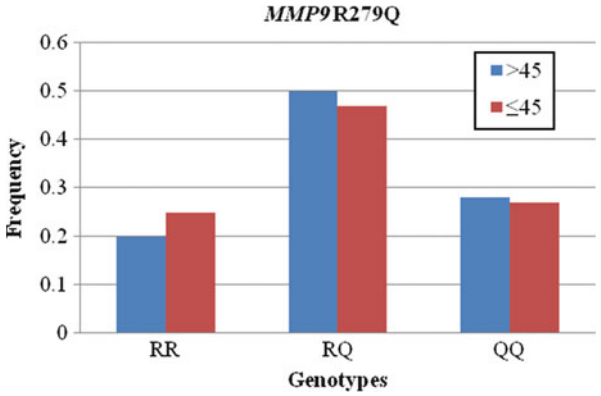

Fig. 17.3 Distribution of *MMP9* R279Q gene polymorphisms in LVD and non-LVD subjects, as the *blue bar* indicates individuals with LVEF >45 % (non-LVD), whereas the *red bar* indicates individuals with LVEF ≤45 % (LVD). The RR, RQ and QQ genotypes correspond to wild-type, heterozygote and variant genotypes, respectively. The corresponding *p*-values are: RQ; *p*-value=0.293, OR (95 % CI)=0.78 (0.49–1.24). QQ; *p*-value=0.379, OR (95 % CI)=0.79 (0.47–1.33)

ejection fraction (≤45 %) as compared to the patients with preserved (>45 %) ejection fraction (*p*-value=0.001, OR=3.48; Fig. 17.1e). As the heterogeneous *MMP9* 668RQ genotype is not found to be significantly different, so we have applied the dominant model (RR vs. RQ+QQ). In dominant model, RQ+QQ was significantly associated with LVD (OR=1.59, *p*-trend=0.030; Table 17.3). However, no significant difference was observed in the distribution of *MMP2* C-735 T, *MMP7* A-181G and *MMP9* R279Q, P574R genetic variants (Table 17.3).

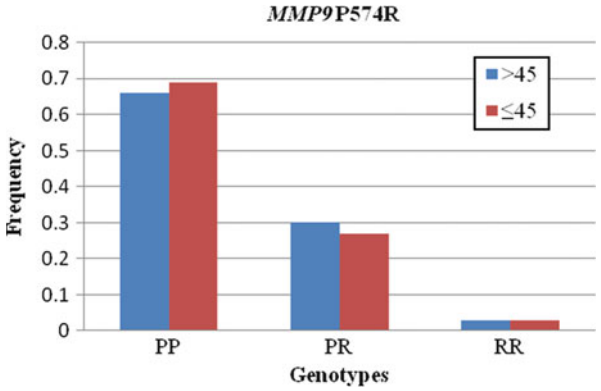

Fig. 17.4 Distribution of *MMP9* P574R gene polymorphisms in LVD and non-LVD subjects, as the *blue bar* indicates individuals with LVEF >45 % (non-LVD), whereas the *red bar* indicates individuals with LVEF ≤45 % (LVD). The PP, PR and RR genotypes correspond to wild-type, heterozygote and variant genotypes, respectively. The corresponding *p*-values are: PR; *p*-value=0.470, OR (95 % CI)=0.86 (0.56–1.30). RR; *p*-value=0.846, OR (95 % CI)=1.12 (0.36–3.45)

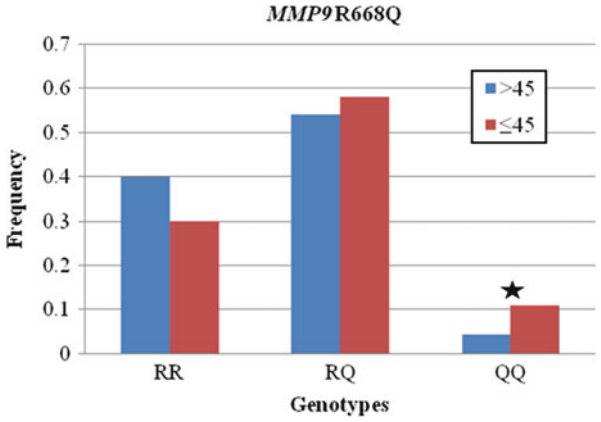

Fig. 17.5 Distribution of *MMP9* R668Q gene polymorphisms in LVD and non-LVD subjects, as the *blue bar* indicates individuals with LVEF >45 % (non-LVD), whereas the *red bar* indicates individuals with LVEF ≤45 % (LVD). The RR, RQ and QQ genotypes correspond to wild-type, heterozygote and variant genotypes, respectively. The corresponding *p*-values are: RQ; *p*-value=0.078, OR (95 % CI)=1.45 (0.96–2.17). QQ; *p*-value=0.001, OR (95 % CI)=3.48 (1.63–7.43). *Significant *p*-value

To further evaluate the role of genetic variants of MMPs in LVD, we calculated a genotype score (G-score) for investigated SNPs. In fact, G-score is a cumulative number that counts the total number of risk-increasing alleles in an individual [16]. An arbitrary value of 3, 2 and 1 was allotted to homozygous variant and heterozygous and homozygous wild-type genotypes, respectively, of each MMP polymorphism.

Table 17.3 Single locus analysis of investigated SNPs between LVD and non-LVD patients

Gene polymorphisms	MAF (%) (LVD)	MAF (%) (Non-LVD)	*p*-value	OR[a] (95 % CI)	OR[b] (95 % CI)	OR[c] (95 % CI)	*p*-trend
MMP2 C-735 T	13.9	10.9	0.170	1.42 (0.89–2.26)	1.28 (0.42–3.92)	1.40 (0.90–2.18)	0.108
MMP7 A-181G	45.4	39.5	0.079	1.26 (0.82–1.93)	1.68 (0.97–2.91)	1.36 (0.90–2.03)	0.159
MMP9 R279Q	50.6	53.6	0.386	0.78 (0.49–1.24)	0.79 (0.47–1.33)	0.78 (0.50–1.21)	0.216
MMP9 P574R	16.4	17.7	0.571	0.86 (0.56–1.30)	1.12 (0.36–3.45)	0.88 (0.59–1.31)	0.554
MMP9 R668Q	40.1	31.6	**0.007**	1.45 (0.96–2.17)	**3.48 (1.63–7.43)**	**1.59 (1.07–2.37)**	**0.030**

Significant values are shown in bold

[a]Odds ratio of heterozygote vs. wild-type homozygote genotypes

[b]Odds ratio of variant homozygote vs. wild-type homozygote genotypes

[c]Odds ratio of heterozygote variant homozygote vs. wild-type homozygote genotypes

Table 17.4 Mean *G*-scores of investigated SNPs in LVD and non-LVD patients with their corresponding *p*-values

Gene polymorphisms	LVD	Non-LVD	*p*-value
MMP2 C-735 T	1.28 ± 0.52	1.22 ± 0.47	0.177
MMP7 A-181G	1.91 ± 0.70	1.79 ± 0.68	0.066
MMP9 R279Q	2.02 ± 0.72	2.07 ± 0.70	0.405
MMP9 P574R	1.33 ± 0.53	1.36 ± 0.53	0.650
MMP9 R668Q	1.81 ± 0.62	1.64 ± 0.56	**0.002**

Significant values are shown in bold

Using these 5 SNPs a G-score was constructed ranging from a minimum of 5 to a maximum of 15 on the basis of the number of risk alleles. For each individual, a consolidated G-score was calculated by adding the values from all 5 SNPs together. The results of G-scores also suggested significant role of *MMP9* R668Q polymorphism in LVD susceptibility (1.81 ± 0.62 vs. 1.64 ± 0.56; $p = 0.002$, Table 17.4).

3.3 Influence of MMP9 R668Q on LV Remodelling

As our results showed that *MMP9* R668Q polymorphism is significantly associated with LVD, hence we compared the distribution of wild-type (RR) and variant (RQ + QQ) genotypes of *MMP9* R668Q polymorphism with clinical characteristics of CAD and other echocardiographic parameter of LV remodelling. There was no significant difference between the frequencies of RR and RQ + QQ with reference to clinical characteristics of CAD. Among echocardiographic traits, mean LVEF (p-value = 0.006), LV mass (p-value = 0.001) and LV dimensions (p-value = 0.010

Table 17.5 Clinical characteristics of patients with *MMP9* R668Q gene polymorphism

Clinical characteristics	RR 191 (%)	RQ+QQ 319 (%)	*p*-value
Age[a] at CAD diagnosis (years)	56.92±9.71	55.59±9.30	NS
Hypertension	79 (42.2)	153 (47.4)	NS
Diabetes	47 (25.1)	101 (31.3)	NS
Smoking	54 (28.9)	79 (24.5)	NS
BMI[a]	24.39±4.49	25.45±5.10	NS
Stable angina	64 (34.2)	101 (31.3)	NS
Unstable angina/non-ST-elevation myocardial infarction (NSTEMI)	46 (24.1)	66 (20.7)	NS
ST-elevation myocardial infarction (STEMI)	76 (39.8)	154 (48.3)	NS
Anterior wall myocardial infarction (AWMI)	38 (20.3)	89 (27.6)	NS
Inferior wall myocardial infarction (IWMI)	38 (20.3)	65 (20.1)	NS
Myocardial infarction (MI)	122 (65.2)	222 (68.7)	NS
Single vessel disease (SVD)	125 (66.8)	217 (67.2)	NS
Double vessel disease (DVD)	40 (21.4)	80 (24.8)	NS
Triple vessel disease (TVD)	22 (11.8)	26 (8.0)	NS
LVEF ≤ 45 (LVD)	49 (25.7)	113 (35.4)	**0.028**
Echocardiographic traits[a]			
Left ventricle ejection fraction (LVEF)	50.92±9.31	48.35±10.53	**0.006**
Left ventricle end diastole dimension (LVEDD), mm	44.7±5.15	46.7±6.15	**0.010**
Left ventricle end systolic dimension (LVESD), mm	29.4±5.38	31.4±6.92	**0.018**
Posterior wall end diastole dimension, mm	9.6±1.52	10.0±1.45	0.130
Interventricular septum end diastole dimension, mm	10.0±1.77	10.3±1.59	0.337
Left ventricle mass (LV mass), gm	151.2±41.43	173.2±49.15	**0.001**

[a]Values in mean±SD
Significant values are shown in bold

and 0.018) were significantly associated with variant (RQ+QQ) genotype (Table 17.5). When CAD patients were stratified on the basis of risk factors like diabetes mellitus, hypertension and smoking status, the *MMP9* R668Q did not modulate the risk of CAD due to these factors.

3.4 Distributions of MMP9 R668Q Genetic Variant in STEMI Subjects Between LVD and Non-LVD Patients

In clinical practice it is a well-known fact that STEMI patients are more prone to develop LVD [17]. We also observed that 73.1 % of STEMI patients had LVD. Therefore, we looked for distribution of *MMP9* R668Q polymorphism in STEMI patients between LVD and non-LVD patients. Our results showed that the subjects with *MMP9*668QQ genotype were more likely to develop LVD as compared to wild-type (RR) genotype (p-value = 0.032, OR = 3.46; Table 17.6). This indicates that CAD patients with severe or large myocardial infarction having *MMP9*668QQ genotype are more susceptible for LVD.

Table 17.6 Distributions of *MMP9* R668Q gene polymorphism in STEMI subjects with LVD and non-LVD patients

Genotypes/alleles	LVD	Non-LVD	*p*-value	OR (95 % CI)
RR	35 (28.5)	51 (41.1)	–	Reference
RQ	76 (61.8)	68 (54.8)	0.071	1.66 (0.96–2.86)
QQ	12 (9.8)	5 (4.0)	**0.032**	**3.46 (1.11–10.76)**

Significant values are shown in bold

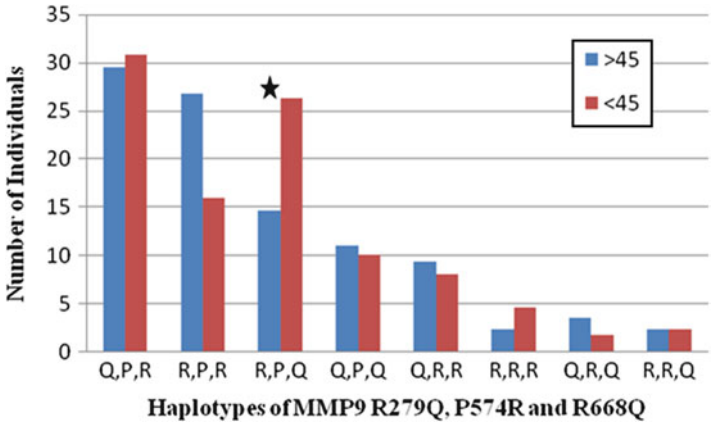

Fig. 17.6 Frequency distribution of haplotypes of *MMP9* R279Q, P574R and R668Q polymorphisms in LVD (LVEF ≤45 %) and non-LVD (LVEF >45 %) subjects. $MMP9R_{279},P_{574},Q_{668}$; *p*-value = 0.008, OR (95 % CI) = 1.83 (1.17–2.86). *Significant *p*-value

3.5 Haplotype Estimation and Their Association with LVD

Haplotype analysis was performed using the online program SNP-stat: a Web tool for the analysis of association studies [18]. Using SNP-stat, eight haplotype combinations were inferred. On analysing the frequency distribution of haplotypes in clinical parameter, we found that the frequency of $MMP9_{279R,574P,668Q}$ haplotype was significantly higher in LVD patients in comparison to non-LVD (26.3 % vs. 14.6 %) and was conferring high risk for LVD ($p = 0.008$, OR = 1.83; Fig. 17.6). It is biologically plausible that these non-synonymous SNPs of *MMP9* gene may work together in LVD development and progression, which is consistent with our observation of an association between combined variant loci and LVD risk. Moreover, *MMP9* 668Q allele or *MMP9* 279R, 574P and 668Q haplotype may be linked to other functional variants such as *MMP9* C-1562 T polymorphism, which has been associated with increased *MMP9* promoter activity. The increased promoter activity leads to higher level of MMP9 which is associated with adverse ventricular remodelling.

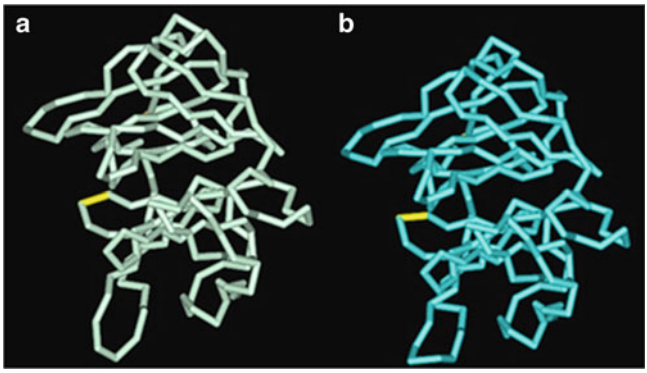

Fig. 17.7 Models derived from in silico molecular modelling of *MMP9* R668Q polymorphism where (**a**) represents the wild type and (**b**) represents the mutated structure (Reproduced with permission from Elsevier [8])

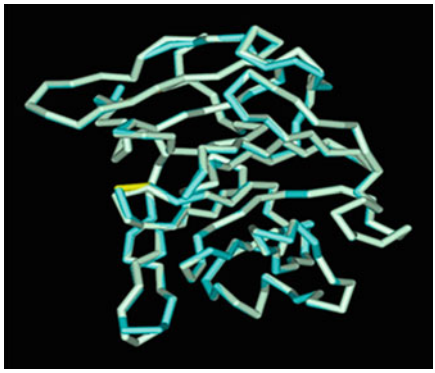

Fig. 17.8 Superimposed structure of *MMP9* R668Q polymorphism (both wild and mutated type), where *yellow colour* represents position of R668Q polymorphism (Reproduced with permission from Elsevier [8])

3.6 Comparative Modelling of MMP9 R668Q Polymorphisms

Since no experimental data and literature on the functional role of *MMP9* R668Q polymorphism exist so to explore the effect of *MMP9* R668Q polymorphisms on 3D structure and consequently function of MMP9 protein, molecular modelling of MMP9 protein was performed. Since MMP9 is 707 amino acid long protein, so the model was generated for the last 200 amino acids which includes R668Q, located in the C-terminal haemopexin-like domain. Molecular modelling was carried out by EasyModeller and 3D-PSSM program. To validate the wild and mutated structures, procheck analysis was performed, followed by Discovery Studio ver. 2.0 for modelled structure superimposition. Potential functional non-synonymous SNPs identification programmes, Polyphen-2, SIFT (Sorting Intolerant From Tolerant) and SNAP were used for the prediction of possible impact of amino acid substitution on protein function for *MMP9* R668Q polymorphism (Figs. 17.7 and 17.8).

Overall, more than 90 % of residues in both the models were in most favoured regions of Ramachandran plot, signifying appropriate modelling. The general root mean square deviation (rmsd) for the wild-type and mutated structure was 0.59A°, suggesting nonsignificant difference between wild and mutated structure due to R668Q polymorphism (Figs. 17.3 and 17.4). Also, R668Q polymorphism predicted to be 'benign' and 'tolerated' by Polyphen and SIFT computational tools, but results of SNAP were contradictory to these findings, although its reliability index was very low, but predicted 'non-neutral', i.e. significant change in structure.

Although in silico molecular modelling failed to pinpoint the exact functional role of *MMP9* R668Q polymorphism in the function of MMP9, it can be mentioned that *MMP9* R668Q polymorphism located in the C-terminal haemopexin-like domain affects both substrate and inhibitor binding [19]. This domain plays a major role in regulating MMP9 activation, recognition and inhibition by binding different regulatory proteins. The above findings indicate significant remodelling in patients with variant genotype (RQ + QQ) in comparison to wild-type (RR) genotype. Moreover, another MMP9 polymorphism, R279Q, occurs in the coding region within the fibronectin type II domains and plays important roles in substrate binding [20] and P574R, also located in the haemopexin-like domain, thought to affect both substrate and inhibitor binding. Therefore, the three variations altogether may potentially alter the protein structure of MMP9 and may have some functional relevance to affect individual's susceptibility to LVD. However, it still remains unclear whether the effect of *MMP9* gene variants is due to its influence on mRNA stability or translation efficiency or the polymorphism is in LD with other nearby functional variants.

4 Possible Mechanism of Heart Failure

The myocardial damage due to ischemic insult results in abrupt infiltration of neutrophils followed by the activation of *MMP9* in extracellular matrix. A single-nucleotide change in *MMP9*, i.e. R668Q, may increase the matrix metalloproteinases activity. Enhanced activity of MMP9 may contribute to degradation of intermyocyte collagen struts. This event creates wall thinning and ventricular dilatation which further elevates stress on ventricular wall. These series of steps worsen the ventricular function which in later stages might progress to heart failure (Fig. 17.9).

5 Conclusions

The present study indicates that *MMP9* R668Q polymorphism increases susceptibility to LVD in CAD patients by modulating disease severity due to increased ventricular remodelling and exacerbating declining LV function. These findings may have important implications in the understanding of pathobiology of ventricular remodelling and heart failure.

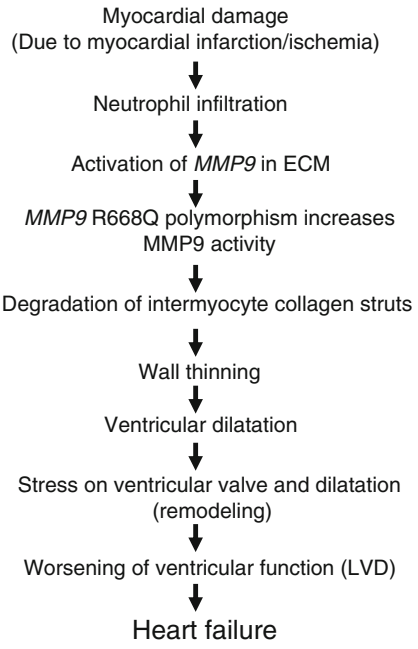

Fig. 17.9 Possible mechanism of heart failure

MMPs have been recognised to play a critical role in the pathological myocardial remodelling processes, so now it is important to link clinical outcome research to fundamental aspects of MMP biology. The new diagnostic and therapeutic targets against MMPs can be tried to reduce LV remodelling process, which potentially could improve outcome for CAD patients suffering from LV dysfunction.

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Chapter 18
Implications of Intracellular Proteolytic Activation of MMP-2 in the Heart

Marcia Y. Kondo and Richard Schulz

Abstract Matrix metalloproteinases (MMPs) are a family of metalloproteases comprised of 25 related members, of which 24 are found in mammals. By cleaving their target proteins, MMPs play regulatory roles in signaling events, control the cellular environment, and modulate many bioactive molecules at the cell surface to influence cell behavior. However, MMPs are also localized inside the cell and can cleave intracellular substrates. In the heart, MMP-2 is widely expressed in nearly all cells and plays important roles in a variety of physiological and pathological processes, ranging from heart development to ischemia–reperfusion (I/R) injury that triggers an acute loss in heart contractile function. MMP-2 is abundantly expressed in cardiac myocytes and is directly activated by oxidative stress. This results in the S-glutathiolation of a critical cysteine in the prodomain which removes its coordination to the catalytic zinc and allows access of substrates to its catalytic domain, resulting in the proteolysis of specific sarcomeric and cytoskeletal intracellular proteins.

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MMP-2 activity is also regulated by its phosphorylation. Intracellular substrates of MMP-2 include troponin I, titin, myosin light chain 1, α-actinin, and glycogen synthase kinase-3β. The hydrolysis of specific sarcomeric and cytoskeletal proteins by MMP-2 contributes to contractile dysfunction after I/R injury pointing towards inhibition of MMP-2 as a possible therapy for the treatment of heart diseases associated with enhanced oxidative stress.

Keywords Matrix metalloproteinase-2 • Intracellular proteolysis • Oxidative stress • Ischemia–reperfusion • Doxycycline

1 Introduction

Proteolytic enzymes, proteases, proteinases, or peptidases are synonymous terms for enzymes that cleave other proteins or peptides. They are currently classified into seven classes according to MEROPS database: serine-, cysteine-, aspartic-, metallo-, threonine-, glutamic-peptidases, and asparagine peptide lyases [1].

The proteases are found in all organisms from viruses to humans, comprise more than 2 % of the human genome, and occur inside or outside of the cell. They control multiple biological processes including the cell cycle, autophagy, signaling, wound healing, inflammation, food digestion, protein and organelle turnover, immune response, infectious diseases, aging, blood pressure, cancer, and degenerative diseases among others [2, 3]. All these characteristics make proteolytic enzymes interesting targets for the development of prognostic biomarkers and drugs to be used in the treatment of many diseases. Some successful medications involving proteases include the captopril-like drugs, inhibitors of angiotensin-converting enzyme which are used in the treatment of hypertension and heart failure; the HIV-1 protease inhibitors present in anti-HIV cocktails; and human kallikrein-3, more popularly known as prostate-specific antigen, used as a diagnostic test for prostate cancer [4].

In the heart, proteases have been reported to participate in numerous processes either in physiological or pathological conditions [5–7]. We describe along this chapter the features of an important group of proteases in the heart, focusing on matrix metalloproteinases (MMPs), especially MMP-2.

2 Matrix Metalloproteinases

A family of structurally related zinc metalloproteases called matrix metalloproteinases (MMPs) is found in all vertebrates. The first report of MMP activity came in 1962 by Gross and Lapiere. They described a collagen-degrading activity in the culture medium of a tadpole undergoing morphogenesis which accounted for the hydrolysis of collagen in the tail as it is resorbed [8].

MMPs therefore play important roles in many physiological processes such as embryonic implantation, development, immune functions, and tissue remodeling but are also involved in pathological conditions including multiple aspects of cancer initiation and progression, inflammation, autoimmune diseases, osteoarthritis, vascular diseases, and neurodegenerative disorders [9–15]. Although MMPs have long been recognized for their ability to catalyze the hydrolysis of extracellular matrix (ECM) proteins, more recently attention has focused on MMPs activity to proteolyze non-extracellular matrix substrates both outside and inside cells [16–18].

There are currently 23 different endopeptidases identified as human MMPs [19]. There are some variations in their structure, but in general, MMPs are multidomain enzymes possessing several common structural characteristics. They have an N-terminal signal sequence which allows for subcellular targeting and extracellular export of the enzyme. However, the signal sequence of MMP-2 was recently found to be very inefficient in targeting the nascent protein to the endoplasmic reticulum for the secretory pathway, resulting in almost 50 % of MMP-2 in the cytosol. A splice variant of MMP-2 lacking the signal sequence was also found; therefore there are at least two intracellular MMP-2 moieties [20].

MMPs are synthesized in an inactive zymogen form with an autoinhibitory propeptide domain. The propeptide domain shields the neighboring Zn^{2+}-containing catalytic domain. The propeptide domain also contains a highly conserved PRCGVPD sequence, which is believed to assist in the binding of the cysteine thiol to Zn^{2+} in the catalytic domain, preventing access of substrate into the catalytic site. The PRCGVPD sequence thus plays an important role in the regulation of MMP activity [21, 22].

MMPs can be classified into five subgroups based on their primary structure, substrate specificity, and subcellular localization as collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-12), matrilysins (MMP-7 and MMP-26), and membrane-type (MT)-MMPs (MT1-MMP through MT6-MMP) [23]. This original system of MMPs classification is now questioned due to the ability of several MMPs to biologically hydrolyze and target various non-matrix substrates both outside and inside the cell [17].

The activities of MMPs are regulated by diverse mechanisms at every step from their induction to their inhibition and degradation [9]. These include (a) gene transcription and translation; (b) posttranslational modifications such as glutathiolation and phosphorylation; (c) interaction with tissue inhibitors of metalloproteinases (TIMPs 1–4), which are their endogenous inhibitors; and (d) by compartmentalization (their intra-/extracellular localization) [24, 25].

2.1 MMPs in the Heart

Information about the role of MMPs in cardiac physiology is still scanty. In normal hearts, MMPs are present predominantly in their zymogen form (proMMPs) and are

often co-expressed in complex with their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [26]. The presence of MMPs in the heart has been implicated in early heart development such as the role of MMP-2 in heart tube formation [27]. MMP-2 has also been shown to play important roles in angiogenesis [28] and heart valve development [29].

Although under pathological conditions the levels of MMPs 1, 2, 3, 7, 8, 9, 12, 13, and 14 have been reported to respond to cardiac tissue repair stimuli after chronic permanent coronary artery occlusion in humans and animal models, MMP-2 and MMP-9 are the most highly studied MMPs reported in heart and cardiovascular diseases including atherosclerosis, hypertension, heart failure, and ischemic heart diseases [30]. MMP-2 mRNA [29] and protein [31] are abundantly expressed in heart tissue [32], whereas MMP-9 is normally associated with activated inflammatory cells such as leukocytes and macrophages and is not present in cardiac myocytes, aortae, or vena cava from healthy humans and rats [33].

3 MMP-2 in the Heart

In the heart, MMP-2 is ubiquitously expressed in normal cardiac myocytes [31, 34] and fibroblasts [35], which implies that it has roles in normal heart physiology and which underlies several reports of its involvement in cardiac pathologies.

MMP-2 like other MMPs is synthesized as an inactive zymogen of 72 kDa. The autoinhibitory, hydrophobic propeptide domain PRCGVPD that shields the catalytic site of the enzyme is present at its N-terminus. The catalytic site contains a Zn^{2+} ion that is essential for its activity. Activation of MMP-2 can occur in the pericellular and extracellular compartments. The proteolytic cleavage of the propeptide domain present in 72 kDa MMP-2 occurs by MMP-14 in a multistep pathway that also involves tissue inhibitor of metalloproteinase 2 (TIMP)-2, resulting in enzymatically active 64 kDa MMP-2 in the extracellular compartment [36].

However, it is not necessary for 72 kDa MMP-2 to lose its propeptide domain in order to become an active protease. MMP-2 can also be activated upon exposure to reactive oxygen/nitrogen species such as peroxynitrite ($ONOO^-$), an important mediator of oxidative stress injury inside the cell via nonproteolytic modulation of the 72 kDa form via the S-glutathiolation of the cysteine residue in its inhibitory propeptide domain involved in its activation [37]. The cysteine moiety in the PRCGVPD propeptide sequence was found to be S-glutathiolated by low concentrations of $ONOO^-$ (1–10 μM), a modification that changes the conformation of the propeptide allowing contact of the substrate to the catalytic Zn^{2+} domain, thus resulting in its activation [37] (Fig. 18.1). On the other hand, it was also observed that higher concentrations of $ONOO^-$ (30–100 μM) led to MMP-2 deactivation [37, 38].

Phosphorylation is another significant regulator of MMP-2 activity. MMP-2 as expressed in the human fibrosarcoma (HT-1080) cell line is phosphorylated on S32, S160, S365, T250, Y271, and likely also on several other residues.

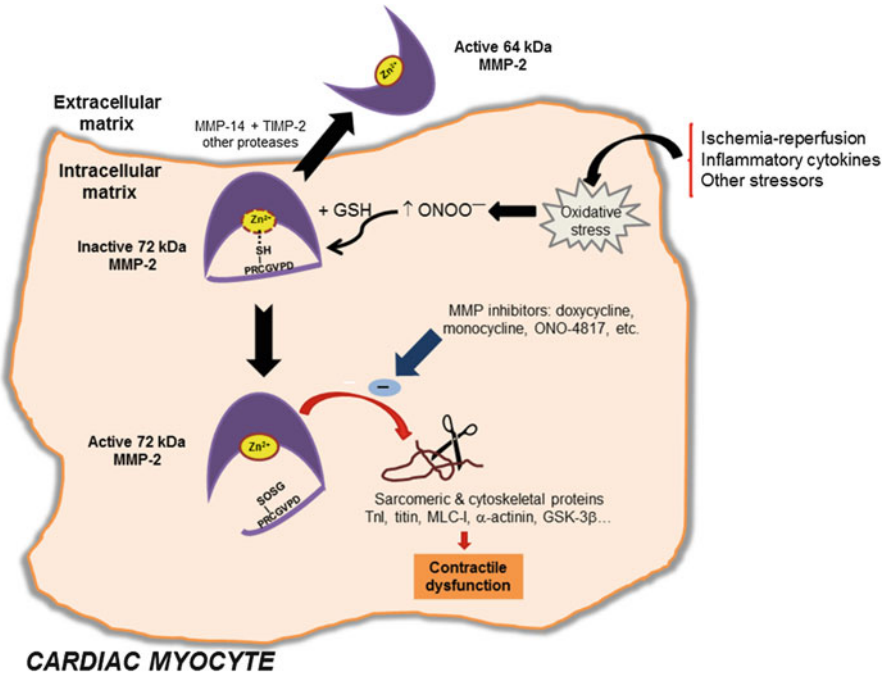

Fig. 18.1 Paradigm of MMP-2 in the cardiac myocyte. 72 kDa MMP-2 can be activated extracellularly by MMP-14 in the presence of TIMP-2 (or by other proteases) by the cleavage of its propeptide domain, yielding an active 64 kDa MMP-2 which acts on the extracellular matrix. I/R injury, proinflammatory cytokines, or other factors can cause an increased biosynthesis of peroxynitrite ($ONOO^-$) inside the cell that in the presence of cellular glutathione can result in the nonproteolytic activation of 72 kDa MMP-2 (without losing the propeptide domain). This activated 72 kDa MMP-2 can act intracellularly to hydrolyze susceptible sarcomeric and cytoskeletal proteins, causing myocardial contractile dysfunction. MMP-2 can be inhibited by sub-antimicrobial dosage of doxycycline, which improves contractile function

Phosphorylation of MMP-2 greatly reduces its enzymatic activity [39]. In silico analysis of the MMP-2 protein sequence shows that several kinases, including protein kinase C, protein kinase A, and glycogen synthase kinase-3, are potentially able to phosphorylate MMP-2 and consequently modulate its activity. However, the protein kinases and phosphatases responsible for MMP-2 phosphorylation in vivo are still unknown. In isolated rat hearts treated with okadaic acid at a low concentration that selectively inhibited protein phosphatase 2A but not protein phosphatase 1 activity, myocardial MMP-2 was kept in a more phosphorylated and less active state, which may account in part for the cardioprotective action observed with this inhibitor in hearts subjected to ischemia–reperfusion (I/R) injury [39]. More information about the phosphorylated and dephosphorylated forms of MMP-2 in the heart is needed; however, these data indicate that various posttranslational modifications in the cytosol can act to regulate intracellular MMP-2 as previously suggested [17, 40].

3.1 Extracellular MMP-2 in the Heart

The extracellular matrix actions of MMP-2 in the heart have been implicated to be crucial in embryonic heart development including angiogenesis, valve development, and heart tube formation [27–29]. In the latter, there seems to be a significant role of MMP-2 as demonstrated in chick embryos, where an MMP-2-neutralizing antibody or a selective MMP inhibitor were shown to inhibit MMP-2 activity and produce severe heart defects, including cardia bifida, abnormal left–right patterning, and a disruption in the looping direction, suggesting a key role of MMP-2 in cell migration and remodeling required for normal heart development [27]. Moreover, MMP-2 knockout mice survive at birth and are viable as adults; however, they display significantly retarded growth in comparison to the wild-type controls [41].

MMP-2 has also been implicated in various heart pathologies including postinfarct extracellular matrix remodeling [42]. In the myocardium of spontaneously hypertensive heart failure rats, there are reports of an increase in MMP-2 activity with age and cardiac hypertrophy [42, 43]. Increase of MMP-2 activity is also reported in parasitic myopathies caused by *Trypanosoma cruzi* (Chagasic cardiomyopathy) and coxsackievirus B3 (coxsackievirus B3 myocarditis) [44, 45].

3.2 Intracellular MMP-2 in the Heart

Ischemic heart disease is the most common cause of death in most western countries. There are many mechanisms that mediate I/R injury including an imbalance in energy substrate preference for ATP production and dysregulation of Na^+/H^+ as well as Na^+/Ca^{2+} exchangers [46]. Moreover, there is accumulation of intracellular Ca^{2+} due to impairment of sarcoplasmic reticulum Ca^{2+} uptake leading to activation of Ca^{2+}-dependent proteases and phospholipases [47, 48]. The sudden reintroduction of molecular oxygen during reperfusion reenergizes mitochondria and reactivates the electron transport chain, causing a rapid and large increase in the biosynthesis of reactive oxygen/nitrogen species including $ONOO^-$, the reaction product of nitric oxide and superoxide which activates MMP-2 [37, 49] (Fig. 18.1). Studies of the pathogenesis of myocardial I/R injury revealed a structural and functional remodeling of intracellular matrix components in the cardiac myocyte [50]. MMP-2 activation by $ONOO^-$ in particular has been shown to play an important role in pathogenic processes of the very early steps of myocardial I/R injury, also known as myocardial stunning [29] when myocardial muscle is still reversibly injured.

Besides the well-known activity of MMP-2 in extracellular matrix remodeling, studies from our group have shown that MMP-2 is localized to specific compartments inside the cell and also has very important intracellular roles [17]. MMP-2 is activated inside cardiac myocytes in injuries associated with increased oxidative stress including I/R injury [31] or exposure to proinflammatory cytokines [51], thus

cleaving susceptible intracellular proteins that are essential for myocyte contractile function, such as troponin I [34, 51], myosin light chain-1 [52], α-actinin [53], GSK-3β [54], and titin [55] (Fig. 18.1).

The cleavage of specific intracellular substrates by MMP-2, as a result of oxidative stress injury in the heart results in acute myocardial contractile dysfunction, demonstrates the importance of an intracellular fraction of MMP-2 that is not secreted from the cardiomyocyte. This results from two events: (1) a splice variant of MMP-2 lacking the signal sequence is expressed in both neonatal and adult human cardiomyocytes making it present exclusively in the cytosol, and (2) the signal sequence of canonical MMP-2 is inefficient in its ability to target newly synthesized MMP-2 to the endoplasmic reticulum and thereby does not restrict it to only the secretory pathway, resulting in a considerable portion of canonical MMP-2 which resides in the cytosol [17, 20, 40, 50].

In fact, there is evidence of diverse forms of intracellular MMPs. Lovett et al. showed a novel intracellular, N-terminal-truncated 65 kDa MMP-2 isoform which clearly distinguishes it from the 64 and 72 kDa MMP-2 forms. The 65 kDa MMP-2 isoform is not found under basal conditions. Redox stress induced by hypoxia or I/R injury activates a latent promoter in the first intron of the MMP-2 gene, generating a truncated mRNA transcript encoding the N-terminal-truncated 65 kDa MMP-2 isoform. This isoform lacks the secretory signal sequence and inhibitory prodomain and is then present in the cytosol and mitochondria as an active enzyme. The 65 kDa MMP-2 isoform hydrolyzes inhibitory IkB-α, thereby activating NFkβ/NFAT mitochondrial-nuclear stress signaling, inducing of an innate immunity transcriptome [56]. Other intracellular MMPs have been reported in different tissues: MMP-26 in breast carcinomas [57], MMP-10 in brain striatal cells [58], and MMP-3 in human liver [59]; however, whether more MMPs are also localized inside the cardiomyocyte, as MMP-2, is unknown.

3.3 Intracellular Targets of MMP-2 in the Heart

Troponin I (TnI)

TnI was the first intracellular MMP-2 substrate identified among the family of MMPs which shows that these proteases may have many yet undiscovered biological actions. TnI is a regulatory protein of actin–myosin interaction present in the thin myofilaments, known to be rapidly proteolyzed during acute I/R injury [34]. Our group showed using immunogold electron microscopy that MMP-2 was localized to the sarcomere in cardiac myocytes and confocal microscopy, and immunoprecipitation experiments revealed that MMP-2 also colocalized with TnI. TnI was highly susceptible to proteolysis by MMP-2 in vitro, and loss of TnI during I/R injury in isolated rat hearts was attenuated by MMP inhibitors, indicating that myocardial stunning injury is caused in part by MMP-2-mediated proteolysis of TnI.

Myosin Light Chain 1

The discovery of the localization of MMP-2 to the sarcomere led us to look for other intracellular MMP-2 substrates in hearts subjected to I/R injury. By using a pharmacoproteomics approach, myosin light chain 1 was identified as an intracellular MMP-2 target [52]. Myosin light chain 1 was reported to undergo proteolytic degradation in hearts subjected to I/R injury [60]. MMP-2 activity was found in preparations of thick myofilaments (which contain myosin light chain 1) prepared from rat hearts; immunogold microscopy localized MMP-2 to the sarcomere in a pattern consistent with the known distribution of myosin light chain 1, and purified myosin light chain 1 was susceptible to proteolysis by MMP-2 in vitro with a cleavage site near the C-terminus identified by mass spectrometric analysis [52].

Titin

Titin is another sarcomeric protein to which MMP-2 is colocalized. It is the largest known mammalian protein (3,000–4,000 kDa) and is found in both cardiac and skeletal striated muscles. It forms an intrasarcomeric elastic filament spanning nearly half the length of the sarcomere, from its N-terminus anchored at the Z-disc to its C-terminus in the M-line region. Titin also has elastic segments in the I band region that allow it to serve as a molecular spring. The titin molecule is the framework for the organized assembly of other myofilament proteins, thus helping to maintain the structural and functional stability of the myocyte [61]. Titin is also the molecular superstructure on which sarcomeric proteins are assembled during sarcomerogenesis in embryonic myocytes. In cardiac muscle, titin is of vast importance since it is a determinant of both diastolic and systolic function and the Frank–Starling mechanism of the heart [62]. We found that MMP-2 localizes to the Z-disc region of titin. Cleavage of titin in perfused rat hearts subjected to I/R injury, or in skinned cardiomyocytes incubated with MMP-2, was prevented with MMP inhibitors *o*-phenanthroline or ONO-4817. Hearts from MMP-2 knockout mice subjected to I/R in vivo did not show loss of titin content [55]. These data indicate that MMP-2 plays an important role in titin homeostasis, which directly affects the contractile function of the heart at the sarcomeric level.

Cytoskeletal Targets

Myocardial stunning injury in isolated guinea pig hearts is accompanied by the degradation of the cytoskeletal proteins desmin, spectrin, and α-actinin, although the protease(s) responsible was not identified [63]. α-Actinin is a cytoskeletal protein found at the Z line of the sarcomere. It connects actin filaments in adjacent sarcomeres and thus serves as a pivotal protein in transmitting the force generated

by the actin–myosin complex. Our group found that α-actinin and desmin (but not spectrin) are susceptible to cleavage by MMP-2 in vitro. Infusion of $ONOO^-$ into isolated, perfused rat hearts caused activation of MMP-2 with concomitant loss of myocardial α-actinin content, which was preventable by a selective MMP inhibitor, PD-166793 [53].

Nuclear Targets

The nuclear matrix has similarities with that of the extracellular matrix with a proteinacious structure that resembles the extracellular matrix in that it imparts structure and organization, as well as provides support for various processes [64], although the exact composition of the nuclear matrix is unknown. Proteolysis of nuclear matrix proteins, such as poly-ADP-ribose polymerase-1 (PARP-1) and X-ray cross-complementary factor 1 (XRCC1), is involved in important cellular processes such as apoptosis and regulation of the cell cycle [65, 66]. Kwan et al. discovered that MMP-2 activity and protein were found in nuclear extracts from human hearts where it co-immunoprecipitates with PARP-1, a chromatin-associated enzyme with multiple functions [67]. MMP-2 was able to efficiently cleave PARP-1 in vitro, and this may be a further means to regulate PARP-1 activity. Indeed, PARP-1 is only one of several putative nuclear targets of MMP-2 identified by unbiased, high-throughput degradomic approaches [18]. Thus MMP-2 is very likely to have several yet undiscovered biological functions in the nucleus.

Other Targets

MMP-2 is also suggested to be involved in myocardial apoptosis. Kandasamy et al. showed that glycogen synthase kinase beta (GSK-3β) is a target of MMP-2. GSK-3β is a serine/threonine kinase abundantly expressed in eukaryotes and is important in regulating glycogen metabolism and processes such as the cell cycle, apoptosis, and cell polarity [68]. GSK-3β is susceptible to proteolysis during oxidative stress and is therefore dysregulated by its increased kinase activity. Incubation assays of MMP-2 with GSK-3β resulted in the time- and concentration-dependent cleavage of GSK-3β, showing that GSK-3β may be a target of MMP-2 and that MMP-2 mediates its activity through cleaving the N-terminal of GSK-3β which contains the autoinhibitory phospho-serine 9 residue. The activity of GSK-3β was significantly enhanced upon incubation with MMP-2 and was prevented by MMP inhibitors GM-6001 or ONO-4817. H_2O_2 stimulated GSK-3β activity in cardiomyoblasts, and this was prevented with MMP inhibitors [54]. This may suggest that cleavage and activation of GSK-3β may be an additional means of the contribution of MMP-2 to oxidative stress-induced cardiac dysfunction. Inhibition of GSK-3β activity can also be cardioprotective by reducing apoptosis in the ischemic heart [54].

3.4 Intracellular MMP-2 vs. Calpain

Besides MMP-2, other proteases have been described to have intracellular roles in the heart, especially the calpain family of enzymes. Calpains are cysteine proteases activated by intracellular Ca^{2+}. Since Ca^{2+} overload is a significant determinant of cardiovascular malfunction, calpains are thought to contribute to heart disease. Calpains participate in a variety of cellular processes including cytoskeletal and sarcomeric remodeling, signal transduction, and cell death. Enhanced calpain activity by increased intracellular Ca^{2+} concentration following loss of Ca^{2+} homeostasis results in tissue damage as seen in myocardial infarct, stroke, and muscular dystrophy [69]. Increase in the intracellular concentration of Ca^{2+} can activate calpains which in turn may hydrolyze troponin I, α-fodrin, and ryanodine receptors and impair L-type Ca^{2+}-channel function [69, 70]. Two of the three ubiquitous forms of calpain are expressed in heart tissue: μ-calpain (calpain-1) requires micromolar Ca^{2+}, and m-calpain (calpain-2) requires millimolar Ca^{2+} for activation. Whether these are found directly in cardiac myocytes is unclear. Both calpains are heterodimeric proteins composed of a large 78–80 kDa subunit and a 29 kDa regulatory unit [71].

Calpain-1 has been implicated in the pathogenesis of myocardial stunning injury. Oxidative stress generated during I/R injury may result in the development of intracellular Ca^{2+} overload and activation of calpain activity in the heart [72]. Oxidative stress can also activate intracellular MMP-2 activity directly in the myocardium. Although the exact role of calpain in acute myocardial I/R is controversial [73], there are evidences of similar target proteins of MMP-2 and calpain such as troponin I, or calpain has been incorrectly identified as the protease responsible for some intracellular proteolytic activities. This can be inferred by the fact that calpain proteolysis of substrates in cardiac cells rests on the use of calpain inhibitors such as calpain inhibitor III (MDL-28170), ALLN, and PD-150606, which were found to also inhibit MMP-2 activity in vitro at commonly employed concentrations [74]. Thus, it would be interesting to investigate the suggested calpain substrates in the myocardium for their susceptibility to cleavage by MMP-2, and much more work is needed on the localization of calpains with their putative substrates.

4 MMP Inhibitors: Evolution and Clinical Application

The involvement of various MMPs in diverse human pathologies has attracted the attention of the pharmaceutical industry for the development of MMP inhibitors. About 60 MMP inhibitors have been pursued as clinical candidates since the first drug discovery program targeting this enzyme family began in the late 1970s. Major targeted indications included various cardiovascular diseases, cancer, arthritis, and chronic obstructive pulmonary disease [75]. Most pharmacological inhibitors of MMPs act by chelating the zinc ion in the enzyme's catalytic site. Such MMP inhibitors include batimastat, marimastat, GM-6001 (ilomastat/gelardin), o-phenanthroline, PD-166793, and ONO-4817. Although these compounds selectively inhibit MMP activity in comparison to other protease classes, they do not preferentially inhibit a

specific MMP [24]. Clinical trials in cancer with early MMPs inhibitors were unsuccessful due to the appearance of unanticipated side effects. However, these trials failed for several reasons including (1) limited target knowledge (the exact MMP to be inhibited was unknown during the development of the drugs), (2) lack of knowledge of intracellular MMP activity which has markedly broadened the biological roles of these proteases, (3) lack of understanding that MMPs can be activated by oxidative stress without requiring proteolytic removal of the propeptide domain, and (4) lack of understanding of MMP phosphorylation and whether drugs could be designed to target phosphorylated versus non-phosphorylated forms.

The role of MMPs has been since greatly expanded especially as a crucial pathological determinant in cardiovascular diseases associated with enhanced oxidative stress. The evidence that MMP-2 and other MMPs can be activated by prooxidant stress in the form of $ONOO^-$ suggests that they mediate some of the earliest detrimental actions of oxidative stress to the heart, particularly through the proteolysis of sarcomeric and cytoskeletal proteins [50]. Since there are currently no safe and therapeutically proven $ONOO^-$ blockers/scavengers, the ability to block the consequences of increased $ONOO^-$ stress using MMP inhibitors is a promising and novel alternative to treat cardiovascular diseases for which oxidative stress is a key component of the pathogenesis.

Tetracyclines are a group of broad-spectrum antibiotics commonly used to treat a variety of bacterial infections. Some members of the tetracycline class (doxycycline in particular) have been shown to have additional pharmacological actions, independent of their antibacterial effects, especially in their ability to inhibit MMP enzymatic activity, which for doxycycline occurs at sub-antimicrobial plasma concentration [76]. Of the tetracyclines, doxycycline, followed by minocycline, are the most potent MMP inhibitors. Doxycycline (in a sub-antimicrobial dose formulation) is the first and only clinically approved MMP inhibitor (by the US Food and Drug Administration and Health Canada) for the therapeutic treatment of periodontitis and rosacea.

An epidemiological study from the United Kingdom showed the possible clinical utility of tetracycline use for cardiovascular disease as it revealed a statistically significant reduction in the risk of first-time acute myocardial infarct seen only in patients who had taken tetracycline class, but not any other antibiotics of several different classes, comparing 3,315 such patients vs. 13,139 control subjects [77]. Thus, the cardiovascular benefits associated with tetracycline-class antibiotics may be connected to the inhibition of pathological MMP activity and suggest a possible therapeutic use. Furthermore, doxycycline was also able to prevent cardiac mechanical dysfunction triggered by endotoxic shock [78] and streptozotocin-induced diabetic cardiomyopathy in rats [79], models of heart failure known to involve increased biosynthesis of $ONOO^-$ [80]. Therefore, doxycycline is an attractive and possible alternative for the treatment of cardiovascular disease given its proven MMP inhibitor profile at sub-antimicrobial doses, low toxicity, excellent safety profile, and low cost. Although doxycycline is a possible candidate for therapy as an MMP inhibitor, the development of a drug which selectively blocks 72 kDa intracellular MMP-2 activated by $ONOO^-$ may become a next generation therapy for the treatment of many cardiovascular and other diseases.

5 Conclusions

Although originally thought to exclusively cleave proteins of the extracellular matrix and contribute to adaptive and maladaptive cardiovascular remodeling, MMP-2 is now also recognized as an important intracellular protease, and an increasing number of novel MMP-2 intracellular substrates and functions for it are being discovered. MMP-2 can be considered as an integral sarcomeric protein with roles in the proteolysis of susceptible sarcomeric and cytoskeletal proteins during injury to thus acutely diminish cardiac contractile function. Evidence suggests that MMP-2 may have biological actions in other subcellular compartments including caveolae, nuclei, and mitochondria. Although the role of MMP-2 in early myocardial I/R injury is becoming clear, more studies are needed to elucidate its role in myocardial cell death pathways and other cellular functions, both in physiological and pathological conditions. Finally, the development of specific intracellular MMP-2 inhibitors should be a possible therapeutic strategy to prevent or treat oxidative stress injury of the heart and other organs.

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Chapter 19
Matrix Metalloprotease-2 in the Development and Progression of Cardiovascular Diseases

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Abstract Matrix metalloproteases (MMPs) are a family of proteolytic enzymes that are regulated by a variety of signals that mediate changes in extracellular matrix (ECM). MMPs are important in the progression of cardiovascular diseases. MMP activation modifies the plaque architecture and may also be involved in the process of plaque rupture. MMPs participate in cardiac remodeling following myocardial infarction and in the development of cardiomyopathy. Among the MMPs, MMP-2 is one of the most ubiquitous members of the MMP family and is expressed in all cells of the heart. In the past two decades, there has been tremendous progress in understanding the role of MMP-2 in the development of cardiovascular pathology. In this review, we discuss the implications of MMP-2 in the progression and development of different types of cardiovascular diseases such as atherosclerosis, myocardial infarction, cardiomyopathy, and heart failure.

Keywords Matrix metalloproteases • Matrix metalloprotease-2 • Cardiovascular diseases • Hypertension • Atherosclerosis • Restenosis • Heart failure • Left ventricular remodeling • Aneurysm • Thrombosis

1 Introduction

Cardiovascular diseases are caused by dysfunction of the heart and blood vessels, which include diseases like atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction, stroke, and aneurysm. Cardiovascular diseases are the number one cause of death that occur globally [1]. The critical factors that

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have been ascribed to the risk of developing cardiovascular diseases include family history, sex, age, high blood pressure, elevated glucose, obesity, tobacco consumption, lack of physical activity, and abnormal blood cholesterol level [2, 3].

Matrix metalloproteases (MMPs), one of the important classes of proteases in biological system, can degrade the ECM and, therefore, may contribute to extensive remodeling of the tissue which leads to the pathological processes leading to cardiovascular diseases. Some excellent insights into the role of MMPs in ECM degradation and progression of cardiovascular diseases are available in the existing literature [4–6]. The role of MMPs in developing cardiovascular diseases such as the formation of atherosclerotic plaque and the development of cardiomyopathies is now well established. The progress of ventricular remodeling after myocardial infarction is also mediated through breakdown of the ECM components by MMPs. The result of this could lead to ventricular hypertrophy, which may ultimately cause congestive heart failure and death [7].

In this review, rather than focusing all MMPs, we restrict mainly in elucidating the role of MMP-2 in cardiovascular diseases. MMP-2 is abundantly and ubiquitously expressed in virtually all types of cells in the heart [8]. Several studies have shown that ECM degradation by MMP-2 plays an important role in the pathogenesis of a wide spectrum of cardiovascular disorders such as atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction, and aneurysm [9–14]. MMP-2 is emerging as an important signaling component implicated in the proteolytic regulation of various intracellular and extracellular proteins in the etiology and pathogenesis of cardiovascular diseases.

2 MMP-2 in Hypertension

A large number of reports are available in the existing literature in regard to the relationship between elevated blood pressure and the role of MMPs in human hypertension [15–18]. Hypertension causes fibrosis in the arterial wall, a process implicating involvement of MMPs [19, 20]. Several groups have shown an increase in MMPs, for example, MMP-2 level in human hypertension [21, 22], while others found a decrease in MMP-2 level, suggesting that the decrease in the protease results in collagen buildup in the arterial wall [23]. It seems that the conflicting reports could be due to differences in factors such as experimental design and the presence or absence of comorbidities and antihypertensive treatments within patient populations. However, the existing belief is in favor of a role of MMP-2 in hypertension.

Recent researches have shown the possibility that MMP-2 in the plasma cleaves substrates involved in the pathophysiology of hypertension and associated diseases. These substrates include membrane receptors such as insulin receptor, β2 adrenergic receptor (β2-AR), and vascular endothelial growth factor receptor-2 (VEGFR-2) [24–27]. These studies have used spontaneously hypertensive rats (SHRs) as the model system. The destruction of insulin receptors may explain insulin resistance in

the SHR and imply links between human hypertension and the metabolic dysregulation. Similar results have elicited for the β2-AR. Destruction of this receptor would lead to increased peripheral resistance and hypertension as the normal functioning of β2-AR mediates vascular smooth muscle cells (SMC) relaxation. This was further supported by the fact that a reduced vasodilation was observed in response to β2-AR agonist in both SHRs [28] and in human hypertension [29]. The origin of elevated MMP-2 activity in the hypertensives, however, remains unknown.

3 MMP-2 in Atherosclerosis

Formation of atherosclerotic plaques and its progression are one of the most prevalent pathologies of cardiovascular diseases and currently considered as the principal cause of mortality and morbidity in urban areas [30]. Atherosclerosis is characterized by the accumulation of lipids (atheroma) and fibrous elements (sclerosis) in the large arteries connecting the heart (coronary arteries) and the brain (carotid arteries) [30]. Proliferation of the intimal SMCs and their migration from the media occurs with accumulation of SMCs within the intima in atherosclerosis. Rearrangement of the surrounding ECM is essential for SMC proliferation or migration. A connection between MMP-2 and SMC proliferation and migration was evident from both in vivo and in vitro studies [31, 32]. The plaque formation in atherosclerosis occurs mainly in three phases: fatty streak formation at the early stage, matured plaque formation at the mid-phase, and plaque progression at the late phase. The rupture of atherosclerotic plaques is a result of several clinical events including myocardial infarction, stroke, and peripheral vascular diseases. The predominant cause of plaque rupture is thought to be the loss of ECM proteins within the lesions, such as the degradation of collagen and elastin, which normally accords with areas of inflammation. It has become increasingly evident that significant alterations in the structure and composition of the ECM play a key role in the atherogenic process. There is growing evidence that MMP-2 plays an important role in all stages of the atherosclerosis process, i.e., from the initial lesion to plaque rupture [33].

At early stage of atherosclerotic plaque formation, monocytes and lymphocytes penetrate into the tunica intima, passing through damaged endothelium caused by factors such as free radicals or shear stress. Monocyte recruitment is thought to be a crucial event in atherosclerosis development. For monocytes to invade tissues, it must be able to degrade the physical barrier represented by the ECM, and, therefore, protease activity is strongly required in which MMP-2 plays a vital role. MMP-2 level is elevated in human atherosclerotic plaques, especially at the macrophage-rich shoulder regions [5]. The activation of MMP-2 in this scenario could be achieved by serine proteases [34]. Thrombin has also been shown to proteolytically activate purified proMMP-2 in vitro and thus could provide cell-independent MMP-2 activation at sites of vascular lesion [35]. Pericellular activation of proMMP-2 can be achieved by MT-MMPs, expressed by vascular endothelial cells

(ECs) and SMCs in response to cytokines and oxidized lipoproteins [22, 36]. The plasminogen cascade represents another proteolytic-activating mechanism of proMMP-2 [22]. Proinflammatory molecules such as IL-1, TNF-α, PAF, LTD_4, and ox-LDL enhance MT1-MMP expression, which may lead to activation of proMMP-2 in SMC and that causes alteration in vascular remodeling in atherosclerotic human arteries compared to the normal one [37]. The most fragile sites in the plaque where most of the ruptures take place termed as shoulder regions. Furthermore, in situ zymography also unveiled that MMP-2 activity is augmented in the shoulder regions of advanced plaques, compared to normal arteries where MMP-2 level has also been detected [38, 39]. Elevated levels of MMP-2 have been shown in prone-to-rupture regions of unstable plaques and are co-localized with cleaved collagen fragments, the main ECM component of the external fibrous cap of plaque [40]. The overall findings uncovered crucial role of MMP-2 expression and its activity in the process of plaque progression, thus clinically significant in patients with advanced atherosclerotic disease.

4 MMP-2 in Myocardial Infarction

The activities of MMPs have been comprehensively evaluated in experimental studies. Myocardial measurements of MMPs in subjects in the context of myocardial infarction (MI) can be tricky. However, an early postmortem study of MMP-2 in human heart samples following a significant MI was performed by zymography, which revealed an increase in MMP-2 level in myocardial extracts taken from the MI region [41]. Zymographic study in the pericardial fluid of patients undergoing cardiac surgery for an MI showed increase in MMP-2 level [42]. Herzog et al. [43] reported that the increase in MMP-2 activity occurs just 1 h after coronary ligation in both the infarcted and noninfarcted zones. Cleutjens et al. [44] demonstrated an increase in MMP-2 activity in the infarcted left ventricle, which began on day two after acute myocardial infarction (AMI), peaked on day seven and thereafter begin to decline. They also demonstrated an increase in TIMP-2 mRNA in the infarcted zone 6 h after AMI, which reached a maximum on day 2 before going to decline. Changes in MMP activity in the remote area were not at all observed. Chen et al. [45] have also reported that MMP-2 level was increased in the myocardium at 1 week after MI and reached a maximum at 2–3 weeks in the infarcted zone; albeit low level of the enzyme activity was detected in the remote area. Serum MMP-2 levels in patients with AMI were found to be elevated twofold than the control subjects and sustained for about a week [46].

In a gene knockout mouse model of experimental MI, targeted deletion of MMP-2 gene attenuates an early cardiac rupture as well as late left ventricular (LV) remodeling after MI [13]. The incident was further supported by Matsumura and his colleagues [47], who undertook a task to look into the mechanistic detail behind the inhibition of LV rupture in MMP-2 knockout mice. Their work revealed that deletion of MMP-2 gene in mice is associated with suppression in degrading ECM

components including laminin and fibronectin in the infarcted myocardium and a marked improvement in their survival rates compared with wild-type mice. In addition, it was also established that selective pharmacological inhibition of MMP-2 attenuates LV remodeling after MI and protects against cardiac rupture, suggesting that inhibition of MMP-2 activity could be a potential therapeutic strategy for patients at risk for development of cardiac rupture after MI [47]. Interestingly, besides direct inhibition of MMP-2, its activity can also be blocked by targeting its upstream activators in post-MI model. Treatment with an endothelin A (ET_A) receptor antagonist sitaxsentan 3 days after MI prevented an increase in the level of MMP-2 and attenuates LV dilation [48]. Prediction in LV remodeling could also be possible by assessing circulating level of MMP-2, which was also correlated positively with changes in LV remodeling from 2 weeks to 6 months in AMI patients with successful coronary angioplasty [49]. Cell-based gene therapy for effective inhibition of MMP expression has also been a subject of improvement of cardiac function after MI. Upregulation of TIMP-3 expression via cell-based gene therapy contributed additional regulation of MMP level after MI, thereby boosting the structural and functional effects of cells transplanted at 3 or 14 days after MI in rats [50]. TIMP-2, the specific endogenous inhibitor of MMP-2, has been found to play protective role when administered externally to improve post-MI cardiac function. Adenoviral-mediated overexpression of TIMP-2 has also been shown to improve survival and preserve cardiac function and limit cardiac dilation and infiltration in a murine model of MI [51].

5 MMP-2 in Cardiomyopathy

Cardiomyopathy refers to diseases of the myocardium (heart muscle) in which the muscle becomes enlarged, thick, or rigid and may lead to irregularities in heart beats called arrhythmias. Several studies have investigated the role of MMP-2 in the pathology of cardiomyopathy. Dilated cardiomyopathy (DCM) is a common form of cardiomyopathic disease that is characterized by significant left ventricle (LV) chamber dilation and severe systolic dysfunction.

A major initial contribution made to understand the relationship between myocardial MMP-2 level and end-stage DCM was that of the Woessner's laboratory [52]. They have studied MMP-2 activity in myocardial extracts from DCM hearts and found a marked increase in MMP-2 activity in DCM patients, suggesting the role of MMP-2 in deteriorating the mechanical strength of the heart that leads to its weakening and subsequently dilation [52]. Another study by Rouet-Benzineb et al. [53] proposed that MMP-2 also contributes to proteolytic activation of bioactive signaling molecules in addition to its role in matrix degradation. By employing in situ zymography and confocal microscopy in homogenates of human hearts, the investigators showed that MMP-2 activity was associated within the cardiomyocytes in the DCM hearts compared to that observed for MMP-2 activity in the periphery of cardiomyocytes in the control hearts. The localization of MMP-2

activity within cardiomyocytes of DCM hearts suggests a role in its degenerescence [53]. These investigators also provided evidence that MMP-2 may degrade myosin heavy chain, which may affect the myocytes structure and function as well as cardiac contractility in the DCM hearts [53].

Downregulation of TIMP-2 at protein and mRNA levels also suggested an imbalance between MMP-2 and its inhibitors in cardiomyocytes of patients with cardiomyopathy [53]. A previous report also suggested that high plasma MMP-2 level is responsible for LV dilation and deteriorated LV systolic function in hearts of patients with hypertrophic cardiomyopathy (HCM) [54, 55]. The study of Kitaoka et al. [56] revealed that high MMP-2 level is associated with reduced LV systolic function in HCM patients suggesting MMP-2 may be involved in LV remodeling in HCM patients [56]. Inhibition of MMP-2 activity with doxycycline was shown to protect, at least partially, the development of diabetic cardiomyopathy [57]. The report shows that doxycycline prevents proteolytic cleavage of troponin I (TnI) facilitated by MMP-2, localized in the cardiac sarcomeres, and is responsible for improvement in contractile function of the heart. In a mouse model, increased expression and activity of MMP-2 in the heart after experimental *Trypanosoma cruzi* infection and the mortality were markedly reduced by treatment with the MMP-2 inhibitor, suggesting a role of MMP-2 in mediating acute Chagasic cardiomyopathy [58]. Furthermore, doxycycline diminishes cardiac inflammation and prevents death in *T. cruzi*-infected mice. In addition to these findings, there are some contrasting data regarding the role of MMP-2 in the pathogenesis of cardiomyopathy. Matsusaka et al. [59] have reported that selective ablation of the MMP-2 gene exacerbated survival and LV function in transgenic mice overexpressing TNF-α in cardiac tissue [59].

6 MMP-2 in Heart Failure

Regardless of the clinical phase of heart failure, elevated levels of MMP-2 were shown in plasma of patients with heart failure [60]. Later, Yamazaki et al. [12] showed that circulating serum MMP-2 level was increased in patients with congestive heart failure (CHF). Interestingly, among CHF patients, MMP-2 levels were found to be significantly higher in patients with severe CHF than in mild CHF [12]. However, their observation does not correlate the increased level of MMP-2 with the etiology of CHF. In a rat model, increased expression of MMP-2 was found to be associated with decreased cardiac tissue tensile strength and may cause systolic and diastolic dysfunction leading to heart failure [61]. The contribution of MMP-2 in heart failure was further strengthened by the study that MMP-2 protein and mRNA levels were upregulated in the compensatory hypertrophy and the failing LV and treatment with MMP inhibitor preserved LV dilation and systolic function in different animal model systems [62, 63]. LV remodeling known to be a central feature of heart failure progression has been suggested to be mediated through MMP-2 action. An in vivo study in post-infarcted heart shows a significant improvement in

the survival after MI in MMP-2 null mice, which was mainly attributable to the inhibition of the development of subsequent LV dysfunction. After MI, the zymographic MMP-2 level significantly and persistently increased in the LV, which has been suggested to contribute to LV remodeling progression leading to its dysfunction and failure [13].

7 MMP-2 in Restenosis

Restenosis refers to reoccurrence of "stenosis," i.e., narrowing of blood vessel, leading to restricted blood flow. Restenosis usually pertains to an artery or other large blood vessel that has become narrowed, received treatment to clear the blockage, and subsequently become narrowed again. MMP-2 can modulate matrix degradation and SMC migration and might thereby play a role in the pathogenesis of restenosis. Plasma MMP-2/TIMP-2 ratio as well as MMP-2 activity in the coronary sinus blood has been shown to be significantly increased within 24 h in patients with ischemic heart disease, who underwent percutaneous coronary angioplasty [64]. In addition, plasma MMP-2 levels were significantly higher in patients with late restenosis than in those without restenosis. Elevated serum level of MMP-2 has been shown to be associated with increased restenosis in patients treated with drug-eluting stents [10]. In a balloon injury rat carotid artery model, histopathological analysis after two weeks of injury showed that MMP-2 activity was significantly increased in the arterial tissue. Pharmacological modifications of cGMP are known to have potential vasodilatory activity [65]. Oral administration of 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), a guanylate cyclase activator, has been shown to prevent neointima formation (a common feature of restenosis) and downregulates MMP-2 activity, suggesting that YC-1 has therapeutic potential for treatment of restenosis after angioplasty [65]. However, the mechanism by which YC-1 inhibits MMP-2 activity is currently unknown.

8 MMP-2 in Aneurysm

Aneurysm, a complex multifactorial disease with life-threatening implications, is a major disease generally of the adult arteries caused by progressive medial degeneration of the blood vessel wall. Aneurism typically has no signs and symptoms, and ruptures of arteries due to aneurysm cause a high mortality rate [66–68].

MMP-2 activation is an important event in the pathogenesis of aneurysm [69]. Aneurism has been shown to be associated with infiltration of inflammatory cells and local destruction of matrix molecules in the blood vessels. The chronic infiltrate is predominantly composed of macrophages and T-lymphocytes. Activated macrophages produce a variety of mediators including TNF-α, which were found in patients with aortic aneurysm [70].

Increasing evidence suggests that tissue damage mediated by phagocytic cells, for example, neutrophils can be initiated by complement fragments, notably C5a, which are potent stimulators of neutrophil superoxide production and adherence to vascular endothelium [71]. In both the classical and alternate pathways, C3 convertase cleave C3 into C3a and C3b. C3a is released into the fluid phase, while C3b may complex with the convertase forming C4aC2aC3b of the classical pathway or C3bBbC3b of the alternative pathway. These two complexes are called C5 convertases, and they cleave C5 into C5a and C5b, which are potent stimulators of neutrophil superoxide production and adherence to vascular bed. The complement cascade is activated during ischemia–reperfusion injury to the vasculature. The C5b in loose association with C3b of the convertase combines sequentially with C6, C7, C8, and C9 and that ultimately forms a ringlike C5a-9 lesion (membrane attack complex, MAC) across the SMC membrane. The severity of aneurysm has been positively correlated with C9 deposition [71–73]. The membrane attack complex (MAC) may induce production of the mediators such as TNF-α, IL-1β, and platelet-activating factor (PAF), thereby causing an increase in vascular permeability with concomitant manifestation of aneurysm [74–76]. Important studies in the recent past have defined mechanisms, depending on the production of mediators, involving MEKK/JNK- and/or NIK/NF-κB-mediated expression and subsequently activation of proMMP-2 leading to aneurismal rupture of vascular cells [70, 77–79]. Figure 19.1 represents schematically aneurysm formation by MMP-2 during ischemia–reperfusion induced complement activation and subsequent production of mediators such as TNF-α, IL-1β, and PAF with concomitant involvement of MEKK/JNK/AP-1 and/or NIK/NF-κB signaling pathways.

9 MMP-2 in Thrombosis

MMP-2 is known to be involved in acute biological reactions associated with discrete cell signaling such as regulation of vascular reactivity, leukocyte activation, and platelet function [80, 81]. Platelets are involved in physiological hemostasis and pathological thrombosis. After accidental or pathological injury, platelets adhere to the damaged portion of the vascular wall initiating an intricate set of reactions that lead via platelet aggregation to the formation of occlusive thrombus [82–84].

Platelet adhesion has been shown to be triggered by Von Willebrand factor [85], and its aggregation by components such as collagen and thrombin leads to liberation of proMMP-2 [86, 87], which then translocated to the platelet surface membrane where they appear to co-localize in areas of cell contact with β2 integrin [88, 89]. The translocation of proMMP-2 to the cell surface is likely to provide stimulus for enzyme activation. Interestingly, proMMP-2 may be activated by the classical MT1-MMP-/TIMP-2-dependent pathway [87] or by a mechanism that does not involve TIMP-2 [90]. MMP-2 primes platelets for adhesion, aggregation, and subsequently thrombus formation, which may lead to thrombosis [90, 91].

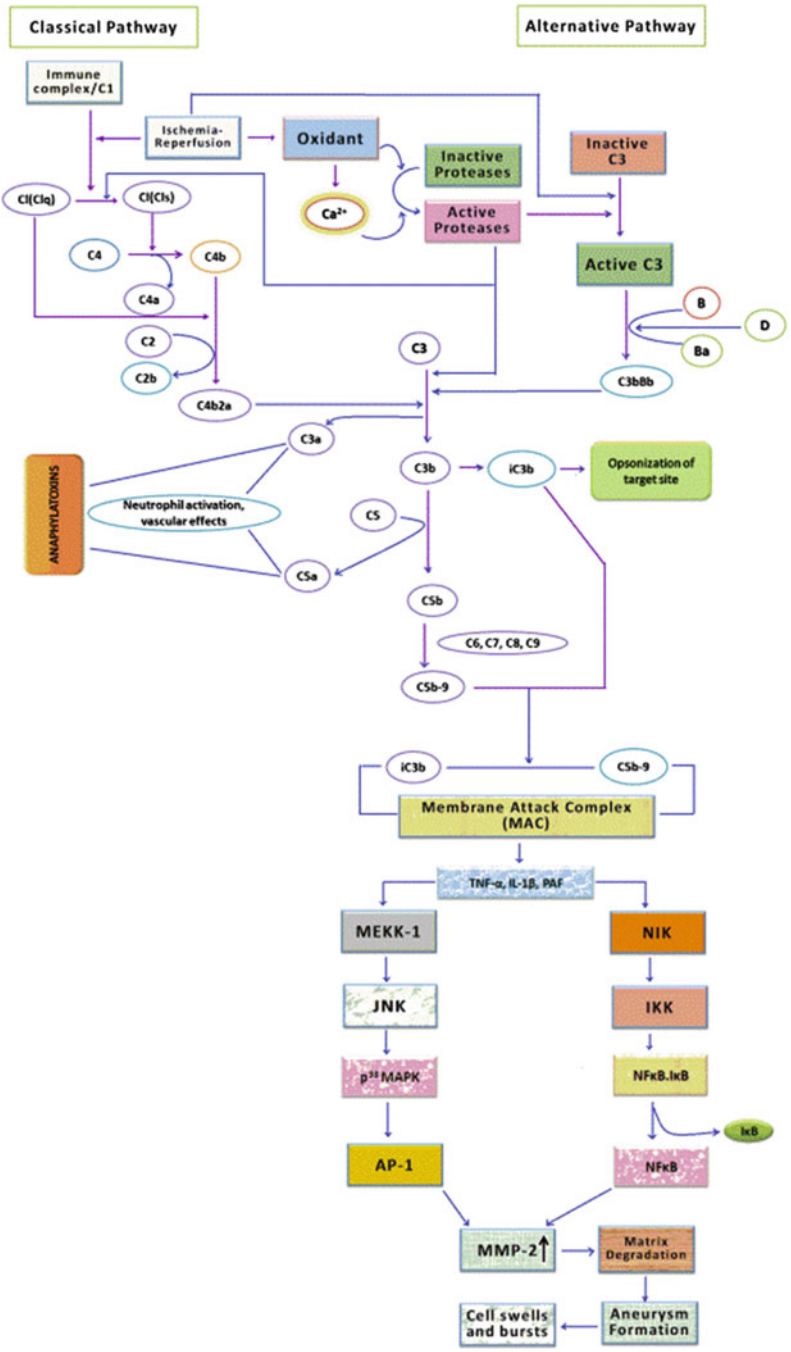

Fig. 19.1 Proposed schema of aneurysm formation by MMP-2 during ischemia–reperfusion induced complement activation and subsequent production of mediators such as TNF-α, IL-1β, and PAF with concomitant involvement of MEKK/JNK/AP-1 and/or NIK/NF-κB signaling pathways in vascular cells. *TNF-α* tumor necrosis factor-α, *IL-1β* interlukin-1β, *PAF* platelet-activating factor, *MEKK* MEK kinase, *JNK* c-Jun N-terminal kinase, *AP-1* activator protein-1, *IκB* nuclear factor κ-light polypeptide gene enhancer in B-cell inhibitor, *IKK* IκB Kinase, *NF-κB* nuclear factor κ-light-chain-enhancer of activated B cells, *NIK* NF-κB-inducing kinase

10 Conclusions

MMPs are important components in many biological and pathological processes because of their capacity to degrade ECM components. It has become evident that the ECM is not only a scaffold for cells but it also facilitates a variety of biological functions that can be relied upon proteolysis mediated by MMPs. Considerable advancements have been made in understanding biochemical and structural aspects of MMPs, including their activation and catalytic mechanisms, substrate specificity, and the mechanism of inhibition by TIMPs. A large number of evidence suggests association of MMP-2 with cardiac diseases like atherosclerotic plaque formation, MI, and heart failure. Several studies using in vitro and in vivo approaches have firmly established that MMP-2 plays an important role in cardiovascular pathologies. The design of specific synthetic inhibitors for MMP-2 is an important future challenge. Such inhibitors are useful not only for gaining insights into the biological roles of MMP-2 but also for the development of therapeutic interventions for diseases associated with unbalanced ECM degradation. Emphasis should be given in designing MMP-2 inhibitors to investigate their ability for treatment of cardiovascular diseases. Several MMP-2 synthetic inhibitors are currently being evaluated for therapeutic use, and their clinical trials are underway to assess their protective effect in heart diseases.

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Chapter 20
Role of Protease Activation in Subcellular Remodeling and Heart Failure

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Abstract Both intracellular and extracellular proteases including matrix metalloprotease (MMP2 and MMP9) and calpains are well known to play a critical role in the development of cardiac remodeling and heart failure due to hypertension, valvular disorders, myocardial infarction, and dilated cardiomyopathies. These proteolytic enzymes are considered to be activated due to the elevated levels of plasma hormones including angiotensin II, catecholamines, endothelin, vasopressin, and serotonin. The activation of these proteases has been shown to produce marked changes in the composition and structure of the extracellular matrix as well as sarcolemma, sarcoplasmic reticulum, myofibrils, and mitochondria. Such alterations in the function of the extracellular matrix and subcellular organelles due to the activation of different proteases appear to occur as a consequence of oxidative stress and intracellular Ca^{2+}-overload, which are considered to result in cardiac dysfunction in the failing heart. The activation of different proteases in the failing hearts also seems to be dependent upon changes in the activities and contents of their endogenous inhibitors such as tissue inhibitors of matrix metalloproteases and calpastatins. It is becoming evident that alterations in the activation of both intracellular and extracellular proteases as well as changes in their endogenous inhibitors depend upon the stage and type of heart failure. Thus, a great deal of attention needs to be paid towards the prevention of protease activation and subcellular remodeling for improving the treatment of cardiac dysfunction in heart failure.

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1 Introduction

Several types of proteases including different isoforms of matrix metalloproteases (MMPs), calpains, cathepsins, and caspases are present in the heart [1, 2]. These proteolytic enzymes are considered to maintain the integrity and function of various types of cells in the myocardium under physiological conditions. However, the activation of these proteases under pathological situations has been observed to result in cardiac dysfunction [3–12]. It has been shown that different isoforms of MMPs and cathepsins are localized in the extracellular matrix of the myocardium, whereas different isoforms of calpains, cathepsins, caspases, and MMPs are located intracellularly in cardiomyocytes. The proteolytic activity of MMPs is kept at a low level by the endogenous tissue inhibitors of matrixmetalloproteases (TIMPs), whereas that of calpain is controlled by its endogenous inhibitor, calpastatin [2, 8, 9]. Thus, an imbalance between the proteolytic enzymes and their endogenous inhibitors can be seen to increase the proteolytic activity in the heart and result in cardiac dysfunction. Although several other mechanisms including the development of intracellular Ca^{2+}-overload and the occurrence of oxidative stress have been proposed for the activation of proteolytic enzymes [13–15], it should be emphasized that the increase in the proteolytic enzyme activities has been observed in various forms of cardiomyopathies [8, 10]. Recently, we have described the role of both intracellular and extracellular proteases in cardiac dysfunction due to ischemia–reperfusion injury [9]. Since several types of cardiomyopathies are associated with heart failure (Fig. 20.1), this article will be focused on the role of different proteases in the development of cardiac dysfunction in ischemic cardiomyopathy, hypertrophic cardiomyopathy, dilated cardiomyopathy, diabetic cardiomyopathy, and genetic cardiomyopathy.

It is now well known that heart failure is invariably preceded by cardiac hypertrophy and is associated with cardiac remodeling as reflected by changes in the size and shape of the heart [16–20]. Cardiac hypertrophy is commonly considered to be an adaptive mechanism to maintain cardiovascular homeostasis under conditions of pressure overload, volume overload, or cardiac muscle loss (myocardial infarction). However, if left unattended, cardiac hypertrophy progresses into heart failure. In fact, the development of cardiac dysfunction is the hallmark of heart failure under a wide variety of pathological conditions [18–20]. The occurrence of cardiac hypertrophy due to increase in protein synthesis seems to be the result of elevated plasma levels of different vasoactive hormones including angiotensin II, catecholamines, vasopressin, endothelin, and serotonin before the occurrence of heart failure (Fig. 20.2). However, prolonged exposure of the heart to the elevated levels of these vasoactive hormones leads to the development of heart failure [19–21]. Although the exact mechanisms for the transition of cardiac hypertrophy into heart failure are

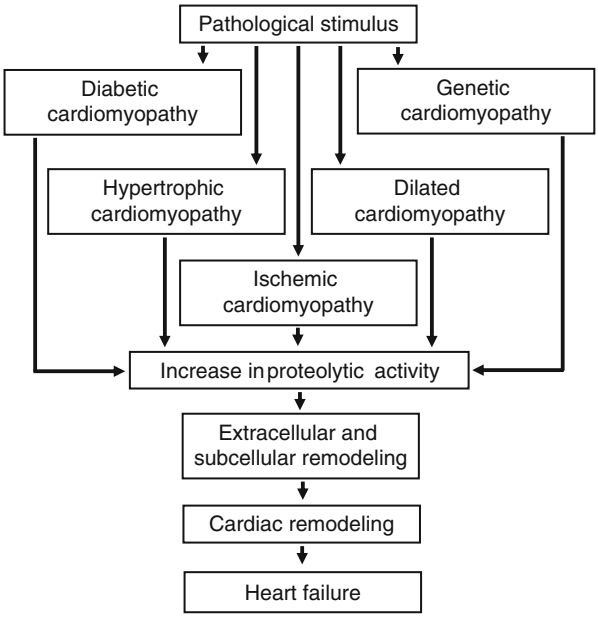

Fig. 20.1 Role of increased proteolytic activity in the development of extracellular and subcellular remodeling, cardiac remodeling, and heart failure in different types of cardiomyopathies

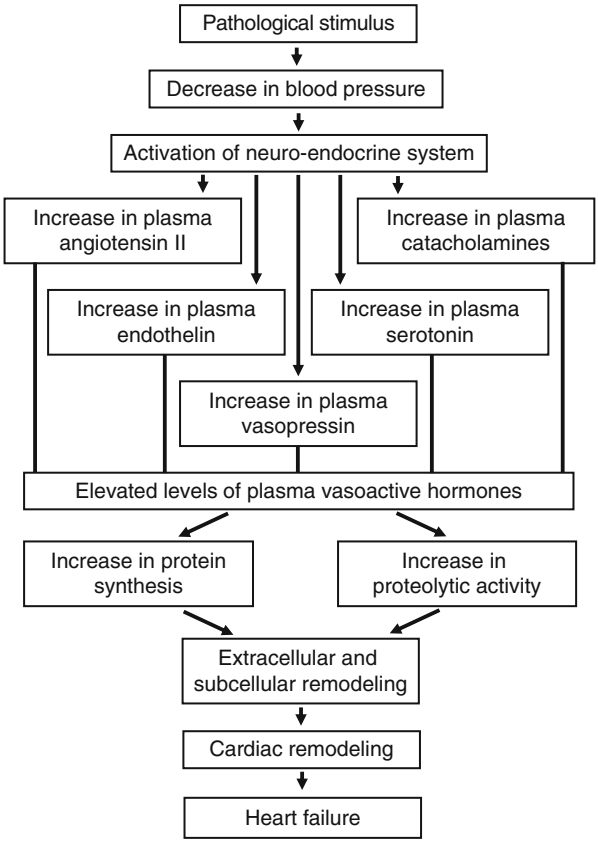

Fig. 20.2 Role of different vasoactive hormones in inducing increase in protein synthesis and proteolytic activity for the development of cardiac remodeling, extracellular and subcellular remodeling, as well as heart failure

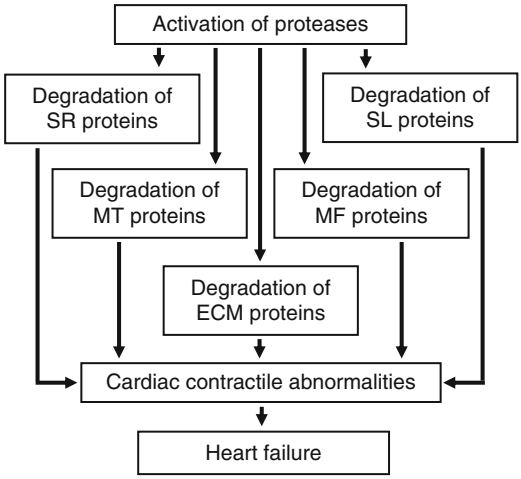

Fig. 20.3 Role of protease activation in inducing subcellular and extracellular defects leading to the development of cardiac contractile abnormalities and heart failure. *SR* sarcoplasmic reticulum, *SL* sarcolemma, *MT* mitochondria, *MF* myofibrils, *ECM* extracellular matrix

not fully understood, alterations in the extracellular matrix due to the activation of different MMPs have been suggested to account for the development of cardiac dysfunction in the failing heart [6, 8, 10, 22–25].

Since the activation of several other proteases including calpains, cathepsins, and caspases has been observed in the failing hearts [4, 8, 10, 16, 26–31], the degradation of subcellular proteins has also been proposed to explain cardiac dysfunction in heart failure [8, 10, 19, 20]. It should be noted that heart failure is invariably associated with defects in Ca^{2+}-handling proteins in the sarcolemma (SL), sarcoplasmic reticulum (SR), and mitochondria (MT) as well as in the processes for energy production by MT and energy utilization by myofibrils (MF) [16, 19, 20]. In fact, it has been proposed that the activation of proteases leads to changes in both extracellular matrix and subcellular during the development of heart failure organelles (Fig. 20.3). Accordingly, we believe that the activation of both intracellular and extracellular proteases [8, 9] is involved in the progression of subcellular defects, cardiac remodeling, and cardiac dysfunction during the development of heart failure. As cardiac remodeling and heart dysfunction seem to depend upon the stage and type of heart failure, this review is intended to emphasize the mechanisms of changes in the proteolytic activities in different forms of cardiomyopathies at different stages of heart failure.

2 Activation of Proteolytic Enzymes in the Failing Heart

2.1 Hypertrophic and Dilated Cardiomyopathies

By employing a Dahl salt-sensitive rat model of dilated and/or hypertrophic cardiomyopathy due to hypertension, it was observed that the MMP-9 protein level was increased in the heart [32]. The activities and mRNA levels of both MMP-2 and

MMP-9 were increased prior to the occurrence of left ventricular dilatation and systolic dysfunction in the Dahl salt-sensitive rat [33]. Furthermore, cardiac remodeling and changes in the proteolytic activity were attenuated by treatment with angiotensin converting enzyme (ACE) inhibitors [33]. Reduced MMP-1 and increased TIMP-1 protein levels in the serum as well as reduced turnover of interstitial collagen in hypertensive patients either with or without cardiac hypertrophy have also been observed [34, 35]. In addition, decreased heart weight and cardiomyocyte area were associated with depressed MMP-2 and MMP-9 expression during hemodynamic unloading upon heterotopic transplantation of hypertrophic hearts [36, 37]. Increased protein level of TIMP-1 was also observed in both hypertrophied and pressure-unloaded hearts [37]. On the other hand, increased protein level of TIMP-4 and decreased protein level of MMP-9 were evident upon attenuating MI-induced cardiac hypertrophy by K^+-ATP channel opener, KUMP-3 [38]. These observations are consistent with the view that cardiac remodeling due to hypertension is associated with an imbalance between MMPs and their endogenous inhibitors, TIMPs [39–44].

Hypertension-induced cardiac hypertrophy was observed to be associated with a reduction in calpain activity in both DOCA-salt hypertensive rat and spontaneously hypertensive rat hearts [45]. On the other hand, both hypertensive patients and rats with heart failure showed elevated mRNA and protein levels of cathepsin isoforms [46]. Calpain activity was increased, and the protein level for its inhibitor, calpastatin, was decreased initially due to pressure overload, but these changes returned to basal values within a week [47]. Increased level of calpastatin due to incorporation into the genome of mice was found to depress calpain activity and prevent angiotensin II-induced cardiac hypertrophy [48]. Caspases-3 activation was also increased upon inducing pressure overload; this increase was attenuated by a caspase inhibitor, calpeptin, whereas the increased calpain activity in hypertrophied heart was unaltered by this treatment [47]. The extent of cardiac hypertrophy and increased calpain activity due to isoproterenol were reduced by treatment with a cysteine protease inhibitor, E64-e [49]. These observations suggest that changes in the calpain activity, in addition to MMPs, are involved in the development of cardiac hypertrophy, whereas alterations in the activities of other proteases such as caspases may play a critical role in the progression of heart failure due to pressure overload. Since the transition of cardiac hypertrophy to heart failure is considered to be associated with subcellular defects [19, 29], it seems likely that the activation of proteolytic enzymes is intimately involved in remodeling of subcellular organelles and extracellular matrix.

The activities of different proteases have also been shown to change depending upon the stage of the development of dilated cardiomyopathy where the myocardium is distended with increases of interstitial fibrosis, wall thinning, chamber dilatation, and contractile dysfunction [50]. In a rat model of dilated cardiomyopathy, induced by an injection of cardiac C protein, inflammation peaked at 2 weeks with corresponding increases in mRNA levels for MMP-2, MMP-9, and TIMP-1 [51, 52]. Increased activities of MMP-1, MMP-13, and MMP-2/9 degrade collagen and proteoglycans and thus result in dilatation of the ventricular chambers [6]. Differential increases in the protein content for MMP-9 and MMP-2 were also observed during the development of cardiac hypertrophy and heart failure upon aortic banding [53]. It should be noted that increased levels of MMP-2 and MMP-3 have been detected

in patients with dilated cardiomyopathy [54–56]. It appears that MMP-3 is critical in cardiac remodeling because it degrades a variety of extracellular matrix components including fibronectin, laminin, and proteoglycans and serves as a biomarker for differentiating dilated cardiomyopathy from other types of cardiomyopathies. There is an increase in the mRNA levels for TIMPs and MMPs in the hearts of patients afflicted with dilated cardiomyopathy [57–61]; however, the protein content of TIMP-1 and TIMP-3 was decreased at advanced stages in dilated cardiomyopathy in humans [56].

2.2 Diabetic Cardiomyopathy

Chronic diabetes is invariably associated with cardiovascular defects or diabetic cardiomyopathy [62]. Cardiac remodeling in diabetic patients with heart failure has been observed in association with shortened left ventricular ejection time, longer preejection period, elevated end-diastolic pressure, and myocytolytic changes [63–65]. Alterations in protein content and activities of different proteolytic enzymes have been shown to participate in remodeling of both extracellular matrix and subcellular organelles [66–68]. Although TIMP-2 expression is increased in diabetic cardiomyopathy, the activity of MMP-2 is decreased due to the reduced expression of MT-MMP [69]. This alteration seems to be specific for diabetic cardiomyopathy because it differs from both dilated cardiomyopathy and ischemic cardiomyopathy [70]. Various other studies have shown that the activities of different MMP are increased in the diabetic heart [71–74] indicating that changes in proteolytic enzymes are dependent upon the stage and severity of diabetes.

Varying degrees of changes in calpains, caspases, and cathepsins have also been observed in the diabetic heart. High concentration of glucose was found to increase the calpain activity and depress SL Na^+-K^+ ATPase and L-type Ca^{2+} channels as well as SR Ca^{2+}-release channels [75]. The activation of calpain was attenuated by calpastatin. Furthermore, there was an increase in caspase activity in diabetic cardiomyopathy, whereas cathepsin protein level was first decreased and then increased [76–79]. These alterations in calpain, caspases, and cathepsin D are considered to explain cardiac muscle degradation apoptosis and cardiac dysfunction in diabetic cardiomyopathy. Although an increase in the calpain activity in diabetic heart can be seen to induce subcellular alterations and cardiac dysfunction, the exact role of the activation of calpain in the development of diabetic cardiomyopathy remains to be established.

2.3 Ischemic Cardiomyopathy

Heart failure due to ischemic cardiomyopathy, as a consequence of myocardial infarction, is a complex process because the viable myocardium reacts first by

compensating the loss of cardiac function and then becomes decompensated [16, 19, 20]. It is the decompensation of the hypertrophied viable myocardium, which results in cardiac dysfunction as a consequence of cardiac remodeling and is dependent upon the infarct size [16, 19, 20]. Thus, it is prudent to discuss the role of protease activation in (1) ischemic regions, which become a scar over a certain time period and trigger all subsequent events; (2) viable myocardium, which becomes hypertrophied and compensates for the loss of myocardial function; and (3) hypertrophied heart, which becomes decompensated and results in heart failure. The involvement of different proteases in all of these aspects of ischemic cardiomyopathy has recently been described in details [8, 9]. However, it is intended to focus briefly on the role of protease activation during the progression of heart failure, because the development of cardiac dysfunction due to myocardial infarction occurs as a consequence of defects in subcellular organelles and extracellular matrix [16, 19, 20]. Nonetheless, it is not our intention to de-emphasize the role of changes in gene expression for defects seen in different cardiac proteins during the development of heart failure [80–92].

Remodeling of the extracellular matrix is mainly associated with increased MMP proteolytic activity and/or decreased protein content for TIMPs in the failing heart [6]. It should be noted that heart failure induced by pacing has also been related to increased MMP-1, MMP-2, and MMP-3 protein content [89]. Elevated levels of MMP-9 have been linked to cardiac dysfunction [93, 94]. Increased amounts of MMP-2, MMP-3, and MMP-9 have also been observed in the plasma of patients with congestive heart failure, whereas plasma TIMP-1 level was decreased [95]. The imbalance between MMP activity and collagen deposition in the extracellular matrix is considered to result in myocardial rupture following myocardial infarction [96, 97]. These observations are consistent with the view that increased proteolytic activity due to the activation of MMPs is responsible for extracellular remodeling and heart failure in ischemic cardiomyopathy.

In addition to increased MMP activity, several other proteolytic enzymes such as calpain, caspase 3, chymase, and cathepsin G are activated in the viable hypertrophied heart following myocardial infarction [8]. Increased levels of calpain and calcineurin have been observed in patients with congestive heart failure [98]. Protein content of calpain, unlike the protein content of its inhibitor calpastatin, was increased in failing hearts following myocardial infarction in rats [99]. Treatment of infarcted rats with a combination of both calpain and caspases-3 inhibitors attenuated changes in cardiac performance as well as blood pressure and heart rate [100]. The level of serine proteases, proteinase 3, was found to be increased and is considered to be an important biomarker for determining the incidence of heart failure as well as mortality in patients with myocardial infarction [101]. Thus the activation of calpain, caspases, cathepsins, and other proteolytic enzymes at different time intervals following myocardial infarction highlights their importance in cardiac remodeling during the progression of heart failure. It is emphasized that the increased proteolytic activity in the hypertrophied heart leads to the development of defects in different subcellular organelles and subsequent cardiac dysfunction in ischemic cardiomyopathy.

2.4 Genetic Cardiomyopathy

Syrian cardiomyopathic hamsters have been shown to be an excellent model of cardiomyopathy and heart failure [102, 103]. In these hamsters, the progression of cardiomyopathy begins with acute focal degeneration of myocardium between 4 and 8 weeks followed by calcification and fibrosis for about 4 weeks and eventual progression to congestive heart failure after about 20 weeks [10]. Some strains (B1O 14.6, UM-X7.1) of cardiomyopathic hamsters develop ventricular hypertrophy, whereas other strains (B1O 53.3, TO and TO-2, and J2N) develop ventricular dilatation. Pathological and biochemical changes with respect to intracellular Ca^{2+}-overload and oxidative stress in the cardiomyopathic hamster hearts are similar to those seen in patients with advanced cardiomyopathy [103–108]. Increased activities of different MMPs including MMP-1, MMP-2, and MMP-9 have been observed in BIO 14.6 and BIO 53.58 strains of cardiomyopathic hamsters [109]. However, both MMP-2 and MMP-9 activities were most elevated during the hypertrophic stage in BIO 14.6 strain of hamsters, whereas these proteases were most activated in the heart failure stage in BIO 53.58 hamsters [109]. Increased activities of MMP-1 and MMP-2 in UM-X7.1 hamster strain did not prevent cardiac fibrosis, but were considered to be involved in the development of heart failure [110, 111].

It is noted that in contrast to cardiomyopathic hamsters, the activities of some of the MMPs are either unchanged or decreased in different human cardiomyopathies [112–115]. Nonetheless, other proteases including cathepsin D, calpains, and caspases were increased, and there was a decrease in the protein content for calpastatin in cardiomyopathic hamsters [116–118]. During both the fibrotic and hypertrophic stages in BIO 14.6 hamster heart, chymase mRNA and its activity were increased [119]. It is pointed out that the activation of chymase, which is associated with the formation of angiotensin II, has also been reported in human cardiomyopathy and heart failure [120, 121]. The increased proteolytic enzyme activities in cardiomyopathic heart result in the alterations of different proteins in subcellular organelles such as SL, SR, MT, and MF [122–126]. Thus, it appears that the activation of calpains, caspases, and cathepsins, in addition to MMPs, plays an important role in remodeling of different subcellular organelles and extracellular matrix for the occurrence of heart failure in genetic cardiomyopathy.

3 Mechanisms of Protease Activation in Heart Failure

From the foregoing discussion, it is evident that both extracellular proteases (mainly various forms of MMPs) and intracellular proteases including calpains, cathepsins, and caspases are activated during the development of heart failure in different types of cardiomyopathies. The activation of these proteolytic enzymes is considered to degrade a wide variety of proteins present in the extracellular matrix and subcellular organelles and result in subcellular defects in cardiac dysfunction during the

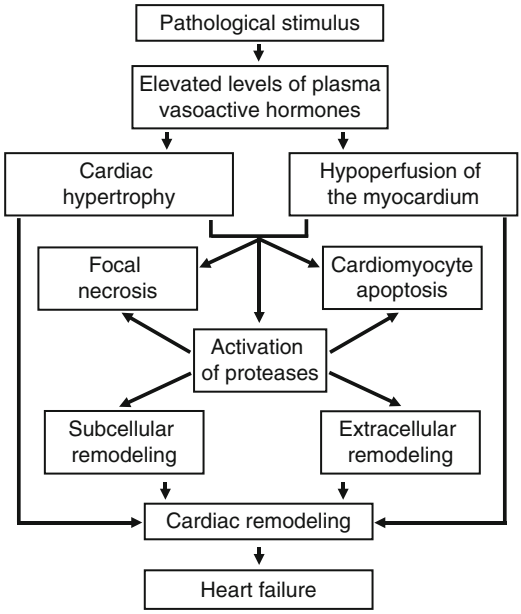

Fig. 20.4 Involvement of elevated levels of plasma vasoactive hormones in the development of cardiac hypertrophy, hypoperfusion of the myocardium, and activation of proteases in cardiac remodeling and heart failure

development of heart failure. The mechanisms of protease activation leading to remodeling of extracellular matrix as well as SL, SR, MT, and MF have been outlined elsewhere [6–11]. It is emphasized that irrespective of the type of cardiomyopathy, heart failure is associated with elevated levels of different vasoactive hormones including catecholamines, angiotensin II, vasopressin, endothelins, and serotonin. All of these vasoactive hormones increase protein synthesis to produce cardiac hypertrophy and constriction of blood vessels to raise blood pressure. The vasoconstriction of the microvasculature in the myocardium induces hypoperfusion of the hypertrophied heart, thereby producing functional hypoxia. We believe, it is this functional hypoxia in the hypertrophied heart which results in the activation of different proteases and is associated with protein degradation, focal necrosis, and apoptosis as well as remodeling of extracellular matrix and subcellular organelles. The consequence of elevated levels of vasoactive hormones, and subsequent hypertrophic response and hypoperfusion of myocardium leading to cardiac remodeling and heart failure are described in Fig. 20.4.

It should be noted that several vasoactive hormones, which are released in the circulation in response to diverse pathophysiological stimuli, play a compensatory role to maintain hemodynamic homeostasis at the initial stages. However, over a prolonged period, these vasoactive hormones produce oxidative stress and intracellular Ca^{2+}-overload, which serve as the major mechanisms for the activation of intracellular and extracellular proteases leading to cardiac remodeling, subcellular defects, and heart dysfunction [6–11]. The events illustrating the role of both oxidative stress and intracellular Ca^{2+}-overload in the activation of different proteases and

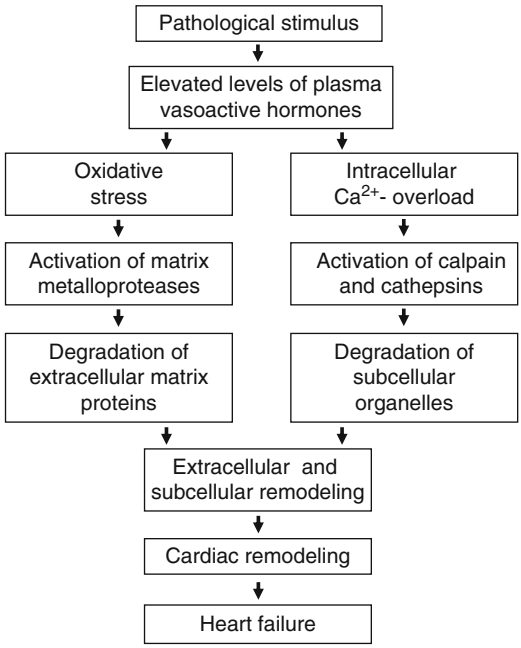

Fig. 20.5 Mechanisms of involving oxidative stress and intracellular Ca^{2+}-overload for the development of extracellular and subcellular remodeling as well as heart failure

development of heart failure are depicted in Fig. 20.5. It should be noted that oxidative stress is known to activate different MMPs, whereas calpains, cathepsins, and caspases are activated by elevated levels of intracellular Ca^{2+}. Since the increase in proteolytic enzyme activities is also caused by an imbalance of protein content of proteases and their endogenous inhibitors, corresponding alterations in gene expression for various proteases and their inhibitors are considered to serve as a mechanism for the increased proteolytic activities and cardiac remodeling in the failing hearts. Such changes in gene expression for calpains and MMPs as well as for calpastatin and TIMPs are also proposed to be due to elevated levels of plasma hormones and subsequent alterations in the concentrations of different intracellular cations, cytokines, and other factors (Fig. 20.6). Thus, it appears that the mechanisms of protease activation leading to the development of cardiac remodeling, subcellular defects, and heart failure are complex in nature, and extensive research is needed to sort out their status for each type of cardiomyopathies.

4 Conclusions

In response to pathological stimuli for the development of different types of cardiomyopathy, various vasoactive hormones are released in the circulation. The elevated plasma levels of these vasoactive hormones stimulate heart function and/or produce cardiac hypertrophy at initial stages as a compensatory mechanism to maintain hemodynamic homeostasis. However, prolonged exposure of the heart to the

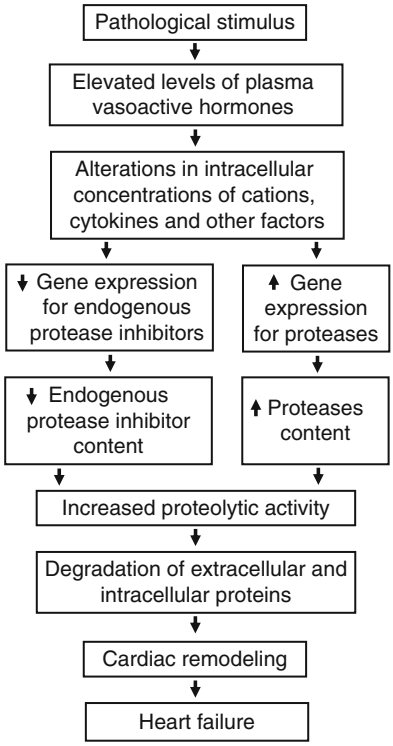

Fig. 20.6 Imbalance of protease content and endogenous protease inhibitors due to changes in gene expression leading to degradation of intracellular and extracellular proteins and heart failure

elevated levels of vasoactive hormones results in the development of heart failure. Although the activities of different proteolytic enzymes such as MMPs and calpains are altered in the hypertrophied heart, it is not clear whether an increase or decrease in their activities leads to the development of cardiac hypertrophy or cardiac dilatation. From the existing literature, it is apparent that dilated cardiomyopathy is associated with an increase in MMPs activity and remodeling of the extracellular matrix. On the other hand, hypertrophic cardiomyopathy may be associated with decreased proteolytic enzyme activities and depositions of collagen in the extracellular matrix. The data at hand seem to suggest that the activation of MMPs is involved in the remodeling of extracellular matrix, whereas the activation of calpains, cathepsins, and caspases as well as MMP-2 is intimately involved in the degradation of subcellular organelles including SL, SR, MT, and MF during the development of heart failure. Although oxidative stress and the occurrence of intracellular Ca^{2+}-overload are two major mechanisms responsible for the observed increase in the proteolytic enzyme activities, the imbalance of protein content for proteases and their endogenous inhibitors may play an important role in increasing the proteolytic enzyme activities in the failing heart. Thus, it is evident that an increase in proteolytic enzyme activities may play a critical role in remodeling of extracellular matrix and subcellular organelles, and thereby these changes lead to the genesis of cardiac dysfunction and heart failure.

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Chapter 21
Proteases as Potential Targets in Left Ventricular Remodeling After Myocardial Infarction

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Abstract Left ventricular (LV) remodeling plays a key role in the march to heart failure after myocardial infarction (MI). The remodeling process involves a complex series of changes that occur at structural and extracellular as well as cellular, subcellular, and molecular levels. Typically, the changes are time dependent, occur in tandem sequence, and span the healing/repair process after MI and extend well beyond. Proteases play a major role in the remodeling that occurs at the extracellular as well as cellular, subcellular, and molecular levels. Evidence since the mid-1970s has established the critical roles of the extracellular matrix (ECM) for maintaining normal cardiac shape and function and the dramatic adverse remodeling post-MI, with LV shape deformation and dysfunction that results from disruption of the ECM driven primarily by an imbalance between the class of proteases known as matrix metalloproteinases (MMPs) and the endogenous tissue inhibitors of the MMPs or TIMPs. Evidence since the 1990s has implicated several other classes of proteases in adverse LV remodeling and dysfunction after MI and the march to HF, disability, and death. Experimental studies have shown that pharmacologic inhibition or genetic deletion of key MMPs can effectively prevent or limit post-MI LV remodeling, and some clinical studies have tested MMP inhibitors. Proteases are therefore attractive as potential targets to limit, prevent, or reverse remodeling and dysfunction after MI. However, further studies are needed to identify the optimal strategy to maximize the benefits of protease inhibitors after MI.

Keywords Extracellular matrix remodeling • Left ventricular remodeling • Matrix metalloproteinases • Myocardial infarction • Matrix proteases • Tissue inhibitors of metalloproteinases • Heart failure

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1 Introduction

A steady flow of evidence from multiple disciplines since the mid-1970s has established that adverse left ventricular (LV) remodeling plays a key role in the march to heart failure (HF) after myocardial infarction (MI) [1–15]. Concurrently, the evidence indicated that the remodeling process involves a complex series of changes that occur at structural and extracellular levels as well as changes at cellular, subcellular, and molecular/biochemical levels [12–14]; importantly, the changes are time dependent, occur in tandem sequence, and span the healing/repair process after MI and extend well beyond [9, 11–14, 16], providing the rational basis for timed and prolonged therapeutic interventions [9, 11]. Other evidence indicated that proteases play a major role in the remodeling that occurs at the extracellular as well as cellular, subcellular, and molecular levels [6, 7, 12–23]. The collective evidence since the mid-1970s has established the critical roles of the extracellular matrix (ECM) for maintaining normal cardiac shape and function [6, 7, 17–25] and the dramatic adverse remodeling post-MI, with LV shape deformation and dysfunction that results from disruption of the ECM driven primarily by an imbalance between the class of proteases known as matrix metalloproteinases (MMPs) and the endogenous tissue inhibitors of the MMPs or TIMPs [12–14, 19–23, 26–28]. Evidence since the 1990s has implicated several other classes of proteases in adverse LV remodeling and dysfunction after MI [14–16, 29–33] and the march to HF, disability, and death [32, 33]. Experimental studies have shown that genetic deletion of key MMPs [34–38] or pharmacologic inhibition [39–44] can effectively prevent or limit post-MI LV remodeling [36–44], and one randomized clinical trial that tested pharmacologic MMP inhibition after MI did not show significant benefit [45]. This chapter focuses on proteases as potential targets to limit, prevent, or reverse remodeling and dysfunction after MI.

2 Heart Failure: A Global Problem

Heart failure is a global healthcare problem that is increasing at an alarming pace [46–50], especially in the older adult and elderly populations [46, 51, 52]. Importantly, the risk of HF increases with antecedent MI and hypertension [46, 47], which are the two leading causes of HF in developed countries. Furthermore, this risk of HF has continued to increase despite current therapies [46–50, 52], indicating the need for new therapeutic targets and strategies. While extensive studies have established the primary role of LV remodeling in the development and progression of HF [4, 5, 12, 52] and identified cardiac ECM remodeling as a key underlying mechanism in the development and march to end-stage HF [12–14, 24], evidence-based therapies that specifically target matrix proteases and ECM remodeling in HF are lacking in the management guidelines [47–50]. As discussed below, HF that follows an MI is quite different from that after hypertension, and the outcome is far more sinister [52].

3 Proteases in Cardiovascular Disease

Proteases, also called proteinases or proteolytic enzymes, are ubiquitous proteins in biological systems of all living organisms. They regulate multiple physiological actions and pathophysiological reactions, several of which are highly relevant for cardiovascular diseases that lead to HF, including MI, hypertension, and cardiomyopathies. This regulation is mediated through the fundamental biochemical process of proteolysis that can be limited or unlimited. Proteases have been classified on the basis of their catalytic active site and conditions of action, and their anchorage depends on the structural conformation of the site and the specific amino acid needed for their action. Of the six main groups of proteases (i.e., serine, threonine, cysteine/thiol, aspartate, glutamic, and metalloproteases or metalloproteinases), the metalloproteinases (MMPs) and serine proteases are especially pertinent for LV remodeling after MI [13, 14, 24].

Typically, proteases digest and degrade the target long-chain proteins and polypeptides and split peptide bonds between amino acids to yield short fragments that may be active or inactive; the exopeptidases (such as MMPs) cleave amino acids at amino or carboxy terminals, while endopeptidases (such as trypsin, chymotrypsin, pepsin, papains, elastase) cleave peptide bonds within the chains. This simple but critical cleavage step may have important physiological or pathophysiological effects.

Proteases play important roles in several major systems, including (1) the blood coagulation cascade (with thrombin, plasmin, Hageman factor, etc.) and the thrombolytic system for lysis of clots; (2) the immune and inflammatory systems (with inflammatory cells such as neutrophils/leucocytes, monocytes and macrophages containing elastase, cathepsin G, etc.); (3) the renin–angiotensin–aldosterone system (RAAS), bradykinin–kallikrein system, and angiotensin II formation/degradation pathways; and (4) the prostanoid and cyclooxygenase-2 (COX-2) pathway [16, 53–56]. Typically, complex series of reactions occur in tandem sequence, resulting in rapid and efficient amplification of physiological signals which is critical for signaling pathways and, if dysregulated, can contribute to pathophysiology.

For example, in the immune system, proteases carry out fragmentation of antibodies or immunoglobulins into fragments that retain antigen-binding capacity; thus, the protease papain yields monovalent F(ab) fragments, while the protease pepsin yields bivalent F(ab′)-2 fragments which are critical for defense. In the early response to infection or injury, activation of plasma proteases including serine proteases of the complement system produces peptides which are chemoattractant for neutrophils and induce proinflammatory cytokines interleukin (IL)-1 and IL-6 within minutes, and failure to resolve the acute inflammation leads to chronic inflammation. Protease-activated receptors (PARs) expressed on blood platelets and vascular endothelium play a critical role in coagulation (via thrombin signaling), inflammation, and vascular homeostasis but also contribute to the pathogenesis of atherosclerosis, restenosis, and thrombosis [29].

In general, protein lifespan is controlled by regulated protein degradation [57] which serves two functions: (1) to remove misfolded, misassembled, or damaged

proteins and (2) to maintain appropriate protein levels for homeostasis. This is achieved through two main pathways: a rapid one involving lysosomal degradation and a slower one involving the more complex proteasome machine with identification of proteins to be degraded by ubiquitin tags [57]. Pertinent for hormones, antibodies, and other enzymes, proteases determine the lifespan of other proteases using the fastest switch-on/switch-off regulatory mechanism in physiology. Proteases themselves undergo degradation by cleavage to other proteases or proteins as an important mechanism for their regulation. This is particularly pertinent for ECM homeostasis and remodeling.

While increased proteolytic activity normally degrades misfolded or malfunctioning proteins in cardiomyocytes [58], MMPs and other proteases such as calpains [59], chymase and cathepsins [60, 61], and caspases [62] have been implicated in remodeling of both ECM and subcellular organelles of cardiomyocytes in failing hearts [30, 31, 59–62]. The proteolytic activity of caspases, calpains, and the ubiquitin–proteasome system (UPS) [63] has also been implicated in cardiomyocyte apoptosis in HF [31]. An imbalance between the activity of proteases and their endogenous inhibitors affects many processes including remodeling of ECM [23, 31, 64], intracellular matrix [65], cellular pathways such as apoptosis [62], and subcellular pathways leading to cardiac dysfunction [31]. In Dahl-sensitive hypertensive rats with increased MMP-9 and decreased TIMP-4, congenic expression of TIMP-4 attenuates hypertrophy and restores function [66]. Endogenous protease inhibitors include the TIMPs [23], calpastatin [59], serpins [67], and lipocalins. The serpin family, also referred to as irreversible serine protease inhibitors, is involved in blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, tumor suppression, and hormone transport and includes α-1-antitrypsin, antithrombin, α-1-antichymotrypsin, trypsin, plasminogen inhibitor-1 (PAI-1), and neuroserpin. It has been studied in vascular remodeling and atherosclerosis [67]. The lipocalins play a role in cell regulation and differentiation and in lipophilic ligands that inhibit tumor proteases, but their possible role in ECM/LV remodeling remains unclear. Of note, proteases have also been classified on the basis of optimal pH into acid, neutral, and basic groups; the neutral proteases, which include calpains [31], also play key roles in the type I hypersensitivity reaction (where they activate complement and kinins), the inflammatory response after injury [16], and the coagulation cascade [16].

4 Proteases in LV Remodeling Post-MI

In general, cardiac remodeling refers to the net result of adaptive and maladaptive changes in cardiac structure, geometric shape, and function that occur over time after an insult and is especially dramatic after MI [1–12]. This concept of *injury → remodeling/dysfunction* is supported by extensive data on cell size, shape, and function in post-MI models of HF and on LV size, shape/structure, and function in patients with post-MI HF. The concept has been reinforced by demonstrations that

biochemical, molecular, cellular, and subcellular changes that affect both heart muscle and matrix mediate the gross changes in structure and function [12]. Pertinent here, evidence indicates that proteases are key not only in ECM homeostasis that is essential for maintaining normal LV shape/function but also in the adverse post-MI ECM remodeling that drives the development and progression of HF [12]. After MI, disruption of the ECM network with dysregulation of ECM homeostasis is a major pathway leading to adverse dilative LV remodeling with shape deformation, dysfunction, and HF with low ejection fraction (HF/low EF) [12, 22, 23].

Typically MI results from coronary artery occlusion secondary to thrombosis on an ulcerating atherosclerotic plaque [56]. The remodeling that follows a large anterior transmural MI or ST-segment-elevation myocardial infarction (STEMI) is both dramatic and highly dynamic; rapid early remodeling of the infarcted wall with thinning and dilatation (i.e., infarct expansion) is followed by progressive dilative remodeling of the whole LV and a march through LV systolic dysfunction, volume overload, wall thinning, eccentric hypertrophy, HF/low EF, and poor outcome with disability and death [1–15]. Clinicopathological studies have established that MI results in early damage of muscle, matrix, and microvasculature via necrosis and apoptosis and triggers a complex healing process, which through a timed sequence of biochemical, molecular, and cellular/subcellular reactions over weeks results in a fibrotic scar [12–14, 17, 18]. During the healing process, timed release of chemokines, cytokines, matrikines, growth factors including transforming growth factor-β (TGF-β), and MMPs/TIMPs and other matrix proteins orchestrate inflammation, remodeling of myocardium and ECM, and fibrosis [12–14, 17, 18]. The sequence of reactions includes acute inflammation, chronic inflammation with granulation tissue formation, tissue repair with fibroblast proliferation, ECM deposition, myofibroblast and scar formation, and structural and functional remodeling of infarcted and non-infarcted myocardium through cardiomyocyte hypertrophy with little regeneration and some angiogenesis [12–14]. An important feature is that post-MI LV remodeling spans all phases of the healing process and is progressive; it begins during the early infarction phase (first 24–48 h in humans), continues during the subsequent healing phase (~6 weeks to 3 months in humans), and extends well beyond (over months to years in humans), and multiple factors modulate the remodeling of myocardium, vascular tissue, and ECM [12–14].

Also typically, coronary reperfusion within 30 min after an STEMI can limit infarct size, LV dilative remodeling, and dysfunction, but few patients present that early. A more common scenario is that STEMI reperfused ≥90 min results in persistent adverse LV remodeling/dysfunction and HF in survivors worldwide [6, 7, 14, 56, 70]. Besides infarct size, the culprits in that saga include reperfusion damage with no-reflow and flow-function mismatch at the microvascular level [14, 15, 56, 68–73], damage to the ECM [6, 7, 12–15, 70], and inflammation in early and late phases of healing [12–15, 70, 74, 75]. Since this adverse remodeling/dysfunction occurs despite medical therapy recommended in management guidelines [14, 56, 68–70], there is critical need for identifying new therapeutic targets in the post-MI remodeling process.

5 MMP/TIMP Balance in Normal ECM Homeostasis and Remodeling

In general, the balance between MMPs and TIMPs is essential for normal ECM remodeling. In the heart, normal ECM remodeling (i.e., ECM synthesis and degradation) occurs continuously and is tightly regulated to maintain homeostasis and cardiac shape/function and prevent excessive ECM degradation. The cardiac ECM consists of a complex 3-dimensional assembly of proteins such as collagen, elastin, and specialized proteins such as fibrillin, fibronectin, proteoglycans, and matricellular proteins that contribute to normal structure and function [12, 13, 22, 24]. The organized interstitial ECM network of collagen fibers provides architectural support for cardiac myocytes and blood vessels as well as the essential milieu for cell migration, growth, differentiation, and interaction. Cardiac fibroblasts that are abundant in the heart regulate ECM synthesis and deposition, mediate ECM degradation and turnover through the MMPs and TIMPs, and maintain necessary tension in the network [76].

6 MMP/TIMP Imbalance in Post-MI ECM and LV Remodeling

As discussed earlier, studies in MI have established four important points: (1) LV remodeling is a major mechanism for LV enlargement and HF; (2) ECM disruption causes and drives LV dilatation; (3) angiotensin II, a primary effector molecule of the RAAS, drives both ECM and LV remodeling; and (4) a major pathway leading to adverse disruptive ECM and dilative LV remodeling involves an imbalance between MMPs and TIMPs reflected in an increased MMP/TIMP ratio [12–14].

The MMPs belong to a family of zinc-dependent endopeptidases that represent one major proteolytic system for ECM degradation. They degrade several ECM components including collagens, fibronectin, proteoglycans, laminin, and gelatin [12, 13]. Nearly 25 MMPs but only 4 TIMPS have been identified, and MMP activity is posttranslationally regulated by the TIMPs [77]. The first four classes of MMPs (collagenases, gelatinases, stromelysins, and elastase) are secreted as latent pro-MMPs that bind ECM proteins and are activated when the propeptide domain is cleaved through a cysteine switch mechanism by the serine proteases, trypsin, chymotrypsin, and plasmin [78]. Several MMPs are also activated by other pro-MMPs and trigger activation of more pro-MMPs thereby triggering a cascade of proteolysis. A fifth class of MMPs includes membrane-type MMPs (MT-MMPs) which are activated upon positioning in the cell membrane and retain the propeptide domain that is needed for both activation and TIMP binding [79]. After injury including MI, the expression of MMPs increases in many cells including myocytes, fibroblasts, myofibroblasts, endothelial cells, and inflammatory cells (i.e., neutrophils, monocytes, and macrophages). While most cells express TIMPs, TIMP-4 expression is

especially highest in human myocardium. While the classic role of MMPs is ECM degradation and turnover, MMPs are multifunctional and play a role in regulation of cell behavior influencing such processes as angiogenesis through proteolytic processing of non-matrix substrates including signaling molecules, growth factors, and cytokines [58]. Along the same vein, while the main role of TIMPs is to inhibit MMPs, they too are multifunctional and exert pro-growth, anti-apoptotic, and anti-angiogenic effects [77].

The key substrates of MMPs in post-MI ECM remodeling are the fibrillar collagens [12–14]. A sharp rise in MMPs occurring within minutes to hours after acute MI leads to a high MMP/TIMP ratio and rapid degradation of mature ECM (mostly cross-linked, type I collagen), followed by decreased collagen content, adverse ECM, and LV remodeling/dysfunction. This rapid degradation of ECM is followed by slow synthesis, with deposition of immature type III collagen and slow maturation during healing, resulting in a time window of vulnerability for adverse LV remodeling [12–14]; this window of several weeks provides an opportunity for interventions to be applied during healing but is also an interval of susceptibility to adverse effects of drugs on the healing/repair and remodeling processes [54, 55, 80]. While MMP and TIMP levels subside over several days, chronically high MMP relative to TIMP levels may result in continued ECM degradation and thereby contribute to dilative LV remodeling, whereas higher TIMP than MMP levels could contribute to increased ECM and fibrosis in the non-infarct and infarct zones and thereby contribute, in the long term, to diastolic dysfunction.

During healing after MI, ECM degradation plays an important role in facilitating diffusion of proteins (i.e., growth factors, cytokines, chemokines, and matrikines) and mobility of cells (i.e., inflammatory cells, fibroblast, and vascular cells) to modulate LV remodeling [12–14, 16]. Growth factors such as TGF-β and proinflammatory cytokines such as angiotensin II, IL-6, and TNF-α that are released into the interstitial space modulate MMP/TIMP imbalance, ECM degradation or interstitial fibrosis, and remodeling [16, 25], whereas the matricellular proteins modulate the cellular responses during healing [16].

7 Pertinent MMPs and TIMPs in LV Remodeling Post-MI

MMPs that have been implicated in post-MI LV remodeling include MMP-1, MMP-2, and MMP-9 (Tables 21.1 and 21.2). Transgenic studies have identified phenotypes of the MMPs 1, 2, 3, 9, and 14 (Table 21.2) and TIMPs 1 and 3 (Table 21.3). The collagenase MMP-1 shows high affinity for fibrillar collagens and preferentially degrades collagens I and III after MI [19], and their disruption results in LV dilation/dysfunction [20]. MMP-1 synthesis is increased in MI [21]. TIMP-1 co-localizes with MMP-1 in normal myocardium, and TIMP-1 deletion results in dilative LV remodeling post-MI in mice [26]. Of note, increased TIMP-1 levels correlate with markers of HF and LV remodeling in patients with MI [27] and hypertension [81, 82]. MMP-2 or gelatinase A [34–37, 83, 84] and MMP-9 or gelatinase B [34, 35, 83–86] have both

Table 21.1 Main matrix metalloproteinases in remodeling post-MI: sources and substrates

Class	MMP	Source	Substrate
Collagenases	MMP-1	Fibroblast	Collagens I, II, III, VII, X; gelatins; proteoglycans; entactin
	MMP-8	Neutrophil	Collagens I, II, III
	MMP-13	Undefined	Collagens I, II, III
Gelatinases	MMP-2	Macrophage Myofibroblast Myocyte	Gelatins; collagens I, II, III, IV, V, VII, XI; fibronectin; laminin; elastin; proteoglycans
	MMP-9	Neutrophil Macrophage Myocyte	Gelatins; collagens I, II, III, IV, V, VII; elastin; entactin; proteoglycans
Stromelysins	MMP-3	Myocyte	Gelatins; collagens III, IV, IX, X; collagen telopeptides; proteoglycans; fibronectin; laminin; MMP activation
Membrane type	MMP-14	Fibroblast Myocyte VSMC	Collagens I, II, III, IV; gelatin; fibronectin, laminin; proMMP-2 and proMMP-13 activation

MI myocardial infarction, *VSMC* vascular smooth muscle cell

Table 21.2 Matrix metalloproteinases in remodeling post-MI: phenotypes

MMP	Cardiac phenotype	
MMP-1	ROE	6 months, LV hypertrophy; 12 months LV dilation Increased collagen degradation with aging LV dysfunction with aging
MMP-2	KO	Decreased LV dilation and rupture post-MI Decreased inflammation post-MI
	ROE	LV contractile dysfunction with aging Dilated cardiomyopathy with aging
MMP-3	KO	No phenotype post-MI Cell proliferation and cytokine release defects Impaired scar maturation post-MI
MMP-7	KO	Increased survival post-MI Reduced degradation of conduction system proteins
MMP-9	KO	Decreased inflammation and rupture post-MI Decreased LV dilation post-MI Decreased collagen deposition post-MI
MMP-14	KO	Dwarfism and connective tissue defects

KO knockout/gene deletion mouse data, *MI* myocardial infarction, *LV* left ventricular, *ROE* restricted overexpression/transgenic mouse data

Table 21.3 Tissue inhibitors of matrix metalloproteinases in remodeling post-MI

TIMP	Source	Cardiac phenotype
TIMP-1	Fibroblast Myocyte	Hypertrophy and adverse LV remodeling post-MI Increased ECM loss post-MI Enhanced LV dilation/systolic dysfunction post-MI
TIMP-2	Fibroblast Myocyte	Not known
TIMP-3	Fibroblast Myocyte	Hypertrophy, LV dilation, contractile dysfunction with aging Increased MMP-2 activation in fibroblasts Increased cytokine processing
TIMP-4	Fibroblast Myocyte	Not known

KO knockout mouse data, *LV* left ventricular, *MI* myocardial infarction

been implicated in post-MI remodeling, but the role of MMP-3 (stromelysin-1) remains unclear [34, 83]. Elastase seems to modulate ECM degradation through activation of MMP-2, MMP-3, and MMP-9 and inactivation of TIMP-1 [87], while elastase inhibition before reperfusion seems to reduce infarct size [88].

8 Role of Protease MMP-9 in LV Remodeling Post-MI

Three findings underscore the role and multifunctionality of MMP-9 in LV remodeling post-MI. First, besides processing collagen that has been cleaved by collagenases such as MMP-1, MMP-9 can process full-length interstitial collagens as well as other substrates without the activation cleavage step for proteolysis [88]. Second, MMP-9 interacts with inflammatory response elements such as activator protein-1, specificity protein-1, and NF-κB and thereby participates in the post-MI inflammatory response [88]. Third, both pharmacologic inhibition and deletion of MMP-9 attenuate post-MI LV dilation and dysfunction [35, 39–42], and deletion stimulates angiogenesis in the infarct zone [38].

9 Role of Protease-Inhibitor TIMP-3 and ADAMs in LV Remodeling Post-MI

Interactions between matrix proteins and inflammatory cytokines may modulate ECM damage. TIMP-3 seems to have an affinity to bind MMP-9, and aged TIMP-3 null mice show increased MMP-9, ECM degradation and LV dilation/dysfunction, and cardiomyocyte hypertrophy [89, 90]. TIMP-3 also regulates inflammation [91] and inhibits ADAM (*a d*isintegrin *a*nd *m*etalloproteinase)-17 and ADAM-10 [89–91]. Both ADAMs 17 and 10 can alter integrins (cell-surface matrix receptors), disrupt cell–matrix interactions, degrade ECM, and contribute to LV dilation [91]. The ADAMs also interact with inflammatory cytokines and alter MMPs and thereby impact dilative LV remodeling and/or injury [90].

10 Role of Inflammation in Healing and LV Remodeling Post-MI

Cumulative evidence indicates that optimal healing/repair of MI is critical for a favorable outcome [12–14] and inflammation is a key factor that modulates the quality of healing/repair after MI and profoundly impacts post-MI matrix and LV remodeling [12–14, 16]. The tight regulation of the inflammatory reaction is essential for adequate healing/repair and formation scar of an adequate scar. Dysregulation, as with excessive activation of proinflammatory mediators and/or prolongation or

expansion of inflammatory response, can result in defective scars and aggravate adverse ECM and LV remodeling [14]. As discussed before, the inflammatory reaction after MI is precisely staged, time dependent, and highly dynamic with two main functions: (1) to clear the MI area of dead cells and matrix debris and (2) to activate the pathways necessary for optimal healing/repair and scar formation. Emerging evidence indicates that these functions are largely modulated by different monocyte and macrophage phenotypes that are clinically highly relevant [16, 74].

In non-reperfused MI, neutrophils infiltrate the site of injury and release four subsets of membrane-bound granules (i.e., secretory vesicles, gelatinase granules, specific granules, and azurophilic granules) in sequence and containing various factors including proteases, ECM proteins, and soluble mediators of inflammation [92, 93]. Gelatinase granules release matrix-degrading enzymes including MMP-9 [86] and other MMPs [94]. Specific granules release neutrophil gelatinase-associated lipocalin (NGAL) which modulates ECM and myocardial remodeling by inhibiting MMP-9 degradation [95, 96]. NGAL levels increase in plasma and both infarct and non-infarct zones of patients with acute MI [97, 98]. Activated neutrophils and monocytes also produce myeloperoxidase (MPO) which enhances remodeling through generation of oxidants including reactive chlorinating species and MMP activation [99]. MPO deletion has been shown to attenuate leukocyte infiltration and LV dilation/dysfunction after MI [100]. Azurophilic granules contain three serine proteases (i.e., proteinase-3, cathepsin G, and elastase) that degrade several ECM components (i.e., elastin, fibronectin, laminin, type IV collagen, and vitronectin) that stimulate the coagulation cascade [101, 102] and serine elastase that activates MMP-9 [86] and promotes both vascular [103] and LV remodeling [104]. Evidence shows that the serine protease proteinase-3 can induce endothelial cell apoptosis through caspase-like activity [105], serine proteases can inactivate proinflammatory IL-6 [106], and cathepsin G can activate platelets [107] and inactivate bradykinin and kallidin [108].

In MI reperfused after 90 min, there is an early surge of neutrophils and apoptosis [12–14, 56]. In the dog model [15], STEMI reperfused at 90 min after coronary occlusion is associated with increased proinflammatory markers such as inducible nitric oxide synthase (iNOS), cytokines IL-6, and TNF-α, anti-inflammatory markers such as TGF-β_1 and IL-10, and markers of cardiomyocyte damage (ischemic injury, infarct size, apoptosis, blood flow impairment, and no-reflow), adverse LV remodeling (LV dilation and dysfunction), and evidence of ECM remodeling with increased expression of MMP-9 and MMP-2 as well as the protease-inhibitor secretory leukocyte protease inhibitor (SLPI), the matricellular proteins secreted protein acidic and rich in cysteine (SPARC) and osteopontin (OPN), and the disintegrin metalloproteinases ADAM-10 and ADAM-17. Studies in the rat model also documented increase in these markers in the later phase of healing after reperfused STEMI [75]. During that later phase, resolution of the inflammatory response is considered critical and involves activation followed by inhibition (negative regulation), containment to the infarct zone, and resolution [16].

11 Role of Monocytes and Macrophages in ECM and LV Remodeling Post-MI

Emerging evidence has clarified the regulatory role of monocytes and macrophages in orchestrating events during early and late phases healing/repair and the concurrent ECM/LV remodeling post-MI [16, 74, 109–113]. In one construct, cues from the tissue environment trigger differentiation of monocytes into two subsets of monocytes or macrophages in a temporally and spatially organized manner through a biphasic response: proinflammatory Ly-6C^{high} monocytes that dominate the early phase and pro-resolution/pro-repair Ly-6C^{low} monocytes that dominate the late phase. Ly-6C^{high} monocytes remove necrotic debris and secrete inflammatory cytokines, reactive oxygen species (ROS), and proteases that degrade ECM, while Ly-6C^{low} monocytes trigger angiogenesis, collagen/ECM synthesis by myofibroblasts, and healthy infarct scar formation. In another construct, macrophages polarize to different phenotypes with different gene profiles and functional characteristics depending on the spatial and temporal cues from upregulated cytokines, chemokines, and growth factors in early and late phases [111]. During the early phase, upregulation of macrophage colony-stimulating factor (MCSF) induces monocyte differentiation [16], with polarization to proinflammatory M1 macrophages that secrete cytokines, ROS and nitrogen intermediates, chemokines, and growth factors, regulate fibroblast function and collagen turnover, modulate angiogenesis, boost inflammation, promote ECM degradation, and clear dead cells and matrix debris [109, 110, 112]. Enhanced M1 macrophage polarization in class A scavenger receptor null mice exacerbates LV dilation/dysfunction and fibrosis; increases production of M1 cytokines including IL-1β, IL-6, and TNF-α; and increases MMP-9 which facilitates secretion of inflammatory cytokines and ECM degradation [113]. Studies of monocyte/macrophage kinetics in early post-MI hearts suggest that macrophages from the spleen migrate to the MI site [114]. During the late phase [112], macrophages polarize to M2 macrophages with enhanced phagocytic activity and high anti-inflammatory activity with increased IL-10, IL-4, and TGF-β1, decoy type 2 IL-1 receptor, and IL-1 receptor antagonist (IL-1Ra), thereby exerting pro-resolution effects with removal of inflammatory leukocytes [115–118] and resolution of ECM deposition, fibroblast proliferation, and angiogenesis [119] which are key for tissue repair [120]. Prolonged and excessive activation of the M1 phenotype enhances inflammation and ECM breakdown and leads to dilative LV remodeling and rupture [16], while persistent M2 activation stimulates excessive fibroblast proliferation, ECM deposition, and fibrosis [121].

12 Fibrosis and ECM/LV Remodeling

Myocardial fibrosis refers to excessive deposition of ECM and collagen. The goal of reparative fibrosis after MI is the formation of a mature infarct scar and preservation of LV geometry and systolic function, while reactive interstitial fibrosis

contributes to increased stiffness with deterioration of diastolic and systolic function [122] and impaired electrical activity [123]. As discussed before, STEMI of moderate size results in predominant dilative remodeling and HF/low EF. While the regulators and suppressors of fibrosis have been reviewed [16, 124, 125], the "braking" and "stop" signals in fibrosis of the infarct and non-infarct zones need study [126]. The traditional concept is that stimulation of fibroblasts by TGF-β after MI leads to differential deposition of ECM, collagens, and fibrosis, with greater amounts in infarct than non-infarct zones, and increasing ratio of collagen fibril type I to type III during the later maturation process [12–14, 16]. While net ECM degradation dominates in the early phase post-MI, net ECM deposition, collagen synthesis, and maturation dominate in the later phase, resulting in differential ECM and LV remodeling during the 2 stages [12–14].

13 Collagen Turnover, ECM Remodeling, and Fibrosis After MI

Collagen turnover in cardiac ECM is regulated by fibroblasts and myofibroblasts [13, 124–126]. Ten main steps in the collagen biosynthesis cascade in these cells [13] include (1) intranuclear pro-α gene transcription, slicing, and modification; (2) intracellular synthesis of pro-α chains in the rough endoplasmic reticulum; (3) hydroxylation of selected prolines and lysines; (4) glycosylation of selected hydroxyl serines; (5) formation of procollagen triple helixes; (6) oligosaccharide processing in the Golgi complex; (7) secretion into the extracellular space; (8) conversion into less soluble molecules by cleavage of propeptides; (9) self-assembly into collagen fibrils; and (10) aggregation of fibrils to form fibers. Spatially, the fibroblasts and myofibroblasts synthesize and secrete preprocollagen types I and III as pro-α-collagen chains which form the triple helix structure of procollagens in the rough endoplasmic reticulum [13, 125]. Procollagen molecules are secreted from the Golgi complex into the interstitial space where cleavage of the end-terminal propeptide sequences enables collagen fiber formation.

Since specific pro-collagen N- and C-proteinases release the two terminal propeptides (i.e., amino (N)-propeptide and carboxy (C)-propeptide) of procollagen molecules into the circulation [127], measurement of the levels of these cleaved propeptides provides an indirect index of fibrillar collagen synthesis and deposition [128]. Markers of synthesis include procollagen type I carboxy-terminal propeptide (PICP), procollagen type I amino-terminal propeptide (PINP), and procollagen type III amino-terminal propeptide (PIIINP). PICP is most commonly used. Serum PICP levels are elevated in MI [128]. While collagens I and III increase within the first 48 h post-MI, type I synthesis persists until 1 month post-MI [129]. While PINP has also been used as a marker of collagen type I synthesis [129], it is not consistently cleaved from the procollagen molecule so that serum levels do not accurately reflect synthesis. PIIINP is used as a marker for type III synthesis and to predict cardiac events and mortality [130]; however, incomplete removal of the N-terminal domain

can lead to incorporation of PIIINP in collagen fibers and underestimation of type III synthesis. Markers of collagen degradation turnover include collagen type I carboxy-terminal telopeptide (CITP) as well as the MMPs. Interstitial collagenase cleaves all three collagen α-chains to form a large telopeptide which remains within the interstitial space and a smaller telopeptide that is released in the blood and can be used as a marker of collagen degradation.

Galectin-3 (Gal-3), a soluble β-galactoside-binding lectin that is expressed by activated macrophages and induces fibroblast proliferation and increased deposition of collagen type I [131], is also emerging as a potential marker of cardiac fibrosis. Gal-3 expression increases in the infarct region of post-MI mice [132] and in both experimental [133] and clinical [134] HF. Evidence shows that Gal-3 is associated with collagen synthesis and deposition, while its genetic disruption or pharmacologic inhibition prevents adverse remodeling, suggesting a role as mediator of HF development and progression [135].

14 Fibroblasts and Myofibroblasts in ECM and LV Remodeling

Fibroblasts which are the predominant non-myocyte cells in the heart regulate ECM homeostasis in normal and diseased hearts. Upon activation by signals after MI, fibroblasts undergo proliferation and synthesize and secrete collagens and other ECM components [12–14, 17], convert into myofibroblasts that express contractile proteins (including α-smooth muscle actin, vimentin, and desmin), and modulate granulation tissue and ECM remodeling and mature scar formation [126, 136, 137]. While ECM degradation dominates in the early phase after MI, ECM deposition dominates the late phase of healing/repair. Importantly in the late phase, myofibroblast apoptosis correlates with progression of granulation tissue into a mature scar, whereas failure of myofibroblast apoptosis may drive the progressive fibrosis.

15 Role of Aging in ECM and LV Remodeling

An important aspect of identifying therapeutic targets for preventing adverse remodeling after MI is that the majority of patients with MI and HF are elderly, aged ≥65 years [14, 46, 51]. Aging of the cardiovascular system is associated with physiologic, biologic, and structural changes that lead to increased ECM and fibrosis, increased ventricular-arterial stiffening, LV diastolic dysfunction, and HF associated with HF/PEF [51, 52, 138, 139]. Emerging evidence suggests that aging impairs healing/repair after STEMI, resulting in augmented adverse LV remodeling [14, 15, 52, 70]. In aging hearts, increased ROS and angiotensin II, through its proinflammatory, pro-oxidant, and pro-remodeling effects, results in increased inflammatory cytokines, oxidative stress markers, MMPs, and other matrix proteins that modulate

healing/repair after MI [14, 15]. Recent data in young and old mice suggests that aging-related adverse LV remodeling may be due to dysregulated ECM remodeling and impaired healing after reperfused MI [140]. Unfortunately, most experimental studies have been done in the young, and therapies are tested mostly in adults and not the elderly.

16 MMP Inhibitors for Preventing LV Remodeling After MI

Few studies have tested inhibitors of collagen synthesis for limiting cardiac fibrosis [13, 14]. Numerous studies have shown attenuation of LV remodeling after MI with broad-spectrum as well as selective MMP inhibitors [23]. A nonselective MMP inhibitor attenuated early LV dilation and dysfunction that developed over 4 days post-MI in mice [40]. An MMP inhibitor given at 5 days post-MI in pigs induced attenuation of LV dilation assessed at 14 days and 8 weeks [39]. Although another preclinical study of an MMP inhibitor in pigs with chronic HF suggested beneficial effects [44], a larger study of the MMP inhibitor PG11680 initiated after acute STEMI failed to limit significantly LV remodeling [45]. Although systemic side effects including a musculoskeletal syndrome have caused concern with their use in cancer patients, the inhibitor was well tolerated after MI [45]. The results of the trial suggest that, although increased MMPs relative to TIMPs is a widely accepted pathway to ECM degradation and LV remodeling, ECM damage may have occurred before initiation of therapy and/or other matrix proteins besides MMPs may have contributed to the ECM damage and adverse LV remodeling.

17 Role of BMP-1/Tolloid-Like Metalloproteinase and SFRP-2

Emerging evidence suggests that several factors modulate ECM/collagen deposition and fibrosis during post-MI healing besides the MMP/TIMP pathway; they include ROS, angiotensin II, the (TGF)-β/SMAD pathway, healing-specific proteins, bone morphogenetic protein (BMP)-1/Tolloid (TLD)-like metalloproteinases, and secreted frizzled-related protein 2 (sFRP-2) [12–14, 23, 32, 126, 141, 142]. Studies of the zebra fish and clawed frog have shown that sFRPs can modulate tissue remodeling by enhancing the activity of the TLD-like metalloproteinases (especially BMP-1), which cleave C-propeptides from procollagen precursors to produce mature collagen fibrils and thereby modulate ECM and LV remodeling [142]. Mammalian sFRP-2 was shown to directly enhance cleavage of procollagen into collagen by TLD-like metalloproteinases in the mouse model [141, 142]. Mammalian sFRP-2 also enhanced BMP-1 procollagen proteinase (pCP) activity [142]. Importantly, in healed cardiac tissue of sFRP-2-null mice, collagen deposition and fibrosis are reduced and systolic function is improved [142].

Previous studies of post-MI healing using other genetic models have identified other proteins that can also be targeted for improving ECM remodeling [141]. In mice with MI, injection of the peri-infarct zone with mesenchymal stem cells overexpressing sFRP-2 resulted in enhanced engraftment and vascular density, reduced infarct size, and improved cardiac function; the sFRP-2 induced cardioprotection evidenced by enhanced survival of mesenchymal stem cells and inhibition of the pro-apoptotic effect of the Wnt-catenin pathway [143].

As discussed previously, fibroblasts and myofibroblasts are the main sources of collagen deposition, and collagen types I and III are the major types of collagen that are upregulated during healing after myocardial infarction. Since activated fibroblasts or myofibroblasts secrete these proteins as precollagens with N- and C-propeptides, these must be removed by proteinases for the formation of mature collagen. Since the proteinase responsible for cleaving C-propeptide is BMP-1/TLD-like metalloproteinase, supporters of sFRP-2-induced inhibition of Wnt signaling and the activity of TLD-like metalloproteinase argue that exogenous sFRP-2 might modulate the accumulation of mature collagen fibrils during cardiac remodeling. In their view, sFRP-2 augmentation rather than inhibition would limit post-infarct fibrosis. The opposite conclusions [142, 144] may be explained by a biphasic effect of sFRP-2 on BMP-1 related to dose. In fact, physiological doses (10–20 nM) of sFRP-2 were shown to increase BMP-1 activity as measured by in vitro procollagen cleavage [142]. It is possible that at higher pathological doses (80–200 nM), sFRP-2 might inhibit BMP-1 activity. In fact, 100-fold upregulation of sFRP-2 in Akt-mesenchymal stem cells suggested that high doses of sFRP-2 may inhibit BMP-1 activity. Notwithstanding the apparent controversy, sFRP-2 is potentially promising as a physiologically active agent that can directly target the collagen maturation in the infarct area during healing after MI. A protein-based therapy has clear advantages over cell-based therapy in that setting.

18 Conclusions

First, the collective evidence indicates that ECM/LV remodeling after MI is a multifactorial, highly dynamic, and time-dependent process in which dysregulation of ECM homeostasis leads to adverse ECM remodeling, adverse dilative cardiac remodeling/dysfunction, and adverse outcome. Typically, ECM/LV remodeling begins early post-MI, spans the equally dynamic and time-dependent early and late phases of the healing/repair process, and continues for years in survivors.

Second, proteases play an important and central role in the post-MI ECM/LV remodeling process. Dramatic ECM/LV remodeling follows STEMIs; a sharp rise in MMPs creates MMP/TIMP imbalance that drives rapid ECM degradation, leading to decreased collagen, adverse ECM/LV remodeling, and dysfunction. Although MMP and TIMP levels subside over days, chronically high MMP/TIMP ratios may promote continued ECM degradation and contribute to the commonly observed progressive LV dilation during healing and remote MI. In contrast, a chronically

low MMP/TIMP ratio may contribute to increased ECM and fibrosis in infarct and non-infarct zones, leading to increased stiffness and diastolic dysfunction and contributing to further systolic dysfunction in the long term. In addition, defective ECM and fibrosis with increased cross-linking can augment adverse LV remodeling and systolic/diastolic dysfunction. Furthermore, decrease in ECM and fibrosis and/or defective ECM (with increased collagen type III and decreased or abnormal cross-linking) can lead to more adverse LV remodeling with predominant systolic dysfunction and rupture.

Third, proteases are therefore considered as attractive potential targets to limit, prevent, or reverse LV remodeling and dysfunction after MI. Although translational studies showing that pharmacologic inhibition or genetic deletion of key MMPs could effectively prevent post-MI ECM/LV remodeling and its complications and established cause and effect and proof of concept, a clinical trial of MMP inhibition failed to limit LV remodeling. Parallel studies suggested that the post-MI ECM/LV remodeling process is more complex than previously thought, and other proteases, matrix proteins, signaling molecules, and pathways besides MMPs participate in the process. These studies underscored the multifunctional aspect of the key players including the proteases, the interactions and cross talk that occur, and the spatiotemporal aspects, which, taken together, would support a combinatory and timed approach to therapy.

Fourth, four key points about the real world need to be considered in efforts to identify optimal strategies to maximize the benefits of protease inhibitors after MI. One, it is combined damage to muscle, ECM, and microcirculation and not just ECM damage that drives progression to dilative LV remodeling/dysfunction with HF/low EF. Two, LV remodeling/dysfunction and HF/low EF occur/persist despite conventional therapies, and several therapies also impact the ECM/LV remodeling process. Three, the majority of patients are elderly, and aging impacts the ECM/LV remodeling process. Four, a sound knowledge of the modulators of ECM/matrix/LV remodeling is critical in efforts to prevent and interrupt/limit/reverse the progression to end-stage HF.

Fifth, identifying a specific therapeutic target may be difficult because the multiple proteases and pathways are involved. For example, at least four ECM proteolytic pathways were identified in our studies [12–15]: (1) the classic one with increased MMPs, ECM turnover, oxidants, proinflammatory cytokines, and growth factors; (2) increased ADAMs 10/17 which have been implicated in LV dilative remodeling by reducing cell–matrix interactions via integrin shedding; (3) increased serine protease-inhibitor SLPI; and (4) the cellular response with different subsets of monocytes and macrophages in early and late phases of healing.

In summary, further studies are needed to identify the optimal strategy for maximizing the benefits of protease inhibitors after MI. Combinatory therapeutic strategies may be needed to halt HF progression. For optimal benefit, therapeutic strategies may need to be appropriately timed and modified for elderly patients.

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Chapter 22
MMPs in Cardiovascular Diseases: Emerging Pharmacological Targets

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Abstract Matrix metalloproteinases (MMPs), a family of proteolytic enzymes important in the degradation and turnover of extracellular matrix (ECM) components, result in geometric and structural changes of the myocardium which has become a major mechanism for the progression of various cardiovascular pathologies including atherosclerosis, hypertension and end-organ damage, myocardial infarction, ischemia-reperfusion injury, and hypertrophic cardiomyopathies which ultimately result in heart failure. The natural MMP inhibitors known as Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) also play a major role in regulating the myocardial remodeling by working in a balance with MMPs. An imbalance between MMPs and TIMPs results in disease condition. Restoration of this balance is therefore a promising therapeutic strategy for the treatment of cardiac diseases. Use of synthetic inhibitors to regulate the MMP activity serves as emerging candidates of clinical utility in order to design effective therapeutic strategies. In this chapter we summarize about the MMP family, their involvement in various cardiovascular disease conditions along with the role of MMP inhibitors. We have also mentioned different techniques which can be useful in identification of MMPs and TIMPs and help unveil the mechanisms related to them in disease conditions.

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1 Introduction

Heart failure is a growing epidemic affecting a wide range of population worldwide in terms of morbidity and mortality [1]. Geometric and structural changes in the myocardia ensuing remodeling are a major mechanism for heart failure progression. Timely degradation of extracellular matrix (ECM) is an important feature of development, morphogenesis, tissue repair, and remodeling [2]. This is accompanied by an invariable hormonal change increasing both the preload and afterload on the heart in different pathologies including hypertension, hypertrophic cardiomyopathy, dilated cardiomyopathy, diabetic cardiomyopathy, and myocardial infarction. The vasoactive hormones accompanied by several other mediators including intracellular Ca^{2+}-overload, oxidative stress, etc., result in the activation of different proteases which are intimately involved in the process of cardiac remodeling and further heart failure [3, 4]. The proteolytic activity is beneficial under normal physiological conditions and eliminates misfolded or malfunctional proteins, but their involvement in biochemical remodeling of subcellular organelles and pathogenesis of heart disease makes them a topic of investigation [5]. Protease families including calpains, cathepsins, and caspases along with the involvement of their endogenous inhibitors like calpastatin have been extensively reviewed for their participation in cardiovascular disease [6, 7]. Matrix metalloproteinases (MMPs) are an upcoming target studied for their proteolytic activities which play an important role in the development of both geometric and biochemical remodeling as well as cardiac dysfunction in heart failure [8].

MMPs are a family of Zn^{2+}-dependent endopeptidases capable of cleaving components of ECM. They usually have low or negligible activities in the normal cell state, but their activity increases in response to stress conditions. Their expression is tightly regulated transcriptionally by inflammatory cytokines, growth factors, hormones, and cell–cell and cell–matrix interaction [9]. Since these proteases are secreted as inactive proMMPs, their activities are regulated by precursor zymogens. An important class of regulators of these proteases is their endogenous inhibitors, Tissue inhibitors of matrix metalloproteinases (TIMPs). A balance between MMPs and TIMPs is critical for the eventual ECM remodeling in the tissue. In this chapter, we aim to summarize the structure and the biology of action of MMPs in cardiac disease conditions. Besides this, we shall be discussing about the role of their endogenous inhibitors of MMPs in determining the extent of proteolytic activity in heart failure. Further, we shift our focus on the synthetic inhibitors of MMPs and the use as potential therapeutic agents in order to target cardiovascular complications. We also describe various assays that can be used for the identification and evaluation of MMP and TIMP activity.

2 MMP: Structure and Function

Currently, 23 MMP genes have been identified in humans, and most are multidomain proteins. A summary of different classes of MMPs based on their domain organization and substrate preference is given in Table 22.1. The essential components of MMP protein structure are a propeptide of about 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide or a hinge region of variable lengths, and a hemopexin (Hpx) domain of about 200 amino acids with an exception of MMP-7 (matrilysin 1) and MMP-26 (matrilysin 2) which lack the linker peptide and the Hpx domain. Additionally, MMP-23 has a unique cysteine-rich and an immunoglobulin-like domain after the metalloproteinase domain, and it lacks the linker peptide and Hpx domain [10].

A large number of 3D structures of MMPs have been determined both by X-ray crystallography and NMR spectroscopy, the earliest reported in 1994. The structure of the prodomain is known for MMP-2, MMP-3, and MMP-9. It consists of three α-helices and connecting loops. The "bait region" is the first loop, located between helices 1 and 2, and it is protease sensitive. Helix 3 extends into a peptide region comprising the conserved cysteine switch and lies in the substrate-binding cleft of the catalytic domain. The orientation of this polypeptide segment is opposite from that of a peptide substrate. This region forms a fourth ligand of the active-site zinc, keeping the zymogen inactive. However, the hydrogen bonds that it makes with the active site are identical to those of a substrate backbone [11].

The polypeptide chain folds of MMP catalytic domains are essentially superimposable. The chain consists of a 5-stranded β-pleated sheet, three α-helices, and connective loops. It contains one catalytic zinc, one structural zinc, and, generally, three calcium ions which stabilize the structure. The docking of substrate is determined by the substrate-binding cleft which is formed by strand IV, helix B, and the extended loop region after helix B. The hydrophobic pocket called the specificity pocket or S1′ pocket, located to the right of the zinc atom, is one of the determining factors of substrate specificity of MMPs. Three histidines coordinate the active-site zinc. The loop region contains the conserved "Met-turn," a base to support the structure around the catalytic zinc [12, 13]. The fourth ligand of the catalytic zinc is a water molecule which is displaced after substrate binding. The glutamic acid adjacent to the first histidine is essential for catalysis. The carboxylate group of the glutamate facilitates peptide bond hydrolysis by drawing a proton from the displaced water making it a polarized nucleophile which attacks the carbonyl group of the peptide bond.

Three repeats of fibronectin type II domains found in MMP-2 and MMP-9 are inserted between the fifth β-strand and the catalytic site helix and consist of two antiparallel β-sheets, connected with a short α-helix and stabilized by two disulfide bonds. NMR studies have indicated that domains 2 and 3 are quite flexible, possibly interacting simultaneously with multiple sites in the ECM [14].

The hemopexin domains have a 4-bladed β-propeller fold, with a single disulfide bond between the first and the fourth blades for stabilization. The center of the propeller generally contains one calcium ion and a chloride ion. β-Propeller domains are also found in other proteins, such as heterotrimeric G proteins, clathrin, and the

Table 22.1 The MMP family

MMP	Alternative name	Major substrates	Molecular weight (kDa)		Motifs
			Pro	Active	
Collagenases					
MMP-1	Fibroblast collagenase	Collagens I, II, III, VII, X; gelatin	55	45	A, B, C
MMP-8	PMNL collagenase	Fibrillar collagens I, II, III	75	58	A, B, C
MMP-13	Collagenase-3	Collagen I	60	48	A, B, C
Gelatinases					
MMP-2	Gelatinase A	Gelatin; collagens I, II, III, IV, VII, X	72	66	A, B, C, D
MMP-9	Gelatinase B	Fibronectin; collagens IV, V	92	86	A, B, C, D
Stromelysins					
MMP-3	Transin-1	Fibronectin; laminin; gelatins I, III, IV, V; collagens III, IV, X, IX; and cartilage proteoglycans	57	45	A, B, C
MMP-10	Transin-2	Fibronectin; gelatins I, III, IV, V; and collagens III, IV, V	57	44	A, B, C
MMP-11	Stromelysin-3	Collagen IV, fibronectin, laminin, aggrecan	51	44	A, B, C
Matrilysins					
MMP-7	Matrin	Casein; gelatins I, III, IV, V; and fibronectin	28	19	A, C
MMP-26	Endometase	Collagen IV, fibronectin, fibrinogen, beta-casein, type I gelatin, and alpha1-proteinase inhibitor	28	19	C
Membrane-type MMPs					
MMP-14	Membrane-type matrix metalloproteinase 1	Gelatin, fibronectin, laminin	66	56	A, B, C
MMP-15	Membrane-type matrix metalloproteinase 2	Gelatin, fibronectin, laminin	72	50	A, B, C
MMP-16	Membrane-type matrix metalloproteinase 3	Collagen III, fibronectin, laminin	64	52	A, B, C
MMP-17	Membrane-type matrix metalloproteinase 4	Fibrinogen, fibrin	57	53	C
MMP-24	Membrane-type matrix metalloproteinase 5	Proteoglycans, fibronectin	57	53	B, C
MMP-25	Membrane-type matrix metalloproteinase 6	Gelatin	34	28	C
Others					
MMP-12	Macrophage elastase	Elastin, fibronectin, collagen IV	54	45, 22	A, B, C
MMP-19	Matrix metalloproteinase RASI	Aggrecan, collagen IV, laminin, nidogen, nascin-C isoform, fibronectin, and type I gelatin	54	45	C
MMP-20	Enamelysin	Amelogenin, aggrecan, and cartilage oligomeric matrix protein	54	22	A, C
MMP-21	XMMP	Alpha1-antitrypsin, aggrecan	62	49	C
MMP-23	Femalysin	Gelatin, casein, fibronectin	28	19	C
MMP-27	–	Elastin, aggrecan	N.D.	N.D.	A, B, C
MMP-28	Epilysin	Casein	56	45	C

[a]MMP-4, MMP-5 and MMP-6 have not been cloned and sequenced
[b]MMP-18 is Xenopus collagenase 4. No human homologue of MMP-18 has been yet identified
[c]MMP-22 has been renamed as MMP-23B, and its sequence is similar to MMP-21
A—Matrixins cysteine switch
B—Hemopexin domain signature
C—Neutral zinc metallopeptidases, zinc-binding region signature
D—Fibronectin type II collagen-binding domain signature

α-subunit of integrins, and mediate protein–protein interactions. The hemopexin-like domain is important for substrate specificity depending on the specific MMP [15, 16].

2.1 Activation of MMPs

MMPs are secreted as proMMPs after the removal of signal peptides from the pre-proenzymes. Proteolytic activation of MMPs is referred to as "stepwise activation" [17]. The bait region is cleaved by plasma proteinases. This destabilizes the rest of the propeptide, including the cysteine switch–zinc interaction, which allows the complete removal of the propeptide via intramolecular processing in trans by the action of the MMP intermediates or by other active MMPs. Thus, the final step in the activation is conducted by an MMP. The activities of these MMPs are regulated by tissue-specific location of the enzyme and inactivation by endogenous inhibitors or proteolysis.

Plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10, and proMMP-13. Plasmin is generated from plasminogen by membrane-associated tissue plasminogen activator and urokinase plasminogen activator creating localized proMMP activation and subsequent ECM turnover [18]. MMPs are also readily activated in vitro by treatment with mercurial compounds, thiol-modifying agents (4-aminophenylmercuric acetate, $HgCl_2$, and N-ethylmaleimide), oxidized glutathione, SDS, chaotropic agents, and reactive oxygen species (ROS) probably due to perturbation of the molecule. Low pH and heat treatment can also lead to their activation [19].

3 MMPs in Cardiovascular Diseases

ECM is essential for the proper functioning of the different organs of the human body, including the heart and blood vessels. The active and continuous changes in cell–cell adhesion, cell migration, cell proliferation, apoptosis, and remodeling are required for normal vascular and heart development. Several studies have shown that ECM degradation by MMPs is involved in the pathogenesis of a wide spectrum of cardiovascular disorders, including hypertension, atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction, and aortic aneurysm. The various mechanisms through which MMPs regulate cardiac remodeling in diseases have been summarized in Fig. 22.1.

3.1 Atherosclerosis and Plaque Rupture

Atherosclerosis is a chronic inflammatory response in the walls of arteries and is caused by the formation of multiple atheromatous plaques which comprise of atheromas, calcification, and underlying cholesterol crystals. These plaques eventually

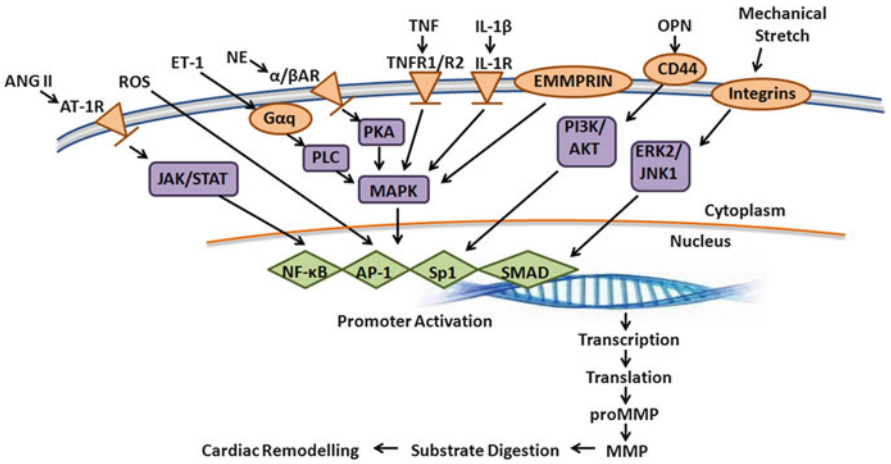

Fig 22.1 Schematic representation of various mechanisms regulating cardiac remodeling. There are various induction factors which give rise to multiple signal transduction pathways ultimately leading to activation of transcription factors which in turn activate the MMP gene transcription on binding to the promoter region upstream of the MMP gene. This is followed by translation producing the inactive proMMPs which on activation result in digestion of their respective substrate in the myocardial extracellular matrix contributing to remodeling of the heart in various cardiovascular pathologies. *ANGII* angiotensin II, *AT-1R* angiotensin-1 receptor, *ROS* reactive oxygen species, *ET-1* endothelin-1, *NE* norepinephrine, *α/βAR* α/β adrenergic receptor, *TNF* tumor necrosis factor, *TNFR* tumor necrosis factor receptor, *IL-1β* interleukin-1 beta, *IL-1R* interleukin-1 receptor, *OPN* osteopontin

rupture leading to superimposed thrombosis, vessel occlusion, and subsequent ischemic end-organ damage. Asymptomatic and stable atherosclerotic plaques are rich in ECM and smooth muscle cells. Several evidences indicate the involvement of MMPs in the formation of atherosclerotic lesions [20]. Local MMP expression within the actual atherosclerotic lesion may be decisive for the role of MMPs, both in differential expression in stable compared with unstable lesions and also in differential expression pattern within specific parts of a particular lesion. They may be involved in the migration of vascular smooth muscle cells to the intimal space where they proliferate and contribute to plaque formation. MMPs may also contribute in degrading the extracellular matrix in the intima thereby diminishing the plaque volume [21].

Galis et al. used an MMP-9 knockout mouse carotid artery model to demonstrate that MMP-9 deficiency leads to a decrease in intimal hyperplasia and lumen loss, but an accumulation of interstitial collagen and speculated enhanced mechanical stability of arteries due to MMP-9 inhibition [22]. MMP-2 and MMP-9 have been shown to be more prevalent in plaques of expansively remodeled vs. constrictive remodeled segments of atherosclerotic coronary arteries [21].

The presence of the MMPs is also associated with sites of potential weakness in the plaque where ruptures tend to occur or where extensive remodeling is taking place. Activation of the MMPs locally, whether through activation of inflammatory signals or free radicals, leads to the degradation of collagen and elastin into peptide fragments, weakens its structure, and permits disruption of plaques [23]. MMPs have also been studied in degradation of fibrous caps of vulnerable atherosclerotic lesions.

The fibrous caps protect the plaques from lesions due to high tensile strength provided by elastin, proteoglycans, and collagens I and III. Macrophage-derived foam cells in the atherosclerotic lesions result in releasing MMPs and hence resulting in thin fibrous cap [24]. MMPs have been implicated in plaque rupture leading to unstable angina where 70 % increase in intracellular MMP-9 levels has been reported [25]. Similarly, MMP-2 has been shown to play a role in plaque calcification [26]. Expression levels of MMP-1 are also increased in regions of high mechanical stress in human coronary lesions [27]. MMP-7 and MMP-12 produced by the foam cells are also involved in plaque rupture. Besides this, studies also show a role of MMP-3, MMP-8, and MMP-14 in plaque rupture and athero-thrombotic events [28–30]. These MMPs further set up a vicious cycle of recruitment of additional inflammation, the increased transformation of cells contributing to the atherosclerotic plaque growth, as well as the promotion of the potential rupture of newly formed plaques. The discovery of new members of the MMP family and the identification of novel substrates for their catalytic activities illustrate the complexity of this pathway.

3.2 Hypertension and End-Organ Damage

Hypertension is a major health problem that is accompanied by enhanced stress of the vessel wall and develops as a disease with organ failure and multifaceted nature of complications that accompany elevated blood pressure. This is usually preceded by hypertensive vascular remodeling which involves degradation and reorganization of the ECM scaffold, as well as hypertrophy and/or hyperplasia of the vascular smooth muscle cells (VSMCs), thus contributing to thickened vessel wall and augmented vascular stiffness. Although initially adaptive, these hemodynamic changes ultimately become maladaptive leading to cardiac and vascular dysfunction that reflects altered cellular phenotypes and ECM composition [31, 32].

Elevation of plasma MMP-9 has been associated with increased arterial stiffness and elevated blood pressure in hypertension, but much of the existing data on plasma MMP levels in hypertension is inconsistent. A functional polymorphism in the promoter of the MMP-9 gene has been identified, which is associated with severity of arterial stiffness and hypertension, and indicates that contributions of MMPs towards hypertension originate at the genetic level [33]. Elevation of plasma MMP-2 and MMP-10 in subjects with hypertensive end-stage renal disease has been observed [34].

2K1C (two-kidney, one-clip) hypertensive rats have been used as a hypertension model to demonstrate the role of MMPs particularly MMP-2, MMP-9, and MMP-14 in increased intima and media thickness of vessels [35, 36]. MMP inhibition was found to reverse the cardiac alterations associated with hypertension [37]. It is postulated that MMPs may promote increased arterial blood pressure through direct interaction with pharmacological receptors or modulation of vasoactive regulatory peptides. Experimental studies have been conducted to evaluate the potential of circulating MMPs as potential biomarkers for hypertension and, therefore, be helpful in its prognosis and also be of clinical utility [38–40]. Inconsistent results with respect to circulating MMPs in hypertensive patients in the studies involving

gestational hypertension have made it difficult to arrive at any conclusion. It is likely that the inconsistencies in reported plasma MMP levels reflect the complex regulation of inflammation by MMPs. It may also be due to differences in study design, severity of hypertension, presence of co-morbidities, use of drugs affecting MMPs, and pre-analytic issues. Thoughtful design and analysis of experiments is, therefore, crucial to interpreting plasma MMP studies.

3.3 Myocardial Infarction

The architecture of myocardium undergoes complex alterations following myocardial infarction which involve dilatation of the left ventricle and infarct thinning, also called infarct expansion. The course of acute myocardial infarction (AMI) is complex and dynamic, involving not only the myocardium but also VSMCs, endothelial cells, and leukocytes [41, 42]. These cells are responsible for the release of MMPs into plasma after AMI. Plasma MMP-2 levels and activity increase gradually after the onset of AMI. Early upregulation of MMP activity after infarction strongly suggests an involvement of MMPs in the repair process of the heart [43]. Inflammation after AMI may enhance production of MMP-1 by peripheral blood mononuclear cells (PBMCs) [44]. These changes may play an important role in the ventricular remodeling that occurs after AMI which leads to loss of the myocardial ECM. Variations in the levels of MMP transcription in patients suffering from MI might contribute to differences in infarct healing, LV remodeling, and the transition to end-stage heart failure or cardiac rupture. MMP-2 and MMP-9 activity increases in a time-dependent manner and is temporally related to cardiac rupture. This deleterious effect of MMPs may be the result of an inappropriate removal of myocardial ECM components and disruption of the myocyte–matrix interface network, causing myocyte misalignment and slippage. MMP-2 and MMP-9 activity is found to decrease though it still remains significantly elevated from normal after reaching its peak within the first 7 days post-MI [43]. MMP-9 levels are significantly reduced at 8 weeks post-MI, whereas MMP-2 levels increase substantially [45]. This clearly indicates the involvement of MMP-2 in early post-MI events. Expression and activity of MMP-1, MMP-3, and MMP-13 increase in the early post-MI events, but MMP-8 which is localized to neutrophils increases starting at 2 weeks post-MI, staying elevated on 5, 8, and 16 weeks post-MI. MMP-8 and MMP-13 remain increased in later stages as well, but MMP-3 is reduced 8 weeks post-MI [46, 47].

3.4 Ischemia/Reperfusion Injury

Stunning injury following reperfusion of the ischemic myocardium is another acute process in which MMPs have been implicated. Cardiac arrest, hemodynamic load, surgical intervention, or multiple organ failure may lead to impairment of one or

more organs causing ischemia/reperfusion (I/R) injury. Restoration of blood circulation after temporary hypoperfusion results in a robust inflammatory response involving cytokines, chemokines, reactive oxygen species that disrupt the cytoskeleton and damage cellular proteins exacerbating tissue damage. I/R injury to the heart is a leading cause of myocardial infarction and one of the important causes of mortality and morbidity in hospitalized patients [48, 49].

The oxidative and nitrosative stress followed by inflammation induce strong transcriptional expression and/or posttranslational activation of MMPs after an ischemic insult [50, 51]. Proinflammatory cytokines such as TNF-α and IL-1β are responsible for transcriptional activation of MMP-2 and MMP-9 [52]. Early upregulation of MMP activation is responsible for mediating myocardial stunning which is reversible and does not cause permanent damage. A long-term consequence of severe ischemia is tissue damage due to imbalance in ECM proteins, such as collagen, laminin, and fibronectin. During the later phases of ischemia-induced tissue injury, enhanced MMP activity seems to be involved in detrimental remodeling. An increase in circulating MMP-2 levels is associated with cardiac dysfunction in patients with ischemic cardiomyopathy. Moreover, increased MMP-14 activity was correlated with the extent of LV remodeling upon cardiac I/R, and MMP-8 expression in the infarct area was upregulated in an ex vivo porcine heart model of I/R. Recently, MMP-7 was shown to cleave fibronectin and tenascin-C in the infarcted LV [53, 54].

3.5 Hypertrophic Cardiomyopathy

Chronic hypertension leads to the occurrence of adaptive developments in the heart muscle that are characterized by hypertrophic changes in the cardiomyocytes. Initially, these developments are compensatory in order to stabilize the tension caused due to the hemodynamic load and achieve a normalized cardiac output. However, a prolonged subjection to stress transforms this physiological response to a pathological one which may ultimately lead to cardiac failure [55].

The cardiomyocytes are terminally differentiated cells, and the number of cardiomyocytes is already laid down during the early phases of fetal development. Hence, these cells increase in size and not in number to combat the prevailing burden and, thus, show a hypertrophic response. The hypertrophic condition is characterized by an enlarged cardiomyocyte size, enhanced protein synthesis (intracellular and extracellular) as well as an altered sarcomeric organization [56]. In addition to this, activation of immediate early response genes, such as c-jun, c-fos, and c-myc, and re-expression of fetal genes like atrial natriuretic factor (ANF), beta-myosin heavy chain (β-MHC), and skeletal alpha actin (SKA) are also witnessed. These fetal genes are regarded as the hypertrophic markers [57, 58]. Furthermore, extensive studies are being carried out to identify certain diagnostic/prognostic markers for hypertrophic conditions, and recent work has reported the possibility of MMPs being established as potent hypertrophic markers [59].

Cardiac hypertrophy involves extensive cardiac remodeling. Induction of hypertrophic conditions causes an excessive synthesis of ECM proteins by the cardiomyocytes where increase in cardiomyocyte size is accompanied with an increase in the ECM surrounding it. Henceforth, there is a rise in collagen deposition along with alterations in other ECM proteins as well as intracellular hormones, cytokines, matrikines, and growth factors [60]. Induction of stress disturbs the intricate balance between formation and degradation of ECM. MMPs serve as crucial mediators, which are overexpressed in the process of ECM remodeling. Time-dependent changes in the myocardial MMP levels have been observed after an acute and prolonged pressure-overload stimulus [61]. Alterations in the protease levels bring about changes in the ECM architecture and so form the basis of hypertrophy.

Studies suggest that out of the entire family of MMPs, only a limited number of specific members are expressed in the failing condition of the hypertrophied heart and also that the increase in the level of MMPs is not uniform. Gelatinases (MMP-2 and MMP-9) have been found to be highly upregulated during hypertrophic conditions and are responsible for ventricular dilatation as well as development of heart failure [62, 63]. Experiments using transgenic mouse models expressing MMP-1 demonstrate that MMP-1 expression brings about changes in the heart and also leads to a systolic dysfunction [64]. Additionally, inhibition of MMP activity has also indicated the attenuation of the onset of left ventricular hypertrophy.

The activity of MMPs is under the control of their endogenous inhibitors, TIMPs that are synthesized locally and bind to MMPs, thus, regulating the proteolytic activity to maintain the ECM integrity. However, during the LV modeling observed in hypertrophic cardiomyopathies, TIMP levels are not found to increase with the same level as that of MMPs [65]. Extracellular matrix metalloproteinase protein inducer (EMMPRIN) has been found to upregulate expression of certain MMPs without increasing the TIMP activity, in parallel [66]. Such preferences, cause imbalances and thus, favor proteolytic activity. The upregulation of MMPs in cardiomyopathies involves binding of cardiac muscle-specific transcription factors to the promoter region of MMP genes. The promoter region of the majority of MMP members contains the activator protein-1 (AP-1) and polyoma enhancer A-binding protein-3 (PEA-3) site interacting with Fos/Jun family and Ets family of transcription factors, respectively. In addition to this, promoter region of MMP-9 gene also contains a nuclear factor kappa B (NF-κB) site [67, 68].

Thus, it can be ascertained that imbalance favors the degradation of ECM proteins by the active MMPs and causes a continued remodeling of ECM.

4 Endogenous Matrix Metalloproteinase Inhibitors

The primary endogenous inhibitors that regulate MMP activity are the TIMPs. Additionally, other proteins including domains of netrins, the procollagen C-terminal proteinase enhancer (PCPE), the reversion-inducing cysteine-rich protein with Kazal motifs (RECK), and tissue factor pathway inhibitor (TFPI-2) have also been

Table 22.2 Overview of the TIMP family

Characteristic	TIMP-1	TIMP-2	TIMP-3	TIMP-4
M.W. (kDa)	28.5	21	27	22
mRNA (kb)	0.9	1.0; 3.5	5.0; 2.4; 2.6	1.4
Chromosome location	Xp11.3-11.23	17q25	22q12.3	3p25
Expression	Inducible	Constitutive	Inducible	Regulated and very restricted
Primary site of action	Reproductive organs	Multiple tissues	Heart, kidney, thymus	Heart and brain
Affinity for MMPs	Low affinity for MT-MMPs	Weaker affinity for MMP-3 and MMP-7	Broad inhibitor	Very less is known

involved; however, their role is not very clear. Proteins like α2-macroglobulin, thrombospondin-1, and thrombospondin-2 have been reported as molecules that remove some MMPs from the extracellular environment.

4.1 TIMP Family

TIMPs, the primary regulators of MMP activity in the ECM, form a 1:1 complex with MMPs through an interaction between the N-terminal domain of TIMP and the catalytic domain of MMP. These proteins have six disulphide bonds and comprise a three-loop N-terminal domain along with an interacting three-loop C-subdomain [69]. Four members in the TIMP family (TIMPs 1–4) with distinct properties are known presently [70]. An overview of the characteristics of TIMP family members is given in Table 22.2. A delicate balance between MMPs and TIMPs under normal conditions is largely responsible for regulating the control of degradation of ECM proteins; however, a dysregulation of this balance arises during the cardiovascular pathologies [71].

4.2 Other Proteins

Molecules like netrins, PCPE, and TFPI-2 share sequence similarity with the specific region of TIMPs for the inhibitory effect and are, therefore, considered as TIMP-like molecules [72, 73]. Inhibition by α2-macroglobulin involves the presentation of a cleavable "bait" region which is proteolytically digested bringing about a confirmational change entrapping the proteinase and leading to a loss of its activity, while the RECK gene encodes for glycoprotein-containing serine protease inhibitor-like domains that are responsible for modulating the MMP activity [74, 75]. Additionally, thrombospondins have been found to modulate MMP function through complex formation which facilitates a scavenger receptor-mediated endocytosis process [76].

5 Synthetic Matrix Metalloproteinase Inhibitors

Disturbances caused in the intricate balance maintained between MMP and its endogenous inhibitors lead to the destruction of the ECM in various cardiovascular pathologies. Extensive research is being carried to develop synthetic inhibitors that shall act as a support to help regain the MMP–TIMP balance. These inhibitors are being designed such that they either inhibit the upregulated MMP activity or increase the local concentration of TIMPs by using recombinant proteins or a gene-based therapy to have a tight control over MMP expression. Use of such synthetic inhibitors can be beneficial to block or reverse the progression of the developing pathological conditions.

A number of MMP inhibitors have been developed over the past few years; however, their design, synthesis, development, and testing have been a big challenge and have still given unsatisfactory results [77]. Out of the many inhibitors that have reached up to the clinical trial phase, only collagenase inhibitor named Periostat (doxycycline hyclate) has been approved by the Food and Drug Administration (FDA) as a drug for treatment of periodontal disease [78]. The prime reasons for these failures have been attributed to the lack of efficacy, broad-spectrum nature, metabolic lability, and toxic side effects of these inhibitors [59]. Almost all broad-spectrum synthetic inhibitors have been found to show musculoskeletal toxicity that ranges from mild myalgia and arthralgia to frank tendonitis and arthritis [79]. Moreover, the role of MMPs is multifaceted, and they play an important role in maintenance of the normal developmental process. Hence, they cannot be suppressed completely, and such inhibition causes complications.

The first-generation synthetic inhibitors are broad spectrum in nature and utilize the zinc-binding group to achieve MMP inhibition. However, they have been found to inhibit even unrelated Zn-dependent proteinases, thus, showing a very poor specificity. Furthermore, the second generation of inhibitors uses a less avid zinc-binding group and has shown a better response. Presently, it is the highly selective third-generation drugs that are being explored upon employing a couple of biophysical techniques and modeling methods [80].

In the current scenario, it is very important to understand and know the biology of MMPs precisely. It is only with an in-depth knowledge of MMPs and their endogenous inhibitors, primarily TIMPs, that much more specific and effective treatments can be investigated upon. The amount of information available on MMPs and their role is increasing and, thus, is paving way for targeting a number of points at which MMP activity is being regulated. Use of a combination therapy or initiation of treatment in the early stage is being evaluated. Also, an integrated approach of theoretical and experimental analysis is being adopted to fully exploit the available information and develop inhibitors of high quality. Thus, it can be concluded that the current need is to design an optimal drug that maintains a delicate balance between efficacy and toxicity.

6 MMP and TIMP Activity Assays

An accurate understanding and a deeper insight of MMPs, TIMPs, and their associated interactions are necessary for synthesizing narrow spectrum inhibitors or high-quality broad-spectrum inhibitors. The following section summarizes assays used for the assessment of MMPs and TIMPs. These techniques have been represented in Fig. 22.2.

6.1 Assays for Assessment of MMPs

Substrate Zymography

It is an electrophoretic technique that involves incorporation of the specific substrate in the polyacrylamide gel and separation of proteins based on their molecular weight under denaturing but nonreducing conditions. Clear bands indicating region of proteolytic digestion against a stained background are obtained which can be quantified by densitometry. Different MMPs, depending on the substrate used, may

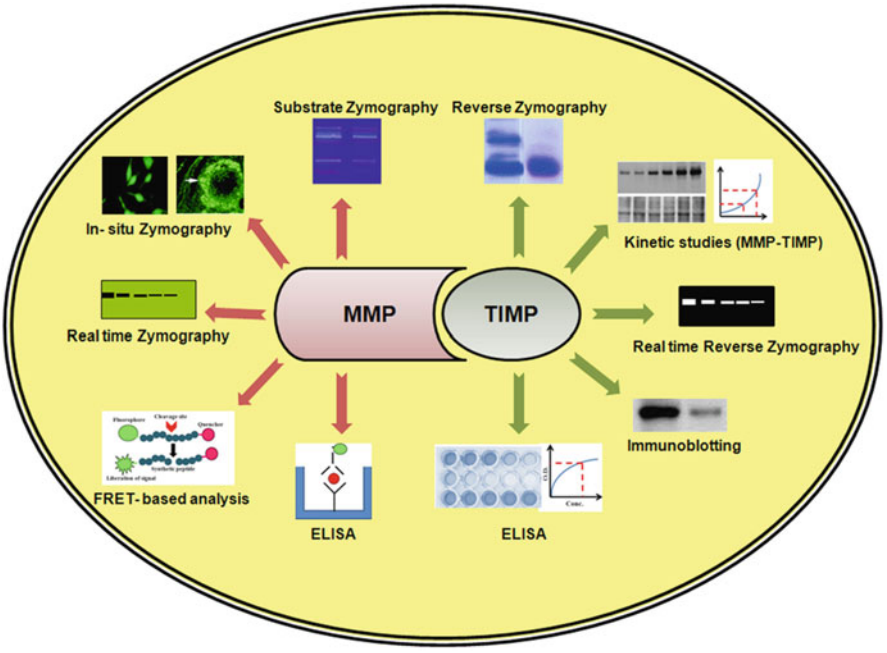

Fig 22.2 Overview of various MMP and TIMP activity assays. A number of techniques have been developed for the identification of MMPs and their endogenous inhibitors, TIMPs. This figure shows the results obtained during the assessment of their activity, expression, and interactions

be detected. Some of the substrate zymographies commonly used include gelatin zymography, casein zymography, collagen zymography, and the heparin-enhanced zymography [81]. Although the protocol is long, both the latent and active forms can be visualized on gel owing to a difference in their molecular weights.

In Situ Zymography

Substrate zymography is a well-established technique used for the identification of MMPs in polyacrylamide gels; however, it cannot be used for localization of this enzymatic activity. Also, certain limitations pertaining to homogenization of samples, incorporation of complex substrates in gel, have been encountered. In situ zymography (ISZ) offers the advantage to assess the functional activity on site within the histochemical or cytochemical sections [82, 83]. The basic principle involves digestion of specific substrate by activated enzymes present at their native location followed by detection of the liberated signal. Based on the nature of substrate, it has been classified as photographic emulsion-based ISZ and fluorescent-labeled substrate-based ISZ [84].

Real-Time Zymography

Substrate zymography involving conventional staining of zymograms with Coomassie stain requires optimization of the digestion time prior to its permanent fixing by the stain. However, real-time zymography, a modification of substrate zymography, can be used to monitor proteolytic activity without terminating the reaction. This technique utilizes a fluorescent substrate (e.g., FITC-labeled casein or collagen) whose digestion is visualized directly under the UV transilluminator where dark bands of the zone of digestion are obtained against a fluorescent background. It has higher sensitivity than conventional methods and gives a semiquantitative representation of the MMP activity [85].

Fluorescence Resonance Energy Transfer (FRET)-Based Analysis of MMPs

Short synthetic peptides that mimic the cleavage site present on the MMP-specific substrate are used for the analysis of MMP activity. A fluorophore and quencher molecule are tagged on the opposite ends of the synthetic peptide for the detection of proteolytic activity which is measured in terms of the fluorescent signal obtained. The amount of fluorescent signal is proportional to the activity of MMP. This assay can be adapted to a high-throughput screening format; however, the sequence of synthetic peptide used is different from the sequence of natural MMP-specific substrate, and therefore, there exists only a partial mimicking of the native interactions. Also, detergents like Brij-35 used in the protocol to minimize the nonspecific interactions with the plasticware can cause interference in the screening procedures [86].

Enzyme-Linked Immunosorbent Assay for MMPs

Enzyme-linked immunosorbent assay (ELISA) kits are available for the quantitative analysis of MMPs. This assay measures the amount of MMP but does not provide an idea about its protease activity. Antibodies are coated on the ELISA plate, and very less quantity of sample for detection of MMP antigen in the sample is required. However, this technique is unable to detect MMP complexes/dimers that have a prominent role in MMP-associated diseases [86].

6.2 Assays for Assessment of TIMPs

Reverse Zymography

It is a technique similar to substrate zymography that involves copolymerization of an MMP and its specific substrate with the polyacrylamide gel under denaturing, nonreducing conditions. Digestion of the substrate by the MMP is observed at all positions except where TIMP has inhibited MMP activity. Hence, a clean gel with blue bands indicating zones of MMP inhibition is obtained. A plain SDS-PAGE gel of the samples is always run as a control. Picogram quantities of TIMP can be analyzed, and the TIMPs can be identified based on their molecular weight [87].

Real-Time Reverse Zymography

This method of identification of TIMPs and assessing their activity is similar to real-time zymography except that the polyacrylamide gel also contains a specific MMP along with its fluorescent-labeled substrate. The activity can be monitored under a UV transilluminator while the reaction is in progress. The gel fluoresces homogenously in the initial phase; however, the fluorescence of the background decreases with time due to MMP activity on the substrate. Fluorescing bands, highlighting zones of MMP inhibition by TIMPs can be observed against the dark background of gel [85].

Kinetic Studies for Assessment of MMP–TIMP Interaction

TIMP molecules bind to MMP following a 1:1 stoichiometry and form a tight, reversible complex that follows model of the type ([MMP] + [TIMP] = [MMP − TIMP]) where K_{asso} is the rate constant for forward reaction, while K_{disso} is the rate constant for reverse reaction. K_i, the equilibrium dissociation constant that indicates the affinity between the inhibitor and target enzyme, is described as K_{asso}/K_{disso} [88]. The enzyme concentrations are kept below K_i such that the association rate is slowed to an extent that is measurable. Equilibrium determination of K_i and progress curve determination of K_i and K_{asso} are carried out as per established protocols and the interactions between MMP and TIMP are assessed.

Immunoblotting

The well-known technique of immunoblotting/Western blotting may also be used for TIMP-based studies where the TIMP molecules in a sample can be identified based on their interaction with a specific antibody as well as molecular weight. In parallel to this, other antigen–antibody interaction-based techniques like immunohistochemistry/immunocytochemistry can be performed as well.

Enzyme-Linked Immunosorbent Assay

This helps in carrying out a quantitative analysis of TIMPs in the biological sample. ELISA kits containing plates with pre-coated specific antibody are generally used. The antigen–antibody interaction is detected via the use of secondary antibody labeled with a chromophore or fluorophore. A number of samples can be processed in parallel using this technique.

7 Conclusion

A prominent role of MMPs in the development and progression of a number of cardiovascular pathologies has been determined. Further studies for a complete characterization of their specific functions must be carried out in the near future. MMPs hold a great potential to be established as predictive biomarkers for various cardiovascular diseases and may also serve as a potent tool in the field of clinical diagnostics. The ongoing research should also focus on development of novel assays or modifications in the existing methodologies for activity and interaction studies that can help in distinguishing between the members of the MMP or TIMP family more distinctly. Use of natural bio-drugs as synthetic MMP inhibitors is an innovative therapeutic strategy which is also being investigated upon and is a prospect for the future.

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Chapter 23
Proteases as Clinical Markers of Adverse Remodeling for Heart Failure in the Aging Population

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Abstract The heart failure (HF) burden, the aging population at large, and the aging population with HF have been increasing concurrently in developed countries worldwide. Epidemiological studies have warned that this trend will continue and lead to bankruptcy in health care unless new effective strategies and therapeutic targets are introduced. Translational studies have shown that adverse left ventricular (LV) remodeling is the principal mechanism for the march to HF after myocardial infarction (MI) and hypertension (HTN), the leading causes of HF in the elderly population aged ≥65 years. Evidence indicates that proteases play an important role in the remodeling that occurs at the extracellular as well as cellular, subcellular, and molecular levels and the march to HF. Besides the matrix metalloproteinases (MMPs) that play a critical role in extracellular matrix (ECM) and LV remodeling, several other proteases have also been implicated in adverse LV remodeling and dysfunction and the march to HF after MI, HTN, and cardiomyopathies. Several are released in the blood and can be imaged in the myocardium. Proteases are therefore attractive as potential clinical markers of adverse remodeling for HF in the aging population, not just for tools in diagnosis and follow-up but also in guiding therapy and prevention.

Keywords Aging • Cardiac remodeling • Heart failure • Markers of adverse remodeling • Matrix remodeling • Proteases • Protease markers

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1 Introduction

The heart failure (HF) burden [1–5], the aging population at large, and the aging population with HF [6, 7] have been increasing concurrently in developed countries worldwide. HF has become a serious cause of disability and death in both adult and elderly populations [1, 6, 7], and the HF burden has continued to increase despite progress and recommended therapy [1–5, 7–11].

Epidemiological studies have warned that this trend will continue with contemporary therapies [1–5, 12] and lead to bankruptcy in health care [13], unless new effective strategies and therapeutic targets are introduced [6, 7]. To stress the magnitude of the problem, the estimated cost of HF for 2010 increased to $39.2 billion in the USA [1]. Translational studies have established that adverse left ventricular (LV) remodeling is the principal mechanism for the development and march to HF after myocardial infarction (MI) and hypertension (HTN), the two leading causes of HF in the elderly population aged ≥65 years [6, 7, 14–16]. Evidence indicates that proteases play an important role in the remodeling that occurs at the extracellular as well as cellular, subcellular, and molecular levels and the march to HF [14–17]. Besides the matrix metalloproteinases (MMPs) that play a critical role in extracellular matrix (ECM) and LV remodeling [14–16], several other proteases have also been implicated in adverse LV remodeling and dysfunction and the march to HF after MI, HTN, and cardiomyopathies [17–19]. Several are released in the blood and can be imaged in the myocardium [19]. Proteases are therefore attractive as potential markers of adverse remodeling, not just as tools for diagnosis and follow-up but also for guiding therapy of HF and in prevention [19, 20].

This chapter focuses on proteases as potential markers of adverse cardiac remodeling for HF in the aging population.

2 Adverse Remodeling in the Development and Progression of HF

Cardiac remodeling refers to the adaptive and maladaptive changes in cardiac structure, shape, and function that occur over time after an insult, including MI [21–28], HTN [29], and various cardiomyopathies [2–5, 9–11]. Gross changes in cell or LV size, shape, and function in HF are associated with biochemical, molecular, cellular, and subcellular changes that affect both cardiac muscle and matrix [5, 12, 13]. The cardiac ECM is critical for maintaining cardiac size, shape, and function, and ECM remodeling with disruption of the network fuels the march to HF, disability, and death [12, 30–40]. Cardiac and matrix remodeling after MI and HTN, the two leading causes of HF, result in two different types of HF, namely, HF with low ejection fraction (HF/low EF) and HF with preserved ejection fraction (HF/PEF) [2–5, 7, 9, 10, 16] that may require different clinical markers [18, 19].

3 Proteases in Cardiovascular Disease

Proteases or proteinases are proteolytic enzymes that regulate multiple physiological and pathophysiological pathways that are highly pertinent for cardiovascular disease (CVD) including cardiac remodeling and HF. Of the six main groups of proteases (i.e., serine, threonine, cysteine/thiol, aspartate, glutamic, and metalloproteases or metalloproteinases), the metalloproteinases (MMPs) and serine proteases are especially pertinent for LV remodeling after MI [14–16, 30, 31, 41].

Typically, proteases digest and degrade long-chain proteins and polypeptides, cleaving peptide bonds between amino acids to yield short fragments that may be active or inactive. Exopeptidases (such as MMPs) cleave amino acids at amino or carboxy terminals, while endopeptidases (such as trypsin, chymotrypsin, pepsin, papains, elastase) cleave peptide bonds within the chains. This simple cleavage step can have important physiological and pathophysiological effects in several major systems, including (1) the blood coagulation cascade and thrombolytic system, (2) the immune and inflammatory systems, (3) the renin–angiotensin–aldosterone system (RAAS), (4) the bradykinin–kallikrein system, (5) the angiotensin II (AngII) formation/degradation pathways, and (6) the prostanoid and cyclooxygenase-2 (COX-2) pathway [38, 42–46]. A characteristic feature of these systems is that a complex series of reactions occur in tandem resulting in rapid and efficient amplification of physiological signals, and dysregulation can contribute to pathophysiology. For example, in the immune system, proteases papain and pepsin yield antibody fragments that are critical for defense against infection. After tissue injury, serine proteases of the complement system produce peptides that attract neutrophils, induce pro-inflammatory cytokines such as interleukin (IL)-1 and IL-6, and secrete MMP-9 within minutes and set up acute inflammation.

The life span of proteins including proteases themselves is controlled by regulated protein degradation by proteases [47]. This serves to remove misfolded, misassembled, or damaged proteins and maintain appropriate protein levels for homeostasis. Degradation is mediated through a rapid pathway involving lysosomes and autophagy and a slower pathway involving the ubiquitin–proteasome system (UPS) which identifies target proteins by ubiquitin tags [47]. Proteases determine the life span of other proteases and their own by the switch-on/switch-off regulatory mechanism. This is pertinent for hormones, antibodies, and other enzymes and for ECM remodeling. While increased proteolytic activity normally degrades misfolded or malfunctioning proteins in cardiomyocytes [48], MMPs and other proteases such as calpains [49], chymase and cathepsins [50, 51], and caspases [52] have been implicated in remodeling of both ECM and subcellular organelles of cardiomyocytes in failing hearts [17, 49–54]. The proteolytic activities of caspases, calpains, and the UPS [55] have also been implicated in cardiomyocyte apoptosis in HF [17]. An imbalance between the activity of proteases and their endogenous inhibitors affects many processes including remodeling of ECM [17, 31, 56], intracellular matrix [57], cellular pathways such as apoptosis [52], and subcellular pathways leading to cardiac dysfunction [17]. After MI, a surge of MMP activity relative

to inhibitors of MMPs (TIMPs) results in ECM degradation [14, 15, 31]. During vascular injury, coagulation proteases that are produced exert their cellular effects by cleaving and activating protease-activated receptors (PARs) expressed on blood platelets, vascular endothelium, and smooth muscle cells; consequent protease signaling leads to coagulation (via thrombin signaling), inflammation, and vascular homeostasis and can contribute to the pathogenesis of atherosclerosis, restenosis, and thrombosis [58]. In vulnerable atherosclerotic plaques, macrophages produce MMPs including MMP-1 [59] which contribute to rupture. MMP-1 is also a PAR-1 agonist and contributes to tumor invasion in cancer [60].

While proteases have been implicated in CVD, protease inhibitors have been proposed for disease therapy. Endogenous protease inhibitors include tissue inhibitors of MMPs (TIMPs) [31], calpastatin [49], serpins [61], and lipocalins [62, 63]. These protease inhibitors normally balance the action of proteases and maintain homeostasis but dysregulation can be harmful. Serpins are serine protease inhibitors that are involved in blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, tumor suppression, and hormone transport; they include α-1-antitrypsin, antithrombin, α-1-antichymotrypsin, trypsin, plasminogen inhibitor-1 (PAI-1), and neuroserpin. Serpin-1 has been shown to attenuate the inflammatory response after vascular injury [61] and limit progression of atherosclerosis [62]. Whether serpins play a role in modulation of LV remodeling after MI needs further study. The TIMPs balance degradation of ECM, and a decrease in TIMPs relative to MMPs can enhance ECM degradation and aggravate HF/low EF, while a relative increase in TIMPs can result in excess fibrosis and promote HF/PEF [14, 15, 30, 31]. Congenic expression of TIMP-4 in Dahl-sensitive hypertensive rats, which have increased MMP-9 and decreased TIMP-4, was shown to attenuate hypertrophy and restore function [63]. Similarly, an imbalance between calpain and calpastatin may aggravate HF; thus, calpain increases in HF and post-MI [64, 65], while calpastatin remains unchanged [66]. The calpains are cytosolic calcium-activated cysteine proteases that contribute to myocardial hypertrophy and inflammation through activation of transcription factors such as NF-κB and to fibrosis through activation of transforming growth factor (TGF)-β [67]. Calpains also form the third proteolytic system for regulated protein degradation and are activated by increase in intracellular calcium.

The lipocalins represent a group of small extracellular proteins that are involved in cell regulation and differentiation and in lipophilic ligands that inhibit tumor proteases, but their possible role in ECM/LV remodeling needs further study. Serum lipocalin-2 levels are elevated in patients with coronary artery disease, suggesting that they may be useful for assessing CVD risk [68]. Lipocalin-2 has also been implicated in the inflammatory response during ischemia reperfusion in mice [69] and apoptosis of rat cardiomyocytes [70]. The finding of increased plasma levels of the neutrophil gelatinase-associated lipocalin (NGAL) in a subgroup of patients who developed renal dysfunction during acute decompensated HF suggested that it may be useful as a marker of kidney injury and poor prognosis [71]. A systematic review concluded that while evidence supports a putative role of NGAL in the pathophysiology of CVD including coronary artery disease and HF, the data was that insufficient to recommend its use for predicting outcome [72]. In a small study of 46 elderly patients with chronic congestive HF, increased plasma NGAL levels

predicted mortality [73]. However in a large study of serum NGAL and clinical outcome in a group of 1,415 patients with chronic HF and aged ≥60 years, NGAL did not provide added information above that provided by N-terminal pro-brain natriuretic peptide (NT-proBNP) and glomerular filtration rate (GFR) in a multivariate model for primary and secondary end points [74].

Proteases have also been classified on the basis of optimal pH into acid, neutral and basic groups; the neutral proteases, which include calpains [17, 54], play key roles in the type I hypersensitivity reaction (where they activate complement and kinins), the inflammatory response after injury [42], and the coagulation cascade [42]. The angiotensin-converting enzyme (ACE) inhibitors that are widely used in the management of HF [2–5, 8–11] and HTN [11] target the proteases ACE and kininase in the RAAS. Other proteases in the non-ACE pathways for AngII generation such as chymase and cathepsin G are pertinent for remodeling and HF.

4 Proteases in Adverse Cardiac Remodeling and HF

In the aging population with HF, evidence suggests that chronic elevations of AngII, reactive oxygen species (ROS), various vasoactive peptides (i.e., endothelins, BNP), and hormones including those in the RAAS (i.e., ACE) and sympathetic nervous system (i.e., catecholamines) lead to enhanced oxidative stress and intracellular Ca^{2+} overload, thereby resulting in a protease-rich environment that fuels the march to progression of adverse remodeling and HF (Fig. 23.1). A host of proteases contribute to adverse remodeling leading to HF due to MI, HTN, and various cardiomyopathies in the aging population. A full discussion of these proteases is beyond the scope of this chapter. The main proteases are listed in Table 23.1. Several of these proteases are elevated in the plasma of patients with those diseases, and some can be used as clinical markers of adverse remodeling for HF (Table 23.2). These will be discussed further.

5 Proteases and Protease Markers in Adverse Remodeling After MI

The remodeling events and proteases after MI have been reviewed [14–16, 21–28, 31–36].

5.1 Adverse LV Remodeling After Non-reperfused and Reperfused MI

Briefly, a large non-reperfused anterior transmural MI or ST-segment-elevation MI (STEMI) results in dramatic and rapid early remodeling of the infarcted zone with thinning and dilation (i.e., infarct expansion) that is followed by progressive dilative

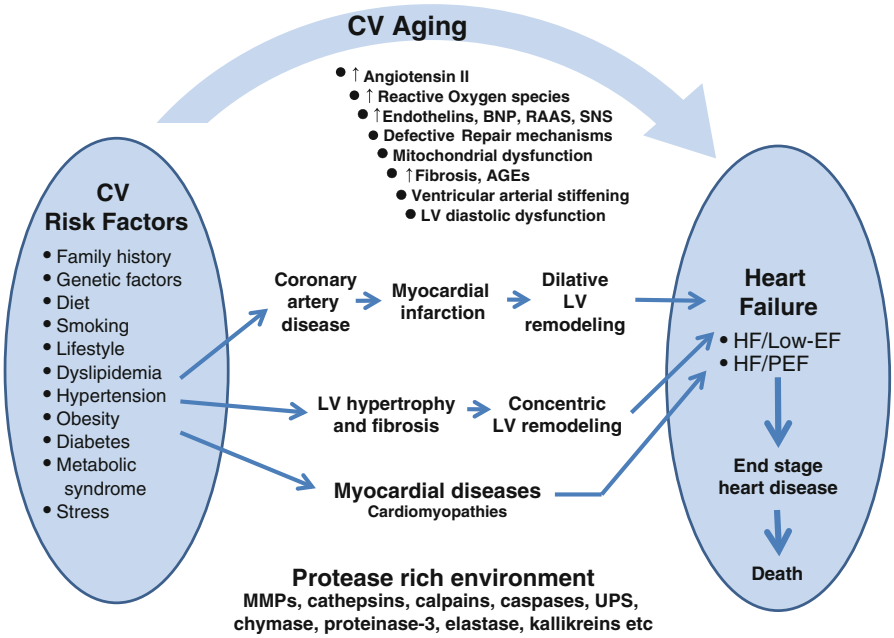

Fig. 23.1 March to the two main types of cardiac remodeling and heart failure in the aging population. Aging is associated with changes that predispose the heart to HF/PEF. Coronary artery disease with myocardial infarction and hypertension in the elderly leads to dilative and concentric remodeling with HF/low EF and HF/PEF, respectively, as in the non-elderly, but both the progression and impact are more severe in the elderly. Besides the harmful effects of the biochemical, cellular, and subcellular abnormalities that develop during aging, the dominant protease-rich milieu contributes to the augmented adverse remodeling found in the elderly. Several of these proteases can provide biomarkers of disease progression and serve as tools to assess response to therapy besides being targets for novel therapies and treatment strategies. *AGEs* advanced glycation end products, *BNP* brain natriuretic peptide, *CV* cardiovascular, *EF* ejection fraction, *HF* heart failure, *LV* left ventricular, *MMPs* matrix metalloproteinases, *RAAS* renin–angiotensin–aldosterone system, *SNS* sympathetic nervous system, *UPS* ubiquitin proteasome system

LV remodeling leading to systolic dysfunction, volume overload, wall thinning, eccentric hypertrophy, HF/low EF, and severe consequences including poor outcome, disability, and death. The MI results in combined damage of muscle with necrosis and apoptosis, matrix, and microvasculature and triggers the healing process, which through a timed sequence of biochemical, molecular, and cellular/subcellular reactions over weeks results in a fibrotic and collagenous scar [14–16, 26–28, 35, 36, 42]. Release of chemokines, cytokines, matrikines, growth factors including TGF-β, and MMPs and other matrix proteins orchestrate inflammation, remodeling of myocardium and ECM, and fibrosis [14–16, 42]. Typically, healing involves a sequence of acute inflammation, chronic inflammation with granulation tissue formation, tissue repair with fibroblast proliferation, ECM deposition,

Table 23.1 Proteases and inhibitors pertinent in adverse remodeling and heart failure

Protease	Reference
MMPs	Extracellular: [14, 16, 30, 31, 41, 83]
	Intracellular: [31, 57]
ADAMs	[16, 34, 79, 136–138, 228]
Cathepsins	[50, 51, 53, 54, 87, 153, 154, 185, 200, 201]
Chymase	[50, 51, 154, 173, 174, 177–184]
Caspases	[17, 52, 187, 189, 190]
Calpains	[17, 49, 54, 64, 65, 67, 170–172, 199, 204]
UPS	[17, 47, 55, 191]
Proteinase-3	[148–152, 186]
Myeloperoxidase	[34, 79, 145–147, 227, 228]
SPARC	[16, 34, 79, 225, 228]
Protease inhibitors	TIMPs: [14, 15, 30, 31, 112]
	SLPI: [16, 34, 79, 228]
	Calpastatin: [17, 49, 64, 66]
	Serpins: [61]
	Lipocalins: [68–74, 143, 144]
	NGAL: [71–74, 130, 142–144]

ADAM a disintegrin and metalloproteinase, *MMP* matrix metalloproteinase, *NGAL* neutrophil gelatinase-associated lipocalin, *SLPI* secretory leukocyte protease inhibitor, *SPARC* secreted protein acidic and rich in cysteine, *TIMP* tissue inhibitor of matrix metalloproteinase, *UPS* ubiquitin proteasome system

myofibroblast and scar formation, and structural and functional remodeling of infarcted and non-infarcted myocardium through cardiomyocyte hypertrophy with little regeneration and some angiogenesis [14–16, 42]. Also typically, remodeling spans all phases of the healing process and is progressive; it begins early during the infarction phase (first 24–48 h in humans) and continues during the healing phase (~6 weeks to 3 months in humans) and extends over months or years [14–16]. Multiple factors modulate myocardial, vascular, and ECM remodeling during the remodeling process post-MI [14–16, 18]. Over time, the remodeling process in MI survivors becomes global, extending to other cardiac chambers, tissues, cells, and molecules and resulting in a vicious cycle leading to end-stage HF.

Although early coronary reperfusion within 30 min of a STEMI can limit infarct size and LV remodeling/dysfunction, most patients present late and survivor of STEMI reperfused ≥90 min from the onset develop persistent adverse dilative LV remodeling/dysfunction and HF [37, 38, 75–77]. Besides infarct size, the culprits in this march of adverse remodeling to HF include reperfusion damage with a surge of apoptosis, microvascular no-reflow and flow-function mismatch [16, 34, 38, 40], damage of the supporting ECM [14–16, 33, 34, 38, 40], and inflammation in early and late phases of healing [14–16, 34, 38, 40, 78, 79]. Importantly, this adverse remodeling and dysfunction occurs despite medical therapy recommended in the management guidelines [2–5, 8–10].

Table 23.2 Pertinent protease markers of adverse remodeling and markers of collagen turnover and fibrosis

Marker	Reference
MMPs/TIMPs	Plasma: [198]
TIMP-1	Plasma: [56, 90, 91, 112, 193]
MMP-2/TIMP-2	Plasma: [112]
MMP-9	Plasma: [113–128]
Cathepsins	Tissue: [17, 54, 153, 154, 185, 200]
Chymase	Tissue: [50, 51, 173, 174, 177–184]
Caspases	Tissue: [17, 52, 187, 189, 190]
Calpains	Tissue: [17, 49, 64, 65, 67, 170–172, 199, 204]
UPS	Tissue: [17, 47, 55, 191]
Proteinase-3	Tissue/plasma: [148–152, 186]
Myeloperoxidase	Tissue/plasma: [145–147, 227, 228]
SPARC	Tissue/plasma: [16, 34, 79, 225, 228]
SLPI	Tissue: [16, 34, 79, 228]
Lipocalins, NGAL	Tissue/plasma: [68–74, 130, 142–144]
Collagen turnover	Plasma: [215–218, 232]
Synthesis	Plasma PICP: [126, 214–216]
	Plasma PINP: [217]
	Plasma PIIINP: [217]
Degradation	Plasma CITP: [218]
Fibrosis	Serum tenascin-C: [205]
	Serum galectin-3: [219, 221–223]
Other	Serum NT-proBNP: [229–231]

ADAM a disintegrin and metalloproteinase, *MMP* matrix metalloproteinase, *NGAL* neutrophil gelatinase-associated lipocalin, *NT-proBNP* N-terminal pro-B natriuretic peptide, *PICP* procollagen type I C-terminal propeptide, *PINP* procollagen type I N-terminal propeptide, *PIIINP* procollagen type III N-terminal propeptide, *SLPI* secretory leukocyte protease inhibitor, *SPARC* secreted protein acidic and rich in cysteine, *TIMP* tissue inhibitor of matrix metalloproteinase, *UPS* ubiquitin proteasome system

5.2 *Adverse ECM Remodeling After Non-reperfused and Reperfused MI*

As reviewed elsewhere, the cardiac ECM consists of a complex three-dimensional assembly of proteins that contribute to the normal cardiac form and function [14, 15, 30, 80]. The proteins include collagen, elastin, and specialized proteins such as fibrillin, fibronectin, proteoglycans, and matricellular proteins. The organized interstitial network of collagen fibers provides a supportive matrix for cardiac myocytes and blood vessels as well as a milieu for cell migration, growth, differentiation, and interaction. Cardiac fibroblasts regulate ECM synthesis and deposition and mediate ECM degradation and turnover through MMPs and TIMPs and maintain tension in the network [81]. Normal ECM remodeling (i.e., synthesis and degradation) is tightly regulated by the balance between MMPs and TIMPs that is critical for maintaining homeostasis and cardiac shape and function and preventing excessive ECM

degradation that leads to adverse remodeling, dilation, and rupture. A major pathway leading to ECM and adverse dilative LV remodeling involves MMP/TIMP imbalance reflected in an increased MMP/TIMP ratio [14–16].

MMPs represent a major proteolytic system for ECM degradation and consist of a family of zinc-dependent endopeptidases that degrade ECM components such as collagens, fibronectin, proteoglycans, laminin, and gelatin [14, 15]. Nearly 25 MMPs but only 4 TIMPS have been described, and MMP activity is posttranslationally regulated by the TIMPs [82]. The first four classes of MMPs (collagenases, gelatinases, stromelysins, and elastase) are secreted as latent pro-MMPs that bind ECM proteins and become active when the propeptide domain is cleaved through a cysteine switch mechanism by serine proteases, trypsin, chymotrypsin, and plasmin [83]. The fifth class of MMPs includes membrane-type MMPs (MT-MMPs) which are activated upon positioning in the cell membrane and retain the propeptide domain that is needed for activation and TIMP binding [84].

5.3 MMPs as Potential Protease Markers of Adverse Remodeling After MI

MMP expression in myocytes, fibroblasts, myofibroblasts, endothelial cells, and inflammatory cells (i.e., neutrophils, monocytes, and macrophages) is increased after injury. Studies in MI have shown that LV remodeling is a major mechanism for LV enlargement, HF, and death and the ECM plays a pivotal role in LV remodeling, whereby ECM disruption causes LV dilation. In addition, studies showed that AngII, a primary effector molecule of the RAAS, drives both ECM and LV remodeling [14–16]. Several MMPs, including MMP-1, MMP-2, and MMP-9, modulate cardiac remodeling (Table 23.1). The fibrillar collagens are key substrates of MMPs in ECM remodeling [14–16]. A sharp rise in MMPs occurs within hours after acute MI and leads to rapid ECM degradation, followed by decreased collagen content, adverse ECM, and LV remodeling and LV dysfunction. Although MMP and TIMP levels subside over several days, chronically higher MMP than TIMP levels under certain conditions may result in continued ECM degradation and thereby contribute to dilative remodeling. In contrast, higher TIMP than MMP levels could contribute to increased ECM and fibrosis in the non-infarct zone and contribute to diastolic dysfunction in the long term. Since MMPs can be detected in blood, they can serve as markers of adverse remodeling ECM/cardiac remodeling after MI.

For example, the collagenase MMP-1 is increased in MI [85, 86] and degrades fibrillar collagens I and III [85]; MMP-1 disruption results in LV dilation/dysfunction [87], and increased MMP-1 expression in transgenic mice inhibits fibrosis and transition to HF in LV pressure overload [88]. Deletion of TIMP-1, which colocalizes with MMP-1 in normal myocardium, results in dilative remodeling post-MI [89], and increased TIMP-1 levels correlate with markers of HF and LV remodeling in patients with MI [90] and HTN [56, 91].

MMP-2 (gelatinase A) [91–97] and MMP-9 (gelatinase B) have also been implicated in post-MI remodeling [92–99]. MMP-9 deletion attenuates LV dilation and collagen deposition after MI [93]. Targeted deletion of MMP-2 attenuates early LV rupture and late remodeling post-MI [95]. However, the role of MMP-3 (stromelysin-1) remains unclear [92, 94]. Elastase appears to modulate ECM degradation through activation of MMP-2, MMP-3, and MMP-9 and inactivation of TIMP-1 [100], and elastase inhibition before reperfusion has been shown to reduce infarct size [101]. Of note, MMP-2 and MMP-9 that degrade denatured interstitial collagen may not be the MMPs that cause its accumulation.

Several studies have shown that decreased collagen integrity after MI [102] can lead to cardiac rupture [103–106]. TIMP-4 knockout mice are also prone to develop rupture after MI [107]. Other MMPs, TIMPs, and proteins have also been implicated in rupture [108, 109], but the theme after MI is usually either enhanced ECM degradation or impaired healing/repair or both. In pacing-induced congestive HF, LV dysfunction is associated with increased MMP-1, MMP-2, and MMP-3 and increased degradation of gelatin and collagen III [110]. Restricted overexpression of MT1-MMP is associated with adverse remodeling post-MI [111].

5.4 Plasma Levels of MMPs as Markers of Adverse ECM/LV Remodeling for HF

Since the mid-1990s, researchers have been searching for MMPs as potential markers of adverse remodeling after MI and HF [20]. Plasma MMP-2 and MMP-2/TIMP-2 levels in veteran athletes correlated inversely with echocardiographic indices of LV hypertrophy, suggesting that MMPs and TIMPs could be potential markers of adaptive remodeling [112]. In a prospective study of 109 STEMI patients who underwent reperfusion by primary coronary intervention (PCI), stenting, and abciximab therapy, high levels of MMP-9 correlated with adverse LV remodeling [113]. In a study of circulating levels of MMPs in 134 patients with cardiomyopathy and LV systolic dysfunction and HF, the diastolic dysfunction by the diastolic mitral inflow E/e' ratio, systolic ejection fraction, and MMP-9 level were significant independent predictors of all-cause mortality on multivariate analysis, and an $E/e' \geq 13$ and a MMP-9 level >89.9 ng/mL correlated with worse survival in Kaplan–Meier curves [114]. In patients with congestive HF, levels of plasma MMP-2, MMP-3, and MMP-9 increased progressively with severity of HF, while TIMP-1 levels decreased and full-length fibronectin levels in LV samples [115]. In a study of patients with MI, increased levels of MMP-8 and MMP-9 correlated with cardiac rupture [116]. One study showed that MMP-9 levels correlated with inflammatory marker levels (IL-6, hs-CRP, and fibrinogen) and CVD mortality [117]. At least six more studies documented and/or correlated increased MMP-9 levels with adverse LV remodeling, dilation, dysfunction, and HF/low EF in patients with acute coronary syndromes and/or MI [118–123]. Other studies have shown that MMP-9 correlates with LV hypertrophy [124], susceptibility to MI [125], HF/PEF [126], and HF/low EF [127, 128]. Together, the evidence suggests that MMP/TIMP balance/imbalance is a

critical issue in ECM/LV remodeling. Furthermore, it suggests that a protease marker of adverse remodeling such as an MMP could be used to follow the progression of the remodeling process after MI and HF. The requirements for markers have been reviewed elsewhere [129].

5.5 MMP-9 as a Protease Marker of Adverse ECM/LV Remodeling for HF

Four lines of evidence favor the use of MMP-9 as a marker of adverse ECM/LV remodeling for HF post-MI. First, MMP-9 can process full-length interstitial collagens as well as other substrates without the activation cleavage step for proteolysis and not just collagen that has already been cleaved by collagenases such as MMP-1[130]. Second, MMP-9 participates in the post-MI inflammatory response through interactions with pro-inflammatory elements such as activator protein-1, specificity protein-1, and NF-κB [130]. Third, both pharmacological inhibition and deletion of MMP-9 attenuate post-MI LV dilation and dysfunction [131–134], and deletion stimulates angiogenesis in the infarct zone [135]. Fourth, as reviewed above, MMP-9 has been implicated as a biomarker of adverse LV remodeling/dysfunction post-MI, HF, and mortality [113–128]. Precautions are needed with assays of MMP-9 [20] and other MMPs [31].

5.6 Interactions Between Matrix Proteins and Inflammatory Cytokines

Increased MMP-9 in aged TIMP-3 null mice is associated with ECM degradation and LV dilation, cardiomyocyte hypertrophy, and LV dysfunction [136]. TIMP-3 also inhibits ADAM (*a d*isintegrin *a*nd *m*etalloproteinase)-17 and ADAM-10 [136–138] and regulates inflammation [138]. Both the ADAM-17 and ADAM-10 can alter integrins (cell-surface matrix receptors), disrupt cell-matrix interactions, degrade ECM, and contribute to LV dilation [138]. They also interact with inflammatory cytokines and alter MMPs and thereby impact LV remodeling and/or injury [137]. Together, the evidence suggests that interactions between matrix proteins and inflammatory cytokines occur and can modulate ECM damage.

5.7 Role of Inflammation in ECM/LV Remodeling During Healing/Repair After MI

The inflammatory reaction post-MI not only modulates the quality of the healing/repair process that is critical for survival [14–16, 139] but also impacts ECM/LV remodeling through production of various proteases and other factors [14–16, 42].

Dysregulation, as with enhanced activation of pro-inflammatory mediators and/or prolongation or expansion of inflammatory response, can result in defective scars and amplified adverse ECM/LV remodeling. As discussed, the inflammatory reaction is a staged, time-dependent, and highly dynamic process with two main functions: clearance of dead cells and debris and activation of the pathways for optimal healing/repair and scar formation. These functions are largely modulated by different monocyte and macrophage phenotypes [42, 78]. Neutrophils that infiltrate the site of MI release four subsets of membrane-bound granules (i.e., secretory vesicles, gelatinase granules, specific granules, and azurophilic granules) containing various factors including proteases, ECM proteins, and mediators of inflammation [140, 141]. The gelatinase granules account for 25 % of peroxidase-negative granules in human neutrophils and release MMPs [142] such as MMP-9 [101]. The specific granules release NGAL which modulates ECM/myocardial remodeling by preventing MMP-9 degradation [99, 130]. In patients with acute MI, NGAL levels increase in plasma and both infarct and non-infarct zones [143, 144]. Activated neutrophils and monocytes also produce myeloperoxidase (MPO), another protease that enhances remodeling through generation of oxidants including reactive chlorinating species and MMP activation [145]. MPO deletion results in attenuation of leukocyte infiltration, LV dilation, and dysfunction after MI [146], and plasma MPO levels in patients predict MI risk and adverse events [147]. The azurophilic granules also contain three serine proteases (i.e., proteinase-3, cathepsin G, and elastase) which degrade several ECM components (i.e., elastin, fibronectin, laminin, type IV collagen, and vitronectin) that stimulate the coagulation cascade [147, 148] and serine elastase which activates MMP-9 [101] and promotes both vascular [149] and cardiac remodeling [150]. Proteinase-3 can induce endothelial cell apoptosis through caspase-like activity [151], while serine proteases can inactivate pro-inflammatory IL-6 [152], and cathepsin G can activate platelets [153] and inactivate bradykinin and kallidin [154].

In STEMI reperfused after 90 min, a surge of neutrophils and apoptosis typically occurs [14–16, 38]; the excess neutrophils participate in myocardial damage as well as ECM damage through MMPs including MMP-9, and enhanced cardiomyocyte apoptosis contributes to irreversible LV dysfunction [16]. In the dog model in our laboratory [34], STEMI reperfused 90 min after coronary occlusion resulted in increased pro-inflammatory markers inducible nitric oxide synthase (iNOS), cytokines IL-6, and tumor necrosis factor (TNF)-α and anti-inflammatory markers TGF-β1 and IL-10, plus evidence of cardiomyocyte damage (ischemic injury, infarct size, apoptosis, blood flow impairment, and no-reflow), adverse LV remodeling (LV dilation and dysfunction), and ECM remodeling with increased expression of MMP-9 and MMP-2 as well as secretory leukocyte protease inhibitor (SLPI), secreted protein acidic and rich in cysteine (SPARC), osteopontin (OPN), and ADAM-10 and ADAM-17. In the rat model, these markers increased in the later phase of healing after reperfused STEMI [79]. During that later phase, resolution of the inflammatory response with activation followed by inhibition (negative regulation), containment to the infarct zone, and resolution is considered critical [42].

5.8 Role of Monocytes/Macrophages in ECM/LV Remodeling After MI

Increase in circulating monocytes after MI has been implicated in adverse LV remodeling [139].

Recent evidence underscores the spatiotemporal regulation of cellular aspects of healing/repair and ECM/LV remodeling in the march to HF post-MI [42, 78, 155–159]. In one construct [78, 157], monocytes/macrophages that dominate the repair/healing phases execute multiple functions [42, 78, 155] in a temporally and spatially organized manner. Depending on environmental cues, monocytes/macrophages differentiate into two subsets of monocytes that exert distinct effects on the inflammatory response during the early and late phases. Ly-6C^{high} monocytes that dominate the early phase are pro-inflammatory, remove necrotic debris, and secrete inflammatory cytokines, ROS, and matrix-degrading proteases. Ly-6C^{low} monocytes that dominate the late phase are pro-resolution and pro-repair and trigger angiogenesis, collagen/ECM synthesis by myofibroblasts, and healthy scar formation. The actions of the two subsets of cells need to be balanced so as not to delay resolution of inflammation, impair healing and ECM remodeling, and weaken the scar. Too little or too much inflammation can be harmful [78, 155].

In the second construct, macrophages polarize to different phenotypes depending on spatially and temporally regulated upregulation of cytokines, chemokines, and growth factors in the early and late phases to modulate the healing/repair process [157]. In the early phase, upregulation of macrophage colony-stimulating factor (MCSF) induces monocyte differentiation [42], with polarization to classical pro-inflammatory M1 macrophages that secrete T1-helper cell Th1 cytokines (interferon-γ, TNF-α); the activated M1 macrophages secrete large amounts of inflammatory mediators, ROS and nitrogen intermediates, chemokines, and growth factors, regulate fibroblast function and collagen turnover, modulate angiogenesis, boost inflammation and promote ECM degradation, and clear dead cells and matrix debris [155, 156, 158]. Persistent M1 macrophage activation can exacerbate LV remodeling/dysfunction. Enhanced M1 macrophage polarization resulted in increased production of M1 cytokines including IL-1β, IL-6, and TNF-α and deterioration of LV dysfunction/dilation and cardiac fibrosis in class A scavenger receptor null mice [160]. In the M1 phase, IL-1β secretion induces MMP-9, which facilitates secretion of inflammatory cytokines and ECM degradation. Monocyte kinetics in post-MI hearts is rapid, with an average residence time of 20 h; after 20 h, most monocytes die and few regress to the circulation, liver, or other organs [161]. Of note, macrophages from the spleen migrate to the LV post-MI [161]. During the late phase [158], macrophages polarize to activated M2 macrophages (akin to Th2 cells) that show enhanced phagocytic activity and high anti-inflammatory activity with upregulation of IL-10, IL-4, and TGF-β1, the decoy type 2 IL-1 receptor, and IL-1 receptor antagonist (IL-1Ra), exerting pro-resolution effects with removal of inflammatory leukocytes [162–165] and resolution of ECM deposition, fibroblast proliferation, and angiogenesis [166] which are key for tissue repair [167].

The timely shift and balance between M1 and M2 macrophages is crucial for stable scar formation. Prolonged and excessive activation of the M1 phenotype yields uncontrolled inflammation and ECM breakdown, which in turn contributes to adverse remodeling and cardiac rupture. Persistent M2 activation stimulates excessive fibroblast proliferation, ECM deposition, and fibrosis, which influences structural, biochemical, mechanical, and electrical properties of the myocardium [168]. Macrophage phagocytosis of apoptotic neutrophils, a marker of the active resolution of acute inflammation, triggers TGF-β production, suggesting that phagocytosis may stimulate conversion to the M2 status [158]. The M1/M2 ratio may be a potential marker for predicting long-term outcome post-MI.

5.9 Other Proteases as Markers of Adverse ECM/LV Remodeling for HF

Since MMP inhibitors do not completely prevent adverse ECM/LV remodeling post-MI and in HF [27, 169], other serine proteases such as plasmin, kallikrein, elastase, chymase, cathepsin G, and others also appear to participate in post-MI ECM/LV remodeling and HF. In addition, several other proteases were found to affect intracellular remodeling and thereby contribute to LV dysfunction [17].

5.10 Calpains as Potential Markers of Adverse ECM/LV Remodeling for HF

As discussed previously, calpains have been implicated in myocardial remodeling and HF [67] and development of ventricular hypertrophy [170]. Calpains are intracellular Ca^{2+}-activated, papain-like, cysteine proteases; they are increased in patients with congestive HF [64] and acute MI [66] and rats with MI [65, 171]. Expression of m-calpain after rat MI increases early at 3 days while μ-calpain peaks later at 2 weeks [171]. An imbalance between calpain and calpastatin may explain tissue damage in early MI and remodeling in late MI. Increase in calpain proteolysis in the face of unchanged calpastatin may contribute to cardiac dysfunction. Calpain inhibition in MI reduces early cardiomyocyte loss and improves LV function [172]. Calpain/calpastatin imbalance may be a marker of risk for HTN [67]. Also in HF patients, increase in ventricular tissue calpain is limited to μ-calpain in mild HF (NYHA class II) and includes both μ- and m-calpains in more severe HF (NYHA classes III–IV), suggesting a role in HF progression [64, 170]. Also in HF, calpain-induced regulatory pathways in transduction of cardiac remodeling included increase in cain/cabin1 cleavage and calcineurin activation [64]. Calpains may be useful as tissue markers for HF.

5.11 Chymase as a Potential Marker of Adverse ECM/LV Remodeling for HF

The finding in the 1990s that non-ACE pathways can generate AngII during ACE inhibition [173, 174] implied that therapy of HF with ACE inhibitors was suboptimal. The serine protease chymase and other non-ACE enzymes can generate AngII from AngI and/or from angiotensinogen via non-renin pathways. Several studies showed that AngII levels persisted during long-term ACE inhibitor therapy and supported the use of AngII receptor blockers (ARBs) for blocking the effects of AngII at the AT_1 receptor [175]. The AngII-forming capacity of chymase from AngI was in fact 20-fold higher than that for ACE [173, 174], and chymase could also activate the kallikrein–kinin pathway [176]. These findings implied that the combination of a chymase inhibitor with either an ACE inhibitor or an ARB might result in added benefits by reducing both AngII and kinins. Experimentally, chymase inhibition was shown to improve diastolic LV function and prevent fibrosis in dogs with HF [177], reduce arrhythmias and LV dysfunction after MI in hamsters [178], attenuate LV remodeling in mice [179], and decrease infarct size through attenuation of MMP-9 and pro-inflammatory cytokines in a pig model of reperfused MI [180]. In mice, attenuation of LV hypertrophy and fibrosis was associated with reduced LV chymase, AngII, oxidative stress, IL-6, TNF-α, and TGF-β [179]. In hamsters with post-MI HF, combined ACE and chymase inhibition reduced infarct size and LV remodeling/dysfunction compared to ACE inhibition alone [181]. In dogs with LV volume overload due to mitral regurgitation, the anti-remodeling effect of chymase inhibition was in part due to inhibition of MMP and kallikrein activation and fibronectin degradation [182], thereby attenuating loss of ECM and cell-ECM connections and cell death. The multiple actions of chymase on tissue remodeling support its role in adverse LV remodeling and HF post-MI [183, 184]. Whether plasma chymase may be useful as a marker of adverse remodeling for HF needs study.

As discussed before, both serine proteases cathepsin G and proteinase-3 degrade ECM; cathepsin G in LV tissue has been implicated in adverse remodeling in end-stage HF [185], while plasma proteinase-3 correlates with prognosis for mortality and HF in patients with acute MI [186] so that they can be used as tissue and plasma markers of remodeling, respectively.

5.12 Caspases as Potential Markers of Adverse ECM/LV Remodeling for HF

Extensive evidence has established the role caspases in apoptosis (programmed cell death) during adverse LV remodeling during MI and HF ([17] for review). In MI, apoptosis is enhanced by reperfusion and occurs in different cell types, including cardiomyocytes as well as cells in the inflammatory response, granulation tissue, and fibrosis during healing/repair. Briefly, caspases are cysteine proteases that, upon

activation through cleavage of aspartate residues in the dominant mitochondrial-mediated and the extrinsic death-receptor pathways, degrade polypeptides in the DNA-repair machine and protein kinases and thereby regulate apoptosis [187]. Cardiac apoptosis can also be caused by caspase-independent pathways including the UPS [55], apoptosis-inducing factor (AIF) [188], and other proteases calpains [17]. Caspases induce damage to intracellular contractile proteins (i.e., actin, myosin, troponin) and destabilize sarcomeric structures and thereby contribute to LV dysfunction that can be prevented by caspase inhibition [189]. Caspases also activate MMPs and contribute to adverse remodeling that is prevented by caspase inhibition [190]. Of note, the UPS regulates apoptosis by degrading caspases [191]. Increased tissue levels of caspases 3, 8, and 9 that have been implicated in apoptosis leading to LV dysfunction and remodeling post-MI [17] could be used as markers.

6 Proteases and Adverse Remodeling After Hypertension

Remodeling in response to chronic LV pressure overload in HTN progresses at a slower pace than after MI and results in concentric, hypertrophic, and non-dilative LV remodeling [29] with excessive deposition of ECM with fibrosis [31, 80], increased LV stiffness, and typically HF/PEF [9, 10]. Over time, that too may progress to LV dilative remodeling with eccentric hypertrophy and HF/low EF [7, 29], followed by chronic decompensation and end-stage heart disease with congestive HF [7]. Various proteases modulate the progressive remodeling after HTN [17].

6.1 Proteases as Markers of Adverse ECM/LV Remodeling for HF After HTN

At least six groups of proteases have been implicated in hypertrophic remodeling during HTN and can serve as biomarkers. First, an imbalance between TIMPs and MMPs with increased TIMP/MMP ratio is expected to be profibrotic and favor ECM accumulation. However, TIMPs are multifunctional with pro-growth, anti-apoptotic, and anti-angiogenic effects [82], and results studies of individual markers are not always consistent. In Dahl salt-sensitive hypertensive rats, increase in MMP-9 and MMP-2 precedes progression to LV dilation/dysfunction [99]; increased MMP-9 is associated with decreased TIMP-4, and congenic transfer of TIMP-4 results in reduced LV hypertrophy and improved function [63]. Patients with hypertensive heart disease show low serum MMP-1 and either high or unchanged [192–196] TIMP-1 levels and reduced collagen turnover [195]. Athletes with LV hypertrophy have high TIMP levels [112] while patients with hypertrophic cardiomyopathy show high MMP-2 and MMP-9 [197]. Some of the discrepant findings may be due to sampling at different stages of disease progression. In a large study of 17 plasma biomarkers, multimarker panels (i.e., MMP-1, MMP-2, MMP-3, MMP-7, MMP-8,

and MMP-9; TIMP-1, TIMP-2, TIMP-3, and TIMP-4; NT-proBNP; cardiotrophin; OPN; soluble receptor for advanced glycation end products; collagen I telopeptide; collagen III N-terminal propeptide [PIIINP]; and 6 min walks) were found to perform better than any single biomarker including NT-proBNP [198]. In that study, plasma biomarkers reflecting changes in ECM fibrillar collagen homeostasis combined into a multimarker panel had more discriminative value in identifying the presence of LV hypertrophy and clinical evidence of diastolic HF [198].

Second, while calpain is increased in patients with systolic HF [64] and MI [65], it is reduced in DOCA salt-sensitive rats and spontaneously hypertensive rat hearts [199], and the calpain/calpastatin ratio may serve as a tissue marker of risk in HTN [67]. Third, cathepsins that degrade fibrillar collagens and elastin and activate pro-MMPs [200] have been implicated in dilative remodeling with end-stage HF [185] and increased cathepsins L and B in dilated cardiomyopathies [17]. However, cathepsin L can also be protective through preservation of lysosomes and LV structure and function as its overexpression in mice is associated with attenuation of activities of caspases 3, 8, and 9 (reviewed in [17]). Nevertheless, cathepsins including cathepsin S and cathepsin K are increased in patients with HTN and rats with hypertensive HF [201]. Fourth, while changes in Na+/K+ ATPase with decreased α2 isoform occur in HF/low EF post-MI [17], hypertrophic hearts in HTN also show changes in Na+/K+ ATPase isoforms with decreased α2 [202] as well as subcellular remodeling with enhanced Cαq signaling [203]. Fifth, while MMPs (i.e., MMP-2, MMP-9, MMP-3, MT1-MMP), calpains, and cathepsins are involved in dilated cardiomyopathies, they are also involved in diabetic cardiomyopathy (review in [17]). High glucose appears to stimulate calpain-1 and thereby decrease Na+/K+ ATPase and trigger cardiomyocyte apoptosis via the AIF [17]. Diabetic cardiomyopathy is also associated with increased caspase-9, decreased cathepsin D and cathepsin L, and decreased ECM. In post-MI HF, treatment with caspase-3 and calpain inhibitors improve function and delay progression of HF [17]. Increased caspase-3 during calpain inhibition by calpeptin may play a role in progression from pressure overload hypertrophy to end-stage HF [204]. Sixth, besides changes in serum MMP-9 and TIMP-1, TIMP-2, and TIMP-4 levels, patients with chronic HTN and LV hypertrophy show differential changes in tenascin-C (Tn-C) splice variants, with higher levels in eccentric than concentric hypertrophy [205], suggesting that serum Tn-C levels may be a useful biomarker of the transition from concentric to eccentric phenotypes.

6.2 Non-protease Markers of Fibrosis and ECM/LV Remodeling

Myocardial fibrosis implies excessive ECM and collagen deposition. In HTN, mechanical and humoral mechanisms mediate fibrosis [206] that results in increased myocardial stiffness, LV diastolic dysfunction, and HF/PEF [126]. After MI, fairly rapid reparative fibrosis attempts to preserve LV shape and systolic function [207], while in HTN, gradual reactive fibrosis increases LV stiffness and contributes to LV

dysfunction [206, 208, 209] and electrical remodeling [210]. After MI, a timed sequence of molecular and cellular events during the healing/repair process modulates infarct zone fibrosis [14–16] in response to various regulators and suppressors (review in [42]). Net ECM degradation dominates early healing/repair, while net ECM deposition, collagen synthesis, and maturation dominate the late phase, resulting in differential ECM and LV remodeling during the two stages. The traditional concept is that fibroblast stimulation by TGF-β after infarction and during early healing leads to differential deposition of ECM, collagens, and fibrosis, with greater amounts in infarct than non-infarct zones, and an increasing ratio of collagen fibril type I/type III during the late maturation process [14–16, 42]. However, the regional "brake" and "stop" signals in the infarct and non-infarct zones still need study.

While protease-mediated collagen turnover, especially via MMP/TIMP imbalance, modulate ECM turnover, the non-protease products of collagen turnover can serve as markers of ECM/LV remodeling. Collagen turnover in cardiac ECM is regulated by fibroblasts and myofibroblasts ([15, 211] for review). Ten steps in collagen biosynthesis in these cells [15] include (1) intranuclear pro-α gene transcription, slicing, and modification; (2) intracellular synthesis of pro-α chains in the rough endoplasmic reticulum; (3) hydroxylation of selected prolines and lysines; (4) glycosylation of selected hydroxyl serines; (5) formation of procollagen triple helixes; (6) oligosaccharide processing in the Golgi complex; (7) secretion into the extracellular space; (8) conversion into less soluble molecules by cleavage of propeptides; (9) self-assembly into collagen fibrils; and (10) aggregation of fibrils to form fibers. Spatially, fibroblasts and myofibroblasts synthesize and secrete preprocollagen types I and III as pro-α-collagen chains which form the typical triple helix structure of procollagens in the rough endoplasmic reticulum [15, 212]. Procollagen molecules are secreted from the Golgi complex into the interstitial space where cleavage of the end-terminal propeptide sequences enables collagen fiber formation. Since specific procollagen N- and C-proteinases release the two terminal propeptides (i.e., amino (N)-propeptide and carboxy (C)-propeptide) of procollagen molecules into the circulation [213], measurement of the levels of these cleaved collagen propeptides can provide an indirect index of fibrillar collagen synthesis and deposition.

Three main markers of synthesis are procollagen type I carboxyterminal propeptide (PICP), procollagen type I aminoterminal propeptide (PINP), and procollagen type III aminoterminal propeptide (PIIINP). First, PICP, the 100 kDa C-terminal propeptide of collagen type I, is the most commonly used marker. Serum PICP levels are elevated in MI [214] and diastolic HF [126] and correlate with the collagen fiber deposition in LV hypertrophy [215]. In one study, collagens I and III increased within the first 48 h post-MI, and synthesis of collagen I persisted until 1 month post-MI [216]. Second, PINP, a 70 kDa propeptide, has been used as a marker of type I synthesis [217]. Because it is not always cleaved from the procollagen molecule, serum levels may not correlate well with synthesis. Third, PIIINP, a 42 kDa N-terminal propeptide from procollagen type III, is used as a marker for type III synthesis [217] and to predict cardiac events and mortality. However, incomplete removal of the N-terminal domain can lead to incorporation of PIIINP in collagen fibers and underestimation of type III synthesis.

Two markers of collagen degradation turnover are collagen type I carboxyterminal telopeptide (CITP) and the MMPs that degrade ECM. Interstitial collagenase cleaves all 3 α-chains of collagen to form a large telopeptide which remains within the interstitial space, and a smaller 12 kDa telopeptide which is released immunochemically intact form in the blood can be used as a marker of collagen degradation. A clinical study showed that high plasma levels of CITP within 72 h post-MI correlates with poor long-term outcome [218]. MMPs and TIMPs have been discussed before.

Galectin-3 (Gal-3) is emerging as another marker of cardiac fibrosis. Gal-3 is a 29–35 kDa soluble β-galactoside-binding lectin that plays a role in the regulation of cardiac fibrosis and remodeling. Gal-3 is expressed by activated macrophages and induces fibroblast proliferation and increased deposition of collagen type I [219]; its expression is increased in the infarct region at 7 days post-MI in mice [220] and in both experimental [221] and clinical [222] HF. In a study of 106 patients with HF/PEF, serum Gal-3 correlated with markers of ECM turnover such as PIIINP, TIMP-1, and MMP-2 as well as the NYHA functional class [223], implying a relation between macrophage activation and ECM turnover.

6.3 Protease Markers of ECM/LV Remodeling in HF During Aging

The majority of patients with HTN, MI, and HF are elderly (age ≥65 years) [1]. As reviewed elsewhere, aging of the cardiovascular system itself is associated with physiological, biological, and structural changes that lead to increased ECM and fibrosis, increased ventricular-arterial stiffening, LV diastolic dysfunction, and HF associated with HF/PEF even in the absence of CVD and/or comorbidities [6, 7, 9, 16, 224]. Multiple pathways modulate age-related fibrosis and include increased ROS, AngII, endothelin-1, TGF-β, recruitment of mononuclear cells, and fibroblast progenitors [16]. SPARC also contributes to increased collagen content in aging hearts via post-synthetic procollagen processing [225]. After STEMI, aging results in defective healing/repair and augmented adverse LV remodeling [7, 16, 34, 40]. Increased myocardial ROS and AngII (via its pro-inflammatory, prooxidant, and pro-remodeling effects) increase inflammatory cytokines, proteases including MMPs, and oxidative stress markers such as MPO in aging hearts all act in concert to modulate healing/repair after MI [34]. Data from young and old mice suggest that aging-related adverse LV remodeling may be due to dysregulated ECM response and impaired healing after reperfused MI [226]. In a study of 512 patients with acute MI aged <80 (mean 62) years, elevated plasma MPO levels as oxidant markers correlated with risk for long-term mortality and added to the prognostic value of NT-proBNP and LVEF [227]. As discussed, aging results in amplified increases in tissue levels of MMP-9 and MMP-2, protease-inhibitor SLPI, matricellular proteins SPARC and OPN, and ADAM-10 and -17 after reperfused STEMI [34, 79, 228]. Since therapy for optimizing post-MI healing remains lacking for both the young

and old [16], use of the aforementioned protease biomarkers in achieving this goal is a potentially important emerging area of research, development, and discovery.

A consistent finding during progression of MI, HTN, and cardiomyopathies to end-stage HF is the increase in protease/proteolytic activity. It should be noted that while MMP/TIMP imbalance is a major pathway in adverse ECM/LV remodeling, MMP inhibition in HF did not completely prevent adverse remodeling [169]. Despite numerous studies showing attenuation of post-MI LV remodeling with broad spectrum as well as selective MMP inhibitors [31] and MMP inhibition attenuated remodeling in mice [133], pigs [132], and chronic HF patients [134], a large study of MMP inhibition initiated after STEMI failed to limit LV remodeling [169]. The negative results suggest that other proteases and matrix proteins and besides MMPs may contribute to adverse ECM/LV remodeling and more research is needed.

6.4 Biomarkers for Monitoring Adverse LV Remodeling and HF with Aging

The growing burden of HF in the aging population suggests that current therapy is not optimal. Since the 1990s, the algorithm for treatment of STEMI survivors has been to use early and prolonged therapy with a few pharmaceutical agents proven to improve outcome in evidence-based randomized clinical trials conducted in mostly non-elderly patients. The time has come for a paradigm shift because there are multiple potential targets for the different (early, late, and remote) stages of the healing/repair process [18, 75–78]. Different sets of multiple markers for different stages may prove more effective for monitoring pathophysiological changes, progression, and response to therapy [19]. For example, in that construct, there is a critical need for biomarkers of adverse post-STEMI remodeling to guide preventive measures, assess efficacy of therapeutic interventions, and improve outcome. Several biomarkers of post-STEMI remodeling have been proposed [19, 229], including NT-proBNP [230], ECM turnover markers [231–233], myocardial contrast echocardiography (MCE) and 3D echocardiography [234], magnetic resonance imaging (MRI) [235, 236], positron emission tomography (PET) imaging [237, 238], cellular/molecular imaging (FMT/MRI) [78], and micro-RNAs [239, 240]. While several novel therapeutic strategies that target inflammatory, cellular, matrix, and vascular components have been proposed, a discussion of these is beyond the scope of this chapter.

7 Conclusions

The aging population with HF is increasing worldwide, and adverse ECM/LV remodeling is the principal mechanism for the march to HF after MI and HTN, the leading causes of HF in the elderly population. Proteases play a critical role in the remodeling at extracellular, cellular, subcellular, and molecular levels. In addition to MMPs and

the MMP/TIMP imbalance in ECM and LV remodeling, several other proteases have also been implicated in adverse LV remodeling and dysfunction and the march to HF after MI, HTN, and various cardiomyopathies. Several proteases are released in the blood and can be imaged in the myocardium and serve as potential clinical markers of the development and progression of adverse remodeling in the march to HF in the aging population, as tools in diagnosis and follow-up and as guides for therapy and prevention. Because cardiac remodeling is a multifactorial, highly dynamic, and time-dependent process in which dysregulated ECM homeostasis leads to adverse ECM/LV remodeling, dysfunction, and adverse outcome, different sets of multimarker panels for different stages of progression might be more effective than isolated markers. Development and progression to HF is more dramatic and rapid after STEMIs than in HTN and certain cardiomyopathies so that appropriate timing of the markers is required. Different sets of potential markers are involved in progression to fibrosis and HF after STEMIs, HTN, and cardiomyopathies. Non-protease products of ECM turnover and markers of fibrosis can also be used clinically to advantage.

While excessive fibrosis is a major concern with the aging heart, decrease in ECM and/or defective ECM (with increased collagen type III and decreased or abnormal cross-linking) is also a concern as it can lead to more adverse LV remodeling with predominant systolic dysfunction and rupture. Markers can be applied to detect this process and take preventative measures. Since both dilative and concentric remodeling and HF progress to end-stage HF, disability, and death despite conventional therapy, markers can be applied for early detection and preventative measures. The effectiveness of plasma markers can be vastly enhanced by use of imaging modalities. For optimal benefit, a combined multipanel marker approach that is appropriately timed is needed for prevention of adverse remodeling and HF in the aging population.

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