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Mechanisms of Activation and Stabilization of Gelatinase A: TIMP-2 Complexes

by

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ABSTRACT

Mechanisms of Activation and Stabilization of Gelatinase A:TIMP-2 Complexes.

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Proteolytic degradation and remodeling of the extracellular matrix (ECM) occurs in both normal and pathological processes. An important group of enzymes necessary for ECM remodeling are the matrix-degrading metalloproteinases. The metalloproteinases are secreted as inactive proenzymes that must be proteolytically modified in order to become active enzymes. Activation cascades involving other proteinases are likely to be important in the regulation of metalloproteinase activity. For example, plasmin can convert both procollagenase and prostromelysin to their active forms. Gelatinase A (72 kDa type IV collagenase) is a metalloproteinase that is expressed by many cells in culture and overexpressed by some tumor cells. The pro-form of gelatinase A has been detected as a bimolecular complex with its inhibitor, TIMP-2, bound at the carboxyl domain at some distance from the active site. TIMP-2 is a member of the Tissue Inhibitors of Metallo- Proteinase family (TIMPs). Although the gelatinase A:TIMP-2 complex can be activated *in vitro* by organomercurial treatment, the *in vivo* activator(s) of gelatinase A have not been identified.

Initial observations suggested that the serine proteinase, neutrophil elastase, might play a role in the activation of gelatinase A. Additionally, it was speculated that non-catalytic interactions between gelatinase A and matrix components might alter potential activation pathways. The question of whether elastase could modify gelatinase A and what effect these modifications would have on the activity of the gelatinase were addressed by substrate zymography, activity assays and amino-terminal sequencing. This study demonstrates that elastase can proteolytically modify gelatinase A:TIMP-2 complexes by cleaving at a number of sites within the gelatinase A. Matrix components can alter the proteolytic modifications in gelatinase A that are mediated by neutrophil elastase. In the absence of gelatin, neutrophil elastase destructively degrades gelatinase A by hydrolyzing at least two bonds within the gelatin-binding fibronectin-like domain of gelatinase A. However, in the presence of gelatin, these two inactivating cleavage sites are protected and cleavage at an alternate site results in a truncated yet active gelatinolytic enzyme. The results suggest a regulatory role for matrix molecules in modifying and stabilizing gelatinase A.

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I. Introduction

1.1 Inflammation and extracellular matrix degradation.

The migration of neutrophils from post capillary venules across basement membranes and into the interstitium is pathognomonic of the acute inflammatory response to cellular injury. The signals that elicit this inflammatory response are most commonly thought to arise from microbial origins, such as the bacterial peptides that are potent chemoattractants for neutrophils. However, many causes of cellular injury may provoke inflammation. These causes include hypoxia, physical or chemical agents, and immunologic reactions. All can result in the release or modification of endogenous molecules that are chemotactic for inflammatory cells. Soluble matrix components can provide the molecular gradient for inflammatory cells to find injured tissue. At sites of cellular injury, inflammatory cells initiate pathways that lead to extracellular matrix remodeling necessary for the resolution and/or organization of the injured tissue.

There are no mechanisms for simply patching or repairing matrix components following disruption due to cellular injury. An initial step necessary for repair is the removal of damaged matrix components. Proteinases play a central role in this process of enzymatic debridement. The proteinases involved with these events are both the products of resident cells within the matrix, such as fibroblasts, endothelial cells, and tissue macrophages and also the products of recruited inflammatory cells. For example, neutrophils contain proteinases capable of directly degrading the components of the matrix. In addition, inflammatory proteinases can affect other molecules within the matrix. Proteolytic activation of other matrix degrading enzymes or proteolytic degradation of endogenous inhibitors can increase the potential for degradation of the extracellular matrix.

Extracellular matrix remodeling during inflammation depends on interactions between different classes of proteinases. The codependency of matrix degradation with both serine proteinases and metalloproteinases has been established. It has been

suggested that these separate proteinase classes can interact in activation cascades (Saksela & Rifkin). The pathways of extracellular degradation may begin in a very specific fashion with the activation of enzymes that have a narrow substrate specificity followed by more generalized and complete proteolysis by the activation of enzymes having a broader substrate specificity. For example, collagenase is a highly specific enzyme that can cleave and partially denature collagen making it susceptible to further hydrolysis by the broadly specific gelatinases.

The subject of this dissertation will be the investigation of how proteinase-inhibitor balance can effect inflammatory reactions. The novel interaction between a metalloproteinase, Gelatinase A, and a serine proteinase, neutrophil elastase is discussed. The role that the matrix plays in this interaction is also analyzed.

1.2 Extracellular Matrix Components Mediate Inflammation.

The extracellular space around parenchymal cells in tissues is filled with a macromolecular network composed of polysaccharides and proteins. This matrix is secreted locally by connective-tissue cells (fibroblasts or the more specialized forms found in bone and cartilage) and assembled by a combination of spontaneous and cell associated mechanisms into an organized structural framework. The functional adaptations to the requirements of a particular tissue are reflected by the diversity of matrix assemblies found throughout the body. It is the variation in the types and amounts of matrix molecules that allow these requirements to be satisfied.

The molecules that make up the matrix include i) the glycosaminoglycans, ii) fibrous structural proteins (collagens and elastins), and iii) adhesive proteins (fibronectins and laminin). The glycosaminoglycans are covalently associated with core proteins to form proteoglycans that make up the bulk of the extracellular space. Glycosaminoglycans are highly sulfated and very hydophilic. The net negative charge on

the surface of these molecules binds osmotically active cations that cause large amounts of water to be drawn into and retained to produce a fluid filled gel that allows for diffusion of water soluble nutrients and other factors. However many of the interactions in the matrix take place at the insoluble interface of the structural and adhesive proteins that are embedded within this gel. Collagen fibers or sheets provide tensile strength to the tissues and help organize the matrix. Elastin fibers provide the tissue with flexibility and resiliency. Fibronectins and laminins provide adhesive sites for other matrix associated molecules and cell-membrane associated receptor-like molecules. The extracellular matrix can be thought of as an affinity support that many secreted and cell-associated proteins can bind to.

A number of studies have established that isolated components of the ECM and proteolytic fragments of these components can stimulate the directed migration and degranulation of inflammatory cells. Fragments from laminin (Teranova et al.) and type IV collagen purified from pepsin digests of placenta (Senior et al., 1989) are chemotactic for neutrophils. The chemotactic activity of these matrix components is nearly equivalent to the formylated peptide, fmet-leu-phe. Although intact fibronectin does not elicit a chemotactic response by neutrophils or monocytes, a fragment produced by proteolysis does induce chemotaxis by monocytes (Norris et al.). Fibronectin also stimulates the phagocytic activity of neutrophils and increased degranulation that results in the release of proteinases (Wachtfogel et al.). Once degradation begins in the matrix the hydrolyzed products of these events elicit further inflammatory responses which in turn lead to increased ECM turnover.

Control of this perpetuating proteolytic cycle is largely regulated through the presence of proteinase inhibitors (section 1.4). Inhibitors of the serine proteinases can limit the extent of proteolysis by the inflammatory serine proteinases. However modified inhibitors may also serve to amplify the inflammatory response.

Proteolytically modified α1-proteinase inhibitor (α1-PI) is a potent chemoattractant for neutrophils (Banda et al.,1988a,b).

1.3 Proteinases Involved in Matrix Degradation.

Proteinases are an important group of effector molecules in the degradation of the ECM (Alexander and Werb). There are four mechanistic classes of proteinases: the aspartic and cysteine proteinases, that exhibit acidic pH optima, and the serine and metallo- proteinases that are active at or near neutral pH ranges. Due to their neutral pH optima the serine and metallo- proteinases have been considered to be the most important group responsible for the proteolysis of substrates within the extracellular matrix. However, proteinases with acidic pH optima (cathepsin D and cathepsin L) may also play a role (Anderson et al.). These normally lysosomal proteinases are found on the outside of anoxic cells and their pH optima may be met by the high lactate levels generated by anoxic cells particularly at wound centers. This discussion, however, will be limited to the serine and metallo- proteinase families of proteolytic enzymes.

The serine proteinases include many of the enzymes responsible for triggering the inflammatory cascades necessary for fibrinolysis, coagulation and complement activation. The serine proteinases that are believed to be involved with degradation of the ECM include plasminogen, the plasminogen activators, that convert the inactive plasminogen to active plasmin, and the neutrophil serine proteinases, elastase and cathepsin G. Plasminogen is ubiquitous in plasma and tissue and, once activated, is characterized by having a broad specificity capable of degrading fibrin and fibronectin. Plasmin can also activate the metalloproteinase, procollagenase. There are two plasminogen activators, the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA) that are secreted by macrophages, fibroblasts, endothelial cells, and neutrophils (Saksela and Rifkin). Neutrophils also contain

elastase and cathepsin G that are packaged into the azurophilic granules. Inflammatory signals induce a series of events that lead to degranulation, the process in which these enzyme laden granules fuse to the plasma membrane and release their contents into the extracellular space. Elastase is named for the ability to degrade elastin, the cross-linked structural protein that is resistant to degradation by most proteinases. But like many proteinases that are named after a single substrate, elastase actually has broad substrate specificity and has activity on other proteins besides elastin. Elastase degrades fibronectin, laminin, collagen (particularly type II), and, as discussed in a later section, can modify the activities of the metalloproteinases. The molar concentration of elastase in a single neutrophil has been estimated to be 0.036-0.058 mM (Travis et al.,1992). Thus 10^6 neutrophils arriving at a site of inflammation have the potential to deliver about 2 μg of neutrophil elastase. Without the modulating influence of serine proteinase inhibitors, neutrophils have the potential to affect extensive matrix damage.

The matrix degrading metalloproteinase family is comprised of at least nine members that include (i) Matrilysin (PUMP-1), (ii) the Stromelysins, (iii) gelatinases (Gelatinase A, 72 kDa, and Gelatinase B, 92 kDa), (iv) collagenases (fibroblast and neutrophil) and (v) a mouse macrophage elastase (Banda and Werb, Shapiro et al.). These proteins consist of distinct domain structures (figure 1.1)(Matrisian 1990,1992). Common to all members of this family is the propeptide which constitutes the amino terminal domain and is required for the latency of the secreted metalloproteinase proenzymes. A sulfhydryl group from a free cysteine residue within the propeptide sequence is thought to coordinate with the active site zinc. The zinc atom is bound to a site within the essential catalytic domain. Although there is no crystallographic data of the mammalian metalloproteinases, sequence homology with other zinc binding enzymes suggests that the histidine residues of the conserved HEXGH sequence constitutes two of the three ligands for binding zinc (McKerrow). The smallest

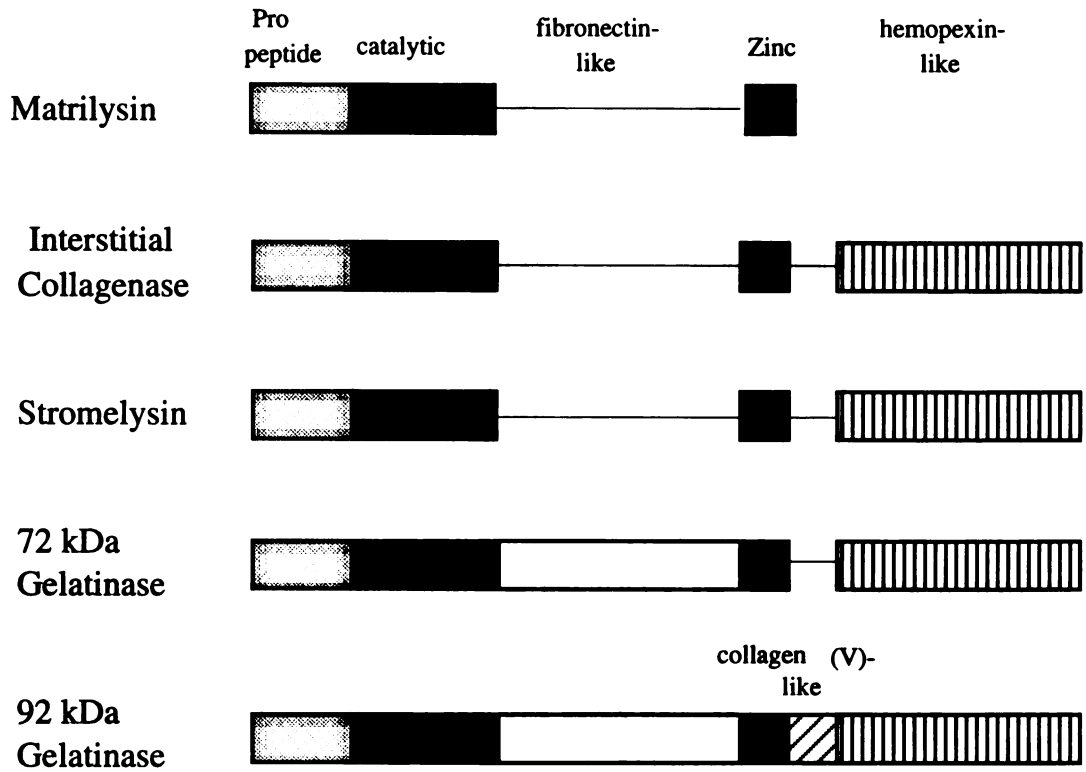


Figure 1.1. Domain structure of the metalloproteinases.

metalloproteinase is matrilysin which is composed of only the catalytic domain and the propeptide. The differences in substrate specificities between the MMPs is reflected by the presence of additional domains. The remaining members of the family have a hemopexin or vitronectin-like domain at the carboxyl terminus which has been suggested to play a role in substrate specificity. In the gelatinases this domain binds one of the metalloproteinase inhibitors, TIMP-1 or TIMP-2. The gelatinases contain an insert in the catalytic domain that is homologous to fibronectin type II domains and confers the gelatin binding properties of the gelatinases.

What are the natural substrates for the matrix metalloproteinases? Most members of the MMP family can degrade gelatin and fibronectin. The stromelysins and matrilysin also degrade the globular domains of laminin and proteoglycan core protein. The collagenases (fibroblast and neutrophil) are very specific and have the unique ability to degrade native interstitial collagen. The gelatinases degrade gelatin, which is partially degraded collagen, and can also degrade type IV collagen and elastin (Senior et al., 1991). The mouse macrophage elastase degrades elastin and other proteins, including α1-proteinase inhibitor (Banda et al.,1980).

1.4 Control of Matrix Degradation

Proteolysis of matrix components is necessary for physiological processes involving cellular migration. Migrating cells must breach many barriers to their movement. Growth and development requires rapid cellular migration with dramatic restructuring of the extracellular matrix. Blastocyst implantation is accomplished with a proteinase assisted invasion into the endometrium. Angiogenesis is dependent upon the release of proteinases by endothelial cells that degrade the endothelial basement membrane and allow the cell to migrate into the perivascular connective tissue and form capillary sprouts. During inflammation a neutrophil must move through the basement membrane of the vascular endothelium, degrade the laminar collagen of basement membranes, and continue moving through the stromal connective tissue. The penetration of cells through tissues requires that the migrating cell is capable of expressing a variety of proteolytic enzymes. The expression of these proteinases must result in sufficient activity to overcome the high levels of endogenous proteinase inhibitors. An appropriate balance between proteinases and proteinase inhibitors is necessary for homeostasis. However, for many processes, this balance must shift in favor of proteolysis. Three ways that proteolysis can be regulated extracellularly are;

1) Regulation of proteinase activation. The matrix degrading metalloproteinases are secreted as latent proenzymes that must be activated.

2) Inactivation of proteinases by proteinase inhibitors.

3) Localization of proteolytic activity. Pericellular compartmentalization of a proteinase focuses the proteolytic activity and can protect an enzyme from inhibition. Examples include the localization of uPA bound to specific receptors on macrophages (Blasi et al.) and neutrophil elastase resistant to inhibition when the enzyme is in direct contact with its substrate beneath the cell (Campbell and Campbell).

1.4.1. Activation of Proteinases

Because many proteinases are secreted as inactive proenzymes, their activation becomes a very important regulatory step. Of the serine proteinases discussed earlier only the neutrophil proteinases, Elastase and Cathepsin G, are released in fully active forms. Plasminogen, the inactive precursor of plasmin, must be activated by uPA or tPA. The regulation of uPA and tPA is largely regulated by the levels of the endogenous serpin, PAI-1.

All of the metalloproteinases are secreted as latent proenzymes and must be activated (Murphy, Ward et al.). The latent state is maintained by the amino terminal propeptide. The metalloproteinases can be activated by many agents *in vitro*, including organomercurials, detergents, and other proteinases (Nagase et al.1991,1992). These agents appear to have in common the ability to disrupt the interaction between the propeptide and the active site and lead to autoproteolytic processing. The perturbation of the cysteine-zinc interaction and subsequent release of the propeptide has been termed the "cysteine switch" mechanism of proteinase activation (Springman et al., Van Wart et al.). Mutational studies of the Cys residues and adjoining residues confirm that the propeptide is necessary for the latent state (Park et al., Sanchez-Lopez et al.). The

mechanism of *in vivo* activation is likely to involve proteolytic processing of the propeptide. Plasmin can convert both prostromelysin and procollagenase to their active forms (He et al.). Activated stromelysin was also shown to "superactivate" collagenase (Suzuki et al.). The *in vivo* activation of the gelatinases remains controversial. The fibrosarcoma cell line HT1080 secretes an active gelatinase A and a membrane preparation from these cells can activate exogenous progelatinase A (Brown et al., Ward et al.). The exact nature of this activation has not been determined.

1.4.2. Proteinase Inhibitors

Serine Proteinase Inhibitors

The importance of controlling serine proteinase activity is reflected by the high amounts of **ser**ine **p**roteinase **in**hibitor**s** , or **serpins**, present in the blood. Nearly 10% of the total protein in plasma are proteinase inhibitors (Travis and Salvesen). The prototype of the serpin family of inhibitors is α1-proteinase inhibitor (also known as α1-antitrypsin), a 55,000 dalton glycoprotein that is the primary inhibitor of neutrophil elastase (Beatty et al.). The plasma concentration of α1-proteinase inhibitor is approximately 2 mg/ml. During the acute phase response, plasma concentrations of α1-proteinase inhibitor increases three-fold to four-fold. The increase of α1-PI in plasma is mainly due to synthesis by hepatocytes but α1-PI is also expressed locally by macrophages. Due to its small size α1-proteinase inhibitor is also found outside the circulation. The serpin family also includes α1-antichymotrypsin, antithrombin, C1-inhibitor, protease nexin-1 (PN-1), heparin cofactor II, α-2-antiplasmin, plasminogen activator inhibitor (PAI-1), angiotensinogen and the noninhibitor members of the family, corticosteroid- and thyroid- binding globulins, and finally ovalbumin.

The individual serpins share more than 30% overall sequence similarity but this rises to 70% when only the internal hydrophobic residues are considered (Carrell and Travis). The conservation of internal sequences is important for the overall tertiary structure that even the noninhibitor members of the serpin family share. The inhibitory specificity of the serpins is primarily determined by the reactive center P_1 position. α1-PI has a methionine at the P_1 position that is cleaved by neutrophil elastase. Substitutions at the P_1 site can dramatically alter the inhibitory properties of the serpins. For example, a fatal bleeding disorder was caused by a mutation of α1-PI in which the reactive site methionine was replaced with an arginine. This mutation produced a complete switch in activity from an inhibitor of elastase to an inhibitor of thrombin (Owen et al.). Because the plasma levels of α1-PI are much higher than that of antithrombin, particularly during the acute phase response, the expression of this mutant inhibitor led to the complete inactivation of thrombin that prevented clot formation.

The inhibition of serine proteinases by the serpins is via a tight 1:1 stoichiometric and presumably covalent complex between the proteinase and the inhibitor. The association between the proteinase and serpins is very rapid with a second order rate constant of 10^5 - 10^7 mol^{-1} s^{-1}. α1-Proteinase inhibitor presents an exposed peptide loop that is accessible to proteases and acts as an irreversible switch following proteolysis. It has been suggested, however, that serpins under limited conditions can reversibly inhibit proteinases without becoming inactivated (Tyagi). Thiol-modified α1-PI derivatives are reversible competitive inhibitors of neutrophil elastase (Tyagi and Simon).

It has been estimated that the normal elimination rate of neutrophil elastase is approximately 250 mg per day (Travis et al. 1992). The clearance of this proteolytic enzyme from serum and tissue spaces, particularly following inflammation, is necessary for the maintenance of homeostasis. The elastase is cleared primarily as an

inactive complex with α1-PI through the serpin receptors in the liver (Ohlsson and Laurell, Pizzo et al.).

Metalloproteinase Inhibitors

There is a family of metalloproteinase inhibitors referred to as the Tissue Inhibitors of Metallo- Proteinases, or TIMPs. Two mammalian TIMPs (TIMP-1 and TIMP-2) have been cloned and an additional member of the family has been cloned from chicken fibroblasts (TIMP-3)(Pavloff et al). The human homologue of TIMP-3 has recently been cloned (personal communication, Susan Hawkes). Additionally at least two lower molecular weight inhibitors (IMPs) have been detected in the conditioned medium of endothelial cells and glioma cell lines (Apodaca et al., Herron et al.1986a,b). TIMP-1 is the most abundant of the TIMPs and is constitutively expressed by fibroblasts and endothelial cells *in vitro*. TIMP-2 and the IMPs are more sensitive to culture conditions and passage number (unpublished observations).

Unlike the serpins, that can form covalent complexes with their target proteinases, the TIMPs bind to the metalloproteinaes in a noncovalent manner. TIMPs can prevent the activation of the metalloproteinase proenzymes and can also inhibit the activated forms of the enzymes. An interesting and unusual characteristic of the gelatinases is that the latent form can be found in cell conditioned medium with the TIMPs bound to a carboxyl terminal site that is separate from the active site. TIMP-2 is complexed to Gelatinase A (Goldberg et al.) and TIMP-1 is found in complex with Gelatinase B. The interaction between Gelatinase A with TIMP-2 is discussed in Chapter three.

The inhibition of proteinase activity in plasma may be primarily by α2-macroglobulin, a large generalized proteinase inhibitor found in blood at a concentration of 2.2 mg/ml. The concentration of α2-macroglobulin in interstitial fluid is

surprisingly high where it has been reported to be near plasma levels (Tollefsen and Salfvedt) and may contribute to the inhibition of proteinases in the matrix.

1.4.3. Localization of Proteinases and Inhibitors

There are several advantages to localizing proteinases at the cell surface or to extracellular matrix components (Moscatelli and Rifkin). Proteolytic activity can be limited to the immediate vicinity of secretion or activation of the proteinase. Bound enzymes may also be more active than enzymes in the fluid phase. Upon binding of plasminogen to endothelial cell matrix its activation by the plasminogen activators is enhanced compared to the activation in solution (Knudsen et al.). Proteinases bound to cells or matrix are also less susceptible to inhibition by proteinase inhibitors. Bound plasmin is not as easily inhibited by α2-antiplasmin as plasmin in the soluble phase (Plow et al.). Elastase is less susceptible to inhibition by α1-proteinase inhibitor when bound to elastin (Campbell et). The metalloproteinases, Gelatinase A and Gelatinase B have collagen binding domains that are homologous to the collagen binding domain of fibronectin. The activation and stability of both of these enzymes are increased when substrate is present *in vitro*.

Inhibitors are also bound to specific sites within the matrix. PAI-1 binds to vitronectin and its stability *in vitro* is dependent upon this binding (Wiman et al.). PN-1 is also bound in the matrix to heparin sulfate proteoglycans. The *in vitro* matrix that is formed from transformed fibroblasts has very high amounts of active inhibitors associated with it, even after the cells have been treated with trypsin and removed. The newly characterized TIMP-3 has been reported to be associated with the matrix (Pavloff).

II. Identification of Proteolytically Modified α1-Proteinase Inhibitor as a Chemoattractant for Neutrophils.

2.1 α1-Proteinase Inhibitor is a neutrophil chemoattractant after proteolytic inactivation by macrophage elastase or neutrophil elastase.

Mouse macrophage elastase, a metalloproteinase, catalytically inactivates human α1-PI by hydrolyzing a single peptide bond between Pro^{357} and Met^{358}. The two resulting fragments (Mr=4,200 and 47,800) remain tightly associated and can only be separated by denaturing conditions. Proteolytically inactivated α1-PI becomes a potent chemoattractant for human neutrophils (Banda et al., 1988a). The chemotactic response is optimal at a concentration of 1 nM and is equivalent to that elicited by the formylated peptide, formyl-Met-Leu-Phe. Purification and separation of the two fragments of proteolytically inactivated α1-PI has allowed the identification of the Mr= 4,200 carboxyl terminal fragment as being the chemoattractant.

Human neutrophil elastase, a serine proteinase, is the principal inhibitory target of α1-PI (Beatty et al.). Unlike macrophage elastase, which does not form a complex with α1-PI, HNE forms a stable stoichiometric complex with α1-PI. The inhibitor-proteinase complex consisting of human α1-PI and human neutrophil elastase (HNE) has also been demonstrated to be chemotactic for neutrophils (Banda et al., 1988b). As with α1-PI that has been inactivated by macrophage elastase, the chemotactic response is optimal at 1nM complex and is associated only with the α1-PI portion of the complex. Neither native α1-PI nor HNE possesses chemotactic activity. However, when HNE is inactivated by α1-PI, the Met^{358}- Ser^{359} bond of α1-PI is hydrolyzed. Analysis of the crystal structure of α1-PI suggests that proteolytic cleavage leads to a conformational change of the molecule and the exposure of a previously unexposed chemotactic signal.

These data suggested that a neutrophil cell surface receptor binds both proteolytically inactivated α1-PI and the α1-PI in the inhibited complex with HNE.

Saturation of the receptor by preincubation with α1-PI:HNE complexes prevents neutrophil chemotaxis toward α1-PI that has been proteolytically modified by macrophage elastase. Saturation of the receptor with the unrelated but well characterized chemoattractant molecule, formyl-Met-Leu-Phe, does not inhibit the migration of neutrophils toward proteolytically modified α1-PI. Therefore, this receptor is distinct from the formylated peptide receptor.

Other studies have also suggested the presence of a cell surface receptor for proteolytically modified α1-PI. In addition to chemotactic activity, α1-PI:HNE complexes have been shown to have other functional activities that are not expressed by the native molecules. Complexes stimulate increased synthesis of α1-PI in monocytes and macrophages (Perlmutter et al., 1988, Perlmutter and Punsal). α1-PI:HNE complexes are subject to rapid in vivo catabolism by the liver (Pratt et al., 1987, 1988, Pizzo et al. 1988) More recent evidence demonstrates that the clearance of α1-PI:HNE complexes can be blocked by other serpin:enzyme complexes (Mast et al. 1991a,b, Joslin et al., 1993) suggesting the existance of a generalized serpin-enzyme receptor. Taken together these observations indicate that the structural rearrangement of the α1-PI molecule, following proteolysis, exposes a domain that is recognized by a cell surface receptor. Whether these activities are mediated through a common receptor or a family of distinct but related receptors is not known.

Crystallographic data of proteolytically modified α1-PI revealed that α1-PI undergoes a striking conformational change upon cleavage from a strained unstable state to a stable inactive state (Loeberman). The Met ^{358} and Ser ^{359} that were formerly joined in the native molecule are now at opposite ends of the cleaved molecule 69Å apart. This conformational change has also been reported in proteolytically modified α1-antichymotrypsin.

2.2. Binding experiments using radiolabeled α1-PI:HNE complexes and peptides.

The initial goal of this project was to characterize the binding affinities and determine the number of receptors on the surface of neutrophils. Radiolabeled complexes and synthetic peptides were used as ligands.

Radiolabeled complexes were prepared by iodination of the elastase molecule followed by stoichiometric addition of α1-PI. The elastase could be iodinated to a higher specific labeling (>50,000 cpm/ng) than the α1-PI and was not susceptable to inactivation from the oxidizing conditions of chloramine-T labeling as was the α1-PI. This avoids the potential oxidation of the reactive site methionine of α1-PI (Johnson and Travis). When the reactive site methionine is oxidized to the sulfoxide, α1-PI becomes an ineffective inhibitor of elastase. The use of the non oxidizing Bolton Hunter reagent ,which iodinates lysine residues, results in lower specific labeling and does not iodinate the carboxyl terminal fragment of α1-PI.

Although these radiolabeled complexes were chemotactic for neutrophils in chemotaxis assays they did not bind in a saturable manner to neutrophils in binding assays. Because the addition of an excess of unlabelled elastase could reduce the nonspecific binding, these α1-PI-elastase complexes may interact with the neutrophil surface glycosaminoglycans.

Additionally, synthetic peptides based on the sequence of α1-PI were prepared. From X-ray crystallographic data (Loeberman et al.) and computer modeling (FRODO program at the MRC) the only accessible region of the carboxyl terminus of α1-PI following proteolytic cleavage was predicted to be a continuous sequence of eight amino acids from Ser^{359} to Phe^{366}. This peptide was synthesized with two additional residues on the carboxyl terminus including a tyrosine residue as a substate for iodination (SIPPEVKFNY). In addition a peptide representing the uncleaved sequence of

α1-PI (AIPMSIPPEV) and a related sequence from Protease Nexin-I were made (SSPPWFIVY).

However these peptides were not chemotactic for neutrophils nor did they desensitize neutrophils from responding to modified α1-PI. Therefore they did not compete with α1-PI-HNE complexes for binding to the receptor. The radiolabeled peptides did not bind to neutrophils.

2.3. Characterization of a serpin-enzyme complex receptor.

After these studies were initiated another group reported the characterization of a serpin-enzyme complex (SEC) receptor on human hepatoma cells (HepG2) (Perlmutter et al. 1990a). A series of synthetic peptides based on the sequence of α1- P I from the carboxyl terminal region inclusive of residues 359 to 374 were produced. One of these peptides (SIPPEVKFNKPFVYLI) was found to bind specifically and saturably to a single class of receptors with a K_d of 4.0×10^{-8}. (The phenyalanine at position 372 was changed to a tyrosine for iodination.) There were 4.5×10^5 receptors per cell as determined by Scatchard analysis. The peptide also stimulated α1-PI mRNA expression at a rate similar to that stimulated by α1-PI:HNE complexes. The binding of this peptide was blocked by α1-PI:HNE complexes but not by either native α1 -PI nor HNE.

More recently it has been demonstrated that there is a time dependent and saturable internalization of the α1-PI:HNE complexes on human hepatoma cells (Perlmuter et al. 1990b). Internalization of these complexes was inhibited by the addition of the synthetic peptide that corresponds to residues 359-374 of α1-PI. In addition the minimal peptide sequence necessary for the induction of the chemotactic response has been determined to be a pentapeptide consisting of residues 370-374 (Joslin et al., 1991a). This region is highly conserved throughout the serpins even in those that do

not possess proteinase inhibitory function, such as ovalbumin (figure 2.1). Whether these serpins also have chemotactic activity has not been determined. Additionally, it has been shown that other unrelated peptides can inhibit neutrophil chemotaxis towards modified serpins. These include amyloid β-peptide, substance P and tachykinin (Joslin et al., 1991b, Joslin et al.,1992).

Figure 2.1. Sequence alignment of serpins near carboxyl terminal chemotactic domain

α1-PI	M	S	I	P	P	E	-	-	-	-	-	V	K	F	N	K	**P**	**F**	V	F	L	M	I	E	Q
Hep Cofact II	L	S	T	Q	V	R	-	-	-	-	-	**F**	T	V	D	R	**P**	**F**	**L**	F	L	I	Y	E	H
AT-III	R	S	L	N	P	N	-	-	R	V	T	F	K	A	N	R	**P**	**F**	**L**	V	F	I	R	E	V
C1-Inhibitor	R	T	L	L	V	-	-	-	-	-	-	F	E	V	Q	Q	**P**	**F**	**L**	F	V	L	W	D	Q
endo PAI	R	M	A	P	E	E	-	-	-	-	-	I	I	M	D	R	**P**	**F**	**L**	F	V	V	R	H	N
Antichymo.	L	S	A	L	V	E	T	R	T	I	-	V	R	F	N	R	**P**	**F**	**L**	M	I	I	V	P	T
Antiplasmin	R	M	S	L	S	S	-	-	-	-	-	F	S	V	N	R	**P**	**F**	**L**	F	F	I	F	E	D
PN-1	R	S	S	P	P	W	-	-	-	-	-	F	I	V	D	R	**P**	**F**	**L**	F	F	I	R	H	N
Ovalbumin	A	S	V	S	-	E	E	-	-	-	-	F	R	A	D	H	**P**	**F**	**L**	F	C	I	K	H	I
BarleyZprot.	M	S	M	P	L	K	V	D	L	V	D	F	V	A	N	H	**P**	**F**	**L**	F	L	I	R	E	D
pep 359-366		S	I	P	P	E	-	-	-	-	-	V	K	F	N	Y									
pep 359-374		S	I	P	P	E	-	-	-	-	-	V	K	F	N	K	**P**	**F**	**V**	Y	L	I			
pep 370-374																		**F**	**V**	Y	L	I			

III. Role of HNE in the Modification of Progelatinase A:TIMP-2 Complexes.

Inflammatory cells migrate into tissue sites and upon stimulation release proteolytic enzymes that can mediate structural and functional changes in the extracellular matrix (ECM). These changes may be the result of direct degradation of ECM components by inflammatory cell proteinases, or be the indirect result of modifications made on other proteinases and proteinase inhibitors that are secreted by resident cells embedded within the matrix, such as fibroblasts or endothelial cells. Both serine and metallo- proteinases are well established as important mediators of ECM degradation (Alexander and Werb). Interactions between these proteinase classes can produce proteolytic cascades in which inflammatory serine proteinases activate latent matrix metalloproteinases (MMP) by proteolytic removal of the amino terminal propeptide (Mignatti et al.). With the exception of interstitial collagenase, most proteinases in the extracellular matrix have more than one potential substrate. The neutrophil serine proteinases, cathepsin G and elastase, can degrade not only collagen and proteoglycans but can also activate the metalloproteinase prostromelysin. Plasmin degrades cartilage proteoglycans and can, in addition, activate both procollagenase and prostromelysin. In addition to amino terminal processing, proteolytic modifications at other sites can alter the activity of the MMPs. Stromelysin can further activate interstitial collagenase by proteolytic cleavage near the carboxyl terminus (He et al.). The concerted interaction between proteinases can lead to proteolytic cascades capable of overwhelming the high level of proteinase inhibitors and effect net degradation of the matrix.

The activation of Gelatinase A is less well characterized compared to the activation of either collagenase or stromelysin. Gelatinase A is secreted by a wide variety of cell types, including fibroblasts (Seltzer et al.), epithelial cells (Collier et al.), endothelial cells (Herron et al. 1986b), mesangial cells (Lovett et al.), and is overexpressed in

several human tumor cell lines (Salo et al., Levy et al.). It has been detected as a bimolecular complex with its inhibitor, TIMP-2, bound to the hemopexin-like carboxyl domain at some distance from the active site. Proteinases that can directly activate the Progelatinase A:TIMP-2 complex have not been reported, although an as yet uncharacterized activation has been shown to be associated with plasma membrane preparations of tumor cells and concanavalin A treated fibroblasts (Brown et al., 1980, Overall and Sodek). Organomercurial treatment of progelatinase A:TIMP-2 complexes yields an active gelatinase on which TIMP-2 stays associated with the hemopexin-like domain. However, upon continued incubation this activated complex loses activity (Kleiner et al.). Deletional mutations of gelatinase A that lack the carboxyl hemopexin-like domain and do not have a bound TIMP-2 are more active and are more resistant to inhibition *in vitro* (Fridman et al.). It is not known if gelatinases with carboxyl terminal truncations occur *in vivo*. Proteinases that can remove the hemopexin-like carboxyl terminal domain of gelatinase A to generate these lower molecular weight gelatinolytic species have not been reported. Progelatinase A can autoactivate if it has been separated from TIMP-2 by reverse phase chromatography in acidic conditions followed by neutralization (Howard et al. 1991a). The products of autoactivation include an active 62 kDa gelatinase that has lost the propeptide and an active 43 kDa fragment that has lost both the propeptide and the hemopexin-like carboxyl terminal domain. This carboxyl hemopexin-like domain of Gelatinase A has been shown to have a binding site for TIMP-2 (Howard and Banda). The specific activity of the autoactivated gelatinase preparation is apparently greater than the complexed gelatinase. Due to the extreme conditions used to generate the free gelatinase it is unlikely that this autoactivation occurs *in vivo*. However it raises the possibility that other proteinases could generate a similarly modified gelatinase.

The initial inflammatory response to cellular injury is the migration of neutrophils to wound sites. The repair of damaged extracellular structures begins with the proteolysis and removal of matrix components. A coordinated spatial and temporal activation of proteinases must occur to exceed the high level of inhibitors that are found in plasma and distributed throughout the matrix. Modifications that can increase the proteolytic potential of enzymes within the matrix may be important in controlling the extent of degradation and rebuilding of the extracellular matrix. This study will test the hypothesis that an inflammatory serine proteinase, neutrophil elastase (HNE), can proteolytically modify gelatinase A:TIMP-2 complexes and thereby alter the potential for matrix turnover.

Matrix components influence protein-protein interactions within the extracellular matrices. An initial observation was made in which the addition of gelatin to free progelatinase A stimulated its autoactivation (fig. 3.1). These studies will test the hypothesis that non-catalytic interactions with matrix components may alter the activation pathways of proteinases.

Materials and Methods

Materials-- Human 72-kDa progelatinase A:TIMP-2 complexes, free progelatinase A and TIMP-2 were purified from serum free conditioned medium of transformed human fibroblasts (AT2SF-395) as described previously (Howard et al. 1991a). Progelatinase A:TIMP-2 complexes were purified by gelatin affinity chromatography. The bound gelatinases (A and B) were eluted with 10% dimethylsulfoxide in 20 mM HEPES pH 7.4, 0.5 M NaCl, 1 mM CaCl. The gelatinases were separated from one another by lentil lectin chromatography which binds the glycosylated Gelatinase B but does not retain the nonglycosylated Gelatinase A. Progelatinase A was separated from TIMP-2 by

reverse phase HPLC on a Vydac C_4 column eluted with a gradient of acetonitrile with 0.1% trifluoroacetic acid.

TIMP-1 was purified from the fibroblast conditioned medium that did not bind to the gelatin affinity resin (Howard et al. 1991b). This material was passed over a zinc chelating column to remove collagenase and the void fraction was applied to a lentil lectin column to bind the glycosylated TIMP-2. Eluted material was purified further by reverse phase HPLC. Protein concentrations were determined colormetrically using the bicinchoninic acid assay (Pierce) with bovine serum albumin as the standard.

Human neutrophil elastase was purchased from Elastin Products Co. (Owensville, Mo.).

Proteinase Activation-- Progelatinase A:TIMP-2 complexes were activated by incubation with 1mM aminophenylmercuric acetate (APMA) for 2-16 h at 37 ^{o}C. Stock solutions of APMA (1 M) were made immediately before use by dissolving dry reagent in 0.1 M NaOH with vigorous shaking. This was added to progelatinase:TIMP-2 containing samples at a dilution of 1:100.

Treatment of Gelatinase A:TIMP-2 Complexes with HNE-- Progelatinase A:TIMP-2 complexes or APMA activated complexes were diluted into 50 mM Tris/ 150 mM NaCl/ 0.02% NaN_3 / pH 7.5 with or without 1 mg/ml gelatin. HNE (1 mg/ml in Tris buffer containing 0.02% Brij) was added at molar ratios ranging from 1:1 (HNE : gelatinase A-TIMP2) down to 1:50 (HNE :gelatinase A-TIMP2) and allowed to react at 37 ^{o}C at various time points from 5 min to 48 h. Digestions were stopped by inhibition of elastase using either 10 mM phenylmethylsulfonyl fluoride (PMSF) or a 5 fold molar excess of a1-proteinase inhibitor. Digestion mixtures were analyzed by substrate gel zymography and by gelatin degradation assays.

Electrophoresis-- SDS-polyacrylamide gel electrophoresis was performed according to the methods described previously (Laemmli). Zymography was carried out on 10% acrylamide gels containing 1 mg/ml gelatin (Sigma; porcine skin, 300 Bloom) under nonreducing conditions as described previously (Heussen and Dowdle, Herron 1986a). After electrophoresis, the gels were incubated for 30 min in 2.5% Triton X-100/ 0.02% NaN_3 at ambient temperature, then incubated from 2-16 h at 37 ^{o}C in substrate buffer (50 mM Tris/10 mM $CaCl_2$/ 0.02% NaN_3 pH 8.0). Gels were stained with Coomassie Blue R250 and destained in 50% methanol in water. For protein blotting and preparation of samples for amino acid sequence analysis electrophoresis was performed on 10% or 12% standard acrylamide SDS gels followed by electrophoretic transfer to PVDF in 10 mM CAPS buffer pH 11.

Proteinase Assays-- Gelatin degradation assays were performed as previously described (Howard et al. 1991a)). Collagen (rat tail type I , Collaborative Research) was reductively acetylated with [^{3}H]acetic anhydride to a specific activity of 5.7 X 10^{6} cpm/mg. This labeled collagen was diluted with nonlabeled collagen and dialyzed against 25 mM Tris, 1 mM CaCl, pH 7.6. The final specific activity of the tritiated collagen was 500,000 cpm/mg. The [^{3}H]collagen was boiled for 5 min. for use in gelatinase assays. Gelatinase samples were diluted in Tris/CaCl buffer with 2 mg/ml ovalbumin and added to an equal volume of the labeled gelatin. After 5 to 60 min. reactions were stopped by the addition of an equal volume of 50% trichloroacetic acid. Supernatants were analyzed for acid solubilized gelatin peptides by liquid scintillation spectrometry.

Protein Radioiodination-- Progelatinase A:TIMP-2 complexes were oxidatively radioiodinated by the chloramine T method (Radioiodination techniques Amersham). Carrier free Na1^{25}I (1 mCi) was added to 10 µl 0.25M Na phosphate buffer pH 7.5 and the following were added in rapid succession with continuous mixing: 1 µg progelatinase

A:TIMP-2 complex, 50 μg chloramine T then 120 μg sodium metabisulphate in 50 mM phosphate buffer. The total volume was brought to 1 ml with 2 mg/ml NaI in 50 mM phosphate buffer and labeled complexes were separated from unincorporated label over a PD10 column (Pharmacia) preequilibrated with phosphate buffer.

TIMP-1 and TIMP-2 (10 μg) in 100 ml 250 mM NaPi, pH 7, were oxidatively radiolabeled for 10 min. with 1 mCi $Na^{125}I$ (Amersham) in tube coated with 100 μg Iodogen (Pierce Chemical Co.). Saturated Tyrosine was added to bind free ^{125}I and the iodination mixture was diluted into buffer containing 1 mg/ml ovalbumin, 1 mg/ml NaI, and the radiolabeled proteins were separated by size exclusion chromatography using PD-10 columns (Pharmacia).

Protein Sequencing-- Amino acid sequence determination was performed by the Biomolecular Resource Center at UCSF. Samples containing 50-250 pmole protein immobilized on PVDF were subjected to Edman degradation using an Applied Biosystems 470A gas-phase sequencer. The PTH-derivatives were identified and quantitated by reverse-phase HPLC using an on -line Applied Biosystems 120A PTH Analyzer.

Cells-- Transformed human ataxia telangiectasia fibroblasts (AT2SF-395) that secrete elevated levels of metalloproteinases were provided by John Murnane (University of California, San Francisco). These cells have been stably transformed with the plasmid pLR309, which contains SV40 sequences with a defective origin of replication. Cells were cultured in DME-H21, 10% heat inactivated fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin. Upon reaching confluence, the cells were washed three times with phosphate-buffered saline, and the medium was replaced with DME supplemented with 0.2% lactalbumin hydrolysate. Medium conditioned by these cells was collected every 2-3 days, filtered through Whatman no. 5 paper, and concentrated by ammonium sulfate precipitation (60%).

HS27 cells and HT1080 fibrosarcoma cells were obtained from the American Type Culture Collection (ATCC). HS27 is a human foreskin fibroblast cell line that was propagated in DMEM with 10% fetal bovine serum. HT1080 fibrosarcoma cell line was propagated in MEM with 10% fetal bovine serum.

Neutrophils were separated on Ficoll-Hypague gradients from peripheral blood obtained from volunteers. Blood was drawn into heparinized tubes, mixed 1:1 with phosphate-buffered saline, and layered over Ficoll-Hypaque (Sigma). (30 ml of blood/PBS was carefully added to 15 ml Ficoll-Hypaque in sterile 50 ml polypropylene centrifuge tubes.) The tubes were centrifuged for 30 min at 500 x g at room temperature in a clinical centrifuge. After removal of the plasma and the mononuclear leukocytes at the interface between plasma and ficoll, the remainder of the ficoll is removed by vacuum aspiration to just above the cell pellet. The pellets containing polymorphonuclear leukocytes and red cells are suspended with 2.5% dextran (250,000 molecular weight, Sigma, in phoshate-buffered saline), and incubated at 37^{o}C for 15 min. The settled cell pellets were resuspended in a 3-fold volume of ice-cold NH_4Cl medium (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA adjusted to pH 7.4 and filter sterilized) for 10 min. to lyse the erythrocytes . Cells were centrifuged at 1,000 rpm for 10 min., washed three times with phoshate-buffered saline, and diluted to a cell concentration of 1-2 x 10^6 cells/ml.

Neutrophil Elastase Assay -- Elastase activity was measured with methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma) at 1 mM substrate concentration. Rate assays were performed at room temperature by following the release of p-nitroaniline at 410 nm (E_{410} 8800). Freshly prepared substrate (10 mM in Dimethyl sulfoxide; 6.22 mg/ml) was diluted 1:10 in 0.1 M HEPES, pH 7.5, containing 0.5 M NaCl, immediately before use.

Peptide Synthesis, Characterization, and Purification-- Peptides were synthesized on a Milligen 9020 manual peptide synthesizer using Fmoc protected pentafluorophenyl esters of the amino acids. Peptides were removed from the polyamide support by 1-2 h. incubation in 100% trifluoroacetic acid (TFA). TFA also removed protecting groups. The crude peptide was precipitated in ice-cold ether, washed three times with ice-cold ether, and dried under vacuum. The peptides were purified by reverse phase high pressure liquid chromatography (C4 and C18). The expected mass of the synthesized peptide amides was verified by mass spectrometry analysis.

Results

An initial observation was made in which the addition of gelatin to free progelatinase A stimulated its autoactivation (fig. 3.1). The activation was increased considerably as detected by substrate gel zymography. A synthetic peptide substrate, PLGLAG, based upon a cleavable sequence in type I collagen derived gelatin, did not inhibit the autoactivation of gelatinase A. This suggested that sites other than the active catalytic domain are involved with the activation pathways and possibly stability of gelatinase A. A synthetic peptide modeled after the site within progelatinase A that must be cleaved to generate the 43 kDa gelatinolytic fragment (GIQELYGAS), also did not inhibit the autoactivation of purified progelatinase A. However, neutrophil elastase does cleave this peptide and could potentially play a role in the activation of gelatinase A. What effect do matrix components have on the interaction between inflammatory proteinases and resident proteins within the matrix?.

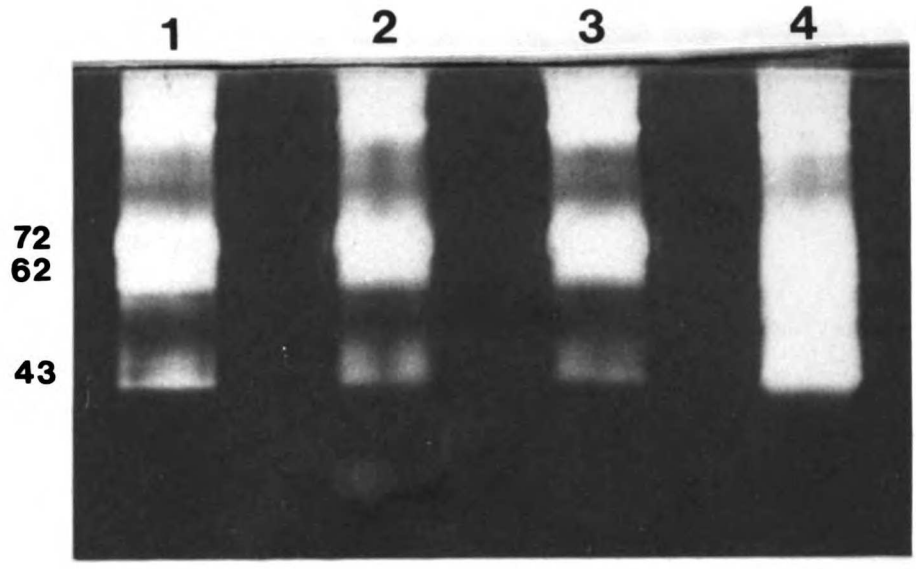
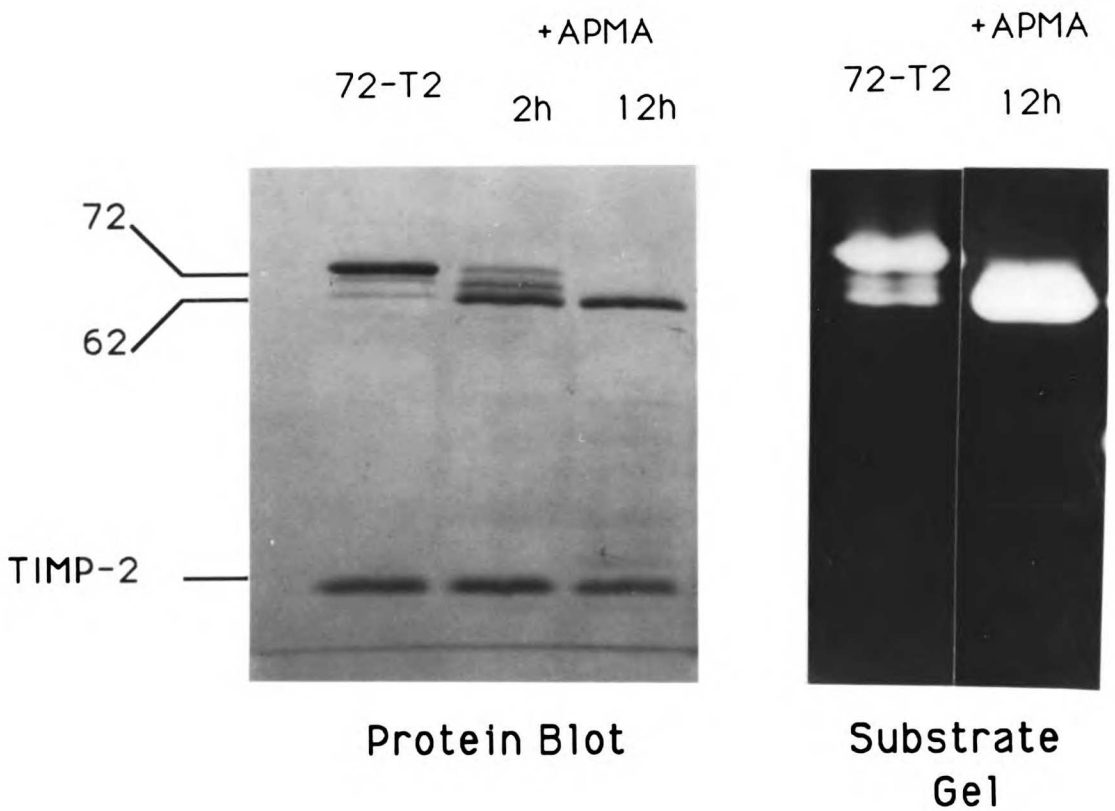


Figure 3.1. **Increased autoactivation of progelatinase A in the presence of gelatin.** Purified free progelatinase (1 μ g) containing 0.1% trifluoracetic acid was dialyzed against 20 mM HEPES/ 0.15 mM NaCl/1 mM CaCl/ pH 7.5 at 37^{0}C for 2 h. The autoactivation of progelatinase was determined in the presence of the peptide amides PLGLAG (1mM) (lane 2), GIQELYGAS (1 mM) (lane 3) and gelatin (porcine skin, 2 mg/ml) (lane 4) and compared to control (lane 1). Samples were analyzed by gelatin substrate zymography.

Progelatinase A:TIMP-2 complexes are activated by treatment with APMA (figure 3.2). The activation corresponds to the proteolytic removal of the 10 kDa propeptide and the resulting shift in molecular weight from 72 kDa to 62 kDa as seen on protein blots and substrate zymography. Progelatinase:TIMP-2 complexes are not active in soluble gelatin degradation assays. However, these complexes do show activity on gelatin zymograms due to the effect of the detergent SDS.

Can HNE Process Gelatinase A:TIMP-2 Complexes Into Lower Molecular Weight Forms That Retain Gelatinolytic Activity? -- The effect of neutrophil elastase on gelatinase A:TIMP-2 complexes was examined. Increasing amounts of HNE were used to digest both 72-kDa progelatinase A:TIMP-2 complexes and 62-kDa gelatinase A:TIMP-2 complexes that result from APMA activation. If progelatinase A:TIMP-2 complexes are completely converted to active 62-kDa gelatinase A:TIMP-2 by APMA, then one major gelatinolytic fragment migrating at M_r 40-kD is produced from digestion by neutrophil elastase. When 72-kDa progelatinase A:TIMP-2 complexes that also contain some 62-kDa gelatinase A are digested by HNE, two products are formed as detected by gelatin zymography. The larger fragment migrates with an M_r of 50-kD. The lower molecular weight fragment is the M_r 40-kD cleavage product of the 62-kDa gelatinase A:TIMP-2 complex (figure 3.3). Because the propeptide adds approximately 10 kDa to the size of the proteinase, it is likely that the 50-kDa form represents a fragment of the 72-kDa progelatinase A that retains the propeptide. If the 50-kDa form does possess a propeptide it should reduce to the 40-kDa form upon treatment with APMA. Indeed, treatment with APMA converts the 50-kDa fragment to the 40-kDa gelatinase A (figure 3.4). The 40-kDa gelatinase A can result from HNE

Figure 3.2. **SDS-PAGE analyses of the activation of progelatinase A:TIMP-2 complexes by APMA.** Progelatinase A:TIMP-2 complexes (0.2 mg/ml) were incubated with 1 mM APMA for the indicated times and analyzed by either coomassie stained protein blots (left panel) or by gelatin substrate zymography (right panel). The techniques are discussed in "Materials and Methods". 72-T2 is the progelatinase:TIMP-2 complex electrophoretically separated. Upon APMA treatment the 72 kDa progelatinase converts to the 62 kDa form.


+APMA
72-T2
2h
12h
72
62
TIMP-2
Protein Blot
+APMA
72-T2
12h
Substrate
Gel

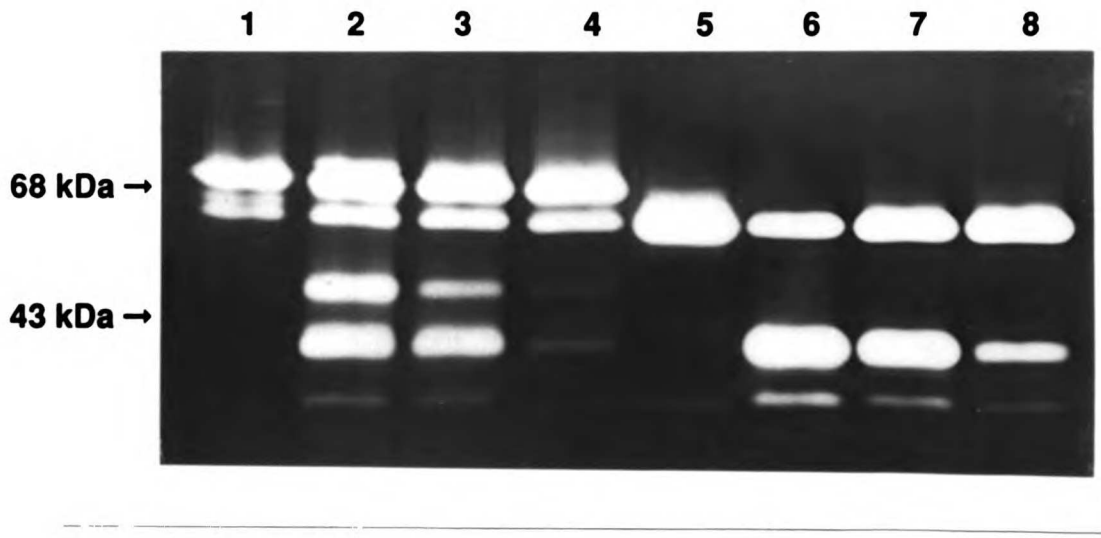


Figure 3.3. **Gelatinase A:TIMP-2 complexes are processed into lower molecular weight forms by elastase.** Progelatinase A:TIMP-2 complexes (2 pmole, lanes 1-4) and APMA activated gelatinase A:TIMP-2 complexes (2 pmole, lanes 5-8) were digested with HNE and analyzed by gelatin zymography as described in "Materials and Methods". Complexes were digested with 2 pmole HNE (lanes 2 and 6); 0.2 pmole HNE (lanes 3 and 7); 0.1 pmole HNE (lanes 4 and 8) and with PMSF inhibited HNE as control (lanes 1 and 5). Bovine serum albumin (68kDa) and ovalbumin (43 kDa) were used as molecular weight markers.

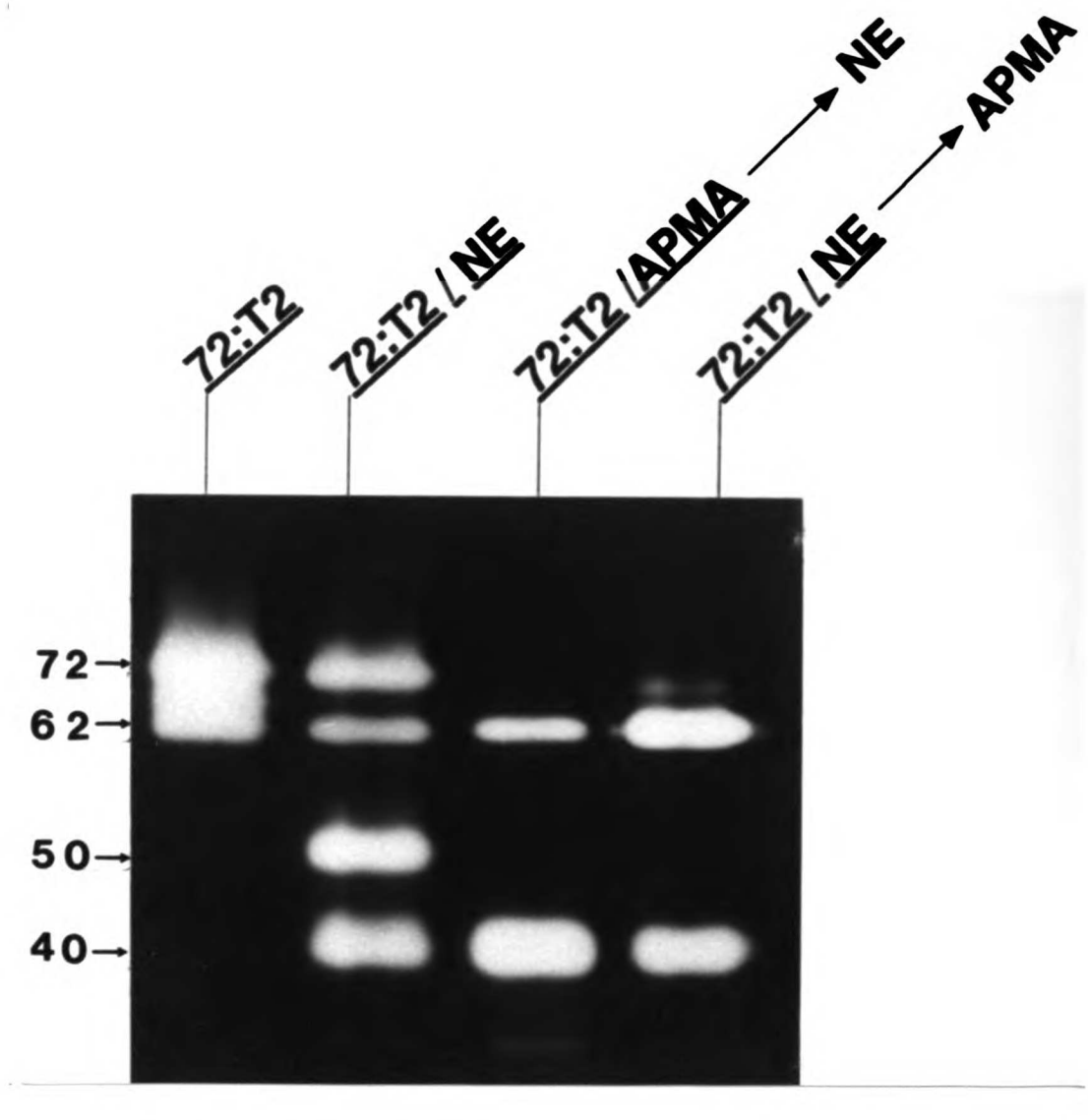


Figure 3.4. **The 40 kDa gelatinase can be generated by two pathways.** Lane 1 (72:T2) is 2 pM of progelatinaseA:TIMP-2 complex. Progelatinase A:TIMP-2 was digested with elastase at a 1:1 molar ratio (lane 2, 72:T2/NE). In lane 3, progelatinase A:TIMP-2 complexes were first treated with 1 mM APMA for 2 h followed by elastase digestion for an additional 2 h. In lane 4 progelatinase A:TIMP-2 complexes were first digested with elastase followed by APMA treatment. Progelatinase and APMA activated gelatinase are indicated by the 72 and 62, respectively. The apparant molecular weights of the truncated gelatinases (50 and 40) were determined by comparison with ovalbumin.

proteolytic processing of the 62-kDa gelatinase A:TIMP-2 complex or, conversely, from APMA treatment of the 50-kDa progelatinase A that is formed from HNE digestion of 72-kDa progelatinase A:TIMP-2 complexes (figure 3.5) .

Does the Modification of Progelatinase A:TIMP-2 Complexes by Sequential Treatment with APMA and HNE Effect its proteolytic Activity?-- To determine if the truncated gelatinases produced by HNE digestion of complexes lead to increased activation, digestion mixtures were assayed for soluble gelatinolytic activity. Treatment of the 72-kDa progelatinase A:TIMP-2 complex with APMA eventually results in the removal of the amino-terminal propeptide yielding a 62-kDa active proteinase which remains associated with TIMP-2. Additional treatment of the activated 62-kDa gelatinase A:TIMP-2 complex with HNE yields an active preparation with a four-fold increase in gelatinolytic activity over APMA treated complex (figure 3.6). Does this increased gelatinolytic activity correspond to the 40 kDa fragment? To test this gelatinase A:TIMP-2 complexes were treated with elastase and at specified times identical samples were evaluated for soluble gelatin degradation assays and gelatin zymograms. The increased gelatinolytic activity that results from HNE digestion of the 62-kDa gelatinase A:TIMP-2 complex is always associated with the appearance of the fragment that migrates at 40-kDa on gelatin zymograms (figure 3.7a,b). The increasing gelatinolytic activity is proportional to the amount of the 40-kDa fragment.

Is HNE Proteolysis of TIMP-2 Necessary for Processing of Gelatinase A?-- HNE can degrade TIMP-2 (figure 3.8a). It is, therefore, possible that HNE must first degrade TIMP-2 before directly attacking gelatinase A. To distinguish immediate cleavage of gelatinase A by HNE from cleavage following TIMP-2 proteolysis, [^{125}-I] radiolabeled progelatinase A:TIMP-2 complexes were digested with HNE. The degradation of TIMP-2 is not a necessary prerequisite for the processing of gelatinase A:TIMP-2 complexes by HNE (figure 3.8b). Additionally, activated gelatinase A might be responsible for some of the proteolysis described above. To distinguish direct cleavage

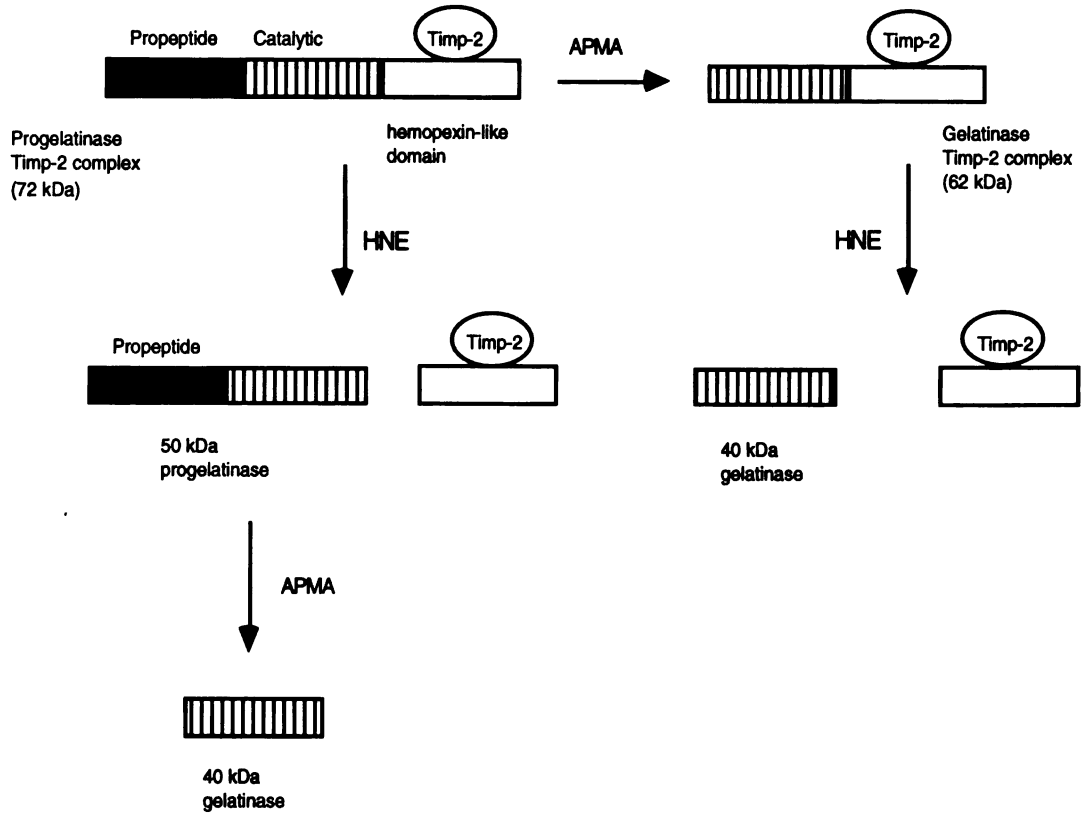


Figure 3.5. **Schematic showing activation of Gelatinase A and modifications made by elastase.**

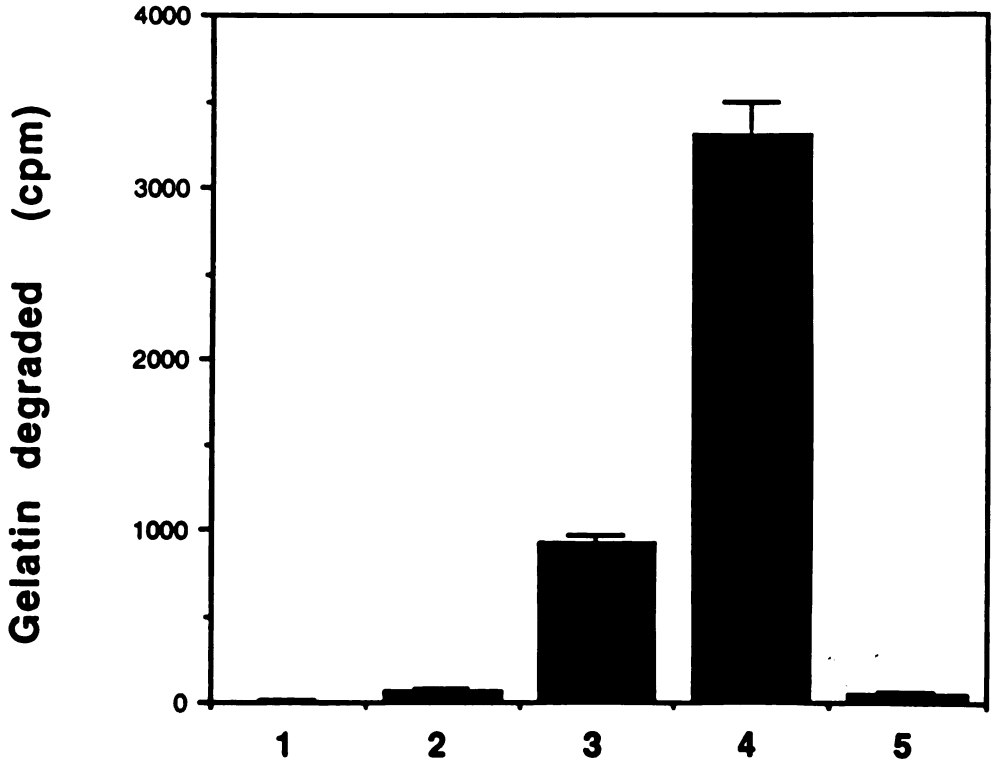


Figure 3.6. **Modification of Gelatinase A by elastase results in increased gelatinolytic activity.** Gelatin degradation assays (Materials and Methods) were performed with the following samples; 1, 72 kDa progelatinase A:TIMP-2 complexes; 2, 72 kDa progelatinase A:TIMP-2 complexes treated with elastase; 3, APMA treated gelatinase A:TIMP-2 complexes; 4, Same as sample 3 followed by elastase treatment; and 5, control with elastase that has been inhibited with 1mM PMSF.

Figure 3.7. **Increased gelatinolytic activity correlates with the 40 kDa gelatinase.** Progelatinase A:TIMP-2 complexes (10 nmole) were activated with 1 mM APMA for 1 h followed by the addition of 2 nmole elastase. At the designated times samples were taken, treated with 1 mM PMSF to inhibit the elastase, and **A**, analyzed by gelatin substrate zymography, or **B**, assayed by gelatin degradation assays. BSA (68 kDa) and ovalbumin (43 kDa) are used as molecular weight markers.

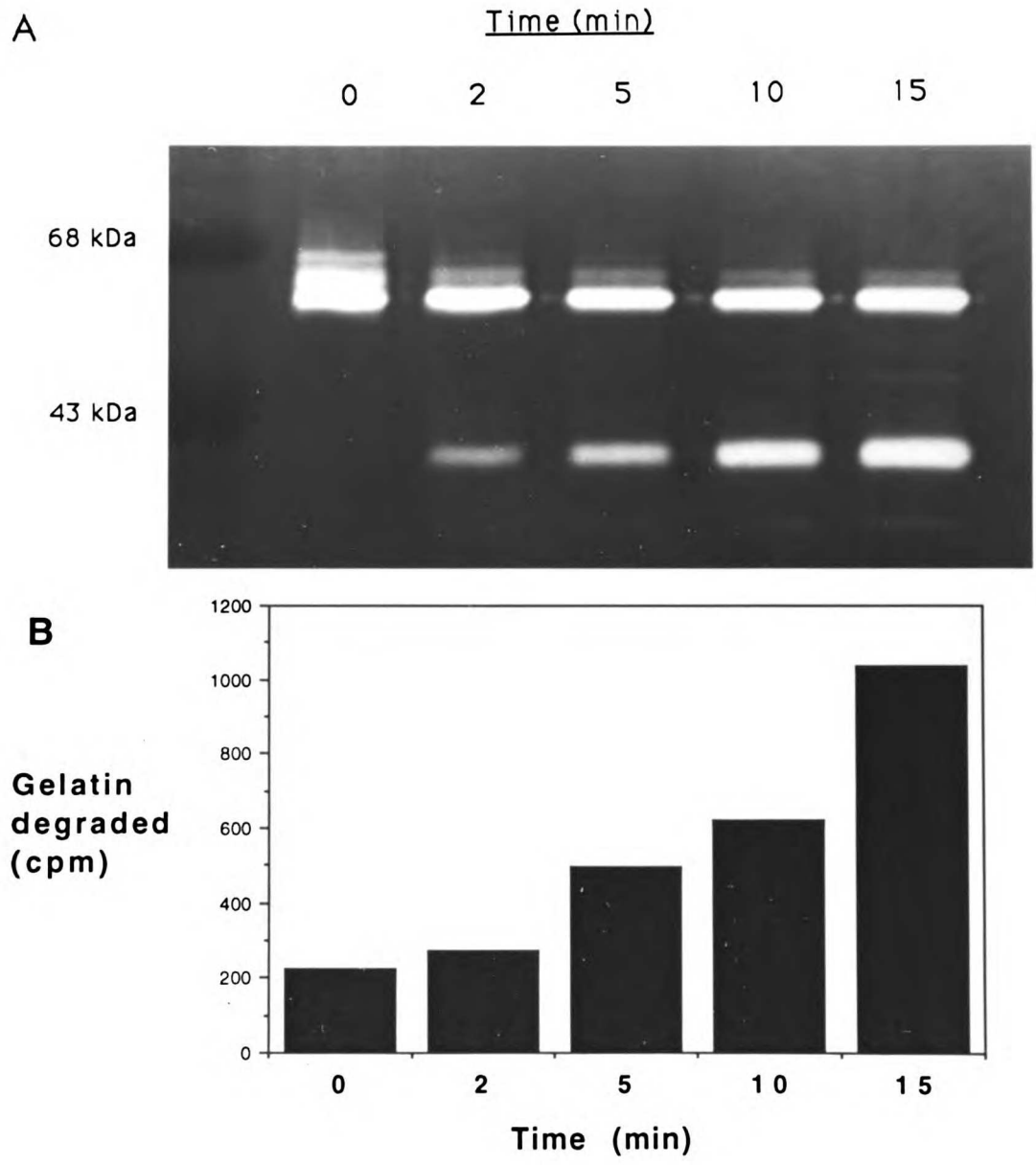
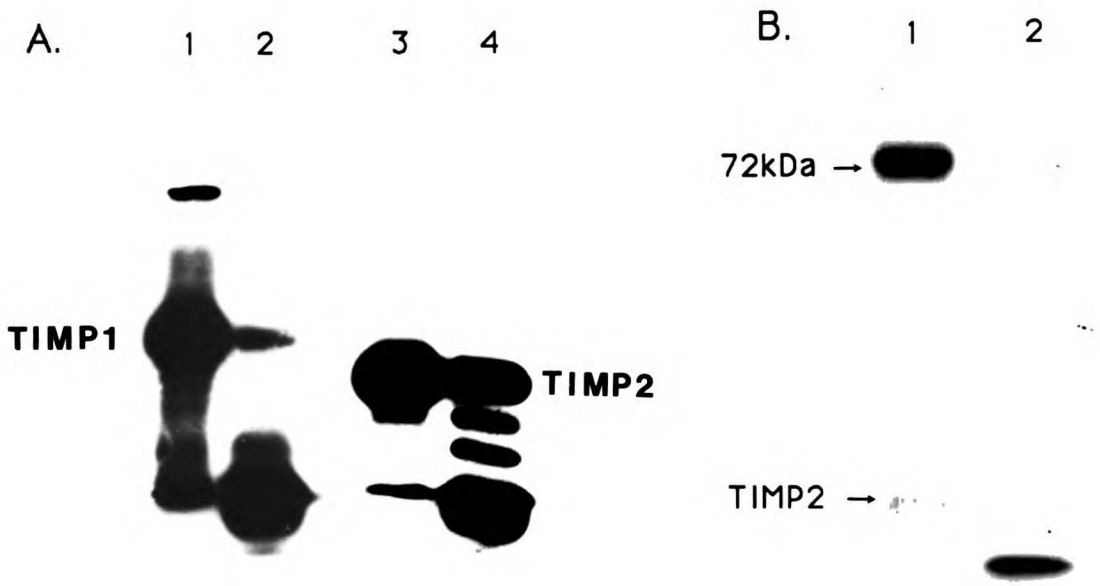

A
Time (min)
0
2
5
10
15
68 kDa
43 kDa
B
Gelatin degraded (cpm)
1200
1000
800
600
400
200
0
0
2
5
10
15
Time (min)

Figure 3.8. **Degradation of TIMP-2 by elastase is not required for processing of gelatinase A.** Panel A) Human TIMP-1 (lanes A1,A2) and TIMP-2 (lanes A3,A4) were oxidatively labeled with $Na^{125}I$ to a specific activity of 29,000 cpm/ng and 19,000 cpm/ng, respectively. ^{125}I-TIMP-1 and ^{125}I-TIMP-2 were incubated with a 10 fold excess of neutrophil elastase for 2 h at 37 C (lanes 2,4). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (12%) followed by autoradiography. Panel B) Progelatinase A:TIMP-2 complexes were oxidatively labeled with $Na^{125}I$ to a specific activity of 15,000 cpm/ng. ^{125}I-Progelatinase A:TIMP-2 complexes (lane B1) were incubated with equimolar neutrophil elastase for 2 h at room temperature. Samples were analyzed as in A.


A.
1
2
3
4
TIMP1
TIMP2
B.
1
2
72kDa →
TIMP2 →

of gelatinase A by HNE from HNE mediated auto-cleavage of gelatinase A, experiments with class specific proteinase inhibitors were performed. If HNE mediates the auto-cleavage of gelatinase A, then the metalloproteinase inhibitors, EDTA, 1,10-phenanthrolene or an excess of TIMP-2 should inhibit the formation of the 40-kDa gelatinase A from HNE digestion of 62-kDa gelatinase A:TIMP-2 complexes. Metalloproteinase inhibitors did not inhibit the formation of the 40-kDa fragment resulting from HNE processing of 62-kDa gelatinase A:TIMP-2 (figure 3.9). Inhibitors of serine proteinases, however, completely inhibited the formation of the 40-kDa species. Both phenylmethylsulfonylfluoride (PMSF) and a1-proteinase inhibitor prevent the conversion of the 62-kDa gelatinase A to the 40-kDa form. Therefore, it is likely that HNE directly enhances the activity of the 62-kDa gelatinase A:TIMP-2 complexes by generating the 40-kDa species.

Where Are The HNE Cleavage Sites Within 72-kDa Gelatinase A?-- HNE can cleave at least three peptide bonds of 72-kDa progelatinase A. Amino acid sequence analysis established that the two bands at 36 kDa and 31 kDa are the carboxyl terminal fragments produced from cleavages made between Ala^{307}-Met^{308} and Ala^{350}-Asn^{351}, respectively. These cleavage sites are within the fibronectin-like domain of gelatinase A and lead to a loss of activity because the zinc binding domain has been separated from the catalytic site. Another cleavage is made between Val^{435}-Thr^{436} that results in the 22 kDa carboxyl terminal fragment that migrates in front of the TIMP-2 and a functional amino fragment (figure 3.10a). These cleavage sites are shown diagrammatically in figure 3.10b.

What is the Role of Matrix in the Interaction of Gelatinase A and HNE?--

The matrix provides a solid phase affinity support for many secreted and cell associated proteins. Gelatinase A has a collagen binding domain that has been exploited for the purification of the gelatinases. Gelatinase A is also found associated with the

Figure 3.9. **Truncated gelatinase formation is the direct result of elastase cleavage.** APMA activated Gelatinase A:TIMP-2 complexes (0.8 pmole) were preincubated for 1 h at 37 degrees C with the following proteinase inhibitors; 1 mM PMSF (lanes 3,4); 1 mM EDTA (lanes 5,6); TIMP-2 (2 pmole); control without inhibitors (lanes 1,2). Samples were incubated with 2 pmole neutrophil elastase for 2 h at 37 C and analyzed by gelatin zymography on10% gels. Gelatinase A is indicated by 62 kDa and the truncated gelatinase migrates at 40 kDa.

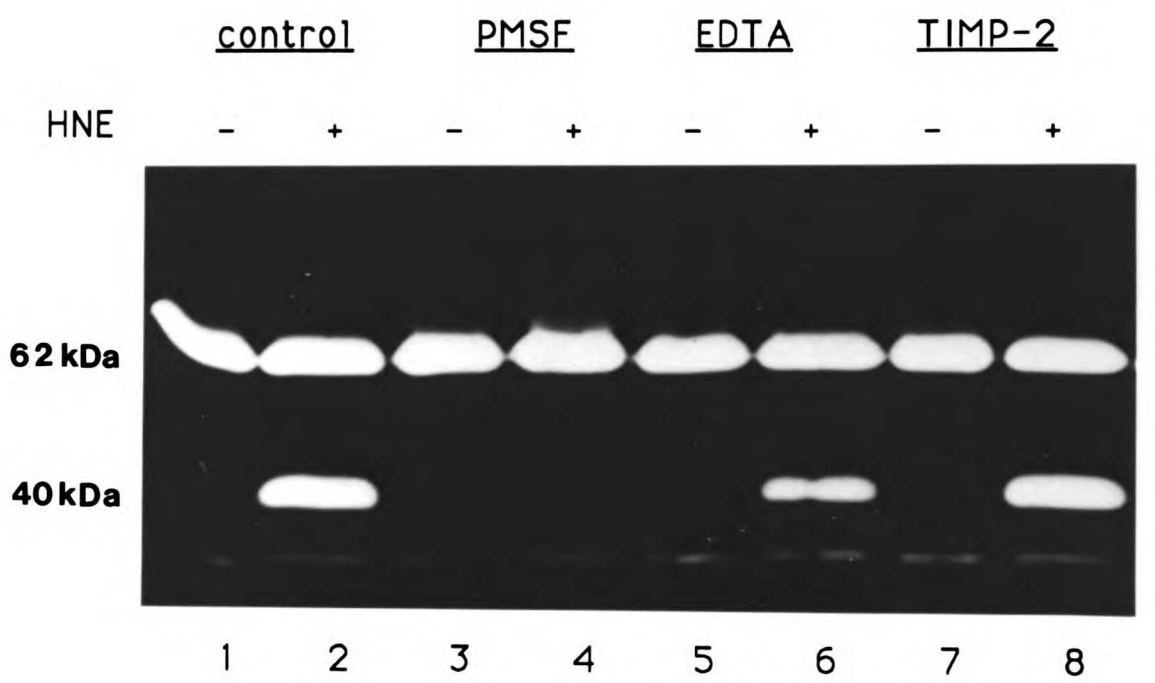
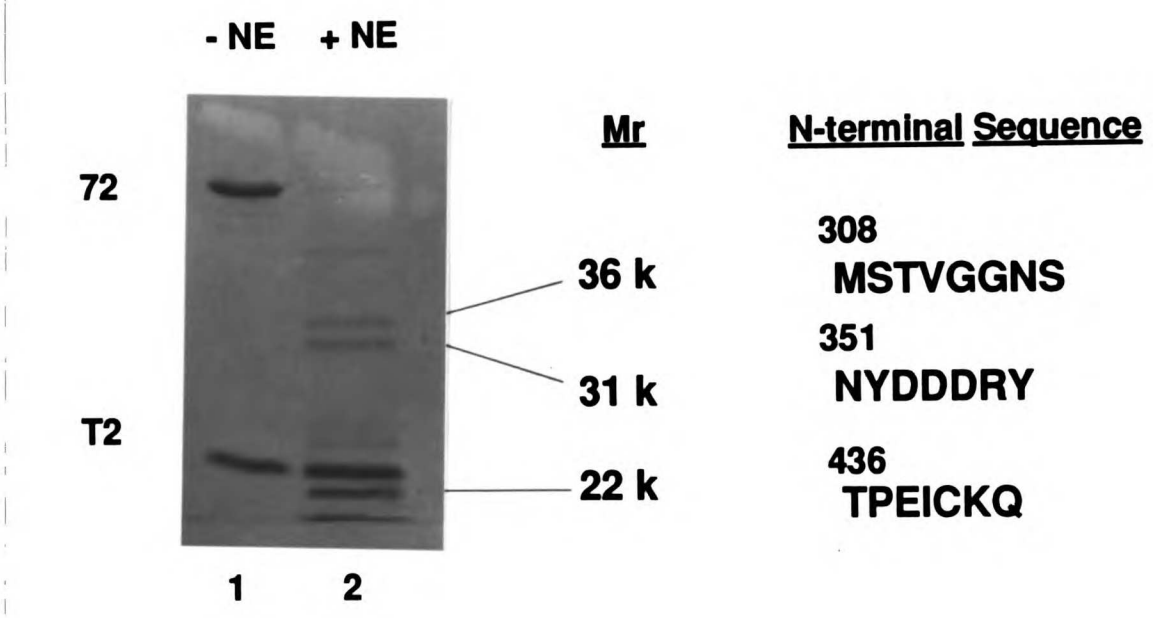
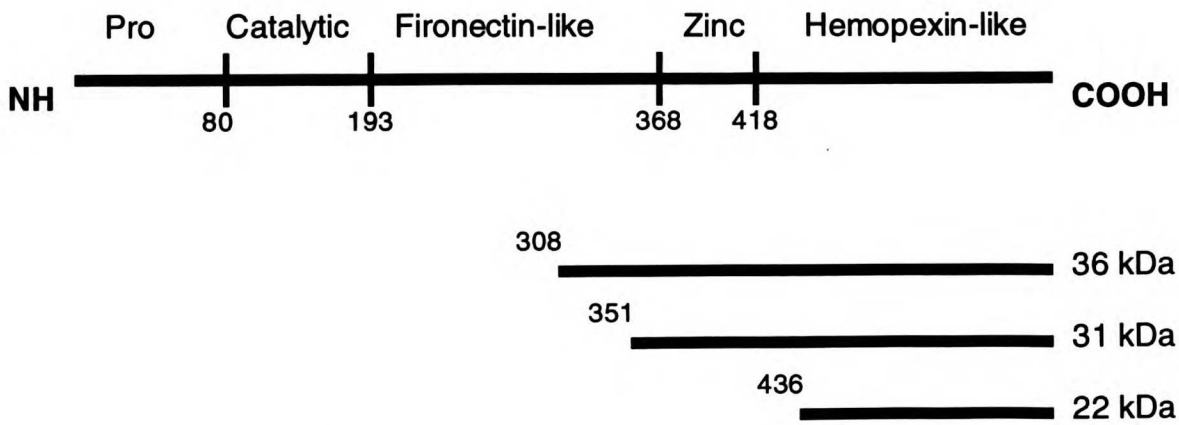

control
PMSF
EDTA
TIMP-2
HNE
- + - + - + - +
62 kDa
40 kDa
1 2 3 4 5 6 7 8

Figure 3.10. **Elastase cleaves Gelatinase A at three sites. A.** Progelatinase A:TIMP-2 complexes were incubated with 3% (wt/wt) neutrophil elastase (-NE/+NE). Samples were electrophoresed on 10% acrylamide gels followed by electrophoretic transfer to PVDF membrane in 10 mM CAPS buffer pH 11.0. The blot was stained with coomassie blue, destained with 50/40/10 methanol/water/acetic acid and subjected to amino acid analysis as described in "Materials and Methods". The size and the amino terminal sequence of the fragments are listed. **B. Map of cleavage sites.** The domain structure based upon the primary sequence of progelatinase A is drawn (not to scale). The three major products that result from elastase digestion of the progelatinase A are shown as black lines with their corresponding molecular weights (as determined by SDS-polyacrylamide electrophoresis).

A.


- NE
+ NE
72
T2
1
2
Mr
36 k
31 k
22 k
N-terminal Sequence
308
MSTVGGNS
351
NYDDDRY
436
TPEICKQ

B.


Pro
Catalytic
Fironectin-like
Zinc
Hemopexin-like
NH
COOH
80
193
368
418
308
36 kDa
351
31 kDa
436
22 kDa

matrix generated by fibroblasts in culture. This *in vitro* affinity for gelatin and collagen is likely to be true for the gelatinases *in vivo*. Activated gelatinase is more stable in the presence of gelatin. Because neutrophil elastase cleaves the gelatinase in its gelatin binding fibronectin domain, the location of gelatinase in the matrix may influence the interaction between elastase and gelatinase.

Destructive degradation of both the 72-kDa progelatinase A and the APMA activated 62-kDa gelatinase A occur when either high HNE concentrations are used or when digestion times are long. However, the presence of gelatin or collagen in the digestion mixture decreases the rate of destructive HNE mediated degradation of gelatinase A:TIMP-2 complexes (figure 3.11). If the digestion of 62-kDa gelatinase A:TIMP-2 complexes by HNE is done in buffer or in the presence of ovalbumin, which can be degraded by HNE, the 40-kDa gelatinase A is detected at 1 h but by 12 h it has been completely degraded. In addition the 62-kDa gelatinase A is also almost completely degraded by 12 h as reported by others (Okada and Nakanishi). However, when gelatin or collagen is included in the digestion mixture, the 40-kDa fragment and the 62-kDa gelatinase A are still detected at 12 h. Because HNE can inactivate the gelatinase A by cleaving at either of the sites within the fibronectin-like gelatin binding domain, addition of gelatin to the digestion should protect the gelatinase A from degradation and reduce the amounts of the 36 kDa and the 31 kDa inactive fragments. To test this possibility, gelatinase A:TIMP-2 complexes were preincubated with gelatin prior to incubation with HNE. This results in a loss of the 36 kDa and 31 kDa fragments presumably because the cleavage sites have been blocked by binding to gelatin (figure 3.12).

Do the Truncated Gelatinases Remain in Complex With TIMP-2?-- TIMP-2 is bound to 72-kDa progelatinase A at the carboxyl terminal hemopexin-like domain. A consequence of HNE cleavage of the Val^{435}- Thr^{436} bond would be the release of this 22-kDa carboxyl terminal fragment together with its bound TIMP-2. Gelatinase

Figure 3.11. **Gelatin protects gelatinase A from destructive degradation by elastase.** APMA activated Gelatinase A:TIMP-2 complexes (10 pmole) were preincubated with; ovalbumin (1 mg/ml); gelatin (porcine skin, 1 mg/ml); collagen (rat tail type 1, 1 mg/ml); or buffer control (20 mM HEPES pH 7.4 /0.2 M NaCl/1 mM Cacl) for 1 h at 37 C. Neutrophil elastase (2 pmole) was added and samples were taken at the indicated times and analyzed by gelatin substrate zymography. Gelatinase is indicated by the **62** and the truncated gelatinase is **40.**

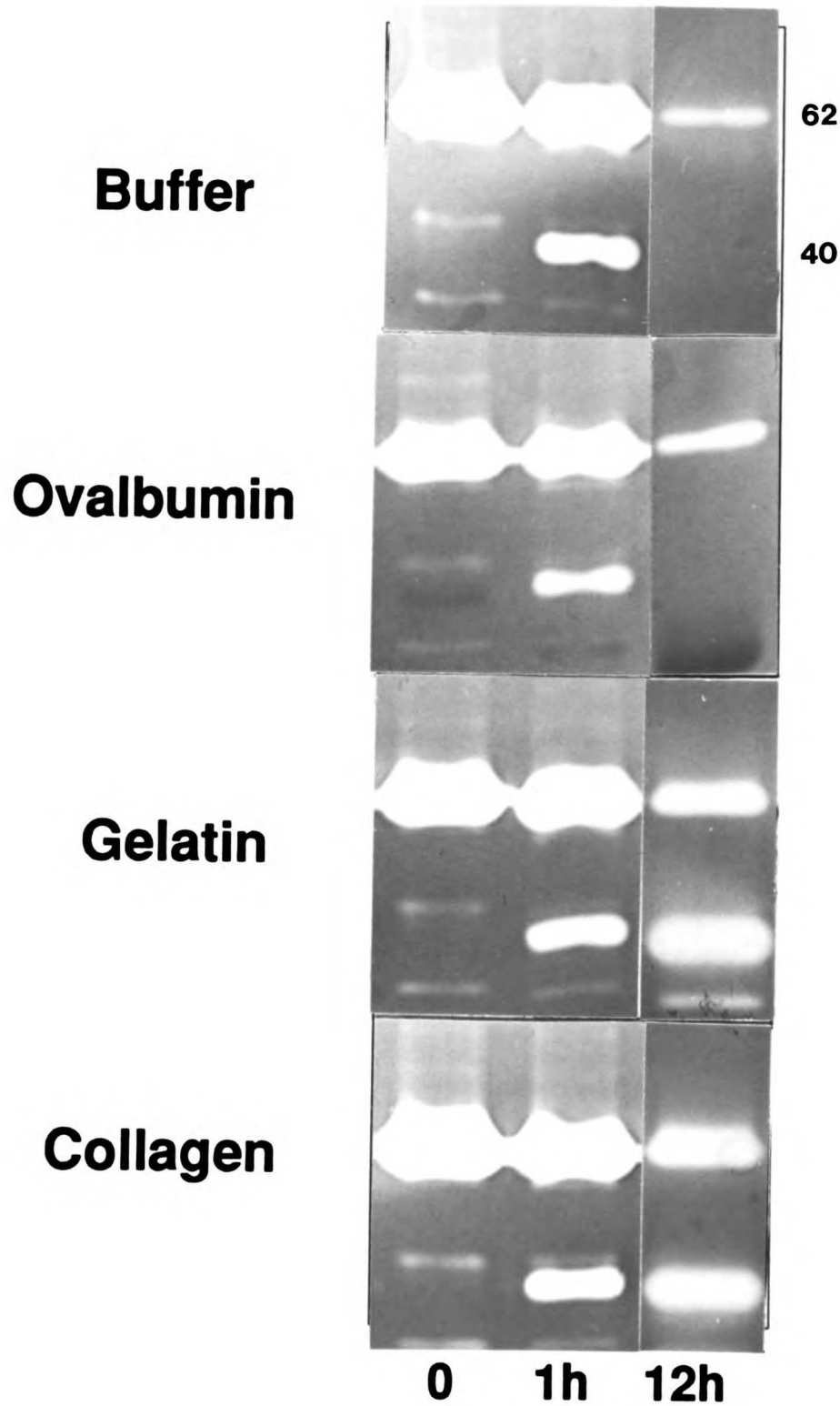
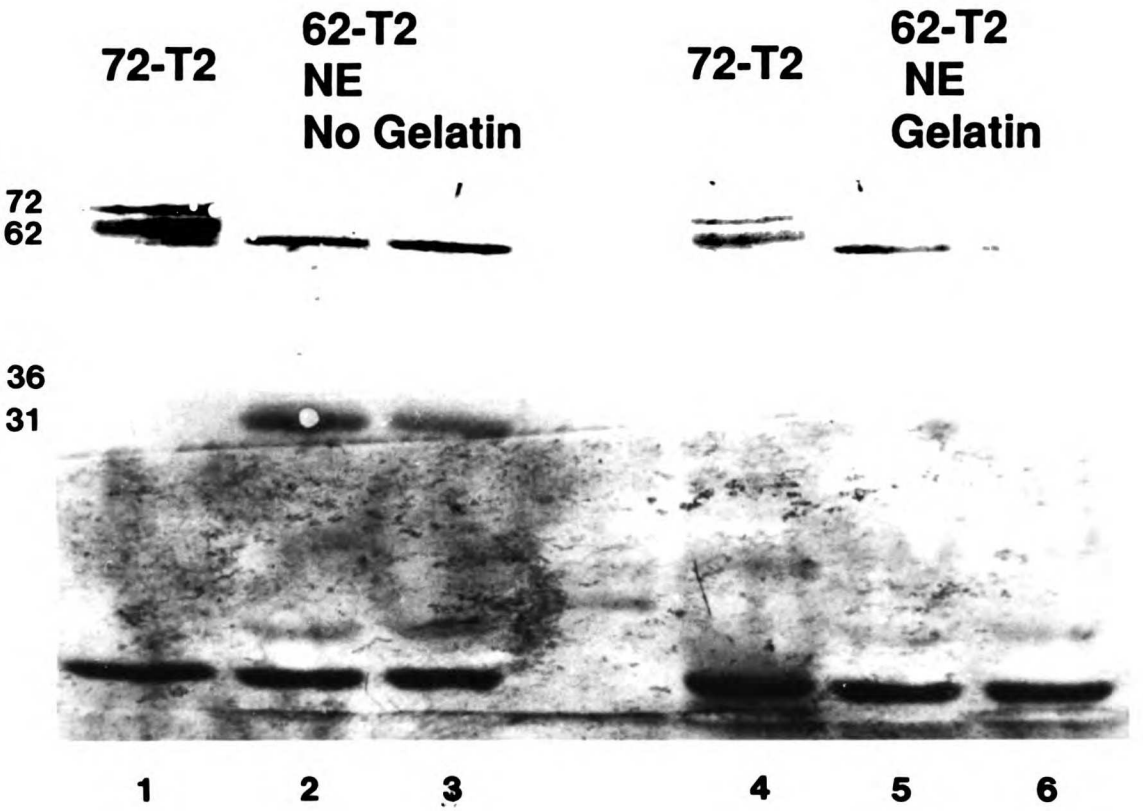

Buffer
Ovalbumin
Gelatin
Collagen
62
40
0
1h
12h

Figure 3.12. **The degradation of gelatinase A by elastase is reduced with the addition of gelatin.** Progelatinase A:TIMP-2 complexes (lanes 1 and 4) were activated with APMA and digested with elastase (1:1 mol/mol) either with (lanes 5 and 6) or without 1 mg/ml gelatin. Samples were analyzed by SDS-polyacrylamide gels and electrophoretically transferred to PVDF followed by coomassie blue staining. **72** and **62** represent 72 kDa progelatinase and the 62 kDa APMA activated gelatinase, respectively. **36** and **31** represent the 36 kDa and 31 kDa inactive fragments of gelatinase as discussed in the text.

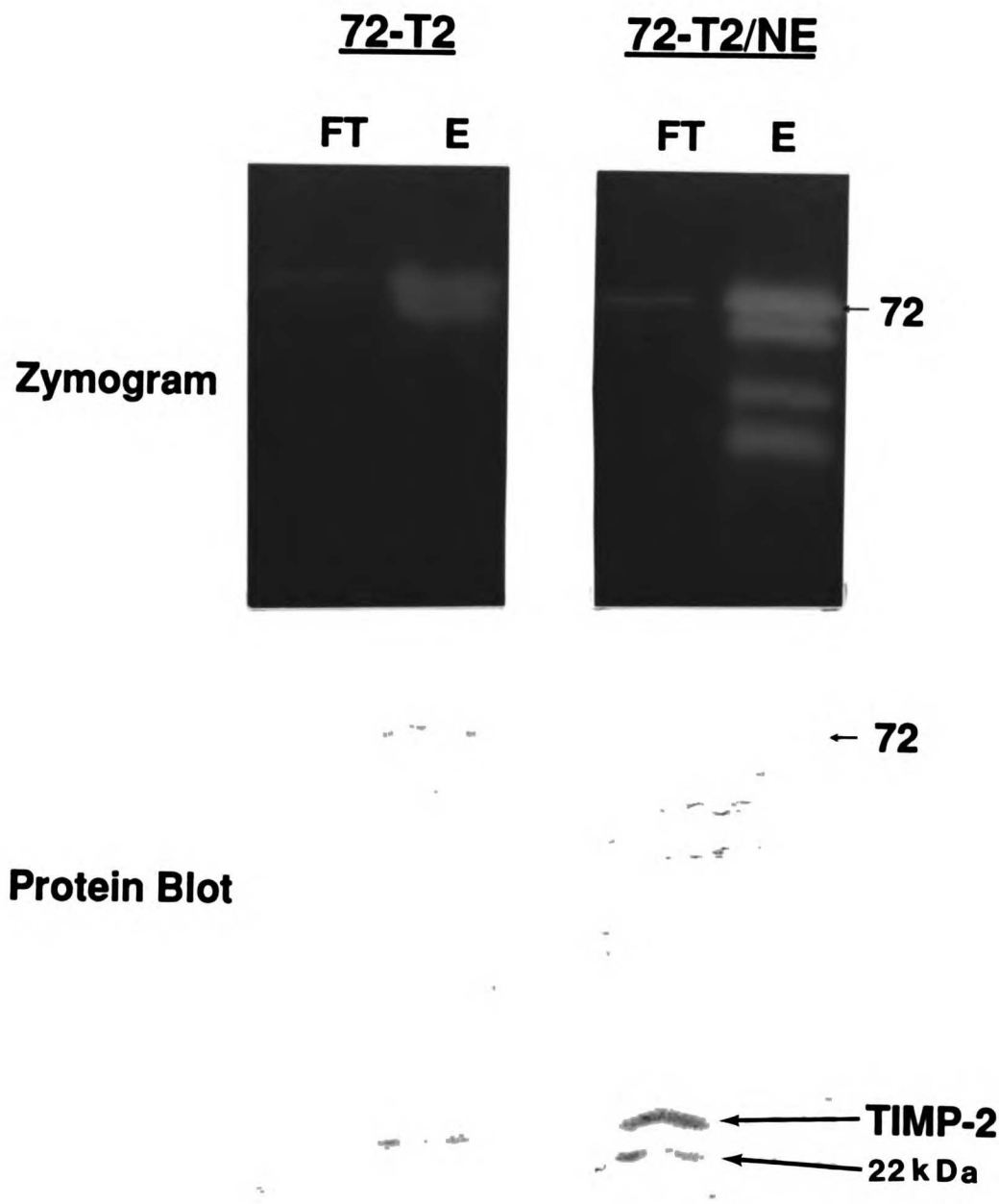

72-T2
62-T2
NE
No Gelatin
72-T2
62-T2
NE
Gelatin
72
62
36
31
1
2
3
4
5
6

A:TIMP-2 complexes bind to gelatin sepharose by the fibronectin-like domain of gelatinase and can be eluted with buffer containing 10% DMSO. Purified TIMP-2 does not bind to gelatin sepharose. When progelatinase A:TIMP-2 complexes are bound to gelatin sepharose and then digested with HNE, the TIMP-2 and the 22-kDa carboxyl terminal fragment of gelatinase appear in the unbound fraction. The truncated gelatinases remain bound to the affinity matrix and can be eluted with DMSO (figure 3.13).

Does the TIMP-2 released by elastase cleavage of gelatinase:TIMP-2 complexes function as an inhibitor? Elastase digestion of gelatinase:TIMP-2 complexes results in the formation of active gelatinolytic species even though TIMP-2 is present in the digestion mixture. Therefore, under these conditions, the TIMP-2 does not inhibit the gelatinase. Purified TIMP-2 / 22 kDa gelatinase hemopexin domain generated from elastase cleavage of gelatinase:TIMP-2 complexes does not inhibit truncated gelatinases. The TIMP-2 may be so tightly bound to the hemopexin domain of gelatinase that it cannot dissociate and act as an inhibitor.

Do Neutrophils Interact With Cells That Produce Gelatinase A? Gelatinase A is expressed in many different tissues and has been isolated from many different cells. Where would neutrophils be expected to interact with gelatinase A that is being secreted or has been secreted and bound to the extracellular matrix? During the course of neutrophil migration out of the blood and into the tissue there are numerous encounters with cells that produce gelatinase A. Endothelial cells secrete gelatinase A as do fibroblasts in the stroma underlying basement membranes. Neutrophils are found in granulation tissue that is richly vascularized and potentially full of bound gelatinase A. Neutrophils respond to an implanting blastocyst, surrounding it and possibly affecting the matrix around the implantation site (Finn and Pope). Mesangial cells have also been

Figure 3.13. **Timp-2 is released from the truncated gelatinase.** Progelatinase A:TIMP-2 complexes (10 mg) were bound to 1 ml gelatin sepharose in 25 mM Tris, 0.15 mM NaCl, pH 7.4. The column was washed with 10 column volumes (10 ml) buffer. The columns were treated for 2 h at 25^{0}C with either 1 ml of 2 mg/ml elastase (right panels, 72-T2/NE) or with PMSF inhibited elastase as a control (left panels, 72-T2). The unbound material was collected and the columns were washed. Bound material was eluted with 10% DMSO in loading buffer. Samples were analyzed by gelatin substrate zymography and by coomassie stained protein blots. The migration of progelatinase A and the TIMP-2 are indicated by the **72** and the TIMP-2. The carboxyl terminal of gelatinases A cleaved off by elastase migrates at 22 kD.


72-T2
72-T2/NE
FT
E
FT
E
Zymogram
72
72
Protein Blot
TIMP-2
22 k Da

shown to secrete progelatinase A (Lovett et al.). Inflammation of the kidney that leads to an accumulation of neutrophils within the glomerulus can potentially influence the activity of Gelatinase A (Johnson et al., 1988).

Is the amount of elastase released by neutrophils sufficient to modify gelatinase *in vivo* in the manner described by these *in vitro* studies? To partially address this question, experiments were carried out with freshly isolated neutrophils that were incubated with purified gelatinase. The amount of elastase released by PMNs was measured. Unstimulated cells (ie. with no additional stimulation besides the effect of separation from blood) released 0.36 μg per 10^6 cells. After stimulation by either 10 nM fMLP or phorbol ester, 1.5 μg of elastase per 10^6 cells was released. This figure is in agreement with the literature value of the estimated total amount of elastase per 10^6 cells at approximately 2 μ g (Travis et al., 1992).

Can Neutrophils Directly Truncate Gelatinase A?-- To determine whether neutrophils could process gelatinase A, purified gelatinase A:TIMP-2 complexes were incubated with freshly isolated neutrophils. The truncated 40 kDa gelatinase is produced by incubation with neutrophils (figure 3.14).

In order to test what effect neutrophil elastase has on secreted gelatinase A, purified elastase was added to gelatinase producing cells in culture. HS27 is a normal human foreskin fibroblast cell line. In culture these cells secrete gelatinase A that can be purified as the 72 kDa progelatinase:TIMP-2 complex. HT1080 is a human fibrosarcoma cell line that also produces gelatinase A but, unlike HS27 cells, can activate Progelatinase A to the 62 kDa active gelatinase. Elastase treatment of HS27 cells produces the 50 kDa truncated progelatinase (fig. 3.15). Elastase treatment of the HT1080 cell line results in the formation of both the 50 kDa and the 40 kDa truncated gelatinase A species.

Figure 3.14. **Neutrophils can modify gelatinase A in a similar manner as purified elastase.** APMA activated gelatinase A (lane 1) was treated with purified elastase (1:1 mole/mole gelatinase to elastase) for 2 h at room temperature in 0.05 M HEPES, pH 7.4, containing 0.15 M NaCl and 1 mg/ml gelatin (lane 2). APMA activated gelatinase A was also added to 100 ml of a suspension of freshly isolated neutrophils (5.0 x 10^6 cells/ml in 0.05 M HEPES, pH 7.4, containing 0.15 M NaCl and 1 mg/ml gelatin for 2 h at room temperature. The digest mixture was centrifuged and the supernatants were analyzed by gelatin substrate zymography (lane 4). **62**- gelatinase A, **40**- truncated gelatinase.

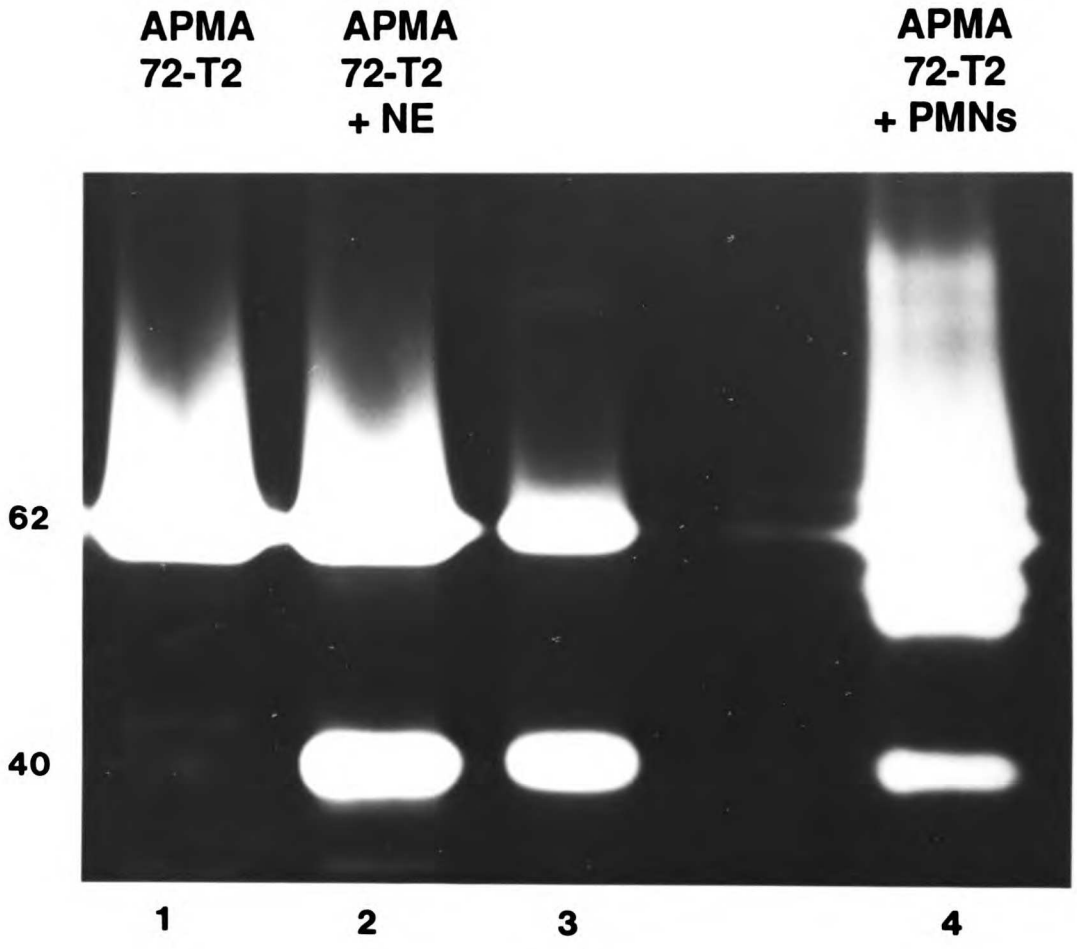
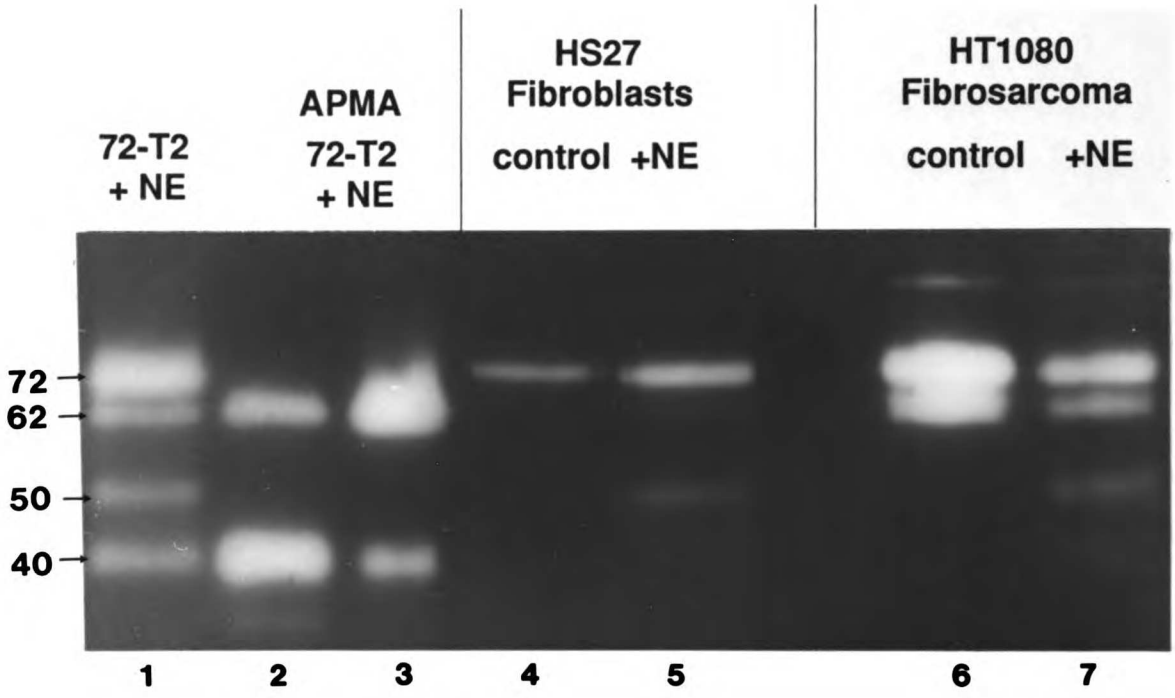

APMA
72-T2
APMA
72-T2
+ NE
APMA
72-T2
+ PMNs
62
40
1
2
3
4

Figure 3.15. **Purified elastase can generate truncated gelatinases from fibroblasts and fibrosarcoma cells in culture.** HS27 human foreskin fibroblasts and HT1080 fibrosarcoma cell lines were grown to confluence in 12 well plates that had been coated with Vitrogel. The growth medium was removed, the cells were washed 3 times with PBS, and 0.5 ml DME was added back with or without purified elastase (10 mg/ml). After 8 h the conditioned medium was collected, centrifuged, and analyzed by substrate zymography. HS27 cell conditioned medium is in lane 4 and elastase treatment is lane 5. HT1080 conditioned medium with and without elastase is shown in lanes 6 and 7. Purified progelatinase A:TIMP-2 complexes and APMA treated gelatinase A:TIMP-2 complexes digested by elastase were run as controls (lanes 1,3)


72-T2
+ NE
APMA
72-T2
+ NE
HS27
Fibroblasts
control +NE
HT1080
Fibrosarcoma
control +NE
72
62
50
40
1
2
3
4
5
6
7

Discussion

The data presented here demonstrates how an inflammatory serine proteinase, neutrophil elastase, can proteolytically modify gelatinase A:TIMP-2 complexes. HNE can cleave at a site within the hemopexin-like carboxyl terminal of either the 72-kDa progelatinase A:TIMP-2 complex or the partially activated APMA treated complex producing a 50-kDa or 40-kDa gelatinase, respectively. The completely activated 40-kDa form of gelatinase A can be produced by treatment of the 72-kDa progelatinase A:TIMP-2 complexes with APMA followed by limited proteolytic digestion by HNE, or conversely, by first digesting the progelatinase complexes with HNE and then treating with APMA. The 40-kDa form of gelatinase appears to have a higher specific activity than that of the APMA activated gelatinase A:TIMP-2 complex. These truncated gelatinases no longer have the carboxyl terminal hemopexin-like domain nor are they associated with the formerly bound TIMP-2.

The presence of matrix components effect the modifications that HNE can make on gelatinase A:TIMP-2 complexes. In the absence of gelatin or collagen, HNE hydrolyzes gelatinase A:TIMP-2 complexes at two sites within the fibronectin-like gelatin binding domain of gelatinase. This fibronectin-like domain is inserted between the catalytic domain and the zinc binding domain and cleavage at these sites results in loss of activity of the enzyme. These findings are consistent with those previously reported that HNE does not activate but rather degrades gelatinase A in the absence of gelatin (Okada and Nakanishi). However, when gelatin is bound to the fibronectin-like domain these potential cleavage sites become more resistant to proteolysis by HNE and the cleavage between Val^{435} and Thr^{436} that produces the truncated gelatinolytic enzyme is more prevalent.

Increased proteolytic potential can also result from proteolytic degradation of inhibitors. HNE has been reported to degrade TIMP-1 (Okada et al., 1988), a metalloproteinase inhibitor related in structure and function to TIMP-2. HNE might

also degrade TIMP-2 and that the enhanced activation of 72-kDa progelatinase A:TIMP-2 complexes by HNE may be due to an autolytic event subsequent to TIMP-2 degradation in a manner similar to that seen with autoactivated 72-kDa progelatinase A (Howard et al. 1991a). HNE does degrade TIMP-2 but more slowly than it degrades TIMP-1. When HNE is incubated with 72-kDa progelatinase A:TIMP-2 complexes for a time sufficient for the complete degradation of progelatinase A the TIMP-2 remains intact. Also the formation of the 40-kDa gelatinase A from HNE digestion of APMA treated gelatinase A:TIMP-2 complexes occurs in the presence of metalloproteinase inhibitors suggesting that HNE directly generates the 40-kDa form of gelatinase A.

An additional consideration is the structure of the fibronectin-like gelatin binding domain of gelatinase A. It is composed of three head-to-tail repeats of the type II structures that are also found in fibronectin (Holland et al.) and the seminal plasma protein PDC-109 (Banyai et al.). Each type II structure is approximately 40 amino acids in length and includes four cysteines that, in fibronectin and PDC-109, are found to be completely oxidized with two disulfide bonds connecting cysteine residue pairs 1-3 and 2-4 (Constantine et al.,1991&1992). The cleavage of gelatinase A by HNE at Ala^{307} is between the second and third repeat of the type II structures. The cleavage at Ala^{350} is within the third type II repeat between cysteine residues 346 and 361. The two most hydrophilic regions of Gelatinase A are within the fibronectin-like domain. with the highest value centered at residue 352. This region corresponds to the predominant destructive cleavage by elastase. The carboxyl terminal products of these cleavages are the inactive 36-kDa and 31-kDa fragments that can be detected with both reduced and unreduced samples. These data suggest that, unlike fibronectin and PDC-109, there is not a disulfide bond between the second and fourth cysteine residues of the third type II repeat in gelatinase A. Since this region is immediately adjacent to the zinc binding domain there may be structural constraints that would limit the necessary proximity of the cysteine residues for disulfide bond formation between them.

The fate of secreted progelatinase A:TIMP-2 complexes is unknown. When secreted by normal fibroblasts or endothelial cells these complexes become, in effect, part of the matrix by binding to either native or denatured collagens and can be activated by cell associated mechanisms. How long gelatinase A:TIMP-2 complexes remain active in tissues is not known but *in vitro* studies have shown these complexes to become inactive thereby limiting the extent of proteolytic degradation of the matrix. The present studies indicate that neutrophil elastase can modify substrate bound gelatinase:TIMP-2 complexes such that they become both more active and less inhibitable by the TIMPs. During an acute inflammatory response these modifications may facilitate a more complete restructuring of the ECM. This modification that increases the proteolytic potential of gelatinase A may be limited by the available collagenous substrates. When the substrates have been degraded free Gelatinase:TIMP-2 complexes may be quickly degraded into inactive fragments by HNE. The carboxyl terminus of gelatinase A may be necessary for cell associated activation (Murphy, Willenbrock et al.). Whether the 50 -kDa progelatinase A that results from HNE digestion of Progelatinase A:TIMP-2 complexes can be activated by this mechanism is not known.

Conclusion

The study set out to test two hypotheses. The first hypothesis was that an inflammatory proteinase, neutrophil elastase could proteolytically modify the metalloproteinase Gelatinase A and thereby alter the potential for matrix turnover. The second hypothesis was that non-catalytic interactions with matrix components might alter activation pathways and the stability of Gelatinase A. Both of these hypotheses have been successfully tested and shown to be valid.

An early stage in wound healing involves the infiltration of neutrophils into sites of cellular injury. These inflammatory cells release proteolytic enzymes that help initiate the debridement of damaged tissue. The modifications that inflammatory proteinases make on other proteins within the matrix may alter the pathways of degradation and, ultimately, the repair of tissues. In these studies, neutrophil elastase was shown to proteolytically modify the metalloproteinase, gelatinase A. The nature of these modifications depend on whether the gelatinase A:TIMP-2 complexes are free in solution, or bound to gelatin. Free gelatinase:TIMP-2 is destructively degraded by elastase. However when the gelatinase A is bound to its substrate, elastase cleaves preferentially at a site that generates a truncated, yet more active gelatinase. Although these studies were done using purified components *in vitro*, interactions between elastase and gelatinase A are likely to occur *in vivo*. Some potential sites of interaction were discussed earlier.

If neutrophil elastase can, by itself, degrade most if not all the components of the matrix what would be the advantage of increased proteolytic potential? As long as the proteolytic activity is restricted to the immediate cascade perhaps there can not be too much proteolysis especially when digesting the damaged tissue at a wound. Gelatinase A when bound to gelatin can be superactivated by elastase. When substrate is degraded and the gelatinase is free it can be quickly degraded by elastase. The elastase and the

gelatinase work in a concerted fashion to completely degrade damaged macromolecular substrates. Because the Gelatinases (both A and B) can degrade elastin, elastase and gelatinase A may again act in a concerted fashion to degrade elastin.

Proteinases from other inflammatory cells may also be involved with the modification of proteinases or inhibitors within the matrix. Although these studies only examined the effects of neutrophil elastase on gelatinase, proteinases from macrophages may also be important in altering pathways of protease activation that lead to the restructuring of the matrix. Wound healing can occur even in the absence of neutrophils, albeit more slowly and perhaps at greater risk of infection (Leibovich and Ross). There are inherent redundancies in most systems and that is most certainly true for proteinases.

Understanding how neutrophils modify the extracellular matrix during migration may shed some light on the invasive properties of malignant tumor cells. Invasive growth of tumors is dependent on degradation and remodeling of the stromal architecture. Many tumor cell lines secrete elevated amounts of proteinases in culture. Among other proteinases, gelatinase A has been implicated in the metastatic process because of its ability to degrade basement membrane ECM. HT1080 fibrosarcoma cells secrete and activate high levels of gelatinase A. The invasive properties of these cells may be in part due to this increased expression of proteolytic activity. Proteolytic processing of gelatinase A to a more active form by non tumor cells might increase the metastatic potential of tumor cells.

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