Interstitial Collagenase (Matrix Metalloproteinase-1) Expresses Serpinase Activity

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Abstract

Human endothelial cells treated with either interleukin-1β, tumor necrosis factor-α, or phorbol myristate acetate secreted a metalloproteinase that hydrolyzed and inactivated the two major serine proteinase inhibitors (Serpins) found in plasma, α_1-proteinase inhibitor and α_1-antichymotrypsin. Surprisingly, the responsible metalloproteinase was identified as human interstitial collagenase (matrix metalloproteinase-1), an enzyme whose only known physiologic substrate has heretofore been believed to be the extracellular matrix molecule, collagen. The metalloproteinase inactivated the Serpins by cleaving peptide bonds at sites unrelated to those hydrolyzed in collagenous macromolecules. NH_2-terminal sequence analysis localized the cleavage sites in the Serpins to regions near their respective reactive site centers at three distinct peptide bonds on the amino-terminal side of bulky, hydrophobic residues. Together, these data indicate that matrix metalloproteinase-1 displays an expanded substrate repertoire that supports the existence of a new interface between connective tissue turnover and Serpin function. (*J. Clin. Invest.* 1991. 88:2258–2265.) Key words: endothelial cells • inflammation • collagen • α_1-proteinase inhibitor • α_1-antichymotrypsin

Introduction

Members of the superfamily of serine proteinase inhibitors (i.e., Serpins) play a central role in regulating the proteolytic processes associated with coagulation, fibrinolysis, kinin formation, complement activation, and connective tissue turnover (1–3). The inhibitory profile of individual Serpins is dictated primarily by a reactive site center that lies within an exposed loop of the carboxy terminal domain wherein the sequence and conformation of the aligned residues match the substrate specificities of the cognate proteinases (1–3). In this manner, binding of the hydroxyl group of the catalytic serine of the proteinase to a specific region at the reactive center of the Serpin results in the formation of an enzyme-inhibitor complex (1–3).

The biologic importance of Serpins is readily appreciated under those conditions in which the aberrant synthesis or accelerated turnover of a proteinase inhibitor precipitates life-threatening thrombotic or hemorrhagic events, attacks of episodic edema, or emphysema (1–3). Although defects in Serpin synthesis or function are frequently associated with inherited disorders, acquired deficiencies arise when Serpins are attacked in regions near their reactive site centers by proteinases that they are unable to inhibit (for recent examples see references 3–6). Recently, it has been reported that human neutrophils contain a number of serine- and metalloproteinases that are capable of inactivating Serpins under physiologic conditions (3–8). However, because these proteinases are, for the most part, uniquely distributed in neutrophils (small quantities are found in human monocytes), it is frequently assumed that the potential to inactivate Serpins at inflammatory sites is restricted to these human cell types (4–9). Nonetheless, it is interesting to note that perturbations in the Serpin-serine proteinase balance have been speculated to participate in the pathogenesis of disease states not necessarily associated with a neutrophil influx (e.g., references 10–12). Hence, increased interest has focused on determining whether other populations of human cells synthesize alternate proteinases which express an as yet uncharacterized ability to inactivate Serpins. In this regard, while examining the ability of human endothelial cells to secrete metalloproteinases in response to proinflammatory cytokines, we now find that these cells secrete a Serpin-cleaving enzyme (herein termed Serpinase activity) capable of inactivating both α_1-proteinase inhibitor (α_1PI)[1] and α_1-antichymotrypsin (α_1ACHY), the two most abundant proteinase inhibitors found in human plasma. Further analysis resulted in the identification of the Serpinase as human interstitial collagenase (matrix metalloproteinase-1; MMP-1), an enzyme whose synthesis is widely distributed among endothelial, epithelial, and mesenchymal cells, but not neutrophils (13–15). Although the only physiologic substrate heretofore identified for MMP-1 has been the extracellular matrix molecule, collagen, our data indicate that the enzyme expresses an expanded substrate and sequence specificity that allows it to attack and inactivate human Serpins.

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1. *Abbreviations used in this paper:* α_1ACHY, α_1-antichymotrypsin; α_1PI, α_1-proteinase inhibitor; APMA, 4-aminophenylmercuric acetate; CAT G, cathepsin G; DFP, diisopropylfluorophosphate; HNE, human neutrophil elastase; MMP-1, matrix metalloproteinase-1; TIMP-1, tissue inhibitor of metalloproteinase-1.

Methods

Endothelial cell culture. Human umbilical vein endothelial cells were cultured on gelatin-coated dishes (100 mm; Corning Glass, Inc., Corning, NY) as described previously (16). Confluent monolayers (5–7 d-old) were then incubated with 5 U/ml natural human IL-1β (Genzyme, Boston, MA), 100 U/ml recombinant human TNF-α (Amgen Biochemicals, Thousand Oaks, CA), or 0.1 μg/ml PMA (Sigma Chemical Co., St. Louis, MO) for 24 h in serum-free M199 (Gibco Laboratories, Grand Island, NY) supplemented with 0.2% lactalbumin hydrolysate.

Incubation conditions. Conditioned media from the endothelial cell cultures (~ 40 ml) was concentrated 10-fold by ultrafiltration and dialyzed against 50 mM Tris-HCl, 150 mM NaCl, 4 mM $CaCl_2$, 0.05% Brij-58 (pH 7.5). Aliquots (100 μl) of the supernatant were then incubated with either 2.5 μg bovine trypsin (Sigma Chemical Co.) or 0.5 mM 4-aminophenylmercuric acetate (APMA) for 15 min at 25°C to activate latent MMPs (13, 14, 17). To inhibit trypsin completely, samples were then treated with 2 mM PMSF and either 25 μg α_1PI (Calbiochem-Behring Corp., La Jolla, CA) or 10 μg soybean trypsin inhibitor (Sigma Chemical Co.) for 15 min at 25°C. The trypsin or APMA-treated samples were analyzed for their ability to degrade rat tail type I collagen (Collaborative Research, Inc., Bedford, MA), guinea pig skin type I collagen (18), or Serpins in the absence or presence of 1 mM diisopropylfluorophosphate (DFP), 1 mM *N*-ethylmaleimide, 0.1 mM pepstatin A (Sigma Chemical Co.), 10 mM EDTA, purified human fibroblast tissue inhibitor of metalloproteinases-1 (TIMP-1; prepared and provided by Dr. A. Galloway of G. D. Searle, High Wycombe, UK), or human fetal type V collagen (Calbiochem-Behring Corp.). In selected experiments, conditioned media from the endothelial cell cultures were depleted of type IV collagenase by gelatin-affinity chromatography (19), then treated with MAb VI-3 or MAb 2C5 which specifically react with MMP-1 at sites that inhibit collagenolysis or with MAb III-7 which reacts with a nonfunctional epitope (antibodies provided by Dr. H. Birkedal-Hansen, University of Alabama at Birmingham) (20).

Characterization of endothelial cell-derived MMPs. MMPs secreted by the endothelial cells were identified by substrate gel electrophoresis as described (13, 14, 21). Briefly, conditioned media were electrophoresed under nondenaturing conditions in SDS-polyacrylamide gels (8.5%) impregnated with either 1 mg/ml gelatin or 1 mg/ml β-casein (Sigma Chemical Co.). The gels were then washed in 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100 (vol/vol), pH 7.6, for 15 min and then rinsed in buffer without Triton X-100. The MMPs were then activated in a buffer containing 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.5 mM APMA, 1% Triton X-100, 0.02% NaN_3, pH 7.6, for 4 h at 37°C. The gels were stained with Coomassie Brilliant Blue and destained as described (21). Zones of enzymatic activity are demarcated by negative staining.

To determine the type I collagen-, gelatin-, and α_1PI-hydrolytic activity of the MMPs visualized in the SDS-substrate gels, conditioned media were electrophoresed on an 8.5% polyacrylamide gel in the presence of SDS (21). The gel was washed as described above and sliced into 2-mm pieces which were then incubated with 75 μg gelatin for 6 h at 37°C, 25 μg rat tail type I collagen for 20 h at 25°C, or 12.5 μg α_1PI for 20 h at 37°C in 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.5 mM APMA, 1% Triton X-100, 0.02% NaN_3, pH 7.6, in a final volume of 50 μl. The reaction products were analyzed on an 8.5% polyacrylamide gel.

Analysis of Serpin activity. The neutrophil elastase-inhibitory activity of α_1PI and the cathepsin (CAT) G-inhibitory activity of α_1ACHY (Calbiochem-Behring Corp.) were quantitated spectrophotometrically in amidolytic assays using methoxysuccinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide (Calbiochem-Behring Corp.) for human neutrophil elastase (HNE) and succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide (Calbiochem-Behring Corp.) for human CAT G (7, 22). The α_1PI and α_1ACHY were > 95% pure by amino-terminal sequencing while the HNE and CAT G (both obtained from Calbiochem-Behring Corp.) were > 95% pure as assessed by SDS-PAGE.

Purification of MMP-1. MMP-1 was purified to homogeneity from human skin fibroblasts by sequential chromatography over Zn^{+2}-chelate-Sepharose and Cibacron Blue-agarose, followed by molecular sieve chromatography according to the technique of Roswit et al. (23). MMP-1 was also purified from human gingival fibroblasts by a sequence of heparin-Sepharose, Zn^{+2}-chelate-Sepharose, and molecular sieve chromatography (20), and was kindly provided by H. Birkedal-Hansen. Because MMP-1 from endothelial, epithelial, and mesenchymal cells is a single gene product (13–15, 17), the enzyme preparations were used interchangeably.

NH_2-terminal sequence analysis. Native or proteolyzed Serpins were dialyzed against water, lyophilized, and sequenced as described previously (7).

Results

Proteolytic activity of endothelial cell-derived metalloproteinases. In the presence of IL-1β, TNF-α, or PMA, endothelial cells secrete collagenolytic metalloproteinases (24, 25). To activate the latent proenzymes ex situ, conditioned media were briefly incubated with trypsin and the reaction terminated by the addition of PMSF and α_1PI (see Methods). As expected, the activated metalloproteinases completely degraded type I collagen at 37°C (Fig. 1). Unexpectedly, however, we noted that α_1PI was also cleaved into a major fragment (α_1PI$_x$) with an M_r ~ 4,000 less than the native molecule (Fig. 1). Trypsin was not involved in the degradation of α_1PI because identical results were obtained when supernatants were treated with APMA, an organomercurial activator of MMPs (17) (Fig. 1). However, if either the endothelial cell supernatant, trypsin, or APMA were omitted from the reaction mixture, neither collagen nor α_1PI were hydrolyzed.

To determine the relationship between the endothelial cell proteinases involved in collagen degradation and α_1PI cleavage, supernatants were incubated with type I collagen and α_1PI in the presence of inhibitors of serine proteinases (1 mM DFP), cysteine proteinases (1 mM *N*-ethylmaleimide), acid proteinases (0.1 mM pepstatin A), or metalloproteinases (10 mM

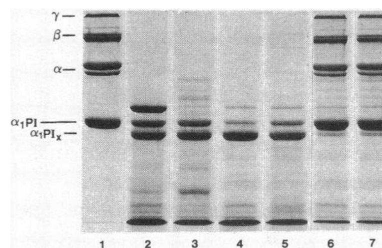

Figure 1. Proteolytic activity of endothelial cell metalloproteinases. Conditioned media were collected from IL-1β, TNF-α, or PMA-treated monolayers and MMPs activated with trypsin. After 15 min, the trypsin was inhibited with 2 mM PMSF and 25 μg α_1PI, and the reaction mixture incubated with 25 μg rat tail type I collagen for 20 h at 37°C in the presence of 100 mM arginine (to prevent collagen fibril formation) (17). Samples were reduced, heat-denatured, and analyzed on an 8.5% SDS-polyacrylamide gel. Native type I collagen was incubated alone (lane *1*) or with the trypsin-activated media from IL-1β-(lane *2*), TNF-α-(lane *3*), or PMA-(lane *4*) stimulated endothelial cells. If APMA, rather than trypsin, was used to activate MMPs in the media recovered from PMA-stimulated monolayers (lane *5*), identical results were obtained. Trypsin-activated media from PMA-stimulated cells did not degrade either collagen or α_1PI in the presence of 10 mM EDTA (lane *6*) or 1 μg TIMP-1 (lane *7*). Collagen trimers, dimers, and monomers are depicted as γ, β, and α respectively. The protein band migrating above α_1PI in lane *2* is bovine serum albumin, which is present in the commercial preparation of IL-1β as a protein stabilizer.

EDTA) (26). Under these conditions, only EDTA prevented either collagen or α_1PI from being hydrolyzed (Fig. 1). Similarly, if trypsin-activated endothelial cell supernatants were incubated with collagen and α_1PI in the presence of TIMP-1, a physiologic MMP inhibitor (15, 27), neither substrate was degraded (Fig. 1). Together, these results indicate that MMPs not only participate in the degradation of triple-helical collagen, but in the cleavage of α_1PI as well.

Proteolytic inactivation of α_1PI and α_1ACHY. To determine the functional status of the hydrolyzed α_1PI, the ability of the Serpin to complex its target proteinase, human neutrophil elastase (1-3) was assessed by SDS-PAGE. In contrast to the ~ 80-kD complex detected when native α_1PI and HNE were combined (Fig. 2), the hydrolyzed Serpin did not form SDS-stable complexes with the proteinase and was further degraded into smaller fragments (Fig. 2).

Although no mammalian metalloproteinase is known to cleave multiple Serpins, the homology that exists between members of this superfamily raised the possibility that other proteinase inhibitors might be susceptible to hydrolysis. Hence, α_1PI was replaced with α_1ACHY, a Serpin whose target enzymes are believed to include chymotrypsin-like enzymes found in neutrophils, mast cells, and the brain (i.e., cathepsin G, chymase, and clipsin, respectively) (1, 28). In the presence of the activated endothelial cell supernatant, native α_1ACHY (~ M_r 68,000) was hydrolyzed into a product migrating with a slightly lower apparent M_r (α_1ACHY$_x$) (Fig. 2). Furthermore, when incubated with CAT G, stable complexes were not formed, and as observed with hydrolyzed α_1PI, the major α_1ACHY fragment was degraded further (Fig. 2).

The inability of the cleaved α_1PI or α_1ACHY to bind target enzymes in an SDS-stable fashion suggested that the Serpins had completely lost their proteinase inhibitory activity. Indeed, neither hydrolyzed α_1PI nor α_1ACHY retained any detectable inhibitory activity against HNE or CAT G, respectively, in amidolytic assays (n = 3). Kinetic analyses of Serpin inactivation revealed that at physiologic concentrations of α_1PI or α_1ACHY (i.e., ~ 25 and 7 μM, respectively), supernatants recovered from ~ 75,000 PMA-stimulated endothelial cells inactivated 85.3±0.9% of the α_1PI and 76.5±4.2% of the α_1ACHY in the course of a 20-h incubation at 37°C (mean±1 SD, n = 3) (Fig. 2). If Serpins were incubated with the endothelial cell supernatant in the presence of TIMP-1, α_1PI-HNE, and α_1ACHY-CAT G complexes were formed and full proteinase inhibitory capacity was retained (Fig. 2).

Characterization of endothelial cell-derived MMPs. The MMP family is comprised of at least seven distinct gene products that are capable of degrading components of the extracellular matrix (15). To identify the endothelial cell MMPs, conditioned media were analyzed by SDS-substrate gel electrophoresis (17, 21). On a β-casein-impregnated gel, two closely spaced bands of enzymatic activity were detected at an M_r of ~ 55,000 and ~ 52,000, which correspond to the glycosylated and unmodified forms, respectively, of MMP-1 (13, 14). In turn, gelatin zymography revealed the presence of a major band of activity at an M_r of ~ 72 K which has been characterized previously as a type IV collagenase (Fig. 3) (19). Consistent with previous reports (29), stimulated endothelial cells did not secrete detectable quantities of the MMP, stromelysin, as assessed by either SDS-substrate gels or enzyme-linked immunosorbent assay (data not shown). To determine whether Serpinase activity was associated with either of the detected metallo-

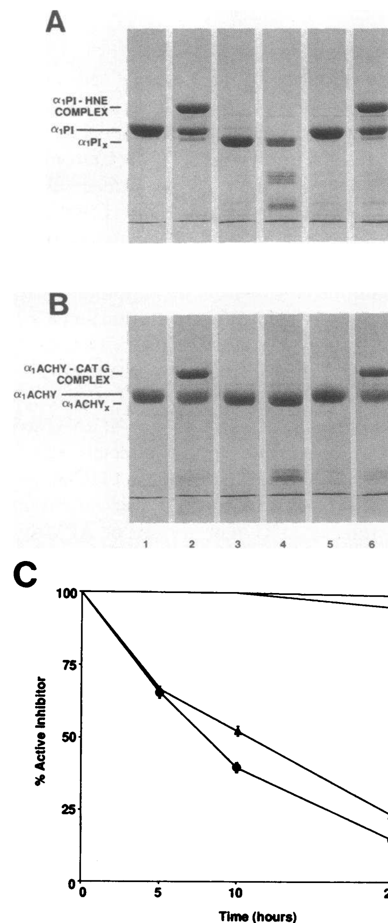

Figure 2. Proteolytic inactivation of α_1PI and α_1ACHY. (*A*) Native α_1PI (lane *1*) or α_1PI cleaved by the endothelial cell metalloproteinase (lane *3*) were incubated alone or with purified HNE (lanes *2* and *4*, respectively) at an HNE/α_1PI molar ratio of 0.4:1 for 5 min at 25°C and analyzed by SDS-PAGE. α_1PI incubated with the endothelial cell metalloproteinase in the presence of TIMP-1 was not cleaved (lane *5*) and formed the expected complex with HNE (lane *6*). (*B*) Native α_1ACHY, α_1ACHY cleaved by the endothelial cell metalloproteinase, or α_1ACHY incubated with the endothelial cell metalloproteinase in the presence of TIMP-1 were incubated alone (lanes *1, 3,* and *5*, respectively) or with purified CAT G (lanes *2, 4,* and *6*, respectively) under conditions identical to those described above. (*C*) The proteinase inhibitory capacity of α_1PI or α_1ACHY (● and ▲, respectively) that had been hydrolyzed by the endothelial cell metalloproteinase or incubated with endothelial cell metalloproteinase in the presence of 1 μg TIMP-1 (○ and △, respectively) was monitored as a function of time. Results are expressed as the mean±SD of three experiments.

proteinases, conditioned media were subjected to SDS-PAGE, the gels sliced, and the fractions incubated with native type I collagen, gelatin, or α_1PI. Under these conditions, Serpinase activity comigrated with MMP-1 activity. That is, fractions 7-10 not only contained the bulk of the Serpin-cleaving activity, but also the majority of MMP-1 activity as defined by the enzyme's known ability to both cleave native type I collagen at a single locus into ¾ and ¼-sized fragments (termed TC$_A$ and TC$_B$) and to preferentially degrade the α_2 chain of gelatin (18, 30) (Fig. 3). In contrast, type IV collagenase, an enzyme characterized by its strong gelatinolytic activity (19), was localized to lanes *5* and *6* where only small amounts of Serpinase activity were detected (Fig. 3). Finally, consistent with the fact that almost all Serpinase activity comigrated with interstitial collagenase, endothelial cell supernatants depleted of type IV collagenase by gelatin-affinity chromatography (19) retained full Serpinase activity with either α_1PI or α_1ACHY as substrates (data not shown).

Although MMP-1 and Serpinase activity comigrated on SDS-PAGE gels, MMP-1 is believed to express a restricted substrate spectrum with physiologic targets limited exclusively to collagen types I, II, III, VII, and X (17, 31). If, however, the substrate spectrum of MMP-1 extends to Serpins, then α_1PI

hydrolysis should be sensitive to both competitive and noncompetitive inhibitors of the metalloproteinase. Indeed, when endothelial cell supernatants depleted of type IV collagenase were incubated with α_1PI in the presence of native type I collagen, α_1PI cleavage was almost completely inhibited (Fig. 4). Inhibition was not detected, however, when α_1PI was incubated with native type V collagen, a collagen type unrecognized by MMP-1 (17) (Fig. 4). Furthermore, the hydrolyses of α_1PI and type I collagen were inhibited in tandem fashion after the addition of MAb VI-3, which reacts specifically with MMP-1 in a region near its catalytic site (20) (Fig. 4). When a monoclonal antibody raised against a nonfunctional epitope on the collagenase molecule (MAb III-7) (20) was used, the hydrolysis of the substrates was unaffected (Fig. 4). Interestingly, MAb VI-3 did not block α_1ACHY hydrolysis (data not shown). However, when MMP-1 was incubated with MAb 2C5 (20 μg/ml), a second antibody that specifically inhibits type I collagen hydrolysis (20), the inactivation of α_1ACHY was inhibited by 68% (n = 2).

Because these results were consistent with the possibility that MMP-1 itself expresses Serpinase activity, the proteinase was purified to homogeneity by two independent techniques (see Methods) and examined for its ability to cleave α_1PI and α_1ACHY. As predicted, MMP-1 purified by either technique cleaved α_1PI in a manner indistinguishable from that described with the endothelial cell supernatants (Fig. 4). Similar results were obtained when the purified metalloproteinase was incubated with α_1ACHY (data not shown). Interestingly, neither α_1PI nor α_1ACHY was hydrolyzed by purified Clostridium histolyticum collagenase (data not shown). Hence, Serpinase activity is not a general characteristic of collagenases per se, but rather a specific characteristic of the mammalian enzyme. At physiologic concentrations of α_1PI or α_1ACHY (25 μM or ~ 750 μg/0.5 ml and 7 μM or ~ 250 μg/0.5 ml, respectively), 1

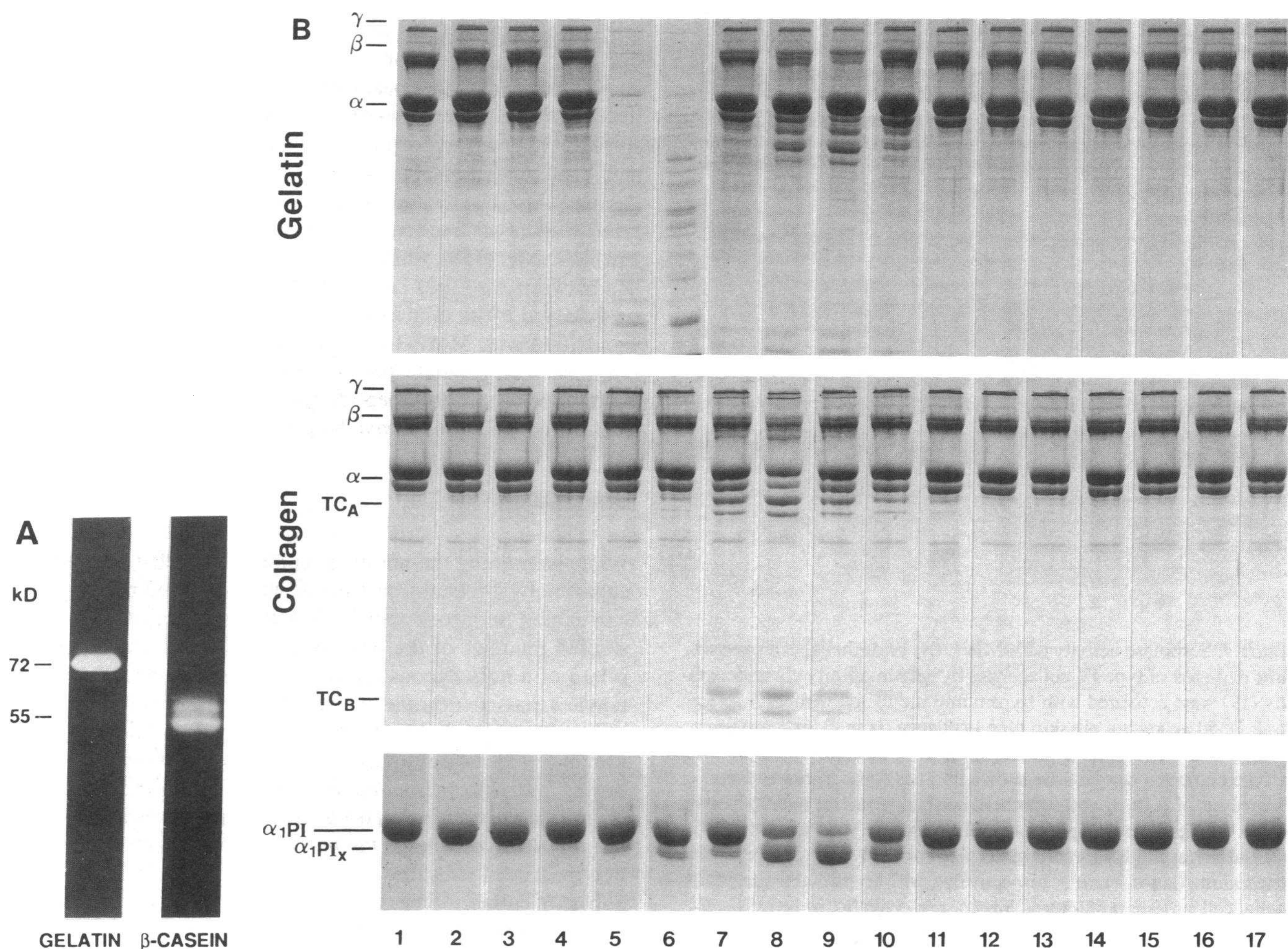

Figure 3. Characterization of endothelial cell metalloproteinases. (*A*) Endothelial cell supernatants were loaded onto an 8.5% SDS-polyacrylamide gel impregnated with either 1 mg/ml gelatin or 1 mg/ml β-casein and electrophoresed as described (21). Zones of enzymatic activity are indicated by negative staining. (*B*) Supernatants were fractionated by SDS-PAGE on an 8.5% gel at 4°C. After electrophoresis, triplicate lanes were sliced into 2-mm sections and incubated with either 75 μg gelatin for 6 h at 37°C, 25 μg type I collagen for 20 h at 25°C, or 12.5 μg α_1PI for 20 h at 37°C in the presence of 0.5 mM APMA. Gelatin, collagen, and α_1PI cleavage products generated from each fraction were analyzed by SDS-PAGE. Serpin cleaving activity (as defined by the presence of α_1PI cleavage product, α_1PI$_x$) was detected in fractions *7–10.* This activity comigrated with MMP-1 activity which was characterized by the formation of tropocollogen (TC$_A$) and (TC$_B$)fragments of collagen and the preferential hydrolysis of the α_2 chain of gelatin. The gelatin degrading activity localized to fractions *5* and *6* corresponded to the 72-kD type IV collagenase, an enzyme characterized by its strong gelatinolytic activity.

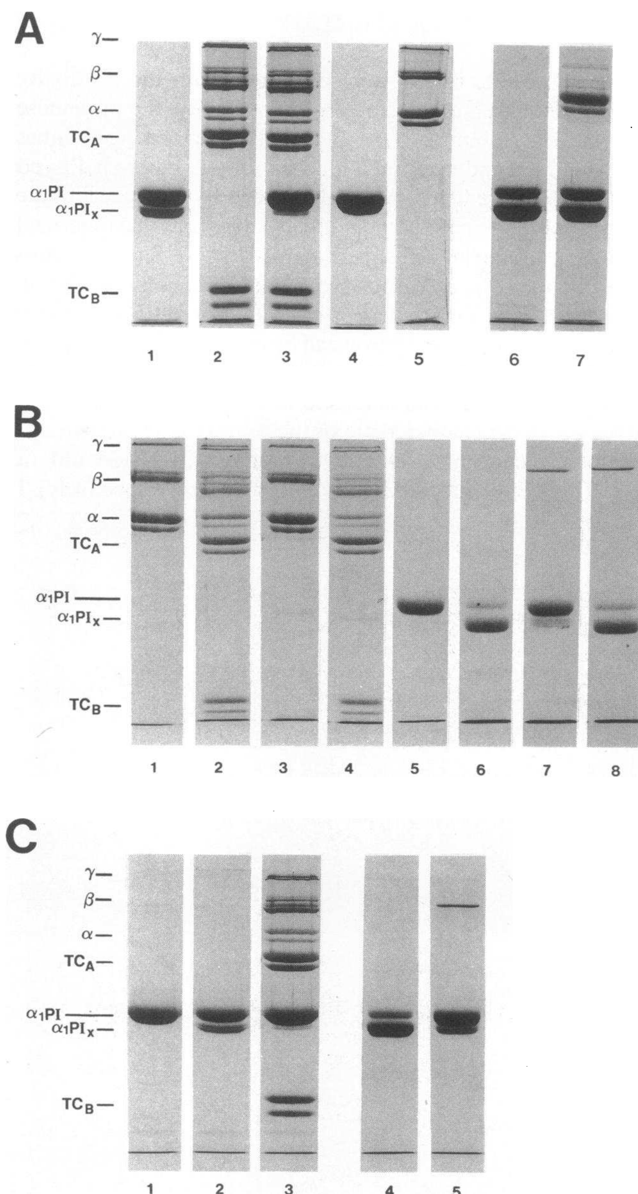

Figure 4. Serpinase activity of MMP-1. (*A*) Endothelial cell supernatants depleted of type IV collagenase by gelatin-affinity chromatography (19) were activated with trypsin and incubated with 12.5 μg α_1PI (lane *1*), 50 μg guinea pig skin type I collagen (lane *2*), or a mixture of α_1PI and type I collagen (lane *3*) for 10 h at 27.5°C. Native α_1PI or type I collagen controls are shown in lanes *4* and *5*, respectively. Hydrolysis of α_1PI by the endothelial cell supernatant at 32.5°C (lane *6*) was unaffected by the presence of 12.5 μg type V collagen (lane *7*). (*B*) Native rat tail type I collagen (75 μg) or α_1PI (12.5 μg) were incubated alone (lanes *1* and *5*, respectively), with trypsin-activated endothelial cell supernatant (lanes *2* and *6*, respectively), or trypsin-activated supernatant that had been pre-treated with 20 μg/ml Mab VI-3 (lanes *3* and *7*, respectively) or Mab III-7 (lanes *4* and *8*, respectively) for 30 min. Incubations with type I collagen or α_1PI were performed at 25°C and 37°C, respectively, for 20 h. (*C*) Native α_1PI (12.5 μg) was incubated alone (lane *1*), with 150 ng of trypsin-activated purified human MMP-1 (lane *2*), or with MMP-1 in the presence of 50 μg guinea pig skin type I collagen (lane *3*) for 10 h at 27.5°C in a final volume of 75 μl. At 37°C, the hydrolysis of α_1PI by purified MMP-1 (lane *4*) was almost completely inhibited by pretreating the enzyme with 20 μg/ml Mab VI-3 (lane *5*). Mab III-7 did not affect α_1PI hydrolysis.

μg of purified MMP-1 hydrolyzed 389.5 μg α_1PI and 55.6 μg α_1ACHY over a 10-h period at 37°C in a final volume of 0.5 ml (n = 2). If the concentrations of α_1PI or α_1ACHY were increased to levels found under acute phase conditions (~ 100 and 30 μM, respectively), 1 μg MMP-1 hydrolyzed 1488.0 μg α_1PI and 235.2 μg α_1ACHY (n = 2). At the physiologic and acute phase concentrations, the calculated turnover numbers of MMP-1 on α_1PI (43 and 165 molecules degraded per molecule collagenase/h, respectively) and α_1ACHY (5 and 20 molecules degraded per molecule collagenase/h, respectively) were either comparable to, or exceeded, that reported for MMP-1 on fibrillar type I collagen (i.e., 25 molecules degraded per molecule collagen/h) (18).

Identification of MMP-1 cleavage sites in α_1PI and α_1ACHY. The inactivation of α_1PI or α_1ACHY by MMP-1 is consistent with hydrolysis occurring at site(s) located within the reactive site loop (1–3). However, MMP-1 only cleaves collagen substrates at Gly-Leu or Gly-Ile bonds, and similar sequences are not found in the active site loop of either Serpin (1–3). Recently, however, MMP-1 has been shown to catalyze intramolecular autolyses at additional sites containing bulky, hydrophobic residues on the amino-terminal side of the cleaved bond: sites that bear little resemblance to those hydrolyzed during collagenolysis (32). Accordingly, sequence analysis of the hydrolyzed Serpins revealed cleavage sites within the active site loop domain that were similar to those generated during MMP-1 autocatalysis (Fig. 5). That is, α_1PI was hydrolyzed with equal frequency between Phe (352)-Leu (353) and Pro (357)-Met (358), while α_1ACHY was hydrolyzed at a single site between Ala (362)-Leu (363). Interestingly, neither Serpin was cleaved if the inhibitors were heat denatured before their incubation with MMP-1 or if the Serpins were allowed to first complex their cognate proteinases (data not shown). Thus, MMP-1 relies on both peptide sequence and conformational cues to recognize and cleave Serpin targets.

Discussion

While examining the ability of endothelial cells to synthesize collagenolytic metalloproteinases, we discovered that MMP-1 can exhibit Serpinase activity. MMP-1 is the most thoroughly studied member of the human MMP family, which is comprised of a homologous set of at least seven zinc metalloproteinases that can degrade one or more components of the extracellular matrix (15, 17). MMP-1 participates in diverse events ranging from the physiologic remodeling of tissues that occurs during morphogenesis and wound healing to the pathologic destruction of tissues that occurs in inflammatory disease states (15, 17). In vivo, MMP-1 has been believed to express a remarkably restricted substrate specificity that has been limited to a subset of collagen types (13–15, 17). Furthermore, in addition to its substrate specificity, MMP-1 also displays a tightly regulated sequence specificity wherein susceptible collagen types are only cleaved at either Gly-Leu or Gly-Ile bonds localized to one or two distinct regions of the collagen triple helix (17, 18, 31). More recent studies, however, have demonstrated that the repertoire of sequences cleaved by MMP-1 can be expanded to include peptide bonds in which the amino terminal side contains any one of a number of large, hydrophobic residues (32, 34, 35). Nonetheless, because the only substrates cleaved by MMP-1 at these sites include (*a*) the collagenase molecule itself

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                1    2        351   ↓                     ↓   *
α1PI       NH2-GLU-ASP---------MET-PHE-LEU-GLU-ALA-ILE-PRO-MET-SER-ILE-ARG-PRO

               1    2         353                            *        ↓
α1ACHY     NH2-HIS -PRO----------THR-ALA-VAL-LYS-ILE-THR-LEU-LEU-SER-ALA-LEU-VAL

                1    2         83↓            98     ↓   ↓   ↓        269↓
Collagenase NH2-MET-HIS----------THR-LEU-LYS------ALA-GLN-PHE-VAL-LEU--------PRO-ILE
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Figure 5. MMP-1 cleavage sites in α_1PI and α_1ACHY. 50 μg α_1PI or α_1ACHY were incubated alone or with either trypsin or APMA-activated MMP-1 for 20 h at 37°C, dialyzed against water, lyophilized, and sequenced. α_1PI was cleaved at two sites with equal frequency while α_1ACHY was cleaved at a single site (cleavage sites identified with arrows). Asterisks indicate the active site of each Serpin. Purified α_1ACHY contained two isoforms, one lacking a His-Pro dipeptide (33). Sequence analyses were performed with cleaved α_1PI and α_1ACHY in at least three experiments with a repetitive efficiency of > 85%. Autocatalytic cleavage sites identified previously in human fibroblast collagenase (20, 32) are shown for comparison.

as it undergoes autolytic intramolecular processing, (*b*) rat α-macroglobulins, and (*c*) synthetic peptides, the physiologic significance or relevance of this expanded sequence specificity has remained unclear (32, 34, 35). The only "exception" to this rule is the interaction of MMP-1 with its major physiologic inhibitor, α_2-macroglobulin (27, 34). This proteinase inhibitor is unrelated to Serpins and displays the unique ability to inhibit serine-, cysteine-, aspartate-, and metalloproteinases by allowing the target enzymes to cleave peptide bonds located in a 30-amino acid residue bait region (1). After proteolysis within this region of α_2-macroglobulin, the inhibitor rapidly undergoes a conformational change that irreversibly entraps the responsible proteinase (1). Thus, unlike the Serpins, α_2-macroglobulin is an MMP-1 inhibitor and is not considered a substrate since attacking proteinases can only cleave a single molecule of α_2-macroglobulin before being irreversibly inhibited. Our findings demonstrate that the ability of MMP-1 to cleave biologically relevant substrates is no longer restricted to collagen and that the expanded sequence specificity of the enzyme can now be applied to at least two members of the Serpin family. Finally, we note that activated MMP-1 can slowly break down into a small fragment ($M_r \sim 22$K), which expresses caseinase and gelatinase activity, while losing collagenolytic activity as well as sensitivity to inhibition by TIMP-1 (36). However, when active MMP-1 was incubated alone for 18 h at 37°C and then α_1PI added to the reaction mixture, Serpin hydrolysis remained inhibitable by both type I collagen and TIMP-1 (data not shown). Hence, Serpinase activity is a property intrinsic to the active, parent molecule.

Perturbations in the Serpin-serine proteinase balance have been linked to a wide variety of pathologic conditions affecting coagulation, fibrinolysis, and inflammation (1–8, 10–12). Hydrolyzed, inactive Serpins as well as active MMP-1 have been detected in vivo and their presence is believed to reflect the generation of a proinflammatory environment wherein unregulated serine proteinases could express their digestive functions in a pathologic manner (1, 4, 12, 37). Together, α_1PI and α_1ACHY are quantitatively the two major proteinase inhibitors found in human plasma and their inactivation by MMP-1 should allow at least two of their cognate proteinases, i.e., neutrophil elastase and cathepsin G, to attack host tissues. However, given the fact that α_1PI also functions as a primary inhibitor of protein C (38), while α_1ACHY can inhibit chymotrypsin-like enzymes found in brain and mast cells (1, 28), the consequences of Serpin inactivation are clearly not restricted to effects on neutrophil enzymes or function. Indeed, in preliminary studies, we have found that MMP-1 can cleave a number of additional Serpins including α_2-antiplasmin and plasminogen activator inhibitor-2 (unpublished observation).

Cleavage within the active site loop of a Serpin is invariably associated with an irreversible loss in its proteinase inhibitory activity (1–3). Hence, the biological consequences of Serpin inactivation are usually predicted from the perspective of the substrate spectrum of the affected cognate proteinases. However, recent studies indicate that hydrolyzed Serpins can express additional activities distinct from those associated with the intact molecule. For example, α_1PI cleaved within its active site loop can act as a powerful neutrophil chemoattractant at nanomolar concentrations (39). In addition, Perlmutter and colleagues have identified a Serpin-enzyme complex receptor on human monocytes and hepatoma cells that recognizes hydrolyzed Serpins and mediates increases in α_1PI gene expression (40). Together, these data suggest that even when the MMP-1-dependent hydrolysis of a given Serpin fails to proceed to a degree that would significantly increase the half-life of the cognate proteinase, the hydrolyzed molecule could exert a number of important effects on the progression of the inflammatory response.

In attempting to gauge the potential importance of MMP-1-dependent hydrolysis of Serpins in vivo, it should be noted that the proteinase is not only synthesized by endothelial cells, but also by fibroblasts, synovial cells, smooth muscle cells, keratinocytes, monocytes, and macrophages (13, 14, 17, 23). Thus, our data suggest that multiple cell types could potentially create proinflammatory sites throughout the body by initiating Serpin hydrolysis. However, several lines of evidence suggest that other human MMPs which display a more selective pattern of distribution could similarly participate in these events. First, stromelysin (MMP-3) is an omnivorous proteinase that can degrade a wide variety of connective tissue substrates (e.g., fibronectin, laminin, cartilage proteoglycans, etc.) (29, 41). Although stromelysin does not share MMP-1's ability to cleave interstitial collagens within their triple helical domains, the rabbit enzyme has been reported to cleave human α_1PI at an uncharacterized site (41). The substrate specificity of the rabbit enzyme is not identical to that of the human enzyme (42), but we have found that purified human stromelysin can also inactivate α_1PI and α_1ACHY (unpublished observation). Although a distinct macrophage metalloelastase that can cleave α_1PI has also been isolated from animal macrophages, a human homologue of this enzyme has not been identified (43). Second, we have demonstrated previously that human neutrophils can be triggered to release and oxidatively activate a latent metalloproteinase capable of hydrolyzing α_1PI at one of the two cleavage sites identified as products of the MMP-1 reaction (7). Although the neutrophil enzyme(s) responsible for α_1PI cleavage could not be identified in that report, we (manuscript in preparation) as well as others (44, 45) have recently found that both human neutrophil collagenase (MMP-8) and neutrophil gelatinase are the principal mediators of the hydrolytic process. Human neutrophil collagenase is a distinct gene product that is only found in this single cell type and which displays only $\sim$ 50% homology to MMP-1 (46). Similarly, neutrophil gelatinase displays size heterogeneity as well as other unique post-translational modifications that distinguish it from all other

MMPs (15, 21). Thus, these data suggest that multiple MMPs could participate in Serpin hydrolysis in vivo depending on the composition of both the resident and infiltrating cell populations. Nonetheless, because many of the cell types capable of synthesizing MMP-1 can secrete large quantities of this proteinase (e.g., endothelial cells or fibroblasts release $\sim 6\ \mu g/10^6$ cells/24 h [13]), we predict that MMP-1 plays a predominant role in these events.

In conclusion, we have demonstrated that MMP-1, an enzyme whose range of physiologic targets was heretofore believed to be restricted to collagenous macromolecules, can also cleave Serpins. Taken together, our data provide strong evidence that an unsuspected interface may exist between MMP-1, collagen turnover, and Serpin function. In both physiologic as well as pathologic states, the regulation of MMP-1 as well as other MMPs could exert effects on the progression of the inflammatory response that are more complex than appreciated previously.

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