Biology of Extracellular Matrix

William C. Parks Robert P. Mecham *Editors*

Extracellular Matrix Degradation



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Preface

The extracellular matrix is complex in its composition, wide ranging in its deposition, and diverse in how it shapes cell behavior and tissue organization. Critical to matrix function is the balance between deposition and turnover of its many, varied protein components. Indeed, the spatially and temporally precise removal and remodeling of connective tissue is critical to several developmental, homeostatic, and reparative processes. However, if matrix turnover and degradation are excessive and unregulated, as occur in many inflammatory conditions, bad things can happen. Because of the marked chemical diversity of matrix proteins - inclusive of glycoproteins, proteoglycans, and insoluble hydrophobic polymers, among other components - it is not surprising that the endopeptidases implicated in matrix turnover are equally diverse, both in their makeup and function. For example, the large and physiologically important serine proteinase family, which includes leukocyte elastase, plasminogen, and its activators, among many others enzymes, mediates a variety of activities, from clot dissolution to tissue destruction. Matrix metalloproteinases (MMPs), which compose a large subfamily within the even larger metalloproteinase family, have a specialized function in turnover of some extracellular matrix proteins, but as is discussed in more than one chapter in this volume, these enzymes are also effectors in other functions, particularly repair and immunity.

Essentially, all proteins transition through inactive, active, and finally deactivated states, and various posttranslational modifications mediate these changes. Proteolysis is one of several posttranslational mechanisms that regulate protein activity, and it is the principal way for ending a protein's time and recycling its amino acids for reuse. Evolution has provided us with six families of proteinases, defined by the amino acid or cofactor that catalyzes the nucleophilic attack on the peptide backbone of substrate proteins. Proteinases are found and operate both inside and outside of the cell. Many members of the cysteine, serine, and metalloprotienase families function extracellularly, and thus members of these three families have been implicated or demonstrated to function in turnover and degradation of the matrix. Hence, this volume focuses on these groups of enzymes.

Over the years, matrix metalloproteinases (MMPs) have gained considerable attention due to their suspected causative role in many destructive conditions, and this association has led to the oft-held idea that MMPs are the principal mediate of matrix turnover and degradation. However, in Chap. 1, Sean Gill and William Parks discuss that this assumption may not be as accurate as assumed and propose that MMPs and inhibitors have many diverse functions. If MMPs are not degrading as much matrix as we think, then who is? In Chap. 2, Dieter Brömme and Susan Wilson make a case for cysteine proteinases as major effectors of matrix remolding. Indeed, cysteine proteinases not only have been long known to degrade matrix (think osteoclasts), but they also make up much of the proteolytic machinery of lysosomes. Related this in both Chaps. 3 (Thomas Bugge and Niels Behrendt) and 8 (Vincent Everts and Paul Saftig), the authors discuss the important role of internalization to lysosomes in turnover of matrix, a process that has been too neglected in this field. In Chap. 4, Judith S. Bond, Timothy R. Keiffer, and Qi Sun discuss the membrane-bound serine proteinases and the essential roles these interesting enzymes serve in processing matrix and other extracellular proteins.

The next two chapters focus on structural mechanisms of how proteinases – with an emphasis on MMPs – interact with matrix substrates. In Chap. 5, Hideaki Nagase and Robert Visse discuss their interesting work on how MMP-1 can loosen a collagen triple helix making it more accessible to fit within the catalytic pocket of a metalloproteinases. In Chap. 6, Steven Van Doren presents sophisticated structural data on how the noncatalytic exosite regions of MMPs direct their interaction with matrix substrates.

The remaining chapters address on the role of matrix degradation in disease processes. In Chap. 7, James Quigley and Elena Deryugina discuss how MMPs promote multiple stages of tumor progression, from early transformation to metastasis, by acting on a variety of cell surfaces and matrix proteins to mediate different responses. As mentioned, in Chap. 8 Vincent Everts and Paul Saftig emphasize internalization as a critical component of matrix breakdown and turnover and so in the context of bone homeostasis and disease. In Chap. 9, A. McGarry Houghton, Majd Mouded, and Steven D. Shapiro focus on what is causing the breakdown of elastin, which occurs in devastating conditions such as emphysema and aneurysm formation.

We thank all authors for their effort and time and for their thoughtful and interesting contributions. We hope this volume on *Degradation of the Extracellular Matrix in Health and Disease* is of value for the many investigators and students with an interest in matrix biology, proteinases, and related topics.

Seattle, WA, USA St. Louis, MO, USA William C. Parks Robert P. Mecham

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Chapter 1 Matrix Metalloproteinases and Their Inhibitors in Turnover and Degradation of Extracellular Matrix

Sean E. Gill and William C. Parks

Abstract As their names imply, matrix metalloproteinases (MMPs) are thought to degrade or turnover extracellular matrix (ECM) proteins. Although some MMPs have prominent roles in ECM metabolism, most members of this large gene family, however, have either limited or no demonstrated roles in ECM turnover. In vivo studies of MMP function have revealed that matrix degradation per se is neither the sole nor predominant function of these proteinases and that these proteinases act on a variety of extracellular protein substrates, often to mediate gain-of-function processing. Because their substrates are diverse, MMPs have emerged as critical effectors in a variety of homeostatic functions, such as bone remodeling, wound healing, metabolism, and several aspects of immunity and inflammation. However, MMPs are also involved in a number of pathological processes, such as tumor progression, fibrosis, chronic inflammation, and vascular disease. In this chapter, we discuss the role of MMPs in affecting ECM metabolism and aspects of how their catalytic activity is regulated, with an emphasis on the action of the tissue inhibitors of metalloproteinases (TIMPs).

1.1 Matrix Metalloproteinases

1.1.1 Introduction

Like serine and cysteine proteinases, the other families of proteinases discussed in this volume, metalloproteases comprise a large family of endopeptidases. All metalloproteinases contain an active site Zn^{2+} (hence the prefix "metallo"), and the nearly 200 members found in mammals (Puente et al. 2003) are divided into subfamilies or clans based on evolutionary relationships and structure of the

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catalytic domain. The metzincin subfamily of metalloproteases is characterized by a 3-histidine zinc-binding motif and a conserved methionine turn following the active site (Bode et al. 1993) and includes the reprolysins or ADAMs (A Disintegrin And Metalloproteinases), serralysins, astacins, and matrixins (aka: matrix metalloproteinases; MMPs) (Stöcker et al. 1995). In this chapter, we focus on MMPs, their roles in turnover, remodeling, and degradation of extracellular matrix (ECM) proteins, and how their catalytic activity is governed by the action of the tissue inhibitors of metalloproteinases (TIMPs).

1.1.2 The Defining Features of MMPs

MMPs comprise a family of 25 related, yet distinct gene products, of which 24 are found in mammals (Table 1.1). The structural features of MMPs have been thoroughly discussed in several reviews (Maskos and Bode 2003; Massova et al. 1998; Nagase et al. 2006; Page-McCaw et al. 2007), which have all highlighted the presence of two conserved motifs, the pro- and catalytic domains, that are common to all but one member (Fig. 1.1). The prodomain is about 80 amino acids and contains the consensus sequence PRCXXPD. The exception to this rule is MMP23, in which the critical cysteine is found within a distinct run of amino acids (Velasco et al. 1999). The catalytic domain contains three conserved histidines in the sequence HEXXHXXGXXH, which ligate the active site Zn^{2+} . The glutamate residue within the catalytic motif activates a zinc-bound H₂O molecule providing the nucleophile that cleaves peptide bonds.

The cysteine thiol and zinc ion interaction keeps proMMPs in a latent state, and this linkage must be disrupted for the enzyme to gain catalytic activity (Van Wart and Birkedal-Hansen 1990). About one-third of MMPs, including all membranebound MMPs, contain a furin-recognition sequence between the pro- and catalytic domains and are activated intracellularly before secretion (Illman et al. 2003; Kang et al. 2002; Pei and Weiss 1995; Sato et al. 1996). For the other MMPs, the mode of activation is more presumed than proved, and as we have discussed in an earlier review (Ra and Parks 2007), the in vivo mechanism for the activation of most non-furin cleaved proMMPs is unknown.

With the exceptions of MMP7, 23, and 26, MMPs have a flexible proline-rich hinge region and a hemopexin-like C-terminal domain, which is thought to function in interactions with other macromolecules, such as protein substrates or anchoring factors. Other domains are restricted to subgroups of enzymes. For example, four membrane-type MMPs (MMP14, 15, 16, and 24) have transmembrane and cytosolic domains, whereas MMP17 and 25 have C-terminal hydrophobic extensions that act as a glycosylphosphatidylinositol (GPI) anchoring signal. The two gelatinases (MMP2 and MMP9) have gelatin-binding domains that resemble similar motifs in fibronectin. In addition to a remarkably common 3D structure (Massova et al. 1998), MMPs share a similar gene arrangement suggesting that they arose by duplications of an ancestor gene. At least eight of the known human MMP genes

Designation ^a	Common name(s)	ECM or ECM-	Citations
e		related substrates ^b	
MMP1	Collagenase-1	Fibrillar collagens	Pilcher et al. (1997) and many
	Mcol-A, Mcol-B ^c	C C	others
MMP2	Gelatinase-A	Fibronectin	Kenny et al. (2008)
	72-kD gelatinase	Vitronectin	•
MMP3	Stromelysin-1	Laminins	Sympson et al. (1994)
	-	Type IV collagen	• •
MMP7	Matrilysin	Syndecan-1	Li et al. (2002)
		Elastin	Filippov et al. (2003)
MMP8	Collagenase-2		
	Neutrophil collagenase		
MMP9	Gelatinase-B	Fibrin	Lelongt et al. (2001)
		NG2 proteoglycan	Larsen et al. (2003)
	92-kD gelatinase	Elastin	Kenny et al. (2008), Longo et al. (2002), Pyo et al. (2000)
		β-dystroglycan	Michaluk et al. (2007)
MMP10	Stromelysin-2		
MMP11	Stromelysin-3		
MMP12	Metalloelastase	Elastin	Hautamaki et al. (1997)
			Houghton et al. (2006)
MMP13	Collagenase-3	Fibrillar collagens	Stickens et al. (2004)
		(type II)	Inada et al. (2004)
MMP14	MT1-MMP	Fibrillar collagens	Holmbeck et al. (1999), Hotary et al. (2000), Hotary et al. (2002)
		Fibrin	Hotary et al. (2002)
		Syndecan-1	Endo et al. (2003)
MMP15	MT2-MMP	Fibrin	Hotary et al. (2002)
MMP16	MT3-MMP	Fibrillar collagens	Shi et al. (2008)
		Fibrin	Hotary et al. (2002)
		Syndecan-1	Endo et al. (2003)
MMP17	MT4-MMP		
MMP19	RASI-1		
MMP20	Enamelysin	Amelogenin	Caterina et al. (2002)
MMP21			
MMP22			
MMP23	CA-MMP		
MMP24	MT5-MMP		
MMP25	Leukolysin		
MMP26	Endometase, Matrilysin-2		
MMP27			
MMP28	Epilysin		

 Table 1.1
 Mammalian matrix metalloproteinases

^aMMP4, 5, and 6 turned out to be either MMP2 or MMP3. MMP18 (collagenase-4) has only been cloned from *Xenopus*. A mammalian homologue has not been found

^bIf left blanked, then a physiologic ECM substrate has not been identified and validated by genetic approaches or detailed cell-based models

^cMcol-A and -B are murine homologues of MMP1 (Balbin et al. 2001)



Fig. 1.1 Domain Structure of MMPs. The common motifs of MMPs are the pro- and catalytic domains. The prodomain of a typical MMP is about 80 amino acids and contains the consensus sequence PRCXXPD (except for MMP23). The catalytic domain contains three conserved histidines in the sequence HEXXHXXGXXH, which ligate the active site Zn^{2+} . Several MMPs have a furin-recognition site within the C-terminal half of the prodomain allowing activation of zymogen by proprotein convertases within the secretion pathway. As MMPs function in the extracellular space, they each have a signal peptide (SP), the exception again being MMP23, which has an N-terminal signal anchor. With the exceptions of MMP7, 23, and 26, MMPs have a flexible proline-rich hinge region and a hemopexin-like C-terminal domain. Other additions include transmembrane and cytosolic domains to the membrane-type MMPs, a glycosylphosphatidylinositol (GPI) anchoring signal to MMP17 and 25, and gelatin-binding domains that resemble similar motifs in fibronectin (FN) in MMP2 and 9

(MMP1, 3, 7, 8, 10, 12, 13, and 20) are clustered on chromosome 11 at 11q21-23. Other MMP genes are scattered among chromosomes 1, 8, 12, 14, 16, 20, and 22 (Puente et al. 2003).

1.2 Lexicon of Proteolysis

1.2.1 Breaking Down ECM

Three terms are often used when discussing proteolysis of ECM: turnover, degradation, and remodeling. Although each term implies proteolysis, their meanings within a broader biologic context are distinct. Thus, before we move on to discussing how MMPs function in matrix metabolism, we wish to define these terms and how we will use them to convey distinct biologic processes. *Turnover* (or accretion) refers to the normal physiologic replacement of protein (or any molecule) under homeostatic conditions. For example, type IV collagen is deposited in a basement membrane, and after some period of time, it is degraded and replaced with new type IV collagen. Many proteins, particularly intracellular proteins such as transcription factors and signaling factor, have relatively short half-lives, on the order of 48 h or less. In contrast, many ECM proteins, especially large, insoluble polymers, such as elastin and interstitial collagens, have extremely long half-lives, which has led to the application of archeological methods to measure their turnover rate (Shapiro et al. 1991). Although turnover does involve degradation (it is the balance between synthesis and degradation), we will use the term *degradation* to indicate untimely or excessive matrix destruction as seen in disease, typically chronic inflammatory conditions such as arthropathies, emphysema, and vascular disease. *Remodeling* will be used to indicate the breakdown of matrix in tissues undergoing normal architectural changes, such as clearance of tissue in the formation of digits, involution of the postpartum uterine, and scar resorption in a healed wound bed.

1.2.2 Gain-of-Function Processing

The three terms discussed above – turnover, degradation, and remodeling – all indicate catabolism of ECM, in essence, a loss-of-function process. However, proteolysis does not need to be defined as simply destruction. Another term – *processing* – refers to the proteolytic posttranslational modification of (typically) latent proteins. Proteolysis is a common mechanism used to control the activity of numerous, diverse proteins that function in the extracellular space (e.g., the coagulation and complement cascades, latent cytokines, prohormones, neuropeptides, digestive enzymes, and processing of ECM precursors). Thus, proteolysis – either inside cells or out – can be anabolic mediating gain-of-function processing. As we have argued elsewhere (Gill et al. 2010b; Parks et al. 2004), we propose that the processing of latent proteins is the predominant function of MMPs.

A related term is *cleavage*, which indicates proteolysis at a single site (or more) generating two (or more) discreet protein fragments. Cleavage can involve either loss-of-function proteolysis, such as the single-site splitting of fibrillar collagen by MMP1, preparing the substrate for more thorough proteolysis by other enzymes, or gain-of-function proteolysis, such as removal of propeptide from a latent growth factor or shedding of bioactive ectodomains.

1.3 Do MMPs Function in Proteolysis of the Extracellular Matrix?

The short answer to this question is *yes, some do.* However, it has become clear that ECM turnover or degradation is neither the sole nor the predominant function of these proteinases. So how did the assumption, and one that has reached dogmatic status, arise that MMPs are the main matrix degrading enzymes in our body? The answer lies in the history of the MMP field. The first MMP was discovered by Gross and Lapiere (1962). At the time, the only known collagenolytic enzymes were cysteine proteinases, such as those produced by osteoclasts for turnover of organic bone matrix. However, cysteine proteinases function at an acidic pH (see Chap. 2), yet it was known that fibrillar collagen also are turned over in tissue where the

interstitial pH was closer to neutral. In a quite clever and simple experiment, Gross and Lapiere reasoned that a collagenase had to be involved in the regressing tadpole tail where bone, cartilage, tendon, vascular, and dermal fibrillar collagen stores are being rapidly resorbed. Indeed, they discovered and purified a metallocollagenase that functions at neutral pH. (Although the first MMP discovered, the *Xenopus* collagenase that Gross and Lapiere isolated, was likely what is now called MMP18, or collagenase-4, for which a mammalian homologue has not been found.) Following this lead, essentially all MMPs isolated so far have been shown to be capable of degrading various protein components of the ECM.

As we discuss, there are actually very little data that directly support the contentions that MMPs are the main enzymes that degrade the ECM and that breakdown of ECM is the main function of MMPs. In numerous studies conducted over the last 13 years with mice lacking specific MMPs, the first MMP knockout paper was published in 1997 (Wilson et al. 1997); the data indicate quite convincingly that these proteinases act on a variety of extracellular proteins, such as cytokines, chemokines, antimicrobial peptides, and yes ECM proteins too. Consequently, MMP carry out important effector roles in a variety of biologic and disease processes, with possibly inflammation and immunity being the most common (Parks et al. 2004). The importance of the findings generated in MMP null mice cannot be understated. Understanding what an MMP actually does was not achievable until the consequence of enzyme depletion could be studied in genetically modified mice. In the following sections, we discuss different criteria, expression patterns, pericellular location, substrate verification, and established in vivo observations that are important in understanding and uncovering the functions of MMPs.

1.3.1 Where Do MMPs Function?

An important and simple concept is that MMPs are secreted and anchored to the cell surface, thereby confining their catalytic activity to membrane proteins or proteins within the secretory pathway or nearby extracellular space. About 10% of our genome encodes for proteins with a signal peptide (Clark et al. 2003), leading to an extensive array of potential MMP substrates. Thus, it is not surprising that MMPs evolved to function in a variety of physiologic and disease processes (Cauwe et al. 2007; McCawley and Matrisian 2001; Parks et al. 2004; Stamenkovic 2003).

A clear division among MMPs, and one that impacts our thinking on function, is between MMPs with a transmembrane domain (the MT-MMPs) and those without. MMPs without a transmembrane domain are often called "soluble MMPs," but this label is a bit misleading. Modeling and biochemical studies of granular serine proteinases released by neutrophils demonstrate that proteinases rapidly lose effective catalytic ability as they diffuse from the cell surface (Campbell et al. 1999). In contrast, at the cell surface, enzymes (and other proteins) can be oligomerized into locally high concentrations. As we have discussed in detail elsewhere (Ra and Parks 2007), we propose that "soluble" MMPs are anchored to the cell surface by an exosite interaction with a membrane component and that it is in this compartmentalized state that the proteinases act on their target substrates. The MT-MMPs have a built-in means to be compartmentalized at the cell surface. If this anchored-to-thecell concept is indeed true, then the substrates must also be nearby. Indeed, many of the confirmed, physiologic substrates of MMPs are membrane proteins themselves.

1.3.2 Identifying Substrates

To define the function of a given MMP produced by a specific cell type, it is critical to both identify its protein substrate and determine the consequence of that substrate being proteolyzed, be it gain- or loss-of-function. Because MMPs do not act on consensus cleavage sites, candidate substrates cannot be selected by in silico analysis. Identifying MMP substrates has been accomplished using various strategies. Possibly the most common approach has been to incubate a purified active MMP with a purified candidate substrate under optimal in vitro conditions and assess if the target protein is cleaved or degraded. However, this approach tells us only what an MMP can do, not what it does do. The shortcoming of relying on this approach is that in a test tube most MMPs are nonspecific and can cleave proteins they may not encounter in vivo. (Papain is an illustrative example of this concern. This enzyme is used to cleave animal proteins in a lab setting, although it is unlikely that papain evolved in papaya to perform such functions in the living plant.) Although in vitro proteolysis assays are easy and an essential tool for verification, most of the proteins identified as MMP substrates only by this approach probably are not physiologic targets of the enzymes.

1.3.3 In Vivo Observations

A striking observation with many MMP knockout mice is that, with very few exceptions, they do not reveal profound developmental phenotypes. In contrast, mice that lack key structural ECM components and related proteins, such as type I or IV collagens, elastin, fibronectin, various laminin chains, and lysyl oxidase, among others, have profound tissue defects or, for many, embryonic lethality, mirroring the severity of human conditions with mutations in these genes (Byers 1990; Curren et al. 1993; Gubler 2008; Li et al. 1998; Nicolae and Olsen 2010). In contrast, most MMP null mice have no to only minor phenotypes in the unchallenged state and to date, no human condition has been linked to mutation in an MMP gene (Puente et al. 2003). If MMPs are indeed critical for remodeling in development (as MMP14 is, see below) or for ECM turnover in homeostasis, then more profound phenotypes would be expected if they are mutated.

The generalized lack of developmental phenotypes among most MMP knockout mice is not surprising. Typically, MMPs are not expressed in normal, healthy

tissues, or with notable exceptions; their production and activity are at nearly undetectable levels. In contrast, some level of MMP expression is seen in any repair or remodeling process and in any diseased or inflamed tissue. Although the qualitative patterns and quantitative levels of MMPs vary among tissues, diseases, tumors, inflammatory conditions, and cell lines, a reasonably safe generalization is that activated cells express MMPs. Overall, it appears that the MMP family expanded and evolved to function in the host response to environmental stress. Supporting this idea, knockout of many genes whose products function primarily in immunity also does not lead to developmental phenotypes. A key exception to this generalization is MMP14 and to a lesser extent MMP13. Mice lacking this proteinase suffer from extensive bony defects and die some weeks after birth, and these severe phenotypes arise due to impaired collagen turnover (Holmbeck et al. 1999; Holmbeck et al. 2004; Zhou et al. 2000), as would be expected when ECM metabolism is altered. Similar to $Mmp14^{-/-}$ mice, $Mmp13^{-/-}$ mice reveal defects in endochondral bone formation which point clearly to an inability to remodel type II collagen (Inada et al. 2004; Stickens et al. 2004). Although mice deficient in either MMP2, 9, 12, 13, or 20 have permanent tissue defects, most are not severe and do not grossly affect fertility, growth, or lifespan.

1.3.4 Is the Lack of Overt ECM Phenotypes Is Due to Compensation or Redundancy?

This is a common assumption, but one that is not supported by data. In vivo evidence for compensation among MMPs, that is, the activity of one MMP making up for the loss of another, is lacking (Parks et al. 2004; Zeisberg et al. 2006). Although increased expression of MMP3 and MMP10 is seen in the uteri of some $Mmp7^{-/-}$ mice (Rudolph-Owen et al. 1997), these two MMPs are expressed in compartments distinct from where MMP7 is produced. Because MMP7 functions in mucosal immunity (Parks et al. 2004), the increased expression of MMP3 and MMP10 may represent an altered host response and not compensation. Importantly, most phenotypes reported in challenged MMP null mice are both qualitatively and mechanistically distinct, and the fact that many different roles for specific MMPs and TIMPs have been reported argues against compensation and redundancy within the family. Furthermore, if one thinks about it, compensation is complex mechanism. A cell would need to sense that it lacks some protein, then either upregulate or induce de novo expression of another, related protein to carry out the missing task.

Redundancy is distinct from compensation and refers to different mechanisms that carry out the same biological process. An example of redundancy is the many ways in which an epithelium can ward off invading pathogens: exfoliation, mucociliary clearance, production of a variety of antimicrobial factors (a significant

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degree of redundancy itself), and recruitment and homing of leukocytes. Could redundancy explain the overall lack of developmental phenotypes among MMP null mice? This possibility is suggested by the in vitro observations that multiple MMPs can cleave the same substrate. For example, at least nine different MMPs can cleave or degrade fibronectin in vitro. The efficacy, however, with which these MMPs act on fibronectin in vitro differs among enzymes. If the principal MMP that cleaves fibronectin in vivo is deleted (and it is not yet clear if fibronectin turnover is solely the responsibility of an MMP), it is possible that another MMP may degrade fibronectin but at a slower rate. In a wild-type animal, this lower affinity MMP would likely not interact with fibronectin, as fibronectin would have already been bound and processed by the higher affinity MMP; however, in the knockout mouse, the substrate is suddenly made available, and, as observed in vitro, the lower affinity MMP subsequently cleaves fibronectin providing redundancy for the system and leading to a lack of identifiable phenotypes as reduced processing of a substrate may not be manifest by an overt phenotype. Recently, neutrophil elastase, a serine proteinase, was suggested to serve a redundant function with MMP9 in facilitating neutrophil efflux (Kolaczkowska et al. 2008), but other than this report, evidence for redundancy among MMP function is lacking.

1.4 Some MMPs Do Act on ECM

1.4.1 Collagen

Elsewhere in this volume, the role of MMPs in degradation of type I collagen (Chap. 5) and elastin (Chap. 9) is discussed in detail. Metallocollagenases cleave the triple helix of fibrillar collagen types I, II, and III and possibly others. Although three human MMPs are called collagenases (MMP1, MMP8, and MMP13), several other MMPs, especially MMP14 and MMP16 (two of membrane type MMPs), have been demonstrated to function as physiologic collagenases in vivo (Chun et al. 2004; Holmbeck et al. 1999; Holmbeck et al. 2004; Hotary et al. 2003; Sabeh et al. 2009; Shi et al. 2008). Like the classic collagenases, MMP14 cleaves native type I collagen at a single locus (Gly⁷⁷⁵–Ile⁷⁷⁶ in the α 1 chain; Gly⁷⁷⁵–Leu⁷⁷⁶ in α 2), which is located about 3/4 the distance from the N-terminus of the collagen molecule. At physiologic temperature $(37^{\circ}C)$, the 3/4- and 1/4-length fragments of collagenase digestion denature spontaneously into randomly coiled gelatin peptides and can be further attacked by a variety of enzymes, including other MMPs, such as MMP2 and MMP9, or, in the case of MMP13 and the MT-MMPs, by the collagenase itself. Furthermore, by being a transmembrane protein, MMP14, as well as the other membrane-type MMPs, is in an advantageous position to affect cell movement, and its role in promoting migration and invasion of various cell types has been reviewed in detail (Seiki 2002).

1.4.2 Elastin

Elastin is a remarkably durable ECM polymer, but in chronic inflammatory conditions, such as emphysema and aneurysm formation, it is degraded leading to severe consequences. Studies with mouse models have implicated macrophage-derived MMP9 and MMP12 as the destructive elastases in these conditions (Curci et al. 1998; Hautamaki et al. 1997; Houghton et al. 2006; Longo et al. 2002; Pyo et al. 2000). Surprisingly, neither MMP9 nor MMP12 released by macrophages is effective at degrading elastin in cell-based models (Filippov et al. 2003). Despite this concern, the data strongly indicate that excess production of certain MMPs, as occurs in nonresolving inflammation, does contribute to elastin breakdown.

1.4.3 Basement Membrane

As discussed extensively in Chap. 7, cancer cells break down basement membrane during invasion and metastasis. Although this process is thought to be mediated by MMPs, typically MMP2 and MMP9, the specific enzymes responsible for basement membrane dissolution have not been demonstrated. MMP2 and MMP9 were initially called type IV collagenases because they could degrade this abundant basement membrane component in vitro (Collier et al. 1988; Wilhelm et al. 1989). However, this concept has been challenged (if not, in fact, debunked) by data, largely negative data, with genetically modified mice (Parks et al. 2004; Stetler-Stevenson and Yu 2001). Furthermore, as thoroughly discussed and referenced in a recent review (Rowe and Weiss 2008), several studies found that MMP2 and MMP9 are actually weak type IV collagenases, and no basement membrane defects have been reported in mice lacking either or both of these proteinases. Although the lack of basement membrane effects can be attributed to redundancy or compensation, the fact that $Mmp2^{-/-}$ and $Mmp9^{-/-}$ mice do reveal distinct, nonoverlapping phenotypes in other processes (Cauwe et al. 2007; Parks et al. 2004) argues that these MMPs evolved to carry out specific functions, an argument, we believe, that can be made for all MMPs.

1.5 Silencing MMP Activity

1.5.1 TIMPs

As for all secreted proteinases, the catalytic activity of MMPs is regulated at four points: gene expression, compartmentalization (i.e., pericellular accumulation of enzyme), proenzyme (or zymogen) activation, and enzyme inactivation. MMP activity is further controlled by substrate availability and affinity. TIMPs comprise

a family of four (TIMP1, 2, 3, and 4) glycoproteins that inhibit MMP and ADAM activity by binding to the catalytic site of these enzymes (Baker et al. 2002; Brew et al. 2000). Through their ability to inhibit MMPs, TIMPs have always been thought to balance ECM remodeling and turnover by MMPs. For this to be true, however, two things need to be demonstrated. One, that TIMPs do inhibit MMPs and ADAMs in vivo, and two, that inhibition by TIMPs impacts matrix degradation and deposition.

1.5.2 TIMP–MMP Interactions In Vivo

TIMPs differ in their affinity for specific metalloproteinases in vitro. Despite the fact that TIMPs are effective inhibitors of MMP activity in vitro (e.g., the K_i for TIMP1-MMP1 interaction is about 10⁻¹¹ M), only one group has yet demonstrated a direct interaction between a TIMP and an active MMP in vivo (Chen et al. 2008). In their studies, Chen et al. found that TIMP1 was complexed with MMP7 in alveolar fluid following lung injury, although an impact on ECM deposition was not demonstrated. In another study of wound fluids containing both active MMP1 and TIMP1, all recovered MMP1 was complexed to α_2 -macroglobulin (Grinnell et al. 1997), a serum protein that can inhibit the activity of a wide range of proteinases. Furthermore, although TIMPs are effective inhibitors in vitro, the most clearly demonstrated in vivo functions of TIMPs include activation of MMP activity (Caterina et al. 2000; Wang et al. 2000) and other actions and interactions unrelated to MMP inhibition (Ahonen et al. 1998; Jung et al. 2006; Lambert et al. 2004; Qi et al. 2003; Seo et al. 2003). Indeed, binding of TIMP2 to the hemopexin-like domain of MMP2 is actually required for activation of the enzyme (Caterina et al. 2000; Wang et al. 2000). Thus, there is little direct evidence that the primary function of TIMPs in vivo is to mediate MMP activity.

Recent data, however, with mice lacking TIMP1 or TIMP3 provide compelling, though indirect, data that these two proteins do function to silence MMP activity. For example, in mice deficient in TIMP1, acute lung injury is characterized by accelerated reepithelialization and increased influx of neutrophils into the alveolar space (Chen et al. 2006; Kim et al. 2005), phenotypes that are essentially the opposite of those seen in challenged $Mmp7^{-/-}$ mice (Li et al. 2002). Similarly, $Timp3^{-/-}$ mice develop a spontaneous enlargement of their alveolar space (Leco et al. 1994), and such emphysematous-like changes are often attributed to aberrant MMP activity. Indeed, one of us (SEG) demonstrated increased gelatinase activity in *Timp3^{-/-}* lungs (Gill et al. 2003) and partially rescued the developmental lung phenotype with a synthetic MMP inhibitor (Gill et al. 2006). Similarly, Khokha and coworkers have reported that other phenotypes in *Timp3^{-/-}* mice, such as increased apoptosis during mammary gland involution (Fata et al. 2001) and spontaneous cardiomyopathy (Fedak et al. 2004), are associated with increased MMP levels and are partially reversed by global nonspecific inhibition of metalloproteinase activity. Furthermore, TIMP3 seems to govern tumor necrosis factor- α (TNF α) levels by

moderating ADAM17/TACE activity (Mohan et al. 2002), and in the absence of TIMP3, constitutive levels of TNF α are elevated due to increased basal activity of ADAM17 (Mohammed et al. 2004). Together, these data indicate strongly that TIMPs do in fact inhibit metalloproteinase activity in vivo, for at least some metalloproteinases.

1.5.3 TIMPs Impact on ECM

The role of TIMPs in regulating ECM remodeling in vivo is not so clear; however, there is some in vivo evidence with $Timp3^{-/-}$ mice that suggests TIMPs can impact ECM metabolism via their ability to silence MMP activity. The absence of TIMP3 from the developing lung leads to an increase in total metalloproteinase activity and increased fibronectin degradation, which is reversed by the addition of a synthetic inhibitor of metalloproteinases (Gill et al. 2003; Gill et al. 2006). Furthermore, mature lungs from $Timp3^{-/-}$ mice have not only reduced fibronectin abundance, but also decreased levels of interstitial collagen, both of which have been attributed to enhanced metalloproteinase activity (Leco et al. 2001; Martin et al. 2003). Additionally, decreased collagen abundance is also observed in the aged hearts of $Timp3^{-/-}$ mice, and enhanced fibronectin degradation is present in the involuting mammary glands of $Timp3^{-/-}$ mice (Fata et al. 2001; Fedak et al. 2004). Collectively, these data suggest that TIMP3 functions to govern *normal* matrix remodeling and turnover.

Matrix remodeling is also a component of repair following tissue injury; however, aberrant degradation and/or deposition can result in diseases such as pulmonary fibrosis, which is the result of parenchymal lung scarring due to excessive extracellular matrix deposition (Chua et al. 2005). Members of both the MMP and TIMP families have been implicated in the genesis of pulmonary fibrosis, with TIMPs initially considered to be profibrotic and MMPs considered to attenuate fibrosis (Cabrera et al. 2007; Manoury et al. 2006; Selman et al. 2000; Swiderski et al. 1998). This idea arose from the generally held, though, as discussed, now largely disputed, idea that MMPs degrade ECM, and from correlative observations that all TIMPs are overexpressed in pulmonary fibrosis and bleomycin-injured mice (Garcia-Alvarez et al. 2006; Madtes et al. 2001; Selman et al. 2000; Swiderski et al. 1998). However, experimental observations do not support the idea that excess TIMPs block the ability of MMPs to resolve fibrosis. Indeed, the use of a broadacting metalloproteinase inhibitor or overexpression of TIMP1 resulted in decreased fibrosis in bleomycin-treated mice (Corbel et al. 2001; Fattman et al. 2008), and in the absence of TIMP3, collagen deposition, and hence fibrosis, is increased following bleomycin-induced injury (Gill et al. 2010a). Furthermore, fibrosis is enhanced following heart injury in $Timp3^{-/-}$ mice due to increased TGF β 1 activity (Kassiri et al. 2009), suggesting that if TIMPs function to balance MMP activity following injury, they may do so when the proteinases perform some of their many non-ECM-related functions.

Thus, the role of TIMPs in matrix remodeling is unclear and varies depending on the TIMP in question. TIMP3 appears to restrict ECM, specifically collagen and fibronectin, remodeling during development and under normal physiological circumstances, and as a result, the absence of TIMP3 leads to increased matrix degradation (Fig. 1.2). Following injury, however, TIMPs may not function to regulate ECM remodeling directly, but instead may mediate processing of profibrotic factors by metalloproteinases, which subsequently results in enhanced ECM deposition (Fig. 1.2).

1.5.4 Other Natural Protein Inhibitors

Other proteins are known or suspected inhibitors of MMPs. Possibly the most important among these is α_2 -macroglobulin, a broad-spectrum inhibitor that traps active enzymes of several classes in the circulation, subsequently mediating their uptake by scavenger receptors on macrophages. RECK, a membrane-bound glycoprotein, inhibits MMP2, 9, and 14 (Oh et al. 2001). Because targeted mutagenesis of the gene results in embryonic lethality, RECK likely has other, non-MMP functions. CT-PCPE, a C-terminal fragment of procollagen C-terminal proteinase enhancer, functions as an MMP inhibitor in vitro (Mott et al. 2000), but in vivo verification of this property has not been shown.

1.5.5 Oxidative Modifications

Oxidants can both activate (via oxidation of the prodomain thiol followed by autolytic cleavage) and subsequently inactivate MMPs (via modification of amino acids critical for catalytic activity). For example, hypochlorous acid (HOCl), a product of myeloperoxidase (MPO), and hydroxyl radicals activate proMMPs1, 7, and 9 (Fu et al. 2001; Michaelis et al. 1992; Peppin and Weiss 1986; Weiss et al. 1985), and peroxynitrate can activate proMMP1, 2, and 9 (Okamoto et al. 1997). In addition to promoting autolytic activation, where low concentrations of HOCl activate proMMP7, higher concentrations or extended exposure potently inactivate the active enzyme (Fu et al. 2001; 2003; Fu et al. 2004). Thus, it is possible that the pericellular production of HOCl by phagocytes provides a physiological mechanism for regulating both the activation and inactivation of MMPs within an inflammatory setting.

1.5.6 Endocytosis

Whether MMPs are silenced in the extracellular space by oxidants, TIMPs, or other interactions, how the inhibited enzymes are eventually cleared and broken down is



Fig. 1.2 TIMPs use distinct mechanisms to regulate ECM turnover in the normal and injured lung. (a) TIMP3 directly inhibits matrix degradation by MMPs during lung development and throughout life. In the absence of TIMP3, increased MMP activity leads to enhanced ECM degradation, which results in impaired bronchiole branching morphogenesis, as well as enlarged airspaces in the aged lung. (b) Following injury, TIMP3 regulates matrix deposition by controlling the release and activation of profibrotic factors, such as TGF β , by MMPs. In *Timp3^{-/-}* mice, fibrosis is enhanced in the heart and lung following injury due to increased TGF β signaling

not well understood. Most likely, as occurs for essentially all extracellular proteins (see Chap. 8), inhibited MMPs would be internalized, sorted to lysosomes, and degraded. Indeed, preformed complexes of MMP2/TIMP2 are internalized by low density lipoprotein receptor-related protein (LRP) (Emonard et al. 2004), a member of the low density lipoprotein receptor superfamily that mediates endocytosis of a variety of extracellular proteins. But could endocytosis also be used to directly silence MMP activity? The answer is clearly yes. In particular, internalization, sorting, and recycling are critical mechanisms controlling the activity of transmembrane MMPs at the cell surface (Deschamps et al. 2005; Galvez et al. 2004; Jiang et al. 2001; Lafleur et al. 2006; Osenkowski et al. 2004; Wang et al. 2004). Furthermore, MMP2, MMP9, MMP13, and MMP14 are internalized by LRPmediated endocytosis (Barmina et al. 1999; Hahn-Dantona et al. 2001; Van den Steen et al. 2006; Yang et al. 2001), and by other cell surface-associated proteins as well. In fact, as discussed in Chap. 8, it is quite likely that endocytosis is the principal means for turnover, remodeling, and clearance of ECM, and the function of MMPs, as we have proposed, is more confined to processing steps to control protein activity.

1.6 Significance

Because they are thought to have the ability to degrade ECM, MMPs have been often implicated as contributing to tumor progression and metastasis, supposedly by breaking down tissue barriers that would otherwise restrain invasion (Coussens et al. 2002; Egeblad and Werb 2002). Consequently, many pharmaceutical companies developed programs to target MMPs in cancer. Several drugs, all designed to directly block MMP catalytic activity, were tested in phase III clinical trials; however, none were effective (Coussens et al. 2002). In fact, in one study, patients taking the MMP inhibitor died at a rate higher than those on the placebo.

The key shortcoming of the MMP inhibitor trials is that these drugs lack specificity. They not only block the activity of all MMPs, but also inhibit the activity of the related ADAM and ADAMTS proteinases as well as other metal-loenzymes and even unrelated enzymes (Saghatelian et al. 2004). Despite the largely disappointing clinical trials, several studies in mice and cell models have convincingly demonstrated that *specific* MMPs are indeed essential mediators of tumor progression and invasion (Coussens et al. 2002; Hotary et al. 2002; Hotary et al. 2003; Wilson et al. 1997). In a complex tissue environment, like cancer, several cell types (resident, inflammatory, tumor) express several, different MMPs, and these proteinase can either promote or restrain disease or repair processes by affecting multiple and apparently opposing processes by the same cell at the same time (Coussens et al. 2002; Egeblad and Werb 2002; Parks et al. 2004). Clearly, some of the functions of MMPs involve their ability to act on ECM, but considering the wide range of potential substrates outside of the cell, and the power of

proteolysis as an effective means of posttranslational modification to regulate activity, it is equally clear that MMPs cannot be thought of only as ECM-degrading enzymes.

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Chapter 2 Role of Cysteine Cathepsins in Extracellular Proteolysis

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Abstract Cysteine cathepsins are lysosomal proteases with housekeeping as well as highly specialized functions. Although their activities are optimal at lysosomal acidic and reducing conditions, cathepsins can significantly contribute to the degradation of the extracellular matrix. This may happen under physiological conditions as in cathepsin K-mediated bone resorption or under pathological conditions. Extracellular matrix degradation can occur extracellularly by the secretion of cathepsins or intracellularly following the endocytosis of matrix material. Under physiological conditions, the extracellular matrix is safeguarded against cathepsin activities by its neutral pH, oxidative environment, and high levels of potent endogenous cathepsin inhibitors. However, these barriers can be overcome by pericellular acidification and pathophysiologically reduced anticathepsin concentrations. Whereas matrix metalloproteases are primarily responsible for the homeostasis of the extracellular matrix, cysteine proteases contribute to its destruction under disease conditions. The development of cathepsin inhibitors as anti matrixdegrading drugs appears to be a successful strategy.

2.1 Introduction

Proteases represent 1–4% of the genes per genomes sequenced to date and are found in all known life forms from viruses to mammalia. The human genome expresses more than 670 proteases from which about 31% are serine proteases, 25% cysteine proteases, 33% metalloproteases, and 4% aspartic proteases. The remainder are threonine proteases and proteases of other or unknown mechanisms (http://merops. sanger.ac.uk). Their functions include food processing in the gastrointestinal system (e.g., digestive proteases in saliva, stomach, and intestines), intracellular housekeeping (e.g., lysosomal proteases), one-way signal transduction (e.g., caspases

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in apoptosis), regulation of blood coagulation and the complement system, the processing of precursor proteins (e.g., proenzymes, prohormones, and antigen processing), and the physiological or pathological degradation of the extracellular matrix (ECM) (see reviews Brix et al. 2008; Turk et al. 2001; Vasiljeva et al. 2007). ECM degradation can be catalyzed by membrane-bound and secreted proteases. Historically, matrix metalloproteases (MMPs) have been considered as the main actors of ECM degradation (Brinckerhoff and Matrisian 2002; Burrage et al. 2006; Parsons et al. 1997; Shapiro 1994). This was justified by their cell membrane association or extracellular localization, their neutral pH activity optima, and their ability to degrade structural extracellular proteins such as collagens, elastin, and proteoglycans. Furthermore, their association with various diseases where ECM degradation is a prominent feature such as arthritic joint erosion, atherosclerotic plaque formation, tumor invasion and metastasis has supported the notion that MMPs are pivotal under pathological conditions and thus represent excellent targets for therapeutic interventions.

However, various studies seemed to contradict the central and critical role of MMPs in ECM degradation. For example, stromelysin (MMP3)-deficient mice exhibited an increased arthritic phenotype (Mudgett et al. 1998). MMP inhibitors in cancer treatment trials failed dramatically despite the prominent role given to these proteases in the progression of tumor growth and metastasis (Turk 2006; Zucker et al. 2000). These unexpected results might have been caused by selecting the wrong matrix metalloprotease targets or by the insufficient specificities of the inhibitors used. These results may also indicate that the main function of MMPs lies outside of bulk matrix degradation and more in the highly regulated processing of extracellular proteins. The laboratory of Overall has pioneered MMP substrate identification methods, which revealed a multitude of nonmatrix proteins as MMP targets (Butler and Overall 2009; Morrison et al. 2009). This included the activation and inactivation of various growth factors and other "signaling" proteins, which clearly indicated that MMPs might have a critical regulatory role in ECM metabolism (McQuibban et al. 2000; Overall and Blobel 2007). Moreover, the cleavage specificities of classical matrix metalloproteases such as collagenases and aggrecanases are highly specific and cleave their target substrates only at single or a very limited number of peptide bonds. For example, MMP collagenases cleave specifically a single peptide bond in type I and II collagens and generate typical 1/4 and 3/4 fragments. Aggrecanases hydrolyze specifically one or two peptide bonds between the G1 and G2 interdomain of the major cartilage resident proteoglycan (Tortorella et al. 2000; Westling et al. 2002). On the other hand, ECM-degrading pathologies such as osteoporosis and arthritis suggested a more aggressive and nonspecific proteolytic action. The involvement of proteases other than MMPs in matrix degradation was indicated early on in experiments using cysteine protease inhibitors. For example, it was shown that E64, a pan cysteine cathepsin inhibitor, strongly inhibited osteoclast-mediated bone resorption (Everts et al. 1988). Similarly, cysteine protease inhibitors proved highly potent in proteoglycan degradation experiments (Buttle et al. 1992, 1993) and tumor/metastasis related assays (Jedeszko and Sloane 2004). Various cathepsin knockout mice models revealed decreased disease

susceptibility or less severe phenotypes in models of arthritis, atherosclerosis, and various cancers (de Nooijer et al. 2009; Lutgens et al. 2006; Nakagawa et al. 1999; Palermo and Joyce 2008; Samokhin et al. 2008; Schedel et al. 2004). This indicates that cysteine proteases may play a critical role in ECM degradation. This chapter will focus on the involvement of cathepsins in ECM degradation and its pathophysiological implications.

2.2 Cysteine Cathepsins (Classification, Structure, Specificity)

The term "cathepsin" was derived from the Greek word for "digesting" and dates back 80 years (Willstaetter and Bamann 1929). Originally, it described acidic proteases isolated from the stomach mucosa. It should be noted that the umbrella term "cathepsin" comprises proteases from three different mechanistic classes: cysteine, serine, and aspartic proteases. In this chapter, we will only discuss cysteine cathepsins, which are also known as papain-like thiol proteases. Papain-like thiol proteases are the largest subfamily (C1) among the cysteine protease clan CA. Eleven human thiol-dependent cathepsins are expressed in the human genome (cathepsins B, L, K, S, V, F, W, H, X, C, and O). The mouse genome contains ten of the human orthologues and eight additional cathepsins in the placenta (Mason 2008). Interestingly, mice do not express cathepsin V.

Cathepsins are expressed either ubiquitously or with tissue and cell-type specificity (Lecaille et al. 2002b). Cathepsins B, L, and H are found in most if not in all cell types and tissues and have been attributed to nonspecific bulk protein degradation in lysosomes. Cathepsins S, K, V, F, C, and W, in contrast, are more selectively expressed and exhibit cell-type specific functions. Cathepsins S, F, and V are highly expressed in macrophages, dendritic cells, and/or thymic cortical epithelial cells and are involved in antigen processing and presentation (Riese and Chapman 2000; Shi et al. 2000; Tolosa et al. 2003). Cathepsins C and W are also expressed in immune-related cells. The function of cathepsin C (DPPI) is likely the processing of diverse precursor proteins including those of serine proteases such as granzyme zymogens (Pham and Ley 1999). Cathepsin W is specifically expressed in CD8 and natural killer cells (Linnevers et al. 1997; Wex et al. 2001) but its biological function still remains elusive. Cathepsin O has been described to be highly expressed in colon cancer cells (Velasco et al. 1994).

All papain-like cysteine proteases consist of a signal peptide, a propeptide, and a catalytic domain with the latter representing the mature proteolytically active enzyme (Fig. 2.1A). Signal peptides, which are responsible for the translocation into the endoplasmic reticulum during mRNA translation, are on average between 10 and 20 amino acids in length. Propeptides are of variable length between 36 amino acids in human cathepsin X and 251 amino acids in cathepsin F and have at least three known functions (Wiederanders et al. 2003). First, the propeptide acts as a scaffold for protein folding of the catalytic domain; second, the prodomain acts as a chaperone for the transport of the proenzyme to the endosomal/lysosomal


Fig. 2.1 (A) Schematic representation of the protease domains of human cathepsins. All cysteine cathepsins are expressed as polypeptides consisting of a signal sequence, a propeptide, and a catalytic domain. The length of the domains is depicted in the graph. The signal peptide is cleaved off at the site of the translocation into the endoplasmic reticulum. The propeptide is cleaved in the increasingly acidic environment of the endosomal/lysosomal system resulting in a fully active catalytic domain of the cathepsins. (**B**a) Ribbon structure of cathepsin K revealing the L and R domain organization of cathepsin molecules. The active site is located between both domains with the L domain harboring the active site cysteine and the R domain containing the histidine residue. (**B**b) View on the top of the cathepsin K molecule showing the catalytic diad

compartment; and third, the propeptide acts as a high-affinity reversible inhibitor preventing the premature activation of the catalytic domain.

The catalytic domains of human cathepsins are between 214 and 260 amino acids in length and contain the highly conserved active sites consisting of a

cysteine, a histidine, and an asparagine residue. The cysteine residue (Cys²⁵ based on papain numbering) and the histidine residue (His¹⁵⁹) form a catalytic thiolateimidazolium ion pair (Mellor et al. 1993; Polgar and Halasz 1982). The cysteine thiolate acts as a nucleophile for the attack of the carbonyl carbon atom of the scissile peptide bond.

With the exception of those of cathepsins O and W all other human cathepsin three-dimensional structures have been solved: cathepsin B (Musil et al. 1991), cathepsin L (Guncar et al. 1999), cathepsin K (McGrath et al. 1997), cathepsin S (McGrath et al. 1998), cathepsin V (Somoza et al. 2000), cathepsin F (Somoza et al. 2002), cathepsin H (Guncar et al. 1998), cathepsin X (Guncar et al. 2000), and cathepsin C (Molgaard et al. 2007). The overall fold of cathepsins is highly conserved and consists of L and R domains of similar size where the active site cysteine residue is located in a structurally conserved α -helix of the L domain whereas the histidine residue is in the R domain (Fig. 2.1B). The propeptide is less structured and runs in inverse orientation through the substrate-binding cleft. A comprehensive review of cathepsin structures has been published by McGrath (McGrath 1999).

Five cathepsins are strict endopeptidases (cathepsins L, S, K, V, and F) and four cathepsins are exopeptidases (cathepsins B, C, X, and H). Cathepsins X and B are carboxypeptidases with cathepsin X cleaving single amino acids and cathepsin B cleaving dipeptides from the C-terminus of peptide substrates. Cathepsins H and C are the appropriate amino and dipeptidyl peptidases. It should be noted that cathepsin B can also act as an endopeptidase. To date, little to none is known about the specificities of cathepsins O and W.

2.3 Factors Affecting the Activity of Cathepsins in the ECM

Cathepsins are known as lysosomal cysteine proteases involved in the bulk degradation of intracellular and endocytosed proteins. Their lysosomal localization requires that cathepsins are active at slightly acidic pH (pH activity optimum is about 5). Moreover, the reducing environment present in the endosomal lysosomal compartment prevents the oxidation of the thiol group of the active site cysteine residue and thus the inactivation of cathepsins. Therefore, it was historically assumed that cathepsins are inactive at neutral pH and in an oxidizing milieu typical for the cytosolic and the extracellular space. The pH and redox status-dependent activity of cathepsins thus limits their proteolytic efficacy to the endosomal lysosomal compartment and protects the cytosol and the extracellular space against accidental release of cathepsins. Numerous studies, however, have indicated that cathepsins are involved in ECM degradation (Table 2.1). How can these opposing findings be reconciled? First, not all cathepsins have a strict acidic pH optimum. The pH optimum of cathepsin S is about 6.5 (Bromme et al. 1993; Kirschke et al. 1986), and it still retains its potent activity at neutral to slightly alkaline pH values. The main physiological function of cathepsin S is likely the processing and presentation of antigens in antigen-presenting cells within the less acidic endosomal compartment

ECM protein	Cathepsin
Proteoglycan:	Cathepsin B, L (Maciewicz and Wotton 1991; Roughley and Barrett 1977)
Aggrecan	Cathepsin K, L, S (Hou et al. 2003; Nguyen et al. 1990)
	Cathepsin B (Fosang et al. 1992; Mort et al. 1998; Nguyen et al. 1990)
Fibers: Collagen	Cathepsin S (Maciewicz and Etherington 1988; Xin et al. 1992)
Fibrillar (type I, II, III, V, XI)	Cathepsin B, L: coll II, IX, XI (Maciewicz et al. 1990)
Facit (type IX, XII, XIV)	Cathepsin B: coll IV, X coll (Buck et al. 1992; Sires et al. 1995)
Short chain (type VIII, X)	Cathepsin K: coll I, II (Brömme et al. 1996; Garnero et al. 1998;
	Kafienah et al. 1998; Nosaka et al. 1999)
Basement membrane (type IV)	Cathepsin L: coll I (Nosaka et al. 1999)
Other (type VI, VII, XIII)	
Elastin	Cathepsin S (Shi et al. 1992; Xin et al. 1992)
	Cathepsin L (Kitamoto et al. 2007; Mason et al. 1986)
	Cathepsin V (Yasuda et al. 2004)
	Cathepsin K (Brömme et al. 1996)
Fibronectin	Cathepsin B (Buck et al. 1992; Isemura et al. 1981)
	Cathepsin L (Ishidoh and Kominami 1995)
	Cathepsin S (Taleb et al. 2006)
Laminin	Cathepsin B (Buck et al. 1992)
	Cathepsin L (Ishidoh and Kominami 1995)
Osteocalcin	Cathepsin B; Arg44–Phe45 (Baumgrass et al. 1997)
	Cathepsin S, H, B; Gly7–Ala8 (Baumgrass et al. 1997)
	Cathepsin L; Gly7–Ala8, Arg43–Arg44 (Baumgrass et al. 1997)
	Cathepsin D; Ala41–Tyr42 (Baumgrass et al. 1997)
Osteonectin	Cathepsin K (Bossard et al. 1996)
	Cathepsin B (Page et al. 1993)

 Table 2.1 Extracellular matrix proteins degraded by cathepsins

(Riese et al. 1998). Cathepsin S also exhibits a potent elastolytic activity (Bromme et al. 1993; Shi et al. 1992). Its high expression levels in macrophages and the accumulation of these cells at sites of inflammation and tissue destruction made cathepsin S an early candidate for ECM degradation (Shi et al. 1992). Furthermore, cathepsin S expression is also induced in kerotincytes and smooth muscle cells in the presence of proinflammatory stimuli (Schonefuss et al. 2009; Watari et al. 2000) that seem to trigger the secretion of cathepsin S (Lackman et al. 2007).

A second explanation for how cathepsins can degrade ECM is that the activity of secreted cathepsins in the ECM is facilitated by the acidification of the peri- and extracellular space under inflammatory conditions. pH values as low as 5 have been reported at sites of cartilage erosion in arthritic joints and in atherosclerotic plaques (Konttinen et al. 2002; Naghavi et al. 2002). Pericellular pH values in inflamed tissues or tumors can be significantly below the physiological pH of 7.4 and thus allowing pH labile cathepsins to be active. In particular, macrophages tolerate pH changes in the pericellular environment (Silver 1975; Silver et al. 1988). Moreover, pericellular acidic pH values seem to increase the redistribution of lysosomes to the cell surface and the subsequent secretion of lysosomal proteases as it has been demonstrated for cathepsin B (Rozhin et al. 1994). Extracellular acidification is not

exclusively associated with pathological phenotypes but it is also observed under physiological conditions. Bone-resorbing osteoclasts form a sealed and acidified space between the osteoclast and the bone surface, called the resorption lacuna. Vacuolar ATPase-driven acidification leads to the demineralization of the bone matrix and thus to the exposure of the type I collagen scaffold. This acidification also provides optimal pH conditions in the resorption lacuna for cathepsin K, the predominant collagenase of osteoclasts (Brömme et al. 1996; Xia et al. 1999). Cathepsin K-dependent bone resorption will be described in more detail in Sect. 2.5.1.

Besides the neutral pH, the oxidative environment outside of lysosomes is thought to be a major predicament for the extracellular activity of cathepsins. It was thought that the active site cysteine residue of cathepsins is rapidly oxidized leading to the irreversible inactivation of the proteases. However, recent studies have shown that cathepsins can retain significant catalytic activities under oxidative stress. Thyroglobulin was degraded by cathepsins B, L, K, and S at pH 7.4 and under oxidative conditions (Jordans et al. 2009). It was also shown that H_2O_2 oxidation of cathepsins is partially reversible. For example, about 30% of cathepsin K activity could be restored by dithiothreitol after exposure to H_2O_2 (Godat et al. 2008).

Overexpression of cathepsins is frequently accompanied by the secretion of procathepsins. A typical example is the massive secretion of the major excreted protein (MEP) from 3T3 fibroblasts which was subsequently identified as cathepsin L (Gal et al. 1985; Mason et al. 1987). These findings are primarily derived from cell culture studies and the analysis of the culture media. As discussed above, procathepsins are catalytically inactive, and thus a significant contribution of secreted cathepsins to ECM degradation was doubted. However, this argument can be dismissed when an acidic peri- or extracellular pH is considered. Under acidic pH conditions, procathepsins are effectively processed into catalytically mature proteases either autocatalytically (Pungercar et al. 2009; Vasiljeva et al. 2005) or by other proteases. Moreover, extracellular components such as polysaccharides can facilitate the processing of cathepsins as shown for procathepsin L (Mason and Massey 1992). Furthermore, the pericellular mobilization of active cathepsins by macrophages seem to be facilitated by the presence of an elastin-containing ECM that could act in a positive feedback mechanism to increase the pathophysiological remodeling of the ECM (Reddy et al. 1995).

Extralysosomal cathepsin activity is tightly controlled by endogenous inhibitors such as cystatins (Abrahamson et al. 2003). Cystatins are small protein inhibitors of approximately 10–13 kDa. These inhibitors primarily protect against the accidental release of cathepsins from lysosomes into the intracellular cytosolic or extracellular environment. Thus cells are protected by various intracellular cystatins such as cystatins A and B and extracellular cystatins such as cystatin C. Cystatin C is prevalent in serum and other body fluids. Cystatins are highly selective against papain-like cathepsins exhibiting inhibitor constants in the picomolar range and represent a major safeguard against an unwanted extracellular matrix degradation by cathepsins. However, it has been reported that cystatin can be downregulated in

disease. For example, cystatin C levels are significantly reduced in atherosclerotic and aneurysmal aortic lesions (Shi et al. 1999). Cystatin C deficiency in a mouse model of atherosclerosis significantly increased the tunica media elastic lamina fragmentation, decreased medial size, and increased smooth muscle cell and collagen content in aortic lesions (Sukhova et al. 2005). This indicates that extracellular cathepsin activities increase when the levels of their endogenous extracellular inhibitors decrease.

ECM degradation does not have to occur extracellularly (Everts et al. 1996). Various cell types are effective in endocytosing ECM proteins (e.g., macrophages, histiocytes, antigen presenting cells, and multinucleated cells including osteoclasts, fibroblasts, endothelial and epithelial cells). Endocytosis is discussed in detail in Chap. 4. ECM-containing phagosomes fuse with cathepsin-containing lysosomes, which leads to a rapid degradation of the matrix material. Inhibition of lysosomal cathepsins in phagocytes leads to an accumulation of undegraded matrix within the endosomal/lysosomal compartment. Osteoclasts and fibroblasts deficient in cathepsin K, for example, or cells treated with cathepsin K inhibitors accumulate non-degraded collagen fibrils detectable by electron microscopy (Everts et al. 2003). It is likely that the uptake of ECM is at least partially preceded by an extracellular predigest of the matrix by either secreted cathepsins and/or matrix- and membrane-associated metalloproteases. Figure 2.2 summarizes the interactions between cathepsins and the ECM under physiological and pathological conditions.



Fig. 2.2 Schematic representation of extracellular matrix conditions supportive or prohibitive for cathepsin activity. Neutral pH, oxidative environment, and the abundance of endogenous cathepsin inhibitors such as cystatins prevent or strongly limit the activity of secreted cathepsins (*left side*). Under certain pathological (inflammatory) conditions the extracellular matrix is acidified and cystatin expression is downregulated (*right side*). This provides optimal condition for cathepsins to be active and allows the processing of secreted procathepsins into catalytically active forms. Phagocytosis is possible under both conditions and allows indirectly the degradation of the extracellular matrix by lysosomal cathepsins

2.4 Cathepsins and Collagen Degradation

Triple helical type I and II collagens are highly resistant to proteolysis. Few collagenases such as the matrix metalloproteases MMP1, MMP8, and MMP13 and the cysteine protease, cathepsin K, are able to hydrolyze peptide bonds in the triple helical domain of these collagens. MMPs employ a specific structural element, called the hemopexin domain, to partially unfold the triple helix and cleave at a single site (Chung et al. 2004, 2000). This mechanism is discussed in more detail in Chap. 5. Cathepsins do not possess such a specific "unwinding" domain and most of them are only capable of cleaving in the nonhelical telopeptide regions of collagens (Etherington 1972; Etherington and Evans 1977). The only exception is cathepsin K. Cathepsin K cleaves peptide bonds at multiple sites within the triple helical domain (Garnero et al. 1998; Kafienah et al. 1998). This unique specificity is facilitated by the formation of an oligomeric complex between cathepsin K molecules and extracellular matrix-resident glycosaminoglycans (Li et al. 2000). Molecular weights of the complex are between 200 and 300 kDa, depending on the size of the participating glycosaminoglycans (Li et al. 2002). A recently solved structure of a chondroitin sulfate/cathepsin K complex revealed the formation of a "beads on a string"-like conformation (Li et al. 2008) (Fig. 2.3a). Critical interactions between cathepsin K and chondroitin sulfate molecules exploit a positively charged patch of lysine and arginine (R₈K₉K₁₀; K₁₉₁) residues close to the N- and C-termini of the cathepsin K amino acid sequence. This glycosaminoglycan-binding site is distant from the active site of the protease. Indeed, the binding between cathepsin K and chondroitin sulfate occurs on the "back side" of the protease, which may explain why complex formation with glycosaminoglycans does not interfere with the efficacy and specificity of cathepsin K regarding noncollagen substrates (Li et al. 2008). There is no significant difference between the kinetic parameters for the cleavage of synthetic peptide substrates and the efficacy to cleave gelatin between the monomeric and complex form of cathepsin K (Li et al. 2000, 2002). However, in the absence of the complex, monomeric cathepsin K, like other cathepsins, exhibits only the telopeptide cleavage capability and lacks its collagenase activity (Li et al. 2002). It is assumed that the complex functions to unfold triple helical collagen, as does the hemopexin domain in MMPs. The exact mechanism of unfolding is presently being investigated.

The collagenase activity of glycosaminoglycan/cathepsin K complexes appears to depend on the nature and concentration of participating glycosaminoglycans. At fixed weight per volume concentrations, certain glycosaminoglycans such as chondroitin and keratan sulfates promote the collagenase activity of cathepsin K, whereas dermatan and heparan sulfates inhibit the collagenase activity (Li et al. 2004). This may imply different binding modes for different glycosaminoglycans. Moreover, a significant molar excess of any glycosaminoglycans over cathepsin K inhibits the collagenase activity as well. This may have important implications for the regulation of cathepsin K activity and may explain certain bone phenotypes in diseases where the accumulation of glycosaminoglycans is causative as in



Fig. 2.3 (a) "Beads on a string"-like conformation of multiple cathepsin K molecules on a cosinelike waved chondroitin sulfate molecule. The main interaction between cathepsin K and chondroitin sulfate occurs at a single turn alpha helix from Asp 6 to Lys10 (marked by K9). The E64-containing active site is on the opposite side from the glycosaminoglycan binding site and thus freely accessible to bind substrates for hydrolysis. (b) The *left panel* shows a surface representation of cathepsin K and the binding site of chondroitin 4-sulfate. The *left panel* shows the front site of cathepsin K with the inhibitor E64 bound in the active site. The *blue* surface represents positively charged amino residues and the red surface negatively charged residues. Basic residues R8, K9, K10, and K191, which interact with the negatively charged chondroitin sulfate, are marked in the left panel (modified after Li et al. 2008)

mucopolysaccharidoses (Wilson et al. 2009a). This will be discussed in more detail in Sect. 2.5.1.

As the collagenase activity of cathepsin K depends on the formation of the complex, a disruption of complex formation may represent a novel approach to specifically inhibit the collagenase activity of this enzyme without affecting the general peptidolytic activity of the protease. It was shown that negatively charged molecules such as oligonucleotides and polyglutamic acids specifically inhibit the collagenolytic but not the gelatinolytic activity of cathepsin K (Selent et al. 2007).

High salt concentrations, which interfere with the ionic binding between the negatively charged glycosaminoglycans and the positively charged cathepsin K binding site, also inhibit the collagenase activity of cathepsin K (Li et al. 2002). The formation of cathepsin/glycosaminoglycan complexes is mostly specific for cathepsin K. The only exception is cathepsin V which also forms weak complexes with glycosaminoglycans but lacks a collagenase activity (Brömme et al. 1999; Yasuda et al. 2004). This indicates that the formation of the protease/glycosaminoglycan complex is not the only prerequisite for the potent collagenase activity of cathepsin K. Highly repetitive motifs in triple helical collagens are Gly-Pro-X and Gly-X-Hyp with X and Y representing various amino acids. About 17% of all amino acid residues are proline or hydroxyproline. Identified cathepsin K cleavage sites within the type I and II collagens revealed the acceptance of proline residues in the S1 and S2 subsites of the substrate binding area of the protease (Garnero et al. 1998; Kafienah et al. 1998). This is a unique feature for cathepsin K as other cathepsins exclude proline from these subsites (Choe et al. 2006). Consequently, the mutation of the S2 subsite into a cathepsin L-like one which excludes the binding of proline significantly reduced the collagenase activity of the cathepsin K variant (Lecaille et al. 2002a). This may explain the lack of a collagenase activity of cathepsin V despite its ability to form a complex with chondroitin sulfate. Cathepsin V does not accept proline in the P2 position of substrates (Choe et al. 2006).

2.5 Role of Cathepsins in Extracellular Matrix Degradation

2.5.1 Bone and Cartilage (Collagenolytic and Proteoglycan-Degrading Cathepsins)

Bone and cartilage contain specialized ECM components, which give strength and structural qualities. Bone organic matrix contains predominantly type I collagen (90%). The rest of the bone is composed of inorganic mineral components such as hydroxyapatite and noncollagenous proteins such as osteopontin, osteocalcin, osteonectin, fibronectin, thrombospondin, and bone sialoprotein.

During bone resorption, the degradation of type I collagen is essential; many enzymes such as MMP collagenases are present but the majority of the degradation is performed by cathepsin K. Cathepsin K was originally cloned from rabbit osteoclasts where it was suggested to have a role in bone remodeling and bone diseases (Tezuka et al. 1994). Specialized bone resorbing cells named osteoclasts have since been shown to express a high level of cathepsin K (Bromme and Okamoto 1995; Drake et al. 1996; Kamiya et al. 1998; Littlewood-Evans et al. 1997). Osteoclasts are able to acidify an isolated area between the cell and bone matrix named the resorption lacuna (Silver et al. 1988). This results in the dissolution of the mineral component releasing the matrix collagen and provides an acidic environment for secreted cathepsin K (see also Chap. 8). Cathepsin K was found to

be essential for normal osteoclast-dependent bone resorption. Without its activity such as in patients with the autosomal recessive disorder, pycnodysostosis (Gelb et al. 1996), undigested collagen fibrils were found to accumulate in lysosomes within the osteoclast (Everts et al. 2003).

The role of cathepsin K as the critical bone-degrading protease became apparent when it was noted that, unlike MMPs which cleave collagen creating typical 1/4 C-terminal and 3/4 N-terminal fragments, cathepsin K can cleave collagen at multiple locations resulting in a more complete degradation (Garnero et al. 1998; Kafienah et al. 1998). MMPs had previously been suggested to play a role in bone degradation; however, MMPs expressed by osteoclasts are now considered to play a more regulatory role governing migration and the initiation of resorption (Engsig et al. 2000; Holliday et al. 1997; Parikka et al. 2001; Sato et al. 1998). It should be noted that in calvarial bone, collagenolytic MMPs may play a greater role in collagen digestion (Everts et al. 1999). Figure 2.4 depicts the action of cathepsin K in osteoclastic bone resorption.

The ability of cathepsin K to degrade type I collagen and elastin more effectively than other collagenolytic or elastolytic enzymes (Brömme et al. 1996; Garnero et al. 1998; Kafienah et al. 1998) has led to many investigations into its inhibition for pathological conditions. Collagen fragments created by cathepsin K can be detected



Fig. 2.4 Schematic representation of bone resorbing osteoclasts. (a) The secretion of active cathepsin K into the resorption lacuna where the degradation of type I collagen occurs. Endocytosed collagen fibrils are rapidly degraded intracellularly. Digested collagen fragments can be transcytosed through the cell and released on the apical site of the cell. (b) A cathepsin K-deficient osteoclast which is unable to degrade the bone collagen matrix. As a consequence, endocytosed collagen fibrils accumulate within the cell. However, the cell remains capable to demineralize the bone matrix using the vATPase system

in the serum and urine, providing a useful markers for bone resorption (Atley et al. 2000). Cathepsin K has also been shown to be capable of cleaving SPARC/ osteonectin, a glycoprotein involved in calcium binding (Bossard et al. 1996). Potential functions of this cleavage will be discussed below.

The cleavage of type I collagen by cathepsin K may also create bioactive peptides. Type I collagen contains seven cryptic RGD sequences known to be important for cell attachment. The ability of an osteoclast to form the resorption lacuna depends on its ability to form actin rings, which requires an interaction between $\alpha v\beta 3$ integrin receptors and RGD epitopes in the matrix. In vitro experiments revealed that wildtype osteoclasts plated on type I collagen could create actin rings; however, cathepsin K-deficient osteoclasts were severely restricted in their ability to form actin rings (Wilson et al. 2009b), suggesting that the cryptic RGD sequences within type I collagen require proteolytic exposure by cathepsin K before osteoclast $\alpha\nu\beta3$ integrin-dependent attachment. The exposure of RGD motifs by the proteolytic activity of cathepsin K must be an extracellular event. Further, digestion of type I collagen by cathepsin K led to the generation of soluble peptides and resulted in the inhibition of resorption when added to murine osteoclast cultures in a manner similar to synthetic RGD peptides (Wilson et al. 2009b). The in vivo generation of small RGD peptides is likely an extra- as well as intracellular event. RGD sequences have been shown to be important for wound repair response and malignant tumor growth, suggesting a potential role for cathepsin K in the release of these potent cell signals in different systems. Figure 2.5 summarizes the effect of cathepsin K on the activation of osteoclasts via RGD peptide processing.

Type II collagen is a major component of articular cartilage and is the main protease target for conditions such as arthritis. As with type I collagen, collagenolytic MMPs have been shown to cleave type II collagen at a specific site in the C-terminus resulting in the release of 1/4 and 3/4 fragments (Miller et al. 1976). Cathepsin K has been shown to be capable of cleaving type II collagen within the helical region of the N-terminus, a unique capacity for this protease in collagen digestion (Kafienah et al. 1998).

Cathepsins K, S, and L are also capable of cleaving cartilage-residing proteoglycans such as aggrecan and link protein (Hou et al. 2003). This cleavage aids in the destabilization of cartilage and also releases glycosaminoglycans, which, as previously mentioned, complex with cathepsin K enabling the degradation of collagen (Hou et al. 2003). Cathepsin K is now thought to play a major role in the degradation of cartilage in osteoarthritis as its expression is increased in chondrocytes of patients with osteoarthritis and has also been located to osteoclasts, synovial fibroblasts, and macrophages in osteoarthritis and rheumatoid arthritis (Dejica et al. 2008; Gravallese 2002; Hou et al. 2002, 2001; Konttinen et al. 2002; Vinardell et al. 2008). Lower pH in the extracellular space of osteoarthritic patients suggests that cathepsins are the main target proteases over other collagenases (Konttinen et al. 2002). The inhibition of cathepsin K has been suggested as a potential therapeutic target for arthritis (Svelander et al. 2009). Cathepsins B and L expressed by chondrocytes are also thought to be involved in cartilage destabilization in arthritis (Maciewicz and Wotton 1991).



Fig. 2.5 Schematic representation of osteoclast regulation by cathepsin K-mediated RGD peptide generation. Limited proteolysis of type I collagen by cathepsin K exposes cryptic RGD motifs, which allows the cell to interact with the bone matrix and to obtain the bone resorbing status. Further hydrolysis of bone collagen by cathepsin K leads to the release of soluble RGD motif containing peptides which causes the disruption of the $\alpha\nu\beta3$ integrin interaction with the bone surface and subsequently to the inactivation of the osteoclast

Another site of cartilage digestion by cathepsin K-containing osteoclasts occurs at the growth plate during endochondral ossification. During this process, the cartilage scaffold laid down by chondrocytes is degraded by osteoclasts before new bone can be deposited by osteoblasts. This process is essential for new long bone formation. Malfunction of the matrix in certain pathological conditions such as in mucopolysaccharidosis diseases and its effect on cathepsin K activity is thought to contribute to the severe skeletal phenotype observed. MPS I is characterized by an accumulation of heparan and dermatan sulfate in the matrix. Studies have shown that these glycosaminoglycans not only colocalize with cathepsin K but are also accompanied by decreased cathepsin K-mediated type II collagen digestion (Wilson et al. 2009a). The ability of excessive dermatan and heparan sulfates to inhibit cathepsin K activity (Li et al. 2002, 2004) suggests that the activity of cathepsin K during endochondral ossification is essential to long bone development.

2.5.2 Blood Vessels (Elastolytic and Collagenolytic Cathepsins)

The ECM of the blood vessel wall contains both elastin and collagen. In addition to providing physical strength to the arteries, these proteins also act as a matrix for

smooth muscle cells and endothelial cells. Cardiovascular diseases are characterized by the unwanted degradation of the ECM. MMPs contribute to the ECM degradation in cardiovascular diseases (Keeling et al. 2005; Lijnen 2003; Newby 2006) but cannot account for all of it. Due to their potent collagenolytic and elastolytic potential, cysteine cathepsins have been implicated (Liu et al. 2004; Lutgens et al. 2007). An increased expression of cathepsins has been found in different cell types in atherosclerotic lesions. Smooth muscle cells express cathepsins K, L, and S, macrophages express cathepsins B, K, S, and V, and epithelial cells express cathepsins K and S (Buhling et al. 2004b). Increased expression of cathepsins L, K, S, and V is thought to lead to ECM degradation. Cathepsin K has a strong elastolytic potential at neutral pH and collagenolytic potential at a slightly acidic pH (Brömme et al. 1996), cathepsin V has been shown to be the most potent elastase known so far (Yasuda et al. 2004), cathepsin S is a strong elastase and is active at neutral pH (Shi et al. 1992), whereas human cathepsin L is a relatively weak elastase. It should be noted that mouse cathepsin L, which is more closely related to human cathepsin V than to human cathepsin L, is a potent elastase (Yasuda et al. 2004). In cultured fibroblasts, elastin degradation takes place both intracellularly and extracellularly by cathepsin K, S, and V (Yasuda et al. 2004).

In cultured smooth muscle cells, cathepsin S inhibition prevents the majority of elastin degradation and cell invasion through an elastin gel (Cheng et al. 2006; Sukhova et al. 1998). Cathepsin K, S, and L deficiencies in Apo-/- mice all revealed a reduction in medial elastic lamina breakdown, suggesting the involvement of these elastolytic cathepsins in atherosclerotic blood vessel damage (de Nooijer et al. 2009; Kitamoto et al. 2007; Lutgens et al. 2006; Samokhin et al. 2008). Figure 2.6a depicts a multinucleated cell expressing cathepsin K adjacent to an elastic lamina break. A high-resolution electron microscopy image reveals the accumulation of intercellular vesicles at the cell membrane close to the elastin break site (Fig. 2.6b). Cathepsin levels are also increased in aortic aneurisms and neovascularization and have been linked to ECM degradation (Shi et al. 2003). Cathepsin K expression appears to be regulated by shear stress suggesting that it has a role in arterial remodeling (Lutgens et al. 2006; Platt et al. 2007).

ECM degradation by cathepsins may also regulate angiogenesis. The degradation of the terminal end of collagen XVIII results in the creation of the antiangiogenic factor endostatin (Platt et al. 2007). Collagen XVIII is a component of vascular and epithelial basement membrane. Cathepsins L, B, and K have been shown to be capable of creating endostatin proteins, with cathepsin L the most efficient protease and cathepsin K the least efficient at releasing peptides (Felbor et al. 2000; Platt et al. 2007). The proteases were also shown to degrade the endostatin fragment with cathepsins L and B the most efficient. Certain MMPs can create endostatin but cannot degrade it; therefore, the ability of cathepsins to create and degrade endostatin factors could have implications for tumor metastasis and growth.



2.5.3 Lung (Collagenolytic and Elastolytic Cathepsins)

Lung fibrosis is a pulmonary disorder characterized by ECM deposition, alveolar epithelial injury, and scar tissue formation. Lung fibroblasts from fibrotic tissue have increased cathepsin K activity and it is speculated that cathepsin K is needed to fight the excessive collagen deposits (Buhling et al. 2004a). Cathepsin K-deficient mice are more prone to develop bleomycin-induced lung fibrosis. They deposit significantly more ECM when compared with wild-type mice, suggesting a role for cathepsin K in the regulation of lung matrix, likely due to the lack of collagen degradation in fibroblasts (Buhling et al. 2004a). More recently, it was also shown that the overexpression of cathepsin K in a pulmonary fibrosis mouse model reduced lung collagen deposition and improved lung function (Srivastava et al. 2008).

In inflammatory conditions such as emphysema the lung ECM is destroyed by unwanted proteolytic action. Both MMP and cathepsin (B, S, L, H, and K) expression has been shown to be increased in mouse models of IL-13-stimulated chronic obstructive pulmonary disease (COPD) (Zheng et al. 2000). Inhibition of these enzymes was found to decrease the emphysema and inflammation. Increased cathepsin L activity found in alveolar macrophage and bronchoalveolar lavage fluid has also been linked with promoting emphysema (Takahashi et al. 1993). In other inflammatory conditions such as silicosis and sarcoidosis, mature active cathepsins (B, H, K, L, and S) have been found in the bronchoalveolar fluid suggesting that they play a role in ECM degradation and therefore disease pathogenesis (Perdereau et al. 2006; Serveau-Avesque et al. 2006).

2.5.4 Skin (Collagenolytic and Elastolytic Cathepsins)

Cathepsins were not found to have high expression in normal skin fibroblasts although their expression was found to be much higher in scars (Runger et al. 2007). Wound healing and scar formation requires a tightly controlled equilibrium between synthesis and degradation of ECM proteins. After the completion of wound healing, an antifibrotic state is needed to return the scar to a normal state. Cathepsin K has been suggested to be important during this antifibrotic activity due to its high expression in the dermal fibroblasts of scars and high collagenolytic and elastolytic activities (Runger et al. 2007). Cathepsin K was found in fibroblast lysosomes and is thought to act in the endocytic degradation pathway rather than in the extracellular pathway. Collagens I and IV were found to be internalized into lysosomes for internal degradation (Quintanilla-Dieck et al. 2008).

2.5.5 Cancer

Cancer progression is characterized by the degradation of the ECM with MMPs, cysteine, and serine proteases. Many cathepsins have now been suggested to play a role in tumor progression, including cathepsins B, L, H, X, S, and K contributing to invasion/metastasis and angiogenesis; however, most work has focused on cathepsins B and L (Coulibaly et al. 1999; Mohamed and Sloane 2006; Roshy et al. 2003). Cathepsins have been described to have an increased expression and activity in tumor cells and being involved in cancer cell invasion and migration through ECM components in a variety of cancer types. Cathepsins B and L have been shown to degrade collagen IV, fibronectin, and laminin components of the basement membrane (Buck et al. 1992; Ishidoh and Kominami 1995; Lah et al. 1989). Several in vitro experiments have shown the inhibition of specific cysteine cathepsins to decrease the invasion of tumor cells through matrigel or ECM in a range of cancers, including melanoma, glioblastoma, colon, prostate, and lung cancers (Coulibaly et al. 1999; Levicar et al. 2002). Studies have shown that both intracellular and extracellular inhibitions of cathepsins can inhibit invasion, suggesting that both the secretion of active enzyme and intracellular degradation pathways are important. In prostate, colon, and breast cancer cells, intracellular type IV collagen degradation

was found to be an important factor in invasion (Bervar et al. 2003; Premzl et al. 2003; Sameni et al. 2003; Szpaderska and Frankfater 2001). In many cancers the ratio of cathepsin to endogenous inhibitor is used as a prognostic indicator of the tumor invasive and metastatic potential.

Cathepsin B has also been shown to degrade the ECM protein tenascin C, thereby promoting angiogenesis in gliomas (Mai et al. 2002; Mai et al. 2000). Cathepsin S has been suggested to promote angiogenesis and tumor progression through the degradation of collagen type IV-derived fragments canstatin and arresten (Wang et al. 2006). These antiangiogenic peptides are released through the degradation of type IV collagen in the basement membrane (Colorado et al. 2000; Kamphaus et al. 2000). The degradation of these antiangiogenic peptides by cathepsin S could promote tumor growth, as the presence of canstatin and arresten have been shown to function as tumor growth inhibitors (Colorado et al. 2000; He et al. 2003).

Cathepsin S can also release cryptic bioactive peptides from the lamin-5 complex, another component of the basement matrix. It was shown to produce proangiogenic fragments: $\gamma 2'$ and $\gamma 2 \chi$. These fragments have been shown to promote cancer cell migration and invasion (Seftor et al. 2001).

A recent study has shown that the degradation and processing of bone matrix protein SPARC (secreted protein acidic and rich in cysteine) and osteonectin by cathepsin K promotes tumor progression in the bone (Podgorski et al. 2009). SPARC is a matrix protein, which undergoes proteolytic processing resulting in a variety of physiological and pathological functions (Lane and Sage 1994; Tai and Tang 2008). Biological processing unveils numerous cryptic functions distinct from those of the native protein. Cathepsin K has been previously shown to cleave this protein; however, new studies have suggested that this cleavage may propagate bone tumor progression, a frequent site of pancreatic tumor metastasis. Although the mechanism is unclear, it is thought that cathepsin K is involved in the colonization and growth of tumors in the skeleton through its cleavage of SPARC which results in the release of proinflammatory factors into the tumor microenvironment (Podgorski et al. 2009). The role of cathepsins in cancer has been recently extensively reviewed (Gocheva and Joyce 2007; Gocheva et al. 2006; Palermo and Joyce 2008).

2.6 Cathepsins as Drug Targets to Control ECM Degradation

The first potent cathepsin inhibitors were isolated from various microbial strains about 40 years ago (Aoyagi et al. 1969; Hanada et al. 1978; Umezawa 1982). Classical examples are peptide aldehydes such as leupeptin and peptidyl epoxides such as E-64. E64, a pan-cysteine cathepsin inhibitor, showed some efficacy to slow down muscle wasting in muscular dystrophy in animal models but failed in human trials (Satoyoshi 1992). Preclinical trials using cathepsin inhibitors were also expanded into the cancer and arthritis fields. The main problem of the earlier trials was the lack of specificity of the inhibitors used as well as a limited understanding of

which of the cathepsins would be an appropriate target. A breakthrough in the field was achieved when cathepsin K was identified as the major bone-degrading protease in osteoclasts. Here, a defined target with a clear pathophysiological function became available. Serious efforts in cathepsin K inhibitor design led to the generation of highly specific cathepsin K inhibitors with excellent pharmacodynamic features (Deaton and Tavares 2005; Kim and Tasker 2006; Yamashita and Dodds 2000). At least four different cathepsin K inhibitors entered clinical trials with one compound, odanacatib, presently in phase III clinical testing (reviewed in Bromme and Lecaille 2009: Rodan and Duong 2008). Phase II clinical evaluation of odanacatib demonstrated a dose-dependent reduction in bone resorption markers and an increase in bone mineral density (BMD) at the total hip, lumbar spine, and femoral neck in postmenopausal women with low BMD when given at doses of 10, 25, or 50 mg per week. BMD increased for 24 months of the 2-year study (Rodan and Duong 2008). It should be noted that at least one clinical trial was terminated due to potentially adverse skin and lung side effects of the cathepsin K inhibitor, balicatib. Balicatib, similar to odancatib, is a nitrile-based peptide derivative. Though it showed a similar high selectivity for cathepsin K as odanacatib in in vitro enzyme assays, it lost significant selectivity in cell-based assays due to its lysosomotropic properties (Falgueyret et al. 2005). An increased accumulation of a cathepsin inhibitor in the acidic lysosome/endosome compartment may lead to the inhibition of related cysteine proteases such as cathepsins L, S, and V. In contrast, odanacatib is a nonbasic inhibitor which still maintains its potency and selectivity against individual cathepsins as well as their efficacy in cell-based assays (Desmarais et al. 2008). Besides potential off-target effects of lysosomotropic inhibitors, their lysosomal accumulation may also inhibit cathepsin K in cells other than osteoclasts such as fibroblasts. This may account for the adverse (fibrotic?) side effects of balicatib in skin and lungs.

Cathepsin K inhibitors may also be effective in the treatment of rheumatoid and osteoarthritis, certain bone cancers, and atherosclerosis. In all these diseases, cathepsin K plays a critical role in ECM degradation (for review see Podgorski 2009; Yasuda et al. 2005). It should be mentioned that cathepsin inhibitors, in particular cathepsin S inhibitors, are in preclinical trials for the treatment of immune system-related diseases including rheumatoid arthritis. Here, the pharmacological target is not the degradation of ECM by cathepsin S but its function (and potentially those of cathepsins L and V as well) in antigen processing and presentation (Yasuda et al. 2005). However, cathepsin S inhibitors may also be tested to control cathepsin S-mediated ECM degradation in the near future, as new research supports a direct role of this enzyme in extracellular matrix destruction (de Nooijer et al. 2009).

2.7 Conclusion

Cysteine cathepsins are known as lysosomal proteases responsible for the degradation of intracellular and endocytosed proteins. Their enzymatic characteristics make them well adjusted to the acidic and reducing environment of lysosomes, whereas the extralysosomal neutral pH, oxidative and endogenous inhibitor-rich milieu is prohibitive for cathepsin activity. However, under conditions that allow an acidic pH in the ECM, such as within the resorption lacuna underneath osteoclasts, a potent extracellular activity is possible. Moreover, certain pathologies show an overexpression of cathepsins, and when accompanied by pericellular acidification and a reduction of extracellular cystatin expression this can lead to a highly destructive cocktail of conditions which may cause dramatic tissue damage. Selective cathepsin inhibitors might thus be highly beneficial to control these "out-of-place" proteases.

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Chapter 3 Cooperation Between Proteolysis and Endocytosis in Collagen Turnover

Thomas H. Bugge and Niels Behrendt

Abstract Studies conducted as of today collectively suggest that the degradation of collagen in mammals is carried out in equal parts within the extracellular environment, via the action of a limited number of secreted or plasma membrane proteases with the unique ability to cleave native collagen, and intracellularly, via an assortment of lysosomal cathepsins that degrade collagen made available through its uptake by specific collagen endocytic receptors. Emerging evidence indicates that these two principal mechanisms for collagen degradation cooperate to form a single pathway that executes the sequential and complete degradation of collagen during both physiological tissue remodeling and pathological remodeling, including tumor progression. The chapter summarizes the prevailing paradigms regarding the turnover of this abundant extracellular matrix molecule.

3.1 Introduction

The collagens constitute as much as 90% of the extracellular matrix of a tissue and are by far the most abundant components of the interstitial extracellular matrix and basement membrane of the vertebrate body. They represent a structurally unique group of proteins that are composed of three polypeptide chains, each with a single long uninterrupted section of Gly-X-Y amino acid repeats, which intertwine to produce a superhelix that buries the peptide bonds within the interior of the helix. The fibrillar collagens spontaneously self-associate to form fibrils that range in diameter from 10 to 300 nm, while basement membrane collagens form complicated sheets

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with both triple-helical and globular motifs. After being laid down during embryonic development or postnatal tissue remodeling, collagen bundles, networks, and sheaths are strengthened by extensive intra- and intermolecular crosslinking catalyzed by the enzyme lysyl oxidase and by nonenzymatically crosslinks formed between glycated lysine and hydroxylysine residues (Linsenmayer 1991; Reiser et al. 1992; van der Rest and Garrone 1991). In this way, the collagens form covalently linked structures that are multiple orders of magnitudes larger than the individual cells that laid them down (Birk and Brückner 2011).

The unique supramolecular organization of the collagens makes them remarkably resistant to proteolytic degradation by the complement of about 300 proteolytic enzymes that reside within the extracellular and pericellular environments, and the half-life of collagen in some adult tissues is measured in years or even decades. However, embryonic development, postnatal growth, tissue remodeling, and tissue repair all require the targeted remodeling of existing interstitial and basement membrane collagen to allow for organ growth, cell migration, and translation of contextual clues that are embedded within the extracellular matrix. Furthermore, a number of degenerative and proliferative diseases, such as osteoarthritis, osteoporosis, rheumatoid arthritis, and neoplasia, are characterized by the extensive and detrimental turnover of collagen-rich tissues, which has spurred considerable interest in understanding the mechanics of collagen degradation and exploring molecules involved in collagen degradation as therapeutic targets. Studies spanning a wide range of scientific disciplines have amalgamated over the last 40 years to delineate these collagen turnover pathways and identify specific proteolytic enzymes and cellular receptors that mediate both the normal (physiological) and the abnormal (pathological) degradation of collagen. One of the identified pathways involves a group of secreted or membrane-associated matrix metalloproteinases, the collagenases, and takes place within the pericellular/extracellular environment. A second pathway is mediated by the cysteine cathepsins and takes place in the acidic microenvironment that is created at the osteoclast-osteoid interface specifically during bone turnover (Gelb et al. 1996; Saftig et al. 1998). A third pathway is intracellular and involves the binding of collagen fibrils to endocytic cell surface receptors, followed by the cellular uptake and proteolytic degradation of the internalized collagen in the lysosomal compartment (Everts et al. 1996). This chapter will briefly describe each of these collagen degradation pathways, and it will summarize the limited knowledge as well as current speculations as to how these systems functionally cooperate to facilitate the stepwise and complete turnover of this abundant extracellular matrix component.

3.2 Extracellular Pathways for Collagen Degradation

3.2.1 Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) constitute a family of 25 structurally related secreted and membrane-bound metalloenzymes (Parks and Mecham 1998). All MMPs

have essential zinc and calcium ions in their proteolytic domains, are synthesized as inactive zymogens, and are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Birkedal-Hansen et al. 1993; McCawley and Matrisian 2000; Werb 1997). MMP zymogens are activated by breaking an intramolecular contact between an unpaired cysteine residue in the propeptide domain and the zinc ion in the active site, which usually is achieved by regulated auto- or heterocatalytic removal of the propeptide by proteolysis, which can be triggered by a variety of external factors (Ra and Parks 2007; Van Wart and Birkedal-Hansen 1990). Only a subset of the MMPs have the capacity to cleave native collagens in vitro or in cellbased assays at physiological pH and, thus, have the potential to participate in collagen turnover in vivo (Fig. 3.1). This subset includes the membrane-type metalloproteinases, MMP-14, -15, and -16 (MT1-MMP, MT2-MMP, and MT3-MMP) (Hotary et al. 2006; Shi et al. 2008); the soluble collagenases, MMP-1, MMP-8, and MMP-13 (collagenase 1-3) (Krane and Inada 2008); and the gelatinase, MMP-2 (gelatinase A) (Aimes and Quigley 1995). MMP collagenases all cleave fibrillar collagens at a single conserved Gly-Ile or Gly-Leu bond that is located three quarters of the distance from the N-terminus toward the C-terminus of the triple helix, thereby generating the telltale 1/4 and 3/4 fragments after gel electrophoresis. Although other Gly-Ile or Gly-Leu peptide bonds occur in several places in fibrillar collagen, only this site is susceptible to cleavage by MMP collagenases for reasons that are not yet clear (Krane and Inada 2008). It has been proposed that peptide bond hydrolysis is preceded by the local unwinding of the triple helix by the hemopexin-like domain of the MMPs, providing a general mechanism for the action of MMP collagenases (Chung et al. 2004). This initial MMP collagenase cleavage leads to the rapid denaturation of collagen, which then makes the triple helix in principle susceptible to enzymatic attack by a number of other proteolytic enzymes, such as MMPs with gelatinolytic activity and non-MMP proteases. This action also generates soluble or partially soluble forms of collagen that are susceptible to complete degradation by the endocytic pathways of collagen turnover (see Sect. 3.3). As a potential "fly in the ointment" regarding this otherwise comprehensive model for the turnover of fibrillar collagens, it needs to be mentioned that mice with a point mutation introduced in type-I collagen, designed to prevent collagenase cleavage (Wu et al. 1990) and which leads to the production of type-I collagen by these mice that is completely resistant to MMP collagenase cleavage in vitro, are viable and display a phenotype that is milder than that of mice with individual or combined mutations in MMP collagenases (Holmbeck et al. 1999; Shi et al. 2008; Zhou et al. 2000), suggesting that other mechanisms for initial proteolytic attack of native interstitial collagens may exist.

Delineation of the specific roles of each MMP collagenase in physiological collagen turnover has been greatly advanced by loss of function studies in mice and by the development of cell-based assays of native collagen fibril degradation (Windsor et al. 2002). Caution should be exerted as to the direct translation of results from these studies, as mice and humans may not use the equivalent complement of MMP collagenases for the same purposes, and because collagen degradation ex vivo may not accurately reflect in vivo conditions. For example, congenital MMP-2 deficiency



Fig. 3.1 Mammalian collagenases. The figure depicts the domain structure of the seven mammalian proteases with validated roles in physiological turnover of native collagen. The specific domain types are indicated. The six MMP collagenases all cleave fibrillar collagens at a single conserved Gly-Ile or Gly-Leu bond that is located three quarters of the distance from the N-terminus toward the C-terminus of the collagen triple helix. Cathepsin K cleaves the collagen at multiple sites within the triple helix and also degrades the nonhelical N- and C-telopeptide regions. Proteases capable of cleaving collagens in their nonhelical globular regions, after denaturation of collagen caused by initial collagenase cleavage, or proteases engaged in bulk collagen turnover within the lysosome are not depicted [modified from Parks et al. (2004)]

in humans and mice has different consequences (Itoh et al. 1997; Martignetti et al. 2001), and no true ortholog of human MMP-1 has been found in the mouse (Puente et al. 2003). Nevertheless, a coherent and somewhat unexpected picture has emerged, in which the membrane-bound MMP collagenases (MMP-14, MMP-15, and MMP-16) appear to be the enzymes that are most important for collagen

turnover during both the development and the maintenance of connective tissues (Holmbeck et al. 2004; Krane and Inada 2008). This was first evidenced by the severe defects in the development and maintenance of bone, cartilage, and connective tissues of MMP-14-deficient mice, and by the associated failure of mesenchymal cells residing in the tissues of these mice to degrade fibrillar collagen in vitro (Holmbeck et al. 1999; Zhou et al. 2000). MMP-14 function in part may overlap that of MMP-16, as mice deficient in the latter collagenase display the similar spectrum of defects, though milder, which are greatly exacerbated by the combined absence of both MMP collagenases (Shi et al. 2008). Analysis of mice with null mutations in the soluble MMP collagenases has also affirmed their role in physiological collagen turnover, with the possible exception of MMP-8 (Balbin et al. 2003). Thus, mice with single deficiencies in MMP-2 and MMP-13 display skeletal defects that likely can be directly attributed to impaired collagen turnover (Inada et al. 2004; Inoue et al. 2006; Mosig et al. 2007; Stickens et al. 2004). Cell-based assays of fibrillar collagen degradation have also lent support to the notion of membrane-bound collagenases as the principal mediators of the initial stages of collagen turnover. A series of elegant loss of function and gain of function studies have revealed that only deficiency in membrane-bound MMP collagenases can abrogate the collagen degrading and collagen invasive activity of a variety of cultured mesenchymal cells, and, conversely, only gene transfer of membrane-bound MMP collagenases can confer collagen degrading capacity to cells that are normally unable to do so (Chun et al. 2004; Hotary et al. 2000, 2003, 2006; Sabeh et al. 2004; Shi et al. 2008; Wagenaar-Miller et al. 2007). One exception to this pattern is cultured human and mouse keratinocytes, whose collagen degrading capacity is independent of membrane-bound MMP collagenases, but appears to be strictly dependent on, respectively, MMP-1 and MMP-13 (Netzel-Arnett et al. 2002; Pilcher et al. 1997).

3.2.2 The Cathepsin K System

Vertebrate bone is among the tissues with the highest content of collagen, which constitutes about 90% of the total protein, mainly in the form of type-I collagen fibers that form a tight network enmeshed in the bone mineral matrix (Linsenmayer 1991; van der Rest and Garrone 1991). Bone is characterized by a relatively high rate of turnover, even under homeostatic conditions, with new bone being continuously laid down by osteoblasts and subsequently resorbed by osteoclasts (Zaidi et al. 2003). This osteoclast-dependent collagen breakdown is mediated not primarily by MMP collagenases, but rather by members of the cysteine proteinase family of lysosomal cathepsins, in particular the enzyme cathepsin K (Lecaille et al. 2008; Segovia-Silvestre et al. 2009). Cathepsin K is a typical cysteine cathepsin with a catalytic Cys, His, Asn triad located in the active site. The protease is synthesized as a 37-kDa glycosylated proenzyme (zymogen) that is activated by proteolytic removal of the propeptide induced by low pH (Lecaille et al. 2008). Osteoclast-dependent collagenolysis is a complex, yet fairly well understood, process that is initiated by

the extremely tight attachment of the osteoclast to the bone, which allows for the subsequent formation of an osteoclast lacuna or resorption pit at the bone–osteoclast interface, wherein an acidic microenvironment (pH 4.5) can be generated by the active secretion of H⁺ ions by vacuolar-type ATPases and by the passive transport of Cl^- ions through chloride channels (Roodman 2006). Unlike MMP collagenases, cathepsin K is active at a low pH, cleaves the acid-exposed collagen at multiple sites within the triple helix, and also degrades the nonhelical N- and C-telopeptide regions (Bossard et al. 1996; Garnero et al. 1998; Kafienah et al. 1998). The cleavage sites for cathepsin K do not overlap the MMP collagenase cleavage site. The partially degraded collagenaceous material that is generated by cathepsin K is then released from the osteoclast into the extracellular environment by the process of transcytosis (Nesbitt and Horton 1997; Salo et al. 1997), and it is further degraded by cellular uptake and lysosomal degradation via specific endocytic collagen degradation pathways (see Sect. 3.4).

The above model for cathepsin K-mediated collagen degradation during bone resorption is supported by studies of cathepsin K deficiency in humans (pycnodysostosis) (Gelb et al. 1996). Cathepsin K-deficient individuals have greatly diminished capacity to remodel bone, which leads to osteopetrosis, tooth retention, failure to close cranial sutures, and short and brittle bones (Helfrich 2003). Osteoclasts from pycnodysostosis patients also display a distinct accumulation of collagenaceous material within the resorption lacunas as well as in intracellular vesicles engaged in collagen transcytosis, and the release of collagen fragments to the blood stream is greatly diminished (Everts et al. 1985). The above findings from studies of human patients are largely replicated in cathepsin K-deficient mice (Gowen et al. 1999; Saftig et al. 1998). Interestingly, whereas the concentration of cathepsin-generated collagen fragments in the blood stream is low in cathepsin K-deficient individuals, the concentration of MMP collagenase-generated collagen degradation products is increased, suggesting a partial functional overlap between the two principal pathways for extracellular collagen turnover (Nishi et al. 1999). See Chap. 2 of this volume for a review of cathepsin proteases.

3.2.3 Extracellular Collagen Degradation in Cancer

In the transition from local to metastatic disease, carcinoma cells must transverse the basement membrane underlying the site of origin, as well as the subadjacent interstitial matrix. Both are rich in crosslinked collagens that form a physical barrier that must be proteolytically degraded for transversion by tumor cells. Indeed, it was an early realization that tumor dissemination was a proteolysis-dependent process and that it might be controlled by the inhibition of the proteases responsible for basement and interstitial matrix degradation, which has spurred an intense effort over the last four decades to identify these enzymes [reviewed in Dano et al. (1985) and Liotta et al. (1982)]. These studies have revealed that scores of soluble and membrane-associated proteolytic enzymes are abnormally expressed in either tumor cells or

tumor-associated stromal cells, where they have critical and diverse roles in tumor progression [reviewed in Bugge (2003)]. Nevertheless, at least as defined by studies of established tumor cell lines engaging native basement membrane or interstitial collagen in culture, the complement of proteolytic enzymes that mediate the degradation of basement membrane and interstitial collagens appears to be no different from that used by normal cells to degrade collagen in a physiological context and it seems to be limited to the membrane-type MMP collagenases, MMP-14, MMP-15, and MMP-16 (Hotary et al. 2006; Sabeh et al. 2004). However, the cell-based assay system from which these conclusions were derived does not address the contribution of tumor-associated stromal cells known to express a number of extracellular matrix degrading enzymes, including secreted collagenases, such as MMP-2 and MMP-13. Furthermore, tumor cells adopted for continuous growth in culture may be quite different from their native counterparts in terms of protease expression. Therefore,

the paradigm of tumor cell-produced membrane-type MMPs as the proteolytic enzymes solely responsible for extracellular collagen degradation in the context of tumor progression, although both attractive and well supported by data, awaits definitive confirmation in an in vivo setting [reviewed in Rowe and Weiss (2009)].

3.3 Endocytic Degradation of Collagen

Evidence for the existence of an endocytic pathway for collagen turnover was obtained already four decades ago by the identification of intracellular inclusions of phagocytosed collagen in mesenchymal cells associated with cancer, rheumatoid arthritis, emphysema, and periodontal disease (Cullen 1972; Harris et al. 1977; Neurath 1993; Soames and Davies 1977). Indeed, the endocytic pathway has been proposed to be the major clearance mechanism for collagen under steady-state conditions (Everts et al. 1996). It is primarily operative in mesenchymal cells and ultimately results in the complete degradation of the internalized collagen by lysosomal proteases within the acidified lysosomal environment (Kielty et al. 1993; Lee et al. 1996; Segal et al. 2001). Endocytic collagen turnover is a receptor-dependent process and decisive new insight into endocytic collagen turnover was made by the identification of a subset of members of the mannose receptor family and collagen-binding integrins that act as collagen internalization receptors (Fig. 3.2).

3.3.1 Mannose Receptor Family Pathways

The mannose receptor family is a group of four type-I membrane proteins with a highly conserved domain composition [reviewed in East and Isacke (2002)]. All of these proteins are endocytic receptors with constitutive internalization through



Fig. 3.2 Collagen endocytosis receptors. Cell surface receptors capable of binding and internalizing collagen belong to either the mannose receptor family (uPARAP/Endo180 and mannose receptor) or the integrin family ($\alpha 1/\beta 1$ integrins and $\alpha 2/\beta 1$ integrins). uPARAP/Endo180 and mannose receptor engage collagen via their fibronectin type-II domain, and $\alpha 1/\beta 1$ integrins and $\alpha 2/\beta 1$ integrins engage collagen via the I domain located in their $\alpha 1$ or $\alpha 2$ subunits. The minor integrins, $\alpha 10/\beta 1$ and $\alpha 11/\beta 1$, which also bind collagen and may function as collagen endocytosis receptors, are not depicted. Five other proteins, the M-type phospholipase A2 receptor, discoidin domain receptor 1, discoidin domain receptor 2, platelet glycoprotein VI, and leukocyte-associated IG-like receptor-1, are also cell surface receptors that bind collagen, but a collagen endocytosis function has not been established. *PSI* plexin–semaphorin–integrin, *EGF* epidermal growth factor [modified from Behrendt (2004) and Leitinger and Hohenester (2007)]

clathrin-coated pits. The four members are the (macrophage) mannose receptor (MR), the uPAR-associated protein (uPARAP/Endo180), the M-type phospholipase A2 receptor (PLA2R), and the dendritic cell receptor DEC-205/gp200-MR6 (in the following designated DEC-205). All of these receptors contain a fibronectin type II domain, a domain type that is frequently associated with collagen interactions. Binding and internalization of collagen/gelatin have been positively demonstrated for the first two of these members, as detailed in the following.

3.3.1.1 uPARAP/Endo180

The most well-characterized endocytic collagen receptor is uPARAP/Endo180, also designated MRC2 or CD280. This receptor is a membrane protein of $M_r \sim 180,000$ with an amino acid sequence clearly revealing that it belongs to the MR protein family (Behrendt et al. 2000; Sheikh et al. 2000; Wu et al. 1996). The molecular properties of uPARAP/Endo180 have been reviewed recently (Engelholm et al. 2009). It contains ten extracellular domains, of which only two have an identified function. These are the fibronectin type II domain, which takes part in collagen/ gelatin recognition (Wienke et al. 2003) and an active C-type lectin domain recognizing N-acetylglucosamine, fucose, and mannose residues (East et al. 2002). This lectin domain is the second out of eight sequential lectin-like domains, but appears to be the only one of these to possess a carbohydrate-binding activity. In addition to the collagen interactions to be detailed below, uPARAP/Endo180 has been found in a complex with urokinase and the urokinase receptor (Behrendt et al. 1993). Various cellular functions, unrelated to matrix turnover, have been assigned to interactions between uPARAP/Endo180 and the urokinase system (Sturge et al. 2003), but they will not be included in this discussion.

The binding of collagen to uPARAP/Endo180 has been observed on whole cells (Behrendt et al. 2000) as well as in a purified system (Madsen et al. 2007). There is a binding preference for denatured collagen and for collagen that has been subjected to specific cleavage by MMPs (Madsen et al. 2007). uPARAP/Endo180-dependent cellular internalization of collagen has been observed with all collagen subtypes so far tested, including the major fibrillar (type I) and sheet-like (type IV) collagens (Engelholm et al. 2003; Kjoller et al. 2004; Wienke et al. 2003). In fibroblasts when assayed with solubilized collagen, there is a total dependence of this receptor for cellular collagen uptake, as shown by a complete lack of collagen internalization in fibroblasts from uPARAP/Endo180-deficient mice (Engelholm et al. 2003). uPARAP/Endo180 also appears to be engaged in collagen uptake in cultured hepatic stellate cells (Mousavi et al. 2005), osteoblasts, and chondrocytes (Wagenaar-Miller et al. 2007) and, at least in the collagen-binding step, in cultured osteosarcoma and fibrosarcoma cells (Wienke et al. 2003).

uPARAP/Endo180 is constitutively internalized from clathrin-coated pits, typically with an internalization half-life of just a few minutes (East et al. 2002) and is recycled from the early endosomal compartment to the plasma membrane
(Howard and Isacke 2002). Following uPARAP/Endo180-dependent internalization, the collagen ligand is routed to the lysosome and degraded in a cysteine cathepsin-dependent manner (Kjoller et al. 2004).

uPARAP/Endo180 is predominantly expressed by mesenchymal cells. Although the picture is far from being complete, various localization studies have been performed in healthy tissues, using immunohistochemistry and/or in situ hybridization. In the normal human breast, uPARAP/Endo180 is present in some fibroblasts and myoepithelial cells (Wienke et al. 2007). In mouse embryos and the growing bones of young mice, strong uPARAP/Endo180 expression is observed at primary ossification sites with prominent expression in osteoblasts, proliferative chondrocytes, and resting zone chondrocytes (Wu et al. 1996; Engelholm et al. 2001; Wagenaar-Miller et al. 2007). Chondrocytes of articular cartilage are also uPARAP/Endo180-positive (Howard et al. 2004). Additional sites of expression include dermal macrophages (Sheikh et al. 2000) and mesenchymal cells of the villous stroma in the human placenta (Engelholm et al. 2001). The latter study of placenta also revealed expression of uPARAP/Endo180 in the endothelium of the larger microvessels, although endothelial cells are in most cases uPARAP/ Endo180-negative. In marked contrast to the generally restricted expression pattern of this receptor observed by most investigators, uPARAP/Endo180 in one study was found to be expressed in several cell types in both normal and wounded human gingiva, including epithelial cells (Honardoust et al. 2006). However, an independent study, in which the specificity of immunostaining was rigorously controlled, did not observe staining in the epithelial compartment of the oral cavity, in line with the general absence of uPARAP/Endo180 expression in the epithelial compartment (Sulek et al. 2007). A strongly altered expression pattern is evident in cancer tissue. This will be discussed below.

uPARAP/Endo180-deficient mice have been generated in two independent studies (East et al. 2003; Engelholm et al. 2003), and in both cases, the deficiency has been found to have very limited consequences in unchallenged mice. Thus, mice were born in the expected Mendelian ratio, had a normal weight gain and lifespan, and were able to reproduce. The uPARAP/Endo180-deficient mice did, however, show a reduced growth of the long bones in early postnatal life (Wagenaar-Miller et al. 2007). This effect on bone growth is not unexpected in the light of the prominent expression of the receptor in osteogenic cells and the importance of collagen remodeling in the osteogenic process.

A recent independent work, which focused on the cause of a hereditary bone defect in cattle, has shed additional light on the role of uPARAP/Endo180 in bone growth. The defect in question had occurred spontaneously within a partially inbred strain, "belgian blue" cattle, which are homozygous for a defective myostatin gene, leading to oversized muscle tissue in otherwise healthy animals. The hereditary bone defect was mapped in an unbiased search using chromosomal mapping, SNP analysis, and gene sequencing, identifying a frameshift mutation in the uPARAP/Endo180 gene as being the necessary and sufficient factor to explain all of the observed cases of the defect (Fasquelle et al. 2009). The diseased condition, referred

to as the "crooked tail syndrome," is a severe phenotype, which, in addition to the abnormal tail, includes general growth retardation and an abnormal skull shape.

It is not known whether this pronounced phenotypic consequence of uPARAP/ Endo180 deficiency, as compared with the situation in the mouse, reflects a difference in the physiological importance of the receptor in the two species, or whether other factors are to be considered. Such factors could be the "challenge" in these cattle represented by their oversized muscles, an unrecognized redundancy with myostatin or even additional (unrecognized) redundant gene defects that may be revealed in this inbred cattle strain as a result of a high degree of general homozygosity. It may be noted that the crooked tail syndrome also includes an aberrant muscular phenotype and that even cattle heterozygous for uPARAP/Endo180 deficiency displayed a partial effect in this regard (Fasquelle et al. 2009). This, however, is not easily interpreted when considering the defective myostatin gene present in the whole population of this strain of cattle.

A number of studies have addressed the expression of uPARAP/Endo180 in cancer tissue. In invasive ductal carcinoma of the human breast, a pronounced upregulation of uPARAP/Endo180 was evident in myofibroblasts and the receptor was also present in some tumor-associated macrophages (Schnack Nielsen et al. 2002). Importantly, the tumor cells were in all cases uPARAP/Endo180-negative in this study. However, in a rare type of cancer, basal-like breast cancer in which the tumor cells have a protein expression pattern resembling that of myoepithelial cells, the cancer cells were found to be positive for uPARAP/Endo180 in some cases (Wienke et al. 2007). This is in line with the finding that myoepithelial cells are indeed uPARAP/Endo180-positive in ductal carcinoma in situ (Schnack Nielsen et al. 2002) and, in some cases, also in normal breast tissue (Wienke et al. 2007). The expression of uPARAP/Endo180 has also been studied in head and neck squamous cell carcinoma. In this case, an upregulation of the protein was found in fibroblast-like stromal cells, with the poorly differentiated tumors showing the stronger stromal expression (Sulek et al. 2007). In prostate cancer, an immunofluorescence-based study pointed to an upregulation of uPARAP/Endo180 in both the epithelial and the stromal compartment although the specificity of immunostaining was not rigorously verified (Kogianni et al. 2009).

The localization of uPARAP/Endo180 in the tumor stroma, as well as the importance of matrix turnover during cancer invasion, has prompted studies on the functional role of this receptor in cancer invasion. In one investigation, uPARAP/Endo180-deficient mice were crossed into a genetic tumor model, the MMTV-PymT system, in which mice develop spontaneous malignant mammary tumors. When comparing uPARAP/Endo180 expressing and uPARAP/Endo180-deficient PymT mice in a littermate-controlled setting, the deficiency proved responsible for a significant decrease in primary tumor growth (Curino et al. 2005). Whereas this delay was only moderate, the same study revealed a very striking difference by histological examination of the tumors. The uPARAP/Endo180-deficient mice contained large accumulations of collagen in the tumors, whereas this material had been efficiently cleared in the uPARAP/Endo180-expressing littermates.

A different study, based on transplanted tumors, has addressed the function of uPARAP-expressing tumor cells. After transfection of uPARAP/Endo180-negative human MCF-7 cells with a uPARAP/Endo180 expression vector and injection into immunocompromised mice, it could be shown that tumors formed by the receptor-transfected cells grew more rapidly than mock-transfected cells (Wienke et al. 2007).

3.3.1.2 Mannose Receptor

The MR (CD206) has a protein sequence and domain characteristics very similar to those of uPARAP/Endo180, although the active carbohydrate-binding domain is, in this case, the fourth of the eight lectin-like domains [reviewed in East and Isacke (2002)], and the sequence elements in the cytoplasmic domains that govern endocytosis of the two receptors are different (Howard and Isacke 2002; Schweizer et al. 2000). The MR has a fibronectin type II domain with a high sequence homology to that of uPARAP/Endo180, and this domain is likely to be crucial for the collagenbinding activity of MR (Napper et al. 2006). The present discussion of the activity of MR will be limited to this type of interaction, although the major part of the studies performed on this receptor to date has focused on the lectin activity, the binding to pathogens, and additional roles in the immune system [reviewed in Taylor et al. (2005)].

In a purified system, the extracellular part of MR can bind to gelatin–agarose (Napper et al. 2006), and Fc fusion proteins containing the N-terminal part (including the fibronectin type II domain) of MR have been shown to bind to collagens of several subtypes, and to denatured collagen (gelatin). Furthermore, in the latter study it was shown that the endogenous MR on mouse bone marrow macrophages is solely responsible for the ability of these cells to internalize gelatin and collagen type IV because this ability was lost in macrophages from MR-deficient mice. MR is widely expressed on macrophages but also occurs on hepatic (see below) and lymphatic endothelial cells and in a few other cell types [reviewed in Taylor et al. (2005)]. Evidence for the function of MR in collagen uptake in vivo is presented in Sect. 3.4.2.

3.3.1.3 A Function for PLA2R and DEC-205 in Collagen Internalization

For the last two protein family members, PLA2R and DEC-205, very little is known about any roles in collagen endocytosis. The two receptors are mentioned here, mostly because they show a pronounced structural similarity (including their fibronectin type-II domains) with uPARAP/Endo180 and MR [reviewed in East and Isacke (2002)]. An early finding with PLA2R, however, was indeed a collagen interaction directed to its fibronectin type-II domain (Ancian et al. 1995). PLA2R-deficient

mice have been generated and show no obvious abnormalities, although no specific studies of their collagen uptake have been reported (Hanasaki et al. 1997).

3.3.2 Integrin-Dependent Pathways

Integrins, heterodimeric membrane proteins involved in cell adhesion and signaling, have been well characterized as the "classical" collagen-binding proteins on cell surfaces [reviewed in Leitinger and Hohenester (2007)] with β 1-integrins, notably $\alpha 2\beta$ 1, being dominant binding components. Whereas their role in collagen adhesion is well established, their function in endocytic events still needs to be fully elucidated. This is because the role of integrins in this respect is strongly dependent on the physical state of the collagen ligand and, possibly, on the assay system employed.

With solubilized collagen, integrins do not seem to be critical for the endocytic process. Thus, the internalization of this material by newborn mouse fibroblasts has been shown to be exclusively dependent on uPARAP/Endo180 and is insensitive to blocking of β 1-integrins (Engelholm et al. 2003). In contrast, the uptake of collagen-coated (1-µm diameter) beads by gingival fibroblasts was markedly reduced in the presence of a blocking antibody against $\alpha 2\beta 1$ integrin, suggesting a major role of this integrin, at least in the initial collagen-binding step preceding this uptake event (Arora et al. 2000; Segal et al. 2001). In the same system, the vesicles surrounding the collagen beads after cellular uptake were isolated and analyzed at various time points. This investigation revealed several characteristics consistent with a classical phagocytic process, including a maturation of phagosomes to phagolysosomes. Thus, there was an initial association of the bead vesicles with β -actin and the actin-binding protein, gelsolin, a subsequent fusion with particles containing cathepsin B and lysosomal markers and an ultimate degradation of the collagen material (Arora et al. 2000). A functional importance of gelsolin in this phagocytic process was revealed in a subsequent work, which also demonstrated its dependence on Rac activation (Arora et al. 2004). The collagen-binding matrix proteoglycan, decorin, may act as a regulator of collagen phagocytosis through this mechanism because collagen-coated beads that included decorin displayed reduced cellular binding in the same system as described above (Bhide et al. 2005).

These studies may help to clarify several mechanisms lying behind collagen phagocytosis by fibroblasts, a phenomenon that has been rigorously demonstrated by electron microscopy in early work [reviewed in Everts et al. (1996)]. However, an integrin-mediated collagen uptake in some phagocytic processes does not exclude a role of the MR receptor family members in the same type of events. In the above-mentioned study on genetically (MMTV-PymT) induced mammary tumors in uPARAP/Endo180-deficient mice, electron microscopy revealed an absence of intracellular vesicles with banded collagen in the tumor-associated fibroblasts. In the tumor fibroblasts from uPARAP/Endo180-expressing littermates,

intracellular banded collagen could indeed be demonstrated, reflecting a preceding phagocytic uptake event (Curino et al. 2005).

3.4 Collaboration Between Pericellular and Endocytic Collagen Degradation Pathways

That the extracellular MMP collagenase/cathepsin K systems and the endocytic collagen degradation pathways that have been described in the preceding sections would act in concert to organize the complete degradation of collagen appears intuitively obvious. Mature collagen structures are orders of magnitudes larger than individual cells and, thus, the cellular internalization of collagen by either mannose receptor family members or collagen-binding integrins would be predicated on initial cleavage of collagen by pericellular/extracellular collagenases to generate collagen fragments of a manageable size for the cellular endocytosis apparatus. Conversely, MMP collagenases or cathepsin K is incapable of completely degrading collagen, but rather facilitates the formation of partially degraded collagen fragments that are released into the interstitium or the circulation, where they would become targets for endocytic uptake by cells. This notion of a tight integration of extracellular and intracellular collagen degradation pathways is further supported by in vivo localization studies revealing that cells that are actively engaged in collagen turnover express both collagenases and collagen endocytic receptors during physiological as well as malignant tissue remodeling (Curino et al. 2005; Schnack Nielsen et al. 2002; Wagenaar-Miller et al. 2007), and by the burgeoning functional studies described below.

3.4.1 Collagenases and uPARAP/Endo180-Mediated Collagen Endocytosis

As both uPARAP/Endo180 and MMP-14 have important functions in collagen turnover in connection with bone growth, a genetic study was performed to reveal the mutual dependence of their associated functions. A littermate-controlled program was set up to compare wild-type mice and mice that were homozygously deficient for either uPARAP/Endo180 or MMP-14, or homozygously double deficient (Wagenaar-Miller et al. 2007). Even compared with the clearly compromised MMP-14-deficient mice, the double-deficient mice turned out to display a strongly worsened phenotype. They had a lifespan of less than 3 weeks and very severe skeletal defects, including a lack of cranial closure and delayed bone growth. Since all phenotypic consequences were compatible with defects in bone collagen remodeling, it appears that the turnover mechanisms supported by uPARAP/Endo180 and MMP-14, respectively, can to some extent complement each other. On the

other hand, as MMP-14 acts in partial redundancy with MMP-16 to degrade collagen during osteogenesis (Shi et al. 2008), the very severe phenotype of uPARAP/Endo180 and MMP-14 double-deficient mice could, alternatively, be a consequence of the combined impairment of sequential and interdependent pericellular and endocytic collagen degradation pathways. Indeed, studies with isolated cells have indicated a functional association between MMP-14 and uPARAP/ Endo180. When grown on a reconstituted type-I collagen matrix, wild-type fibroblasts successfully degraded this matrix whereas MMP-14-deficient cells failed to do so. Fibroblasts deficient for uPARAP/Endo180 did degrade the matrix, but in these cultures defined collagen fragments accumulated in the culture medium (Madsen et al. 2007). Since the accumulated collagen was efficiently cleared by wild-type cells, these data point to a sequential reaction where the first collagen cleavage step is performed by MMP-14, after which the initial cleavage products are endocytosed by uPARAP/Endo180 for intracellular degradation. This is in line with the high uptake efficiency of uPARAP/Endo180 for defined collagen fragments mentioned above.

3.4.2 Clearance of Circulating Bone-Derived Gelatin by the Mannose Receptor

The remodeling of bone by osteoclasts in a normal individual is estimated to lead to the release of more than half a gram of partially degraded collagen into the bloodstream per day (see Sect. 3.2.2 and Chap. 2 in this volume), and this material is efficiently cleared by hepatic sinusoidal endothelial cells. The MR appears to be a dominant endocytic receptor in the uptake of collagen/denatured collagen fragments from the blood. Thus, when labeled denatured collagen was injected intravenously into MR-deficient mice, these mice displayed a greatly delayed clearance of this material, as compared to wild-type mice, and MR deficient sinusoidal endothelial cells were unable to internalize the same material when explanted in culture (Malovic et al. 2007). A probably unrelated endocytic activity of the MR is directed to the C-terminal procollagen propeptides, liberated by MMPs or cathepsin K cleavage during collagen turnover, which, most likely, bind through the lectin function of the MR. Strikingly, these prodomains accumulate specifically in the blood of MR-deficient mice (Lee et al. 2002; Sturge et al. 2007).

3.4.3 MT-MMP and Integrin Cooperativity in Collagen Internalization

As evident from the discussion above, cooperative actions between MMPs and receptors of the mannose receptor family can lead to collagen cleavage into defined

fragments and subsequent cellular uptake and complete degradation, without a strict need for a particle-directed (phagocytic) uptake mechanism. However, even in the case of a phagocytic uptake process, initial enzymatic cleavage steps are likely to be required, considering the very large size of intact collagen fibers. In accordance with this view, the β 1 integrin-dependent phagocytosis of fibrillar collagen by gingival fibroblasts was found to be increased after overexpression of MMP-14 and decreased after MMP-14 downregulation (Lee et al. 2006). Thus it appears that, not unexpectedly, MMP-14 undertakes important steps of collagen cleavage, even in this system.



Fig. 3.3 *Cooperation between proteolysis and endocytosis in collagen turnover*. Collagen fibers, being very large structures, are initially fragmented into large cleavage products before cellular uptake or further degradation in the extracellular space. MT1-MMPs play a central role in this initial fragmentation (1). Alternatively, this cleavage may be accomplished by soluble collagenases (1A). The resulting large products may be taken up by some cell types in a phagocytic process, dependent on β 1 integrins (2). Alternatively, further cleavage of the triple-helical collagen is accomplished by MT-MMPs (3) or soluble collagenases (3A), in both cases resulting in specific "1/4" and "3/4" fragments that gradually undergo denaturation and acquire a gelatin-like state. The gelatin-like products are efficiently endocytosed by mannose receptor family receptors such as Endo180/uPARAP (4), routed to lysosomes and degraded. Alternatively, further degradation of the gelatin material may occur extracellularly, catalyzed by gelatinases (5) or other proteases. Symbols depicting integrin and mannose receptor-type receptors and MMPs are the same as in Figs. 3.1 and 3.2. (Black arrows) proteolytic attack, (*red arrows*) cellular uptake. Although in this figure all membrane proteins are depicted on a continuous "plasma membrane," this does not implicate that the active players must necessarily reside on the same cell

3.4.4 Regulation of Collagenase Expression by Collagen Endocytic Receptors

Finally, in addition to redundant functions and integrated reaction sequences, regulatory events may lead to mutual dependence between different mechanisms in collagen turnover. For example, the signaling activity of collagen-binding integrins and the collagen-binding receptor tyrosine kinases, DDR-1 and -2, can lead to the altered expression of MMPs [reviewed in Leitinger and Hohenester (2007)]. Furthermore, regulation may occur at the protein localization and enzyme activity level. Although this is still a quite open area, a recent study examined the effect of siRNAmediated downregulation of uPARAP/Endo180 in cultured fibrosarcoma cells. Not surprisingly, this led to a reduced clearance of collagen in the cell culture and to the accumulation of collagen material in the culture medium. This, however, in turn led to an increased cell surface expression of MMP-14, an increased MMP-14 activity and increased activation of pro-MMP-2 (Messaritou et al. 2009).

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Chapter 4 Pericellular Proteolysis

Judith S. Bond, Timothy R. Keiffer, and Qi Sun

Abstract The immediate environment of cells is a rich source of proteolytic enzymes due to plasma membrane and secreted proteases, and many important physiological and pathological processes take place pericellularly. Meprin metal-loproteases that are membrane-bound and secreted act pericellularly to modify proteins and peptides, and there is increasing evidence that these modifications have important implications in the hematological and immune systems.

4.1 Introduction

"Pericellular proteolysis" is proteolytic degradation surrounding a cell. This can occur as a consequence of the action of plasma membrane proteases or proteases secreted from cells that act in the immediate environment of the cell. There are numerous examples of proteases that engage in pericellular proteolysis (including serine, cysteine, and aspartic and metalloproteases); metalloproteases are one of the major classes that act in the environment surrounding cells. The plasma membrane is a rich source of proteases, and well-studied examples include angiotensinconverting enzyme (ACE), tumor necrosis α converting enzyme (TACE), membrane-type matrix metalloproteinases (MT-MMPs), and meprins (Fig. 4.1). These enzymes may be quite selective for substrates (e.g., ACE) or nonspecific for substrates (e.g., meprins), but their action is limited by their location under normal conditions (at specific plasma membranes or in polarized cells at specific parts of the membrane, e.g., apical or basement membrane). There are also numerous examples of secreted proteases that act pericellularly, including collagen-processing enzymes

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Fig. 4.1 Examples of proteolytic enzymes that act pericellularly and processes that they affect

such as bone morphogenetic protein-1 (BMP-1 or procollagen C-endopeptidase), matrix metalloproteinase 1 (MMP-1, collagenase), blood coagulation proteinases that act at a cell surface (thrombin), and complement proteases that form complexes at cell surfaces. Mobile cells (e.g., leukocytes, cancer cells) often express membrane-bound and secreted proteases that act pericellularly as they migrate and move through epithelial barriers and the vasculature during extravagation and metastases stages. In this chapter, we will mainly discuss meprin metalloproteinases. These proteinases have membrane-bound and secreted forms and are highly expressed in both polarized cells (kidney proximal tubule cells, and epithelial cells of the intestine and skin) and leukocytes. The structure, function, and tissue expression of meprins have been recently reviewed (Sterchi et al. 2008), and thus these aspects will be only briefly discussed in this chapter while advances in the last 2 years will be presented more thoroughly.

4.2 Meprin Isoform Structure and Relation to Other Proteases

4.2.1 Discovery of Meprins

The meprins were discovered in mouse kidney and in human intestine in the early 1980s (Beynon et al. 1981; Sterchi et al. 1982). At that time, there were several proteases known to be expressed in both these tissues, but the meprins had distinct

properties from all the known proteases. For example, in the kidney they were the only membrane-associated proteases that cleaved proteins at alkaline pH values. There were many known lysosomal proteases that act on proteins at acidic pH values, and proteases in other cellular compartments and membranes that were exopeptidases (removed one or two amino acids from the carboxy- or amino-terminus of a protein) or could degrade small peptides, but meprins were unique in their ability to degrade proteins such as azocasein and gelatin at neutral and alkaline pH values. In addition, meprin specific activity against azocasein was $10 \times$ higher in the C57Bl/6 mouse kidney than in any other tissue analyzed (including liver, brain, spleen, muscle). In the human intestine, meprins were discovered as proteases that could degrade *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (PABA-peptide), a substrate that was used clinically at the time to determine pancreatic function because of the ability of chymotrypsin to hydrolyze it. From these beginnings, meprins were purified, cloned, and sequenced, and found to be rather unique in the world of proteases.

4.2.2 Structure of the Meprin Domains and Oligomers

The meprins are composed of two subunits, α and β , that are approximately 50% identical in their primary amino acid sequence and very similar in their domain structure (Fig. 4.2). The subunits are coded for on separate chromosomes; meprin α on human chromosome 6, and mouse chromosome 17; meprin β on chromosome 18 of both the mouse and human genome. Both subunits contain a signal sequence (S) that targets the protein to the secretory pathway, a prosequence (Pro) that keeps the enzyme inactive until it is proteolytically removed (usually by a trypsin-like enzyme), a catalytic (protease) domain that contains a deep cleft with zinc at the active site, MAM and TRAF domains that are protein-protein interaction domains important for protein folding, an epidermal growth factor (EGF) domain, a transmembrane domain (TM), and a small cytoplasmic tail. The α subunit contains an inserted domain (I) not present in the β subunit. The presence of the I domain enables the α subunit to be proteolytically cleaved in the endoplasmic reticulum during biosynthesis. Thus, the α subunit loses its transmembrane domain and is not found at the plasma membrane unless associated with a β subunit. The β subunit is found as a type 1 protein at the cell surface unless it is shed from the surface or redistributed, as in some pathological situations (e.g., after kidney ischemia, see Fig. 4.7).

The mouse meprin subunits have a molecular mass of 75–85 kDa depending on whether they are activated or not, and they are highly glycosylated (Bertenshaw et al. 2003; Kadowaki et al. 2000). Glycosylation accounts for approximately 20% of the molecular mass (Tang and Bond 1998). The glycans contribute to folding, activity, stability, and oligomerization (Ishmael et al. 2006). *N*-Glycans in the protease domain of meprin α are important to the formation of an active, stable



Fig. 4.2 Domain and oligomeric structures of meprins A and B. The meprins are multidomain proteases that contain a signal sequence (S), prosequence, protease domain, meprin A5 protein tyrosine phosphatase μ (MAM) domain, tumor necrosis factor receptor associated factor (TRAF) domain, EGF-like domain, transmembrane (TM) domain, and a cytosolic (C) domain. The meprin α subunit contains an additional inserted (I) domain between the EGF and TRAF domains. While the meprin β subunit is a type 1 membrane-bound protein, the meprin α subunit is proteolytically processed during biosynthesis at the I domain (*black arrow*) during biosynthesis and loses its transmembrane domain. Meprins, which exist in both membrane-bound and secreted forms, form oligomers that are unique in the astacin family of proteases. Dimers of membrane-bound β subunits are referred to as meprin B, whereas tetramers of α and β subunits are referred to as heteromeric meprin A and are membrane-bound due to the presence of the β subunit. The secreted form of meprin, homomeric meprin A, is capable of forming large multimers up to 8 MDa in size (Yura R, Penn State Thesis)

meprin A oligomer with correct disulfide bridging between the subunits of the MAM domain and stability of the oligomer.

Meprins are quite unique in their oligomeric structure. They exist in several isoforms as a consequence of its two subunits: homomeric meprin A (composed of only α subunits), heteromeric meprin A (composed of α and β subunits), and homomeric meprin B (composed of only β subunits). The subunits form disulfide-linked homo- or heterodimers. The meprin α dimers are homophilic and tend to form high molecular mass oligomers as they are secreted from cells. The molecular masses of homomeric meprin A are generally between 1 and 6 million Daltons, among the largest proteases known. The meprin β dimers, on the other hand, do not form larger oligomers, while the heteromeric forms of meprin A tend to exist as tetramers (disulfide-linked heterodimers that form tetramers (β – $\alpha\alpha$ – β)). The homophilic properties of meprin α allow the secreted homomeric meprin A to exist in the extracellular spaces at very high local concentrations. The membrane-bound

meprin B and heteromeric meprin A also tend to be found at high concentrations in the brush border membranes of the kidney and the intestine.

4.2.3 Evolution and Relation of Meprins to Other Proteases

Meprins are members of the "astacin family of metalloproteinases" (Clan MA(M)-M12A) and the "metzincin" superfamily. Extensive information about these families can be found at the MEROPS database (http://merops.sanger.ac.uk). The astacin evolutionary family was named after the crayfish enzyme; it is the smallest member of the family, contains only a prosequence and the protease domain, and it is the only member of the family for which there is a crystal structure to date. There are hundreds of members of the astacin family that have been identified in hydra to mammals. Bone morphogenetic protein-1 (BMP-1) is one of the family members and one of the proteinases important to the processing of collagen. Meprins are unique in the family in terms of the COOH-terminal domains, disulfide bridging of the subunits to form dimers, higher order oligomerization, and enzymatic activities (Bertenshaw et al. 2003; Sterchi et al. 2008).

The metzincin superfamily contains several evolutionary families in addition to the astacins. The other families include the matrixins, the ADAMs, serralysins (bacterial proteases), pappalysins (pregnancy-associated plasma proteins), and leishmanolysins (protozoan proteases). The different families have very little primary sequence identity, but their protease domains contain strikingly similar threedimensional structures, and they have a conserved zinc-binding sequence and a methionine-turn (Met-turn) underlying the active site. These features gave rise to the name "Metzincins" for the superfamily.

4.3 Meprin Substrates and Inhibitors

4.3.1 Peptide Bond Specificity of the Subunits

Meprin α and β have different peptide bond specificities (Fig. 4.3). The β subunit has a preference for cleaving peptide bonds flanked by negatively charged amino acids (Asp, Glu), whereas the α subunit prefers neutral, aliphatic, and aromatic amino acids (e.g., serine, leucine, tyrosine, phenylalanine). The α subunit also has a preference for proline residues one amino acid removed from the cleavage site. This latter preference may be important for the ability of meprin A to hydrolyze extracellular proteins such as collagens and gelatins. Because of these very different peptide bond specificities, the homomeric isoforms of meprin A and B tend to have distinct peptide substrates. For example, gastrin-17 that has a cluster of Glu residues is a good substrate for mouse meprin B, but is not hydrolyzed by homomeric meprin A. Whereas the nine-amino acid bradykinin peptide is cleaved by homomeric meprin A at a Phe-Ser bond, but the peptide is not cleaved by meprin B.

a Preferred amino acids in substrate subsites P1' to P4' for meprin A



b Preferred amino acids in substrate subsites P1' to P4' for meprin B



Fig. 4.3 Peptide bond specificities of homomeric meprin A and meprin B. (a) An acetylated dodecamer peptide library mixture (1 mM) containing roughly equimolar amounts of all amino acids at each position, except cysteine, was incubated with meprin B (10 nM) until the library was

4.3.2 Substrates Cleaved by Meprin A and B (In Vitro and In Vivo)

Meprins are capable of cleaving a wide variety of substrates including extracellular proteins, cytokines, hormones, and growth factors. All isoforms of meprins are capable of hydrolyzing some extracellular matrix (ECM) proteins such as collagen IV, gelatin nidogen, fibronectin, and laminins. These proteins are probably not normally substrates for the kidney and intestinal meprins because they are not present at brush border membranes at the apical surface of epithelial cells or in the lumen of these organs where meprins are localized. However, there are several circumstances where the meprins are redistributed to other portions of the cell, and then ECM substrates are relevant. In addition, in cancer cells and immune system cells that move through cell barriers, ECM and other extracellular proteins (junctional proteins) become physiologically relevant substrates.

There is evidence implicating meprins in the hydrolysis of cell junctional proteins from cell-based studies. E-cadherin was shown to be a meprin B substrate in a cell culture system, and cells expressing meprin B exhibited weaker intercellular contacts (Huguenin et al. 2008). Meprin B also hydrolyzes sites in tenascin-C, an adhesion-modulating ECM protein, and hydrolysis interferes with the oligomerization and adhesion properties of the protein (Ambort et al. 2010). There have been attempts to use proteomic analysis to find novel meprin substrates by looking at protein changes between cells expressing meprin vs. cells with no meprin expression. In one such approach, 17 potential novel meprin substrates have been identified (Ambort et al. 2008). For example, clusterin, lysyl oxidase, and vinculin were identified as meprin substrates. These substrates implicate meprins in processes such as immune response, tissue remodeling, and cell maintenance and growth.

Many bioactive peptides including several naturally present in the gastrointestinal tract are substrates for the meprins. For example, gastrin-releasing peptide fragment (GRP-14–27) and sulfated cholescystokinin (sCCK8_{NH2}) are substrates for both meprins A and B (Bertenshaw et al. 2001). The Km values are in the range of 50–350 μ M, and the kcat/Km range from 0.6 to 7 $\times 10^5$ M⁻¹ s⁻¹. These affinity and specificity constants are not particularly high; however, considering the high concentrations of meprins at the cell surface, the substrates are likely hydrolyzed if

Fig. 4.3 (continued) between 5 and 10% digested. The incubation was in 25 mM HEPES, 100 mM NaCl, 5 mM CaCl₂, pH 7.4, at 37°C. Amino-terminal sequencing of the resulting products allowed for the elucidation of amino acid preference for meprin B at each primed site. The data in each sequencing cycle were normalized to the total molar amount of amino acids in that cycle so that a value of 1 indicates the average value. (b) The same acetylated dodecamer peptide library mixture (1 mM) was incubated with meprin A (33 nM) until the library was between 5 and 10% digested. Amino-terminal sequencing of the resulting products allowed for the characterization of amino acid preference for meprin A at each primed subsite (Bertenshaw et al. 2001)

found in the vicinity of the brush border membrane of the kidney or intestine. Gastrin-17 has been shown to be one of the best substrates for homomeric meprin B with a Km of 1–7 μ M; kcat/Km values of 17–105 $\times 10^5$ M⁻¹ s⁻¹ (Villa et al. 2003).

There is an increasing volume of data on meprin hydrolysis of immune-modulating proteins such as the cytokines. The cytokine osteopontin was one of the first cytokine substrates identified for the meprin B isoform (Bertenshaw et al. 2001). There is also some evidence that meprin α truncates several chemotactic cytokines, such as MIP-1 α , MIP-1 β , RANTES, and MCP-1 at their N-terminal end. These truncations are predicted to lower the bioactivity of these cytokines (Norman et al. 2003b). Two members of the IL-1 β cytokine family, proIL-1 β and proIL-18, have also been identified as meprin substrates. Both meprin A and meprin B convert proIL-1 β to a biologically active cytokine while only meprin B has been shown thus far to convert proIL-18 to an active form (Herzog et al. 2005, 2009; Banerjee and Bond 2008).

In vitro and in vivo studies imply that proIL-18 is a physiologically important substrate for meprins A and B. Studies with meprin B in vitro indicate that the Km for proIL-18 is 1.3 μ M and the kcat/Km value is 52 \times 10⁵ M⁻¹ s⁻¹. This makes proIL-18 one of the best substrates identified for meprins, and the kinetic values are comparable to those for TACE and stromelysin for their physiological substrates. The initial product of degradation of proIL-18 by meprin B results from hydrolysis at an Asn-Asp bond (shown in Fig. 4.4). This product is biologically active in a cell-based system. Moreover, studies with meprin α and β knockout (KO) mice with an experimental model of inflammatory bowel disease (IBD) indicate that active IL-18 is decreased in the serum of meprin BKO mice and increased the serum of α KO mice compared to wild-type mice (Fig. 4.5). These in vivo studies support the contention that meprin β activates proIL-18 in vivo and that meprin α inactivates the interleukin. Caspase-1, an intracellular protease, is known to be able to generate biologically active IL-18. Our data indicate that meprin B is also able to generate biologically active IL-18 in vivo, and we suggest that it hydrolyzes the proform of IL-18 that is secreted from cells.

Meprins have also been implicated in regulating renal function in vivo by hydrolysis of B-type natriuretic peptide (BNP). BNP is expressed by a 108 amino acid precursor that is subsequently processed into a biologically active 32 amino acid product. The mature form of BNP is important for renal and cardiovascular

			caspase-1	putative PR-3	meprin B
1 <u>0</u>	20	30	<u> </u>	5 <u>0</u>	
MAAMSEDSCV	NFKEMMFIDN	TLYFIPEENG	DLESDNFGRL	HCTTAVIRNI	NDQVLFVDKR
70	caspase-3 80	90	100	110	120
QPVFEDMTDI	DQSASEPQTR	LIIYMYKDSE	VRGLAVTLSV	KDSKMSTLSC	KNKIISFEEM

Fig. 4.4 Hydrolysis of proIL-18 by meprin B. The meprin B cleavage site is indicated, and the nine amino acid sequence that was identified by MS/MS is *bolded*. Caspase-1 and putative PR3 cleavage sites are also indicated. The region of caspase-3 cleavage is *italicized* (Banerjee and Bond 2008)



Fig. 4.5 Active interleukin-18 (IL-18) in the serum of meprin-deficient and wild-type mice after intestinal exposure to DSS. Intestinal bowel disease (IBD) was induced in mice by administering 3.5% DSS in the drinking water for 4 days and water on the fifth day. Controls (*Con*) were given water all days. Serum was collected on days 3, 4, and 5, and the levels of active IL-18 were measured by enzyme-linked immunosorbent assay. (**a**) WT and βKO mice had increased levels of active IL-18 compared with their respective control groups (*n* = five/group; *, *p* < 0.02, DSS treated WT vs. control; #, *p* < 0.0002, DSS-treated βKO vs. control). Meprin βKO mice treated with DSS showed significantly lower levels of serum IL-18 compared with WT mice given DSS treatment (**, *p* < 0.05). (**b**) Meprin αKO mice showed significantly elevated levels of IL-18 compared with WT mice after the same DSS treatment (*n* = seven/group; **, *p* < 0.0015). Both DSS-treated groups showed significant elevation in their serum IL-18 levels compared with the respective control populations (*, *p* < 0.0002, DSS-treated WT vs. control; #, *p* < 5 × 10⁻¹¹, DSS-treated αKO vs. control) (Banerjee and Bond 2008)

homeostasis. Meprin α can cleave mature BNP at the N-terminal tail into a truncated form which is then more readily degraded by neprilysin (Pankow et al. 2007). Meprin-truncated BNP also has reduced renal bioactivity, although meprin-truncated

BNP has similar hemodynamic properties as full-length mature BNP (Boerrigter et al. 2009). These findings indicate that meprin plays a role in modulating renal function through hydrolysis of BNP. It has been suggested that inhibiting meprin α can augment renal function in vivo through preventing degradation of mature BNP.

The finding that cytokines are meprin substrates is important for elucidating the role of meprin in the pathogenesis of several inflammatory diseases: acute renal failure (ARF) (Bylander et al. 2008) and urinary tract infections (UTI) (Yura et al. 2009). We hypothesize that meprins influence the inflammatory response by affecting the inflammatory environment through activating or degrading immune-modulating proteins such as the cytokines and by allowing immune cells to migrate through the ECM and cell junctions to sites of inflammation. There is some evidence of the former with IL-18, as meprin α knockout (KO) mice had significantly higher active IL-18 in their serum compared to wild-type and meprin β KO mice in an experimental model of IBD (Banerjee and Bond 2008). Differential cytokine profiles of different meprin KO mice vs. their wild-type counterparts have also been seen in several other animal models including the ARF and UTI models. However, the cytokines that were significantly decreased in meprin KO mice using the ARF and UTI models (IL-6 and TNF- α , respectively) have not yet been shown to be direct meprin substrates (Bylander et al. 2008; Yura et al. 2009).

4.3.3 Meprin Inhibitors

The best meprin inhibitor of meprins identified thus far is actinonin, a bacterial peptidyl hydroxamate. Actinonin has a Ki for human meprin α of 5–20 nM (Kruse et al. 2004; Bylander et al. 2007). Meprins are not inhibited by tissue inhibitors of metalloproteinases (TIMPS), but they have various susceptibilities to the synthetic inhibitors developed against MMPs for clinical trials (Bylander et al. 2007). Mannose binding lectin (MBL) has been suggested as an endogenous inhibitor of meprins A and B (Hirano et al. 2005). However, in studies in our laboratory, we have found little, if any, inhibition of mouse meprin isoforms by MBL (Keiffer and Bond, unpublished data).

4.3.4 Activation of Meprins

Meprin subunits are synthesized as proproteins, as most proteinases, and the prosequence must be removed for the meprins to be enzymatically active (Johnson and Bond 1997). The physiological activating enzymes have not been identified as yet. Meprins are not self-activating proteases, and the best candidates for activators in vivo are trypsin-like enzymes. In the intestinal tract, it is likely that trypsin is the activator. It has been demonstrated that plasmin is capable of activating meprin α at

cancer sites, and kallikrein-4 is capable of activating human meprins with a preference for meprin β activation (Becker et al. 2003; Becker-Pauly et al. 2007).

4.4 Disruption of the Meprin Genes in Mice

In an effort to investigate in vivo functions of meprins, meprins α and β null mice (α KO and β KO) were generated by targeted disruption of the mouse genes (Norman et al. 2003a; Banerjee et al. 2009). Meprin double KO (dKO) mice have also been established (Sun et al. 2009). The KO mice generally do not exhibit any gross pathological phenotype, with normal histological structures of the kidney and intestine where meprins are abundantly expressed. The litter numbers are somewhat smaller than the wild type, but growth, development, and fertility are normal.

However, in response to different challenges, the meprin KO mice exhibited a variety of abnormalities. For example, meprin β KO mice are less vulnerable to kidney damage in the model of ARF induced by surgical ischemia and reperfusion (Fig. 4.6). Renal ischemia/reperfusion results in extensive redistribution of heteromeric meprin A in the mouse C57BL/6 mouse kidney, and the lack of the membrane-bound meprins in the β KO results in much less damage to the kidney

Fig. 4.6 Kidney damage in mice deficient in meprin β and wild-type mice after ischemia and reperfusion. Blood urea nitrogen (BUN; a) and plasma creatinine levels (**b**) following renal ischemia/ reperfusion (I/R) in wild-type (WT) and meprin (Mep) β knockout (KO) mice. Male WT and meprin BKO C57BL/ 6×129 F2 mice, 8–10 weeks old, were subjected to 26 min of warm renal ischemia followed by up to 72 h of reperfusion. (filled square) WT mice; (filled triangle) meprin βKO mice. Values are means \pm SE; n = 13-14 and 6–7 for each group in **a** and **b**, respectively. *p < 0.02; **p < 0.01; ***p < 0.001(Student's t test) (Bylander et al. 2008)





Fig. 4.7 Confocal microscopy of corticomedullary region after 3 h of reperfusion in WT mice. Kidney sections were immunostained with fluorescent antibody to detect meprin β (*red*) and Hoechst to detect nuclei (*blue*). The image depth is 0.35 µm. (a) in sham-operated WT mice, meprin is localized to brush-border membranes extending into the tubular lumen. (b) 3 h after reperfusion in ischemic mice, meprin localized in luminal regions of the tubules (*asterisks*) and in the cytoplasm surrounding nuclei of intact cells (*arrows*) (Bylander et al. 2008)

(Fig. 4.7). Meprin β KO mice were also less vulnerable to intestinal damage in a model of experimental IBD induced by dextran sulfate sodium (DSS) (Bond et al. 2006). Meprin α KO mice are more susceptible to severe colitis in the DSS model (Banerjee et al. 2009). In contrast, endotoxin (lipopolysaccharide, LPS) induces a less severe inflammatory response in the α KO than the wild-type mice in the model of urinary tract infection (Yura et al. 2009). Thus, depending on the type of challenge, the timing (acute vs. chronic), and the meprin isoform lacking, the response to a challenge in meprin null mice can be more or less severe than the wild-type mice.

From the mouse KO investigations, we conclude that meprins act in pericellular proteolysis in the movement of leukocytes to sites of damage or infection, and in the cytokine activation/inactivation systemically and/or at site of damage or infection. For these reasons, we have explored the role of meprins in the hematological system (leukocytes) and in affecting cytokine catabolism in the in vivo models with the meprin KO mice.

4.5 Meprins Role in the Distribution of Leukocytes

The recently available meprin knockout mice have provided much of the information known regarding the function of the enzymes in vivo, and a picture of the pathological consequences of lacking these enzymes is emerging. Quantitative cytological studies revealed that meprins are involved in the hematological system. Interestingly, meprin dKO mice exhibit a disturbance of monocytes and natural killer cells (Sun et al. 2009). Flow cytometrical analysis showed that deficiency in both meprins results in an underpresentation of the CD11b+/CD14+/Ly6c- resident monocytes (R-MC) in the peripheral blood (Fig. 4.8). In correlation with this abnormality is an increase in the prevalence of CD11b+/CD14+/Ly6c+ inflammatory monocytes (I-MC) in the bone marrow. R-MC are mature monocytes which are mostly developed from the I-MC (Auffray et al. 2009). Interestingly, NK cells in the dKO mice are also decreased in blood and increased in the bone marrow, but T and B lymphocytes are not significantly affected by meprin deficiency.

The exact mechanisms underlying the disturbed distribution of these select leukocytes remain undetermined. The accumulation of meprin deficient leukocytes in the bone marrow was not due to a block of the leukocyte development in bone marrow. Along the process of NK cell development, progenitors of the NK lineage sequentially acquire the expression of CD122, NK1.1, and CD49b, which provides



Fig. 4.8 Monocyte changes in blood and bone marrow of dKO mice compared to WT mice. In comparison to WT mice, double knockout (dKO) mice had low $CD14^+/Ly6c^-$ resident monocytes in blood, but high $CD14^+/Ly6c^{++}$ inflammatory monocytes in bone marrow. Peripheral blood and bone marrow cells were stained with CD14-fluorescein isothiocyanate, Ly6c-phycoerythrin, and CD45-peridinin chlorophyll, followed by flow cytometry. *Representative dot plots* (**a**–**c**) are to show cell populations compared in (**d**). *Darker events* are the gated cells in CD14/Ly6c plots. Granulocytes were excluded by gating in the FSC/SSC plots (not shown). Data were from two independent experiments with a total of seven mice in each group (Sun et al. 2009)

markers to distinguish NK cells of different differentiation stages. It is expected that if meprin deficiency impairs the development of bone marrow NK cells at a given step, the prevalence of NK cells at development stages prior to this step would be differentially affected, with the accumulation of NK cells expressing distinct patterns of the above markers. The studies showed that the accumulation of NK cells with the phenotype of CD122+/NK1.1+/CD49b+, but not the immature NK precursors, implying that NK cell development from the immature to mature forms is intact in the meprin-deficient mice (Sun, unpublished observation).

The inversely correlated distribution of monocytes and NK cells in blood and bone marrow of dKO mice may indicate that meprins are required by these cells for efficient egress from bone marrow to blood. Monocytes and NK cells in blood require continuous replenishment from the bone marrow for homeostatic maintenance. Egress of monocytes from bone marrow is a chemotaxis process mediated by multiple cytokines and involving multiple steps including interaction between cellular adhesion molecules and pericellular proteolysis of interstitial ECM (Ebnet and Vestweber 1999). The critical roles of chemokines in egress of bone marrow leukocytes are exemplified by CCR-2, the receptor for monocyte chemoattractant proteins (MCP). CCR-2 knockout mice exhibit the phenotype of low monocytes in blood, with simultaneous accumulation of the same cells in bone marrow (Serbina and Pamer 2006; Tsou et al. 2007). The bone marrow egress of NK cells involves chemotaxis driven by chemokines mediated by CCR-5 and others in response to inflammation (Inngjerdingen et al. 2001), as well as by lysophospholipid sphingosine 1-phosphate (S1P) at the steady state (Jenne et al. 2009; Walzer et al. 2007). However, the defective egress of monocytes and NK cells from bone marrow in meprin dKO mice appeared not to result from compromised chemokine receptors. Expression of CCR-2 and CCR-5 was comparable in the wild-type and meprin KO monocytes (Sun, unpublished observation). One possible alternative for the mechanism involving chemotaxis is that meprin knockout mice might have a lower level of systemic homeostatic chemokines. Consistent with this notion, blood levels of several cytokines, including chemokine MCP-1, were lower in meprin dKO mice than in wild-type mice (Sun, unpublished observation). The definitive evidence for the above hypothesis would be accumulation of monocytes and NK cells in the bone marrow of several established lines of MCP-1 knockout mice (Lu et al. 1998: Takahashi et al. 2009).

The accumulation of meprin-deficient leukocytes in the bone marrow may result from compromised capacity of the leukocyte to overcome the physical hindrance from the interstitial ECM. ECM proteins form a continuous meshwork consisting of distinct structural proteins including laminin, type IV collagen, entactin, and proteoglycans in endothelial basement membrane, and type I and III febrile collagens and fibronectin (FN) in the interstitial matrix (Galis et al. 1994; Laurie et al. 1982). This framework provides the structure critical for cell survival, development, and locomotion (Marastoni et al. 2008), and also poses as a barrier for leukocyte migration (Schenkel et al. 2004).

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Multiple proteases have been implicated in leukocyte migration. MMP-9 efficiently degrades ECM proteins laminin and collagen IV in vitro and is involved in migration of Langerhans cells and dendritic cells (DC) in the absence of ECM (Baratelli et al. 2004; Ratzinger et al. 2002; Tojo et al. 1999; Xie et al. 1998). The degradation of fibronectin by MMP-14 (TM1-MMP) has been correlated with monocyte migration (Matias-Roman et al. 2005), and MMP-12 null mice exhibit delayed recruitment of macrophages into subcutaneous sponge (Shipley et al. 1996). In addition to ECM degradation, MMPs are well established for transmigration of monocytes through endothelial barriers by interacting with intracellular adhesion molecule-1 (ICAM-1) (Matias-Roman et al. 2005; Sithu et al. 2007) and by disrupting the tight junctions formed by occludin molecules (Reijerkerk et al. 2006). In addition, plasminogen/plasmin-mediated pericellular proteolysis play critical roles for migration of blood monocytes to inflammation sites (Gong 2008; Wygrecka et al. 2009), which may work synergically with MMP-9 (Gong 2008). There is evidence that meprin is involved in the trans-ECM migration of leukocytes. Ample in vitro biochemical studies showed that meprins are capable of degrading ECM proteins (Bylander et al. 2007; Kaushal et al. 1994; Kruse et al. 2004). Furthermore, in vitro studies revealed that macrophages from meprin BKO mice are defective in the trans-ECM migration (Crisman et al. 2004) (Fig. 4.9), and human breast cancer cells treated with the meprin inhibitor actinonin are less invasive (Matters et al. 2005).

The mechanisms responsible for the abnormal hematology in meprin-deficient mice remain to be determined; however, meprin deficiency appears pathologically relevant, at least in animal models. Indeed, mice deficient in both α and β meprins exhibited overactive febrile reactions in the model of thioglycollate-induced peritonitis, implying meprins' role in inflammation process (Sun et al. 2009). In addition, meprin α KO mice are predisposed to colitis with systemic alterations in cytokine responses to intestinal stimulation by DSS (Banerjee et al. 2009), a model for IBD. This is consistent with the critical roles of monocytes in the innate immunity as well as in adaptive immune responses, and it is reasonable to predict that meprin deficiency is associated with compromised immunity in human pathological conditions yet to be recognized.

4.6 Meprins Have Been Implicated in a Number of Diseases

Meprins have been implicated in several inflammatory diseases and in cancer. The expression of meprins in several types of cancer cells (intestinal, breast, bone) has implicated these proteases in the growth and metastases of tumors (Bond et al. 2005; Matters et al. 2005). The targets for meprins in cancer cells, as for immune system cells, are likely cytokines, adhesion proteins, and ECM proteins, and this enables mobility of these cells to secondary sites.

The meprin KO mouse studies support the contention that meprins have a functional role in inflammatory diseases. In the case of experimental IBD, a lack of meprin α increases the severity of this disease. Interestingly, there is also strong



Fig. 4.9 In vitro matrigel assays to compare the ability of meprin β null and wild-type leukocytes to infiltrate matrigel. Meprin β null (*filled square*) or age- and strain-matched wild-type (*open square*) mesenteric lymph node leukocytes (1.7×10^5 /well) were incubated in the upper well of triplicate matrigel chambers, with 10 nM MCP-1 (**a**) or 10 nM MIP-1 α (**b**) in the lower reservoir of the matrigel chamber. After 18 h, the filters were stained to reveal those cells that infiltrated through the matrigel and onto the filter. The infiltrating cells were counted in 5–20 fields (*, p < 0.05, n = 15). (**c**) Matrigel assays were performed to detect meprin β and the macrophage-specific protein F4/80 on wild-type leukocytes infiltrating matrigel. The filters were incubated with PE-conjugated anti-F4/80 mAb (*red*, **c**) or isotype control (*red*, **d**) and rabbit antimeprin β antisera (**c**) or rabbit nonimmune sera (**d**), followed by FITC-conjugated donkey antirabbit IgG (*green*). Horscht stain was used as a nuclear counterstain (*blue*) (Crisman et al. 2004)

evidence that polymorphisms in the human meprin α gene are associated with IBD, especially ulcerative colitis (Banerjee et al. 2009). These findings coupled with the observation that meprin α mRNA is significantly lower in inflamed tissue of IBD patients, strongly implicates a lack of meprin α in the pathogenesis of the disease.

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Polymorphisms in the human meprin β gene are associated with diabetic nephropathy in the Pima Indians (Red Eagle et al. 2005). Pima Indians have an extraordinarily high incidence of type II diabetes, and one of the most serious and common side effects of the disease is nephropathy. The single polymorphism in the meprin β gene with the strongest association with the disease results in an amino acid change in the COOH-terminal tail of the meprin β protein. The consequences of this change could result in abnormal interactions with cytosolic or membrane proteins and affect processes that lead to fibrosis.

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Chapter 5 Triple Helicase Activity and the Structural Basis of Collagenolysis

Hideaki Nagase and Robert Visse

Abstract The degradation of interstitial collagens I, II and III is an integral part of many biological events such as embryonic development, organ morphogenesis, tissue remodelling and repair, and in diseases such as arthritis, cancer, atherosclerosis, aneurysm and fibrosis, but these collagens are resistant to most proteolytic enzymes due to their triple helical structures. In vertebrates, the enzymes that degrade interstitial collagens are collagenases that are members of the matrix metalloproteinase (MMP) family, and cathepsin K, a lysosomal cysteine proteinase. Non-vertebrate collagenases include collagenolytic serine proteinases in crustacea and *Clostridium histolyticum* collagenases, also called "bacterial collagenases." This chapter describes the unique properties of these collagenolytic proteinases and the current proposal as to how they recognize triple helical structures and cleave them. Potential enzymatic pathways that may be involved in the degradation of collagen fibrils are also discussed.

5.1 Introduction

Collagens are the major structural proteins in connective tissues of the vertebrates such as tendon, skin, bone, cartilage and blood vessels. Collagens I, II and III are the most abundant, and they form the scaffold of the tissue and guide cells to migrate, proliferate and differentiate. The degradation of these macromolecules is therefore essential in biological processes such as embryogenesis, organ morphogenesis, tissue remodelling, angiogenesis and wound healing that require replacement of old extracellular matrix molecules with new molecules (Woessner 1998; Sternlicht and Werb 2001). These collagens consist of three α chains of approximately 1,000 residues with repeating Gly-X-Y tripeptides, where X and Y are often Pro and Hyp,

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respectively. Due to a high content of imino acids and repeated Gly in every third residue, the α chain adopts a left-handed poly-Pro II-like helix and three left-handed chains intertwine to form a right-handed superhelix (Ramachandran and Kartha 1955; Rich and Crick 1961; Kramer et al. 2001). Such triple helical structures make interstitial collagens resistant to most proteolytic enzymes. Only a limited number of enzymes can degrade native interstitial collagens, and "collagenolysis" often refers to degradation of types I, II and III collagens, as other types of collagens are degraded by a broader spectrum of proteinases.

The first collagenolytic activity was discovered in the culture medium of Clostridium histolyticum, a pathogenic anaerobic bacterium that causes gas gangrene (Jennison 1947; Mandl et al. 1953). The enzyme is referred to as bacterial collagenase or *Clostridium* collagenase. The discovery of vertebrate collagenase had to wait until 1962 when Gross and Lapiere reported collagenase activity in tadpole tail skins, gut and gills undergoing metamorphosis (Gross and Lapiere 1962). While bacterial collagenase cleaves triple helical collagen I at multiple sites, the tadpole enzyme cleaves long filamentous collagen I at a single site 3/4 away from the N-terminus and generates characteristic ³/₄ and ¹/₄ fragments (Nagai et al. 1966). This discovery encouraged many researchers to search for collagenase activity in mammalian tissues as it was considered to be important not only in normal tissue remodelling and repair, but also in diseases such as skin ulceration, arthritis, cancer, atherosclerosis and periodontitis. Human collagenase was first purified from the medium of rheumatoid synovium (Woolley et al. 1975); this is now called collagenase 1 or MMP-1 as the first member of the matrix metalloproteinase (MMP) family, which encompasses a large number of members including 23 MMPs in man. However, MMPs that have collagenolytic triple helicase activity are limited. Other mammalian collagenases are MMP-8 (collagenases 2 or neutrophil collagenase) and MMP-13 (collagenase 3). Xenopus has MMP-18 (collagenase 4), but its counterpart has not been found in mammals. Rheumatoid synoviocytes in culture also produce MMP-2 and MMP-3 (Okada et al. 1986). MMP-2 (gelatinase A) was originally characterized as a gelatinase (Murphy et al. 1985; Okada et al. 1986), but in 1995 Aimes and Quigley reported its collagenolytic activity (Aimes and Quigley 1995). Another major collagenolytic MMP is membrane-type-1 MMP (MT1-MMP or MMP-14). It is a major pericellular collagenase, and mice lacking MMP-14 exhibit skeletal abnormalities due to the lack of tissue collagenolytic activity (Holmbeck et al. 1999). MMP-14 also activates the zymogen of MMP-2 (pro-MMP-2) on the cell surface (Sato et al. 1994). Thus, MMP-2 and MMP-14 exhibit important pericellular collagenolytic activity. These MMPs are produced by many cell types such as fibroblasts, epithelial cells, macrophages, leukocytes, endothelial cells, smooth muscle cells, chondrocytes and osteoblasts, and they have been characterized to have the ability to cleave collagen I similar to tadpole collagenases, at neutral pH.

For bone to be remodelled, the non-mineralized collagen I matrix must be cleaved, and the lysosomal cysteine proteinase, cathepsin K, is considered to be the major collagenolytic enzyme (Gelb et al. 1996; Hou et al. 1999). It is mainly produced in osteoclasts (Brömme et al. 1996), but also in other cell types such as

fibroblasts (Hummel et al. 1998). It is only active in an acidic environment and therefore the sites of its action are specialized in resorbing bones and intracellular digestion of phagocytosed collagens (Everts et al. 2003).

This chapter reviews the structure and function of collagenolytic MMPs and what is currently known about the structural basis of those enzymes to confer triple helicase activity. We will also discuss how insoluble collagens may be digested by vertebrate collagenases. We also describe how cathepsin K, crab collagenase and bacterial collagenases degrade interstitial collagens.

5.2 Domain Structures of Collagenolytic MMPs and Their Activation Steps

5.2.1 Domain Compositions

Six MMPs have been characterized as enzymes with triple helicase activity. Based on domain structure arrangement, they are grouped into the so-called "collagenases" (MMP-1, -8, -13 and -18), gelatinase A (MMP-2) and membrane-anchored MMP (MT1-MMP or MMP-14). All have a signal peptide and four collagenases and gelatinase A are secreted from the cell as inactive proenzymes.

Secreted collagenases consist of a propeptide domain, a catalytic metalloproteinase (Cat) domain, a linker (also called "hinge") region and a hemopexin-like (Hpx) domain. Pro-MMP-2 has three fibronectin type II motifs that are inserted in the catalytic domain, otherwise its domain arrangement is similar to other MMPs. The pro-domain contains a characteristic three-helix bundle with a left-handed twist which is also observed for those of MMP-1,-2, -3 and -9 (Jozic et al. 2005; Becker et al. 1995; Elkins et al. 2002; Morgunova et al. 1999). This is a compact, stable structure supported by the hydrophobic core formed within the bundle. It has the socalled "cysteine switch" sequence PRCGXPD whose cysteine residue coordinates with the catalytic Zn^{2+} ion bound to three histidines, the HEXGHXXGXXH motif, conserved in all MMPs (Van Wart and Birkedal-Hansen 1990). The Cat domain consists of three α helices and five β -strands with one catalytic zinc ion, and one structural zinc and three calcium ions which stabilize the 3D structure. The overall peptide folds of the Cat domains of MMPs are essentially superimposable. The Hpx domain is an ellipsoidal shape consisting of a four-bladed β -propeller structure with a single disulphide bond between the first and the fourth blades. Each blade is made up of four antiparallel β -strands and the four blades have similar scaffolds arranged almost symmetrically around the central core axis. One calcium ion and a chloride are found in the centre of the propeller. The linker regions of MMPs, which connect the Cat and the Hpx domains, are variable in length.

MMP-14 is plasma membrane-bound through its transmembrane domain and activated intracellularly by a furin-like pro-protein convertase (Sato et al. 1996). Thus the active MMP-14 is expressed on the cell surface as a type I transmembrane
protein with extracellular N-terminal metalloproteinase and Hpx domains and with a short intracellular C-terminal domain.

5.2.2 Activation of pro-MMP-1, -8 and -13

The activation of pro-MMPs is an important regulatory step for collagenolysis and depending on the form generated, collagenolytic activity varies five- to tenfold. As demonstrated for many secreted pro-MMPs, procollagenases are activated by a stepwise activation mechanism (Woessner and Nagase 2000) (see Fig. 5.1). This may be initiated by proteolytic attack by a number of tissue or plasma proteinases that cleave near the middle of the propeptide, the so-called "bait" region. This partially activates procollagenase. Final removal of the propeptide is either by autocatalysis or by other MMPs. In the case of MMP-1 and MMP-8, the second processing step dictates the level of enzyme activity depending on the sites of cleavage. The bait region-cleaved MMP-1 autocleaves the Phe⁸¹-Val⁸² or Val⁸²- Leu^{83} bond (residues are numbered taking the first residue of the proenzyme as 1), but the forms generated express only 10-20% of full collagenolytic activity (Suzuki et al. 1990). In the presence of pro-MMP-3, which is activated by similar proteinases, the Gln⁸⁰-Phe⁸¹ bond is cleaved, and this form with N-terminal Phe⁸¹ exhibits full collagenolytic activity (Suzuki et al. 1990). The action of MMP-3 on MMP-1 activation can be substituted by MMP-2, -7 or -10. Therefore, the availability of these MMPs in the tissue influences the local collagenolysis of the tissue.

Partial and full activation have also been described for pro-MMP-8 (Knäuper et al. 1993b). After initial activation of pro-MMP-8 by proteinases, MMP-8 undergoes autolysis and generates forms with Met⁸⁰ or Leu⁸¹ at the N-terminus that are



Fig. 5.1 Propeptide sequence and the cleavage sites identified during activation of pro-MMPs. See the text for details

only partially active. For full activation, the Gly⁷⁸-Phe⁷⁹ bond needs to be cleaved by MMP-3 or MMP-10 (Knäuper et al. 1996b). Crystal structures of the fully activated catalytic domain of MMP-8 showed that the ammonium group of Phe⁷⁹ forms a salt bridge with the carboxylate of Asp^{232} (Reinemer et al. 1994), but this salt bridge fails to form in the partially activated forms. How this salt bridge affects the collagenolytic activity is not understood. For pro-MMP-13, MMP-3, MMP-14 and plasmin can cleave the bait region, but in this case full activation of MMP-13 is attained by autolysis by generating the enzyme with the N-terminal Tyr⁸⁵ (Knäuper et al. 1996a, c).

An organomercurial compound (e.g., 4-aminophenyl mercuricacetate) is commonly used to activate a number of pro-MMPs including above collagenases. Glutathione-dependent activation of pro-MMP-1, -8 and -9 is induced by peroxynitrite (ONOO⁻), an oxidizing agent formed during inflammation (Okamoto et al. 2001). These activation systems perturb the proenzyme structure possibly by reacting with the cysteine residues in the cysteine switch. However, in the case of MMP-1 and MMP-8, they are only partially activated due to autolysis.

5.2.3 Activation of pro-MMP-2 by MMP-14 on the Cell Surface

MMP-2 cleaves a number of ECM macromolecules, but its activity on heatdenatured collagens, gelatin, is most prominent. Because of this activity it is called gelatinase A, MMP-9 being gelatinase B. In 1995, Aimes and Ouigley reported that MMP-2 cleaves type I collagen in a similar manner as MMP-1 (Aimes and Quigley 1995). While the biological activator of pro-MMP-2 had long been an enigma, Sato et al. (1994) cloned MMP-14 and shown it to be activator of pro-MMP-2. This activation requires the formation of a tetramolecular complex of pro-MMP-2-TIMP-2-(MMP-14)₂. TIMP-2 is one of four paralogous tissue inhibitors of metalloproteinases (TIMPs), endogenous inhibitors of MMPs and pro-MMP-2 in the medium can be bound to TIMP-2 through interaction between the Hpx domain of pro-MMP-2 and the C-terminal domain of TIMP-2 (Fridman et al. 1992; Willenbrock et al. 1993). Since this complex is capable of binding and inhibiting active MMPs, it binds to the catalytic domain of MMP-14 on the plasma membrane, which presumably presents the propeptide of pro-MMP-2 to an adjacent MMP-14 for hydrolysis of the Asn³⁷-Leu³⁸ bond in the propeptide. Subsequently, it is fully activated by autocleavage of the Asn⁸⁰-Tyr⁸¹ bond. Fully activated MMP-2, however, may be gradually inhibited by TIMP-2 present in the activated complex (Itoh et al. 1998). Thus, activated MMP-2 may participate in pericellular collagenolysis for a limited period of time. MMP-14 itself has collagenolytic activity (Ohuchi et al. 1997) and can also activate pro-MMP-13 (Knäuper et al. 1996c). Therefore, MMP-14 appears to be one of the key enzymes controlling pericellular collagenolysis. This is evident from MMP-14-null mice, which exhibit impaired collagenolytic activity (Holmbeck et al. 1999).

5.2.4 Structural Changes of pro-MMP-1 upon Activation: From "Closed" to "Open" Conformation

In pro-MMP-1 the bait region is located between the first and second helices in the pro-domain, but it is missing in the 3D crystal structure, suggesting that this region is flexible. When the bait region is cleaved, removal of the N-terminal helix has an impact on the stability of the two remaining helices and the peptide bond around the junction of the pro-domain and the Cat domain becomes exposed. This allows MMP-3 to specifically cleave the Gln⁸⁰-Phe⁸¹ bond, which fully activates MMP-1. Another important feature of pro-MMP-1 is that the pro-domain interacts not only with the catalytic domain, but also with the Hpx domain (Jozic et al. 2005). This keeps the Hpx domain closer to the Cat domain, resulting in a "closed" configuration. After removal of the pro-domain during activation the structure of MMP-1 was found to relax to an "open" configuration (Fig. 5.2a, b). Such structural changes explain the biochemical observation that proenzymes of MMP-1 and MMP-8 do not bind to collagen I, but activated forms of these enzymes do (Knäuper et al. 1993a; Murphy et al. 1992; Welgus et al. 1985). Docking of the molecular structure of native collagen with this open configuration of MMP-1 suggests that the groove formed by the two segments of the catalytic domain and the two blades of Hpx domain may be a site where collagen initially binds in MMP-1 (Fig. 5.2c, d). Hydrogen/deuterium exchange-mass spectrometry studies of MMP-1 with a triple helical peptide containing the collagenase-cleavage site and mutagenesis studies have suggested that Ile²⁷⁰ and Arg²⁷¹ contribute to the recognition of triple helical peptide (Lauer-Fields et al. 2009), supporting the above hypothesis. On the other



Fig. 5.2 Putative interaction site of triple helical collagen in MMP-1. (**a**) The ribbon structure of pro-MMP-1 (1SU3.pdb). The pro-domain is shown in *red*, the catalytic domain in salmon, the linker region in *blue* and the Hpx domain in *green*. The relative arrangement of the Cat and Hpx is compact, due to a direct interaction between the pro and Hpx domains. (**b**) Superimposition of human MMP-1 (linker-Hpx domain in *blue*; 2CLT.pdb) and porcine MMP-1 (linker-Hpx domain in *red*; 1FBL.pdb) on pro-MMP-1 (linker-Hpx domains observed in available crystal structures. In the active forms a cleft opens up between the Cat and Hpx domains. (**c**, **d**) Active human MMP-1 shown as a surface structure. Type I collagen was positioned in the cleft between the catalytic and Hpx domains. Regions of potential contact with collagen are shown in *blue*. Y191 is highlighted in *cyan*

hand, pro-MMP-13 binds to collagen I with a slightly lower affinity than MMP-13 (Knäuper et al. 1997a), suggesting that the Hpx of pro-MMP-13 may bind to collagen.

5.3 Involvement of the Catalytic, Linker and Hpx Domains in Collagenolysis

Clark and Cawston reported that MMP-1 autocleaved the linker region when concentrated and the Cat domain without the Hpx was no longer able to cleave collagen I, although its activity for non-collagenolytic proteins such as casein or synthetic peptides was retained (Clark and Cawston 1989). Requirement of the Hpx domain in collagenolytic activity was shown for MMP-2 (Patterson et al. 2001), MMP-8 (Knäuper et al. 1993a), MMP-13 (Knäuper et al. 1996a) and MMP-14 (Itoh et al. 2006).

To gain further insights into the structural requirements for collagenolysis, a series of chimeras of MMP-1 or MMP-8 with MMP-3 was generated as MMP-3 has a similar domain structural arrangement as collagenases and human MMP-1 and human MMP-3 shares 54% identity in amino acid sequence, but does not cleave type I and II collagens. Hybrid enzymes consisting of the Cat of MMP-1 and the linker and Hpx of MMP-3 and the Cat of MMP-3 and the linker and Hpx of MMP-1 did not exhibit collagenolytic activity (Murphy et al. 1992). These results indicate that the Hpx domain is not the sole component that is responsible for the expression of collagenolytic activity. A similar type of chimeric study with MMP-8 and MMP-3 by Hirose et al. (1993) suggested that the Cat and the length of proline-rich linker of MMP-8 are important for the expression of collagenolytic activity. Substitution of the linker region of MMP-8 with that of MMP-3 inactivated the collagenolytic activity. Knäuper et al. (1997b) subsequently reported that the replacement of three prolines of the MMP-8 linker with alanine reduced the collagenase activity down to 1.5%. A single mutation of Gly²⁵¹ of the linker of MMP-1 to Asp reduced its collagenolytic activity to 13% (Tsukada and Pourmotabbed 2002). These results suggest that the linker region is flexible, and it must correctly coordinate the movement of the Cat domain and the Hpx domain.

In a study with MMP-3/MMP-1 chimeras, Chung et al. (2000) mapped the RWTNNFREY (183–191) loop located between the fifth β -strand and the second α -helix in the Cat domain of MMP-1 as another important component in expressing triple helicase activity. While the polypeptide folds of all catalytic domains of MMPs are essentially identical (Bode and Maskos 2003), the regions corresponding to RWTNNFREY (183–191) are highly variable both in sequence and structure among MMPs. The FNII repeats in MMP-2 and MMP-9, which influence their substrate specificity for gelatin, type IV collagen and elastin (Murphy et al. 1994; Collier et al. 1992; Shipley et al. 1996), are also attached to this loop. This region is located close to the S₃' subsite of the substrate binding groove, and Tyr¹⁹¹ in the

RWTNNFREY of MMP-1 is particularly important (Chung et al. 2000). A single mutation of Tyr¹⁹¹ to Thr (the corresponding residue in MMP-3) reduced collagenolytic activity to less than 25% and activities against synthetic substrates and gelatin by more than 90% (Chung et al. 2000). The peptide bond between Glu¹⁹⁰ and Tyr¹⁹¹ is in an unusual *cis* configuration (Spurlino et al. 1994) and Tyr¹⁹¹ is conserved in all collagenolytic MMPs except MMP-14 (Woessner and Nagase 2000). Mutation of the corresponding Tyr¹⁸⁹ of MMP-8 to Phe similarly reduced collagenolytic activity, but in this case the activity towards a synthetic substrate was not altered (Pelman et al. 2005). In addition, Gly²¹⁴ located three residues before the conserved Met in the catalytic domain is important for collagenolysis. Mutation of this residue to Glu (the corresponding residue in MMP-9) reduces collagenolytic activity to 0.13% without changing the ability to cleave gelatin (O'Farrell et al. 2006). It is considered that Gly²¹⁴ provides the flexibility necessary to accommodate collagen.

5.4 Collagen Type Preference of MMPs

Among six collagenolytic MMPs, MMP-1, -8 and -13 were characterized for their collagenolytic activities on type I, II and III collagens. MMP-1 cleaves in order of preference (bovine) type III >> type I (guinea pig) > type II (bovine), MMP-8 in order of preference type III > type I > type II and MMP-13 cleaves type II = type I > type III (Tominga, Visse, Nagase, unpublished results). MMP-13 has been considered to be an important collagenase in cartilage degradation, as the major fibrillar collagen is type II collagen and Mitchell et al. (1996) reported that MMP-13 is a fivefold better enzyme than MMP-1 in digesting type II collagen. However, the MMP-1 used in Mitchell's study was activated from the proenzyme by using an organomercurial compound, 4-aminophenylmercuric acetate, an agent which generates only 10–20% active MMP-1 (Suzuki et al. 1990). When fully activated, MMP-1 has similar collagenolytic activity on type II collagen as MMP-13. None-theless, MMP-1 cleaves type III and type I collagens much more readily than type II collagen. Collagen III is therefore much more readily cleaved by MMP-1, but it is a poor substrate for MMP-13.

Knäuper et al. (1996a) reported that MMP-13 cleaved type II collagen about five times faster than type I collagen. We, however, found that MMP-13 cleaves collagen I (guinea pig) and collagen II (bovine) at a similar rate. This discrepancy in the two studies may be due to the difference in species of those collagens. Welgus et al. (1981a) found that the k_{cat}/K_m value of human MMP-1 against human collagen I was 66.8 μ M⁻¹ h⁻¹, whereas that against guinea pig collagen I was 21.7 μ M⁻¹ h⁻¹. A more striking difference was observed between human and guinea pig collagen III with the former being cleaved 16 times more readily than the latter. Thus, not only collagen types but also species differences for each type of collagen need to be taken into consideration to study the preferences for the different types of collagen.

5.5 The Triple Helicase Activity of Collagenases

When the 3D structures of the catalytic domains of MMP-1 and MMP-8 were reported in 1994 and the full-length pig MMP-1 in 1995 (Li et al. 1995), it became apparent that the active site of the enzyme cannot accommodate the triple helical collagens because the entrance of the active site is only 5 Å wide, and the diameter of the collagen triple helix is 15 Å (Bode 1995). Simple docking of a triple helical peptide based on type III collagen (Kramer et al. 2001) covering the C-terminal of the collagenase-cleavage site does not allow the peptide bond to access the catalytic zinc ion. The closest peptide bond is \sim 7 Å away from the catalytic zinc atom. In addition, due to the spatial orientation of three left-handed α chains, each single α chain in the native collagen does not engage into the subsite pockets of the enzyme. Effective peptide hydrolysis by MMPs utilizes extended substrate binding subsites (Nagase 2001) and favours a β-strand-like structure which forms numerous hydrogen bonds with the enzyme (Grams et al. 1995). It has therefore been postulated that either the active site of collagenase undergoes large conformational changes to accommodate triple helical collagen or the collagen needs to be unwound for a single α chain to be presented to the active site to initiate collagenolysis. A number of hypotheses have been proposed for the latter (Bode 1995; de Souza et al. 1996; Gomis-Rüth et al. 1996; Ottl et al. 2000; Overall 2002). This includes the "proline zipper" model (de Souza et al. 1996) in which the proline-rich linker of collagenases interacts with collagen and unwinds the triple helical collagen, and the "collagen-trapping" model in which the Hpx folds over the Cat domain sandwiching collagen (Gomis-Rüth et al. 1996). Other possibilities are that collagen may unwind spontaneously around the cleavage site or unwinding may be induced by collagenases. These two models are currently being considered.

5.5.1 The Collagenase-Induced Collagen Unwinding Model

The evidence that collagenase unwinds triple helical collagen before hydrolyzing the peptide bond was presented by Chung et al. (2004) using the MMP-1 and guinea pig collagen I system at 25°C as a model. They postulated that if MMP-1 actively unwinds triple helical collagen, the MMP-1 mutant that lacks the ability to cleave peptide bonds should retain collagen-unwinding activity. If so, collagen I interacting with such a mutant becomes susceptible to non-collagenolytic proteases. The mutation of the catalytically essential Glu200 of MMP-1 to Ala [MMP-1(E200A)] inactivated its peptidolytic activity. The catalytic domain of MMP-1 (MMP-1Cat), MMP-3Cat and a serine proteinase, neutrophil elastase does not cleave collagen I unless triple helical chains are unfolded and could be used as a cutter enzyme of unwound collagen. Their studies showed that, in the presence of MMP-1(E200A), non-collagenolytic proteinases cleaved collagen I into typical ³/₄ and ¹/₄ fragments in a dose- and time-dependent manner. The study has also suggested that MMP-1

preferentially interacts with the $\alpha 2(I)$ chain, as $\alpha 1(I)$ chain was cleaved more readily under the above experimental conditions. The unwinding activity of MMP-1(E200A) detected with neutrophil elastase as a cutter was blocked by an active site-directed hydroxamate inhibitor of MMPs, suggesting that the active site of the enzyme is necessary for the unwinding activity, presumably as one of adaptors of the unwound $\alpha 2(I)$ chain.

5.5.2 The Stultz Model

The collagenase-induced collagen unwinding model proposed by (Chung et al. 2004) was challenged by Stultz and colleagues (Nerenberg et al. 2008). Based on their theoretical molecular simulation, they propose that collagen triple helix around the collagenase-cleavage site is less stable than the rest of the molecule and tends to be partially unfolded. Such unfolded "vulnerable" state is considered to be sufficient for collagenases to recognize and hydrolyzes the peptide bonds. Their theoretical calculation of free energy of collagen-like triple helical peptide (THP) containing the collagenase-cleavage site sequence of collagen III suggests that it adopts both native and partially unfolded states (Stultz 2002), and computational simulations of the collagenase-cleavage site of collagen I suggest that the unfolding of the $\alpha 2(I)$ chain is energetically favoured relative to the unfolding of $\alpha 1(I)$ chains (Nerenberg and Stultz 2008). Higher mobility of polypeptide backbones around this site has been predicted as it has less imino acids compared to the rest of the collagen chain (Brown et al. 1977; Fields 1991). This notion is also supported by the study of Fiori et al. (2002) with a synthetic heterotrimeric triple helical peptide of the $\alpha 1(I)$ and $\alpha 2(I)$ sequences around collagenase-cleavage site of collagen I. The structure of fibrillar type I collagen of rat tail tendon determined at room temperature reported by Orgel et al. (2006) also indicates that the triple helical structure around the collagenase-susceptible region is relaxed compared to the rest of the triple helical collagen. However, the alignment of the $\alpha 2(I)$ chain of Orgel's model to the active site of MMP-1 shows a number of serious molecular clashes, and for collagenases to cleave the $\alpha 2(I)$ chain and $\alpha 1(I)$ chain considerable rearrangements of the polypeptide chain fold need to take place (see below).

5.5.3 Further Insights into Triple Helicase Activity Provided by the Catalytic Domains of Collagenase Mutants

If the vulnerable state were sufficient for collagenase to recognize and cleave triple helical collagen, it should then be susceptible to the catalytic domains of MMP-1, MMP-3 and possibly other non-collagenolytic proteinases, even though their activities on collagen may be considerably low. Furthermore, the ratio of collagenolytic

activity of the catalytic domain over that of the full-length enzyme should be similar among different collagenases and their mutants. To address these questions, we first examined whether the catalytic domain of MMP-1 (MMP-1Cat) alone can cleave collagen into $\frac{3}{4}$ and $\frac{1}{4}$ fragments at 25°C. We found MMP-1Cat could cleave collagen time and dose dependently, but the activity was only 1/60,000 of that of full-length MMP-1. A similar minute collagenolytic activity was recently reported for the catalytic domain of MMP-8 which was roughly 20-fold higher than that of MMP-1Cat (Salsas-Escat et al. 2010). When the catalytic domains of various MMP-1 mutants including MMP-3/MMP-1 chimeras were compared with MMP-1Cat, there were only small changes in collagenolytic activity, but much larger reductions with full-length mutants although they were still 12,000–38,000-time more active than the respective Cat domains (Fig. 5.3). These results suggest that the vulnerable state of collagen is present, but it alone is not sufficient for effective catalysis of triple helical collagen. Non-catalytic domains need to participate in collagenolysis by making the collagen more susceptible by presumably altering the triple helical structure. This latter aspect is supported by a study with the inactive E200A mutant of MMP-1. In the presence of 3 µM MMP-1(E200A), the collagenolytic activity of MMP-1Cat ("cutter" activity) increased about tenfold (Chung et al. 2004). Neither MMP-3 or MMP-3Cat had collagenolytic activity, but MMP-3Cat cleaved $\alpha 1(I)$ and $\alpha 2(I)$ chains into $\frac{3}{4}$ and $\frac{1}{4}$ in the presence of MMP-1(E200A). Variable increases in cutter activity of MMP-1 mutants and MMP-3/MMP-1 chimeras (e.g., a 4.5-fold increase with MMP-1(Y191T) and a 58-fold increase with LC3 mutant) were also observed in the presence of MMP-1(E200A) (Fig. 5.3). These results may be interpreted as indicating that considerable structural changes are induced in $\alpha 1(I)$ and $\alpha 2(I)$ chains of collagen I by interacting with MMP-1(E200A). If MMP-1(E200A) were to simply stabilize the vulnerable state of collagen without causing structural changes in triple helical collagen, it would give a similar fold increase in collagenolytic activity among these mutants as it only affects the concentration of the substrate in a susceptible state, but not substrate specificity.

5.5.4 Docking of Collagenase-Cleavage Site of Collagen I into the Collagenase Active Site

To assess the conformation of the α chain that collagenases can accept in the active site, the $\alpha_2(I)$ chain of triple helical collagen I reported by Orgel et al. (2006) was docked into the active site cleft of MMP-1 (see Fig. 5.4). In this model, the carbonyl oxygen of Gly⁷⁵⁷ of the Gly-Leu bond was directed to the catalytic zinc and the Leu⁷⁵⁶ to the S1' pocket, which gives a good fit of the side chain of the P₁' Leu to the S1' pocket. However, P₂ (Gln) and P₃ (Pro) are in a different orientation from those of the peptide substrate modelled onto crystal structures of MMP-8Cat (Grams et al. 1995). In addition, although $\alpha_2(I)$ has a slightly open structure around the cleavage



Fig. 5.3 Triple helical collagen does not fit in the active of MMP-1. (**a**) Stereo image of the catalytic domain of MMP-1 was first docked with the peptide substrate PLGFA (a stick model in *yellow*) in the active site of the enzyme using Autodock. The S₃ (pro), S₂ (Leu), and S₁' (Phe) sites are indicated. The α 2(I) chain in the native conformation determined by Orgel et al. (2006) (in stick model) containing the MMP cleavage site sequence (PGPQG~LLGAP) was then super-imposed on the cleavable peptide bond of the PLGFA peptide. In this conformation α 2(I) severely clashes with the MMP-1 catalytic domain (indicated as *red* surface). This demonstrates that the α 2(I) chain cannot fit into the MMP active site in the native conformation. Y191 is shown in *dark blue*. (**b**) Stereo image of MMP-1 docked with the two α 1(I) chains in addition to the α 2(I) chain. The colour coding is the same as in A, except that α 2(I) chain is in *red* and additional clashes by the α 1(I) chains are shown as *green surfaces* (MMP-1 structure is 1FBL.pdb; collagen structure courtesy of J.P.R.O. Orgel; figure made with Pymol.)

site, it is still in its polyproline type II-like conformation and there are several molecular clashes elsewhere in the active site cleft. The P_4' (Ala) is away from the enzyme, and there is no contact with Tyr¹⁹¹ of MMP-1, which affects its collage-nolytic activity (Chung et al. 2000). When the two $\alpha 1(I)$ chains were added to $\alpha 2(I)$ chain, they made a few additional clashes with MMP-1. This modelling exercise indicates that naturally occurring unfolding of $\alpha 2(I)$ at room temperature is a very poor substrate for MMP-1 and that considerable conformational changes need to occur for an effective hydrolysis of triple helical collagen.



Fig. 5.4 Collagenase, unwinding and "cutter" activities of MMP-1, MMP-3 and MMP-1/MMP-3 chimeras. Schematic representations of MMP-1, MMP-3 and MMP-1/MMP-3 chimeras are shown on the left. Light grey represents MMP-1 derived sequence, dark grey is sequence originating from MMP-3. At the fusion point the sequence residues are numbered according to the sequence of MMP-3 and MMP-1, respectively. The table shows various activities of each construct and values are in relative activity. All assays were carried out at 25°C. The collagenolytic activity of fulllength MMPs and of the catalytic domain of MMP-1 were measured by quantifying ³/₄ fragments generated after SDS/PAGE. The values given are relative activities taking each activity of MMP-1 as 100. The collagenolytic activity of the catalytic domain of MMP-1 is 1/60,000 (0.0017%) of that of full-length MMP-1. The collagen "unwinding" activity was determined as the ability of each full-length MMP whose catalytic Glu was mutated to Ala to make triple helical collagen I susceptible to 3 µM MMP-1Cat. The raw values obtained as per cent collagen cleaved/h/M MMP(E200A) mutant were converted to relative values taking the activity of MMP-1(E200A) as 100. The "cutter" activity is expressed as the ability of the catalytic domain of each MMP (or MMP variant) to cleave triple helical collagen I in the presence of 2 μ M MMP-1(E200A). The raw values measured as per cent collagen cleaved/h/M MMPCat was converted to relative values taking MMP-1Cat as 100

5.5.5 Multiple Steps are Involved in Collagen Unwinding by Collagenases

The analyses of the unwinding activity of collagen and the ability to hydrolyse peptide bonds (cutter activity) of MMP-1 and of its mutants have provided further insights into triple helicase activity (Fig. 5.3). The collagen unwinding activity was measured for the ability of catalytically disabled full-length enzyme (Cat–Link–Hpx) to allow MMP-1Cat to cleave $\alpha 1(I)$ chains as described by Chung et al. (2004), and the "cutter" activity as the ability of the Cat domain to cleave $\alpha 1(I)$ chains in the presence of full-length MMP-1(E200A). The latter activity was measured under conditions where the Cat domain alone does not cleave collagen. Figure 5.3 summarizes the results. In general, when MMP-1 was mutated at a single site, collagen unwinding activity and collagenolytic activity correlated well, suggesting that the unwinding activity is a rate-limiting step for collagenolysis.

However, chimeras consisting of the N-terminal part of the MMP-3 catalytic domain and the rest of MMP-1 (LC3) exhibited about fourfold higher unwinding activity and about three- to fivefold higher cutter activity, but the active full-length enzyme was a very poor collagenase, suggesting that the unwound α -chains are probably not aligned well within the active site of the chimeric full-length enzymes. Thus, we postulate that for a collagenase to cleave collagen, multiple steps are required during the unwinding process before it finally cleaves the peptide bonds. These steps may be important in collagen specificity, which is dictated by both the collagen sequence and enzyme's unwinding properties. Recent studies of Han et al. (2010) have shown that $\alpha 1(I)_3$ homotrimers are far more resistant to collagenases than $\alpha 1(I)2 \alpha 2(I)$ heterotrimers and this is due to less efficient unwinding of homotrimers by MMP-1. The multiple step model of collagen unwinding is illustrated in Fig. 5.5 together with the model proposed by Stultz and colleagues (Nerenberg et al. 2008).



Fig. 5.5 Models of collagen cleavage. (a) A collagenase-induced unwinding model. This model involves multiple steps: (i) Collagenase (*light grey* represents the catalytic domain and *dark grey* the Hpx domain) binds to native triple helical collagen with the cleft formed between the Cat and Hpx domains; (ii) the collagen is locally unwound though the contact with collagenase; and (iii) the fitting of the unwound strand to the active site. This is followed by cleavage of the three strands of collagen. (b) The Stultz model. In this model, local unfolding of collagen around the cleavage site allows collagenase to bind and cleave

5.6 MMP-2

MMP-2 contains three repeats of a fibronectin type II (FNII) domain. This domain binds to collagen I with an apparent K_d in a low micromolecule range, but less tightly compared to gelatin (Steffensen et al. 1995). The main MMP-2-binding site in native collagen I is the telopeptide region and pepsin-treated collagen, which lacks the telopeptides, does not bind to the FNII domain at 20°C (Steffensen et al. 1995). Therefore for MMP-2 to cleave collagen I particularly at around 25° C, the FNII domain is unlikely to interact with the collagenase-cleavage site of type I collagen, but it may interact with the triple helical region of collagen I after denaturation. In fact, MMP-2 lacking the FNII domains retains collagenolytic activity at 25°C, although it is about 50% of that of full-length MMP-2 (Patterson et al. 2001). Putting back the FNII domain enhances this activity about twofold. This slight enhancement of collagenolytic activity may be due to increased collagen unwinding. The FNII domain is located between Gly¹⁹¹ and Tyr³⁶⁶ in the loop between the fifth β -strand and the third α -helix of the catalytic site of MMP-2. Tyr³⁶⁶ corresponds to Tyr¹⁹¹ of MMP-1, one of the key residues involved in triple helicase activity. Mutation of Tyr¹⁹¹ to Thr reduces collagenolytic activity by 80% and collagen unwinding activity by more than 90% (see Fig. 5.3). Therefore, it would be interesting to compare the collagen unwinding activity of MMP-2 and that of MMP-2 (Δ FNII).

Gioia et al. (2007) measured native bovine collagen I cleavage by MMP-2 at 37°C, and reported that MMP-2 degraded $\alpha 1(I)$ chain about five times more readily than $\alpha 2$ (I) chain, suggesting that the two chains are independently cleaved. On the other hand, they found that collagenase (MMP-8) and the ectodomain of MMP-14 cleaved three α chains almost simultaneously, producing $\frac{3}{4} \alpha 1(I)$ and $\alpha 2(I)$ fragments in the ratio of 2:1. At 37°C the CD spectrum of collagen I showed relaxed triple helicity and the addition of two molar excess MMP-2 which was fully inactivated by a low molecular weight synthetic inhibitor further relaxed to an almost completely unwound state. These changes were interpreted to be mediated by the FNII domain. However, since the FNII binds to the helical region of collagen only when collagen is denatured (Steffensen et al. 1995), the effect of FNII on collagenolysis largely depends on the state of triple helicity of the collagen. The study of Leikina et al. (2002) demonstrated that human lung collagen I gradually unfolds at 37°C (completely after a couple of days) and even below 36° C, and the equilibrium of collagen denaturation could not be extrapolated and complete unfolding was observed even below 36°C. However, refolding of full-length collagen could occur below 30°C. Therefore, the degree of unfolding of the triple helical structure must be greater at 37°C than at 25°C.

5.7 MMP-14 (MT1-MMP)

MMP-14 was first discovered as a pro-MMP-2 activator (Sato et al. 1994) and its activity against interstitial collagens was reported by Ohuchi et al. (1997). Unlike soluble collagenases such as MMP-1, -8 and -13, MMP-14 needs to dimerize on the

cell surface and this dimerization, and perhaps oligomerization, is mediated by the interaction of two neighbouring Hpx domains (Itoh et al. 2006). The reason why the dimerization is required for this activity is not known, but it was speculated that the freedom of the enzyme movement is restricted on the cell surface and/or that MMP-14 needs to interact with other cell surface molecules which present the partner MMP-14 to fibrillar collagen on the cell surface. In addition, for membraneanchored collagenase to cleave immobilized fibrillar collagen, a correct positioning of the enzyme may be necessary to access the single cleavage site within the long filamentous molecule. Dimerization of the enzyme or oligomerization therefore may enhance such action on collagen fibrils. Dimerization or oligomerization of MMP-14 to the front of migrating cells is directed by Rac 1 small GTPases and by the rearrangement of the cytoskeleton (Itoh et al. 2001). MMP-14 colocalized with a2\beta1 and a3\beta1 integrins (Ellerbroek et al. 2001; Wolf et al. 2003). The tetraspanin CD151 (Tspn 24) may also be a key regulator of MMP-14 function at the surface of endothelial cells (Yañez-Mó et al. 2008). MMP-14 colocalizes with tetraspanin CD151 and forms a ternary complex of $\alpha 3\beta 1/CD151/MMP-14$, and CD151-deficient cells showed diminished collagenolytic activity, which is observed in confined areas around the cell periphery. Interestingly, when MMP-13 was expressed as a transmembrane enzyme, it failed to cleave solid-phase collagen, but when fulllength MMP-13 was fused with the Hpx of MMP-14, it dimerized and it regained collagenolytic activity (Itoh et al. 2006). Similar restrictions for collagenolysis may apply even to MMP-13 when it is anchored in the cell membrane. Collagen fibrils degraded by MMP-14 expressed in human fibroblasts are phagocytized for further degradation (Lee et al. 2006). Cathepsin K is the key enzyme for intracellular degradation of collagen fibrils (Everts et al. 2003) (see below). These events appear to be closely coordinated for pericellular collagenolysis, an event critical for cell migration (Sabeh et al. 2004).

5.8 Degradation of Collagen Fibrils

Interstitial collagens in the tissue form insoluble fibrils with a characteristic axial D periodicity of 67 nm. Fibrils are arranged with different suprafibrillar architectures with diameters up to 500 nm (Parry and Craig 1984). Reconstituted acid-soluble collagen I fibrils are more resistant to collagenolysis by MMP-1, compared with monomeric collagen I in solution due to accessibility (Welgus et al. 1980). The activation energy for fibrillar collagen is 119 kcal mol⁻¹ compared to 49 kcal mol⁻¹ for monomeric collagen (Welgus et al. 1981b). Rat tail tendon collagen is even more resistant to MMP-1 and MMP-13. The resistance of collagen fibrils is also explained by the three-dimensional structure obtained by fibre diffraction of an intact collagen I fibre from rat tail tendon solved by Orgel et al. (2006). The naturally occurring unit cell corresponds to one D period of the mature collagen consisting of segments of five triple helical collagen molecules (Fig. 5.6). The



Fig. 5.6 Structure of a collagen fibril. The structural organization of a collagen fibril is shown. (a) Schematic representation of a fibril showing the staggered arrangement of individual collagen molecules. One molecule is highlighted in a rainbow colour scheme from blue at the N-terminus to red at the C-terminus. (b) The above stagger gives collagen fibrils/fibres their characteristic D-periodicity banding pattern. (c) Orgel et al. (2006) solved the structure of rat tail type I collagen fibrils by fibre diffraction. This gave direct insight in the fibril organization and its accessibility for collagenase (Perumal et al. 2008). The structure is of a D-repeat in an orientation as if one is looking down onto the fibril. The colour-coding is the rainbow scheme as before with each collagen molecule *blue* at the N-terminus and *red* at the C-terminus. (d) The grey panels represent each cut-through section through the fibril showing the orientation of the individual collagen molecules relative to each other using the same colour scheme. The MMPcleavage site is indicated by "X." This site is mostly covered by the C-terminal telopeptides of the adjacent collagen molecule, thereby severely limiting the access for collagenase. (e) The view of five collagen molecules within the D5 period. The top is the fibrilar surface. Note that the N-terminus (dark blue) of collagen molecule is below the surface of the fibril and gradually reaches to the surface in the gap region (orange) and terminates with the C-terminal telopeptide (red). The collagenase-cleavage site (shown is while in the orange molecule) is covered by the C-terminal telopeptide by another collagen molecule about it. The figure was made with Pymol (Coordinates courtesy of J.P.R.O. Orgel.)

molecules are arranged to form a supertwisted, discontinuous, right-handed microfibril that interdigitates with neighbouring microfibrils (see Fig. 5.6c–e). This structure indicates that the collagenase cleavage site in the collagen molecule is largely blocked by the C-telopeptide of the neighbouring collagen molecules (Perumal et al. 2008). This suggests that proteinases that have telopeptidase activity such as MMP-2, MMP-3, MMP-13, cathepsin B, cathepsin L, neutrophil elastase and cathepsin G may be important components for fibrillar collagen hydrolysis. Such action would also remove crosslinking sites from the fibrils (Wu et al. 1991). Alternatively thinner fibrils or mechanically damaged collagen fibrils may expose the collagenase cleavage sites and initiate collagenolysis.

Saffarian et al. (2004) reported that active MMP-1 moves in one direction on reconstituted collagen fibrils, like a molecular ratchet which is driven by proteolysis. This movement may be explained from the Orgel model of collagen fibrils: Upon removal of the C-telopetide or damaging of a part of fibrils, collagenase can cleave and remove the C-terminal 1/4 fragment, including the C-telopeptide of the collagen molecule, which then reveals the C-terminally adjacent collagenase cleavage site. Subsequent cleavage of this site will expose another cleavage site on the C-terminal of the previous cleavage site, as illustrated in Fig. 5.7. Such directional movement of collagenase may be associated with directional cell movement in the tissue when the cells express MMP-14 or soluble collagenase attaches to the cell surface. For example, MMP-1 binds to $\alpha 2\beta 1$ integrin (Stricker et al. 2001) and to Emmprin (CD147/Basigin) (Guo et al. 2000). The pericellular cleavage of collagen fibrils by MT1-MMP appears to give directional cell movement on collagen fibrils (Wolf et al. 2007). Cells such as inflammatory cells and keratinocytes may use such mechanisms and move along in one direction depending on the orientation of collagen fibrils. According to Orgel's model, the $\alpha 2\beta 1$ integrin binding site located in the D3 is also blocked by the adjacent collagen molecule (Sweeney et al. 2008). To make these sites available to the integrins, collagenolysis and denaturation of the ³/₄ fragment may be necessary. Further investigation is necessary to ascertain how the cell moves along the collagen fibrils and its association of pericellular collagenolysis. In addition, the small



Fig. 5.7 Directed collagenolysis of collagen fibrils. The Orgel model of rat tail tendon collagen fibrils indicates that collagenase-cleavage sites in collagen fibrils are largely blocked by C-telopeptides, suggesting that either telopeptidases need to cleave the C-telopeptide or the structure needs to be distorted or damaged. This will initiate collagenase cleavage of the collagen fibrils and remove the C-terminal ¹/₄ fragments, which causes the subsequent cleavage site located to the C-terminal to become exposed to collagenase. Thus, a single collagenase molecule moves towards the C-terminal direction driven by proteolysis. The Orgel model hence explains the ratchet movement of collagenase reported by Saffarian et al. (2004)

leucine-rich repeat proteoglycans such as lumican, decorin, biglycan and fibromodulin that bind to collagen fibrils and limit their diameters may also limit the accessibility of collagenases to cleave collagen fibrils.

5.9 Cathepsin K

Cathepsin K is a cysteine proteinase predominantly expressed in osteoclasts and to a lesser extent in other cell types in soft tissues such as fibroblasts (see Chap. 3). A unique property of the enzyme is its ability to cleave type I and II collagens in their helical regions at an acidic pH (Kafienah et al. 1998; Garnero et al. 1998a). Collagenolytic activity of cathepsin K is considered to be important in osteoclastic bone resorption, as evidenced by the observation that deficiency in cathepsin K activity causes a rare bone sclerosing disorder, pycnodysostosis (Gelb et al. 1996), and that the disease-related mutation in the cathepsin K gene leads to a loss of its collagenolytic activity (Hou et al. 1999). Patients with pycnodysostosis typically have decreased bone resorption, enhanced bone density and dwarfism (Fratzl-Zelman et al. 2004). An osteopetrotic phenotype was reported in cathepsin K-deficient mice (Saftig et al. 1998). Studies of Everts et al. (2003) have shown that cathepsin K is also important in lysosomal degradation of phagocytosed collagens in soft connective tissue fibroblasts.

Biochemical studies of collagenolytic activity of cathepsin K have demonstrated that the enzyme cleaves at multiple sites, primarily near the N-terminal side in triple helical regions of interstitial collagens (Kafienah et al. 1998; Garnero et al. 1998b). However, its collagenolytic activity is negligible without the presence of glycosaminoglycans (Li et al. 2000). The most effective glycosaminoglycan is chondroitin 4-sulphate (C4-S) with which the enzyme forms a multimeric complex by associating with a C4-S chain (Li et al. 2002). Li et al. (2008) have recently reported the 3D structure of the complex formed between cathepsin K and C4-S, which revealed a "beads-on-a-string"-like organization. The K_d value of their interaction is about 10 nM. Multiple cathepsin K molecules bound to a single C4-S chain forming a cosine wave-like curve, where one cathepsin K molecule periodically binds at each maximum and minimum of the cosine wave. One molecule of cathepsin K occupies three disaccharide units of C4-S which is bound to the R-domain located on the opposite side of the active site of the enzyme, keeping the enzyme's active site unobstructed. In this area, several positively charged side chains interact directly with the negatively charged groups of C4-S. Another important set of interactions are located in a single turn of the helix, close to the N-terminus of the enzyme, containing a basic amino acid triplet (Arg⁸-Lys⁹-Lys¹⁰) that forms multiple hydrogen bonds with the carboxylate or the four-sulphate groups of C4-S. It is speculated that the collagen triple helix could be accommodated between two spatially adjacent cathepsin K molecules bound to C4-S and one active site of the two enzyme molecules may be directed towards the scissile bond of the collagen. However, cleavage of the triple helical collagen requires a partial unwinding of collagen

substrate. The exact mechanism of collagenolysis by the cathepsin K-C4-S complex needs to be further investigated.

5.10 Non-vertebrate Collagenases

5.10.1 Crab Collagenase

Certain trypsin-like serine proteinases in hepatopancreas of the decapod crustacea such as crab, shrimp, krill, crayfish and lobster have the ability to cleave triple helical collagen I. Those enzymes are called "brachyurins" and they are thought to be involved in the digestion of foods. There are at least three types in the brachyurins: type Ia with broad specificity with activity similar to trypsin, chymotrypsin and elastase; type Ib with broad specificity but less trypsin-like activity; and type II with strictly trypsin-like specificity. Fidler crab collagenases belonging to type Ia and Ib have been characterized for their activities to cleave collagen types I-V (Welgus et al. 1982; Welgus and Grant 1983). At 25°C, all collagen types were cleaved by both types of crab collagenase at multiple sites. However, the main sites cleaved in type I collagen were a few residues in the C-terminal side of the mammalian collagenase cleavage site (Gln-Arg, Arg-Gly, Leu-Gly in the $\alpha 1(I)$ chain and Leu-Gly in the $\alpha 2(I)$ chain), suggesting that looser triple helicity makes it more susceptible to crab collagenase (Tsu et al. 1994). The Leu⁵⁸⁷-Thr⁵⁸⁸ in the $\alpha 1(I)$ chain was also cleaved (Tsu et al. 1994). The 3D structure of crab collagenase has been solved in complex with the *Escherichia coli* serine proteinase inhibitor ecotin (Perona et al. 1997). Ecotin forms a tetrameric complex with the enzyme in a 2:2 molar stoichiometry, and the global structure of crab collagenase is similar to that of vertebrate trypsins with conservation of the double β -barrel core and the catalytic triad (His⁵⁷, Asp¹⁰² and Ser¹⁹⁵). However, the specificity of crab collagenase for collagen substrates is probably due to the subtle modification of several surface loops creating an extended S7-S4' substrate binding site. Molecular modelling indicates that this structure accommodates the collagen sequence with little or no arrangement of side chain groups and of main chain atoms (Perona et al. 1997). An equivalent structure is not found in other serine proteinases, suggesting that crab collagenase has adapted the ability to digest collagens. However, it is notable that collagen concentrations which saturate mammalian MMP-1 failed to saturate crab collagenase, indicating that the affinity of the crab enzyme for native collagen I is considerably lower than mammalian collagenases (Welgus et al. 1982).

5.10.2 Clostridium histolyticum Collagenases

C. histolyticum is an anaerobic spore-forming bacterium that causes gas gangrene. All strains of histotoxic clostridia produce collagenases. There are two types of collagenases in *C. histolyticum* : Class I collagenases (ColG) consist of a metalloproteinase domain followed by one copy of polycystic kidney disease domain and two copies of collagen binding domain (CBD); and class II collagenase (ColH) consists of a metalloproteinase domain, two copies of polycystic kidney disease domain and one copy of CBD (Matsushita et al. 1999). The metalloproteinase domain has the HEYTH zinc ion binding motif, where His⁴¹⁵ and His⁴¹⁹ serve as zinc ligands and Glu⁴⁴⁷ as a third zinc ligand, making them members of the gluzincin metalloproteinase superfamily (Jung et al. 1999). Fulllength enzymes cleave insoluble triple helical collagens at several sites. The enzymatic activity can also be measured with a synthetic substrate, 4-phenylazobenzyloxycarbonyl-Pro-Leu~Gly-Pro-D-Arg (Pz-petide), where ~ is the cleavage site. The enzyme has preference for Gly in P1' and P3 subsites, aromatic residues in P1, and Pro or Ala in P2 and P2' subsites (Van Wart and Steinbrink 1985).

The CBDs consisting of about 110 amino acids are homologous to each other and bind to various types of collagens by recognizing the triple helical conformation of collagen, but not to heat-denatured gelatins (Toyoshima et al. 2001; Matsushita et al. 2001). The CBD-deleted collagenases are not active on collagens, but they have activities against gelatin and Pz-peptide substrate. Ca^{2+} enhances the binding of CBDs to collagen at physiological concentration. In the absence of Ca^{2+} , CBD forms a β -sheet sandwich fold with the linker consisting of 12 amino acids adopting an α -helix. The addition of Ca²⁺ unwinds the linker and anchors it to the distal side of the sandwich as a new β -strand (Wilson et al. 2003). However, the linker is relatively unimportant in collagen interaction. The three conserved Tyr residues (Tyr 970, 994 and 996) are solvent exposed and mutagenesis studies indicate that these residues are involved in collagen binding. In particular Y994F mutation reduced the affinity to triple helical peptide (Pro-Hyp-Gly)₈ by \sim 12-fold and it is considered as the "hot-spot" (Wilson et al. 2003). Since both (Pro-Pro-Gly)₈ and (Pro-Hyp-Gly)₈ bind to CBD tightly, it is likely that the hydroxy group of Tyr⁹⁹⁴ forms a hydrogen bond with the mainchain atoms rather than to the hydroxy group of Hyp. The helix-to-sheet structural change removes the N-terminal region from the collagen binding face (Wilson et al. 2003).

The 3D structures of the catalytic domains of *Clostridium* collagenases have not been solved yet. Such information, however, will assist us to further understand how bacterial collagenases cleave triple helical collagen at multiple sites.

5.11 Conclusions and Perspectives

Interstitial fibrillar collagens are major components of the ECM and they play an important role in maintaining tissues and organs by giving tensile strength. They also are key molecules to create correct cellular environments. Although the turnover of those fibrillar collagens in the steady state adult tissues is very slow,

it is accelerated during the process of tissue remodelling and wound healing. For microbes to invade into host animal tissues, fibrillar collagens are barriers. Effective degradation of native collagens requires enzymes that cleave their triple helical regions, but only a limited number of collagenases are found in vertebrates, e.g., collagenolytic MMPs and cathepsin K in mammals. In non-vertebrates, including microbes, there are a number of proteinases reported to have collagenolytic activity, and the bacterial collagenases and crab collagenases have been well studied. One common feature of these enzymes, regardless of the mechanisms of peptide bond hydrolysis, is that triple helicase activity is assisted by the noncatalytic ancillary domains, as shown for mammalian MMPs and bacterial collagenases. Cathepsin K also needs to be periodically assembled on a chondroitin 4-sulphate glycosaminoglycan chain to be an effective collagenase. Similarly, dimerization or oligomerization of the cell surface-bound MMP-14 is important to be a functional collagenase. Another key aspect of collagenolysis is that collagens need to be partially unwound to be cleaved by those proteinases, as triple helical strands cannot access the active site of the enzyme in the native collagen structure. An exception is crab collagenase, which accommodates of the triple helix in the active site (Perona et al. 1997). While there is a debate as to whether collagenases unwind triple helical collagen before they cleave triple helical chains or a partial unfolding of collagen is sufficient, the currently available data with MMPs suggest that at least vertebrate collagenases appear to have an ability to unwind triple helical structure in full-length forms. The next challenge is to elucidate how these collagenolytic enzymes unwind native collagens.

Thermal stability of collagens is an important factor in collagenolysis. When collagen is completely unwound, many non-specific proteinases can cleave the denatured collagen. Collagenases prefer triple helical structure compared with heat-denatured gelatins. At 37°C native collagen I is much more readily cleaved by MMP-1 than gelatin. However, below 10°C, little collagenolytic activity is detected, whereas gelatinolytic activity can be detected (Chung et al. 2004). The activation energy of human MMP-1 calculated for human type I collagen was 49 kcal mol^{-1} and that for gelatin was 14 kcal mol^{-1} , suggesting that heat makes an important contribution to collagenolysis (Welgus et al. 1981b). Therefore, the induction of a correct secondary and tertiary structure in the substrate is contributed by cooperation between the heat and the full-length collagenase-collagen interaction. Understanding the exact molecular mechanism by which collagenases unwind triple helical collagen requires investigations of where collagen binds to the enzymes and how the $\alpha 2(I)$ chain fits into the active site of the enzyme at different temperatures using the biophysical and structural analyses along with mutagenesis studies.

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Chapter 6 Structural Basis of Extracellular Matrix Interactions with Matrix Metalloproteinases

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Abstract Interactions of the extracellular matrix (ECM) with domains of matrix metalloproteinases (MMPs) increase their proteolytic action upon matrix components. This occurs by multiple structural means. The activating interactions of a partner protein with pro-MMP-9 and of GAGs with pro-MMP-7 were reported. The interactions of fibrillar proteins with catalytic domains can traverse the breadth of the active site cleft and overflow into a neighboring exosite and perhaps beyond. Exosite interactions bear some resemblance to precedents in thrombin complexes. Basket-shaped surfaces on fibronectin II-like modules (inserted into the catalytic domains of MMP-2 and -9) appear to bind protein fibrils, may bend them, and certainly unwind triple helices. C-terminal hemopexin domains are joined loosely to the catalytic domain, which might facilitate positioning and movement across collagen triple helices. At least the first blade of the β -propeller of the hemopexin domain of MMP-1 seems to interact with the triple helix. Unifying themes among diverse interactions of MMPs with ECM polymers are (1) that two domains of the MMP often participate and (2) that the interaction guides the MMP to the site for proteolytic action.

Abbreviations

CBD	Collagen-binding domain composed of three FnII-like modules
CS	Chondroitin sulfate

- ECM Extracellular matrix
- FnI Fibronectin type I
- FnII Fibronectin type II

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GAGs	Glycosaminoglycans
HDX	Hydrogen/deuterium exchange
HDXMS	Hydrogen/deuterium exchange detected by mass spectrometry
HPX	Hemopexin
HS	Heparan sulfate
NMR	Nuclear magnetic resonance
PAR	Protease-activated receptor
SAXS	Small angle X-ray scattering
THP	Triple helical peptide

Two decades of determinations of extracellular protease structures have focused mainly upon discovering their 3D folds, details of their active sites, and how they interact with drug lead compounds (Maskos 2005; Bode 2006). The matrix metalloproteinases (MMPs) are introduced in Chap. 1. Structures of complexes of MMPs with extracellular matrix (ECM) components are few and precious, leaving a large and open frontier regarding the nature of these interactions. Structural insights into MMP interactions with components of the ECM have been gained largely by mapping the interfaces of such complexes by a variety of approaches including NMR spectroscopy, mass spectrometry, X-ray methods, mutagenesis, and comparison of behavior of different combinations of MMP domains. Examples of each of the domains found in MMPs have been found to interact with polymers from the matrix. ECM components considered herein include glycosaminoglycans (GAGs), collagen triple helices, gelatin, and elastin fibrils. Recent structural insights and hypotheses regarding these interactions are surveyed, starting with the prodomain and proceeding through catalytic domain, insertion of fibronectin II-like repeats, and then on to the C-terminal hemopexin (HPX) domain.

6.1 Prodomain and Activating ECM Interactions

6.1.1 Initiation of Activation: Rapid Changes in Coordination of Zinc in Active Site

Two reports have documented cases of disengagement of the prodomain of MMP-9 from its usual cysteine coordination of the zinc in the active site, *without proteolysis* or *prior to proteolysis*. The zymogen of MMP-9 can be activated *without proteolysis* sis upon association with gelatin (Bannikov et al. 2002) or upon association with the irreversibly inactivated serine protease of tissue kallikrein (Rosenblum et al. 2007). Within the first 1.4 s of pro-MMP-9 association with active tissue kallikrein, three successive intermediates appeared prior to the first proteolysis event, rapidly establishing the coordination environment of the active MMP. The intermediates differed in coordination of the active-site zinc relative to both the zymogen and

activated forms of MMP-9 (Rosenblum et al. 2007). Almost immediately upon association, a fifth ligand joined in coordination of the zinc, probably the carboxyl group of the key glutamate in the active site. By a quarter of a second after association, the cysteine *thiolate* coordination of the zinc by the prodomain was lost (Rosenblum et al. 2007). Simulation suggested that this should allow water to penetrate and coordinate the zinc and allow the presumed glutamate carboxylate ligand to dissociate a little more than a second after association with kallikrein. The evidence for adjustable depth of the helix floor of the active site and adjustable breadth of the cleft suggested the dimensions of the active site to be malleable (Bhaskaran et al. 2007). This would provide a context where changes in zinc coordination and distances are plausible. The association of physiological activating partners then appears to have the potential to trigger allosteric and dynamic changes in the active site that could initiate activation of MMPs (Rosenblum et al. 2007).

6.1.2 Activation of Pro-MMP-7 by Glycosaminoglycans

An important means of anchoring MMP-7 (matrilysin) to epithelial cell surfaces appears to be its affinity for GAG such as heparin sulfate (HS) (Yu and Woessner 2000) and HS-containing proteoglycan CD44 (Yu et al. 2002). Heparin from mast cells is more sulfated than HS and affects the activity of several proteases and inhibitors from the blood coagulation cascade (Capila and Linhardt 2002). The autolytic activation and activity of pro-MMP-1 and -2 were found to be enhanced by heparin (Crabbe et al. 1993, 1994). Heparin tightly bound MMP-7 and activated rat pro-MMP-7 (which it bound even more tightly) by greater than 15-fold in vitro while leaving the prodomain intact (Yu and Woessner 2000, 2001). A recent study found that association with heparin, chondroitin-4,6-sulfate (~500 nM K_d values), and dermatan sulfate accelerated the intermolecular autolytic activation of pro-MMP-7 even more dramatically, as well as accelerating activation of MMP-7's processing of pro- α -defensing to their mature antibacterial form (Ra et al. 2009). Chondroitin-4,6-sulfate is a component of serglycin. Although activated MMP-7 retained high affinity (150 nM K_d) for heparin, its affinity for chondroitin-4, 6-sulfate (60 μ M K_d) was much weakened by the absence of the prodomain (Ra et al. 2009). Since activation of a pro-MMP requires disruption of the bond between catalytic Zn²⁺ and conserved cysteine thiolate from the prodomain (Rosenblum et al. 2007), an important question is how does GAG binding promote this activation.

Binding of GAGs was predicted to occur around the swath of basic residues that cradles the catalytic domain of MMP-7 (Yu and Woessner 2000). These lysines and arginines wrap around 60% of the circumference of the catalytic domain most distant from the active site (Fig. 6.1). They are distributed in the amphipathic helices A and C, β -strand II, and six loops (Fig. 6.1). The zymogen form having enhanced affinity for heparin (Yu and Woessner 2000) and 100-fold higher affinity for chondroitin-4,6-sulfate (Ra et al. 2009) suggested that these GAGs interact with



Fig. 6.1 Distribution of positive charge on MMP-7 where sulfated GAGs could bind on (**a**) the catalytic domain (Yu and Woessner 2000) and (**b**) the zymogen with prodomain. Lysine are arginine side chains are colored blue. The label "h" refers to α -helix and "s" to β -strand. The back of the catalytic domain lies in the foreground of panel (**a**) where the swath wraps around it. The active site in the background has zinc ions (*gray*) and calcium ions (*brown*) around it. Panel (**b**) adds the prodomain and its positive charges (upper right) that may account for the higher affinity of the zymogen for heparin and C-4,6-S. The structure of pro-MMP-7 was predicted by homology modeling, using the SWISS-MODEL server (Schwede et al. 2003), using coordinates of the proplus catalytic domains of MMP-3 (PDB accession code 1SLM) as the structural template. The color code ranges from red at the N-terminus (top) of the prodomain to blue at the C-terminus (bottom) of the catalytic domain. The active site is in the center with its helix (*light blue*) just to the left of it

the prodomain. In the structure predicted for pro-MMP-7, the nine positive charges are distributed all over the prodomain (Fig. 6.1b). Seven of these arginines and lysines are found in the three amphipathic helices of the prodomain. Multiple orientations of binding of sulfated GAGs to the prodomain are conceivable. GAG chains can be more confidently envisioned to follow the arc of positive charge around the back of the catalytic domain (Fig. 6.1).

Two potential mechanisms of activation are possible. One would be an allosteric means of activation akin to the allosteric effects of association of either gelatin or tissue kallikrein with pro-MMP-9 that disrupt cysteine coordination of the zinc (Bannikov et al. 2002; Rosenblum et al. 2007), as described in Sect. 6.1.1. An important alternative mechanism would be the concentrating effects of colocalizing pro-MMPs on a GAG chain like the "beads-on-a-string" association of cathepsin K with chondroitin-4-sulfate, observed to bind surfaces of cathepsin K distant from its active site (Li et al. 2008a, b). The multiple sites of potential GAG contact with the prodomain suggest that effects of this might allosterically affect the cysteine thiolate coordination of the zinc at the procatalytic interface. GAG chains might also be able to bridge from prodomain to the cradle behind the catalytic domain in the activation process. The associations of GAGs with pro-MMP-7 might also serve to draw together other molecules of MMP-7, perhaps as beads on GAG string(s), to provide a concentrating effect that would promote bimolecular proteolytic activation. Thus, how GAGs promote activation of pro-MMP-7 remains an open question.

6.2 Catalytic Domain

6.2.1 Full Length of Active Site of MMP-12 May Interact with Elastin

The catalytic domains of MMP-12 (macrophage elastase or metalloelastase) and MMP-7 are distinctive among MMPs in their sufficiency to cleave elastin fibrils (Busiek et al. 1992; Shapiro et al. 1993; Imai et al. 1995; Gronski et al. 1997), which are long-lived and resistant to proteolysis and acid hydrolysis. At least three dozen of the sites that MMP-12 hydrolyzed within insoluble elastin from human skin were identified by mass spectrometry. The sites clustered near the termini and most commonly at G-L peptide bonds, as well as at G-V and A-L linkages (Taddese et al. 2008). The docking to MMP-12 of peptide sequences encompassing several of these cleavage sites was simulated with guidance by ambiguous NMR chemical shift mapping of effects of dilute, solubilized elastin on NMR spectra. The simulated structural models place the peptides linearly across the active site cleft with four to six residues on the unprimed ("left") side of the scissile bond and three or four on the primed ("right") side (Bertini et al. 2009, 2009b). Such models are conventional and consistent with the previous models of peptides docked to MMPs such as MMP-12 (Lang et al. 2001; Bertini et al. 2006). Several MMP-12 residues along the length of the active site cleft often formed hydrogen bonds to peptides from elastin in the structural simulations (Bertini et al. 2009, 2009b) (Fig. 6.2). Recent NMR and protein engineering measurements corroborate elastin interactions with these residues but implicate Thr210 and Gly227 in the active site cleft as well (Palmier et al. 2010) (Fig. 6.3).



Fig. 6.2 Chemical groups of the backbone of MMP-12 predicted to form hydrogen bonds with linearized peptide fragments of elastin. This is based on structural simulations of how the peptides may dock when guided by NMR chemical shift mapping (Bertini et al. 2009, 2009b). The active site cleft is shown in the standard orientation. The color code is *gray* for the active site zinc, *brown* for the calcium ion nearest the active site, *red* for carbonyl oxygen hydrogen bond acceptor, *cyan* for carbon, *white* for amide hydrogen bond donor, and *blue* for amide nitrogen. The chemical groups are plotted largest when engaged in a hydrogen bond with the elastin peptides in almost all the structural models, with medium thickness H-bonded in a majority of structural models, and smallest thickness when H-bonded in a significant minority of the cases. The NMR structure represents the ligand-free state of human MMP-12(E219A) (Bhaskaran et al. 2007)



Fig. 6.3 Exosite and ten residues on the periphery of MMP-12 active that tune specificity for and interact with soluble elastin species and a collagen V-derived triple helical peptide. The sites of interaction were mapped as burial within NMR-detected interfaces between the substrates and inactivated MMP-12 and subsequently confirmed by mutagenesis to modulate specific activity for these substrates (Bhaskaran et al. 2008; Palmier et al. 2010). Inactivated MMP-12(E219) is shown as a surface plot with the active site running horizontally at left and the newly recognized exosite at right. The backbone of the enzyme is faintly visible as a ribbon underneath the molecular surface. Conservative mutations of red residues impair K_m for an elastin substrate and of *yellow residues* impair rate of catalytic turnover k_{cat} (Palmier et al. 2010). Each lesion also impairs k_{cat} for the THP substrate, and six of them impair K_m as well (Palmier et al. 2010)

6.2.2 Exosites of Catalytic Domains

6.2.2.1 Exosites in a Catalytic Domain Exemplified by Thrombin Complexes

The serine protease thrombin offers the perspective of a varied, rich, and thoroughly characterized array of interactions with its positively charged, anion-binding exosites I and II, also known as the "fibrinogen recognition exosite" and "heparinbinding site", respectively (Bode 2006). Interactions at both of these exosites may prove to be illustrative and conceptually relevant to matrix interactions with the catalytic domain of MMPs. The binding of multiple partners makes complementary contacts with exosite I. Complexes with thrombin inhibitors of hirudin, rhodniin, and ornithodorin demonstrated each of the inhibitors to extend out of the active site to wrap its acidic segments across basic exosite I, and make hydrophobic contacts there too (Bode 2006). Peptide epitopes from protease-activated receptors (PARs) that are substrates similarly wrap from the active site around exosite I. Mutations of either active site or exosite I affect thrombin specificity among PARs (Ayala et al. 2001). This behavior of substrates and inhibitors extending from active site and wrapping around a corner to reach an external site may be relevant to how some MMPs might grip protein fibrils for cleavage (see Sects. 6.2.2.2, 6.2.2.3, 6.2.2.4, and 6.3.1).

Like the hirudin complex, the haemadin complex is a case were the inhibitory polypeptide reaches from N-terminal interactions with the thrombin active site to its C-terminal acidic tail interaction with basic exosite. However, the exosite is exosite II near the "top" of the protease in the standard view (Richardson et al. 2000). The binding of heparin by exosite II of thrombin facilitates the binding of antithrombin or protein C inhibitor by bridging between exosite II and the serpin's positively charged heparin-binding site, which is especially clear in the ternary complex with antithrombin (Dementiev et al. 2004; Li et al. 2004, 2008a, b). The concept of GAG chain bridging between the positive patch of a protease and the positive patch of a partner may be relevant to GAG-dependent stimulation both of activation of pro-MMP-7, -1, and -2 (Sect. 6.1.2) and of MMP-7 activity upon pro- α -defensins (next section).

6.2.2.2 Matrix-Interacting Exosites in Catalytic Domain Enhance Proteolysis

One early hint for the possibility of allosteric effectors for MMPs was heparinincreased activity of MMP-7, -1, or -13 in zymography (Yu and Woessner 2001). While the activation mechanism remains unclear, in the case of MMP-7, association with heparin around the distal positively charged cradle of residues is likely (Fig. 6.1). The GAG-dependent activation of MMP-7 in the maturation of pro- α -defensins (Ra et al. 2009) has the additional mechanistic possibility of simply bringing the protease and substrate together as described above. The importance of the V-B loop in collagenolysis by MMP-1 and -8 (Chung et al. 2000; Pelman et al. 2005) (at right in Fig. 6.2) outside the conserved heart of the active site cleft is consistent with an exosite in that region, as described below. Evidence of peptide substrates "turning a corner" to interact with this region is implied by the influence of the P4' and even P5' and P6' positions in MMP-2 cleavage of peptides from libraries (Schilling and Overall 2008).

6.2.2.3 Elastin Interactions Beyond the Active Site

The concept of an exosite from thrombin research was recently established both in structural and functional terms within the catalytic domain of MMP-12. The catalytic domain of MMP-12 is the predominant, mature form observed in vivo, making it like MMP-7 in practice. Much of the active site cleft where elastin peptides were computationally docked with MMP-12 (Bertini et al. 2009) is highly conserved. That conservation raises the question of what determines MMP-12's relatively high specific activity toward elastin? At least one *exosite* and ten residues of five loops

around the periphery of the MMP-12 active site (Fig. 6.3) have been implicated in both structural interaction with solubilized, cross-linked elastin and hydrolysis of it (Palmier et al. 2010). The principal exosite includes residues F202, T205, H206, and T210 of human MMP-12 (Fig. 6.3), at sequence positions that distinguish MMP subfamilies. The NMR evidence of numerous, multifaceted interactions with solubilized α -elastin could explain why MMP-12 was observed bound to digested elastin fibrils in the human aorta, even when TIMP is bound across its active site (Curci et al. 1998; Liang et al. 2010).

Also supporting the high activity of the catalytic domain of MMP-12 appears to be its relative rigidity and trading of some of its marginal stability for greater activity (Liang et al. 2010). Those authors hypothesized that several residues distal from the active site could modulate the stability of the catalytic domain.

6.2.2.4 Triple Helical Peptide Interactions Beyond the Active Site

Locations of exosite interactions with elastin appear to be relevant to triple helical peptidase activity. MMP-12 had not been considered a collagenase. Nonetheless, it hydrolyzed the following triple helices of fibrillar collagens: collagen V into peptide fragments (Fu et al. 2001), a triple helical peptide (THP) mimic of the MMP-2 and -9 cleavage site in collagen V (dubbed $\alpha 1(V)$ 436–447 fTHP) (Bhaskaran et al. 2008), as well as skin collagens I and III at several sites (Taddese et al. 2010). $\alpha 1(V)$ 436–450 THP protected three major surfaces of MMP-12 from NMR line broadening by a small inert probe molecule: (1) the primed (right) side of the active site cleft (center of Fig. 6.3), (2) the main exosite mentioned above (at right in Fig. 6.3), and (3) a distal patch on the β -sheet (not shown) (Bhaskaran et al. 2008). Eight to ten residues around the active site cleft and at the main exosite were found important in digestion of the triple helices from the $\alpha 1(V)$ fTHP mimic of collagen V and from labeled collagen IV, based on structurally guided site-directed mutagenesis. All ten conservative mutations impaired rate of catalytic turnover of the THP without harming catalytic efficiency for a linear peptide substrate, meaning that they selectively perturb turnover of the bulky miniprotein substrates (Palmier et al. 2010). Six of the lesions also impaired apparent affinity K_m for the THP substrate.

These results highlight the importance of the V-B loop region (F202, T205, and H206 in human MMP-12) in triple-helical peptidase activity (Bhaskaran et al. 2008) (Palmier et al. 2010). This joined the prior evidence implicating the V-B loop of MMP-1 and -8 in collagenolysis (Chung et al. 2000; Pelman et al. 2005) or triple-helical peptidase activity (Minond et al. 2006). Thus, the aforementioned exosite for elastin (Fig. 6.3) is also important in the recognition of triple helical substrates by MMP-12, -1, and -8. T210 at the beginning of active site helix C is also near this patch considered to be the main exosite (Fig. 6.3). It was proposed that the α 1(V) THP bent from the MMP-12 active site across the exosite that includes the V-B loop (Bhaskaran et al. 2008). This interpretation has the merits of accounting for (a) the protection of the exosite(s) external to the active site, (b) the

functional importance in specificity of the exosite nearest to the active site, and (c) unwinding of the triple helix for cleavage (Bhaskaran et al. 2008). The hypothesis of bending-induced unwinding of the triple helix was likened to transcription factors' bending of DNA that promotes underwinding of the duplex and strand separation to open a transcription bubble (Bhaskaran et al. 2008).

Most consequential to triple-helical peptidase activity was the introduction of a mere hydroxyl group by the F185Y substitution of MMP-12 (in the IV–V loop) that knocked down the catalytic efficiency of 19-fold toward the $\alpha 1(V)$ 436–447 fTHP and seven-fold toward fluorescently labeled collagen IV (Palmier et al. 2010). Also supporting triple peptidase activity are locations in neighboring loops such as the S-shaped III–IV loop (G178 and I180 in MMP-12), the B-C loop (G227), and the S1' specificity loop (T239 and K241) (Palmier et al. 2010). How much these sequence positions might support the triple-helical peptidase activities of collagenolytic MMPs more generally may prove interesting and relevant for targeting of diagnostic and therapeutic agents.

6.3 Fibronectin-Like Modules: Ties That Bind, Distort, and Unwind

Identification of exosites in MMPs has usually instead referred to their presence in domains other than the catalytic domain, such as in the insertion of modules resembling fibronectin II (FnII) or the C-terminal HPX domain (Overall 2001, 2002; Tam et al. 2004; Gioia et al. 2009). MMP-2 and -9 (72 and 92 kDa type IV collagenases or gelatinases A and B) contain three FnII-like modules of 58 residues each inserted at the aforementioned V-B loop of the catalytic domain. MMP-2 and -9 apparently required their inserts of FnII-like modules to hydrolyze elastin (Shipley et al. 1996), collagen (Collier et al. 1992; O'farrell and Pourmotabbed 1998; Tam et al. 2004), and THP mimics of collagens (Lauer-Fields et al. 2008). The insert of FnII-like modules from MMP-2 is often called its collagen-binding domain (CBD) (Steffensen et al. 1995; Gioia et al. 2009; Xu et al. 2009).

6.3.1 Exosites That Interact with Intact and Denatured Collagen Triple Helices

Early work of Goldberg's group localized a gelatin-binding exosite in the second FnII-like module of MMP-9 (Collier et al. 1992); this maps in crystal structures to the cyan-colored residues at top in the view of Fig. 6.4. Compared with MMP-9, the CBD domain of MMP-2 generally binds protein fibrils more tightly with dissociation constants appearing to be in the low μ M range for both intact and denatured forms of collagens I, IV, and V, as well as elastin (Steffensen et al. 1995).



Fig. 6.4 Binding sites and proposed path of triple helix and gelatin past FnII-like modules of type IV collagenases. The crystal structure of MMP-2 (Morgunova et al. 1999) (PDB code 1CK7) is plotted with catalytic domain (*ice blue*) in standard orientation and prodomain omitted. The most extensive patch of contacts with triple helices and gelatin is indicated by *green residues* with thick side chains in module 2 of the FnII-like insert, the second main site of contacts with *blue side chains* of medium thickness in module 3, and ancillary contacts with *red* and thin side chains in module I. *Cyan-colored residues* mark the site of MMP-9 mutations in its FnII-like module II that disrupt interactions with gelatin (Collier et al. 1992). The area corresponding to the confirmed exosite colored *dark red* in Fig. 6.3 lies to the right of Ca⁺⁺1 under this hypothesized path of a collagen triple helix (*gold*). The crystal structure of a collagen triple helix (Bella et al. 1994) (*gold*, 1CAG.pdb) has been docked by hand into the groove between the FnII-like modules (*brown*) and the catalytic domain; the linear path shown across the HPX domain is not proposed but is simply the complication of the linearity of triple helical coordinates available

This agrees with low $\mu M K_m$ values of MMP-2 hydrolysis of placental type IV collagen enriched in $\alpha 1$ chains (Monaco et al. 2007; Gioia et al. 2009). These authors also measured K_m values as much as 100-fold weaker for placental type IV collagen enriched in $\alpha 2$ chains. By contrast, MMP-9 has (a) K_m values for intact or

denatured α 1 chain-enriched chains of this collagen IV that are one to two orders of magnitude *weaker* than MMP-2, but (b) a K_m for intact α 2 chain-enriched collagen IV that is one order of magnitude *tighter* than MMP-2 (Gioia et al. 2009). The higher affinity of MMP-2 for most protein fibrils evaluated could result from additional contacts made by two to three of the FnII-like modules (Briknarova et al. 2001; Gehrmann et al. 2004; Xu et al. 2009) (Fig. 6.4).

The mapping of sites within the basket-shaped FnII-like modules of MMP-2 that may contact triple-helical and gelatin-like peptides was performed by NMR chemical shift mapping of single FnII-like modules from MMP-2 (Briknarova et al. 1999, 2001) or the complete CBD of MMP-2 (Gehrmann et al. 2004; Xu et al. 2009), and mutagenesis of the CBD (Xu et al. 2009). Residues identified in at least two of these studies have been plotted in Fig. 6.4. These interfacial residues form a bowl in each module where a tryptophan side chain forms its hydrophobic floor above a twostranded β -sheet (Fig. 6.5). Each bowl may be able to accommodate one or perhaps two bulky and hydrophobic amino acid side chains. Triple helix or gelatin strands can run horizontally, with only a modest bend, from the active site at left to reach the binding site in module 3 (blue) at right in Fig. 6.4. For triple helix or gelatin strands to run from the active site to the principal binding site in module 2 (green at top in Fig. 6.4) or the lesser binding site in module 1 (red in background at right in Fig. 6.4) requires considerable bending of the chains. One potential path for gelatin or a triple helix to traverse from the principal binding site in FnII-like module 2 to the active site is the channel between catalytic and FnII-like insert domains, in either MMP-2 or -9 (Fig. 6.4). A path through this groove is attractive because it passes by the main binding site in module 2 (top in Fig. 6.4) and across the exosite proposed to lie in the catalytic domain to the right of Ca⁺⁺1 (center of Fig. 6.4 and dark red in



Fig. 6.5 The bowl-like arrangements of residues that were reproducibly found to interact with gelatin-like or triple-helical peptides (see text) from FnII-like modules 2 and 3 are shown in panels **a** and **b**, respectively. The module 2 residues are plotted with *green side chains* (**a**) and module 3 with blue side chains (**b**). The color code of the transparent surface is *green* for polar residues, *white* for hydrophobic residues, *blue* for basic residues, and *red* for acidic residues
Fig. 6.3) in MMP-12 (Bhaskaran et al. 2008; Palmier et al. 2010), and probably also in MMP-1 and -8 (Chung et al. 2000; Pelman et al. 2005; Minond et al. 2006) (see Sect. 6.2.1.4). Regarding the hypothesis of gelatin or triple helix occupying this groove, reaching the catalytic site would require the triple helix to be bent by $>90^{\circ}$. While some readers may object to the possibility of a triple helix undergoing such dramatic bending, the unwinding implied by a bending mechanism (see Sect. 6.2.2.4) has the potential to explain the requirement of the FnII-like modules for unwinding of the triple helix (Tam et al. 2004). A denatured or more intact triple helix would also have to bend to reach the mouth of each interfacial bowl in FnII-like modules 2 and 1. The distant separation of the main binding sites for gelatin and triple helices in modules 2 and 3 of the CBD of MMP-2 (Fig. 6.4) has the potential to keep individual chains separated to expedite proteolysis.

MMP-2 and -9 were recently proposed to cooperate in type IV collagenolysis: MMP-2 CBD binding of type IV collagen facilitates MMP-9 cleavage of an independent site in type IV collagen (Gioia et al. 2009). Binding of the type IV collagen to two of the binding sites in the CBD (Fig. 6.4) could in principle introduce a loop and bending to the triple helix. This might underwind and melt it, thereby making it more vulnerable to MMP-9 scission of the individual, freed strands.

6.3.2 Do FnII-Like Modules Add a Strand from a Triple Helix, as Does ⁸⁻⁹FnI?

A recently hypothesized mode of triple helix binding to the fibronectin-like modules is considered next. Gelatin-binding fragments of fibronectin, particularly ⁸⁻⁹FnI, were reported to bind two hotspots in the collagen triple helix, including the $\frac{3}{4}$ cleavage site (Erat et al. 2009). The peptide fragment from the $\alpha 1(I)$ strand of collagen I bound in linear fashion as an additional β-strand, in antiparallel orientation with the β -sheet of ⁸FnI (Erat et al. 2009). The polyproline II helix conformation of each chain of the collagen triple helix is very similar to the extended conformation of a β -strand, making it not so big a change in conformation. The more significant change accompanying the addition of a β -strand to the β -sheet of ⁸FnI is the separation and unwinding of the chains of the triple helix necessary for this addition. Indeed, the association with ⁸⁻⁹FnI destabilizes a THP model of homotrimeric type II collagen, corroborating the view that the association unwinds and melts the triple helix (Erat et al. 2009). By linearizing a chain of the triple helix and increasing exposure of the other two chains, fibronectin module binding (Erat et al. 2009) may meet the apparent need for unwinding of the triple helix for collagenolysis (Chung et al. 2004). Fibronectin binding and destabilization of this site can alternately be considered either to facilitate or sterically hinder cleavage (Erat et al. 2009). Since the RGER sequence adjacent to the $\frac{3}{4}$ collagenase cleavage site interacts with ⁸⁻⁹FnI (Erat et al. 2009) and potentially with the HPX domain of MMP-1 (Perumal et al. 2008), a functional overlap between the fibronectin II-like

insert modules and the C-terminal HPX domain was postulated at this key site (Erat et al. 2009).

Each FnII-like module of MMP-2 and -9 does have an exposed edge that in principle could add a β -strand (Fig. 6.4). However, further inspection of the interface mapping results on FnII-like insert modules of MMP-2 and -9 presents a conceptual obstacle in drawing an analogy between ⁸⁻⁹FnI and CBD binding of triple helices. The CBD residues interacting with triple helical or gelatin-like polypeptides have reproducibly mapped to bowl-like clusters above the small β -sheets (Fig. 6.5) (Collier et al. 1992; Briknarova et al. 1999, 2001; Gehrmann et al. 2004; Xu et al. 2009). These interaction bowls appear too distant from the edge strands of the β -sheets (Figs. 6.4 and 6.5) to support the β -strand addition hypothesis of triple helix interaction and disruption.

6.4 C-Terminal HPX Domain

6.4.1 Requirement for Collagenolysis

Loss of the C-terminal HPX domain was reported to abrogate collagenolysis by MMP-1 (Clark and Cawston 1989; Murphy et al. 1992), MMP-8 (Knauper et al. 1993), MMP-13 (Knauper et al. 1997), and MMP-2 (Patterson et al. 2001). The HPX domain of MMP-1 supports unwinding of the triple helix for its ensuing hydrolysis, strand by strand, by the catalytic domain (Chung et al. 2004). The nature of structural and functional coordination between the catalytic and HPX domains of collagenolytic MMPs is then a major question for interactions with protein fibrils from the ECM such as collagens.

6.4.2 Loose Tethering Between Catalytic and HPX Domains

In the crystal structure of full-length MMP-12, the position and orientation of the HPX domain is far removed (120°) from that in full-length MMP-1 and -2 structures (Fig. 6.6) that agree with each other (Bertini et al. 2008). This possibility was not surprising in view of early NMR and crystallography evidence of the mobility and disorder of the linker between catalytic and HPX domains (Gooley et al. 1993, 1994; Van Doren et al. 1993; Li et al. 1995; Van Doren et al. 1995). Loose tethering in solution was clearly demonstrated recently in full-length MMP-12 where the HPX and catalytic domains rapidly reorient relative to each other, again facilitated by the mobility of the linker region (Bertini et al. 2008). Fitting of SAXS data confirmed that full-length MMP-12 samples a multitude of interdomain orientations. About half of these agree with the compact orientation in crystals, while the other half have greater separation of the domains that sample diverse orientations, none



Fig. 6.6 The HPX domain freely and rapidly reorients relative to the catalytic domain in fulllength MMP-12 (*green*) (Bertini et al. 2008) and to a lesser extent in full-length MMP-1 (*red*, *blue*) (Bertini et al. 2009, 2009b). The HPX domain has the β -propeller fold at left and right. The coordinates plot the catalytic domains (center) near the standard orientation using PDB accession code 3BA0 for MMP-12, 2CLT for MMP-1 (*red*), and 1SU3 for the zymogen form of MMP-1 (*blue*). The zinc in the active site is plotted as a *black sphere*

of which agree with the crystal structures of other full-length MMPs (Bertini et al. 2008).

Full-length MMP-1 exhibited NMR relaxation evidence of similarly rapid reorientation between the catalytic and HPX domains (Bertini et al. 2009, 2009b). Moreover, the interface between two domains in the crystal structures of full-length MMP-1 (Figs. 6.6 and 6.7) was exposed to a probe molecule that broadens the NMR peaks, suggesting that in solution the two domains were separated at least part of their lifetimes (Bertini et al. 2009b). Fits of SAXS data suggest that for about two-thirds of their lifetimes MMP-1 molecules were similarly compact as the crystal structure (red in Fig. 6.6) (Bertini et al. 2009b). The smaller proportion of extended MMP-1 molecules, compared with MMP-12, might result from the linker being two residues shorter in MMP-1 (Bertini et al. 2009b). It could also be related to the contacts between its catalytic and HPX domains that are absent in MMP-12 (Fig. 6.6).

The linker between HPX and catalytic domains is longest in MMP-9. This length promoted very loose tethering, judging from SAXS data and single-molecule imaging. All dimensions of wild-type MMP-9 in solution were estimated to be broad, such as the most populated 78 Å distance between catalytic and C-terminal lobes, whereas all dimensions were much smaller with the linker removed



Fig. 6.7 Regions of MMP-1 (inactivated by E200A) with enhanced protection in HDXMS experiments in the presence of a THP model of the MMP-1 cleavage in type I collagen (Lauer-Fields et al. 2009). The backbone ribbon color ranges from *red* at the N-terminus to *white* to *blue* at the C-terminus. The protected peptide fragments from residue 287 to 295, 304 to 316, and 439 to 457, are colored *yellow*, *orange-yellow*, and *green-yellow*, respectively, upon the structure of the active form of MMP-1 (Iyer et al. 2006). The R291A substitution diminished MMP-1 activities toward THPs and collagen I (Lauer-Fields et al. 2009)

(Rosenblum et al. 2007). These observations suggest that reorientation between HPX and catalytic domains should be inherent to all MMPs in solution. This reorientation should range from somewhat restricted in the collagenases with shortest linkers (MMP-1, -8, and -13), to slightly restricted when the linker is a little longer as in MMP-12, to largely independent in most MMPs, to maximally independent in MMP-9.

6.4.3 Possible Implications of Loose Tethering for Proteolysis of Fibrils

The longer and looser tethers of MMPs might serve simply to extend their reach from the cell surface for pericellular proteolysis (Rosenblum et al. 2007). 1D diffusional locomotion was observed for MMP-1 traveling rapidly in one direction along collagen fibrils (Saffarian et al. 2004). In this context, loose tethering of

catalytic to HPX domains could enable the MMP to spread out across collagen fibrils. Inchworm-like movement could then be possible if the domains were to approach each other; this could provide a way for an MMP to ratchet its way processively down the lengthy fibril to scan for sites suitable for hydrolysis (Saffarian et al. 2004; Overall and Butler 2007). Understanding of the structural organization of the collagen I fibril (Orgel et al. 2006) led to an important recent model for initiation of collagenolysis in which proteolytic removal of the C-terminal telopeptide from the surface exposes the 3/4 to 1/4 cleavage site (Perumal et al. 2008). The resulting groove accommodates the splayed out orientation of the catalytic and HPX domains upon the exposed and more vulnerable $\alpha 2$ chain (Perumal et al. 2008). Inchworm-like ratcheting motion of the collagenase can then be envisioned within the groove.

6.4.4 Mapping of Sites of Collagen Triple Helix Binding in HPX Domain

E200A-inactivated, full-length MMP-1 (having both catalytic and HPX domains) was incubated with a THP model of the MMP-1 cleavage in type I collagen designated $\alpha 1(I)$ 772–786 THP. Association with this THP protected three segments of the HPX domain in hydrogen-deuterium exchange mass spectrometry (HDXMS) of peptide fragments of MMP-1(E200A) (Fig. 6.7) (Lauer-Fields et al. 2009). This suggests that the hydrogen bonding of these three epitopes of the HPX domain was stabilized by direct contact with $\alpha 1(I)$ 772–786 THP, or alternatively by indirect effects of its association. THP-protected Ile287-Met295 lie within the innermost β -hairpin of the first blade of the four-bladed β -propeller fold of the HPX domain (Fig. 6.7). THP-protected Arg304-Phe316 form the outer edge of this first blade. THP-protected Phe439-Leu457 form a large section of the adjoining fourth blade (Fig. 6.7). R291A substitution in the first blade impaired the catalytic efficiency of MMP-1 toward three THP substrates and collagen I, confirming the participation of that part of the first blade in collagenolysis (Lauer-Fields et al. 2009).

In crystals, active MMP-1 had a more wide open arrangement (Fig. 6.7) than the latent proform in which the loop of the HPX domain containing Phe308 especially was rotated toward the catalytic domain, as if it pivoted about Arg300 (Fig. 6.7) (Iyer et al. 2006). Reflecting upon this proposed pivoting, the significant interdomain mobility in solution (Bertini et al. 2009b) and the THP-protected surfaces across HPX β -propeller blades 1 and 4 (Lauer-Fields et al. 2009) raises the question of whether the catalytic and HPX domains might rotate so as to clamp down upon an exposed collagen triple helix. Such a rotation and grip would be consistent with the original hypothesis of the catalytic and HPX domains sandwiching the triple helix between them (Bode et al. 1994; Bode 1995). Such a rotation could be postulated to be upward and counterclockwise in the view of Fig. 6.7, about an

apparent fulcrum of Arg300. This would be further in the same direction of the relative rotation described for the zymogen form relative to the activated, mature form (Iyer et al. 2006). Such large rotations of the HPX domain relative to the catalytic domain may well be attainable in solution. However, in the context of early stages of collagenolysis where the exposure of the $\frac{3}{4}$ to $\frac{1}{4}$ cleavage site is limited by the packing of the triple helices all around it (Perumal et al. 2008), the utility of rotations and displacements of the domains may instead be sterically restricted to inchworm-like ratcheting movements.

How the HPX domain might enhance collagenolysis remains an elusive question. The MMP-1 HPX domain has been proposed to align the triple helix properly for cleavage, particularly for longer triple helices of greater similarity to collagen (Lauer-Fields et al. 2009). One possibility for how the HPX domain might align the triple helix is suggested by THP-induced HDX protection of the outer edge of the first propeller blade (Fig. 6.7). Among the suggested sites of contact with the triple helix (Lauer-Fields et al. 2009), this exposed β -strand of the first blade is nearest to the active site cleft. It is conceivable that this proximal β -strand presents its available edge to add a β -strand derived from a lower stability segment of the triple helix, just as ⁸FnI adds a β -strand from the collagenase cleavage site in collagen I (see Sect. 6.3.2). Such a hypothetical linearization by the first blade, if it were to occur, could in principle foster strategic unwinding of the triple helix.

In summary, structural aspects of diverse interactions of matrix components (GAGs, collagen triple helices, gelatin, and elastin) with the pro-, catalytic, FnIIlike, and HPX domains of MMPs have been considered. These typically increase MMP activity by proper localization or positioning, but potentially by allosteric effects as well. Concepts operable in exosite-mediated interactions of thrombin with heparin, inhibitors, and protein substrates may in time prove to be relevant to the interactions of MMPs and other extracellular proteases with ECM components. Qualitative similarities among diverse interactions of the MMP and how that interaction guides the MMP to the place to stage its proteolytic attack.

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Chapter 7 The Role of Matrix Metalloproteinases in Cellular Invasion and Metastasis

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Abstract Within the last two decades of research, the accumulated evidence unequivocally demonstrates that proteolytic degradation and modification of ECM proteins and proteolytic remodeling of stromal tissue play critical roles in cell invasion. With regard to cancer biology, a particular class of proteolytic enzymes, i.e., matrix metalloproteinases (MMPs), is considered highly important in facilitating overall tumor progression and metastasis. As the metastatic cascade represents a continuum of distinct steps, some of which appear to be rate-limiting, the contribution of select MMPs at different stages of malignant disease could be appreciated. Thus, secreted MMPs such as MMP-9 coming from nontumor cells have been functionally linked to tumor progression, while other MMPs have been shown to be potent in cell-cell contact dissolutions during the early epithelialto-mesenchymal transitions. The membrane-tethered MMPs such as MT1-MMP appear to govern directional invasion of tumor cells across basement membranes into surrounding collagen-enriched stroma. However, the identification of MMPs which preferentially facilitate the later steps of the metastatic cascade, especially the transition from micrometastases to macrometastases, remains an unanswered issue. In addition, progress in cancer stem cell (CSC) biology, including the formation of the premetastatic and vascular niches and mechanisms of CSC trafficking, has indicated a high potential of MMP involvement. This review is focused mostly on recent advances in our understanding of MMP physiology in metastatic spread, but also provides an outlook on the experimental findings which paved the ground for newly developed concepts and hypotheses about the roles of MMPs in cancer progression especially in regard to stromal tissue and matrix remodeling.

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7.1 Introduction

The complex process of metastasis includes very distinctive invasion steps where proteolytic enzymes, especially matrix metalloproteinases (MMPs), have been demonstrated to play critical roles. Multiple invasion events occur during both early and late stages of tumor progression, including (1) initial escape of tumor cells from the primary site where MMPs are believed to dissolute cell-cell contacts between tightly adherent carcinoma cells, degrade the epithelial BM, and initiate matrix proteolysis and remodeling; (2) directional invasion of individual or cohort tumor cells into the surrounding stroma, which is specifically modified and remodeled by MMPs; (3) tumor angiogenesis during which endothelial cells, activated by proangiogenic factors released and/or processed by MMPs, invade tumor stroma and form a new vessel network; (4) intravasation and extravasation of tumor cells, during which MMPs are thought to proteolytically degrade the basal membrane at the abluminal surface of blood vessels; (5) establishment of secondary metastases, including MMP-mediated preparation of metastatic niches; and (6) expansion of the established micrometastatic foci, where MMPs can assist the onset of a new invasive metastatic cycle.

Conceptually, the link of MMPs to the most early invasion steps of tumor progression, i.e., escape from the primary tumor and invasion of surrounding stroma, has been the most logical and comprehensible of all the linked associations between proteases and malignancy. The need for proteolytic digestion of the extracellular matrix (ECM) during transition from "carcinoma in situ" to "invasive carcinoma" became the basis for evoking a contributory role for those MMP proteases that can digest the components of BM. The same logic was imposed on the concept that infiltrating cancer cells need to pass through the dense fibrillar network of the ECM and thus specific ECM-degrading proteases, i.e., most members of the MMP family, were linked to the invasive cancers.

In the 1980s and 1990s, the above concepts were fueled by the findings that certain members of the MMP family were expressed at higher levels in the more invasive tumors and more aggressive tumor cell lines. Substantial evidence regarding the role of MMPs in cellular invasion had been accumulated by using broad-range MMP inhibitors, which generally dampened tumor invasion within many in vitro settings and tumor progression within a few in vivo model systems. These studies justified the use of MMP inhibitors in clinical trials, the failure of which, however, forced a substantial reevaluation of the role of MMPs in tumor progression.

During and immediately after the clinical trials of MMP inhibitors, several significant concepts had emerged in the MMP field. One notion has been founded on experimental data indicating that host- or stroma-derived MMPs were functionally important in tumor progression, possibly more than tumor cell-derived MMPs (Egeblad and Werb 2002; Folgueras et al. 2004; Deryugina and Quigley 2006; Jodele et al. 2006). The second concept was the recognition that the cell membrane-anchored MMPs, i.e., membrane-type MMPs, or MT-MMPs, were potent cell

surface proteases and collagenases capable of localizing matrix degrading activity to the pericellular space (Seiki 2002; Holmbeck et al. 2004; Sounni and Noel 2005; Itoh and Seiki 2006). Additional information was accumulated following the clinical trials, which indicated that the early events of tumor progression such as tumor-induced angiogenesis were closely linked to MMP functions (Folgueras et al. 2004; van Hinsbergh et al. 2006; van Hinsbergh and Koolwijk 2008; Deryugina and Quigley 2010). Also, it became apparent that some MMPs can have protective roles in cancer progression (Fingleton 2006; Lopez-Otin and Matrisian 2007; Martin and Matrisian 2007), and if they are mistakenly targeted for inhibition, tumor metastasis might inadvertently be promoted (Overall and Kleifeld 2006; Fingleton 2008). Finally, specific roles of MMPs during formation of metastatic and vascular niches (Lapidot et al. 2005; Psaila and Lyden 2009; Butler et al. 2010) as well as proteolytic functions of MMPs in tumor self-seeding (Norton and Massague 2006; Kim et al. 2009) and cancer stem cell (CSC) physiology (Kucia et al. 2005; Neth et al. 2007; Laird et al. 2008; Zhou et al. 2009) are now in a spotlight of intensive investigations.

This review will be centered mainly on the evidence directly linking proteolytic functions of MMPs with matrix invasion of tumor cells during distinct steps of the metastatic cascade.

7.2 The Onset of Matrix Invasion in Tumor Progression: Epithelial–Mesenchymal Transitions

The conversion of early stage adenomas into invasive carcinomas involves epithelial-mesenchymal transitions (EMT), ultimately resulting in tumor cells accumulating the ability to invade the stroma, reach the circulation, and develop secondary foci (Kang and Massague 2004; Polyak and Weinberg 2009). This concept of conversion of epithelial cells into distinct mesenchymal cell lineages has been persuasively challenged as lacking convincing in vivo histopathological evidence in clinical samples of human and animal tumors (Tarin et al. 2005). Nevertheless, EMT with an emphasis on gene expression transitional changes rather than true cell lineage conversions is still generally regarded as one of the earliest and critical steps in tumor progression required for efficient dissemination of tumor cells.

One of the major hallmarks of EMT contributing to malignant phenotype in experimental model systems is the loss of E-cadherin, the molecule responsible for adherens junctions between epithelial cells and thus tight intercellular organization of carcinomas (Thiery and Sleeman 2006; Yilmaz and Christofori 2009). Repression of E-cadherin occurs under control of several transcriptional factors, such as Twist, Snail and SIP1, overexpression of which triggers EMT in a subset of premalignant epithelial cells (Yang et al. 2006). Concomitant with the repression of E-cadherin and other epithelial markers is the induction of the mesenchymal markers, e.g., vimentin, fibronectin, smooth muscle actin, and N-cadherin in carcinoma cells undergoing EMT (Yang et al. 2004). The mesenchymal phenotype of

the cells that have accomplished EMT is also closely associated with their enhanced invasiveness in vitro and in vivo, hence evoking the functional contribution of MMPs known to assist matrix invasion (Gilles et al. 2005).

Among the aforementioned transcriptional factors, Snail induces expression of MMPs capable of basement membrane (BM) degradation (Thiery et al. 2009). Thus, MMP-13 (collagenase 3) expression was shown to be upregulated and maintained in the NBT-II bladder carcinoma cells exposed to FGF-1, a potent inducer of EMT (Billottet et al. 2008). In agreement with the suggested induction of MMPs during EMT, soluble E-cadherin fragments induced expression of several MMPs, including MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MMP-14 (MT1-MMP), in lung carcinoma cells and promoted their invasion into chick heart and into collagen type I gels (Nawrocki-Raby et al. 2003a). Conversely, overexpression of E-cadherin in highly invasive bronchial tumor cells, which would cause dampening of EMT, resulted in decrease of β -catenin transcriptional activity, decreased invasion, and a concomitant decrease of MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1), MMP-9, and MT1-MMP, at both mRNA and protein levels (Nawrocki-Raby et al. 2003b). Recently, MT1-MMP and MT2-MMP (MMP-15) were demonstrated to cooperatively function as direct-acting, proinvasive factors in Snail1-triggered breast carcinoma MCF-7 cells employed in the chick embryo CAM model (Ota et al. 2009). The expression and proteolytic activity of these two membrane-anchored MMPs were required for the EMT invasion program induced by Snail1, while a number of secreted MMPs, including MMP-1, MMP-2, MMP-3, MMP-7 (matrilysin), MMP-9, and MMP-13, were dispensable and indicated to be irrelevant in this experimental model system. However, there are also documented cases where cancer cells, e.g., generated from spontaneously developed murine mammary tumors, had undergone an EMT, but were still noninvasive and nonmetastatic despite the expression of vimentin and MT1-MMP (Tester et al. 2000). Therefore, EMT-induced expression of MMPs might be insufficient to assure the metastatic potential of cancer cells in vivo.

Alternative to the notion that MMPs are induced after the onset of EMT, some MMPs were shown to function as stabilizers or even triggers of EMT. However, the evidence for a *proteolytic* role of MMPs in the *induction* of EMT which would facilitate metastasis in vivo remains rather circumstantial. Thus, expression of MMP-3 in the mammary epithelial cells in transgenic mice resulted in the formation of invasive mesenchymal-like tumors (Sternlicht et al. 1999). On the other hand, MMP-3 null animals demonstrate an increased sensitivity to the development of squamous cell carcinoma and, conversely, overexpression of MMP-3 specifically targeted to keratinocytes reduces tumor multiplicity in transgenic mice (McCawley et al. 2008). This apparent discrepancy highlights contrasting roles of the same tumor MMP depending on the experimental setting or specifics of mammary gland tumorigenesis (Almholt et al. 2007) or skin carcinogenesis (Martin and Matrisian 2007). In vitro, treatment of SCp2 mouse mammary epithelial cells with exogenous MMP-3 caused a loss of intact E-cadherin, increased motility and invasiveness, downregulation of epithelial markers, and upregulation of mesenchymal markers, including Snail1 (Przybylo and Radisky 2007). Furthermore, proteolytic disruption of E-cadherin by MMP-3 has been shown to directly mediate EMT in renal tubular epithelial cells (Zheng et al. 2009).

EMT also could be induced by overexpression of MMP-9 and MMP-28 (epilysin). In SCp2 mammary cells, MMP-9-induced EMT was associated with the loss of cytokeratin and acquisition of vimentin in a subset of scattering, motile cells (Orlichenko and Radisky 2008). Stable and irreversible EMT was shown to be triggered by the overexpression of proteolytically active MMP-28 in lung A549 adenocarcinoma cells (Illman et al. 2006). Coordinately, MMP-28-stimulated EMT was accompanied by the loss of cell surface E-cadherin, increased levels of bioactive TGF β due to proteolytic processing of latent TFG β complexes, upregulation of MT1-MMP and MMP-9, and increased cell invasion of collagen matrices. Interestingly, the onset of EMT was sensitive to a general MMP inhibitor GM6001, but once TGF β -dependent EMT had occurred, the cell mesenchymal-like phenotype became MMP-independent (Illman et al. 2006). In a similar fashion, once initiated, the progression of invasive mammary carcinomas in transgenic MMP-3 mice becomes independent of continuous MMP-3 expression (Sternlicht et al. 1999).

Therefore, select MMPs can be induced during EMT or directly trigger EMT or EMT-related processes (Fig. 7.1). Regardless of the ambiguous causality in EMT–MMP relations, EMT-associated MMPs are likely to proteolytically degrade cell–cell junction proteins and catalytically modify ECM at the tumor/stroma border, thereby assisting invasion of malignant cells at the leading edge of infiltrating carcinomas.

7.3 Escape from the Primary Tumor: Breaking the Integrity of the Epithelium and Its Basement Membrane

The detachment of aggressive tumor cells from the primary tumor is regarded as an early step in the metastatic cascade and often as a prerequisite to metastatic dissemination via hematogenous or lymphatic routes. For this reason, it is plausible to expect that the MMPs induced during EMT would be capable of proteolytic degradation of tight cell–cell contacts involving E-cadherin and the BM underlying the epithelium. Indeed, constitutive shedding of E-cadherin ectodomain by proteolytically active MMP-3 and MMP-7 was demonstrated from the surface of MCF-7 and MDCK in vitro (Noe et al. 2001) and injured lung epithelium in vivo (McGuire et al. 2003). Inhibition of E-cadherin-mediated cell aggregation and induction of collagen invasion by the released cadherin ectodomain strongly suggested that select MMPs can proteolytically regulate cell invasion in vivo. In addition to MMPs, E-cadherin can be proteolytically shed by other proteases, including ADAM10 (Maretzky et al. 2005) and kallikrein 6 (Klucky et al. 2007).

Another mechanism of tumor disaggregation in vivo involves shedding of α 3 integrin ectodomain by MT1-MMP, recently demonstrated for the ovarian carcinoma cells (Moss et al. 2009). MT1-MMP-mediated spontaneous release of



Fig. 7.1 MMP-mediated mechanisms involved in early stages of carcinoma progression. The schematic highlights the critical events where MMPs have been shown to induce, mediate, or govern specific mechanisms during early stages of carcinoma progression, i.e., from the carcinoma in situ to definitively invasive carcinoma. Carcinoma in situ is regarded as incipient carcinoma lacking visible signs of epithelial cell invasion beyond the boundaries of basement membrane that fully surrounds epithelial cell acini. It has been demonstrated that certain MMPs can induce the invasive phenotype and behavior in carcinoma cells leading to the process collectively described as epithelial-mesenchymal transition (EMT). Alternatively, MMPs can be induced as a result of EMT. At this very early carcinoma progression stage, stromal fibroblasts can be already activated and become myofibroblasts that express proinflammatory factors attracting myeloid cells and ECMmodifying MMPs. Early invasive stage is manifested by the breaching of basement membrane by singular MMP-expressing carcinoma cells that escape the primary tumor and actively degrade and modify (pattern) the surrounding ECM. Migration and invasion of leading carcinoma cells could be guided by MMP-expressing activated fibroblasts. This stage of carcinoma progression involves the recruitment of inflammatory myeloid cells, e.g., neutrophils and monocytes/macrophages delivering MMP-9 that triggers the angiogenic switch. The invasive carcinoma stage is associated with MMP-mediated transition from individual cell migration to collective cell invasion, manifested by "Indian files" or multicellular strands. The proteolytic activity of MMP-14 (MT1-MMP) has been most clearly implicated in this transition. The induction of tumor angiogenesis by proteolytic release and activation of proangiogenic factors has been linked mainly to the enzymatic activity of inflammatory MMP-9

individual cells and cell aggregates in this study was linked to the ability of ovarian carcinomas to form secondary lesions. The invasive spread of carcinoma included the initial formation of cell aggregates and their subsequent adherence to the mesenteruim, followed by disaggregation and development of the invasive foci. Interestingly, in this sequence of apparently opposing events, i.e., detachment–aggregation–disaggregation, the individual underlying processes were similarly dependent on the proteolytic activity MT1-MMP (Moss et al. 2009).

Dissolution of tight cell-cell junctions in the developing carcinoma is accompanied by the proteolytic degradation of the BM, allowing for initiation of tumor cell invasion into the surrounding stroma. The major components of the BM include laminins, type IV collagen, nidogen and proteoglycans (Kalluri 2003; Rowe and Weiss 2008), most of which constitute multiple proteolytic targets of many MMPs in vitro, including MMP-2 and MMP-9, the two type IV collagenases. The functional contribution of MMPs to the proteolysis of epithelial BM in vivo is somewhat restricted and sometimes is attributed to only membrane-anchored MMPs, in particular MT1-MMP, overexpressed in various cell types, including fibrosarcoma cells, carcinoma cells, fibroblasts, and bone marrow mesenchymal cells (Sabeh et al. 2004; Hotary et al. 2006; Li et al. 2008; Ota et al. 2009; Rowe et al. 2009; Lu et al. 2010). However, the putative degradation and remodeling of epithelial BMs in vivo by MT1-MMP is conceptually compromised by the absence of in vivo and even in vitro biochemical data which would demonstrate and characterize that the specific BM components, including type IV collagen, are indeed cleaved by MT1-MMP in situ. Nevertheless, in an ex vivo model employing epithelial BMs recovered from the rat or mouse mesentery, it was the three MT-MMPs, i.e., MT1-MMP, MT2-MMP, and MT3-MMP (MMP-16), which conferred the transfected cells with the ability to perforate BMs (Hotary et al. 2006). Furthermore, in the chick embryo CAM model, invasion in vivo of various cells through the BM underlying the chorionic ectoderm was demonstrated to solely depend on the proteolytic activity of MT1-MMP and MT2-MMP, but not on the activity of MT3-MMP or a number of secreted MMPs produced by the tested cells, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13 (Sabeh et al. 2004; Hotary et al. 2006; Li et al. 2008; Ota et al. 2009; Rowe et al. 2009; Lu et al. 2010). In particular, MT1-siRNA targeting confirmed that expression of MT1-MMP in human HT-1080 fibrosarcoma cells was critical for CAM invasion (Sabeh et al. 2004). However in the same CAM model system, the role of MT1-MMP was not demonstrated to be essential for the ability of a highly disseminating variant of HT-1080 fibrosarcoma to penetrate the undamaged chorionic ectoderm and epithelial BM and invade the CAM mesoderm (Deryugina et al. 2005). Moreover, the specific targeting (by both siRNA and neutralizing antibody approaches) of several HT-1080-derived MMPs, such as MMP-1, MMP-2, and MMP-9, actually facilitated HT-1080 fibrosarcoma cells to

breach the CAM and disseminate to other tissues (Deryugina et al. 2005; Partridge et al. 2007), apparently indicating that some of the tumor-derived MMPs can have protective roles in tumor progression. Consistent with the above suggestion, data obtained in several mammalian model

Consistent with the above suggestion, data obtained in several mammalian model systems demonstrate that exclusive expression of MT1-MMP in tumor cells might actually be insufficient for tumor growth and proliferation in vivo. Thus, the transplants from colon carcinoma cells, generated from MT1-MMP-deficient mice and transfected with inducible MT1-MMP, grew slowly in MMP-2-deficient mice despite the presence of induced MT1-MMP. However, transplantation of the same tumor cells into MMP-2-expressing recipients or transfection of tumor cells with MMP-2 or coimplantation of tumor cells with MMP-2-expressing fibroblasts all resulted in a rapid growth of transplants upon MT1-MMP induction and concomitant

activation of MMP-2 (Taniwaki et al. 2007). These findings demonstrate that stroma-produced MMP-2, which is a true type IV collagenase, could be the actual MMP that upon MT1-MMP-mediated activation degrades in vivo deposits of type IV collagen, a major component of tumor-associated basement membrane. That MMP-2 can function as a critical protease in BM degradation and remodeling was shown in another in vivo experimental system, where proteolytic activity of MMP-2 has been directly linked to the structural alterations in the BM of the renal epithelium (Cheng et al. 2006).

7.4 Individual and Collective Cell Invasion at the Primary Tumor Site

Morphologically, tumor cells escaping from the primary tumor and invading surrounding stroma constitute individual cells or the cells assembled in single cell chains (i.e., Indian files) or multicellular strands (i.e., cohorts). These clustered cells are frequently regarded as collectively migrating/invading cells. The cohort cell invasion can be viewed as a consequence of incomplete EMT, which resulted in a partial dissolution of intercellular contacts at the leading edge of solid tumors and therefore in the directional migration of cancer cells still connected to each other. Although being visualized in histological sections of carcinomas for decades (Tarin et al. 2005), collective cell invasion has recently become a focus of intensive experimental reinvestigations aimed to elucidate its mechanisms in solid tumor dissemination (Gaggioli et al. 2007; Friedl and Wolf 2008, 2010; Wolf and Friedl 2009).

In tissue culture, directed carcinoma cell invasion requires generation of a free edge, allowing the tumor cells to move coordinately in a direction of free space formed in the matrix (Hall 2009). Therefore, one might assume that collective cancer cell invasion would require proteolytic preparation of the ECM by the leading single cells. These invasive cells, individually released from the primary tumor, are usually viewed as nonpolarized mesenchymal-type cells infiltrating the stroma and creating the chemoattractive pathways for the ensuing cells, which invade the stroma in cohorts (Yilmaz and Christofori 2009). However, the directionality of tumor cell infiltration poses a question as to how initially nonpolarized carcinoma tumor cells eventually get polarized and exploit their complex polarity machinery to sense the topography of the ECM and perform persistent migration and invasion.

Only recently, has the multistep processes employed by tumor cells during transition from individual to collective cancer cell invasion started to be addressed mechanistically. Using time-resolved multimodal microscopy in vitro, Wolf et al. (2007) have demonstrated that invasive HT-1080 fibrosarcoma and MDA-MB-231 breast carcinoma cells first break down the sterically impeding collagen fibers using the collagenase activity of MT1-MMP and then realign the remodeled fibers into

single-cell size microtracs. This ECM micropatterning assists further cell locomotion and cell-mediated expansion of matrix platforms capable of accommodating collective cell migration. This transition is controlled by multistep pericellular proteolysis executed by cell surface proteases, particularly by MT1-MMP, in tight coordination with β 1 integrins and F-actin. In collagen-rich matrices, the widening of the ECM tracks, i.e., macropatterning, and the induction of multicellular invasion also depend on MT1-MMP-mediated collagenolysis (Wolf et al. 2007).

It appears from these models that individual MT1-MMP-expressing tumor cells that have escaped from the edge of a solid tumor and infiltrated surrounding stroma would govern the subsequent collective tumor cell invasion. This scenario, however, could be attributed to the mesenchymal origin of the HT-1080 fibrosarcoma cells or upregulation of mesenchymal markers by the MDA-MB-231 carcinoma cells. In conditions, more closely mimicking the physiological situation, i.e., when tumor cells retain their epithelial markers such as E-cadherin and p120 catenin in SCC12 and A431 squamous cell carcinomas, the collective invasion in 3D collagen model system was shown to be invariably led by stromal fibroblasts (Gaggioli et al. 2007). Moreover, carcinoma cells invading collagen gels as collective chains also maintained their epithelial markers and were crucially dependent on close contacts with fibroblasts. The leading fibroblasts were demonstrated to generate tracks in the matrix, suggesting the role for proteases and MMPs in collective cancer cell invasion; however, the nature of these proteases or the identity of the specific MMPs was not elucidated in this study (Gaggioli et al. 2007), but it is possible that some or all of these matrix-degrading MMPs may come from the leading fibroblasts or tumor-activated fibroblasts.

7.5 Secreted vs. Cell Surface-Anchored MMPs

An important aspect of MMP-mediated cellular invasion is whether secreted MMPs, which do not contain a transmembrane domain or membrane-anchoring sequence, have any significant role during tumor invasion or whether cell surface MMPs are the sole MMPs that execute pericellular proteolysis. Historically, the elevated expression of secreted MMPs has been demonstrated in metastatic carcinomas, especially at the leading edge, resulting in the concept that secreted MMPs might indeed play a substantial role in tumor spreading. However, cell surface bound MMPs are the only proteases which logically accommodate the directionality of tumor invasion.

Given the ability of many secreted MMPs to bind to cell surface-docking molecules, paradoxically, it might not matter whether MMPs are intrinsically tethered to the plasma membrane (such as MT-MMPs) or initially secreted into the intercellular space (such as MMP-2 or MMP-9) (Fig. 7.2). Importantly, upon binding to the cell surface, secreted MMPs frequently process and activate their respective membrane receptors and also initiate signal transduction pathways determining the survival of tumor cells or efficiency of their migration and invasion. Secreted MMP-1 produced by stromal fibroblasts was shown to bind to and cleave the protease-activated receptor 1 (PAR-1) on breast carcinoma cells, generating



Fig. 7.2 Mechanisms of cell surface docking of secreted MMPs. The schematic depicts the molecular mechanisms providing the means for cell surface docking of secreted MMPs and focalizing soluble MMP activity to the pericellular space. Secreted MMPs can directly bind to their cell surface target molecules, e.g., MMP-1 binds to PAR-1 to proteolytically activate the latent receptor. Alternatively, secreted MMPs can be processed by the membrane-anchored MMPs, e.g., MMP-2 proenzyme is activated by cell surface MT1-MMP. Secreted MMPs can also dock via their respective PEX domains to the corresponding cell surface molecules, e.g., MMP-1 binds to $\alpha 2\beta 1$ integrin, MMP-2 binds to $\alpha \nu \beta 3$ integrin, and MMP-9 binds to CD44 and $\beta 1$ and $\beta 5$ integrin subunits. A unique mechanism of cell surface docking is represented by MMP-2 activation that requires binding of MMP-2 proenzyme to the C-terminal region of TIMP-2, which in turn is bound via its N-terminal portion to the catalytic domain of MT1-MMP

PAR1-mediated signal transduction and inducing cell migration and invasion in vitro and tumor growth in the orthotopic implantation in vivo (Boire et al. 2005). Synergistic blocking of MMP-1 proteolytic activity and MMP-1/PAR-1 signaling in MDA-MB-231 xenograft tumors almost completely abrogates their growth and, correspondingly, metastasis (Yang et al. 2009). Both the collagenase activity of MMP-1 and MMP-1-mediated PAR-1 signaling were demonstrated to be necessary for matrix invasion and metastatic dissemination of melanoma cells (Blackburn et al. 2009). Secreted MMP-1 also binds to $\alpha 2\beta 1$ integrin on keratinocytes, assisting simultaneous collagen-mediated migration and collagen cleavage at the sites of $\alpha 2\beta 1$ -mediated adhesion (Dumin et al. 2001).

The secreted MMP-2 proenzyme binds to $\alpha\nu\beta3$ integrin (Brooks et al. 1998), which localizes MMP-2 to specific sites involved in cell adhesion but does not mediate the proenzyme activation (Deryugina et al. 2001). Instead, MMP-2 activation requires the formation of proMMP-2/TIMP-2/MT1-MMP complex, in which TIMP-2-bound proMMP-2 is activated by another TIMP-free MT1-MMP molecule (Strongin et al. 1993, 1995; Sato et al. 1994). Since MT1-MMP binds and proteolytically activates $\alpha\nu\beta3$ (Deryugina et al. 2002; Ratnikov et al. 2002), the formation of MMP-2/MT1-MMP/ $\alpha\nu\beta3$ ternary or MMP-2/TIMP-2/MT1-MMP/ $\alpha\nu\beta3$ quaternary complexes provides a potent means for highly localized proteolysis at the sites of MMP-mediated substrate degradation and $\alpha\nu\beta3$ -mediated cell adhesion and migration. In vivo, the cooperation of $\alpha\nu\beta3$ with MT1-MMP in MCF-7 breast carcinoma cells increased the growth of orthotopic xenografts (Borrirukwanit et al. 2007). Secreted MMP-2 can also bind to the cell surface-tethered MT2-MMP, which activates the MMP-2 proenzyme in a TIMP-2-independent manner (Morrison et al. 2001).

Specific binding of secreted MMP-9 to the hyaluronan receptor CD44 and the formation of stable cell surface MMP-9/CD44 complexes provide a mechanism for CD44-mediated carcinoma and melanoma cell invasion of collagen IV matrices in vitro and tumor invasion in vivo (Yu and Stamenkovic 1999) and represent another example when the proteolytic activity of a secreted MMP is localized to the pericellular environment. On the other hand, CD44 functions as the docking molecule for MMP substrates, e.g., heparin-binding precursor of EGF processed by MMP-7 (Yu et al. 2002) or heparin-bound TGF β processed by MMP-9 (Yu et al. 2002), or serves as a direct substrate of MT1-MMP (Seiki 2002). In addition, MMP-9 can dock on $\alpha 4\beta 1/CD44$ complexes on the surface of leukemic cells (Redondo-Munoz et al. 2008, 2010) or directly bind to $\beta 5$ (Bjorklund et al. 2004) and $\beta 1$ integrins on invasive tumor cells (Radjabi et al. 2008; Redondo-Munoz et al. 2008).

The mechanisms that direct secreted MMPs to the cell surface often involve their corresponding hemopexin (PEX) domains (Piccard et al. 2007). The schematic representation of various PEX domains mediating cell surface binding of select secreted MMPs is depicted in Fig. 7.2. Thus, complex formation between $\alpha 2\beta 1$ and MMP-1 requires the linker and hemopexin (PEX) domains of the proenzyme (Dumin et al. 2001). The hemopexin domain is obligatory for complex formation between MMP-9 and its docking CD44 and B1 integrin molecules (Yu and Stamenkovic 1999; Bjorklund et al. 2004; Redondo-Munoz et al. 2010). PEXmediated binding has also been implicated in the cell surface localization of MMP-28 in lung carcinoma A549 cells (Illman et al. 2006), although the cell surface receptor of MMP-28 was not identified. The involvement of PEX domains of secreted MMPs during their cell surface binding or during homodimerization of membrane-type MMPs (Itoh et al. 2006) may mechanistically link the ability of PEX to guide MMPs and adapt substrate recognition by MMPs (Cha et al. 2002; Mori et al. 2002; Van den Steen et al. 2006; Piccard et al. 2007) with the requirement of directional matrix proteolysis and remodeling during cell invasion. This notion is supported by a number of in vitro and in vivo studies where expression of PEXmutants or delivery of soluble PEX domains of select MMPs, e.g., MMP-2 (Brooks et al. 1998; Pfeifer et al. 2000), MMP-9 (Roeb et al. 2002; Burg-Roderfeld et al. 2007; Mantuano et al. 2008; Ezhilarasan et al. 2009), or MT1-MMP (Cao et al. 2004), effectively suppressed tumor growth and angiogenesis and inhibited cell migration and matrix invasion by tumor and endothelial cells.

7.6 Pericellular Proteolysis and Directional Cell Invasion Mediated by Specialized Membrane Structures

The main mechanism underlying pericellular proteolysis by MMPs includes their recruitment to the sites of cell receptor–substrate contacts to provide focalized degradation of specific ECM molecules (Fig. 7.3). The focalized matrix proteolysis occurs at highly specialized cell adhesion-motility structures, including lamellipodia, filopodia, invadopodia, and podosomes, where MMPs act in tight coordination



Fig. 7.3 Cell membrane migration/invasion structures involving MMP matrix proteolysis and remodeling. The schematic depicts major types of cell membrane structures implicated in cell migration and invasion, i.e., sheet-like lamellipodia, rod-like filopodia, shallow podosomes, and deeper penetrating invadopodia. The association of these structures with MMPs is highlighted by the cell surface tethered MT1-MMP alone or in complex with CD44 or by focalized binding of secreted MMP-2 and MMP-9 to MT1-MMP and CD44, respectively

with the crucial elements of migration machinery such as integrins, F-actin, cortactin, and RhoGTPases (Chen and Wang 1999; Wolf et al. 2007; Wolf and Friedl 2009; Yilmaz and Christofori 2009). In the flat, sheet-like lamellipodia regarded as the main organelle for cell locomotion, MT1-MMP was identified directly bound via its hemopexin-like domain to CD44, mediating its proteolytic cleavage and stimulating cell migration due to disrupted hyaluronan-mediated adhesion (Mori et al. 2002). Matrix proteolysis mediated by MT1-MMP and MMP-2 and MMP-9 gelatinases has been linked to the filopodia, rod-like cell extensions, the presence of which correlates with the invasiveness of angiogenic endothelial (De Smet et al. 2009) and metastatic cells (Coopman et al. 1998). The podosomes found in nontransformed but highly migratory cells are believed to be formed when cell adhesion junctions and matrix should be degraded concomitantly (Linder 2009; Yilmaz and Christofori 2009). Not surprisingly, membrane-bound MT1-MMP has been implicated in such coordinated proteolysis (Linder 2009), e.g., by dendritic cells (West et al. 2008) or endothelial cells (Tatin et al. 2006; Varon et al. 2006). In addition to MT1-MMP, both MMP-2 and MMP-9 have also been localized to podosomes in different cell types including activated endothelial cells (Tatin et al. 2006; Varon et al. 2006; Wang et al. 2009).

Invadopodia are formed mostly by invading carcinoma cells and are considered the transformed counterparts of podosomes (Yamaguchi et al. 2005; Linder 2007, 2009). These cell structures play an important role in directional tumor invasion and also in tumor cell intravasation into blood and lymphatic vessels and tumor cell extravasation at secondary sites (Yilmaz and Christofori 2009). Invadopodia-mediated matrix proteolysis involves close coordination of MT1-MMP with actin and cortactin to recruit MT1-MMP to cell-ECM contacts (Artym et al. 2006) and also with caveolin-1 to traffic MT1-MMP to lipids rafts (Yamaguchi et al. 2009). When invadopodial localization of MT1-MMP was disrupted, overexpression of MT1-MMP in melanoma cells did not facilitate ECM degradation or cell invasiveness (Nakahara et al. 1997), further emphasizing that both structural and enzymatic coordinations are required during invadopodia function. However, the structural organization of invadopodia per se appears to be MT1-MMP-independent. Thus, the invadopodial actin-cortactin structures were formed in MDA-MB-231 breast carcinoma cells upon inhibition or depletion of MT1-MMP, but were unable to degrade matrix and assist tumor cell invasion (Artym et al. 2006). Initially, MT1-MMP and also its direct target, MMP-2, were found to be the major MMPs responsible for mediating matrix degradation by the proteolytically active invadopodia of breast carcinoma cells (Kelly et al. 1998). The active form of MMP-9, closely associated with CD44, was also implicated in the invadopodia-associated matrix degradation and tumor cell migration during breast cancer progression (Bourguignon et al. 1998). Recently, the functional proteolytic activity of invadopodia has started to be attributed exclusively to MT1-MMP (Rowe and Weiss 2008). Nevertheless, in addition to MT1-MMP, both MMP-2 and MMP-9 have been repeatedly found central to invadopodia-mediated matrix degradation (Linder 2009).

Spatial control of MT1-MMP distribution and pericellular proteolysis at the leading edge of an individual cell has been shown to create a complex sensor axis, actually guiding cell migration in a certain direction (Packard et al. 2009). Although it is still not completely clear which proteases and substrate molecules are responsible for the very *initial* induction of a unidirectional proteolysis and chemoattractive cell migration, it has become clear that MT1-MMP and native and denatured collagens play a critical role in mediating these processes (Friedl and Wolf 2009). The recent insights gained by reexamining collective cell cancer invasion and the evocation of directed pericellular proteolysis of matrix components at the leading edge provide a new look at the original pathway-clearing aspects of tumor-derived MMPs and bring these early simplistic in vitro notions to the very complex multimolecular and multicellular aspects of in vivo malignant invasion.

7.7 The Cross Talk Between Tumor and Stromal MMPs During Tumor Invasion and Angiogenesis

The apparent sufficiency of MT1-MMP to govern most of the critical programs exploited by tumor cells to invade the surrounding stroma (Hotary et al. 2006; Rowe and Weiss 2008) would imply that highly invasive tumor cells should express this MMP in vivo within the confinements of the developing primary tumor. However, MT1-MMP (or any other MMP for that matter) was not identified in

the final gene expression signature associated with cell motility and invasion when breast carcinoma cells were collected directly from live animals using an in vivo invasion assay and subjected to gene expression analysis in comparison to the general population of tumor cells (Wang et al. 2004b, 2005). Although MT1-MMP was initially identified as a part of a 24 gene signature predicting the invasiveness of human breast cancer cell lines (Zajchowski et al. 2001), more recent gene profiling of 51 breast carcinoma cell lines did not identify MT1-MMP or other MMP genes as associated with the invasive behavior or mesenchymal appearance of tumor cells in culture (Neve et al. 2006). In cancer patients, the expression of MT1-MMP in primary tumors was also not validated as a potential biomarker, either diagnostic or prognostic, in a variety of cancer types (Roy et al. 2009), with the exception of one recent study of clear cell ovarian carcinoma (Adley et al. 2009).

In addition to the demonstrated necessity for the proteolytic activity of MT1-MMP and a few other tumor MMPs in directional matrix proteolysis by tumor and endothelial cells, an overwhelming body of evidence has accumulated also pointing to the functional contribution to tumor invasion and metastasis of another class of matrix-degrading MMPs, i.e., MMPs associated with the tumor microenvironment. These MMPs are produced by different types of cells comprising tumor stroma, including fibroblasts and inflammatory cells. Some of the genes for stromal cell MMPs might not show up in the gene signatures of select tumor variants or tumors from patients. Mutual induction of MMP expression in juxtapositioned tumor and stromal cells is well documented in numerous in vitro and in vivo studies and summarized in a number of comprehensive reviews (Elenbaas and Weinberg 2001; Egeblad and Werb 2002; Lynch and Matrisian 2002; Almholt and Johnsen 2003; Mueller and Fusenig 2004; Deryugina and Quigley 2006, 2010; Jodele et al. 2006).

7.7.1 Tumor-Associated Fibroblasts and Their MMP-Mediated Functions

Tumor-associated fibroblasts (TAFs) or cancer-associated fibroblasts (CAFs) represent a particular stromal cell type because these cells can function both as the active ECM producers and as the active modifiers of the deposited ECM. In vivo, fibroblasts are regarded as slow or nonmigratory cells responsible for the structural organization and stability of connective tissues. Nevertheless, if exposed to haptotactic factors and/or chemoattractants, fibroblasts exhibit remarkable migration and invasion activities in experimental in vitro or in vivo settings and often exhibit traits of myofibroblasts in development. Thus, soluble factors secreted by breast carcinoma SUM102 cells stimulate the expression of MMP-1 in normal human mammary fibroblasts and induce their migration and invasion (Eck et al. 2009). However, when cultured in vitro alone, without any associated tumor cells, isolated mouse fibroblasts were surprisingly shown to be capable of invasion into type I collagen matrices, and this invasive behavior required exclusive expression and proteolytic activity of only one MMP, i.e., MT1-MMP (Sabeh et al. 2004; Zhang et al. 2006; Rowe et al. 2009).

The role of fibroblast-derived MT1-MMP in complex in vitro and in vivo settings appears to be less exclusive and the contribution of other MMPs can be demonstrated. Thus, the invasion into type I collagen gels of human head and neck squamous cell carcinoma tumor cells, triggered by cocultured wild-type murine fibroblasts, was abrogated when the fibroblasts were deficient in MT1-MMP, but was also reduced by 50% if the fibroblasts were lacking MMP-2 (Zhang et al. 2006). Moreover, in an orthotopic model where tumor cells were coinjected with wild-type or MMP-deficient fibroblasts, tumor growth was significantly diminished not only when fibroblasts did not express MT1-MMP, but also if they lacked MMP-2 and MMP-9 (Zhang et al. 2006).

The importance of matrix remodeling by MT1-MMP expressed in TAFs has been recently extended to metastatic spread from the primary tumor. To assess the ability of MT1-MMP-deficient tumor cells to proliferate and metastasize in vivo, MT1-MMP knockout mice were crossed to MMTV-PyMT mice (Szabova et al. 2009). When mammary glands from MT1-MMP-deficient/MMTV-PyMT or their wild-type MMTV-PyMT littermates were orthotopically transplanted into cleared mammary fat pads of wild-type recipients, tumorigenesis was significantly accelerated in the absence of MT1-MMP, a finding which is in apparent contrast with the previously demonstrated cell proliferation controlling function of tumor MT1-MMP in 3D collagen (Hotary et al. 2003) and the reported defects of collagen remodeling in MT1-MMP-deficient mice (Holmbeck et al. 1999). Surprisingly, the levels of lung colonization in the recipients bearing orthotopic MT1-MMP-deficient tumors were 50% lower compared with wild-type tumors, indicating that MT1-MMP expression is essential for full metastatic spread in this model. Since in MMTV-PyMT-driven mammary carcinomas, MT1-MMP is exclusively expressed in the stromal fibroblasts, the findings suggest that the lack of stromal collagen remodeling in primary tumors by MT1-MMP-deficient TAFs is responsible for the reduction of metastatic spread (Szabova et al. 2009). These findings provide an excellent example of how stromal MMPs can profoundly influence metastasis and how the lack of proper matrix remodeling in the primary tumor can inhibit dissemination of actively proliferating tumor cells.

Critical importance of CAFs in cancer progression has been demonstrated by their ability to influence the development and progression of carcinomas via fibroblast-derived growth factors and cytokines and MMPs (Bhowmick et al. 2004; Orimo and Weinberg 2006). Thus, a number of studies show that CAFs promote tumor growth and angiogenesis through secretion of SDF-1, which directly stimulates growth of CXCR4-expressing carcinoma cells and attracts endothelial progenitor cells (Orimo et al. 2005) or mesenchymal stem cells (Karnoub et al. 2007). In mouse models of skin, mammary, and pancreatic cancer, CAFs were demonstrated to promote tumor growth by orchestrating the recruitment of inflammatory cells and stimulating angiogenesis (Erez et al. 2010). Importantly, activated CAFs expressing proinflammatory gene signatures,

including overexpressed *MMP3* and *MMP12*, were identified in the earliest incipient neoplasias and persisted during neoplastic progression to invasive cancer (Erez et al. 2010).

7.7.2 Contributions of Inflammatory Cell MMPs to Tumor Invasion and Angiogenesis

Inflammatory cells infiltrating primary tumors, i.e., tumor-associated monocytes/ macrophages (TAMs), tumor-associated neutrophils (TANs), mast cells, and lymphocytes, represent other important types of cells which produce or deliver MMPs into the tumor microenvironment (Coussens and Werb 2002; Balkwill et al. 2005; van Kempen et al. 2006; De Nardo et al. 2008; Hojilla et al. 2008; Murdoch et al. 2008; Aggarwal and Gehlot 2009).

Histologically, TAMs are the most frequently observed inflammatory cells as they almost invariably are found at the periphery of solid tumors. In addition to potent proangiogenic cytokines and growth factors, such as VEGF, TNF α , IL-8, and FGF-2, TAMs release a number of MMPs, including MMP-2, MMP-7, MMP-9, and MMP-12 (macrophage metalloelastase), all of which might assist in tumor invasion (Pollard 2004; Lewis and Pollard 2006; Solinas et al. 2009). It appears that among all MMPs produced by TAMs, MMP-9 has become obligatory in many processes involved in tumor progression, especially in tumor angiogenesis. Thus, MMP-9 produced by monocytes/macrophages releases and makes bioavailable VEGF sequestered in the ECM, thereby triggering the onset of angiogenesis in primary tumors developing *de novo* in genetically engineered mice (Bergers et al. 2000). This angiogenic switch, which is regarded as a critical event in turning benign adenoma into malignant adenocarcinoma, has been initially attributed to MMP-9 delivered exclusively by tumor-infiltrating monocytes/macrophages (Bergers and Benjamin 2003; Giraudo et al. 2004).

Recently, MMP-9 derived from another inflammatory cell type, i.e., TANs, earned its spotlight in cancer progression (Murdoch et al. 2008; Mantovani 2009). In several cancer models, neutrophil infiltration of developing primary tumors was linked to their accelerated growth, angiogenesis, and metastasis (Fridlender et al. 2009; Tazzyman et al. 2009). Importantly, MMP-9-delivering TANs become not just an alternative, but a major inflammatory cell type that supports tumor angiogenesis and progression when tumor-promoting macrophages are suppressed, e.g., in CCR2 null mice (Pahler et al. 2008). In addition, MMP-9-delivering neutrophils have been the largest contributor facilitating the development of lung metastases in the transgenic mammary gland model (Martin et al. 2008).

At first glance it should not matter whether secreted MMP-9 is produced by monocytes/macrophages or any other cell type, including tumor cells. However, a distinct neutrophil origin of MMP-9 has proved to confer this MMP with unique qualities. It has been demonstrated that MMP-9 released by neutrophils is an



Fig. 7.4 Mechanisms involved in the activation of proMMP-9 produced by different tumorassociated cell types. The schematic depicts secretion and subsequent activation of TIMP-free proMMP-9 by tumor-associated neutrophils (TANs) and TIMP-complexed proMMP-9 by tumorassociated macrophages (TAMs) and fibroblasts (TAFs). Activated TANs are the only abundant inflammatory cell type that is capable of rapid release of secretory granules containing prestored proMMP-9. Moreover, since neutrophil MMP-9 is uniquely produced as a TIMP-free proenzyme, it is readily available for activation. Other cells types, including tumor-associated monocytes/ macrophages (TAMs) and likely tumor-associated fibroblasts (TAFs) synthesize MMP-9 de novo following cell activation and secrete proMMP-9 in a stoichiometric complex with TIMP-1. Complexed TIMP-1 delays activation of proMMP-9 and can also inhibit activated MMP-9 enzyme

exceptionally potent proangiogenic MMP and that this potency of neutrophil MMP-9 is attributed to its TIMP-free status (Fig. 7.4). While all thus far tested cells, including monocytes/macrophages and tumor cells, produce proMMP-9 complexed with TIMP-1, which severely dampens its activation and proteolytic activity, inflammatory neutrophils rapidly release proMMP-9 free of TIMP (Opdenakker et al. 2001). Therefore, in a given inflammatory environment, neutrophil MMP-9 would be immediately available for activation to rapidly execute proteolytic matrix degradation and remodeling at the sites of angiogenesis or tumor development (Ardi et al. 2007, 2009). Remodeling of the ECM by neutrophil MMP-9 is accompanied mainly by the release of sequestered FGF-2 (Ardi et al. 2009), while MMP-9 produced by macrophages is usually associated with mobilization of VEGF (Bergers et al. 2000; Giraudo et al. 2004). Since both FGF-2 and VEGF are major proangiogenic factors (Ferrara et al. 2003; Presta et al. 2005; Ellis and Hicklin 2008;

Murakami and Simons 2008), inflammatory cell MMP-9 becomes a major trigger of the angiogenic switch during tumor progression.

The expression of MMP-9 was validated in a variety of cancers, including breast, pancreatic, lung, bladder, colorectal, ovarian, prostate, and brain cancers, making this MMP one of the most consistent MMP biomarkers (Aggarwal and Gehlot 2009; Roy et al. 2009). Nevertheless, the role of MMP-9 in tumor progression remains controversial, as in different settings the overexpression or downregulation of MMP-9 might result in the retardation or acceleration of metastasis. Thus, the genetic ablation of MMP-9 in the multistage mammary MMTV-PyVT tumor model was associated with 80% decrease in lung metastases (Martin et al. 2008), while the delivery of MMP-9 adenovirus to the estradiol-induced mammary xenografts in nude mice resulted in increased MMP activity in vivo, but decreased tumorigenesis and angiogenesis (Bendrik et al. 2008). The overall inhibitory effects of *MMP9* gene therapy were attributed to the MMP-9-mediated release of endostatin, a potent inhibitor of angiogenesis in vivo (Nilsson and Dabrosin 2006) but might also be attributed to the MMP-9-mediated generation of tumorangiogenesis (Hamano et al. 2003).

7.7.3 MMP-Governed Angiogenesis

Following the initial MMP-9-mediated switch, the formation of the angiogenic network in vivo would require disaggregation of the endothelial cell layer, induction of endothelial cell migration and invasion, and finally lumen and tubule formation. Collectively, these processes were consistently shown to depend on the expression and matrix-remodeling activity of MT1-MMP within various in vitro settings employing collagen and fibrin gels (Lafleur et al. 2002; Chun et al. 2004; Davis and Senger 2005; van Hinsbergh and Koolwijk 2008; Iruela-Arispe and Davis 2009). More detailed analyses employing ex vivo angiogenesis models demonstrated that MT1-MMP expression is largely confined to the sprouting tip of the developing vasculature and that this site-specific expression of MT1-MMP depends on a cross talk between endothelial cells and mural, vascular smooth muscle cells (Yana et al. 2007). Furthermore, MT1-MMP expressed in the endothelial cells is required for initial proteolytic generation of vascular guidance tunnels in 3D collagen matrices to allow for subsequent lumen and tube network formation (Stratman et al. 2009). These findings highlight a previously unrecognized step in vascular morphogenesis, i.e., creation of physical ECM spaces via MT1-MMP proteolysis, and also reinforce the previously demonstrated dependence of collagenous matrix remodeling on MT1-MMP during directional migration and invasion of tumor cells (Wolf et al. 2007; Sabeh et al. 2009). An important aspect of MT1-MMP expression during neovascularization in vitro is that its de novo expression in endothelial cells can directly be triggered by different cytokines, e.g., GM-CSF (Krubasik et al. 2008), and therefore MT1-MMP can be a downstream effector molecule within cytokine regulatory pathways.

The requirement of MT1-MMP in angiogenesis in vivo is more difficult to validate since MT1-MMP knockout mice are not compromised in vasculogenesis during embryogenesis and do not live long enough to allow for studying tumorinduced angiogenesis (Holmbeck et al. 1999). The lack of MT1-MMP in null mice results in severe impaired vascular invasion of cartilage during early postnatal development (Zhou et al. 2000). In addition, MT1-MMP-deficient mice do not exhibit angiogenic response to FGF-2 in a corneal model (Zhou et al. 2000). The abnormal vessel wall morphology in the brain of MT1-MMP-deficient mice is associated with a significant reduction in vessel coverage with mural cells (i.e., pericytes and vascular smooth muscle cells), rather than with direct abnormalities in the endothelial cells (Lehti et al. 2005). Although MT1-MMP appears to regulate mural cell *coverage* of brain microvasculature, pericyte *recruitment* to the sites of tumor neovascularization requires the proteolytic activity of MMP-9. Thus, the angiogenic vasculature in orthotopic or s.c. human neuroblastoma xenografts in MMP-9 knockout mice displays fewer and smaller blood vessels and significant decrease in pericytes along microvessels, indicating that stroma-derived MMP-9 contributes to tumor angiogenesis and vessel maturation (Chantrain et al. 2004). These architectural defects in tumor vasculature observed in the MMP-9 deficient microenvironment could be rescued by transplantation of bone marrow from wild-type donors, and therefore it is stromal MMP-9 produced by bone marrowderived cells (BMDCs), namely infiltrating leukocytes, that critically contributes to the formation of a mature vasculature with efficient pericyte coverage (Jodele et al. 2005). Furthermore, wild-type bone marrow derived myelomonocytic cells contribute to tumor growth and vasculogenesis specifically by supplying their MMP-9 to the MMP-9-deficient microenvironment of knockout recipients (Ahn and Brown 2008).

7.8 Intravasation: MMP-Dependent Proteolysis of the Basement Membrane at the Abluminal Surface

The intravasation step is the least studied in the metastatic cascade, possibly because there are basically no available in vitro models and only a few in vivo models for this complex step. Although there is evidence that tumor cells might shed passively into the blood or lymphatic vessels (Wong and Hynes 2006; Bockhorn et al. 2007), in general intravasation is viewed as an active process whereby tumor cells escaping from the primary tumor enter the circulation. To accomplish this, intravasating cells must first breach the BM of blood or lymphatic vessels and then traverse the endothelial cell layer. It is assumed that breach points in the vascular BM are proteolytically created by MMPs or other proteases produced by tumor cells or tumor-activated stromal cells, inflammatory cells, and angiogenic endothelial cells. The basic components constituting vascular BM include laminins, type IV and XVIII collagens, and nidogen-1 (Kalluri 2003; Davis and Senger 2005; Rowe and Weiss 2008), relative representation of which is different in the BM of

blood vs. lymphatic vessels (Vainionpaa et al. 2007). BM constituents in normal and angiogenic vessels also can differ quantitatively and, maybe more importantly, qualitatively, e.g., can include new proteinaceous molecules (Cao 2005; Vainionpaa et al. 2007). This broad compositional array suggests that more than a few MMPs could be essential for degradation of any number of BM components to create the points of entry for intravasating cells.

Since endothelial lumen and tube formation within collagenous matrices in vitro critically depend on MT1-MMP, the notion that MT1-MMP governs proteolysis of vascular BM in tumors in vivo has been widely accepted. Despite overwhelming evidence for the role of MT1-MMP-mediated localized matrix proteolysis and guided invasion of tumor and endothelial cells through fibrillar collagens, there is little evidence that the proteolytic activity of MT1-MMP expressed by either tumor or stromal cell types is responsible for the breaking of vascular BM in vivo. Although essential components of migration/invasive machinery are expressed in aggressive tumor cells, causal links between these markers and proteolytic functions of specific MMPs precisely during intravasation have been addressed in rather limited number of metastasis studies.

The mammalian in vivo model of intravasation, employing intravital multiphoton microscopy and advanced optical technology, has allowed for direct observation of tumor chemotaxis toward blood vessels and rare events of tumor cell entry into blood vessels in real time (Wyckoff et al. 2000; Condeelis and Segall 2003), but did not provide data on the contribution of MMPs to this process. The avian, chick embryo CAM model represents a unique quantitative intravasation model system (Fig. 7.5), where MMPs and serine proteases have been repeatedly proved to play critical roles by demonstrating that the broad-range MMP and serine protease inhibitors significantly inhibit intravasation and subsequent dissemination of fibrosarcoma as well as carcinoma cells (Kim et al. 1998; Deryugina et al. 2005; Madsen et al. 2006; Conn et al. 2009). However, it remains controversial which MMPs are critically required for cancer cells to penetrate though the BM and endothelial layers of the CAM blood vessels. Thus, downregulation of MT1-MMP by specific siRNA constructs either led to complete abrogation (Sabeh et al. 2004; Li et al. 2008) or was without any negative effects on intravasation of the HT-1080 fibrosarcoma in the chick embryo CAM model of spontaneous metastasis (Deryugina et al. 2005; Partridge et al. 2007). Moreover, specific downregulation of MMP-2 and MMP-9 by siRNA did not inhibit intravasation of HT-1080 cells, but unexpectedly resulted in a several fold increase in their dissemination (Deryugina et al. 2005; Partridge et al. 2007). Making it more complicated, the intravasation of several carcinoma cell types, including breast, prostate, and epidermoid carcinomas, was initially reported to critically depend on MMP-9, but in uPA/uPAR-dependent fashion (Kim et al. 1998). It is also important to keep in mind that the proteolytic breaching of vascular BM by tumor cells during their intravasation has never been formally demonstrated by any group employing the CAM model system. In contrast, the initial invasion of tumor cells through intact chorionic epithelium was shown to be accompanied by the active retraction of the epithelial BM into compact islands with active invasion of cells into the mesoderm occurring between the islands (Armstrong et al. 1982).



Fig. 7.5 Tumor cell invasion and intravasation and inflammatory cell influx in the CAM spontaneous metastasis assay. The schematic on the left depicts tissue composition of the CAM at the time of tumor cell application on day ten of chick embryo incubation. From 0.5 to 2×10^6 tumor cells (green) are applied in a volume of 20–25 µl to the top of dropped/lowered CAM. The dropped CAM is not subsequently injured and remains covered with the ectoderm (the layer of *blue cells*), which is underlined with the basement membrane (grey) and ectoderm capillary plexus (red irregular circles filled with nucleated erythrocytes). Larger terminal capillaries (bulb-like structures) lay right beneath ectoderm plexus and protrude to the mesoderm filled with the ECM fibrils, stromal fibroblasts, and arterial and venous blood vessels containing erythrocytes, monocytes, and neutrophils. The mesoderm is underlined by the thin endoderm, which separates CAM from the allantoic cavity. Within 5-7 days (right), the applied tumor cells can form a large tumor mass, which compresses the CAM and makes it much thinner than the original membrane. The staining for blood vessels allows for visualization of the compressed ectoderm plexus at the tumor border and also for larger vessels in the mesoderm and angiogenic vessels in the tumor mass. Tumor cells with high potential of dissemination and metastasis appear to escape from the primary by breaching the ectoderm layer, the basement membrane, and ectoderm plexus. The escaped tumor cells invade the mesoderm and appear to congregate and wrap around blood vessels and capillaries and sometimes are visualized intravascularly within the blood vessels. Although traversed with numerous angiogenic blood vessels, the density of tumor cells usually does not allow for unambiguous identification of intravascular tumor cells within the primary tumor. Importantly, tumor development is associated with the influx of inflammatory, MMP-delivering neutrophils (MMP-9) and monocytes/macrophages (MMP-13), which can be identified both within the primary tumor and at the tumor/CAM border by a corresponding MMP staining. Most of the intravasated tumor cells are captured at the capillary plexus throughout the CAM serving as the lung analog of the adult animal, but can also be identified as spontaneously metastasized to different internal organs, including liver, lungs, and brain

Therefore, intravasation of tumor cells measured as an overall outcome of cell dissemination from primary tumor cells placed atop the CAM cannot be unequivocally attributed to the MMP-dependent proteolysis of BMs underlying the CAM vessels.

That intravasation could be significantly dampened by general MMP inhibition, but not by specific targeting of tumor-derived MMPs, provides strong evidence for the regulation of tumor cell intravasation by stromal, most probably inflammatory MMPs. Data in favor of this notion in mammalian model systems are scarce, but some elegant studies demonstrate that macrophages often assist tumor cell entry into blood vessels in orthotopic and transgenic breast cancer models (Wyckoff et al. 2007). In a Transwell model in vitro, transendothelial migration of breast carcinoma MDA-MB-231 cells was shown to be facilitated by neutrophils (Wu et al. 2001). Unfortunately, the role of MMPs released by macrophages or neutrophils, known producers of MMP-9 and other MMPs that could function catalytically in mediating the intravasation events, has not been addressed in these studies. Therefore, it is still largely unknown precisely where and exactly how tumor cells intravasate and even less clear which MMPs with regard to their cell origin or biochemical nature are likely involved in the degradation of vascular BM during intravasation.

In the chick embryo CAM model, where intravasation could be significantly diminished by broad-range MMP inhibitors but not by inhibition of tumor-derived MMPs (Deryugina et al. 2005; Partridge et al. 2007), our preliminary data indicate that MMPs delivered by inflammatory cells could be positively linked to the intravasation event. Thus, there are strong positive correlations between the levels of spontaneous intravasation with the levels of MMP-9-delivering neutrophils and MMP-13-positive macrophages infiltrating the stroma of primary tumors derived either from HT-1080 fibrosarcoma or PC-3 prostate carcinoma cells. Inhibition of such inflammatory cell influx into primary carcinoma or fibrosarcoma tumors with anti-inflammatory agents, such as anti IL-8 blocking antibody or specific antibodies blocking CXCR2-ligand interactions, results in significant and concomitant decrease of tumor angiogenesis, intravasation, and metastasis without having any significant effect on primary tumor size (Fig. 7.6). Although formally not proved, the angiogenic vessels are generally regarded as conduits for tumor cell intravasation. In our CAM studies, the strong positive correlations between the levels of tumor angiogenesis and tumor cell intravasation and dissemination provide additional evidence that angiogenic vessels are indeed those exploited by escaping tumor cells as conduits for vascular dissemination.

7.9 MMP-Mediated Proteolysis During Late Steps of Tumor Progression

Following successful intravasation, cancer cells are believed to circulate in the blood or lymphatic system. This stage might be considered a period between the early and late events of metastasis. Intravasated cells must survive the hostile nature of the circulation, avoid clearance, and also avoid the onslaught of apoptotic and anoikis signals that target unanchored tumor cells in the circulation. It is uncertain how long it takes for *spontaneously* intravasated tumor cells to get arrested in the capillary bed of the secondary organ. In contrast, the arrest of tumor cells inoculated as a single cell suspension intravenously or intracardially appears to be a relatively rapid process, taking possibly only a few minutes. In the chick embryo, tumor cells injected into the allantoic vein are trapped in the terminal arterioles of the CAM





within 10 min (Deryugina et al. 2009; Subauste et al. 2009), most probably due to size restriction. The arrest of intracardially inoculated tumor cells in liver and lung of rats appears to occur in less than 20–30 min, but without size restriction,

suggesting the involvement of specific tumor cell–endothelial cell interactions (Wang et al. 2004a; Schluter et al. 2006). Lewis lung carcinoma cells injected intra-arterially into the heart were visualized in hepatic sinusoids by fluorescence intravital microscopy within 30 min following inoculation (McDonald et al. 2009). Interestingly, this initial arrest of tumor cells on liver sinusoidal endothelium was enhanced by inflammation and facilitated by adherent neutrophils in the inflamed sinusoids.

It is still unclear whether any specific MMP directly assists tumor cell arrest on the vascular endothelium, but interactions between tumor cells and platelets facilitate the formation of tumor cell/platelet emboli, which is believed to enhance intravasation via induced expression of tumor cell MMP-9 (Lindenmeyer et al. 1997). The interactions of arrested tumor cells with platelets and fibrinogen in the lung capillaries have long indicated that coagulation facilitates tumor spreading in the pulmonary vasculature (Im et al. 2004). Therefore, both coagulation and fibrin proteolysis appear to be essential for tumor cells to efficiently interact with the endothelial cell layer and initiate extravasation, which is considered the beginning of the late steps in the metastatic cascade. The biochemistry of fibrin proteolysis would mainly indicate the involvement of the tPA/uPA/plasminogen activation cascade and plasmin-mediated degradation. Since MT-MMPs expressed on the endothelial cells were shown to be capable of degrading fibrinrich matrices both in vitro and in vivo (Hiraoka et al. 1998; Ratel et al. 2007), it is possible that dissolution of fibrin clots containing tumor cells might at times be actually performed by the MT1-MMP expressed on the surface of tumor cells or endothelial cells at the site of tumor cell transmigration. It should be kept in mind, however, that dissolution of the fibrin clot by MT1-MMP would likely not be as efficient as the well-established, natural fibrinolytic protease, plasmin.

Endothelium-attached tumor cells can also proliferate within the capillaries of the secondary organs, thereby providing an intravascular source of metastasis (Al-Mehdi et al. 2000). In an orthotopic model of human prostate cancer, in vivo imaging in live mice allowed for the real-time characterization of cancer cell-endothelium interactions during spontaneous metastatic colonization. Prior to detection of extravascular metastases, GFP-tagged PC-3 carcinoma cells were observed residing inside the blood vessels of the liver and the lung, where they proliferated and exhibited MMP proteolytic activity, detected by an MMPSense probe sensitive to MMPmediated cleavage. These results demonstrate that the intravascular microenvironment could provide a critical staging area for the development of spontaneous metastasis (Zhang et al. 2010). It would be of interest to identify whether expression of tumor MMPs in such intravascular metastases would have specific spatiotemporal regulation, i.e., be attenuated at the early phases of tumor foci development and intensified at the later stages to assist in tumor cell shedding into the vascular circulation. In general, however, it is accepted that if survived in the circulation, arrested tumor cells cross the endothelial cell-BM barrier, i.e., extravasate into the parenchyma of the distant organ.

7.10 Extravasation: Breaching the Basement Membrane Following Transendothelial Migration

After arrest at specific sites on subluminal surfaces of hematogenous or lymphatic vessels, tumor cells have to first migrate through the endothelial cell barrier and then penetrate underlying vascular BM in a process long referred to as extravasation. There is no consensus as to whether extravasation is a rate-limiting step in the metastatic cascade since it is a highly efficient process when evaluated in experimental metastasis models where tumor cells are injected directly into the circulation (Chambers et al. 1992; Koop et al. 1995).

A few models have been introduced to study extravasation in vitro allowing for detailed mechanistic analysis of critical events involved in this process, i.e., interaction of tumor cells with the endothelial layer, transendothelial migration, and invasion of the matrix underlying the endothelium. In a Transwell model, downregulation of MMP-1 in conjunction with one or more genes significantly inhibited transendothelial migration of breast carcinoma cells (Gupta et al. 2007; Kim et al. 2009). Combined with confocal microscopy, 3D extravasation models demonstrate that transendothelial migration is complete within a few hours after tumor cells attach to the endothelium and involves pseudopodia formation (Qi et al. 2005, 2006). Therefore, a contribution of invadopodia-associated MMPs, e.g., MT1-MMP, in the degradation of vascular BM might be anticipated. During transendothelial migration in vitro, tumor cells were observed to irreversibly damage endothelium at the site of migration (Brandt et al. 2005). Whether the integrity of the layer, and possibly of the underlying matrix, were breached in an MMPdependent manner was not addressed in these studies, but the finding of structural damage to the endothelium might suggest a mechanism for the efficient collective extravasation of tumor cell cohorts at an individual point of exit.

Tissue specifics of endothelia and endothelial BMs in distant sites may require differential expression of molecules which would in one or another way assist in extravasation at different secondary tissues or organs. The structure of blood vessel or lymphatic endothelia in different organs varies significantly with regard to their level of fenestration (Strell and Entschladen 2008) or continuity of the BM (Pepper and Skobe 2003), which might explain different rates of colonization by extravasating cells. Thus, discontinuous endothelium in the liver sinusoids with large gaps that expose the underlying ECM (Aird 2007a, b) might explain high levels of liver colonization observed in some metastatic cancers and experimental metastasis model systems (Kuo et al. 1995; Kobayashi et al. 1998; Conn et al. 2009; Deryugina et al. 2009), while incomplete BM of lymphatic capillaries might explain high propensity of certain aggressive tumor cells for lymph node metastases and lymphatic route of dissemination.

Following transendothelial passage, the MMPs must proteolytically degrade the endothelial BM, and in principle, these MMPs might functionally overlap with those participating in the degradation of vascular BM proteins during intravasation. Thus, type IV collagen degradation indicating the breaching of vascular BM was
detected by a cryptic site-specific HU126 monoclonal antibody around blood vessels in the colonized lungs at 24 h and 7 days after i.v. inoculation of melanoma cells into the chick embryo (Roth et al. 2006). The involvement of type IV collagen degradation in extravasation in a mammalian system was demonstrated indirectly by significant inhibition of lung colonization with the same HU126 during experimental metastasis in mice (Roth et al. 2006). The nature of the proteases degrading collagen IV, unfortunately, was not identified in this study.

When colonization levels of target organs such as liver, brain, or bone marrow are used as an end point of experimental metastasis, it is difficult to dissect the extravasation process per se from the overall experimental metastasis process to specifically address the putative contribution of specific MMPs. Therefore, it could be the survival and proliferation capabilities of the extravasated tumor cells which would determine the overall outcome of colonization. In this regard, MMPs expressed by extravasated tumor cells might be more important in enhancing their survival, i.e., avoiding an apoptosis onslaught by mediating distinct migratory–adhesive interactions in a new matrix microenvironment. In this case, the invasive aspects of extravasation would be reflected in the ability of tumor cells to degrade and remodel the ECM of the secondary site by possibly exploiting their own MMPs or the stromal MMPs in their microenvironment. Thus, stromal MMP-9 supplied by bone marrow derived cells, and most likely by neutrophils, was implicated in the early survival and establishment of lung metastatic foci in mice following i.v. inoculation of human lung carcinoma cells (Acuff et al. 2006a).

7.11 Contribution of MMPs to Primary Tumor Self-Seeding: Another Extravasation Event

Recent studies from Massague's laboratory provided experimental evidence for the self-seeding concept in cancer progression (Kim et al. 2009). In particular, this concept accommodates the view that primary tumors can be reinfiltrated by circulating tumor cells (CTCs), which extravasate back to the primary tumor bed after their initial escape, intravasation, and circulation (Norton and Massague 2006). Self-seeding by aggressive breast cancer CTCs was demonstrated for several tumor types, including breast and colon carcinomas and melanoma tumors developing in mice (Kim et al. 2009). Similar to the gene signatures associated with organmetastasis to the lungs, bone, and brain (Nguyen and Massague 2007; Bos et al. 2009; Nguyen et al. 2009), the MMP1 gene was identified in a gene signature associated with the propensity of breast carcinoma CTCs to efficiently reenter their primary tumors. Together, MMP-1 and fascin-1 expressed by breast cancer CTCs act as mediators of transendothelial migration involved in tumor self-seeding. The MMP-1/fascin-1-mediated extravasation of CTCs into the tumor of origin, in turn, is driven by chemoattractants, such as IL-6 and IL-8, produced by the primary tumor. Complementing this vicious cycle, the reinfiltrated CTS accelerate growth of the original tumor, enhance tumor angiogenesis, and induce recruitment of neutrophils and macrophages through "seed-derived" factors such as CXCL1, a mediator of leukocyte influx into sites of inflammation (Kim et al. 2009).

An important implication of the above-described findings is that MMP-1-expressing breast cancer cells might be a part of the mechanism for local recurrence following tumor excision (Kim et al. 2009). It would imply that enhanced MMP-1 expression might be part of a signature in primary tumors from patients that will develop local recurrence. However, a recently published analysis of MMP/TIMP expression in breast cancer patients who have undergone mastectomy demonstrated lower expression levels of several MMPs, including MMP-1, MMP-7, and MMP-9, in primary tumors from those patients who developed local recurrence compared with those without local recurrence (Del Casar et al. 2010). These data may indicate that a subset of aggressive MMP-expressing tumor cells responsible for recurrence could be very small and would not show up in a straightforward signature of primary tumors in patients. The patient data might also indicate that MMP/TIMP signatures in the primary tumors may not be predictive of mechanisms or events that occur later in the metastatic cascade.

7.12 Involvement of MMPs in the Establishment of Metastases at the Secondary Site

The fact that millions of cells could be shed from primary tumors but only a few are capable of establishing metastases led to the notion that spontaneous metastasis via hematogenous route could be a very inefficient process (Bockhorn et al. 2007). This inefficiency is attributed in part to the low rate of cell survival in the circulation and the possibility that extravasated cells enter a dormant state in the unfavorable environment of the secondary organ (Suzuki et al. 2006). Based on the failure of approximately 98% of solitary tumor cells to initiate growth in secondary sites and the failure of a majority of micrometastases to give rise to secondary tumors, there is a notion that the transition of micrometastases to macrometastases and the maintenance of metastatic growth into clinically relevant metastases are the only principal rate limiting steps in the whole metastatic cascade (Croker and Allan 2008). If the dormancy is overcome and spontaneously metastasized cells initiate proliferation, MMPs could be active players in communications between metastatic tumor cells and the stroma of the secondary site (Cairns et al. 2003). The importance of stromal MMPs in influencing the fate of extravasated cells was initially demonstrated in an experimental metastasis model, where colonization of intravenously injected melanoma and lung carcinoma cells was significantly inhibited in MMP-2 knock-out mice (Itoh et al. 1998).

During spontaneous metastasis, however, the proteolytic functions of tumor MMPs in organ-specific homing and the establishment of secondary lesions remain poorly defined despite their well-appreciated role in matrix degradation and tumor invasion. The expression of a secreted collagenase, MMP-1, at both the gene and protein levels, was repeatedly associated with the propensity of MDA-MB-231 cell variants to specifically metastasize to lung, brain, and bone (Kang et al. 2003; Minn et al. 2005a, b; Bos et al. 2009). Since these cell variants were originally derived following experimental metastasis, thereby bypassing early steps of the metastatic cascade, the requirement of MMP-1 expression in the selected breast carcinoma cells capable of distant metastasis likely indicated the involvement of this MMP either during extravasation at distant sites or during reseeding of primary tumors (as discussed in the previous section) or during establishment of the micrometastatic foci. By RNA interference, MMP-1 in conjunction with other factors was demonstrated to functionally mediate tumor angiogenesis and extravasation of circulating tumor cells from the lung capillaries (Nguyen and Massague 2007). In the case of the bone-homing variant of the breast carcinoma MDA-MB-231 cell line, MMP-1 also was shown to orchestrate a paracrine signaling cascade modulating bone microenvironment to favor osteoclastogenesis and breast cancer metastasis (Lu et al. 2009).

The search for genetic determinants of cancer metastasis in patients reconfirmed the presence of MMP1 gene in the 18-gene signature associated with breast cancer metastatic disease. MMP1 expression was also validated in the set of 63 genes associated with the progression and metastasis of advanced cervical cancers (Harima et al. 2009). On the other hand, extensive gene expression profiling of primary carcinomas of diverse types vs. metastases, performed in search of universal genetic differences predictive of metastases in patients, did not identify any genes encoding for MMPs within the refined gene-expression signature associated with metastasis and poor clinical outcome (Ramaswamy et al. 2003). Profiling of genes differentially expressed between human bladder cancer cell lines sequentially selected for increased ability to colonize mouse lungs after i.v. inoculations did not identify any MMP genes which would individually be associated with increased metastatic potential (Nicholson et al. 2004). Once again, it has to be considered that gene expression profiling may not yet be optimal for characterizing relationships between primary tumors and metastasis (Klein 2009). In the case of MMP gene signatures, this indeed has to be taken into account.

In the microenvironment of the secondary organ, individual MMPs can actually suppress the development of metastases. Thus, MMP-11 (stromelysin 3) was shown to function as a repressor of distal invasion of spontaneously disseminating breast cancer cells from mammary tumors developing in the MMP-11 deficient transgenic MMTV-ras mice (Andarawewa et al. 2003). Remarkably, the same MMP-11 functions as an inducer of local invasion in the primary tumors, suggesting the contribution of other metastasis mechanisms leading to cancer progression in this model system. In addition, in an experimental metastasis model, the elimination of MMP-12-mediated production of the antiangiogenic peptide, angiostatin, in MMP-12-deficient mice was implicated in elevated angiogenesis and increased size of tumor colonies developing from extravasated Lewis lung carcinoma cells (Acuff et al. 2006b).

At the metastatic site, tumor cells are believed to undergo mesenchymal–epithelial transition (MET), which is regarded as an important event in the metastatic cascade (Chaffer et al. 2007; Hugo et al. 2007). Along with the overall reversal nature of MET features such as reversal toward reexpression of E-cadherin (Auersperg et al. 1999; Wells et al. 2008), it is plausible to suggest that MMPs, which have been initially induced during EMT at the primary tumor and overexpressed during early invasion steps, would be downregulated during the establishment of metastases as the colonizing cells undergo MET. In agreement with this view, a comprehensive comparative analysis performed to identify molecular signatures associated with nodal metastasis in breast cancer demonstrated significant downregulation of MMP-10 (stromelysin 2), MMP-13, and MT3-MMP in lymph node tumors vs. corresponding primary breast tumors. Furthermore, genes expressed at higher levels in metastases were mainly those involved in transcription, signal transduction, and immune response, providing metastasized cells with proliferation and survival potential (Ellsworth et al. 2009).

7.13 MMPs and Formation of the Premetastatic Niche

The mechanisms by which MMPs assist the arrival, survival, and proliferation of disseminating tumor cells to the secondary site involve the formation of premetastatic niches at distant organs. The original niche model has postulated the existence of a specific microenvironment locale that maintains and regulates stem cells (Morrison and Spradling 2008). A relatively novel model for metastatic niches suggests the existence of a suitable conducive microenvironment that allows for tumor cell engraftment and micrometastatic-to-macrometastatic transition (Psaila and Lyden 2009). The induction of new niches or the adaptation of preexisting niches is now regarded as a central mechanism of tumor cell metastasis to distant organs.

Despite considerable progress in elucidating the underlying mechanisms of niche formation, the knowledge of functional contribution of specific MMPs in this process is mainly limited to the initial findings demonstrating the involvement of MMP-9 (Kaplan et al. 2005). In the premetastatic lung, VEGFA specifically induces MMP-9 expression in endothelial cells and also attracts MMP-9-producing MAC1-positive and VEGFR 1-positive myeloid cells (Hiratsuka et al. 2002; Kaplan et al. 2005). The proteolytic activity of MMP-9 in turn is believed to release growth factors and cytokines, including soluble Kit ligand, which recruits tumor cells and additional BMDCs that express c-Kit, the soluble Kit receptor. The central role played by MMP-9 in these processes was demonstrated by a genetic approach whereby the suppression of MMP-9 induction by using MMP-9 null mice significantly impaired niche formation and tumor engraftment (Hiratsuka et al. 2002; Kaplan et al. 2005).

Priming of premetastatic niches for tumor engraftment should involve local MMP-mediated tissue remodeling which has been confirmed in several model

systems. Thus, proteolytic degradation of vascular BMs in the premetastatic sites was demonstrated in the lungs of mice orthotopically implanted with human breast cancer cells (Erler et al. 2009). Specifically, lysyl oxidase secreted by primary tumor cells was shown to accumulate at premetastatic sites and cross-link type IV collagen, facilitating the recruitment of BMDC and their production of MMP-2. Myeloid cell-derived MMP-2 in turn cleaves collagen, thereby enhancing homing of spontaneously metastasizing tumor cells to prepared metastatic sites (Erler et al. 2009). In a separate model, it was another gelatinase, i.e., MMP-9 produced by a subpopulation of CCR5-positive mesenchymal cells, that was shown to cause changes in the stroma of premetastatic lung and functionally contribute to melanoma B16/F10 engraftment (van Deventer et al. 2008). It has also been reported that the primary B16/F10 melanoma tumor upregulates MMP-3 and MMP-10 and angiopoetin 2 in the lung at the premetastatic stage, leading to the increased permeability of pulmonary vasculature and subsequent extravasation of circulating tumor cells (Huang et al. 2009). In an orthotopic model of breast cancer, knocking down of MMP-3, MMP-10, and angiopoetin 2 significantly inhibited spontaneous lung metastasis of an MDA-MB-231 cell variant in nude mice (Huang et al. 2009). Therefore, MMP-mediated vascular destabilization is likely to constitute an important mechanism in the formation of premetastatic niches, where extravasation of tumor cells is specifically promoted to facilitate the establishment of metastatic lesions.

7.14 Involvement of MMPs in Cancer Stem Cell Mobilization and Homing

With the recognition of CSCs and their role in tumor progression, many of the questions posed for the roles of MMPs in tumor cell invasion and metastatic dissemination have been applied to CSC biology. It has also been suggested that some aspects of CSC physiology could be similar to those demonstrated for normal stem cells and therefore might involve already established cellular mechanisms, including MMP-mediated stem cell release, trafficking, and homing (Kucia et al. 2005; Li and Neaves 2006).

Mobilization of BMDCs and hematopoietic stem cells (HSCs) from their quiescent niches in bone marrow has been shown to critically depend on the enzymatic activity of MMP-9. It was demonstrated that MMP-9 proteolytically releases soluble Kit ligand, thereby promoting the recruitment of c-Kit-positive stem/BMDCs in mice (Heissig et al. 2002). During bone marrow ablation, MMP-9-mediated release of Kit ligand has been shown to be dependent on activation of proMMP-9 by tissue-type plasminogen activator, indicating that MMP-9 can be a downstream target molecule in the plasminogen activation cascade (Heissig et al. 2007).

The physiology of HSCs is critically dependent on their interaction with stromal matrix and stromal cells, including multipotent stromal cells (MSCs), frequently

referred as to mesenchymal stem cells. MSCs, originally isolated from bone marrow as stromal progenitor cells with high proliferative and differential potentials (Pittenger et al. 1999), have no self-renewal capacity and therefore do not meet stringent criteria for functional stem cells. Mobilized into the circulation, MSCs were demonstrated to infiltrate numerous secondary sites, including primary tumors and metastatic sites, where they integrate into tumor stroma and vasculature, differentiate into cells of different lineages, and affect progression of carcinogenesis (Lazennec and Jorgensen 2008).

Several MMPs have been implicated in different aspects of MSC physiology. Inflammatory cytokines upregulate MMP-2, MT1-MMP, and MMP-9 production in MSCs, thereby providing potential mechanism for MSC recruitment and extravasation (Ries et al. 2007). Passage through BM may involve MT1-MMP and MMP-2, individual knock-down of which substantially impaired MSC invasion through Matrigel (Ries et al. 2007). Differentiation of MSCs into osteoblasts is accompanied by the upregulation of MMP-2, MMP-3, MMP-8 (neutrophil collagenase), MMP-13, and MT1-MMP (Parikka et al. 2005; Neth et al. 2006; Kasper et al. 2007), but functional contribution was validated by RNA silencing for only MMP-13 (Kasper et al. 2007) and MT1-MMP (Lu et al. 2010).

Considering that the expression and proteolytic activity of MMPs and especially of MT1-MMP is required for highly invasive tumor cells, it has been expected that MMPs would also regulate metastatic dissemination of CSCs as they traffic in vivo. The majority of studies addressing physiology of CSCs, however, employ established tumor cell lines exhibiting some levels of stem cell markers (such as CD133) and the ability to form spheres in vitro. The efficiency of sphere formation and sphere invasiveness in 3D matrices are used operationally as a measure of "stemness" of such tumor cell lines. Thus, neurosphere formation by the medulloblastoma DAOY cell line was accompanied by increased gene expression of CD133, MT1-MMP, and MMP-9 (Annabi et al. 2008). Indicating the functional importance of these two MMPs, gene silencing of either MT1-MMP or MMP-9 by siRNA reduced the formation of neurospheres and concomitantly abrogated their invasion in vitro, while overexpression of MT1-MMP triggered neurosphere formation and concomitant activation of proMMP-2. In a similar fashion, neurospheres formed by glioma U87 cells selected for high levels of CD133⁺ had enhanced the expression of MT1-MMP, which was functionally important for COX-2 or S1P signaling (Annabi et al. 2009a, b). These findings allowed for the suggestion that increased MT1-MMP might promote brain tumor infiltration, whereas the increase in MMP-9 may contribute to the opening of the blood-brain barrier (Annabi et al. 2008), although it is debatable how much the behavior of spheres generated by cells from established tumor cell lines reflects the actual physiology of CSCs.

Circulating stem cells are believed to release MMPs that will enable them to cross the endothelial barrier and home to distant organs (Kucia et al. 2005). It has been shown that human bone marrow and peripheral blood CD34⁺ cells comprising a subset of stem cells manifest a differential production of MMPs and TIMPs in response to chemokines such as SDF-1, MIP-1 α , and IL-8, driving their invasion through the subendothelial BMs in vitro (Janowska-Wieczorek et al. 2000).

However, it is still unclear which MMPs might be differentially produced by functional CSCs to specifically support their extravasation during metastasis in vivo. Since trafficking and homing of CSCs is governed by the CXCR4/SDF-1 axis, it is possible that MMPs directly induced by SDF-1 or delivered by SDF-1-responsive, CXCR4-positive cells could be those MMPs mechanistically assisting in invasion/intravasation and extravasation/colonization of noncancerous stem cells (Lapidot et al. 2005; Li and Neaves 2006). In this regard, MMP-9 is induced in HSC niches in bone marrow (Heissig et al. 2002) or in premetastatic niches at the secondary site (Kaplan et al. 2005) in an SDF-1-dependent fashion.

7.15 Selective Inhibition of Specific MMPs vs. Overall Inhibition of MMP Functions in Metastasis

The pleiotropic nature of MMPs in cancer, reflected in the promoting or inhibiting activities of different MMPs or by the dual functionality of individual MMPs, might lead to uncertain outcomes when MMPs are blocked with broad-range, indiscriminatory inhibitors (Kruger et al. 2009). Therefore, the development of inhibitors, which would target the MMP of interest with high selectivity and potency, has become a priority in pharmacological intervention into metastatic disease (Ra and Parks 2007; Morrison et al. 2009). In addition, inhibitors specifically targeting proteolytic functions of MMPs involved in a particular physiological process (e.g., inflammation) or produced by particular types of cells (e.g., inflammatory cells) have become a focus of recent therapeutic approaches (Hu et al. 2007).

To determine which individual steps of the metastatic cascade are specifically affected by MMP blocking, a complex, spatiotemporal analysis of inhibitory effects in several independent in vivo models is required. Recently, such analysis was performed during validation of a highly selective, fully human recombinant MT1-MMP inhibitory antibody DX-2400 that specifically blocks the catalytic activity of MT1-MMP (Devy et al. 2009). Discriminatory blockage of MT1-MMP by DX-2400 significantly slowed tumor growth and angiogenesis and significantly reduced the levels of gelatinolytic activity in the MDA-MB-231 s.c. xenografts. Blocking MT-MMP also retarded the formation of metastatic lesions in the lungs and liver when MDA-MB-231 cells were implanted orthotopically, suggesting that the early steps of metastatic dissemination were targeted by DX-2400. In addition, examination of lung colonization after inoculation of B16F1 murine melanoma cells indicated that the late steps of metastatic cascade were also susceptible to the inhibitory effects of DX-2400 antibody. Most alarming, however, was the demonstration that the gelatinolytic activity in xenografts of mice that received a combination of DX-2400 and the anti-VEGF blocking antibody bevacizumab was not inhibited. Such lack of inhibition was due to an unexpected fourfold increase in gelatinase activity in the xenografts caused by VEGF blocking (Devy et al. 2009), emphasizing that the inhibition of MMP functions amidst complex anticancer therapy should be implemented with extreme caution.

7.16 Emerging Perspectives

Rapidly emerging concepts of cancer progression have illuminated new, unexpected mechanisms, which might govern metastatic disease along different pathways, thereby challenging the conventional views. One such notion is based on findings demonstrating the presence of cancer cells in distant organs at very early stages of cancer progression or even in the absence of detectable primary tumors (Pantel and Brakenhoff 2004; Dalerba et al. 2007; Husemann et al. 2008). This point of view challenges the widely accepted prevailing scenario postulating that dissemination of metastatic cells occurs from well-established, invasive primary tumors, where aggressive tumor cells and engaged stromal cells are endowed over time with the traits necessary for ensuing metastasis, including disregulated MMPs. New studies might validate functional, proteolytic roles of MMPs in the very early dissemination of metastatic cancer cells from those primary sites which even lack visual signs of EMT, invasive fronts or angiogenesis.

A special emphasis may also be placed on addressing whether individual MMPs other than MMP-9 contribute to the formation of premetastatic niches especially if the size of the primary tumor is negligible or too small to induce cytokines and other factors at systemic levels necessary for recruitment of MMP-9 delivering BMDCs to distant sites. Clearly, the establishment of a niche in a premetastatic site will require tissue or matrix remodeling and likely some proteolytic activity of yet-tobe-defined enzymes. In addition, validation of functional roles of specific MMPs in the important transition of micrometastases into macrometastases could constitute a focus of studies aimed to elucidate this rate-limiting, final step of the metastatic cascade (Croker and Allan 2008). These roles might include MMP-triggered neovascularization and matrix remodeling at the secondary site probably by MMP-delivering or MMP-producing BMDCs that influx into metastatic foci and facilitating their sustained growth.

Tumor self-seeding (Kim et al. 2009) is another recent notion in cancer progression, which could represent a general phenomenon during metastasis. This model illuminates the possibility that aggressive CTCs that had escaped from a fairly developed tumor can reseed the primary site and establish therein a selected subset of highly aggressive tumor cells responsible for overt metastases and local recurrence. This model also points to reseeding CTCs as a possible source of those heretofore undefined cells that could actually be responsible for recruitment of bone marrow-derived progenitor cells to developing primary tumors. In addition, this model also raises the possibility that CTCs might reenter their original and familiar microenvironment and therefore not slip into dormancy, but on the contrary, actively proliferate and induce MMP-dependent stroma remodeling through either their own MMPs, such as MMP-1 [already identified in the reseeding signature (Kim et al. 2009)] and MT1-MMP (one of the important cell surface tethered tumor MMPs), or stroma-engaged MMPs, such as MMP-9 produced by newly recruited inflammatory neutrophils and macrophages. The functional contribution of both tumor and stromal MMPs in granting the aggressive CTCs access back to the primary tumor thereby reinforcing its malignant behavior will definitely constitute the scope of upcoming research.

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Chapter 8 Degradation of Bone and the Role of Osteoclasts, Bone Lining Cells and Osteocytes

Vincent Everts and Paul Saftig

Abstract Resorption of bone depends on the combined action of osteoblast-like bone lining cells and osteoclasts. The bone lining cell plays an essential role in modulating the formation of the osteoclasts and in the mean time prepares the bone surface for these multinucleated bone resorbing cells to attach to. The osteoclast attaches to the bone and the resorption starts with lowering the pH at the resorption site, a site that is secluded from the rest of the bone surface and the surrounding environment. The lowered pH results in dissolution of the mineral component, thus exposing the matrix. The bone matrix is subsequently digested by proteolytic enzymes, among which the cysteine proteinase cathepsin K appears to be essential. Recent findings indicate that not all osteoclasts are alike, different bones may harbour osteoclasts that differ in several aspects, among which the proteolytic enzymes used for resorption. In this chapter we will discuss the different steps that result in the degradation of bone, the onset of new bone matrix deposition and the presence of different osteoclasts at various bone sites.

Abbreviations

AE2	Anion exchanger 2
CC19	Chemokine ligand 9
CIC7	Chloride channel 7
DC-STAMP	Dendritic cell-specific transmembrane protein

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ICAM-1	Intercellular-associated molecule 1
IL-1, -6	Interleukin-1, -6
LAMP-2	Lysosome-associated membrane protein 2
LFA-1	Leukocyte function-associated protein 1
M-CSF	Monocyte colony stimulating factor
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T cells
NHA2	Sodium-proton antiporter 2
NO	Nitric oxide
OPG	Osteoprotegerin
PA (tPA, uPA)	Plasminogen activator (tissue, urokinase)
PEDF	Pigment epithelium derived factor
PGE ₂	Prostaglandin E ₂
PTH	Parathyroid hormone
RANK(L)	Receptor activator nuclear kappa (ligand)
ROS	Reactive oxygen species
SlC4a4	Solute carrier 4a4
Spp24	Secreted phosphoprotein 24
TGFβ	Transforming growth factor β
ΤΝFα	Tumour necrosis factor α
TRACP	Tartrate-resistant acid phosphatase
V-ATPase	Vacuolar ATPase

8.1 Introduction

Bone is a unique tissue since it not only consists of extracellular matrix constituents like collagen, but also contains high levels of the mineral hydroxyapatite. Degradation of bone requires therefore cells that have the capacity to digest both mineral and collagen. The multinucleated osteoclasts are equipped for this task. These cells are generated locally at the site where resorption has to occur, a process organized and modulated by osteoblast-like cells, the bone lining cells. Mononuclear osteoclast precursors are attracted by the bone lining cells, attach to these cells and due to the interaction with the bone lining cells, transform into osteoclasts. In the mean time the bone lining cells clean the mineralized bone surface from non-mineralized collagen fibrils thus paying way for the osteoclast to attach to the bone. The osteoclast firmly adheres to the bone surface and isolates an area of this surface from the rest of the extracellular environment. In this secluded area the actual resorption takes place. First, the pH is lowered due to the release of H⁺ by proton pumps present in the resorption membrane. The lower pH results in dissolution of the mineral component of the bone. Next, proteolytic enzymes are secreted in the resorption area. These proteolytic enzymes, of which cathepsin K is one of the most

important ones, digest the different matrix components. Following resorption, the osteoclast migrates to another area and the bone lining cells enter the site left by the osteoclast. The bone lining cell cleans the bottom of the pit and deposits a thin layer of proteins after which osteoblasts start with the deposition of new bone. In this chapter we will discuss each of the different steps that result in the degradation of bone and the onset of new bone matrix deposition.

8.2 Definition of the Different Bone-Associated Cells

In this chapter we will use the following definitions for the different cell types:

Osteoclast is a multinucleated cell that resorbs mineralized tissues, such as bone. *Osteoblast* is the cell type that actively deposits bone and it has a cuboidal shape. These cells cover the osteoid layer of newly formed bone.

- *Bone lining cell* is the cell localized at the site where (1) no bone remodelling activity is found or (2) resorption will occur or (3) resorption is occurring. This cell is osteoblast-like since it expresses relatively high levels of the enzyme alkaline phosphatase but is has a flattened morphology and covers the bone surface from which some non-mineralized bone collagen fibrils protrude.
- *Osteocyte* is derived from the osteoblast and is, during the formation of bone, incorporated in bone and finally completely embedded within this tissue.

8.2.1 The Bone Lining Cell and the Attraction of Osteoclast Precursors

8.2.1.1 The Role of the Osteocyte in Steering the Site Where Resorption Has to Occur

Of all bone-associated cell types far out the most frequently occurring one is the osteocyte; being approximately 80–90% of all bone cells. In the past the osteocyte was not considered to have an important function in processes related to bone remodelling. Nowadays this view has changed dramatically. Ample data indicate an essential role of the osteocyte in steering both resorption and deposition of bone (Burger et al. 1995; Bonewald and Johnson 2008).

The osteocytes are surrounded by bone tissue and their cell bodies are localized in lacunae. The cells contact each other through small channels, canaliculi, in which their numerous cell extensions are running. The osteocyte has been proved to be extremely sensitive to small changes in strain exerted upon it. Differences in strain are sensed by the osteocyte by the flow of fluid surrounding the cellular extensions and the cell body. This results in synthesis and secretion of a variety of signaling molecules, like nitric oxide (NO), prostaglandins (e.g., PGE₂) and sclerostin (Klein-Nulend et al. 1997). The different signaling molecules then modulate the activity of cells at the bone surface. Formation of bone is modulated by PGE_2 and sclerostin, NO inhibits the activity of osteoclasts. The latter cells are attracted to the resorption site by signals sent by apoptotic osteocytes (Bronckers et al. 1996; Burger et al. 2003; Gu et al. 2005). Also a direct cell–cell contact between osteocyte extensions and the bone lining cells occurs and probably results in a localized activation of the bone lining cell to attract the osteoclast precursor.

8.2.1.2 Chemokines Attract the Mononuclear Osteoclast Precursors

The bone lining cells secrete chemokines, like CCl9, that attract mononuclear precursors of osteoclasts (Yu et al. 2004). The release of the chemokines probably results in the activation of the endothelial cells of blood vessels in the near vicinity of the bone surface. The activated endothelial cells then attract the mononuclear cells (e.g., monocytes) that are primed to become osteoclasts. These monocytes leave the blood vessel and migrate towards and attach to the bone lining cell.

8.2.2 The Generation of Osteoclasts and the Role of the Bone Lining Cell

8.2.2.1 Interaction of the Precursor with the Bone Lining Cell Induces Essential Modifications

The attachment of the mononuclear osteoclast precursor is mediated by intercellular cell-associated molecule 1 (ICAM-1) expressed by the bone lining cell and leukocyte function-associated protein 1 (LFA-1) present on the membrane of the mononuclear cell (Gao et al. 2000; Kurachi et al. 1993; Tanaka et al. 1995; Bloemen et al. 2009). Following this attachment, the interaction between the two cell types results in significant changes in gene expression of each cell type. The expression of genes associated with attachment, like ICAM-1, increases synergistically (Bloemen et al. 2010). A similar phenomenon is also found for the expression of genes responsible for the formation of the multinucleated osteoclasts, RANK, RANKL and M-CSF. In the mean time OPG is strongly upregulated. Together these findings suggest that an efficient osteoclastogenesis occurs when the cell is in direct contact with the bone lining cell. In the mean time a high level of OPG prevents formation at sites where such a close cell-cell relationship does not occur. This cell-cell interaction eventually results in the formation of tartrate-resistant acid phosphatase (TRACP) positive mononuclear osteoclasts (Perez-Amodio et al. 2004).

8.2.2.2 Role of RANK, RANKL and OPG

As indicated in Sect. 8.2.2.1, molecules like RANKL and RANK play an important role in the generation of osteoclasts (Khosla 2001). RANKL is expressed by the bone lining cell and its ligand RANK by the mononuclear precursor. Following the initial attachment mediated by ICAM-1/LFA-1 the synthesis of RANKL and RANK is induced. In the microenvironment created by the close interaction of the two cell types, RANKL and RANK bind to each other which results in a series of intracellular signaling activities that steer the mononuclear cell in the direction of the osteoclast (Teitelbaum 2007). This transition is characterized by a sequence in the expression of different molecules like NFAT, DC-STAMP, cathepsin K, calcitonin receptor, and TRACP. Several of these proteins (e.g., TRACP, calcitonin receptor, cathepsin K) are relatively unique for the osteoclast. The RANKLmediated osteoclastogenesis is blocked by the ligand OPG. OPG is produced by the bone lining cells; it binds to RANKL and prevents thereby RANKL-RANK interaction. In this way OPG plays an essential role in limiting formation of osteoclasts; high expression results in a low number of osteoclasts, whereas low expression promotes osteoclastogenesis (Yasuda et al. 1998).

When the mononuclear precursor has been transformed into a mononuclear osteoclast that expresses enzymes like cathepsin K and TRACP, it induces the withdrawal of the bone lining cell (Perez-Amodio et al. 2004). This process of withdrawal not only does make space for the osteoclast at the bone surface to attach to, but also pin points the site where resorption is needed.

8.2.3 Preparation of the Bone Surface Prior to Osteoclastic Resorption

8.2.3.1 Resorption of Collagen Fibrils by Bone Lining Cells

During the process of withdrawal from the bone surface the non-mineralized components like collagen fibrils that still protrude from the surface have to be removed (Chambers and Fuller 1985); the osteoclast attaches to the mineral component of the bone surface. Removal of these proteins by mammalian collagenase was shown to induce attachment of the osteoclast and subsequent resorption (Chambers et al. 1985); without this cleaning activity osteoclasts do not or hardly adhere to the bone surface. In subsequent ultrastructural studies the digestion of the collagen fibrils protruding from the surface was shown to involve engulfment of these fibrils by cytoplasmic extensions of the bone lining cell (Everts et al. 2002; Takahashi et al. 1986a, b) (Fig. 8.1). Digestion of the fibrils at this site proved indeed to be metalloproteinase (MMP)-dependent (Everts et al. 2002), a class of enzymes that includes the collagenases. Although the exact MMP involved in this cleaning process has not been elucidated yet, several findings suggest a role for

Fig. 8.1 A bone lining cell engulfs non-mineralized collagen fibrils protruding from the bone surface and cleans this surface prior to osteoclastic bone degradation at that site



MMP-13 (Behonick et al. 2007; Inada et al. 2004) and/or MMP-14 (Andersen et al. 2004) herein.

The removal and degradation of the collagen fibrils probably results in the release of small fragments. Since Holliday et al. (1997) and more recently Eck et al. (2009) demonstrated that MMP-1 and the fragments of type I collagen generated by the activity of this enzyme had an activating and differentiating effect on osteoclasts and their precursors, it is tempting to suggest that such a process occurs on the bone surface and is mediated by the action of the bone lining cells.

8.2.4 Attachment of the Osteoclast to the Bone Surface

8.2.4.1 Formation of Sealing Zone and Ruffled Border

Attachment of the osteoclast to the bone surface depends on the presence of the integrin $\alpha_v \beta_3$, which is highly expressed by these cells (Helfrich et al. 1996; Lakkakorpi et al. 1991) (Fig. 8.2). The integrin attaches to proteins like osteopontin (Horton et al. 1995), a protein abundantly present in bone. The moment the osteoclast is attached to the bone, the cell starts expressing some unique properties. The cell becomes highly polarized and forms first the so-called sealing zone, an area characterized by a high density of actin filaments and attachment sites. This zone more or less creates in its centre an area adjacent to the osteoclast that is secluded from the rest of the extracellular environment. The central area thus created is characterized by deeply indented membrane folds and is called the ruffled border (Fig. 8.3). This membrane is formed by fusion of lysosomal vacuoles with the outer membrane and is considered a secondary lysosome. Proteins characteristic for the lysosomal membrane protein 1 (LAMP-1), LAMP-2 and V-ATPase (reviewed in (Everts and Beertsen 2005)).

Adjacent to this membrane the actual resorption takes place, first by lowering the extracellular pH and then by secreting proteolytic enzymes in this area. Studies by

Fig. 8.2 Localization of the integrin $\alpha_v \beta_3$ in osteoclasts generated in vitro by culturing bone marrow cells in the presence of M-CSF and RANKL. Note the very strong staining of the membrane of the multinucleated osteoclast. Nuclei are *blue* (Micrograph donated by Carmeliet and Torrekens, Leuven, Belgium.)



Fig. 8.3 Electron micrograph and a schematic overview of an osteoclast with its functional membrane parts involved in the resorption of bone and the subsequent transport of matrix fragments through transcytosis from the ruffled border site to the functional secretory domain (FSD). See (Mulari et al. 2003b; Palokangas et al. 1997; Salo et al. 1997) for detailed descriptions of the different functional domains of an osteoclast. SZ sealing zone, RB ruffled border, Ca mineralized cartilage, Bo bone, N: nucleus [From Everts et al. (2009).]



the group of Väänänen (Mulari et al. 2003a, b; Palokangas et al. 1997; Salo et al. 1996, 1997) have shown the presence of different functional regions of the ruffled border membrane. The middle portion of the ruffled border appears to be specialized in the uptake of partially digested constituents after which these components are

transported through the cytoplasm to the opposite part of the cell (Salo et al. 1997), the functional secretory domain ((Mulari et al. 2003b), FSD; Fig. 8.3). The parts of the ruffled border that surround the endocytic central part are primarily involved in secretion of enzymes needed for resorption (Mulari et al. 2003a).

8.2.5 Lowering of the pH Results in Dissolution of Mineral

8.2.5.1 Role of Different Membrane Pumps in the Ruffled Border

Lowering of the pH depends on the presence of specialized pumps in the ruffled border membrane. The V-ATPase is the membrane-bound pump that releases H^+ into the extracellular space. The proton is derived form carbonic acid which is converted by carbonic anhydrase II into bicarbonate and H^+ . To achieve electroneutrality, a chloride channel (ClC7) transports Cl^- into the extracellular area facing the ruffled border. Also ClC7 is localized in the membrane of the ruffled border (Hall and Chambers 1989; Karsdal et al. 2005; Kornak et al. 2001). Each of the three molecules mentioned, V-ATPase, carbonic anhydrase II and ClC7, have been shown to be crucial for the proper functioning of the osteoclast and thus for the degradation of bone. In the absence of each of these proteins the osteoclast can no longer degrade the bone, resulting in osteopetrosis (Helfrich 2003). Under these conditions osteoclasts are formed in normal or even higher numbers, they attach to the bone, but they do not form a ruffled border which is essential for the resorption to occur (Fig. 8.4).

8.2.5.2 Role of Pumps Localized in the Baso-Lateral Membrane

Secretion of H^+ and the presence of increased levels of bicarbonate severely influence the intracellular pH. To create a physiological intracellular pH,

Fig. 8.4 Light micrograph of a bone biopsy of a patient suffering from osteopetrosis. Note the large number of osteoclasts (OC) but absence of active resorption by these cells [From Everts et al. (2009).]



Fig. 8.5 Immunolocalization of anion exchanger 2 (Ae2) in the baso-lateral membrane of a mouse osteoclast. Note the high level of staining exclusively present in the baso-lateral membrane. The resorption zone (RB) is devoid of the exchanger (Micrograph donated by Bronckers, ACTA, Amsterdam, The Netherlands.)



bicarbonate has to be extruded from the cell and in the mean time Cl^- has to be transported into the cell. For this activity an anion exchanger (AE) is necessary. Recent findings indicate that of the four known anion exchangers, AE2 is the one used by the osteoclast for this activity (Josephsen et al. 2009; Wu et al. 2008; Jansen et al. 2009). The exchanger is localized in the baso-lateral membrane of the osteoclast (Fig. 8.5), and in its absence, the osteoclast does not form a ruffled border (Jansen et al. 2009), a phenomenon also seen with the other pH-modulating proteins as mentioned in 8.2.5.1.

Of considerable interest is the finding that in mice, osteoclasts at different bone sites appear to differ with respect to the exchanger they use. Osteoclasts that harbour long bones depend solely on $Ae2_{a,b}$, whereas those present in the skull use next to this exchanger also the sodium transporter SIC4a4 (Jansen et al. 2009). The differences among osteoclast subpopulations will be discussed in more detail below (see Sect. 8.2.6.5).

8.2.5.3 Effect of pH Changes on Osteoclastic Activity

Slight changes in the pH of the environment greatly affects the activity of osteoclasts (Arnett 2003, 2008). A lower pH simulates resorption significantly. Muzylak et al. (2007) found that a relatively small decrease in pH, from pH 7.25 to 7.15, resulted in a highly significant increase in the size of the osteoclasts. Under these conditions osteoclasts were eight times larger and contained more nuclei. Others have shown that the level of resorption is related to the size of the osteoclast; the larger the osteoclast is, the more bone is resorbed (Hu et al. 2008; Lees et al. 2001). So, it appears that the size of the osteoclast and thus the fusion of mononuclear precursors are modulated by the pH. Somehow the cell senses changes in pH and these changes highly influence the osteoclast formation and subsequent bone degradation.

8.2.6 Release of Enzymes Results in Digestion of the Bone Matrix

Next to the dissolution of the mineral of bone, the second major constituent, the matrix, has to be digested. The matrix of bone contains a variety of proteins, of which collagen type I is the one far out the most abundantly present. Some proteins that are relatively bone specific are bone sialoprotein, osteocalcin and osteopontin. These proteins play an essential role in the modulation of mineral crystal formation (Jahnen-Dechent et al. 2008). Finally, the matrix contains a relatively low level of proteoglycans. All these components are digested in the ruffled border area by a variety of enzymes of which the proteolytic enzymes are probably the most important ones.

The proteolytic enzymes which have been shown to be functional in the process resulting in the degradation of the matrix are members of the cysteine proteinases, the matrix metalloproteinases and the serine proteinases (Delaisse et al. 2000). Next to enzymes essential for protein degradation, a high level of TRACP is secreted in the ruffled border area. The role of these different enzymes will be discussed in the following paragraphs.

8.2.6.1 Cysteine Proteinases, in Particular Cathepsin K, and Their Role in Bone Degradation

Delaisse et al. (1984) were the first to demonstrate the involvement of cysteine proteinases in osteoclast-mediated degradation of bone. These authors used selective inhibitors of this class of enzymes and demonstrated both in vivo and in vitro that blockage of the activity of cysteine proteinases resulted in a decreased degradation of bone.

What occurs at the cellular level in the absence of cysteine proteinase activity was found initially in bone biopsies obtained from patients suffering from the rare osteopetrosis-like disease pycnodysostosis. Adjacent to the osteoclasts of these patients a high amount of non-digested bone matrix was found ((Everts et al. 1985), Fig. 8.6). Demineralization proved to continue but the cells did not have the capacity to digest the matrix. This observation suggested that enzymes involved in matrix degradation were not expressed or not active. Subsequently by using selective inhibitors of cysteine proteinases in an in vitro bone explant system, it was shown that similar non-digested but demineralized areas were present next to actively resorbing osteoclasts (Fig. 8.7, (Everts et al. 1988)). Collectively these findings provided proof for the participation of cysteine proteinases in osteoclast-mediated bone matrix resorption.

In the mean time some groups detected that osteoclasts express a relatively unique member of the group of cysteine proteinases, cathepsin K, an enzyme that proved indeed to be essential for bone resorption by osteoclasts (Bromme et al. 1996; Xia et al. 1999). Mice deficient for this enzyme were characterized by a mild



Fig. 8.6 Osteoclasts in a bone biopsy obtained from a patient suffering from pycnodysostosis. Note the demineralized area (DA) adjacent to the ruffled border of the two osteoclasts [From Everts et al. (1985).]



Fig. 8.7 Mouse bone explants were cultured for 6 h in the presence of a selective inhibitor of cysteine proteinases and during this period the osteoclast demineralizes the bone but degradation of the matrix is blocked. Microscopic examination shows huge areas of demineralized bone matrix (DA) adjacent to the osteoclast. (a) Light micrograph of a section stained with the trichrome Goldner's stain. The *green areas* denote bone and the *orange* stained area is demineralized bone. (b) Electron micrograph of a comparable area. *Blc* bone lining cell, *Ocy* osteocyte, *RB* ruffled border, *DA* demineralized area

osteopetrosis, and adjacent to the osteoclasts areas of demineralized non-digested bone matrix were readily found (Fig. 8.8; (Saftig et al. 1998; Gowen et al. 1999), thus mimicking pycnodysostosis. Gelb et al. (1996) and Johnson et al. (1996) demonstrated that patients suffering from pycnodysostosis had a defect in the gene responsible for the expression of cathepsin K, as a consequence these patients lacked active cathepsin K. Finally, selective inhibitors and interference with the

Fig. 8.8 Demineralized area (DA) of bone matrix adjacent to an osteoclast in bone from a cathepsin K-deficient mouse. Section stained with the trichrome Goldner's stain. The *green areas* denote bone and the *orange* stained area is demineralized bone



expression of the enzyme resulted in a decreased resorption of bone (Xia et al. 1999; Gowen 1997; Ishikawa et al. 2001; Stroup et al. 2001). All data together clearly show the important role played by cathepsin K in osteoclast-mediated resorption of bone.

Not all parts of the skeleton, however, proved to be similarly affected when cathepsin K was inactive. The long bones showed a more severe osteopetrotic phenotype than the bones of the skull (Gowen et al. 1999). A plausible explanation for this difference in response is the finding that calvaria osteoclasts express a lower level of cathepsin K activity and use next to this enzyme other enzymes to digest the matrix of bone (Everts et al. 1999a), see below.

Recently it was shown that also other cysteine proteinases participate in bone resorption by osteoclasts (Everts et al. 2006). By using mice deficient for cathepsin B and/or cathepsin L, involvement of cathepsin B could not be shown but cathepsin L appeared to play a role in the degradation by modulating the activity of matrix metalloproteinases (MMPs). The use of a panel of enzyme inhibitors that were selective for different cysteine proteinases demonstrated that in addition to cathepsin K yet unknown cysteine proteinases are involved in osteoclast-mediated bone matrix degradation (Everts et al. 2006).

8.2.6.2 Matrix Metalloproteinases and Bone Matrix Degradation

In a series of studies by Delaisse et al. (1985), next to the participation of cysteine proteinases also the role of MMPs was investigated. They showed that MMPs were indeed somehow involved in the sequence of events that results in bone degradation. It was not clear, however, what their exact role was and which member of this class of enzymes participates in the different steps of the resorption process.

By using isolated osteoclasts or osteoclasts that were generated in vitro, in various studies it was shown that the actual resorption was not affected when MMP-activity was blocked (Holliday et al. 1997; Delaisse et al. 1987; Fuller and Chambers 1995). These findings strongly suggested that osteoclast-mediated resorption did not involve the activity of MMPs. Yet, some data appear to suggest differently. The osteoclasts used for the analyses mentioned above were isolated

from long bones or they were generated in vitro using long bone marrow cells. Also osteoclasts generated from peripheral blood were recently shown to resorb bone without MMP involvement (Fuller et al. 2007). However, by using the calvaria system it became obvious that MMPs do participate in osteoclast-mediated bone matrix degradation (Everts et al. 1999b). This prompted us to compare both types of bone, long bone and calvaria. In this study (Everts et al. 1999a) it was shown that bone site-specific differences exist between osteoclasts and their use of MMPs in the process of bone resorption. Osteoclasts of calvaria use next to cysteine proteinases also MMPs whereas those harboring the long bones use only cysteine proteinases for matrix degradation (Everts et al. 1998, 1999a, 2006).

Which MMP does play a role in this process is still not clear. Although osteoclasts highly express MMP-9 (Delaisse et al. 2000), its involvement in bone matrix degradation has not been shown yet. In a very extensive study on the presence of a large number of different MMPs, Andersen et al. (Andersen et al. 2004) demonstrated high levels of MMP-13 in the resorption pits of osteoclasts, suggesting that this enzyme may participate in digestion. The enzyme was not present in the osteoclast and therefore the authors suggested that neighboring bone lining cells produced this enzyme which was subsequently used in the resorption lacuna (Andersen et al. 2004).

How can, in the calvaria system, MMPs act at the low pH present in the ruffled border area during resorption? At the onset of the resorption process the pH is lowered to a pH of about 4 (Silver et al. 1988), an optimal situation not only to dissolve the mineral but also for cysteine proteinases to exert their activity. This group of proteinases expresses its highest activity at a relatively low pH, being around a pH 4–5. MMPs, however, act optimal at a more physiological pH. We proposed the following sequence to occur during resorption of calvaria bone by the osteoclasts at that bone site (Fig. 8.9, (Everts et al. 1998)). First, the pH is lowered resulting in the dissolution of mineral; at the same time the cysteine proteinases are active and digest in particular non-collagenous proteins. The release of mineral as well as the release of fragments of the digested proteins not only results in an increased activity of the cysteine proteinases involved (Li et al. 2004) but also increases the pH (Etherington and Birkedahl-Hansen 1987). This change in pH is favourable for the MMPs to digest the rest of the matrix, in particular the collagen (Everts et al. 1998).

8.2.6.3 Role of Other Proteinases, in Particular Plasminogen Activators

Next to the proteolytic enzymes mentioned in the previous chapters, participation has been shown for some members of the class of serine proteinases in the process of bone degradation (Tumber et al. 2003). In mice deficient for the two types of plasminogen activators (PAs), uPA and tPA, osteoclastic bone degradation was delayed but not inhibited (Everts et al. 2008). It was suggested that in the absence of these enzymes a proper attachment of the osteoclast to the bone surface was hindered. In line herewith, the localization of the integrin $\alpha_v \beta_3$ was shown to be


Fig. 8.9 Schematic presentation of the proposed sequence of activities of calvaria bone degradation as depicted from (Everts et al. 1998). *Step 1*: lowering of the pH results in dissolution of the mineral; *step 2*: cysteine proteinases digest non-collagenous proteins; *step 3*: matrix metalloproteinases digest the collagenous matrix. Between step 2 and 3 the pH increases to a level favourable for MMPs to exert their activity

disturbed. Several studies demonstrated a close association between this integrin and PA in the membrane. Which of the plasminogen activators is most important in this process is not known yet, but Kubota et al. (Kubota et al. 2003) showed a very strong upregulation of uPA during osteoclast formation of RAW cells. This may suggest that uPA is the more important of the two activators.

8.2.6.4 TRACP and Bone Degradation

TRACP is highly expressed by the osteoclast and used as a marker enzyme to localize these cells in sections and cell cultures. Yet, its role in the digestion of bone appears to be rather limited. Mice deficient for TRACP are characterized by a mild osteopetrotic phenotype (Hayman et al. 1996), indicating a slightly disturbed resorption. Osteoclasts are present in normal numbers and they resorb bone. In a subsequent study in which not only TRACP but also lysosomal acid phosphatase was deleted, high levels of non-digested osteopontin were noted next to the resorbing osteoclasts (Suter et al. 2001). In line with other studies (Andersson et al. 2003) where TRACP was shown to dephosphorylate non-collagenous proteins like osteopontin, it was concluded that TRACP is primarily involved in this process.

Next to dephosphorylation TRACP can also generate reactive oxygen species (ROS) (Vaananen et al. 2000). Data suggest that ROS may induce fragmentation of matrix components ingested by the osteoclast (Halleen et al. 1999, 2003).

Not all osteoclasts express the same level of TRACP. Those harboring the calvaria express much higher levels of this enzyme than osteoclasts in long bones

(Perez-Amodio et al. 2006a). Even within a long bone, data indicate site-specific differences in expression of TRACP (Zenger et al. 2007).

8.2.6.5 Resorption Machinery Differs at Different Sites of The Skeleton

As pointed out in some of the previous paragraphs not all osteoclasts are alike. Osteoclasts at different sites of the skeleton differ with respect to the enzymes used for resorption (see Everts et al. 1999a, 2006; Shorey et al. 2004), the level of TRACP (see Perez-Amodio et al. 2006a; Zenger et al. 2007) and the expression of the anion exchanger and sodium transporter (Fig. 8.10; see (Jansen et al. 2009)). In line with the latter finding are data presented by Pham et al. (2007). These authors showed that the sodium-proton antiporter NHA2 was highly expressed by osteoclasts. The expression proved to be extremely high in the jaw and calvaria but much lower in the femur. This finding suggests that also for this transporter bone-site specific differences exist.

In addition, also differences in the size between osteoclasts were noted. Osteoclasts present in the calvariae were larger than those present in long bones in bone biopsies obtained from pycnodysostosis patients (Everts et al. 2003). Such differences in size can be related to the bone resorbing activity of the cells. Ample data indicate that larger osteoclasts resorb more bone than smaller ones (Lees et al. 2001; Lees and Heersche 2000). Whether this also counts for the osteoclasts at different locations of the skeleton has not been investigated yet.



Taken together these findings indicate differences among osteoclasts and in a recent paper we have proposed that each bone site has its own specific set of osteoclasts (Everts et al. 2009).

8.2.6.6 Osteoclasts Prefer "Old" Bone

It is of considerable interest that osteoclasts seem to prefer to resorb "older" bone (Henriksen et al. 2009). Such a preference appears to make sense in the remodelling of bone; old bone is removed and replaced by new bone. The mechanisms behind this preference are not elucidated yet, but it could be related to the level of mineral in bone of different ages. Since it is well now that the amount of mineral increases age-dependently and osteoclasts prefer to attach to mineralized substrates, the level of mineral may thus modulate the activity of the osteoclast.

8.2.6.7 Differences in the Composition of Bone at Different Skeletal Sites

Recently it was shown that considerable differences exist between the composition of bones at different skeletal parts. Van den Bos et al. (2008) compared calvaria and long bone and noted differences in the amount of collagen (higher in calvaria), non-collagenous proteins and the level of collagen cross-links (higher in calvaria) (Fig. 8.11).

These differences can provide an explanation for the differences in osteoclasts and their activity; a different substrate needs another cellular activity to resorb such substrate. Whether the difference in substrate does indeed modulate the osteoclast has not been investigated yet.

Recently it was demonstrated that for dentin a difference in osteoclastic activity may be induced by the substrate. Dentin obtained from permanent teeth was compared with dentin from deciduous teeth and the former dentin proved to inhibit osteoclastic activity (Sriarj et al. 2009).

8.2.7 Modulation of Resorption by Cytokines, Growth Factors and Hormones

Osteoclastic bone resorption is modulated by a wide variety of compounds like the cytokines IL-1, IL-6 and TNF α , the growth factor TGF β and the hormones PTH and calcitonin (Chambers 2000). With the exception of calcitonin, these compounds do not directly affect the activity of the osteoclasts. The osteoblast-like bone lining cell that is localized next to the osteoclast responds to the compounds and then signals the osteoclast. For a review on the role of cytokines in osteoclastic bone resorption, the reader is referred to (Teitelbaum 2007).



Fig. 8.11 The composition of calvaria bone differs considerably from that of long bone as depicted in this figure. The fold difference is shown for a number of bone-associated proteins. *PEDF* pigment epithelium derived factor, *Spp24* secreted phosphoprotein 24 [Data derived from Van den Bos et al. (2008).]

8.2.8 The Osteoclast Retracts and Moves Away to Another Site

The osteoclast resorbs the bone up to a certain depth and then moves away from that site. Under normal conditions the cell does not continue resorbing the bone at one site, for yet unknown reasons it stops eating at a certain time point. One possible explanation has been recently suggested by Wilson et al. (2009). These authors noted that collagen fragments generated by cathepsin K were able to inhibit the resorption of the osteoclast. It could thus be that compounds released by the osteoclast during the resorption process modulate its activity.

8.2.9 Cleaning of the Resorption Pit by Bone Lining Cells

Following resorption the osteoclast leaves the resorption pit, the Howship's lacuna. During normal bone remodelling the lacuna is subsequently occupied by bone forming osteoblasts and new bone is deposited at that site (Parfitt 2000). Prior to the bone-forming activity, however, bone lining cells enter the lacuna and clean the

bottom of the pit from matrix remnants left by the osteoclast (Everts et al. 2002). The bone lining cells are attracted to the resorption site and it has been suggested that TGF β released from the bone matrix during resorption plays a role in this process. Alternatively, it has been proposed that TRACP attracts the bone lining cells (Perez-Amodio et al. 2006b). When the osteoclast leaves the resorption pit a high level of TRACP is found left behind in the pit and this enzyme may attract the bone lining cells after which the enzyme is endocytosed and subsequently blocked in its activity.

Following entering the pit, the bone lining cell starts cleaning the bottom. The cleaning involves engulfment of the demineralized collagen fibrils protruding from the bottom and subsequent digestion of these fibrils (Everts et al. 2002). This degradation is mediated by MMPs, probably MMP-13 (Zhao et al. 1999) or MMP-14 (Andersen et al. 2004), both enzymes being highly expressed by bone lining cells. In line with the view that MMP-14 may be important in the digestion of non-mineralized collagen protruding from the bone surface is the very high amount of non-digested fibrillar collagen enclosed in vacuoles in the bone lining cells as found in bone samples of mice deficient for MMP-14 (Fig. 8.12). This observation suggests that the cell can engulf the collagen but digestion is hampered, comparable to the digestion of connective tissue collagen by the phagocytic activity of fibroblasts. In the absence of MMP-14 huge amounts of non-digested fibrillar collagen are present in fibroblasts (Beertsen et al. 2002).

The cleaning of the bottom is followed by the deposition of a thin layer of collagen and osteopontin, the cement line (McKee and Nanci 1996). Following this activity osteoblasts deposit new bone in the lacuna (Mulari et al. 2004).



Fig. 8.12 Non-mineralized collagen fibrils protruding from the bone surface are engulfed by a bone lining cell (BLC). Some of these fibrils are indicated with an *arrow*. The bone (calvaria) was obtained from a mouse deficient for MMP-14 (Holmbeck et al. 1999). Note the high number of collagen fibrils engulfed by the bone lining cell

If cleaning is inhibited or prevented, new bone deposition does not occur (Everts et al. 2002).

The importance of the cleaning activity was emphasized in pycnodysostosis patients and in mice deficient for cathepsin K. In both situations the osteoclast is not able to digest the matrix thus leaving behind large areas of demineralized nondigested bone matrix. If these matrix remnants were not removed, deposition of new bone would not have occurred at these sites thus hampering the remodelling of bone. The bone lining cell appears to rescue this. These cells digest the matrix left over by the osteoclast and since this degradation depends on MMP activity and not on the activity of the enzyme that is lacking, cathepsin K, the remodelling of bone is delayed but not absent (Everts et al. 2002). In this respect it is of interest to note that in mice lacking cathepsin K the expression of different MMPs increased, not only by osteoclasts but also by osteoblast-like cells (Kiviranta et al. 2005). Thus a lower level or absence of cathepsin K is compensated by the organism by expressing higher levels of another group of matrix-degrading enzymes, the MMPs (Everts et al. 2006; Kiviranta et al. 2005).

8.3 Summary

The sequence of activities involved in osteoclast-mediated bone degradation during normal remodelling of the bone can be depicted as follows:

- 1. The osteocyte signals the bone lining cell to attract osteoclast precursors.
- 2. The precursor attaches to the bone lining cell and differentiates into a mononuclear osteoclast-like cell.
- 3. The bone lining cells clean the bone surface from non-mineralized material (e.g., collagen fibrils).
- 4. The mononuclear osteoclast-like cell migrates to this cleaned bone surface and fuses with other mononuclear osteoclast-like cells to form the actual osteoclast.
- 5. The osteoclast forms an attachment zone (sealing zone) and in its centre a ruffled border; the actual resorption starts.
- 6. The pH adjacent to the ruffled border is lowered and the mineral is dissolved.
- 7. Proteolytic enzymes, in particular the cysteine proteinase cathepsin K, are released in the resorption area and the matrix is digested. [At different bone locations different sets of enzymes are used, see (Everts et al. 2009).]
- Matrix fragments are endocytosed and by transcytosis released at the opposite baso-lateral – side of the osteoclast.
- 9. The osteoclast stops resorbing the bone and leaves the Howship's lacuna.
- 10. The bone lining cell enters the lacuna and cleans the bottom and deposits a cement line.
- 11. The lacuna is then filled with new bone by osteoblasts.

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Chapter 9 Consequences of Elastolysis

A. McGarry Houghton, Majd Mouded, and Steven D. Shapiro

Abstract Elastic fibers are an essential and irreplaceable structural component of lung tissue, blood vessels, and skin. Degradation of elastic fibers in these organs results in emphysema, aortic aneurysm, and solar elastosis (aging skin), respectively. Enzymes capable of degrading elastin have been termed elastases. This chapter will discuss the properties of the elastases, discuss the consequences of elastic fiber breakdown (elastolysis), and review the emerging reports of none-lastolytic functions of elastases in health and disease.

9.1 Introduction

Elastin is one of the most long-lived and inert substances known. Few proteinases have the capacity to degrade elastin, and many that do are termed "elastases," to highlight their potency. The name should not suggest that elastin degradation is a physiologic function of the enzyme. For one, it is difficult to understand under what circumstances degrading elastin would be beneficial given the importance of elastin to the structural and functional integrity of the vascular system, lung, and skin. Second, elastases have been around quite a long time, being present in prokaryotes. Elastin, on the other hand, has evolved relatively recently. Elastin is a vertebrate-specific protein dating to time of the sharks that allowed for the transition from an open to a closed, pulsatile circulatory system. Elastin also was critical for the lung to evolve from itself being a muscular pump to being an elastic organ that functions within a pump (the diaphragm and other respiratory muscles). Hence, it is unlikely that elastases were meant to degrade a protein yet to evolve. Luckily, it is difficult to degrade elastin, and its proteinase inhibitors, such as alpha₁-antitrypsin (A1AT), are highly expressed in the blood and tissues to prevent uncontrolled elastolysis. As a

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result, most diseases characterized by elastolysis such as emphysema, aortic aneurysm, and "aging skin" take decades to manifest.

Unfortunately, as difficult as it is to degrade elastin, it also appears that elastin has a very limited capacity for repair. Elastic fiber assembly requires the coordinated secretion of tropoelastin onto a microfibrillar scaffold accompanied by cross-linking of elastin by lysyl oxidases. Adult vertebrates appear to have limited capacity to temporally and spatially coordinate this complex process following growth and development.

Interestingly, as a group, the elastases appear to have a variety of interesting and unique functions beyond their capacity to degrade matrix components. Moreover, elastin fragments themselves have a variety of important biological functions. This chapter will discuss both the importance of elastin and the direct and indirect effects of elastolysis as well as other novel properties of elastases.

9.2 Elastin

The mechanical properties of many tissues, including the lungs, large arteries, ovaries, tendons, and skin, are determined by the matrix that composes them. Elastic fibers are a unique component in those organs that allow for a more uniform distribution of stress along tissue, storage of energy, and maintenance of form. Elastic fibers are key to repeated stretch and recoil of the organs mentioned above. They are formed during development and persist with minimal turnover throughout adulthood. Their synthesis is complicated, and destruction or dysfunction of these fibers has been linked to several adult diseases. Furthermore, the complexity of structure and assembly makes the repair of these fibers difficult leading to permanent, progressive disease.

9.2.1 Elastic Fiber Components

Elastic fibers are the largest structures found in the extracellular matrix. They are primarily composed of two components, elastin and microfibrils, with several associated proteins that further compose the fiber. Elastin is the primary component (~90%). It is formed by the cross-linking of a monomeric secreted form of the protein called tropoelastin (Sandberg et al. 1971). This cross-linking forms an amorphous elastin polymer that comprises the core of the fiber. The second components, the microfibrils, are small 10–15 nm fibers that are composed primarily by fibrillins (Wagenseil and Mecham 2007). The microfibrils are felt to provide a scaffold for the tropoelastin cross-linking and for the further cross-linking of elastin to itself by lysyl oxidase enzymes (Csiszar 2001; Lucero and Kagan 2006). In addition to the microfibrils, multiple proteins associate with the either the fibrils or the elastin itself (see below). They are found both within and surrounding the elastin core.

9.2.2 Tropoelastin/Elastin

Tropoelastin is the monomer that composes elastin, arising from one gene in all species. The sequence includes several exons and introns, which allows for multiple mRNA splice variants within the same organ. Additionally, there may be different splice variants produced in adulthood versus development resulting in different isoforms as a function of age (Rosenbloom et al. 1993).

Tropoelastin, a ~70-kDa monomer composed of alternating hydrophobic and lysine domains (Gray et al. 1973), is produced by fibroblasts and smooth muscle cells (Pasquali-Ronchetti and Baccarani-Contri 1997). These lysine residues are cross linked by lox family enzymes to other tropoelastin fibers to form elastin (Lucero and Kagan 2006). Of the approximately 40 residues, all but ~2–5 are cross-linked. It is this degree of cross-linking that gives elastin its stability and insolubility (Wagenseil and Mecham 2007). A hypothesized model of elastic fiber assembly is discussed below.

Elastin-deficient $(Eln^{-/-})$ mice display mortality within a few days of birth, which is due to vascular obstruction from smooth muscle cell overproliferation and disarrangement (Li et al. 1998a, b). $Eln^{+/-}$ mice, however, have a normal life span but are hypertensive with smaller blood vessels. Surprisingly, they have increased layers of elastin associated with smooth muscle (lamellar units) (Li et al. 1998a, b; Faury et al. 2003). Recent data looking at aortic development in mice show that these changes in structure occur in the few days before birth (E18) to birth (P0), as pressure and blood flow through the vessel increases (Wagenseil and Mecham 2007).

9.2.3 Fibrillins

The microfibrils that compose the elastic fiber are largely composed of fibrillins (Sakai et al. 1991). These are large (~350 kDa) cysteine-rich glycoproteins with calcium binding epidermal growth factor (EGF)-like domains that provide the majority of the structure. There are currently three fibrillins in humans that have been identified (Fib1, -2, and -3) although, in mice, the third fibrillin has been disrupted due to chromosomal rearrangement (Corson et al. 2004; Kielty 2006). Fibrillin-1 and -2 are known to bind tropoelastin in solid phase binding assays (Trask et al. 2000a, b). They are expressed in similar organs in mice but with some regional and quantitative differences. In addition, fibrillin-2 is expressed earlier in development (Zhang et al. 1995). These proteins are produced by fibroblasts and contain conserved Arg-Gly-Asp (RGD) sequences that interact with integrins (Sakamoto et al. 1996; Bax et al. 2003). In addition they have heparin binding domains that interact with cell surface heparin sulfate proteoglycans (Tiedemann et al. 2001; Ritty et al. 2003). These interactions may be essential to guiding in vivo assembly and may also serve as a signal to cells. In fact, the addition of heparin to cell cultures prevented the assembly of microfibrils (Tiedemann et al. 2001).

In humans, mutations in fibrillin-1 leads to Marfan's syndrome, while fibrillin-2 mutations cause congenital contractural arachnodactyly (Lee et al. 1991). $Fbn1^{-/-}$ mice die within 2 weeks of birth secondary to pulmonary and cardiac complications including diaphragmatic collapse, enlarged airspaces, and ruptured aortic aneurysms (Carta et al. 2006). $Fbn2^{-/-}$ mice develop syndactyly but otherwise have a normal life span without pulmonary or vascular defects (Chaudhry et al. 2001; Carta et al. 2006). Mice deficient in both Fbn1 and Fbn2 die in utero with minimal elastic fibers between smooth muscle layers without organization (Carta et al. 2006).

9.2.4 Microfibrillar-Associated Glycoproteins (MAGP1 and -2)

MAGP1 (Mfap) and MAGP2 (Mfap5) are small (~20 kDa) glycoproteins localized to beaded areas of the microfibrils (Henderson et al. 1996; Wagenseil and Mecham 2007). MAGP1 is the only protein besides the fibrillins that is constitutively part of the microfibril (Gibson et al. 1986). It binds fibrillin-1, -2, and tropoelastin (Brown-Augsburger et al. 1994; Trask et al. 2000a, b; Jensen et al. 2001). It has therefore traditionally been considered an important component in elastic fiber assembly by serving as an intermediate between fibrillin and tropoelastin. However, mice deficient in MAGP1 ($Mfap2^{-/-}$) do not have structural abnormalities in elastin, although they have a tendency for bleeding and altered wound healing. Thus it appears that MAGP1 is not essential in elastic fiber formation. Interestingly, MAGP1 binds TGF- β and BPM-7 suggesting that it may modulate microfibrilmediated growth factor signaling (Weinbaum et al. 2008). MAGP2 is found in most microfibrils and binds fibrillin-1 and -2 (Penner et al. 2002). It possesses an RGD cell recognition motif with which it binds integrins. MAGP2-deficient mice have not been described. However, its restricted pattern of localization and expression suggest that it may be more important in cell signaling (Kielty et al. 2005).

9.2.5 Key Associated Proteins

There are additional proteins associated with elastic fibers, including the fibulins, latent TGF- β -binding proteins (LTBPs), and elastin microfibril interface located proteins (EMILINs). These components contribute to the structural and bioactive properties of mature elastic fibers. They have recently been reviewed in detail (Kielty 2006; Wagenseil and Mecham 2007).

9.2.6 Elastic Fiber Assembly

Microfibril assembly is beyond the scope of this review. Suffice it to say that there are complex modifications and trafficking of fibrillin along with interactions with

MAGP, other microfibril components, and components of the basement membrane. We will briefly discuss the assembly of the amorphous elastic fiber and its interactions with microfibrils.

9.2.6.1 Lysyl Oxidases

Lysyl oxidases are a class of copper-requiring enzymes that help to cross-link both collagen and elastin. There are five members of this group. In addition to LOX there are four lysyl oxidase-like proteins (LOXL1–4) that contain a conserved amino oxidase domain. There appear to be two subfamilies based on homology: (1) LOX and LOXL1 and (2) LOXL2, -3, and -4 (Lucero and Kagan 2006). LOX and LOXL1 are the only two that have been shown to cross-link elastin (Borel et al. 2001). LOX and LOXL1 enzymes are essential in the assembly phase of tropoe-lastin/elastin and with linking to the microfibrils (Hayashi et al. 2004; Kielty 2006). They are released as a proform and require processing by bone specific proteases such as bone morphogenic protein-1 (BMP-1) (Borel et al. 2001).

Elastin cross-linking occurs at lysine residues that are modified to form bi-, tri-, and tetrafunctional cross-links (Wagenseil and Mecham 2007). Desmosine is a crosslink of three allysyl side chains with one unaltered lysyl side chain from the same or neighboring polypeptide, and isodesmosine is a lysine derivative whereby four lysines are formed into a pyridinium ring. Both of these are unique to elastin and due to lysyl oxidase cross-linking. LOX can cross link tropoelastin directly whereas LOXL1 can interact with fibulin-5 also (Liu et al. 2004). Interestingly, the prodomain of these enzymes directs them to elastic fibers in vitro and may account for some of the differences between the two enzymes (Thomassin et al. 2005). $Lox^{-/-}$ mice die at the end of gestation or quickly after birth with a rtic aneurysms and diaphragm collapse. Furthermore, there is an approximate 60% reduction in desmosine cross-links in the aorta and lungs. These mice also show impaired collagen development (Maki et al. 2002; Hornstra et al. 2003; Maki et al. 2005). $LOXL1^{-/-}$ mice have a less severe phenotype. They develop pelvic prolapse, loose skin, congenital emphysema, and vascular deformities, but have a normal life span (Liu et al. 2004).

9.2.6.2 Development

Most elastin is formed during embryogenesis and early in life. Both carbon and racemization dating in human lungs show that fibers dated to the patient's age without evidence of significant turnover (Shapiro et al. 1991). Analysis of elastin protein in mice shows that most of the elastic fiber proteins are expressed during the second half of embryogenesis and continue through the early postnatal period (approximately P7–14) (Kelleher et al. 2004). In the lungs, however, there are two peaks of elastogenesis in mice occurring at day E18 and between days P10–14. The former peak is felt to coincide with vascular development in the lungs and the

latter with alveogenesis. Elastin expression drops off rapidly after day P14 and remains low throughout adulthood. Microfibrillar protein expression is more variable throughout (Mariani et al. 2002). Complicated patterns of gene expression imply unique roles for these proteins during elastin fiber synthesis (Wagenseil and Mecham 2007). Finally, as implied above, elastin fibers may not only supply a structural role but may be important in cellular organization. As noted, $Eln^{-/-}$ mice displayed significant vascular structural abnormalities including subendothelial cell proliferation and reorganization of smooth muscle prenatally. The lack of inflammation and endothelial disruption implies that this is not just due to increased strain in the arteries, but rather due to fundamental regulation of these cells by the fibers. The mechanism is unknown, however (Li et al. 1998a, b).

9.2.6.3 Role of Cells in Elastin Formation

Cells participate in the assembly of extracellular matrix components including elastic fibers (Czirok et al. 2006; Kozel et al. 2006). Although the precise mechanisms are unclear, cells organize elastin aggregates into linear structures and deposit them onto preexisting fibers. This aggregate formation is termed "microassembly." The transfer of these aggregates onto fibers is referred to as "macroassembly." Both these processes appear to involve cell movement. As the fiber grows, the motion becomes more organized demonstrating the need for interaction and coordination of multiple cells (Czirok et al. 2006; Kozel et al. 2006).

The mechanism by which cellular interaction occurs is unclear. Possible sites of interaction include the elastin binding protein, integrins, and cell surface glycosaminoglycans (Hinek et al. 1988; Rodgers and Weiss 2004; Broekelmann et al. 2005). Imaging studies utilizing fluorescently labeled antibodies showed that elastin fiber formation occurred sequentially: fibronectin, fibrillin-1, MAGP1, fibulin-5, and elastin fibers, whereby each fiber appears to replace or form in the location of the preexisting fiber. Curiously, fibronectin is necessary for type I and type III collagen matrix assembly, but not considered a component of the elastic fiber. However, it may be essential for LOX processing from its proform to active form (Kozel et al. 2006).

9.2.6.4 Model of Elastic Fiber Assembly

Wagenseil and Mecham have proposed a hypothetical model of elastic fiber assembly (Fig. 9.1) based on the studies described above. The model uses a celldependent method of assembly as observed with live imaging studies. Briefly, tropoelastin is secreted by a cell (either smooth muscle or fibroblasts) and crosslinked into aggregates on the cell surface via LOX. Fibulin-4 and/or -5 interacts with the aggregates on the cell's surface to allow for optimal cross-linking and to regulate the size of the aggregate. The cell continues to secrete and link tropoelastin until the aggregate size is optimal. These aggregates are then transferred to a



Fig. 9.1 Hypothetical model of elastic fiber assembly. (1) Tropoelastin is secreted by the cell and transported to the assembly site on the cell where it is organized into small aggregates that are cross linked by LOX. Cell surface-binding proteins may serve to facilitate movement of the aggregates. Interactions with fibulin-4 and/or -5 facilitate cross-linking and formation of propersized aggregates. (2) Aggregates on the cell surface continue to assemble with new tropoelastin. (3) Aggregates are transferred to extracellular microfibrils that are held in proximity to the cells by integrins. Fibulin-4 and/or -5 may assist in the transfer and cross-linking of the aggregates to the microfibril. (4) Elastin aggregates on the microfibril continue to form larger structures. (5) Elastin aggregates are further cross-linked by LOX/LOXL to form the elastic fiber (Reproduced with permission from Wagenseil and Mecham, 2007.)

microfibril, which are composed of fibrillin-1 and/or -2 and possibly other associated proteins such as the MAGPs. Once in close contact, fibulin-4 and -5 within the aggregates can bind with the fibrillin-1 of the microfibril thereby transferring the elastin aggregate onto the microfibril. The aggregates form larger and larger structures and are further cross-linked by LOX and/or LOXL. Of note, the authors hypothesize that the fact that many of these proteins are not detected by antibodies in the mature elastic fiber may represent that they are coated with elastin during fiber assembly, that they are displaced to the outside of the fiber, or that they are degraded during the process (Wagenseil and Mecham 2007).

9.2.6.5 Structural Contributions

The ideal elastic fiber uses the relaxation phase to fully recover the energy invested into it during extension. This is essential since there is fairly low elastin turnover and therefore the fibers from birth are exposed to the same stretch–relaxation cycles. As such, by the time individuals are entering their seventh decade of life, their aorta has been subject to approximately a billion stretch–relaxation cycles (Urry and Parker 2002).

The elastic forces in the fiber are mostly due to entropic forces (Urry and Parker 2002). As we have stated, elastin is insoluble and is composed of alternating hydrophobic and hydrophilic sequences. The longest repeating sequence is that of $(GVGVP)_{11}$ although there are other less extensive repeats also present (Sandberg et al. 1981; Sandberg et al. 1985; Urry and Parker 2002). Despite frequent lysine cross-links, only about 5% of the total number of residues participate in cross-link formation. This is very different from rubber, where in the vulcanization process, all of the monomers are potential cross link targets and thus there is cross link formation whenever two chains come into contact (Urry and Parker 2002).

In addition to the large number of hydrophobic residues, there are also very few charged residues in bovine elastin. The hydrophobic interactions are responsible for the interactions between chains and that result in parallel-aligned twisted filaments. The (GVGVP)_n repeats play an essential role. This structure forms a series of β -turns with interactions between the hydrophobic Val¹ to Val⁴. When the temperature is raised, the structure wraps into a β -spiral with hydrophobic spacers between the turns of the β -spiral. The β -spirals then further associate hydrophobically to form filaments (Khaled et al. 1981; Urry et al. 1989; Urry and Parker 2002).

The hydrophobic interaction between turns is what gives the elastic fiber most of its elastance (Fig. 9.2). As the fiber is stretched, water molecules intercalate



Fig. 9.2 *Hydrophobic domains and interactions of tropoelastin.* (**a**) Structure of GVGVP repeats showing the series of β -turns. (**b**) Same as **a** with molecular depiction. (**c**) When the temperature is increased, the β -turns form β -spirals which are depicted schematically. (**d**) Further, more detailed schematic depiction of the β -spirals with the β -turns represented and the V¹ to V⁴ interactions illustrated. (**e**) Molecular depiction of **c** and **d**. (**f**) Hydrophobic folding of the β -spirals to form filaments occurs in a cooperative manner (Reproduced with permission from Urry and Parker 2002.)

between these residues. This lowers the entropy or raises the energy of the system. Once the tension is released on the fiber, the hydrophobic interactions resume. These are further supported by the surrounding β -spirals. This results in the return of the fiber to its higher entropy/lower energy state and original shape (Rosenbloom et al. 1993; Urry and Parker 2002).

9.3 Structural Consequences of Elastin Degradation

Elastin is fairly resilient; however, some proteases can degrade it. The degradation of the elastic fiber is pathologic and results in disease in the various organs. We will briefly focus on elastic fiber degradation in the lung (emphysema), large vessels (aortic aneurysm), and skin (solar elastolysis).

In the lungs, elastin can be found in the arterial vessels of the lung arranged as sheets, surrounding the respiratory bronchioles and alveolar ducts in a helical fashion, and lining the walls of the alveoli (Dunsmore 2008). Elastin provides the lungs with their ability to stretch and relax. The hallmark disease involving elastin cleavage in the lung is emphysema.

When an individual smokes, inflammatory cells are recruited including macrophages, neutrophils, and lymphocytes. As these cells release proteinases, they destroy the basement membrane and matrix resulting in the coalescence of alveoli into larger ones giving the emphysema phenotype (Shapiro and Ingenito 2005). The destruction of alveoli and elastic fibers decreases the elastic recoil making the lungs much more compliant. Since exhalation depends on the elastic recoil of the lung, the loss of elastic recoil causes a decreased ability to expire gas during exhalation. This decreased ability to expire gas is the "obstructed" phenotype characteristic of emphysema. Furthermore, the smaller airways are in contact with the alveoli and are tethered open during respiration. As the elastin and matrix are cleaved, there is small airway closure and collapse, which may further exacerbate the obstructive phenotype. These situations may result in air retention at the end of exhalation, which puts the chest wall and diaphragms at mechanical disadvantage and increases the work of breathing. Furthermore, increased work of breathing, loss of alveoli, and retained air in the lungs can significantly alter ventilation throughout the lungs resulting in inconsistent regions of gas exchange. Restoration of the elastic recoil of the lung could potentially reverse this process, which is the goal of lung volume reduction surgery and bronchoscopic lung volume reduction.

Elastic fibers are required for large arteries to withstand the pulsatile pressure of systole, absorb the energy, and return it so that there is perfusion pressure throughout diastole. The large arteries are composed of three main layers: the intima (innermost layer composed of endothelial cells), media (middle layer composed mostly of smooth muscle cells), and adventitia (outer layer which is mostly acellular). The intima and media are separated by the internal elastic lamina and the media and adventitia are separated by an external elastic lamina (Schoen and Cotran 1999). Furthermore, in the media there are layers of elastin associated with smooth muscle called lamellar units. The adventitia is also rich in sheets of elastin interspersed with collagen that run parallel to blood flow. The inner one-third contains mostly elastin, while the outer layers are primarily composed of collagen (White and Mazzacco 1996). The hallmark disease involving elastin degradation in the vascular system is aneurysm formation.

All aneurysms, regardless of size, have reduced adventitial and medial elastin, suggesting that this is a key initial step in aneurismal formation. Exposure of the external aortic layer of rabbits to elastase uniformly resulted in aneurysms that grew progressively and displayed reduced adventitial and medial elastin (the rest of the aorta was normal) (White and Mazzacco 1996). Loss of elastin caused the stress to be transmitted to the collagen fibers and thus dilation and eventual rupture were caused by progressive collagen degradation after elastin loss.

In the skin, elastin forms a three-dimensional meshwork that extends from the papillary down to the dermis. In the reticular dermis it has branching fibers whereas it is more sheet-like in the deep dermis (Tsuji 1988). As the fibers extend upward to the dermal–epidermal junction they become smaller and lose elastin content until they are almost exclusively fibers of glycoproteins. Although there are several skin disorders involving elastin, the two most common disorders, photo-aging and chrono-aging, both appear fairly similar with more frayed and tortuous elastin fibers (Imayama and Braverman 1989). These findings are more pronounced in sun-exposed skin where there can be destruction of the dermal elastin meshwork (O'Brien and Regan 1991). In addition, sun-exposed skin appears to have more inflammatory cells (macrophages and mast cells) and more elastin deposition that aged skin alone (Bernstein et al. 1994).

9.3.1 Inability to Repair Properly and Findings After Cleavage

The complexity of elastic fiber assembly is likely why adults are incapable of adequately replacing them. Thus, diseases of elastin cleavage are irreversible diseases requiring surgical intervention in the case of aneurysm, or supportive care with possible lung volume reduction or transplant in the case of emphysema. If elastin could be reformed properly, perhaps these diseases could be treated medically or much less invasively.

Attempts to bioengineer elastin or elastin-based matrices are underway and reviewed in Daamen et al. (2007). Approaches include the use of purified elastin preparations, hydrolyzed elastin, protein engineering of either, tropoelastin fragments, elastin-like peptides, and elastin hybrids. In many of these preparations, the amounts of other matrix components such as collagen, glycosaminoglycans, and growth factors can be varied to optimize function and cell adhesion. In addition, amino acid sequences may be modified in some cases to further modify the structure/function. Acellular tissue, as a source of matrix, has shown some promise for reconstitution of bladder, esophagus, skin, and vasculature. Replacement and regrowth of elastic fibers may be the only way to control these destructive diseases.

9.4 Biological Consequences of Elastolysis

Elastic fibers and other matrix proteins have traditionally been considered solely as structural support for surrounding tissues. In addition to the structural abnormalities generated by matrix degradation (above), we now know that products of matrix degradation possess bioactive properties (Maquart et al. 2004). Such matrix-derived peptides have subsequently been termed "matrikines." Elastin fragments (EFs) or elastin degradation products (EDPs) are the best characterized of the matrikines to date and exert diverse biological functions including chemotaxis, gene transcription, and cell cycle regulation (Duca et al. 2004). While it would be advantageous for an injury significant enough to damage resilient elastic fibers to elicit a reparative response, unfortunately, EFs evoke detrimental responses in the setting of chronic, low-grade elastin degradation.

9.4.1 Chemotactic Properties of EFs

Senior and Mecham first described the monocyte chemotactic properties of EFs in vitro in 1980 (Senior et al. 1980), which were independently corroborated by Hunninghake et al. shortly thereafter (Hunninghake et al. 1981). Since that time, EFs have been confirmed to possess in vitro chemotactic activity for fibroblasts (Jacob et al. 1987), smooth muscle cells (Ooyama et al. 1987), endothelial cells (Long et al. 1989), lymphocytes (Hauck et al. 1995), and a number of malignant cell lines including Lewis lung carcinoma (Grosso and Scott 1993a, b).

EFs are now recognized as the major source of monocyte chemotactic activity in cigarette smoke-induced emphysema (Houghton et al. 2006a, b). As discussed above, emphysema is characterized by the degradation of elastic fibers and loss of elastic recoil that is caused by a chronic inflammatory cell infiltrate composed of macrophages, lymphocytes, and neutrophils. While investigating the role of MMP12 in cigarette smoke-induced emphysema, MMP12^{-/-} mice were noted to be protected not only from emphysema but also from the accumulation of macrophages in the lung (Hautamaki et al. 1997). After the identification of EFs in the BALF of WT mice exposed to cigarette smoke, antibodies to EFs were administered to mice demonstrating that both the accumulation of lung macrophages and the subsequent development of emphysema were dependent upon EFs. Based on this work, and other recent studies, the means by which macrophages are recruited to the lung is coming into focus. Upon cigarette smoke-exposure, CD8⁺ lymphocytes release IP-10, a member of the CXC chemokine family (Grumelli et al. 2004). IP-10 interacts with CXCR3 on macrophages, which induces the expression and release of MMP12 (Maeno et al. 2007). These activated macrophages also release neutrophil chemokines that will bring neutrophils, and hence NE, into the lungs. In turn, EFs recruit naïve monocytes to the lung where they differentiate into mature tissue macrophages, ultimately propagating the destruction of lung tissue.

Macrophages accumulate in aortic aneurysms as well, where they damage elastic fibers. The EFs present in human aneurismal tissue also display monocyte chemo-tactic properties and are likely responsible for the accumulation of macrophages via chronic progressive elastin destruction (Hance et al. 2002).

Not all EFs display chemotactic properties. The prototypical EF, VGVAPG, is a synthetic peptide frequently used in in vitro assays of EF activity (Senior et al. 1984). Additional study has revealed that essentially all EFs that include the motifs GXXPG or XGXPG (where X is a hydrophobic amino acid) possess chemotactic properties (Grosso and Scott 1993a, b). These motifs are highly prevalent in tropoelastin, the soluble precursor of elastic fibers, representing in excess of 20% of the entire sequence. It should be noted that the biologic activities of GXXPG containing peptides are not limited to elastin derivation. Laminin, for example, is rich in LGTIPG, a sequence that displays similar bioactivity to the GXXPG peptides derived from elastin (Mecham et al. 1989a, b). Additionally, laminin possesses other unique chemotactic sequences (Adair-Kirk et al. 2003).

It is important to recognize that mature elastic fibers do not display chemotactic properties. The biological properties residing within these short, 5- to 6-residue peptides remain hidden within the hydrophobic mass of an elastic fiber. Because elastic fibers are extensively cross-linked, cleavage of the protein does not generate short, linear, bioactive peptides. EFs liberated into lung lavage fluid after cigarette smoke exposure range from ~35 to 50 kDa (Fig. 9.3). These products are a heterogeneous mixture of degradation products, most of which possess bioactive sequences within them.



Fig. 9.3 Depiction of an elastic fiber. The schematic depicts an elastin fiber that consists of multiple tropoelastin monomers cross-linked together by lysyl oxidase. Potential degradation products and their cross-linked nature are shown (Reproduced with permission by the author AMH.)

9.4.2 EFs Impact Cellular Behavior

Elastin fragments are capable of altering cell behavior by means other than simple chemotaxis. Exposure of human skin fibroblasts to VGVAPG has been shown to induce the expression of both MMP1 and -3 (Brassart et al. 2001). EFs also participate in angiogenesis, the process of new blood vessel formation, in the setting of matrix injury. EFs can induce both cell migration and tubulogenesis of endothe-lial cells (Robinet et al. 2005). These effects are dependent upon the induction of MMP2 and -14. Gene silencing of MMP14 abrogated endothelial cell motility and morphogenesis, in this setting.

EFs likely exert their influence on cell behavior via interaction with 67-kDa elastin-binding protein (EBP) (Mecham et al. 1989a, b). Consistent with this concept, the inhibition of EBP signaling eliminated the effects of VGVAPG exposure with respect to MMP production in the above studies. The EBP is also located on circulating lymphocytes. Interaction with EFs and lymphocyte-derived EBP drives Th1 differentiation of CD4⁺ cells in the setting of aortic aneurysm (Debret et al. 2005).

9.4.3 EFs as Biomarkers of Disease

Elastin degradation is relatively specific to disorders of the lung, skin, and blood vessels. Investigators have attempted to measure EFs in the serum, urine, or BALF as a measure of disease activity or severity, most commonly for emphysema and aortic aneurysm (Luisetti et al. 2008). Mature elastic fibers contain two unusual, tetrafunctional, pyridinium ring-containing amino acids, desmosine (DES) and isodesmosine (IDES) (see above). Assays have been developed that reliably detect both DES and IDES in human serum and urine (Darnule et al. 1982). Early generation assays provided conflicting results with respect to DES as a biomarker for emphysema. These assays were able to distinguish between smokers with emphysema versus smokers without the disease (Davies et al. 1983). More recently, the introduction of HPLC methodology has improved the resolution of these assays although they remain tedious and provide a small signal.

Acute aortic dissection carries a high mortality and requires early surgical intervention. Unfortunately, diagnosis of aortic dissection is extremely difficult and often mistaken for acute coronary syndromes. Shinohara and colleagues recently reported promising results, showing that EFs are present in >60% of patients with acute aortic dissection but less than 5% of patient with acute myocardial infarction (Shinohara et al. 2003). Improvements in assay quality and especially rapidity will be required if EF detection is to be used as a reliable diagnostic marker in patients presenting with acute chest pain.

9.5 Contributions of Elastases to Elastolysis

Proteinases represent the second largest collection of gene products in nature trailing only the ubiquitins in number. Proteolytic enzymes fall into one of five classes, which are based upon the mechanism of catalysis: aspartic, cysteine, threonine, metallo-, and serine (Neurath 1999). There are currently greater than 66,000 identified proteinases subdivided into 50 clans and 184 families. Very few of these enzymes have been invested with the capacity to degrade elastin, limited to just a few members each of the metallo-, cysteine, and serine proteinase classes. Specific contributions of individual proteinases to elastolysis in disease are discussed below.

9.5.1 Serine Proteinases

The serine proteinases comprise greater than one-third of all known proteinases, all of which originate from a single gene that has been extensively duplicated over the course of evolution (Page and Di Cera 2008). They are expressed by virtually all life forms ranging from prokaryotes to mammals. Serine proteinases play diverse roles in humans including the digestion of food, the coagulation cascade, and the degradation of ECM structures. The name of these proteinases derives from the nucleophilic Ser residue that is essential for catalysis. The charge relay system in serine proteinases is composed of conserved His-Asp-Ser residues that functions via transfer of electrons from the carboxyl group of the Asp to the oxygen of the Ser. This Ser group, now a powerful nucleophile, attacks the carbonyl carbon atom of the peptide bond in the substrate (Hedstrom 2002).

The serine proteinases have been subdivided into numerous clans and families. With respect to mammalian serine proteinases, elastolysis is limited to clan PA, family S1, subfamily A, also referred to as trypsin-like serine proteinases. With the exception of pancreatic elastase, these enzymes are, for the most part, neutrophil specific and include neutrophil elastase (NE), cathepsin G (CG), and proteinase-3 (PR3) (Pham 2006). There have also been reports of tumor cell and endothelial cell-derived serine elastases (Rabinovitch 1999); however, these may yet prove to be traditional neutrophil-derived serine proteinases that have been endocytosed by neighboring cells.

The serine elastases are synthesized as pre–pro enzymes in the endoplasmic reticulum before subsequent removal of the signal peptide (pre-) and dipeptide (pro-) by dipeptidyl peptidase I (DPPI or cathepsin C) (Adkison et al. 2002). These active enzymes are then stored in intracellular granules, termed azurophilic granules when located within neutrophils. NE, CG, and PR3 are all approximately 28–30 kDa in size. They are housed together within azurophil granules and secreted together as well. They possess similar substrate specificities although differential potency with NE > PR3 > CG in general. These enzymes are rarely dumped into the ECM, as previously thought. Upon physiological stimulation, PMN mobilize ~12% of their NE content to the cell surface, where it is catalytically active and

resistant to inhibitors (Owen et al. 1995). An additional $\sim 2\%$ of NE content is secreted from the cell in quantum microbursts to transiently overwhelm its inhibitor (Liou and Campbell 1996). Additionally, substrate-bound NE is resistant to A1AT (see below) (Owen and Campbell 1995).

Serum proteinase inhibition is accomplished by a number of specific proteins and by the more general inhibitor, Alpha-2 macroglobulin. This massive protein $(\sim 725,000 \text{ kDa})$, which is restricted to the bloodstream due to its size, basically traps proteinases of numerous classes. Specific inhibition of the serine proteinases is accomplished by the serpins. The serpins, a superfamily of serine proteinase inhibitors, function to inhibit the serine elastases both inside and outside of the bloodstream. Of these, A1AT (or Serpina 1) accounts for most of the antiproteinase activity found in serum (Davies and Lomas 2008). The protein is expressed in humans from two independent alleles of a single gene located on chromosome 14. Cell-specific expression of A1AT in macrophages and hepatocytes is accomplished by using different promoters within the same gene (Perlino et al. 1987). The human alleles for A1AT are highly polymorphic, with over 70 mutants having been identified to date (Brantly et al. 1988). The majority of the population is homozygous for the normal M allele, abbreviated as PiMM. Of the many mutant alleles, disease states are most commonly associated with the PiZZ genotype, where serum levels of A1AT are only 10-15% of normal (Yoshida et al. 1976).

The mature glycoprotein exists as a single polypeptide chain of 395 amino acids with a molecular weight of approximately 52 kDa (Carrell et al. 1982). A strained conformation exists in native A1AT between the Meth³⁵⁸ and Ser³⁵⁹ residues that is relieved when a proteinase cleaves this bond, releasing a C-terminal peptide. The new pocket formed between the Meth³⁵⁸ and Ser³⁵⁹ residues provides an exact fit for the active site of serine proteinases (Loebermann et al. 1984). This new A1AT-serine proteinase complex is rapidly cleared from the circulation, thus the inhibition of a given proteinase is essentially permanent. The major physiologic function of A1AT is to inhibit NE. The "antitrypsin" name has been retained for historical purposes, since trypsin was its first identified substrate. In addition to trypsin and NE, its substrates include chymotrypsin, plasmin, thrombin, and PR3 (Beatty et al. 1980).

There are other biologically relevant serine proteinase inhibitors with the capacity to inhibit the serine elastases. Serine leukocyte proteinase inhibitor (SLPI) is able to inhibit both NE and CG, but not PR3 (Sallenave 2000). In contrast, elafin is a small 6-kDa inhibitor of NE and PR3, but likely not CG (Sallenave and Silva 1993). These inhibitors are able to inhibit NE when in association with its substrate, a property not conferred to A1AT.

NE was first implicated in elastic fiber degradation upon the advent of the proteinase–antiproteinase hypothesis of emphysema pathogenesis. The recognition that patients with A1AT-deficiency were at increased risk for emphysema, coupled with the observation that the plant-derived cysteine protease papain could generate emphysema when instilled into rat lungs (Gross et al. 1965), gave rise to this theory that still remains valid nearly 50 years later. Subsequently, investigators have generated experimental emphysema in rodents using a number of different proteinases including pancreatic elastase (Kuhn et al. 1976), NE (Senior et al. 1977), and PR3 (Kao

et al. 1988), but notably not nonelastolytic bacterial collagenase. Damiano later demonstrated the presence of NE on elastic fibers in human emphysematous lungs using immuno-EM (Damiano et al. 1986). Subsequent discovery of MMPs with elastolytic properties (see below) has placed NE within the proper context, one of a number of proteinases functioning in concert to degrade lung elastic fibers during the course of chronic cigarette smoke-exposure (Shapiro 2005).

The contributions of CG and PR3 to elastic fiber degradation in vivo are considerably less well characterized than those of NE. NE-mediated elastolysis has been implicated in several disease states including emphysema, acute lung injury, acute respiratory distress syndrome, and bullous pemphigoid (Liu et al. 2000; Lee and Downey 2001a, b; Lee and Downey 2001). It is likely that CG potentiates the elastolytic capabilities of NE, although even this minor role for CG has been called into question (Boudier et al. 1981; Reilly et al. 1984). PR3 is a more potent elastase than CG. However, with the exception of intratracheal PR3-induced experimental emphysema, specific elastolytic roles for PR3 are lacking.

9.5.2 Matrix Metalloproteinases

Mammalian-derived elastolytic metalloproteinases are limited to the matrix metalloproteinases (MMPs), a member of the metzincin superfamily of enzymes. Taken together, this family of 23 (in humans) enzymes is capable of processing all components of the ECM (Table 9.1), many of which are essential for tissue development, remodeling, and repair (Sternlicht and Werb 2001). When expressed aberrantly, the MMPs contribute to myriad disease states including cancer (Overall and Kleifeld 2006), arthritis, pulmonary emphysema, abdominal aortic aneurysm formation, bullous pemphigoid, and atherosclerosis (Hautamaki et al. 1997) (Pyo et al. 2000) (Liu et al. 1998). MMPs are endopeptidases that share similar structural features including a prodomain, a Zn-containing catalytic domain, and in most cases, a hemopexin-like carboxy-terminal domain (excluding MMP7, -23, and -28). Some MMPs contain additional domains including a gelatin-binding domain (MMP2 and -9), transmembrane domain (MMP14, -15, -16, and -24), or a GPI-anchor domain (MMP17 and -25).

MMPs are typically secreted as inactive proenzymes, or zymogens. The inactive state is maintained by the coordination of the Zn ion with an unpaired cysteine sulfhydryl group within the propeptide domain. The activation of MMPs usually occurs in the extracellular space upon the cleavage of the propeptide by other activated MMPs or serine proteinases, releasing the zinc ion, the coordination of which is essential for catalysis. There are exceptions to this simple schema, however. The activation of MMP2, for example, is a complex process involving secretion of pro-MMP2 and subsequent binding to tissue inhibitor of metalloproteinase-2 (TIMP2). This surface-bound MMP2, which is resistant to processing by the serine proteinases, is then activated by MMP14 (Strongin et al. 1995).

As is the case for serine proteinases, Alpha-2 macroglobulin functions as a major serum inhibitor of the MMPs. Specific inhibition of the MMPs is accomplished by

Table 9.1 Substrate a	specificity	r of selecter	d elastases								
Substrate	NE	PR3	MMP2	MMP3	MMP7	MMP9	MMP10	MMP12	Cat S	Cat L	Cat K
Elastin	+	+	+	+	+	+	+	+	+	+	+
Aggrecan			+	+	+	+	+	+			
Brevican			+								
Collagen I	+		+	Ι	+	+		+	+	+	+
Collagen III	+		Ι	+	Ι	I	+		+	+	+
Collagen IV	+	+	+	+	+	+	+	+			
Collagen V			+	+	I	+	+				
Entactin	+		+	+	+			+			
Fibrillin			+	+		+		+			
Fibronectin	+	+	+	+	+	I	+	+	+	+	+
Fibulin			+		+						
Gelatin	+		+	+	+	+	+	+	+	+	+
Laminin	+	+	+	+	+	+		+	+	+	+
Vitronectin		+	+	+	+	+		+			
A1AT			+	+	+	+		+			
α2-macroglobulin			+	+	+	+					
Pro-TNFa			+	+	+	+		+			
Plasminogen	+			+	+	+		+			
TIMPs	+										
Reviewed in (Chapme	an et al. 1	997; Sternl	icht and Werb	2001; Hedstr	om 2002)						

the TIMPs, of which there are four members. The TIMPs are 20–29-kDa reversible inhibitors of MMPs whose activity mostly arises from evenly spaced cysteine residues within the N-terminal domain of the protein (Gomez et al. 1997). The individual members of the TIMP family differ in their ability to inhibit specific MMPs. For the most part, TIMP2 and -3 display preferential activity against the membrane-bound MMPs, while TIMP1 inhibits the secreted MMPs, although there are exceptions.

Based upon simple in vitro assays of catalysis, six members of the MMP family (MMP2, -3, -7, -9, -10, and -12) have been shown to cleave elastin (see Table 9.1), although the ability of MMP3 and -10 has been questioned. Pathophysiological roles for these enzymes in elastic fiber degradation appear to be a function of the source of enzyme expression. Macrophage-derived MMPs (9 and 12) are important in elastic fiber degradation occurring within lung whereas MMP2, -3, and -10 are most commonly implicated in vascular elastin degradation. However, MMP9 and -12 have been implicated in both COPD/emphysema and AAA, to varying degrees.

MMP2 (gelatinase A or 72-kDa gelatinase) is a simple hemopexin-type MMP with the addition of a gelatin-binding domain. It can be elaborated from various sources but is primarily fibroblast or mesenchymal cell-derived. MMP2 displays a broad substrate specificity including all structures of the basement membrane and is unique in its ability to degrade both elastin and fibrillar collagens. The predominant pathophysiological role for MMP2-mediated elastic fiber degradation is in the formation and progression of AAA. $MMP2^{-/-}$ mice are protected from the development of aortic aneurysms in the CaCl₂ infusion model (Longo et al. 2002). The contribution of MMP2 was purely mesenchymal cell-derived in AAA formation as bone marrow transfer experiments failed to reverse the phenotype. Furthermore, MMP2 has been identified in clinical AAA specimens using IHC, giving credence to a role for the enzyme in human disease (Goodall et al. 2001).

MMP3 (stromelysin-1) and -10 (stromelysin-2) have also been implicated aneurysm formation. Polymorphisms in each of these genes confer increased risk of AAA, although in different populations (Yoon et al. 1999; Ogata et al. 2005). Expectedly, $MMP3^{-/-}$ mice are protected in experimental models of AAA (Silence et al. 2001). The TIMPs are also required in this setting, as their deletion results in increased aortic diameter upon injury (Silence et al. 2002). Similar to the findings for MMP2, the effects of MMP3 (in this model) are mesenchymal cell- and not inflammatory cell-derived.

The individual contributions of macrophage-derived MMPs to elastolysis have been difficult to tease apart. Both macrophage-derived MMP9 and -12 play significant roles in elastolysis in vivo. MMP9 contributes to aneurysm formation in both the elastase and CaCl₂ models of AAA formation (Pyo et al. 2000; Longo et al. 2002). Bone marrow transfer studies have proved that the contribution of MMP9 in this setting is solely macrophage-derived. The role of MMP12 in AAA remains unclear. Initial studies using $MMP12^{-/-}$ mice in the elastase model of AAA failed to demonstrate a role for this elastase (Pyo et al. 2000). A subsequent study employing the CaCl₂ model, however, suggested that MMP12 is required for macrophage accumulation in AAA (Longo et al. 2005), consistent with EF-mediated monocyte

recruitment, described above. The contribution of elastolytic MMPs to atherosclerotic plaque formation and rupture has also been investigated. Interestingly, MMP3 and -9 are protective against plaque rupture whereas MMP12 was disease promoting (Johnson et al. 2005).

Within the lung, the roles of MMP9 (gelatinase B or 92-kDa gelatinase) and MMP12 (macrophage elastase) appear to be reversed, with MMP12 playing a substantially greater role at least in mice. The majority of elastolytic properties of murine alveolar macrophages can be attributed to MMP12. Human alveolar macrophages may also be more dependent upon MMP9, and likely MMP7, to degrade elastin (Filippov et al. 2003). Nevertheless, MMP12 has consistently been demonstrated as an essential component of emphysema pathogenesis in both mouse and man. First described in 1975 (Werb and Gordon 1975), the subsequent cloning and generation of null-mutant mice for MMP12 has established this largely macrophage-specific elastase as essential for the disruption of basement membrane structures both in vitro and in vivo (Shipley et al. 1996). As discussed above, $MMP12^{-/-}$ mice are completely protected from both the development of airspace enlargement and macrophage accumulation upon exposure to cigarette smoke (Hautamaki et al. 1997). MMP12-mediated degradation of elastic fibers is sufficient to cause both the structural (loss of elastic recoil) and biological (generation of EFs and accumulation of macrophages) consequences of elastolysis (Houghton et al. 2006a, b). Expression profiling has demonstrated that MMP12 is the most upregulated gene in human alveolar macrophages (Woodruff et al. 2005). Most recently, Hunninghake and colleagues demonstrated that polymorphisms in MMP12 influence the prevalence of COPD/emphysema in humans (Hunninghake et al. 2009).

The role of MMP9 in COPD/emphysema is less clear. MMP9 is an elastase, and is easily identifiable in human emphysematous lungs (Ohnishi et al. 1998), being highly expressed by both macrophages and neutrophils. Surprisingly, $MMP9^{-t-}$ mice are not protected from cigarette smoke-induced airspace enlargement (SDS, unpublished). They are protected from the occurrence of subepithelial airway fibrosis within the airways. Thus it is likely that MMP9 is involved in the pathogenesis of COPD/emphysema, and further studies using bone marrow transfer protocols may yet tease apart disparate roles for airway epithelial cell-derived and inflammatory cell-derived MMP9. The major phenotypes of elastase nullmutant mice are reviewed in Table 9.2.

9.5.3 Cysteine Proteinases

The cysteine proteinases are a large group of plant and animal endopeptidases with amino acid homology limited to the active site. The mechanism of catalysis is similar to that of the serine proteinases, utilizing a catalytic dyad in which a His residue donates a proton to the sulfhydryl group of the Cys, which functions as the nucleophile (Wolters and Chapman 2000). There are 11 human cysteine cathepsins, which belong to the papain subfamily of cysteine proteinases. These are

Genotype	Phenotype	Reference
NE ^{-/-}	Reduced bacterial killing	Belaaouaj et al. (1998), Weinrauch et al. (2002)
	Protection from bullous pemphigoid	Liu et al. (2000)
	Partial protection from cigarette	Shapiro et al. (2003)
	smoke-induced emphysema	
	Decreased lung tumor growth	Houghton et al. (2010)
$CG^{-/-}$	Normal neutrophil function	MacIvor et al. (1999)
	Impaired PMN integrin clustering	Raptis et al. (2005)
^a <i>PR3</i> ^{-/-}	Decreased neutrophilic inflammation	Kessenbrock et al. (2008)
$MMP2^{-/-}$	Protected from AAA formation	Longo et al. (2002)
	Decreased tumor growth	Itoh et al. (1998)
<i>MMP3^{-/-}</i>	Protected from AAA formation	Silence et al. (2001)
<i>MMP7^{-/-}</i>	Impaired intestinal clearance of bacteria	Wilson et al. (1999)
	Impaired neutrophil recruitment to lung	Li et al. (2002)
	Decreased tumor growth	Wilson et al. (1997)
$MMP9^{-/-}$	Abnormal bone development	Vu et al. (1998)
	Protected from AAA formation	Pyo et al. (2000)
	Decreased tumor growth	Bergers et al. (2000), Coussens et al. (2000)
	Protection from bullous pemphigoid	Liu et al. (1998)
MMP10 ^{-/-}	Normal adipose tissue development	Lijnen et al. (2009)
MMP12 ^{-/-}	Impaired basement membrane penetration by macrophages	Shipley et al. (1996)
	Protection from cigarette smoke-induced emphysema	Hautamaki et al. (1997)
	Protection from cigarette smoke-induced macrophage accumulation	Houghton et al. (2006a, b)
	Normal response to AAA formation	Pvo et al. (2000)
	Partial protection from AAA formation	Longo et al. (2005)
	Increased susceptibility to lung metastases	Houghton et al. (2006)
	Reduced bacterial killing	Houghton et al. (2009)
Cat $S^{-/-}$	Impaired antigen presentation	Shi et al. (1999)
	Protection from arthritis	Nakagawa et al. (1999)
	Protection from atherosclerosis	Sukhova et al. (2003)
Cat $L^{-/-}$	Impaired skin and hair follicle development	Roth et al. (2000)
Cat $K^{-/-}$	Osteopetrosis	Saftig et al. (1998)
	Protection from atherosclerosis	Lutgens et al. (2006)

Table 9.2 Major phenotypes of elastase null-mutant mice

^aThis description is of an NE/PR3 doubly deficient mouse. The $PR3^{-/-}$ single mutant null mouse has not been described

intracellular enzymes predominantly located within endosomes and lysosomes. They are occasionally secreted into the extracellular space. Four of these enzymes are capable of degrading elastin, cathepsin V, S, L, and K.

Hydrolysis of elastic fibers and other components of the ECM likely occur via both extracellular and intracellular means. Partially degraded collagen fragments, for example, are trafficked to lysosomes where they are further degraded by the intracellular cysteine cathepsins. Elastic fiber degradation by cysteine cathepsins displays a "team" approach, whereby cathepsin K attacks insoluble fibers, and cathepsins S and K prefer soluble fragments. The cathepsins are the most potent elastases known (Chapman et al. 1997; Yasuda et al. 2004), especially cathepsins K and V (Table 9.3). Cathepsin K is most active at acidic pH (within lysosomes), and although found extracellularly on occasion it is not stable at neutral pH. Cathepsins S and L, in contrast, are both stable in the extracellular environment at neutral pH. Perhaps the greatest contribution to elastolysis in vivo is made by the nonelastolytic cathepsin C (DPPI), which is required for the activation of neutrophil-derived serine proteinases. The cysteine cathepsins are inhibited by a superfamily of inhibitors, termed the cystatins, of which cystatin C is the most prevalent (Abrahamson et al. 1987).

The relative contribution of cysteine cathepsins versus MMPs versus serine proteinases both in vitro and in vivo remains difficult to elucidate (Chapman and Stone 1984; Punturieri et al. 2000; Filippov et al. 2003). In vitro assays attempting to determine which macrophage-derived elastases are operative have provided conflicting results. The expression of MMPs versus cathepsins changes over time such that different results will be obtained depending on which time point is used. With respect to emphysema, all four elastolytic cysteine cathepsins (S, L, V, and K) can be expressed by alveolar macrophages and therefore could theoretically contribute to elastic fiber degradation within the lung. IL-13 transgenic mice develop airspace enlargement characteristic of emphysema and display a several-fold increase in cathepsin S expression (Zheng et al. 2000). However, subsequent study of *Cat* $S^{-/-}$ mice in the cigarette smoke-exposure model failed to show protection (SDS, unpublished).

Elastolytic cathepsins significantly contribute to atherosclerotic disease. The in vivo contributions of these enzymes have been best characterized in the $ApoE^{-/-}$ mouse model of atherosclerotic plaque and rupture. $CatS^{-/-}$, $CatK^{-/-}$, and Cystatin C-deficient mice are all involved in plaque formation and rupture in this model (Lutgens et al. 2007). The exact source and contributions of the cathepsins to atheroma remain unclear. Smooth muscle cells, endothelial cells,

		D. (
Proteinase	Rank	Reference
Cathepsin K		
рН 5.5	10	Bromme et al. (1996)
рН 7.4	6	Bromme et al. (1996)
Pancreatic elastase	8	Baugh and Travis (1976)
Cathepsin L		
pH 5.5	5	Mason et al. (1986)
Cathepsin S		
рН 5.5	7	Xin et al. (1992)
рН 7.4	2.5	Xin et al. (1992)
Neutrophil elastase	3	Baugh and Travis (1976)
MMP2	2.5	Senior et al. (1991)
Proteinase-3	1.5	Kao et al. (1988)
MMP7	1.5	Murphy et al. (1991)
MMP12	1	Senior et al. (1991)
MMP9	1	Senior et al. (1991)

Table 9.3 Relative potency of selected elastases

Table reproduced with permission from Chapman et al. (1997)

and macrophages express both cathepsins S and K in this context. Decreased elastin breaks have been documented for both $CatS^{-/-}$ and $CatK^{-/-}$ mice in this context and likely impact macrophage accumulation. Additionally, there may be more sophisticated and nonelastolytic functions for these enzymes in atherosclerotic disease. For a more detailed review of cysteine cathepsins, see Chap. 2.

9.6 Nonelastolytic Contributions of Elastases

Matrix degrading enzymes have traditionally been named for the substrates that they degrade (i.e., collagenases, elastases, gelatinases, etc.). The fact that an enzyme can degrade a given substrate in vitro demonstrates what the enzyme can do, but not necessarily what it does do, in vivo. Elastin is such an inert substrate that elastolysis is an important biological function for essentially all elastases. On the other hand, one could assume that because elastases are such good enzymes, they will possess very broad substrate specificities and thus play important roles independent of elastin. Indeed, novel, nonelastolytic roles continue to emerge for elastases in important aspects of biology including host interactions with invading microbes and tumors.

9.6.1 Antimicrobial Properties of Elastases

Host defense against invading microorganisms is an elaborate process involving several different cell types that make up the innate and adaptive immune system. Macrophages and neutrophils, both of which possess elastolytic enzymes, represent the first line of defense (innate) against infection. Macrophages have been long known to clear invading microorganisms during the initial stages of infection (Green and Kass 1964) but the means by which they do so was poorly understood. Macrophages possess some defensin-like antimicrobial peptides and reactive nitrogen species that display antimicrobial properties (Biggar and Sturgess 1977; Hiemstra et al. 1993; Ganz 1999). It has recently been shown that macrophage elastase (MMP12) also significantly contributes to the microbicidal properties of macrophages (Houghton et al. 2009).

 $MMP12^{-/-}$ mice display increased mortality when challenged with bacteria at macrophage-rich portals of entry, such as the peritoneum and lung. Using macrophage-killing assays, we were able to demonstrate that $MMP12^{-/-}$ macrophages were able to phagocytose bacteria normally, but were unable to kill them. Additional studies revealed that MMP12 is directly bactericidal to both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. MMP12 disrupts bacterial membrane integrity resulting in bacterial cell lysis and death. Interestingly, MMP12 does not require its catalytic domain to kill bacteria. All of the bactericidal activity of MMP12 resides within a 20-residue sequence (SR-20) located in the carboxy-terminal domain (CTD),

a domain that typically enhances the binding of an MMP to its substrate. SR20 is highly conserved within MMP12 across species. However, it is not found in other MMPs. Structural analysis of the MMP12 CTD revealed that SR20 exists in vivo as a β -loop (Fig. 9.4). Since MMP12 CTD is capable of killing bacteria without additional processing, we were able to identify just three regions of the peptide that could theoretically interact with bacteria. One of these sequences, KDEK, was of interest because of its unique composition of two basic residues flanking two acidic ones, occurring on an exposed loop of the protein. The bactericidal properties of SR20 were ascribed to these four residues, as mutating this motif and replacing it with the SGRQ motif found in MMP9, eliminated antimicrobial activity.

MMP12 is the only MMP with direct microbicidal activity. Matrilysin (MMP7) is also essential for host defense against invading pathogens, although in this case, the antimicrobial properties are indirect (Wilson et al. 1999). Alpha-defensins are potent antimicrobial peptides produced by numerous cell types including gastrointestinal epithelial cells. Upon infection, MMP7 is required to cleave and activate alphadefensins prior to their secretion into the extracellular space, where they kill bacteria. This is the only other report of antimicrobial activity by an MMP. Matrilysin is also required for the development of chemotactic gradients that acutely recruit neutrophils to the lung (Li et al. 2002). The neutrophil chemokine, KC, is sequestered within syndecan matrices and requires MMP7 to cleave and release it. This activity is also important for host defense, as proper neutrophil emigration into the lung is a major feature of innate host defense against invading microbes.

Neutrophils also possess toxic substances capable of killing invading pathogens, most notably the reactive oxygen species. Additionally, both CG and NE possess antimicrobial properties (Gabay et al. 1989). Based upon results using gene-targeted mice, it appears that NE plays a greater role than CG in the clearance of bacteria in vivo. $NE^{-/-}$ mice display increased mortality when challenged with Gramnegative infection (Belaaouaj et al. 1998), which is not observed in $CG^{-/-}$ mice.

Upon recruitment to sites of acute infection, neutrophils engulf invading pathogens. They then shuttle preformed and active NE into the phagolysosomes where it directly attacks the outer membrane proteins (Omp) located on Gram-negative



Fig. 9.4 *Macrophage elastase (MMP12) CTD*. Computational three-dimensional model of mouse MMP12 CTD. The antimicrobial SR-20 sequence is located within CTD blade II including β -strands β 2 and β 3, as well as the connecting and flanking loops (green trace) (Reproduced with permission by the author AMH.)

bacterial cell walls. Cleavage of OmpA in *E. coli*, for example, causes bacterial cell lysis (Belaaouaj et al. 2000). The antimicrobial properties of NE have been extended to include the enteritides and other organisms (Weinrauch et al. 2002). Similar to MMP12, it does not appear that NE requires its catalytic domain to kill bacteria. Additionally, NE preferentially kills bacteria within the neutrophil, although it remains microbicidal after secretion into the extracellular space.

9.6.2 Functions of Elastases Within the Tumor Microenvironment

The functions of proteinases within the tumor microenvironment have traditionally been limited proteolysis of ECM structures to allow for expansion and invasion of the tumor. Some elastases perform critical functions both for and against the host with respect to tumorigenesis. These functions do not involve the degradation of elastin, and may or may not involve substrates encountered within the ECM.

Vascular endothelial growth factor (VEGF), commonly found sequestered with the ECM, is an essential mediator of tumor-associated angiogenesis. Although several proteinases are capable of releasing bioactive VEGF (Lee et al. 2005), animal studies suggest that MMP9 is required to perform this function in vivo. Using bone marrow transfer studies, several independent studies have demonstrated that bone marrow derived MMP9 (either from mast cells, macrophages, or neutrophils) is an essential event for tumor-associated blood vessel formation (Bergers et al. 2000; Coussens et al. 2000; Nozawa et al. 2006). In these studies, the inhibition of either MMP9 or VEGF eliminated tumor growth and angiogenesis. Although MMP2 is also capable of releasing VEGF from matrix, this does not appear to be the predominant function of MMP2 in vivo. MMP2 directly ligates and activates the $\alpha_v\beta$ 3 integrin located on endothelial cells and promotes angiogenesis via matrix-independent means (Brooks et al. 1996). Whether or not MMP9 and/or MMP2 mediates degradation of basement membrane structures allowing for tumor invasion and subsequent metastasis remains a topic of debate (Hotary et al. 2006).

In contrast to the tumor-promoting functions of the gelatinases, MMP12 actually functions for the host within the tumor microenvironment. Macrophage-derived MMP12 generates angiostatic factors from tumor-associated matrix. The generation of angiostatin from plasminogen and of endostatin from collagen type XVIII can be performed by MMPs other than MMP12 (Cornelius et al. 1998). However, their generation is dependent upon MMP12 in vivo, as MMP12 null mice display increased tumor growth and vessel density in tumor xenograft models (Houghton et al. 2006a, b).

Neutrophils have been identified at sites of tumorigenesis but their function is poorly understood. Neutrophils are actually recruited to sites of tumorigenesis by tumor cells themselves (Sparmann and Bar-Sagi 2004; Ji et al. 2006). NE has recently been shown to promote tumor growth via a matrix-independent mechanism (Houghton et al. 2010). Tumor-associated PMN release NE near the tumor cell surface in modest concentrations. NE then enters tumor endosomes and can be

located within tumor cells. Once inside tumor cells, NE degrades a novel target substrate called insulin receptor substrate-1 (IRS-1). IRS-1 is a key regulator of the phosphoinositol-3 kinase (PI3K) pathway, which dictates cell proliferation and survival. Upon the degradation of IRS-1, the homeostatic binding partner of PI3K is lost, and PI3K is subsequently free to bind to more potent growth factors, such as the platelet-derived growth factor and receptor (PDGF and PDGFR). PI3K-PDGFR interaction activates PI3K signaling, resulting in tumor cell proliferation. This represents the first description of a secreted proteinase gaining access to another cell and effecting its intracellular signaling. This concept greatly expands the list of potential substrates and functions for secreted proteinases in cancer.

The protumor function of NE also translates to human disease. The inverse relationship identified between NE and IRS-1 in murine tumors also exists in human lung adenocarcinomas. In other words, the presence of NE infiltration in human cancers correlates with absence of its target substrate, IRS-1. NE-inhibition may prove to be a viable therapeutic option for human cancers, as treatment of tumor-bearing mice with a synthetic NE antagonist, ONO-5046, reduced tumor growth in vivo. Part of the failure of clinical trials employing MMP inhibitors as cancer therapy stemmed from the varied prohost and protumor functions of the different MMPs (Coussens et al. 2002). One would not expect similar problems using highly specific single agent therapy against NE as cancer chemotherapy.

9.7 Conclusions

Elastic fibers are unique and highly cross-linked structural proteins that evolved to provide flexibility to tissues and to provide the required resiliency of blood vessels in a closed circulatory system. The creation of a mature elastic fiber is a complex temporal–spatial process involving numerous cell types and proteins that cannot be adequately duplicated after the earliest stages of postnatal life. Elastases were developed prior to elastin, as they appeared much earlier during the course of evolution. Their ability to degrade elastin provides no apparent advantage to the host. The ability to degrade elastin may serve as a "marker" of an extremely effective enzyme with broad substrate specificity. Many of these enzymes perform paramount functions in host defense, which explains why their side effect (elastolysis) would be tolerated.

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