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Matrix Metalloproteinase Dependent and Independent Collagen Degradation

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1. ABSTRACT

Cleavage of the fibrillar collagens occurs during physiological conditions, as well as pathological conditions. The resistance of the fibrillar collagens to degradation is due to their rigid and compact structures. There are only a limited number of proteinases that have the capability to initiate the cleavage of the fibrillar collagens. These include some of the matrix metalloproteinases (MMPs) and cathepsins, as well as a few serine proteinases. The MMPs have long been implicated in the collagen degradation and remodeling that occurs at physiological pHs. The cathepsins, on the other hand, have been implicated in the collagen cleavage that occurs at acidic pHs, particularly the collagen degradation that is mediated by osteoclasts. In addition to the MMPs, a few serine proteinases have been implicated in the collagen degradation that occurs at neutral pH. The characteristics that contribute to the resistance of the fibrillar collagens to cleavage are discussed along with the MMPs, cathepsins, and serine proteinases that can cleave these collagens.

2. INTRODUCTION

A common characteristic of many pathological conditions such as arthritis, periodontitis, arteriosclerosis, and tumor metastasis is the destruction of the extracellular matrix (ECM). As structural components of the ECM, the collagens are responsible for the structural integrity of the ECM. About 20 different collagens have been discovered in mammals. They are formed from a combination of more than 25 distinct collagen chains. After being synthesized as large molecules known as pro-chains by membrane-bound ribosomes in the rough endoplasmic reticulum (1), these peptide chains undergo modifications, self-assembly, and proteolytic processing inside and outside of the cells to form the mature collagens. The typical mature collagen molecule consists of three collagen polypeptide chains with at least one stretch of helical region in which the three chains are wound around one another in a rope-like superhelix. The triple helical molecules aggregate and form cross-links among themselves to form fibrils, which are further cross-linked to form collagen fibers. According

Table 1. Fibrillar Collagens

Type	Chain Composition	Structure Features	Tissue Localization	Cross-link sites	References for cross -link sites
I	$[\alpha1(I)]_2[\alpha2(I)]$	300 nm long fibril	Skin, tendon, bone, ligament, dentin, interstitial tissues	α1(I): $Lys9^N$-$Lys930^N$ $Hyl16^C$-$Lys87^N$ α2(I): $Lys5^N$-$Hyl933^N$	44, 45
II	$[\alpha1(II)]_3$	300 nm long fibril	Cartilage, vitreous humor	C^{87}-N^{930}	46, 47, 48
III	$[\alpha1(III)]_3$	300 nm long fibril	Skin, muscle, blood vessels	$Lys8^N$-$Hyl936^C$	44, 49
V	$[\alpha1(V)]_2[\alpha2(V)]$/ $[\alpha1(V)]_3$/ $[\alpha1(V)][\alpha2(V)][\alpha1(V)]$	390 nm long fibril; often with Type I and III	Cornea, teeth, bone, placenta, skin, smooth muscle, fetal tissues	C-telopeptide of α1(I) and C-terminal helical region of α1(V)	40, 44, 51
XI	$[\alpha1(XI)]_3$/ $[\alpha1(XI)]$ $[\alpha2(XI)]$ $[\alpha3(XI)]$	Often with Type II		α1(XI) helix – $\alpha2(XI)\ 24^N$ α2(XI)helix-$\alpha3(XI)\ 11^N$ α3(XI) helix-$\alpha1(XI)\ 24^N$	51

C: Carboxyl group; N: Amino group.

to their amino acid composition and physical properties, the collagens are grouped as network-forming collagens (Types IV, VII, VIII and X), fibril-associated collagens (Types VI, IX, XII, and XIV), transmembrane collagens (Type XIII and XVII), host defense collagens (collectins, C1q, and class A scavenger receptors), and fibrillar collagens (Types I, II, III, V, and XI; Table 1) (2, 3).

The fibrillar collagens compose 90% of all the collagens in the body and are the primary components of the ECM. They are unique in that they form non-interrupted triple helices that are then cross-linked to each other to form fibrils. This endows the fibrillar collagens with the ability to resist cleavage by most proteinases. Only certain matrix metalloproteinases (MMPs), select cathepsins, and a few serine proteinases have the ability to cleave these triple helical molecules. However, denatured fibrillar collagens (gelatin) are readily degraded by multiple proteinases. For example in osteoarthritis patients, newly synthesized Type II collagen is readily denatured that results in a loss of its ordered fibril structure (4, 5), which makes it readily susceptible to degradation by multiple proteinases.

In this review, the structural characteristics of the fibrillar collagens that make them resistance to general proteinase degradation are discussed along with the enzymes that are capable of degrading these collagens.

3. FIBRILLAR COLLAGENS

3.1. Stability of the triple helix

The mature fibrillar collagens consist of a continuous triple helical structure except for their short amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) regions that are referred to as the telopeptides. These helical molecules then aggregate in a "head-to-tail" pattern with partial overlap and form covalent cross-links and hydrogen bonding, and are therefore characterized by a staggered appearance under the microscope.

Rich and Crick (6) modeled the structure of tightly packed polyglycine peptides and confirmed theoretically that the twisting of three polyglycine molecules together forms a right handed helix with the repeat length of 3.1 Å, which is close to the structure of the triple helix in the fibrillar collagens. It is well known that the repeated amino acid triplet, Glycine-X-Y (X and Y can be any amino acid but are proline or hydroxyproline a high percentage of the time), is essential for the formation of the collagen triple helix. The glycine (Gly) residues are essential in every third position because the close packing of the collagen chains near the central axis does not leave room for larger residues (7-9). Even a single base mutation of Gly for another amino acid in either the Type I collagen α1 (I) or α2 (I) chain can result in distortion of the collagen triple helix that results in misformed collagen and osteogenesis imperfecta (10-12). Moreover, the mature α1 (I) collagen chain contains more than 330 repeating Gly-X-Y triplets.

The stability of the triple helix depends partially on the amount of proline (Pro) and its modified form, hydroxyproline (Hyp), in the X or Y positions of the triplet. In 1964, it was reported that the thermal stability of synthetic collagen peptides is determined by the total number of Pro and Hyp residues (13). When comparing native Type I collagen from the sea urchin to that from rat tail, the rat tail collagen displayed a higher melting temperature (about 38^{o}C) and a higher content of Pro plus Hyp residues (17.9% Pro plus Hyp) than the sea urchin collagen (23^{o}C and 12.9% Pro plus Hyp) (14). The difference between the influence of Pro and Hyp on the thermal stability of collagen has also been examined (15-19). Both of these amino acids have been shown to contribute to collagen stabilization (15-19). The total number of Hyp residues in the Y position in a synthetic peptide fragment displays a linear association with the collagen to gelatin transition temperature (16, 18). The most stable triplet identified in vertebrates by artificial polypeptide studies is Gly-Pro-Hyp, which is the most common triplet in the Type I collagen molecule (20) and occurs about 40 times in the α1 (I) collagen chain.

It has been hypothesized that the gamma-hydroxyl group of Hyp at the third position in the triplet forms interchain hydrogen bonds that stabilizes the helix (21). Studies on collagen-like polypeptides such as a repeating sequence of Gly-Pro-Pro or Gly-Pro-Hyp have confirmed that hydration plays a key role in triple helix stability (22-26). Water molecules form two hydration shells around the artificial polypeptides by interacting with the polypeptide chains and by forming hydrogen bonds with water molecules outside of the first hydration shell (23, 26). The hydration shells contributes to the stability of

the triple helix structure, as well as to the self-assembly of the fibrils. Conditions such as pH and ion concentration affects the amount of water associated with the collagen and thus influences the stability and other properties of the triple helix, as well as affects collagen aggregation (27-29). In a crystal formed by a collagen-like peptide, adjacent triple helices have little or no direct contact (28, 29). The connections are maintained by the hydrogen-bonding water bridges (28, 29). For each $(Gly\text{-}Pro\text{-}Pro)_{10}$ peptide, one water molecule interacts with the carbonyl oxygen of the Gly and two others interacts with the carbonyl oxygen of the Pro in the Y position. With three water molecules bound to a triplet, each peptide occupies a larger volume that results in more distance between the peptides, which makes it difficult for the formation of hydrogen bonds between the peptides. Nevertheless, the contribution of bound water to the stability of the collagen triple helix is still controversial (26, 30).

The utilization of the artificial polypeptide chains has helped to explore the roles that Pro and Hyp have on collagen triple helix stability, but has also established the limits of our understanding of what happens with the real fibrillar collagens. However, evidence from a pathological condition of Type I collagen indirectly supports the role of Hyp on the stability of the triple helix. Scurvy is caused by the severe deficiency of vitamin C (ascorbic acid), a necessary reducing agent of prolyl hydroxylase, and results in a lower melting temperature for the collagens and causes a breakdown of the collagens needed for connective tissue, bones, dentin, and the walls of blood capillaries.

It has been well documented that the matrix metalloproteinases (MMPs), especially the collagenases (MMP-1, -8, and –13), cleave all three α chains of native Type I, II and III collagens at a single locus (Gly-Ile/Leu) located approximately three-fourths of the way down the molecules from the N-terminus (31-34). The Gly-Ile/Leu sequences are not unique in that there are many of these sequences in the native collagen chains that are not cleaved by the MMPs. It is believed that the content of Pro plus Hyp in the collagenase cleavage region is different from these other sites in the collagen chains. Highberger *et al.* (35) compared the helix formation and the amino acid sequences between two 36-residue peptides from the α1 (I) chain. These peptides consisted of a collagen cleavage peptide (with the collagen cleavage site in the center) and a non-cleavage peptide whose helix-coil transition behavior was very similar to the whole collagen molecule. The study demonstrated that the helices around the collagenase cleavage site were less compact than in other portions of the collagen chain. Such laxicity is attributed to the total number of Pro and Hyp in the 36-residue peptides and especially to the number of Hyp in the Y position of the triplet. Crystallographic studies have revealed that a polypeptide of Type III collagen (35-37) containing a Pro plus Hyp free region has a higher degree of flexibility than a peptide that has a Pro plus Hyp residue rich region. The mechanisms of how the MMPs recognize these specific sites are still under investigation.

3.2. Fibril Formation

Fibrils are another distinguishing feature of the fibrillar collagens and also essential to their resistance to general proteinase cleavage. Fibril formation increases the thermal stability of native bovine collagen and recombinant collagen fibrils by about $10^{o}C$ and $6^{o}C$, respectively (38). Fibril formation is a self-assembling process that depends on the intrinsic properties of the collagen molecules such as the persistence of the N- and C-terminal telopeptides, the distribution of polar and hydrophobic residues, and the possible copolymerization of several different collagen types into a single fibril. The mechanisms that drive such self-assembly are far from being fully elucidated. However, the intermolecular cross-links between the N- and C-terminal telopeptides and the triple helical region from another molecule are believed to be vital for the initiation of the "head-to-tail" fibril formation (39-42) while the hydrogen bonds formed by the polar residues further stablize the aggregation. The intactness of the telopeptides has been shown to influence the rate of fibril formation (43) and the fibril pattern (39). The intermolecular cross-links are specifically associated with the lysine and hydroxylysine residues located in the telopeptide regions and in the triple helix (Table 1) (40, 44-51). The positions of the cross-linking sites allow for the formation of bridges between the molecules, which are overlapped or staggered relative to one another by 4D units (D= 67 nm). Inhibition of or an increase in cross-link formation is responsible for the damage seen in the fibrillar collagens that results in the fragile fibrils seen in scurvy and the rigid collagens seen in aging fibrils, respectively (40, 51, 52).

The triple helix and the formation of fibrils are unique features of the fibrillar collagens. Both features ensure that the fibrillar collagens are closely packed, thus resulting in limited attack from general proteinases. The region of triple helix laxicity such as in the Pro and Hyp-poor region and at the sites at which cross-links are located within the collagen molecules such as the non-helical telopeptide regions are considered the weakest points in the structure of the fibrillar collagens. Collagenolytic enzymes routinely take advantage of either one or both of these regions to mediate cleavage of the fibrillar collagens.

4. MATRIX METALLOPROTEINASES

4.1. Matrix Metalloproteinase Family

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases responsible for the degradation of multiple extracellular matrix (ECM) components, especially the collagens and proteoglycans (53). The MMPs are involved in the ECM remodeling that occurs in physiological conditions such as tissue and organ development, morphogenesis, and wound healing. They also play important roles in the regulation of cellular communication, molecular shedding, and immune functions by processing bioactive molecules including cell surface receptors, cytokines, hormones, defensins, adhesion molecules, and growth factors (54, 55). The MMPs have also been implicated in pathological conditions such as

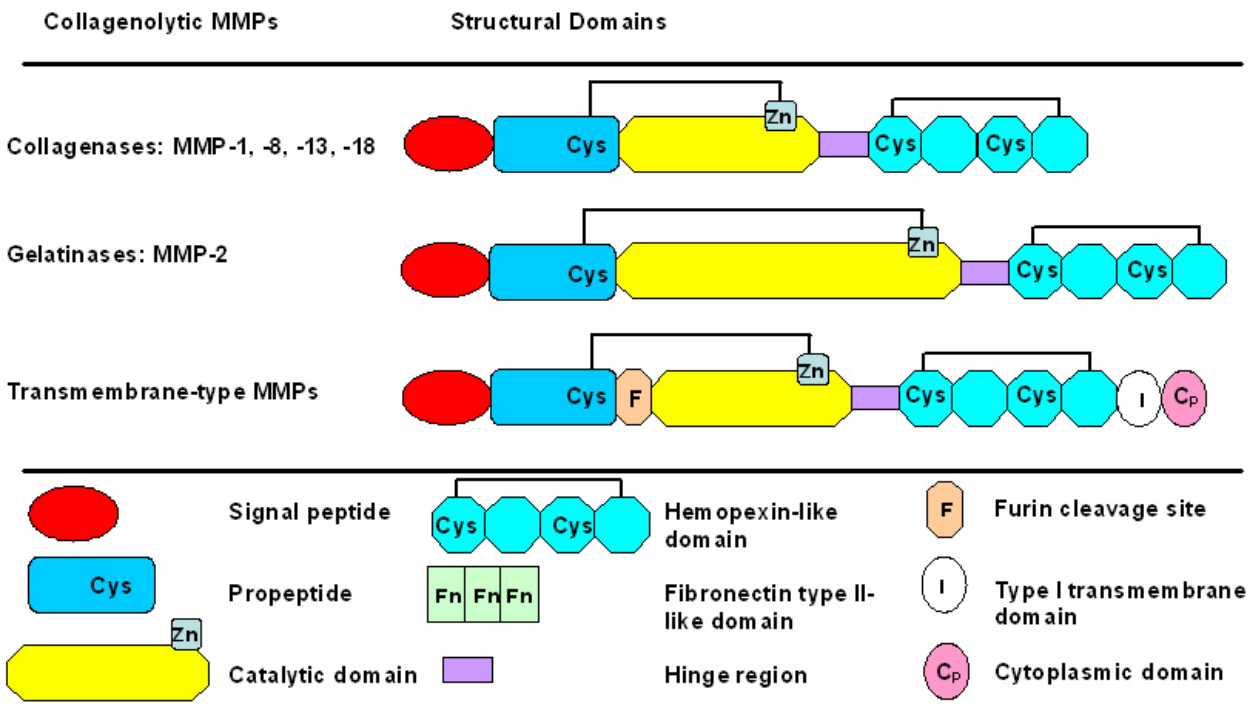

Figure 1. The domain structure of the collagenolytic MMPs. A propeptide domain, a catalytic domain, and a C-terminal hemopexin-like domain are common domains found in the MMPs. In addition to these common domains, the gelatinases contain fibronectin type II-like domains and the MT-MMPs contain transmembrane domains.

arthritis, cancer, asthma, atherosclerosis, and periodontal disease (53, 56, 57).

There are at least 25 members of the MMP family. They can be classified on the basis of substrate specificity and molecular structure into six groups (58). These include the collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), membrane-type MMPs (MT-MMPs) (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), minimal-domain MMPs (MMP-7 and MMP-26), and others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP-27, and MMP-28).

The prototypic MMP contains distinctive structural domains that consist of a signaling peptide, a prodomain, a catalytic domain, a hinge region, and a hemopexin (PEX) domain (Figure 1). The N-terminal signaling peptide directs the newly synthesized pre-proenzyme for secretion and is subsequently removed. The 77-87 residue propeptide of the MMPs contains a "cysteine switch" motif, PRCGXPD, in which the cysteine residue interacts with the catalytic zinc domain to maintain latency (59, 60). The catalytic domain (about 170 residues) contains the zinc-binding motif, HEXGHXXGXXH, in which the three histidine residues bind a zinc ion (61) that is essential for the proteolytic activity of the MMPs. A proline-rich hinge region joins the catalytic and PEX domains. The C-terminal PEX domain (about 200 residues) contains a four-bladed β propeller structure. The PEX domain often mediates protein-protein interactions (62), which is important in part for the substrate specificity of the MMPs. In addition to these domains, MMP-2 and MMP-9 contain three repeats of a fibronectin type II-like domain that are inserted in the catalytic domain (63). These repeats interact with gelatin to enhance MMP-2 and MMP-9 gelatin cleaving ability (64, 65). A transmembrane domain and a furin-activation site are other modifications that are found in other MMPs such as the MT-MMPs (66).

MMP activity is regulated by gene transcription and expression of the proenzymes, activation of the proenzymes, and then their interactions with inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs). Most of the MMPs appear to be secreted as inactive proenzymes, thus making activation a key step in regulating their activity. The disruption of the cysteine-zinc bond between the propeptide and catalytic domain is required for the activation of latent MMPs. The latent MMPs can be activated by proteinases, by non-proteolytic compounds, and by heat treatment (59). Non-proteolytic compounds capable of inducing MMP activation include SH-reactive agents (iodoacetate, 4-aminophenylmercuric acetate, hypochlorous acid, and oxidized glutathione) and denaturants (urea, sodium dodecyl sulfate, and sodium thiocyanate). Several proteinases have been shown to activate multiple latent MMPs *in vitro*. These proteinases include plasmin, trypsin, kallikrein, chymase, and mast cell typtase. The MMPs that contain a furin cleavage site can also be activated by furin. Some MMPs have been shown to be able to activate other members of the MMP family. MMP-14, MMP-15, and MMP-16 can activate MMP-2 (58). MMP-2 and MMP-3 have been shown to activate

Table 2. Cleavage Sites of Fibrillar Collagens Utilized by Collagenolytic MMPs

MMPs	Collagen Type	Cleavage Sites	References
MMP-1/ MMP-8	Human Type I (α1)	Gly^{775}-Ile^{776}	73
	Human Type I (α2)	Gly^{775}-Leu^{776}	73
	Human Type II	Gly^{906}-Leu^{907}	76
	Human Type II	Gly^{775}-Ile^{776}	32
	Human Type III	Gly^{775}-Leu^{776}	32
MMP-13	Human Type II	Gly^{906}-Leu^{907}	76
		Gly^{909}-Gln^{910}	76
MT1-MMP	Guinea pig Type I (α1)	Gly^{775}-Ile^{776}	75
	Guinea pig Type I (α2)	Gly^{775}-Leu^{776}	75
		Gly^{781}-Ile^{782}	75
MT3-MMP	Human Type III (α1)	Gly^{781}-Ile^{782}	77
		Gly^{784}-Ile^{785}	77
MMP-2	Bovine Type I (α1)	Gly^{775}-Ile^{776}	74
	Bovine Type I (α2)	Gly^{775}-Leu^{776}	74
MMP-9	Human Type V (α1)	Gly^{439}-Val^{440}	78
	Human Type V (α2)	Gly^{445}-Leu^{446}	78
	Human Type XI	Gly^{439}-Val^{440}	78

MMP-9. It has been reported that both MMP-3 (67, 68) and MMP-7 (69) can fully activate (superactivate) procollagenase-1 (proMMP-1). MMP-3 has been shown to superactivate MMP-1 by cleavage of the Gln^{80}-Phe^{81} bond in MMP-1 that results in 7-10 fold increase in collagenolytic activity (67, 68). MMP-7 has also been reported to superactivate MMP-1 (69) and the final activation site of proMMP-1 by MMP-7 is the same bond utilized by MMP-3 (69).

4.2. Collagenolytic MMPs and Collagen Cleavage Sites

The fibrillar collagens are resistant to most proteolytic enzymes. Even among the MMP family, only a limited number of them can cleave the highly structured fibrillar collagens under physiological conditions (37^0C and neutral pH). Members of the MMP family that include MMP-1 (collagenase-1 or interstitial collagenase), MMP-8 (collagenase-2 or neutrophil collagenase), MMP-13 (collagenase-3), MMP-18 (collagenase-4 or Xenopus collagenase), MMP-2 (gelatinase-A), and MMP-14 (MT1-MMP) are capable of cleaving Type I, II, and III collagens (70). Although structurally similar, MMP-3 (stromelysin-1) and MMP-10 (stromelysin-2) are unable to cleave collagen Types I or II (71). However, it has been shown that MMP-3 can bind Type I collagen (71). The collagenolytic activity of these enzymes is believed to involve a three-step process (72). First, they must bind the collagen. The unwinding of the triple helix in which the native collagen helices are locally disrupted to provide access follows this binding. The last step is the cleavage of the individual strands of the triple helix. The collagenolytic MMPs can catalyze the initial cleavage of collagens Type I, II, and III at specific Gly-Ile bonds of the α1 chains and a specific Gly-Leu bond of the α2 chain (Table 2) (32, 73-78) that generates the characteristic 3/4 N-terminal and 1/4 C-terminal fragments. These can then be readily denatured at body temperature and further degraded by the gelatinases and other nonspecific proteinases.

MMP-1 and MMP-8 have been shown to cleave the α1 chain and α2 chain of native Type I collagen at Gly^{775}-Ile^{776} and Gly^{775}-Leu^{776}, respectively (73). The same cleavage sites in Type I collagen have been shown to be cleaved by MMP-2 (74). MMP-1 and MMP-8 also cleaves the α-chains of Type II (Gly^{775}-Ile^{776}) and III collagens (Gly^{775}-Leu^{776}) at the same specific sites as in Type I collagen (32). Mitchell *et al*. (76) reported that MMP-1 cleaves Type II collagen into 3/4 and 1/4 fragments at the peptide bond Gly^{906}-Leu^{907}. In the same study, MMP-13 also cleaved Type II collagen at this site. However, MMP-13 also hydrolyzed the α-chains of Type II collagen at a secondary cleavage site of Gly^{909}-Gln^{910}, which is three amino acids C-terminal to the Gly^{906}-Leu^{907} cleavage site (76). The same secondary cleavage site was recently shown to be cleaved by MMP-1 and MMP-8 (79). Moreover a third cleavage site in Type II collagen, three amino acids carboxyl-terminal to the secondary cleavage site, is cleaved by MMP-13 (80). A recent study demonstrated that MMP-13 is an efficient telopeptidase and that this activity is dependent on its catalytic domain and independent of its C-terminal PEX domain (81). This feature distinguishes MMP-13 from MMP-1 and MMP-8 because telopeptidase activity has not been detected in these other two collagenases. The *N*-telopeptidase activity has been suggested to play a role in Type I collagen resorption during embryonic and early adult life, while triple helicase activity is necessary during intense tissue resorption later in life (82).

MT1-MMP is known to have collagenolytic activity. It has been shown to digest collagens Type I, II, and III into the characteristic 3/4 and 1/4 fragments. Type I collagen is cleaved by MT1-MMP at the Gly^{775}-Ile^{776} and Gly^{775}-Leu^{776} bonds of the α1 chain and α2 chain, respectively (75). MT1-MMP also cleaves a secondary site at the Gly^{781}-Ile^{782} bond of the α2 chain of Type I collagen in addition to the initial cleavage at the Gly^{775}-Leu^{776} (75). In addition, MT1-MMP also has an indirect effect on collagen degradation by activating MMP-13 (83) and MMP-2 (83, 84).

A recent study demonstrated that ΔMT3, a truncated form of MT3-MMP lacking the transmembrane and cytoplasmic domains, could cleave Type III collagen into the characteristic 3/4 N-terminal and 1/4 C-terminal fragments by cleaving at the Gly^{781}-Ile^{782} and/or Gly^{784}-Ile^{785} bonds of the α1(III) chains (77). MT3-MMP can not degrade Type I collagen into the typical 3/4 and 1/4 fragments, but it has been reported to cleave the Gly^{4}-Ile^{5} bond within the triple helical portion of α2(I) chain (85). MMP-9 cleavage of native Type V and XI collagen has been reported by Niyibizi *et al*. (78). It cleaves bovine bone Type V collagen at two sites, one near the N-terminus and the other at Gly^{439}-Val^{440} of the α1(V) chain and Gly^{445}-Leu^{446} of the α2(V) chain that results in a 3/5 C-terminal fragment. Type XI collagen has also been reported to be cleaved by MMP-9 between Gly^{439}-Val^{440} of the α1(XI) chain (78).

Table 3. Collagen Substrate Specificity of Collagenolytic MMPs

Collagen	MMPs	K_m (μM)	k_{cat} (h^{-1})	K_m/k_{cat} (μM^{-1} h^{-1})	References
Type I collagen	MMP-1	0.80	53.40	66.70	86
		1.00	16.60	16.60	74
		1.30	22.20	17.10	75
	MMP-8	0.70	6.40	9.14	89
	MMP-2	8.50	16.20	1.90	74
	MT1-MMP	2.90	7.10	2.40	75
Type II collagen	MMP-1	2.10	1.00	0.50	86
		1.00	2.00	2.00	76
	MMP-8	1.10	2.35	2.00	89
	MMP-13	2.00	23.00	11.50	76
Type III collagen	MMP-1	1.40	565.00	403.60	86
	MMP-8	1.80	0.85	0.47	89
	MT1-MMP	0.95	1.25	1.32	77
	MT3-MMP	0.45	3.12	6.93	77

4.3. Collagen Substrate Specificity

The rate of collagen hydrolysis differs among the MMPs and between the different fibrillar collagens (Table 3). MMP-1 has been shown to have similar substrate affinities (K_m = 1-2 μM) for collagens Type I, II, and III (86). In contrast, there are differences in the catalytic rates of MMP-1 for these collagens. The k_{cat} values on human Type I, II, and III collagens for MMP-1 have been shown to be 53.4, 1.0, and 565.0 h^{-1}, respectively (86). Therefore, MMP-1 has the most activity on collagen Type III and then collagen Type I, while activity on Type II collagen is very poor (86). Ohuchi *et al*. (75) also reported that the activity of MMP-1 on Type III collagen is approximately 4.4 and 25.6 fold greater than that on collagen Types I and II, respectively. This may be due to the more local instability of the triple helix at the cleavage site of Type III collagen than Type I collagen (87) and MMP-1 having limited ability to hydrolyze more thermally stable local structures (88).

While MMP-1 prefers Type III collagen as the substrate, MMP-8 cleaves Type I collagen 20-fold better than it cleaves Type III collagen (89). Type II collagen is cleaved 25% more efficient than Type I collagen, but 450% better than Type III collagen by MMP-8. MMP-13 is a very effective collagen-degrading enzyme. Analysis of the substrate specificity of MMP-13 has revealed that Type II collagen is preferentially hydrolyzed 5-6 times more efficient than Type I or III (90). It cleaves Type II collagen at least 10 times faster than MMP-1 (76), but it cleaves Type I collagen with rates comparable to MMP-1 and MMP-8 (90).

MMP-2 is not as efficient as MMP-1 in bringing about collagen Type I dissolution (74). It has been shown that the k_{cat} values of MMP-2 (16.2 h^{-1}) and MMP-1 (16.6 h^{-1}) are basically the same. However, the K_m value for MMP-2 (8.5 μM) is higher than that of MMP-1 (1 μM). The differences in the K_m values makes MMP-2 less efficient in Type I collagen degradation than MMP-1, although the k_{cat} values are similar (74).

The catalytic efficiency on Type I, II, and III collagens by a deletion mutant of MMP-14 (MT1-MMP) lacking the transmembrane domain (ΔMT1) was reported by Ohuchi *et al.* (75). ΔMT1 preferentially digested Type I collagen. ΔMT1 cleaved Type I collagen 6.5 and 4 fold better than Type II and III collagens, respectively. Comparison of Type I collagenolytic activity of ΔMT1 with that of MMP-1 revealed that ΔMT1 was 5-7 fold less efficient than MMP-1 (75). Because of the differences in collagen substrate specificity of MMPs, the types of collagen in tissues or organs may determine the pattern of MMP expression in the area during physiological remodeling or pathological degradation.

4.4. Collagen Binding and Cleavage

Several investigations have attempted to define which MMP domains are required for collagenolytic activity. The catalytic domains of the MMPs are responsible for their catalytic activity. However, the catalytic domain of the collagenolytic MMPs alone can not cleave the fibrillar collagens although it has activity against other substrates (91-93). Tyr^{191} within the catalytic domain is conserved among all the collagenolytic MMPs except for MMP-14, which possesses a Leu at this position (94). A Thr is found in MMP-3 at this position. It has been observed that a substitution of Thr for Tyr^{191} in MMP-1 reduced the collagenolytic and gelatinolytic activity about 5 fold (95). In the same study, the amino acid sequence Arg183-Trp-Thr-Asn-Asn-Phe-Arg-Glu-Tyr191 in the catalytic domain of MMP-1 was identified as being involved in the collagenolytic activity of the enzyme (95). However, the insertion of this sequence in MMP-3 did not result in any collagenolytic activity.

The importance of the hinge region of the MMPs in collagen binding and cleavage has been reported by Hirose *et al*. (96). It has been observed that a 16 amino-acid sequence motif within the 'hinge/linker' region of MMP-8 (residues 259-274) was important for its ability to hydrolyze the fibrillar collagens. The replacement of this hinge/linker region of MMP-8 (16 residues) with that of MMP-3 (25 residues) substantially reduced the proteolytic activity of the enzyme on Type I collagen (96). This study suggests that the length of the hinge/linker region is critical for collagenolysis by MMP-8. However, a MMP-3/MMP-1 chimera produced by Murphy *et al.* (71) had the correct linker peptide of MMP-1 but still did not have any collagenolytic activity. Furthermore, MT1-MMP has a hinge/linker peptide that is considerably longer than in the classical collagenases (97). Thus this suggests that the length of the hinge/linker peptide is not critical for collagenolytic activity in general. A recent study demonstrated the crucial role of Gly^{272} in the hinge region

of MMP-1 for the collagenolytic activity of the enzyme (98). This Gly^{272} residue has been suggested to be responsible for the hinge-bending motion that is essential for allowing the PEX domain to present the collagen to the catalytic site. Substitute of this residue for Asp limited the flexibility of the hinge region and lead to a lack of enzyme specificity (98). A motif within the hinge/linker sequence of the collagenases, Leu263-Ser-Ser-Asn-Pro-Ile-Gln-Pro270 in MMP-8 and Arg262-Ser-Asn-Gln-Pro-Val-Gln-Pro269 in MMP-1, has been hypothesized to be responsible for the ability of these collagenases to bind to and hydrolyze the fibrillar collagens (99). This was later confirmed by Knäuper *et al*. (93) in that replacement of Pro residues in the MMP-8 hinge/linker region with Ala residues reduced the collagenase activity to 1.5%. However, a chimeric mutant prepared from MMP-1 (1–250) and MMP-10 (256–459) that contained the MMP-1 hinge/linker sequence lacked collagenolytic activity (100). The importance of the hinge/linker region of MT1-MMP (MMP-14) has been noted by Tam *et al.* (101). MT1-CD, a recombinant protein containing the PEX domain without the linker region, was able to bind collagen. However, it did not block MT1-MMP mediated collagen cleavage when added in competition experiments nor did it disrupt the secondary structure of the collagen. MT1-LCD, a recombinant protein containing the PEX domain with the linker region, was able to bind collagen, disrupt the secondary structure of collagen, and competitively block MT1-MMP activity (101). Therefore, these studies demonstrated that the hinge/linker region is involved in the collagenolytic activity of the MMPs.

The C-terminal PEX domain facilitates in part substrate binding and the subsequent cleavage by the MMPs. In the collagenases, stomelysin-1, and MT1-MMP, their C-terminal PEX domains bind native collagen (71, 91, 93, 96). However, the C-terminal PEX domain of MMP-2 does not (102). Instead, the native collagen binding ability of MMP-2 is mediated by the fibronectin type II-like domains (64).

The essential role of the C-terminal PEX domain of MMP-1 in the binding and cleavage of the fibrillar collagens was first reported by Clark and Cawston (91). The catalytic domain alone retains proteolytic activities on noncollagenous proteins and peptides, but can not cleave the fibrillar collagens (71, 91). In addition, substitution of the two cysteine with other residues disrupts the C-terminal PEX domain structure and causes the loss of Type I collagenolysis but not proteolytic activity on other substrates (103). The C-terminal PEX domains of other collagenolytic MMPs including MMP-8 (92), MMP-13 (81), and MMP-14 (101, 104) are also important for their ability to bind and cleave the fibrillar collagens. Deletion of the C-terminal PEX domain from MMP-1 (residue 243-450) (71, 91), MMP-8 (residues 243-467) (92), or MMP13 (residues 249-451) (81) results in a loss of their collagenolytic activity. However, MMP-3 has a PEX domain that binds collagen but that does not support collagenolysis. Moreover, replacement of the PEX domain in MMP-3 with that of MMP-1 does not make MMP-3 collagenolytic (71). The PEX domain of MT1-MMP (MMP-14) has been demonstrated to regulate collagen binding and cleavage by this enzyme (101, 104, 105). Recent studies demonstrated that both the catalytic and PEX domains of MT1-MMP are required for effective collagenolysis (105). Exchanging either the catalytic or PEX domain of MT1-MMP with those of MT3-MMP yielded significantly lower collagenolytic activity than wild-type MT1-MMP. Moreover, replacement of the catalytic or PEX domain of MT1-MMP into MT3-MMP failed to generate any significant enhancement of collagenolytic activity compared to wild-type MT3-MMP (105). Both the N- and C-terminal regions of the collagenolytic MMPs contribute to the binding and thus cleavage of the fibrillar collagens. Therefore, the capacity of the collagenases to cleave triple helical substrates is dependent on the specific interactions between the catalytic, hinge, and PEX domains. However, the exact roles of each of these domains with respect to binding and cleavage of the collagen triple helix remain to be further elucidated.

MMP-2 does bind tightly to native and denatured collagen. However, it has been shown that this is mediated by the fibronectin type II-like domains that are localized within the catalytic domain and not by the PEX domain as in the other collagenolytic MMPs (65, 106). Deletion of these fibronectin-like domains abolishes collagen binding and significantly abrogates the gelatinolytic capacity of MMP-2 without affecting its ability to cleave peptide substrates or to bind TIMP-1 and TIMP-2 (107). Recombinant fibronectin type II-like domains, also known as the collagen binding domains, but not the hinge/PEX domains of MMP-2 induce conformational changes in the secondary structure of native collagen that made it more susceptible to cleavage by MMP-1 and MMP-8 (104). Earlier studies suggested that the PEX domain of MMP-2 makes no apparent contribution to Type I collagen binding (65, 106). However, a recent study reported that the initial collagen cleavage that generates the 3/4 and 1/4 fragments is strongly dependent on the presence of the PEX domain, while further hydrolysis is promoted by the fibronectin-like domains thus resulting in the rapid clearance of the initial degradation products (108).

4.5. Collagen Triple Helicase Activity

Three-dimensional structures of the collagenases have shown that the active site clefts are approximate 0.5 nm, whereas the diameter of collagen is about 1.5 nm in diameter (109). Therefore, the active site clefts are too narrow to accommodate the triple helical collagen molecule. Moreover, molecular docking experiments have shown that the closest peptide bond of collagen is about 0.7 nm away from the catalytic zinc atom (36). This suggests that either the collagenases or the fibrillar collagens must undergo considerable changes in conformation to initiate the cleavage of the Gly-Ile and Gly-Leu bonds of α1 and α2 chains of collagen Type I, respectively. It has been suggested that the binding of collagenolytic MMPs induces local unwinding of the collagen triple helix at the cleavage site to allow for the binding of only a single collagen α-chain to the active site cleft for cleavage (7, 87, 110-112).

Several hypotheses have been proposed to explain the interactions between the collagenases and the collagens that results in the destabilization of the triple helix (72, 113-115). The proline zipper model (113) hypothesizes that the proline-rich hinge region of the collagenases interacts with and unwinds the triple helical collagens. This hypothesis is based on the assumption that the proline hinge domain of MMP-1 adopts a collagen-like polyproline helix. With a collagen-like domain, the enzyme is able to disturb the quaternary organization of triple helix in the collagenase-susceptible site. Modeling analyses suggest that an interaction between the prolines of both the collagens and collagenase form a kind of "proline zipper" that is involved in the destabilization step (113). This destabilization makes the triple helical collagens susceptible to the cleavage by the enzyme. The collagen-trapping model (114, 115) proposes that after binding the triple helical collagens that the PEX domain folds over the catalytic site that then traps the substrate at the catalytic site cleft. This interaction causes destabilization or unwinding of the collagens around the cleavable bonds, which allows a single strand to fit into the catalytic site cleft where hydrolysis of each strand then proceeds. Overall (72) has proposed the following molecular mechanism for the triple helicase activity of the collagenolytic MMPs. First, simultaneous binding of the collagen to both the catalytic and the C-terminal PEX domains of the MMPs causes a bend in the collagen structure that distorts the triple helix. The distortion thus allows the triple helix to unfold at the cleavage site and then to be susceptible to the cleavage by the MMPs. An alternative mechanism to the localized melting of the triple helical collagen induced upon binding is the relative movement of the C-terminal PEX domain to the catalytic domain that causes distortion and unwinding of triple helix. Since this is a non-ATP-dependent reaction, the energy for the movement may be derived from the additional contacts of the MMP hinge region (72). The movement of the C-terminal PEX domain after first clamping to the collagen may occur to allow the hinge region to bind or to intercollate with the α-chains. This may laterally pull the collagen apart after a hinge-like action or more likely by a twist of the PEX domain to axially rotate the triple helix open. Compression forces exerted axially along the collagen triple helix might also cause distortion and localized unraveling of the triple helix at the compression bulge (72).

The mechanism of the triple helicase activity of the collagenases and the gelatinases are believed to be different because the PEX domain of MMP-2 can not bind the fibrillar collagens (72). The fibronectin type II-like region may clamp the collagen against the active site to splay or bend open the triple helix in an analogous mechanism to that proposed for the collagenases by Gomis-Ruth *et al*. in the collagen trapping model (114). However, Overall (72) has proposed the model of "strand intercollation" in which the fibronectin type II-like region binds to and interdigitates with the collagen triple helix to splay the three α-chains apart that then allows a single α-chain to bind to and occupy the active site cleft. Also supporting this model is that the fibronectin type II-like region binds to denatured collagen with higher affinity than to native collagen (64). To satisfy a binding preference for single α-chains rather than the native collagen helix, independent modular movement might facilitate penetration of the fibronectin type II-like region into regions of loosened collagen triple helix. This binding could drive the local distortion of the triple helical collagen structure to further loosen the helix to allow for substrate binding to the active site.

The ability of a mutant MMP-1 to unwind triple helical collagen was recently reported by Chung *et al*. (116). The study showed that Type I collagen was cleaved into the typical 3/4 and 1/4 fragments when collagen was incubated with the catalytically inactive MMP-1 mutant (MMP-1 (E200A)) in the presence of the catalytic domain of MMP-1 lacking the PEX domain (MMP-1(ΔC)). However, the MMP-1(ΔC) or MMP-1 (E200A) alone could not cleave the Type I collagen. These results suggested that the MMP-1 (E200A) mutant could locally unwind the triple-helical collagen before it is hydrolyzed by the MMP-1(ΔC) mutant.

Several hypotheses have been proposed as mentioned above to explain the collagenolytic mechanisms utilized by the collagenolytic MMPs. However, more experiments are needed to verify the validity of these proposed hypotheses. As discussed, the collagen binding and cleaving activity of the collagenolytic MMPs are believed to be mediated by interactions between the catalytic domain, the C-terminal PEX domain, and the hinge region of the collagenolytic MMPs. However, the precise location and the exact roles of each of these domains with respect to binding and cleavage of the collagen triple helix remain to be further elucidated.

5. CATHEPSINS

5.1. The cathepsin family

The cathepsins are a group of small proteinases that are most active at acidic pH and are usually characterized as lysosomal proteinases. The cathepsin family can cleave multiple components of the ECM. *In vitro* studies have shown that several members of the cathepsin family are able to cleave the fibrillar collagens (117-125). The cathepsin family consists of at least 16 members and can be subdivided into three distinct groups: serine proteinases (cathepsin A and G), aspartic proteinases (cathepsin D and E), and cysteine proteinases (cathepsins B, C, F, H, K, L, O, S, T, V, W and X). Several of these cysteine proteinases (cathepsins K, S, L, and B) share homology with the structure of papain (126). Cathepsin K shares a substantial degree of structural similarity to cathepsins L and S. Cathepsin K contains a cysteine^{139}, histidine^{276}, and asparagine^{296} in its active site (117). Most of the cathepsins are lysosomal proteinases except for cathepsins E and G (127).

5.2. Synthesis and physiological activity

Cathepsins are synthesized as precursor molecules, which contain N-terminal signal peptides that are cleaved off during transport through the endoplasmic reticulum (128). Pro-cathepsins then undergo proteolytic

processing to the active mature enzyme forms in the acidic environment of late endosomes or lysosomes (129, 130). These processed enzymes contain disulfide-linked heavy and light chain subunits with molecular weights ranging from 20-35 kDa with the exception of cathepsin C, which is a 200 kDa oligomeric enzyme.

The main physiological function of the cathepsins is protein turnover in the lysosomes. Lysosomal cathepsins exist in the intracellular acidic vacuoles and readily cleave the collagen engulfed by the cells. Therefore, cells that are responsible for collagen metabolism such as fibroblasts in various tissues contain these cathepsins. Cathepsin K is a key proteolytic enzyme involved in the digestion of the collagen matrix of bone by osteoclasts (131).

The enzymatic activity of the cathepsins plays a role in human diseases such as arthritis, emphysema, glomerulonephritis, rheumatoid arthritis, osteoporosis, hyperkeratosis, periodontitis, atherosclerosis, and Alzheimer's disease (127). In addition, cathepsins might also be involved in tumor invasion and metastasis (132).

5.3. Enzymatic activity against fibrillar collagen

The cathepsins have a broad range of substrate specificity, although some prefer certain amino acids to others in the target substrate sequence. Most cathepsins are endopeptidases, but a few are exopeptidases (C- or N-peptidases). Exopeptidases (cathepsins B, C, H, and X), in contrast to endopeptidases (cathepsins L, S, V, and F), possess structural features that facilitate binding of C- and N-terminal groups of the substrates into their active site clefts (127).

The function of these enzymes may be altered with changes in pH and their cellular localization. For example, cathepsin B is a carboxydipeptidase but it can also exhibit endopeptidase activity under different conditions (127). At the acidic pH of the lysosomes, cathepsin B is primarily an exopeptidase due to its active site being obstructed by an occluding loop. At higher pHs such as in the endosomes or at neutral pH, the occluding loop is displaced and cathepsin B functions as an endopeptidase. This suggests that substrates for cathepsin B may differ depending on the localization of the enzyme. The fact that cathepsin B is primarily an exopeptidase found within the lysosomes indicates that its function in these organelles is not likely to be extracellular protein turnover (133).

The cathepsins have enzymatic activity against several fibrillar collagens, which include collagen Types I, II, III, and XI. So far, no study has reported the ability of the cathepsins to cleave Type V collagen. Cleavage of soluble collagens Type I and II by cathepsin K has been studied mainly *in vitro* (117, 118). Both cathepsin B and L have been reported to be able to cleave the fibrillar collagens Types II and XI (119). Cathepsin L has also been reported to be able to cleave Type I collagen (120). In addition, cathepsin B may help to mediate fibrillar collagen cleavage indirectly through the activation of collagenase-1 (134). Cathepsin B can also degrade the downstream products of collagenase-1 cleaved collagen, as well as other ECM proteins such as collagen IV, laminin, and fibronectin at both neutral and acidic pHs (135, 136). Aspartic proteinase cathepsin D purified from bovine thymus has limited activity on bovine collagen Types I and III (121). Another aspartic cathepsin, cathepsin E, has similar cleavage characteristics as cathepsin D on the fibrillar collagens (122).

5.4. Optimal pH

Most of the cathepsins, except for cathepsins E and G (127), are lysosomal proteinases. They exist in the intracellular acidic vacuoles and function at acidic pHs. However, some cathepsins can cleave substrates at pHs close to neutral.

Cathepsin B and L degrade insoluble fibrillar collagens maximally at pH 3.5 and soluble monomeric collagen Type I near pH 4.5 (137). Cathepsin L is a much better endopeptidase than cathepsin B on the basis of *in vitro* analyses (138, 139). It degrades insoluble collagen Type I at pH 3.5 over 5-fold faster than at pH 6.0 (139). Cathepsins B and L can degrade cartilage collagen Types II and XI at temperatures from 20° to 37°C and at pH values from 3.5 to 7.0 (119).

Cathepsin K has collagenolytic activity against Type I collagen between pH 5.0 and 6.0 (118, 124). The optimal activity of a recombinant cathepsin K protein was pH 5.5 and it displayed a bell-shaped pH profile (118). Cathepsin K still has collagenolytic activity against Type I collagen even at pH values as high as 6.5 (118) and it has gelatinolytic activity in the pH range of 4.0-7.0 (123). For Type II collagen, the optimal pH of cathepsin K is between pH 5.0 and 5.5 and against denatured Type II collagen is between pH 4.0 and 7.0 (123). Type II collagen could also be efficiently hydrolyzed by recombinant human cathepsin K between pH 5.5 and 6.0 (123).

Cathepsin K is able to hydrolyze fibrillar collagen at pH values above 6 and is also physiologically stable at neutral pH. On the other hand, Cathepsin B and L are unstable at pHs close to neutral. These features may indicate that these cathepsins are involved in different stages of bone collagen cleavage. The most acidic part of the bone resorption lacuna is close to the ruffled border and the interface between the demineralized and mineralized matrix is more likely to be a neutral environment (140). Therefore, cathepsin K may be involved in the initial degradation of bone collagen while cathepsin B and L may be involved in later stages.

5.5. Collagen cleavage patterns and sites

The main cleavage site utilized by the cathepsins in the fibrillar collagens is located within the telopeptides, which then leads to the denaturation of the collagen molecules. The cathepsins can then cleave this denatured collagen at multiple sites in a pattern that is reminiscent of bacterial collagenases, which are able to cleave collagen at multiple points (141, 142) irrespective of its conformation.

Cleavage of the fibrillar collagens by cathepsins B and L occurs only within the non-helical domains unless

Table 4. Collagen cleavage sites utilized by the cathepsins

Cathepsin	Collagen type	Chain	Cleavage sites	References
Cathepsin K	Type I	α1 chain	Gly189-Lys190	123, 124
		α1 chain peptide (157-192)	Met159-Gly160, Ser162-Gly163 , Arg165-Gly166	120
		α2 chain	Arg144-Gly145	123, 124
	Type II	α1 chain	Gly58-Lys59	123, 124
Cathepsin L	Type I	α1 chain peptide (157-192)	Gly166-Leu167, Gln180-Gly181	120
Cathepsin D	Type I	α1 chain	Leu(C6)-Ser(C7)	121
		α2 chain	Phe782-Leu783	

the helix has been destabilized or denatured (119). As mentioned, cathepsin L is a more effective endopeptidase than cathepsin B on the basis of *in vitro* analyses (138, 139). Qualitatively, the action of cathepsin L on collagen is similar to that of cathepsin B that selectively cleaves the telopeptides (139).

The collagenolytic activity of cathepsin K is unique among mammalian proteinases (124). Cathepsin K cleaves native collagens Type I and Type II at both the telopeptides and at various sites within the helical region of the α1 chains (123, 124). Recent studies have demonstrated that the collagenase activity of cathepsin K depends on its complex formation with glycosaminoglycans (GAGs), especially chondroitin-4-sulfate (143-146). Chondroitin-4-sulfate dramatically increases the ability of cathepsin K to cleave soluble, as well insoluble, Type I and II collagens (143). It has been reported that a cathepsin K-specific complex consists of five cathepsin K molecules and five chondroitn-4-sulfate molecules (144). The complex exhibits potent triple helical collagen degrading activity, whereas monomeric cathepsin K has no collagenase activity (144). It has been speculated that the cathepsin K-chondroitin sulfate complexes primarily facilitate the destabilization and/or the specific binding of the triple helical collagen structure. Cathepsin K can be expressed by synovial fibroblasts, which are involved in cartilage and bone degradation (147). It can digest aggrecan aggregates to form the GAGs fragments required for the formation of its collagenolytically active complexes (146). Other GAGs predominantly expressed in bone and cartilage such as chondroitin-6-sulfate and keratan sulfates also enhance the collagenolytic activity of cathepsin K, whereas dermatan, heparin sulfate, and heparin selectively inhibit this activity (145). Moreover, GAGs potently inhibit the collagenase activity of other cysteine proteinases such as cathepsin L and S at 37°C. Therefore, this suggests that cathepsin K might represent the only cathepsin capable of cleaving collagen at or near physiologically relevant conditions (145).

Cathepsin D cleaves the α1 chain in native and denatured Type I and Type III collagen only within the C-telopeptide on the C-terminal side of a lysine residue, which is involved in interchain cross-linking. Cathepsin D is also involved in the processing of procollagen (148). Based upon differences in electrophoretic migration of the cleavage products, cathepsin D appears to cleave the C-propeptides from procollagen at the C-telopeptide at acidic pH and at the C-telopeptide/propeptide junction at pH 6.0 (148).

Specific collagen cleavage sites utilized by the cathepsins have been identified (Table 4). The cleavage sites in collagen were determined by the specific proteinases, rather than by the presence of hyperactive sites in the collagen molecules. The cleavage sites of cathepsin L in Type I collagen have been identified by conformational studies on the cleaved Type I collagen α1 chain (residues 157-192) by proton Nuclear Magnetic Resonance (NMR) spectroscopy (120). The major cleavage sites of cathepsin L were Gly166-Leu167 and Gln180-Gly181. The recognition of these cleavage sites by cathepsin L seems to involve both conformational preference and the presence of specific residues (120). Specific cleavage sites in collagens Type II and XI by cathepsin B and L have not yet been determined (149).

Using synthetic fluorogenic substrates**,** it has been demonstrated that an amino acid with a hydrophilic side chain in the P1 site along with an amino acid having a small hydrophobic side chain in the P2 site are greatly favored as substrates by cathepsin K (117). Substrates with hydrophobic residues or Pro/Hyp at the P2 position have been identified as being required for cleavage by cathepsin K (149, 150). N-terminal sequencing showed that the cleavage site in rat Type I collagen utilized by cathepsin K is at a Gly-Lys bond that is 189 residues from the N-terminus of the helical region in the α1 chain and 144 residues from the N-terminus of the helical region in α2 chain (Table 4). The cleavage site in human and bovine Type II collagen is a Gly-Lys bond that is 58 residues from the N-terminus of the helical domain. The α1 chain cleavage sites in both collagen Types I and II have the specific feature of that the P2 position contains a Pro or Hyp residue. Nosaka *et al.* (120) examined the specific cleavage site of a synthetic fragment of the Type I collagen α1 chain (157-192) by proton NMR spectroscopy. The major cleavage sites for cathepsin K identified were Met159-Gly160, Ser162-Gly163, and Arg165-Gly166. It was concluded that cathepsin K recognizes a consensus sequence in the extended strand of Gly-Pro-Z-Gly-, where Z is Met, Arg, Ser or Gln. It seems that although an amino acid with a hydrophilic side chain is favored in the P1 position, the hydrophobic amino acid Met is also acceptable which questions the restrictiveness of the P1 position. Cathepsin K also has gelatinase activity (118, 124) and is able to cleave denatured collagen (gelatin) at multiple points irrespective of its conformation.

Aspartic cathepsins D and E have cleavage sites in collagen Types I and III within sequences with a general hydrophobic character (122). Both enzymes share the same cleavage motif. It has been reported that positions P1 and P1' of the substrate must be occupied exclusively by hydrophobic amino acids with aromatic or aliphatic side chains. However, Val and Ile residues are not allowed in

the P1 position. Position P2' accepts a broad range of amino acids including charged and polar residues**.** The major cleavage site in the C-terminus has been reported to be between residues Leu6 and Ser7 in the C-telopeptide of the α1 chain of Type I collagen (121). The α2 chain was unaffected in native collagen, but was slowly cleaved between residues Phe782 and Leu783 in the denatured form (121). The very restricted cleavage of peptide bonds in denatured collagens can be ascribed to the infrequent occurrence of groupings of more than two hydrophobic residues and to the high content of the conformation-limiting residues Pro and Hyp.

5.6. Cathepsins in tumor development, bone physiology, and pathology

The activity of cathepsins B, L, D, and K has been linked to several types of cancer. These include cancer of the colon, pancreas, ovaries, breast, lung, and skin (melanoma) (151). Increase in procathepsin L secretion by human melanoma cells strongly increases their tumorigenicity and switched their phenotype from low to highly metastatic (152). The presence of cathepsins in extracellular fluid has been suggested to be useful in determining the tumor invasiveness and the clinical outcome of cancer chemotherapy. Cathepsins secreted by invading tumor cells can degrade collagen and elastin, thereby destroying the basal laminar region (132)**.**

In tumors and cancer cell lines, cathepsin B is upregulated, oversecreted, and redistributed to the cell periphery (153-155). Cell surface cathepsin B may degrade its substrate through the facilitation of annexin II heteotetramer, a docking molecule for proteins such as collagen Type I, tenascin C, plasminogen, and tissue type plasminogen activator. Cathepsin B is secreted from tumor cells by at least two pathways, which are the secretion of procathepsin B by a constitutive pathway and secretion of the active forms of cathepsin B by an inducible pathway (156) possibly through lysosomal exocytosis (157). Tumor cell secreted cathepsin B accumulates extracellularly as inactive/latent enzyme (132). *In vitro* studies have shown that this enzyme can be activated directly or indirectly (through the inactivation of cystatin C) by neutrophil elastase (132)**.**

Cathepsin K is crucial in bone remodeling (158, 159). Cathepsin K has also been referred to as cathepsin O (160, 161), OC2 (162), O2 (163), and X (164) in earlier publications. It is expressed at high levels in osteoclasts (165). As stated earlier, cathespin K is a key enzyme involved in the digestion of the collagen matrix of bone by osteoclasts (131). Osteoclasts are derived from hematopoietic stem cells and function to resorb and remodel bone, as well as play an important role in calcium homeostasis (166). When activated, osteoclasts form an impermeable sealing zone that divides the polarized osteoclasts into basolateral (attached to bone) and apical areas. Cathepsin K has been localized to the ruffled cell border of actively resorbing osteoclasts and also accumulates in specific subcellular compartments, intracellular vacuoles, and lysosomal vesicles that fuse with the ruffled cell border and release cathepsin K into the resorption lacunae (167). The pH in the resoption lacunae is maintained at an acidic pH via a complex acid-transport pathway, which not only demineralizes the matrix but also provides an optimal pH for the degrading enzymes including cathepsin K. Other than Type I collagen, which constitutes 95% of the organic matrix, cathepsin K degrades other bone matrix proteins such as osteopontin and osteonectin (117). Since cathepsin K can also cleave native Type II collagen, it is possible that it is involved in cartilage breakdown in diseases such as osteoarthritis and rheumatoid arthritis (123). Cathepsin K has also been localized in the ovaries, heart, lung, skeletal muscles, and small intestines (163).

Increased cathepsin activity has been linked to degenerative bone diseases including osteoporosis and post-menopausal osteoporosis (166). Overexpression of cathepsins B and K has been observed in rheumatoid arthritis (168) and osteoarthritis (169). Cathepsin K is highly effective in releasing the cross-linked N-telopeptides of Type I collagen neoepitope from bone Type I collagen in osteoclast-mediated bone resorption (150). Cathepsins S, L, and B are less effective and only had 57%, 36%, and 27% of the yield of cathespin K, respectively (150). Increased circulating concentrations of cross-linked C-terminal telopeptide of Type I collagen (ICTP) has been found in several pathological conditions such as bone metastases and rheumatoid arthritis (170). Evidence has shown that cathepsin K cleaves this trivalently cross-linked ICTP structure at two sites between the phenylalanine-rich region and the cross-link. Therefore, the MMPs rather than cathepsin K are more likely to be responsible for the production of ICTP *in vivo* since the MMPs cannot cleave the ICTP fragments (170).

Bone resorption is impaired in situations that have a cathepsin K deficiency (171, 172). Pycnodysostosis is an autosomal recessive osteochondrodysplasia due to various missense, nonsense, and stop codon mutations of the cathepsin K gene (172-175). Clinical manifestations include reduced stature, increased bone density, fragility with high risk of fractures, skull deformity, acroosteolysis of the distal phalanges, clavicular dysplasia, increased mandibular angle, and dental abnormalities. Evidence has shown that there is a huge accumulation of demineralized collagen fibers in the subosteoclastic resorption zones of cathepsin K knockout mice and pycnodysostotic patients (176).

6. SERINE PROTEINASES

6.1. Serine proteinase family

The serine proteinases are members of another enzyme family that are involved in the degradation of the ECM. In mammalian systems, members of the serine proteinase family such as chymotrypsin, trypsin, elastase, and plasminogen activators are believed to play a role in ECM degradation. The primary substrates of these serine proteinases are components of the ECM such as elastin, laminin, fibronectin, and proteoglycans (177, 178).

These serine proteinases all contain a serine residue at their catalytic site that is responsible for the

stabilization of the substrate-enzyme transition state. The underlying mechanism for peptide bond hydrolysis in the serine proteinase superfamily involves a triad of conserved residues, His, Asp (Glu), and Ser (chymotrypsinogen: His57, Asp102 and Ser195) in the enzyme catalytic domain (179, 180). It is believed that the essential Ser forms a covalent acyl enzyme intermediate with the substrate and therefore initiates catalysis. The following step, deacylation, is to hydrolyze the acyl-enzyme intermediate that releases the peptide and restores the Ser-hydroxyl of the enzyme. The His residue serves as a general base to accept the hydroxyl group from the reactive Ser (179, 181, 182).

The serine proteinases exhibit different substrate specificities. Chymotrypsin cleaves after bulky hydrophobic residues, trypsin cleaves after positive charged residues, and elastase is specific for small neutral residues (183). The differences in substrate specificity are determined by the enzyme's S1 positions, which interact with the substrate residues (181, 184).

6.2. Serine proteinases and collagen degradation

Serine proteinases have been shown to be involved in the degradation of fibrillar collagens indirectly via the activation of the MMPs and/or the inactivation of the TIMPs (185, 186, 187). A typical example is the plasminogen-plasmin cascade system. The ability of plasminogen activators (PA) to activate plasminogen to form plasmin that in turn can activate some of the MMPs and inactivate some of the TIMPs has been investigated (188-190). Two immunologically distinct PAs, tissue-type PA (t-PA) and urokinase-PA (u-PA), are responsible for the conversion of plasminogen into plasmin. The plasmin activated by t-PA is involved in the dissolution of fibrin in the circulation, while u-PA binds to a cell surface receptor (u-PAR) that then activates the cell-bound plasminogen. In *in vitro* systems, it has been shown that in concert with membrane type-MMPs, especially MT1-MMP, that plasmin is able to activate proMMP-1, -2, -3, -9, -10, and -13 (189, 190). However, it should not be a surprise that the PA-plasmin system is not the only pathway for the activation of the MMPs *in vivo*. In plasminogen-deficient mice, there is no observed effect on the expression of the MMPs including MMP-1, -3, and -9 by keratinocytes and MMP-2, MMP-11, and MT1-MMP by cells in granulation tissues (191). In addition, evidence from PA/plasmin-deficient mice and *in vitro* studies has not shown that the PAs and plasmin are able to cleave the fibrillar collagen directly. These serine proteinases are capable of cleaving other ECM components such as fibronectin or fibrin (192-194). The PA/plasmin knockout mice display diverse phenotypes in ECM remodeling processes (191, 195-197). The serine proteinases released from neutrophils including neutrophil elastase, cathepsin G, and proteinase-3 has been shown to be capable of activating MMP-2 (198). It is noticeable that membrane type MMPs, especially MT1-MMP, also play a crucial role in the MMP activation processes (198). In a soluble system without MT1-MMP, the activation of MMP-2 by neutrophil serine proteinases was abolished (187, 199), as well as that the plasmin started to directly degrade the MMP-2 (200). It has been speculated that the mechanism by which these serine proteinases activate proMMP-2 is different from that of the trimolecular complex system involving MT1-MMP, proMMP-2, and TIMP-2 in that the catalytic activity of MT1-MMP is not necessary for the proMMP-2 activation by these neutrophil serine proteinases (198).

Studies have shown that some of the enzymes in the serine proteinase family such as neutrophil elastase (NE), trypsinogen-2, and a yet to be identified serine proteinase have the ability to cleave the fibrillar collagen directly. Human NE is about 29 kDa and is primarily stored in the azurophil granules of polymorphonuclear leucocytes (PMNs, 1-3 pg/cell) (201). NE has also been detected in the nuclear membrane, Golgi complex, endoplasmic reticulum, and mitochondria of PMNs, as well as in other blood cells (202, 203). The primary substrates of NE are ECM components such as elastin and proteoglycans (177), as well as any pathogens phagocytized by the PMNs. Generally, NE is believed to recognize peptide bonds on the C-terminal side of amino acid with an alkyl side chain (204). NE cleaves MMP-1, -2, -3, and –9 (205, 206), as well as TIMP-1 (207). This results in MMP activation and/or TIMP inactivation. It has also been demonstrated that human NE can cleave native rat tail Type I (208), bovine Type III, and steer Type XI collagens (209, 210), but not Type II collagen (210). Based on the estimation of the collagen fragment sizes by Western blotting, it has been suggested that the collagen cleavage site utilized by NE is in the same region of helical relaxation as utilized by the MMPs (208, 210).

The trypsinogen family contains four different members, trypsinogen-1, -2, -3, and –4, which are distributed differently in the body. Typsinogen-2 is the most predominant form and is expressed by many tumor cell lines (211). Moilanen *et al.* (212) has demonstrated that tumor cell associated trypsingen-2 from human colon adenocarcinoma cells is able to activate MMP-1, -3, -8, and –13, as well as able to cleave human skin Type I collagen. A recent publication (213) reported the ability of trypsinogen-2 to cleave human Type II collagen. Trypsingen-2 cleaves collagen in a non-specific manner and generates multiple small fragments (212). Mass spectrometric analyses confirmed that the cleavage of Type II collagen by trypsinogen-2 does not occur at the structural relaxed sites in the collagen triple helix or the telopeptide regions, but occurs at trypsinogen-2 specific sites after Arg and Lys residues to release multiple small collagen fragments (213). How trypsinogen-2 accomplishes this requires further investigation.

A novel serine proteinase collagen degradation pathway has recently been described (214). Human temporomandibular joint (TMJ) synovial fibroblasts display an aggressive phenotype in regard to their ability to cleave fibrillar collagen after being cultured for multiple passages (Figure 2). The conditioned media from these TMJ cells is capable of degrading Type I collagen and this degradation could only be slightly inhibited by a MMP inhibitor (1,10-phenanthroline). However, a serine proteinase inhibitor (soybean trypsin inhibitor, STI, Figure

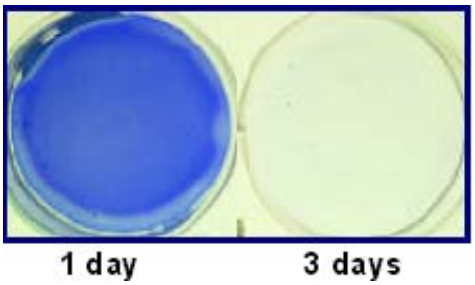

Figure 2. Collagen degradation assay. Each well was coated with 0.45 mg of rat-tail tendon Type I collagen. The plates were incubated with 70,000-90,000 cells seeded in the center of each well in a total volume of 100-150 µl at 37°C. After cell attachment, DMEM media without serum and with 160 nM TPA (12-O-tetradecanoyl-phorbol-13-acetate) was added. At 1 and 3 days the wells were stained with Coomassie blue after the cells were removed to visualize the collagen cleavage in the wells.

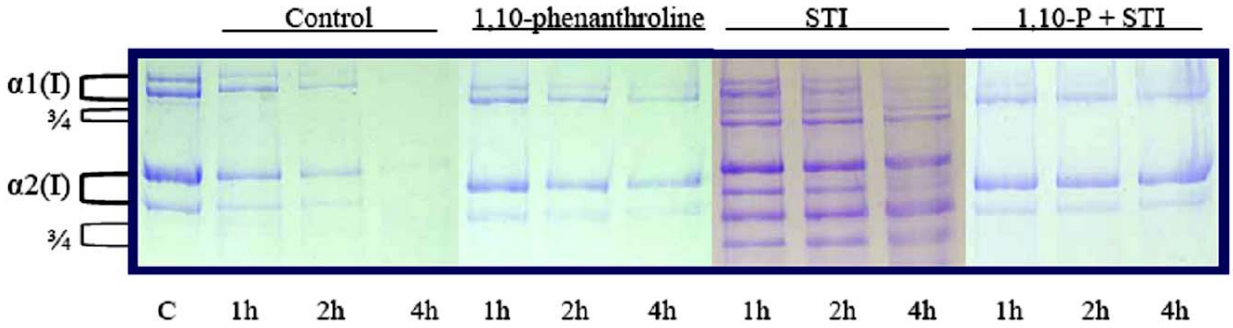

Figure 3. Media mediated collagen degradation. Conditioned media was incubated with rat-tail tendon Type I collagen with or without proteinase inhibitors (Control: without inhibitor, 10 mM 1,10-phenanthroline, 1 mg/ml soybean trypsin inhibitor (STI), 1,10-phenanthroline (1,10-P) plus STI). C: collagen alone..

3) more effectively inhibits the degradation of the Type I collagen by this media. A candidate serine proteinase secreted by these TMJ cells has been identified by zymography of the conditioned media (Figure 4). The identity of this candidate is still under investigation, as well as its role in other physiological and pathological conditions.

Evidence has shown that members of the serine proteinase family have the ability to mediate fibrillar collagen turnover via direct and indirect pathways. The data describing the ability of serine proteinases to directly cleave the fibrillar collagens are still somewhat limited when compared to the data available related to the ability of the MMPs and cathepsins to cleave the fibrillar collagens. The association between NE and a variety of inflammatory diseases including idiopathic pulmonary fibrosis, rheumatoid arthritis, adult respiratory distress syndrome, and cystic fibrosis has been well established (215). The connection between the expression of the trypsinogen-1 and -2 with metastasis of tumors has also been examined (178, 211). The recent demonstration that TMJ synovial fibroblasts utilize a serine proteinase dependent pathway to degrade Type I collagen further implicates these enzymes in fibrillar collagen degradation (214). The challenge now is to demonstrate that these serine proteinases have a role in the fibrillar collagen degradation that occurs *in vivo*. These future studies into the functions of these serine proteinases should further our understanding of the ECM destruction that is associated with pathological conditions, as well as normal conditions.

7. CONCLUSION AND PERSPECTIVE

An understanding of the mechanisms responsible for fibrillar collagen degradation is essential in controlling pathological conditions characterized by excessive collagen degradation. The unique triple helical structure of the fibrillar collagen along with their ability to form fibrils endows these collagens with resistance to cleavage from most proteinases. However, there are regions of the triple helix such as the Pro/Hyp poor region and the sites at which cross-links are formed within the collagen molecules that are accessible to attack by a few select enzymes.

The ability of the collagenolytic MMPs to degrade the fibrillar collagen is linked to their ability to unwind the triple helix in the proteinase susceptible sites. This helicase activity is believed to be mediated by a cooperative mechanism involving the catalytic domain, the C-terminal PEX domain, and the hinge region of the collagenolytic MMPs. Once the triple helix has been destabilize or distorted, cleavage can occur. The MMP family has long been considered to be the major players in the fibrillar collagen degradation that occurs at physiological pH.

The cathepsins, in turn, have been considered to be the major players in the fibrillar collagen degradation that occurs at acidic pHs such as is mediated by osteoclasts. There is limited evidence that select cathepsins such as cathepsin K can hydrolyze fibrillar collagen at pHs close to

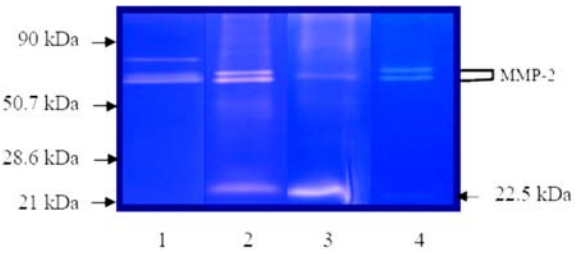

Figure 4. Gelatin zymography. Media collected from the temporomandibular joint (TMJ) fibroblast-mediated collagen degradation plates was resolved in a 10% SDS-PAGE gel containing 1 mg/ml of gelatin (Sigma-Aldrich) at 200 V. The gels were then stepwise washed with solution 1 (2.5% (v/v) Triton-X 100 and 3mM NaN_3), solution 2 (2.5% (v/v) Triton-X 100, 50 mM Tris pH 7.4 and 3mM NaN_3), solution 3 (2.5% (v/v) Triton-X 100, 50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 3mM NaN_3), and solution 4 (50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 3mM NaN_3) for 20 minutes each. The gels were incubated in solution 4 at 37^{o}C overnight with or without the following proteinase inhibitors: 1 mg/mL STI (Sigma-Aldrich) and 10 mM 1,10-phenothroline (Sigma-Aldrich). After staining with Coomassie blue, the proteinases capable of digesting the gelatin were visualized as lytic bands on the blue background of the gel. The molecular weights of the proteinases on the zymogram were estimated by measuring the relative migrations of the standards. 1: conditioned media from TMJ cells before displaying the aggressive collagen cleavage phenotype; 2: conditioned media from TMJ cells displaying the aggressive collagen cleavage phenotype; 3: conditioned media from TMJ cells displaying the aggressive collagen cleavage phenotype incubated with 10 mM 1,10-phenanthroline (MMP inhibitor); and 4: conditioned media from TMJ cells displaying the aggressive collagen cleavage phenotype incubated with STI (serine proteinase inhibitor).

neutral. The major cleavage sites utilized by the cathepsins in the fibrillar collagens are at the telopeptides. Data has shown that the collagenolytic activity of cathepsin K depends on complex formation with glycosaminoglycans. It is speculated that the cathepsin K-chondroitin sulfate complexes primarily facilitate the destabilization and/or the specific binding of the triple helical collagen structure. Other than the fact that cathepsin K is secreted by osteoclasts into the bone absorption lacunae and cleaves collagen extracellularly, most cathepsins cleave their substrates intracellularly.

In vitro studies have shown that certain serine proteinases have the ability to cleave the fibrillar collagens directly at neutral pH, in addition to the established indirect pathways. The ability of certain serine proteinases to directly cleave fibrillar collagens has complicated the understanding of the processes involved in the cleavage of these collagens. It will be challenging to determine if serine dependent collagen cleavage occurs *in vivo*.

Excess degradation of fibrillar collagens occurs in multiple pathological conditions. The MMPs have been targeted for the prevention of this degradation in pathological conditions such as periodontal disease, tumor invasion, and arthritis. It is easy to speculate that better strategies can be developed to control excess collagen degradation in these conditions once the roles that nonMMP proteinases play in *in vivo* collagen cleavage are elucidated.

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