Matrix metalloproteinases and their inhibitors

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The progressive breakdown of connective tissues of the articular joints is a major feature of the arthritides and becomes an irreversible process leading to permanent loss of function and disability. Recent research has been directed at the analysis of the relative roles of different cell types and the different classes of proteinases that they produce in such degradative processes. It is known that proteinases of all mechanistic classes have the potential to degrade individual connective tissue macromolecules in vitro, but the elucidation of their precise role in vivo is more difficult. The current view is that the initial step in the breakdown of the matrix in both physiological and pathological situations is an extracellular process, often involving matrix metalloproteinases (MMPs) which function at neutral pH. In certain special environments, cysteine proteinases with more acidic pH optima are also active and in rapid resorption or inflammatory events, serine proteinases are released by invading cells. Mechanical disruption and the presence of free radicals can also augment the degradative process. Matrix fragments are subsequently phagocytosed for processing intracellularly within the lysosomal system. These processes are normally tightly regulated by a complex interplay of cell-cell and cell-matrix interactions involving the production of proteinases, activators and inhibitors and other regulatory molecules. The accelerated breakdown of connective tissue seen in diseases such as rheumatoid arthritis may be due largely to a breakdown in these regulatory mechanisms.

The matrix metalloproteinases

Although the emphasis on different proteinase activities varies from situation to situation, the MMPs appear to have the most ubiquitous role in matrix turnover, since they are synthesised by endogenous connective tissue cells as well as some types of haematopoietic cell. MMPs are a family of zinc dependent proteinases which are functional at neutral pH, and are regulated by the specific tissue inhibitors of MMPs, TIMPs. Molecular cloning and biochemical studies have allowed the definition of sub-groups, several of which contain a number of enzymes of related structure and substrate specificity (Matrisian 1992, Docherty and Murphy 1990). The collagenases specifically cleave the fibrillar collagens but also exhibit some activity against other matrix proteins; gelatinases cleave denatured collagens and type IV collagen while the stromelysins have more general activity against a number of matrix glycoproteins. However the substrate specificity of the MMPs overlaps considerably in vitro (Table 1), presenting a considerable challenge to the determination of their roles in vivo, as discussed below.

The cDNA predicted amino acid sequences of all the MMPs can be aligned, demonstrating a high degree of conservation between each type of enzyme

Table 1. Biochemical properties of matrix metalloproteinases (human)

Enzyme	MMP No.	Mol. Wt. (proform)	Matrix proteins degraded
Collagenase	1	55000	Single locus in native fibrillar collagens I, II, III. Types VIII, X collagens
	8	75000	Proteoglycan. Gelatins (limited)
	13	65000	Fibrillar collagen
Gelatinase A	2	72000	Denatured collagens (gelatins). Non-helical regions fibrillar collagens
Gelatinase B (type IV collagenase)	9	92000	Specific locus type IV collagens. Types V, VII, XI, collagens. Elastin.
Stromelysin 1	3	57000	Proteoglycan core protein. Non-helical regions type IV collagen (X-links). Types II, IX collagens. Fibronectin. Laminin. Gelatins (limited).
Stromelysin 2	10	57000	Procollagens I, II, III. Collagenase. Gelatinase B.
Matrilysin (pump)	7	28000	Strong stromelysin-like activity. Elastin.
Stromelysin 3	11	51000	Weak stromelysin-like activity
Metalloelastase	12	54000	Similar to matrilysin. Elastin
Membrane-type	14	63000	Progelatinase A

across several mammalian species (about 80% similarity between collagenases and stromelysins). The similarity between types is lower, but still a highly significant 50%. Domains within these sequences can be delineated with apparently specific functions, including a propeptide of 77–87 amino acids lost during activation, a catalytic Zn^{2+} and Ca^{2+} binding domain of 162–173 residues and domains with sequence similarities to a number of extracellular matrix structural proteins (Docherty et al. 1992). Of these the C-terminal vitronectin-like domain of 202–312 amino acids is found on all MMPs except matrilysin. The gelatinases also have a fibronectin-like gelatin-binding sequence inserted into the catalytic domain. 95 kDa gelatinase B has a further collagen-like insertion C-terminal to the catalytic domain. These extra gelatinase domains are contained within a discrete set of exons.

Regulation of matrix metalloproteinase activity

In common with most proteolytic systems, the activities of the MMPs are tightly regulated. A number of cytokines, growth factors and hormones have been shown to modulate synthesis of many of these enzymes and the TIMPs, some of which may be specific to certain cell types and others of which have more general activity (reviewed in Murphy & Reynolds, 1993). Various of these effectors have been shown to be synthesised by connective tissue cells, indicating the importance of autocrine mechanisms in matrix turnover. Most recently, the effects of matrix interactions with cells, in terms of MMP regulation, have been described (Werb et al. 1989, Tremble et al. 1994).

Further levels of regulation of MMP activity occur extracellularly, namely cell or matrix binding, activation and inhibition. We have analysed the ability of individual MMPs to bind to extracellular matrix components, particularly collagen. Collagenase binding to fibrillar (type I) collagen has been observed by a number of groups (Vater et al. 1978, Welgus et al. 1985, Hembry et al. 1986). Interestingly, procollagenase binding is extremely weak, relative to the active form, although the predominant collagen binding site appears to be in the C-terminal domain (Murphy et al. 1992a). In the case of stromelysin we have shown that it binds most strongly to type I or II collagen in the absence of glycoproteins and proteoglycans (Allan et al. 1991). Both the proenzyme and the active enzyme were detected bound to collagen by both immunohistochemical and ELISA methods. Using biochemically prepared fragments of active stromelysin containing either the N-terminal catalytic domain, which is itself and active proteinase, or the C-terminal domain we were able to demonstrate that the stromelysin binding to collagen is also mediated by the C-terminal domain.

Pro and active forms of gelatinases A and B bound to type I and IV collagens, laminin and fibronectin, but binding occurred exclusively via the N-terminal portion of the molecule in both cases. Deletion of the fibronectin-like domain in gelatinase A abolished binding (Allan et al. 1995).

The proteolytic events leading to the loss of the propeptide of MMPs and the generation of the active species are sequential and may be initiated by exogenous proteolytic cleavage in the case of the collagenases, stromelysins-1 and -2, matrilysin and gelatinase B. Nagase et al (1991) have systematically analysed the action of potential physiologic enzyme activators, which include plasmin, plasma kallikrein, cathepsin B, cathepsin G and neutrophil elastase. Plasmin has long been thought to be an important activator of MMPs, and this concept has been supported by a number of studies using cell model systems with both connective tissue and tumour cells (Murphy et al. 1992b). The study of plasmin generation from plasminogen by the action of plasminogen activators is a particularly active and exciting field. It has been shown that plasmin generation and activity is highly focused, largely occurring at the cell surface where both plasminogen and the activators are specifically bound at cell adhesion plaques. It is likely that in localised pericellular regions, the action of $\alpha 2$ antiplasmin is limited because of the location of plasmin on the cell membrane or local excess of free enzyme. Stromelysin-1, which can be sequestered on the collagenous matrix, is particularly susceptible to plasmin activation and once activated can potentiate collagenase activity and act as a gelatinase B activator. Hence, uPA and prostromelysin could represent the key activities in an activation cascade to generate plasmin, active stromelysin and subsequently other active MMPs (Figure 2). TIMPs may regulate the activation process to some extent, since it has been shown that they can slow down or prevent the autocatalytic cleavages that occur after initiation of activation by the exogenous proteinases described above (Murphy et al. 1992b).

Gelatinase A is unlike the other MMPs in that it is regulated differently at the transcriptional level. Furthermore, it differs in its mechanism of extracellular activation. The propeptide of this enzyme has no apparent cleavage site susceptible to plasmin and other proteinases, although it can undergo self-cleavage reactions to lose the propeptide and become active (Nagase et al. 1991, Ward et al. 1991b, Crabbe et al. 1993). However, this enzyme can be activated

Table 2. Potential cell sites for uPA/MMP activation cascades

Cell membrane	Extracellular/collagen
sc uPA to tc uPA (plasmin)	prostromelysin (plasmin)
plasminogen to plasmin (uPA, plasmin)	procollagenase (plasmin/stromelysin)
progelatinase A (MT-MMP, gelatinase A) 0	progelatinase B (stromelysin)
? progelatinase A ? (collagenase, matrilysin, gelatinase A)	

The enzymes which are activated at each site are shown with the putative activators in brackets.
sc UPA: single chain urokinase like plasminogen activator; tc: two chain; MT-MMP: membrane type MMP (MMP 14).

by a fibroblast- or tumour cell membrane-mediated process that is sensitive to metalloproteinase inhibitors. The membrane activator which is specific to gelatinase A and does not activate the other pro MMPs (Ward et al. 1991a) can be induced by a number of effectors including concanavalin A, phorbol esters, and transforming growth factor ß.

Induction of progelatinase activation in cells is accompanied by an increase in binding of progelatinase A to the cell surface. This binding appears to be mediated by the enzyme C-terminal domain, distinct from the fibronectin domain binding to collagen (Murphy et al. 1992c). The C-terminal domain also interacts weakly with TIMP-2 when the enzyme is in its proform and may explain why TIMP-2 is an efficient inhibitor of the cell membrane activation of progelatinase A, relative to TIMP-1 (Ward et al. 1991a, 1994). The nature of the activation mechanism is not clear; although it is known that matrilysin and collagenase can activate progelatinase A, they are not detectable on cell membranes under these conditions. Gelatinase has been shown to undergo autolytic processing if it is concentrated, e.g. by binding to heparin (Crabbe et al. 1993). Hence, it seems likely that some degree of self-cleavage may be involved in the activation process. The recent cloning of the membrane-type metalloproteinase (MT-MMP; MMP-14) and its implication in progelatinase A activation (Sato et al. 1994) has led to the concept that this enzyme may initiate progelatinase A self-cleavages by cleavage of the propeptide at the intermediate site (unpublished observations). This would be analogous to the initiation cleavage of other MMPs by plasmin, prior to further self processing cleavages.

The tissue inhibitor of MMP (TIMP) family appear to be the major local inhibitors of MMPs, although the ubiquitous proteinase inhibitor (2-macroglobulin undoubtedly plays a regulatory role in certain situations. Three forms of TIMP have been unequivocally identified by cloning and sequencing from a number of species. TIMP-1, a 28.5 kDa glycoprotein and TIMP-2, a 21 kDa unglycosylated protein are produced by many cell types and can be demonstrated in body fluids and tissue extracts. Their regulation at the gene level appears to be very different, TIMP-1 expression being very tightly controlled by many cytokines and growth factors whilst little has yet been found that regulates TIMP-2. TIMP-3, a 21 kDa protein which may be glycosylated, is less well understood, although it has been cloned from chicken, mouse and human (Pavloff et al. 1992, Apte et al. 1994a, b). The chicken protein, Chimp-3, was initially reported to be a component of the extracellular matrix of embryo fibroblasts during the early stages of transformation (Staskus et al, 1991). The human and mouse mRNAs for TIMP-3 have been shown to be very widely expressed in connective tissues, including during development (Apte et al. 1994a, b).

Although the individual TIMP forms show as little as 40% sequence identity, they share considerable higher structural similarity (Willenbrock and Murphy 1994). This is notably due to the conservation of 12 cysteine residues that have been shown to form disulphide bonds in TIMP-1, giving a 6 loop structure. They all form high affinity, non-covalent and essentially irreversible complexes with the active forms of MMPs with a 1:1 stoichiometry. It has been shown that active, unmodified TIMP-1 can be recovered from enzyme complexes. TIMP-1 can also bind, albeit rather less tightly to progelatinase B and TIMP-2 to progelatinase A, properties which are thought to allow tight regulation of the activation of these enzymes.

Mutagenesis of both TIMP-1 and TIMP-2 to remove sections of each protein has demonstrated that they consist of two structurally distinct domains; the N-terminal domain consists of 3 loops and the C-terminal domain of the remaining, smaller loops. The N-terminal domain can fold independently of the C-terminal domain to give a functional MMP inhibitor, which interacts with the enzyme catalytic domain.

From our data we can conclude that the mechanism of TIMP action is complex, involving numerous points of interaction with the MMPs (Willenbrock and Murphy 1994). The TIMP C-terminal domain has a number of enzyme binding sites which differ according to the MMP, and act to increase the rate of inhibition. The mechanism for this rate enhancement is by increasing the probability of interaction of the two N-terminal domains. Further analysis of this binding should be facilitated by structural studies of enzyme-inhibitor complexes.

Matrix metalloproteinases in arthritis

There is significant evidence that joint tissues derived from patients with various forms of the arthritides are over-expressing different MMPs. Cultures of cells derived from rheumatoid synovia secreted collagenase into the culture media (Dayer et al. 1976) and collagenase could be detected by immunolocalization at sites of cartilage erosion rheumatoid joints (Woolley et al. 1977). Stromelysin and collagenase have also been demonstrated by immunolocalisation in lining cells of synovia and their respective mRNAs detected by in situ hybridisation (Okada et al. 1990, 1992, Wolfe et al. 1993, McCachren 1991). This was confirmed by a recent immunolocalisation study of MMPs and TIMPs in synovial samples from joints with rheumatoid arthritis or osteoarthrosis (Hembry et al. 1995). The finding of stromelysin 1 in all synovial samples from 10 patients with disparate clinical diagnoses and histories, in contrast to its absence from normal synovia (data not shown), clearly implicates this enzyme in the arthritic process. It was also shown that collagenase, gelatinase A, and matrilysin may have a role in the synovitis associated with rheumatoid arthritis, but are not a significant feature in osteoarthrotic joints. However, marked regional variations were found in the synthesis of these MMPs, indicating that these diseases are episodic and that the control of enzyme synthesis is focal. This indicates the need for further work to colocalise MMP synthesis with cytokine expression in synovia from diseased joints to explore the mechanisms which control the synthesis and degradation of extracellular matrix components in articular cartilage. A better understanding of the control of these processes may indicate ways to down regulate MMP overproduction without compromising normal tissue remodelling. Primary rheumatoid synovial fibroblasts made high levels of stromelysin-1 mRNA, but little stromelysin-2 mRNA (Sirum and Brinckerhoff 1989). MMPs have also been measured in the synovial fluids of rheumatoid and osteoarthrotic knee joints (Walakovits et al. 1992, Lohmander et al. 1993). TIMP-1 has been detected in rheumatoid synovia lining cells (Okada et al. 1990, McCachren 1991, Hembry et al. 1995) and measured in rheumatoid and osteoarthrotic synovial fluids (Clark et al. 1993, Lohmander et al. 1993). Dean et al. (1989) extracted TIMP and MMP activities from osteoarthrotic cartilage and proposed that there was an imbalance in favour of enzyme activity relative to normal tissue.

The MMPs are thought to be involved in cartilage degradation, although there is controversy as to which might be functional against individual matrix components. Interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13) do have the ability to cleave type II, IX and XI collagens (Gadher et al. 1989, 1990, Hasty et al. 1986, V Knäuper, unpublished). It has also been shown that stromelysin-1 can degrade type II, IX and XI collagen in the globular (cross-linking) regions (Wu et al. 1991). This is significant in view of the finding that stromelysin is specifically expressed at high levels in the proximal hypertrophic zone of the developing growth plate of young rabbits (Brown et al. 1989). This is precisely the region where collagen turnover is occurring prior to type I collagen production and mineralisation.

A major focus of interest has been the cleavage of the major cartilage proteoglycan aggrecan which is rapidly released from the tissue following damage. In vitro analyses first revealed that the aggregation core protein is cleaved proteolytically to generate a high molecular weight product that cannot bind to hyaluronic acid. This cleavage is in the region between two globular (G1 and G2) domains (Fosang et al. 1991, Sandy et al. 1991, Flannery et al. 1992), residues Glu 373 and Ala 374 forming the cleavage site. In vitro studies have shown that the interglobular region can be cleaved by many MMPs, including matrilysin, gelatinase A and B, interstitial and neutrophil collagenases and stromelysin-1 and -2 (Hughes et al. 1991, Fosang et al. 1992). We have found that the major cleavage site is Asn 341 - Phe 342 for all these enzymes, however neutrophil collagenase can subsequently cleave at the Glu 373 - Ala 374 site under certain conditions (Fosang et al. 1994). Although proteoglycan loss from cartilage in culture can be prevented by metalloproteinase inhibitors (Buttle et al. 1993) it should be noted that cell-permeating cysteine protein inhibitors are also inhibitory (Buttle et al. 1993). This can be coupled with the old observation that 1 µg/ml cytochalasin B or D, which prevent cell membrane function, prevent aggrecan breakdown (Saklatvala and Sarsfield 1988) and the recent study showing that the antibiotic bafilomycin (which prevents cell vacuolar acidification) has a similar effect (Yocum et al. 1995). These observations indicate that aggrecan cleavage may be a more complex process, possibly involving an endocytic compartment inside the chondrocyte. Cleavage sites identified in aggrecan from diseased cartilage, both in the G1–G2 region and in the chondroitin sulphate rich region have similar characteristics, related to the Glu 373 - Ala 374 described above, suggesting that one common proteinase is responsible (Loulakis et al. 1992, Ilic et al. 1992).

These studies reflect a major goal of current research, to ascertain the precise role of specific pro-

teinases in degradative events in the pathology of the arthritides. The development of antisera to specific cleavage products of matrix proteins will go a long way towards assisting such aims. The recent development of low molecular weight inhibitors to the MMPs and other proteinases and the eventual refinement of their specificity will provide excellent tools for the analysis of the specific role of each enzyme in degradative events. Such developments are aided by the fundamental work on the structure-function relationships of the MMPs and TIMPs and the determination of their regulation.

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