Neutrophil Elastase Processing of Gelatinase A Is Mediated by Extracellular Matrix†

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ABSTRACT: Gelatinase A (72-kDa type IV collagenase) is a metalloproteinase that is expressed by many cells in culture and is overexpressed by some tumor cells. It has been suggested that the serine proteinase neutrophil elastase might play a role in the posttranslational processing of gelatinase A and that noncatalytic interactions between gelatinase A and components of the extracellular matrix might alter potential processing pathways. These questions were addressed with the use of gelatin substrate zymography, gelatinolytic activity assays, and amino acid sequence analysis. We found that neutrophil elastase does proteolytically modify gelatinase A by cleaving at a number of sites within gelatinase A. Sequential treatment of gelatinase A with 4-aminophenylmercuric acetate (APMA) and neutrophil elastase yielded an active gelatinase with a 4-fold increase in gelatinolytic activity. The increased gelatinolytic activity correlated with that of a 40-kDa fragment of gelatinase A. Matrix components altered the proteolytic modifications in gelatinase A that were mediated by neutrophil elastase. In the absence of gelatin, neutrophil elastase destructively degraded gelatinase A by hydrolyzing at least two bonds within the fibronectin-like gelatin-binding domain of gelatinase A. In the presence of gelatin, these two inactivating cleavage sites were protected, and cleavage at a site within the hemopexin-like carboxyl-terminal domain resulted in a truncated yet active gelatinase. The results suggest a regulatory role for extracellular matrix molecules in stabilizing gelatinase A fragments and in altering the availability of sites susceptible to destructive proteolysis by neutrophil elastase.

Inflammatory cells migrate into tissue sites and upon stimulation release proteolytic enzymes that can mediate structural and functional changes in the extracellular matrix (ECM).[1] These changes may be the direct result of degradation of ECM components by inflammatory cell proteinases or the indirect result of modifications of other proteinases and proteinase inhibitors that are secreted by resident cells embedded within the matrix, such as fibroblasts or endothelial cells. Both serine and metalloproteinases are well established as important mediators of ECM degradation (Alexander & Werb, 1989). Interactions between these proteinase classes can produce proteolytic cascades in which inflammatory serine proteinases activate latent matrix metalloproteinases by proteolytic removal of the amino-terminal propeptide (Mignatti et al., 1986). With the possible exception of interstitial collagenase, most proteinases in the ECM have more than one potential substrate. The neutrophil serine proteinases cathepsin G and elastase not only can degrade collagen and proteoglycans but also can activate the metalloproteinase prostromelysin (Nagase et al., 1990). Plasmin degrades cartilage proteoglycans and activates both procollagenase and prostromelysin. In addition to amino-terminal processing, proteolytic modifications at other sites can alter the activity of the metalloproteinases. Stromelysin can further activate interstitial collagenase by proteolytic cleavage near the carboxyl terminus (He et al., 1989). The concerted interaction between proteinases can overcome the high level of proteinase inhibitors and affect net degradation of the matrix.

The activation of gelatinase A (72-kDa type IV collagenase) is less well characterized than that of either collagenase or stromelysin. Gelatinase A is secreted by a wide variety of cell types, including fibroblasts (Seltzer et al., 1989), epithelial cells (Collier et al., 1988), endothelial cells (Herron et al., 1986a), and mesangial cells (Lovett et al., 1992), and is overexpressed by several human tumor cell lines (Salo et al., 1983; Levy et al., 1991). It has been detected as a bimolecular complex with the tissue inhibitor of metalloproteinases (TIMP)-2, bound to the hemopexin-like carboxyl-terminal domain at some distance from the active site. Gelatinase A can be activated by incubation with plasma membrane preparations of tumor cells and concanavalin A-treated fibroblasts (Brown et al., 1990; Overall & Sodek, 1990; Murphy et al., 1992; Strongin et al., 1993). This activation may be mediated by a novel metalloproteinase with a transmembrane domain (Sato et al., 1994). Organomercurial treatment of progelatinase A·TIMP-2 complexes yields an active gelatinase on which TIMP-2 stays bound to the hemopexin-like domain (Howard & Banda, 1991); however, upon continued incubation, this activated complex loses activity (Kleiner et al., 1993). Deletion mutations of gelatinase A that lack the hemopexin-like carboxyl-terminal domain and, therefore, are free of TIMP-2 are more active and more resistant to inhibition in vitro (Fridman et al., 1992). It is not known if gelatinases with carboxyl-terminal truncations are found in vivo. Proteinases that can remove

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[1] Abbreviations: APMA, 4-aminophenylmercuric acetate; ECM, extracellular matrix; HPLC, high-pressure liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; TIMP, tissue inhibitor of metalloproteinases.

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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 in vitro if it has been separated from TIMP-2 by reverse-phase high-pressure liquid chromatography (HPLC) in acidic conditions followed by neutralization (Howard et al., 1991a). The autoactivated products 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 that binds TIMP-2 (Howard & Banda, 1991). Because of the extreme conditions used to generate the free gelatinase, it is unlikely that this autoactivation occurs in vivo. However, these observations raise 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 ECM components. A coordinate spatial and temporal activation of proteinases must occur so that proteinase concentrations exceed the high level of inhibitors that are found in plasma and distributed throughout the ECM. Modifications that can increase the proteolytic potential of enzymes within the ECM may be important in controlling the extent of its degradation and rebuilding. In this study, we tested the hypothesis that an inflammatory serine proteinase, neutrophil elastase, can proteolytically modify gelatinase A, altering the potential for matrix turnover. Because matrix components influence protein−protein interactions within the ECM, we also tested the hypothesis that noncatalytic interactions between gelatinase A and matrix components may alter the activation pathways.

MATERIALS AND METHODS

Protein Purification. 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% dimethyl sulfoxide in 20 mM HEPES, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$. The gelatinases were separated from one another by lentil lectin chromatography, which binds the glycosylated gelatinase B but does not bind 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% trifluoroactic 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 colorimetrically by using the bicinchoninic acid assay (Pierce) with bovine serum albumin as the standard.

Proteinase Activation. Progelatinase A·TIMP-2 complexes were completely activated by incubation with 1 mM 4-aminophenylmercuric acetate (APMA) for 2−16 h at 37 °C. Stock solutions of APMA (100 mM) were made immediately before use by dissolving dry reagent in 0.1 M NaOH with vigorous shaking. APMA was added to samples containing progelatinase A·TIMP-2 at a dilution of 1:100.

Treatment of Gelatinase A·TIMP-2 Complexes with Neutrophil Elastase. Progelatinase A·TIMP-2 complexes or APMA-activated complexes were diluted into 50 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN_3, with or without 1 mg/mL gelatin. Neutrophil elastase (Elastin Products Co., Owensville, MO) (1 mg/mL in Tris buffer containing 0.02% Brij-35) was added at molar ratios ranging from 1:1 to 1:50 (neutrophil elastase:gelatinase A·TIMP-2) and allowed to react at 37 °C from 5 min to 48 h. Digestions were stopped by inhibition of elastase with either 10 mM phenylmethanesulfonyl fluoride (PMSF) or a 5-fold molar excess of α_1-proteinase inhibitor. Digestion mixtures were analyzed by gelatin zymography and by gelatin degradation assays.

Electrophoresis. Sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis was performed according to the methods described previously (Laemmli, 1970). 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 & Dowdle, 1980; Herron et al., 1986b). After electrophoresis, the gels were incubated for 30 min in 2.5% Triton X-100, 0.02% NaN_3 at ambient temperature and then incubated for 2−16 h at 37 °C in substrate buffer (50 mM Tris, pH 8.0, 10 mM $CaCl_2$, 0.02% NaN_3). Gels were stained with Coomassie Blue R250 and destained in 50% methanol in water. Proteinase activity in the sample is detected as a clear zone in a darkly stained background. 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 a poly(vinylidene difluoride) (PVDF) membrane in 10 mM CAPS buffer [3-(cyclohexylamino)-1-propanesulfonic acid], pH 11.

Proteinase Assays. Gelatin degradation assays were performed as described previously (Howard et al., 1991a). Collagen (rat tail type I, Collaborative Research) was reductively acetylated with [^{3}H]acetic anhydride to a specific activity of 5.7×10^6 cpm/mg. This radiolabeled collagen was diluted with nonlabeled collagen and dialyzed against 25 mM Tris, pH 7.6, 1 mM $CaCl_2$. The final specific activity of the ^{3}H-labeled collagen was 500 000 cpm/mg. The ^{3}H-labeled collagen was boiled for 5 min for use in the assays. Gelatinase samples were diluted in Tris−$CaCl_2$ buffer with 2 mg/mL ovalbumin and added to an equal volume of the labeled gelatin. After 5−60 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 (Amersham). Carrier-free $Na^{125}I$ (1 mCi) was added to 10 μL of 0.25 M sodium phosphate buffer, pH 7.5, and the following were added in rapid succession with continuous mixing: 1 μg of progelatinase A·TIMP-2 complex, 50 μg of chloramine T, and 120 μg of sodium metabisulfate in 50 mM phosphate buffer. The total volume was brought to 1 mL with 2 mg/mL NaI in 50 mM phosphate buffer, and radiolabeled complexes were separated from unincorporated ^{125}I over a PD10 column (Pharmacia) preequilibrated with phosphate buffer.

TIMP-1 and TIMP-2 (10 μg) in 100 mL of 250 mM sodium phosphate buffer, pH 7, were oxidatively radioiodinated for 10 min with 1 mCi of $Na^{125}I$ (Amersham) in tubes coated with 100 μg of Iodogen (Pierce Chemical Co.). A saturated solution of tyrosine was added to bind free ^{125}I, and the iodination mixture was diluted into buffer containing 1 mg/mL ovalbumin and 1 mg/mL NaI, and the radiolabeled proteins were separated by size-exclusion chromatography on PD-10 columns (Pharmacia).

Protein Sequencing. Amino acid sequences were determined by the Biomolecular Resource Center, University of California, San Francisco. Samples containing 50−250 pmol of protein immobilized on PVDF were subjected to Edman degradation in an Applied Biosystems 470A gas-phase sequencer. The phenylthiohydantoin (PTH) derivatives were identified and quantified by reverse-phase HPLC on an on-line Applied Biosystems 120A PTH analyzer.

Cells. Neutrophils were separated on Ficoll-Hypaque gradients from peripheral blood obtained from normal human subjects. Blood was drawn into heparinized tubes, mixed 1:1 with phosphate-buffered saline, layered over Ficoll-Hypaque (Sigma), and centrifuged at 2000 rpm for 30 min at ambient temperature. After removal of the plasma and the mononuclear leukocytes at the interface between plasma and Ficoll, the remainder of the Ficoll was removed by vacuum aspiration to just above the cell pellet. The pellets containing polymorphonuclear leukocytes and erythrocytes were suspended with 2.5% dextran (Sigma) in phosphate-buffered saline and incubated at 37 °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 collected by centrifugation, washed 3 times with phosphate-buffered saline, and diluted to a cell concentration of 5×10^6 cells/mL in 0.05 M HEPES, pH 7.4, containing 0.15 M NaCl and 1 mg/mL gelatin.

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 ambient temperature by following the release of *p*-nitroanilide 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. Specific activity measurements showed the elastase to be >95% active.

RESULTS

APMA Activates Progelatinase A·TIMP-2 Complexes. Progelatinase A·TIMP-2 complexes were activated by treatment with APMA (Figure 1). The activation corresponded to the proteolytic removal of the 10-kDa amino-terminal propeptide and the resulting shift in molecular mass from 72 to 62 kDa, as seen by protein blotting and gelatin substrate zymography. The active 62-kDa gelatinase remained associated with TIMP-2. The inactive progelatinase A appears active on the zymogen because of the effect of the detergent SDS (Springman et al., 1990).

Neutrophil Elastase Processes Gelatinase A·TIMP-2 Complexes into Lower Molecular Mass Forms That Retain Gelatinolytic Activity. Neutrophil elastase was used to digest both 72-kDa progelatinase A·TIMP-2 complexes and the 62-kDa gelatinase A·TIMP-2 complexes that resulted from APMA activation (Figure 2). When progelatinase A·TIMP-2 complexes were converted to active 62-kDa gelatinase A·TIMP-2 by APMA, one major gelatinolytic fragment migrating at 40 kDa was produced by digestion with neutrophil elastase. When 72-kDa progelatinase A·TIMP-2 complexes, which also contained some 62-kDa gelatinase A, were digested by neutrophil elastase, two products were formed, as detected by gelatin zymography: The larger fragment migrated at 50 kDa; the smaller fragment was the 40-kDa cleavage product of the 62-kDa gelatinase A·TIMP-2 complex. Treatment of the 50-kDa fragment with APMA produced the 40-kDa gelatinase A, suggesting that the 50-kDa form was a fragment of the 72-kDa progelatinase A that retained the 10 kDa propeptide (Figure 3). Both the 50-kDa and 40-kDa fragments would be free of TIMP-2. Therefore, the 40-kDa gelatinase A can result either from neutrophil elastase digestion of the activated 62-kDa gelatinase A·TIMP-2 complex or, conversely, from APMA treatment of the 50-kDa progelatinase A that is formed from neutrophil elastase digestion of 72-kDa progelatinase A·TIMP-2 complexes (Figure 4).

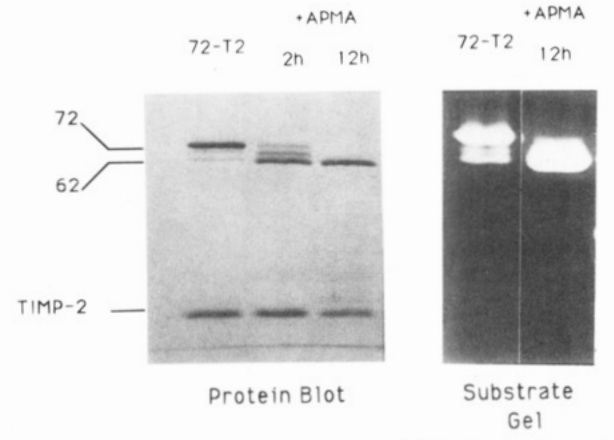

FIGURE 1: Activation of progelatinase A·TIMP-2 complexes by APMA. Progelatinase A·TIMP-2 complexes (0.2 mg/mL) were incubated with 1 mM APMA for 2 or 12 h and analyzed either on Coomassie blue-stained protein blots (left panel) or by gelatin substrate zymography (right panel). 72-T2 is the progelatinase A·TIMP-2 complex after SDS−polyacrylamide gel electrophoresis. Upon APMA treatment, the 72-kDa progelatinase converted to the 62-kDa form.

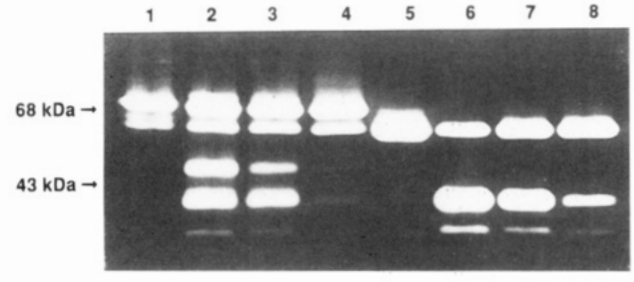

FIGURE 2: Gelatinase A·TIMP-2 complexes are proteolytically processed into lower molecular mass forms by neutrophil elastase. Progelatinase A·TIMP-2 complexes (2 pmol, lanes 1−4) and APMA-activated gelatinase A·TIMP-2 complexes (2 pmol, lanes 5−8) were digested with neutrophil elastase in the presence of 1 mg/mL gelatin for 2 h at 37 °C and analyzed by gelatin zymography. Complexes were digested with 2 pmol of elastase (lanes 2 and 6), 0.2 pmol of elastase (lanes 3 and 7), or 0.1 pmol of elastase (lanes 4 and 8) or with PMSF-inhibited elastase as a control (lanes 1 and 5). Bovine serum albumin (68 kDa) and ovalbumin (43 kDa) were used as molecular mass markers.

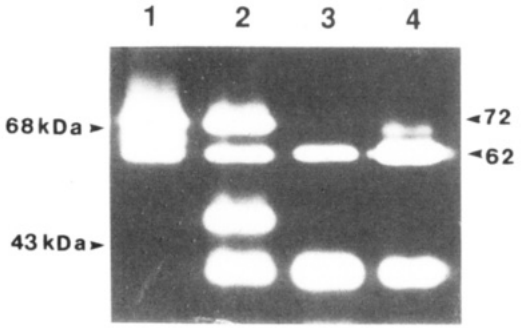

FIGURE 3: 40-kDa gelatinase can be generated by two pathways. Lane 1 contains 2 pmol of progelatinase A·TIMP-2 complex. In lane 2, progelatinase A·TIMP-2 complexes were digested with neutrophil elastase at a 1:1 molar ratio. In lane 3, progelatinase A·TIMP-2 complexes were first treated with 1 mM APMA for 2 h at 37 °C, followed by neutrophil elastase digestion for an additional 2 h at 37 °C. In lane 4, progelatinase A·TIMP-2 complexes were first digested with neutrophil elastase followed by APMA treatment. All reactions contained 1 mg/mL gelatin. Progelatinase and APMA-activated gelatinase are indicated by 72 and 62, respectively. Molecular mass markers are the same as in Figure 2.

Modification of Progelatinase A·TIMP-2 Complexes by Sequential Treatment with APMA and Neutrophil Elastase Increases Gelatinolytic Activity. To determine if the truncated gelatinases produced by neutrophil elastase digestion of complexes led to increased activation, we assayed digestion mixtures for soluble gelatinolytic activity. Digestion of the activated 62-kDa gelatinase A·TIMP-2 complex with neutrophil elastase yielded an active preparation with a 4-fold increase in gelatinolytic activity over that of the APMA-activated complex itself (Figure 5). To determine if this increased gelatinolytic activity corresponded to the 40-kDa fragment, we treated 62-kDa gelatinase A·TIMP-2 complexes with neutrophil elastase, and at specified times evaluated identical samples by soluble gelatin degradation assays and gelatin zymography. The increased gelatinolytic activity that resulted from neutrophil elastase digestion of the 62-kDa gelatinase A·TIMP-2 complex was always associated with the appearance of the fragment that migrated at 40 kDa on gelatin zymograms (Figure 6) and was proportional to the amount of the 40-kDa fragment.

Proteolysis of TIMP-2 by Neutrophil Elastase Is Not Necessary for Modification of Gelatinase A. Because neutrophil elastase can degrade TIMP-2 (unpublished observation), it was possible that the elastase might degrade the TIMP-2 in the complex before degrading the gelatinase A. To distinguish immediate cleavage of gelatinase A by neutrophil elastase from cleavage following TIMP-2 proteolysis, we incubated ^{125}I-radiolabeled progelatinase A·TIMP-2 complexes with neutrophil elastase. The degradation of TIMP-2 was not a necessary prerequisite for the proteolytic modification of gelatinase A·TIMP-2 complexes by neutrophil elastase (data not shown). We then considered whether activated gelatinase A might be responsible for some of the proteolysis. To distinguish direct cleavage of gelatinase A by neutrophil elastase from neutrophil elastase-mediated autocatalytic cleavage of gelatinase A, we performed experiments with class-specific proteinase inhibitors. Metalloproteinase inhibitors (EDTA, or an excess of TIMP-2) did not inhibit the formation of the 40-kDa fragment resulting from neutrophil elastase modification of 62-kDa gelatinase A·TIMP-2 (Figure 7). However, an inhibitor of serineproteinases (PMSF) completely inhibited the formation of the 40-kDa fragment. Therefore, it is likely that the serine proteinase neutrophil elastase did not mediate the autocatalytic cleavage of gelatinase A but directly enhanced the activity of the 62-kDa gelatinase A·TIMP-2 complexes by generating the 40-kDa fragment.

Location of the Neutrophil Elastase Cleavage Sites within 72-kDa Progelatinase A. Neutrophil elastase can cleave at

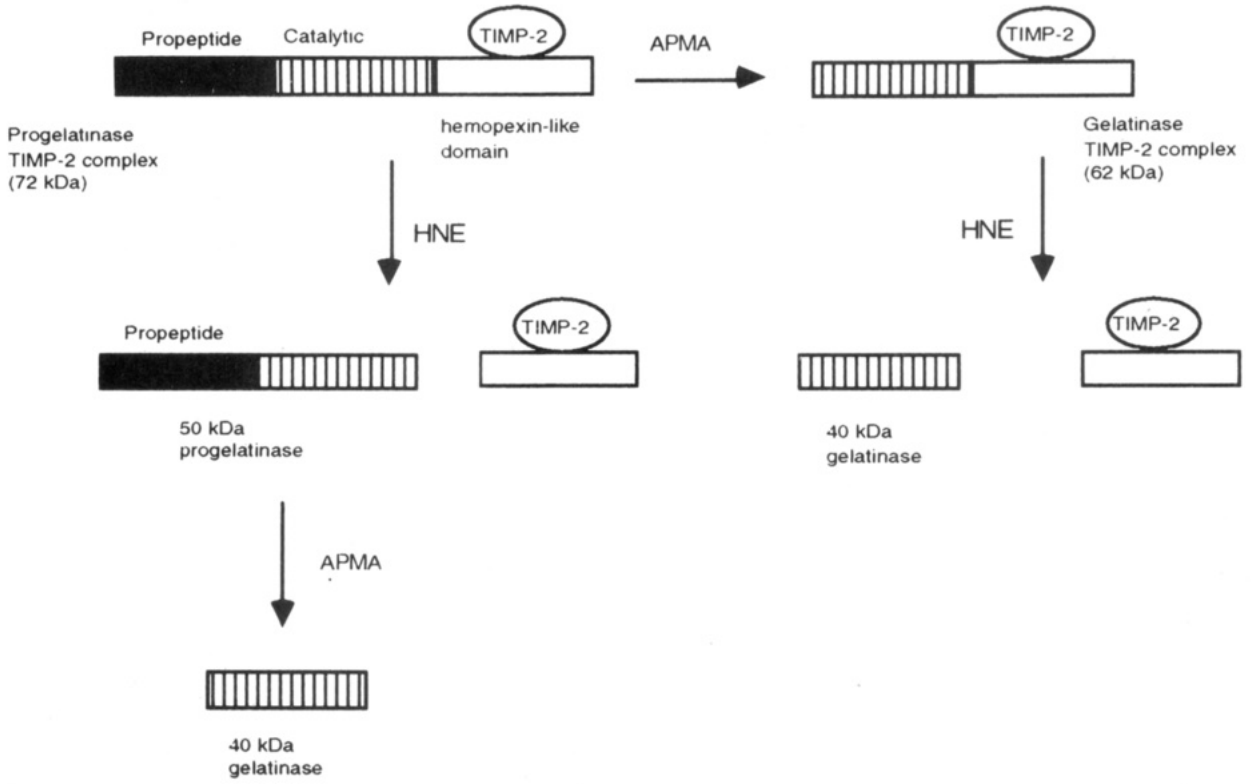

FIGURE 4: Activation of gelatinase A by APMA and modifications made by elastase (HNE).

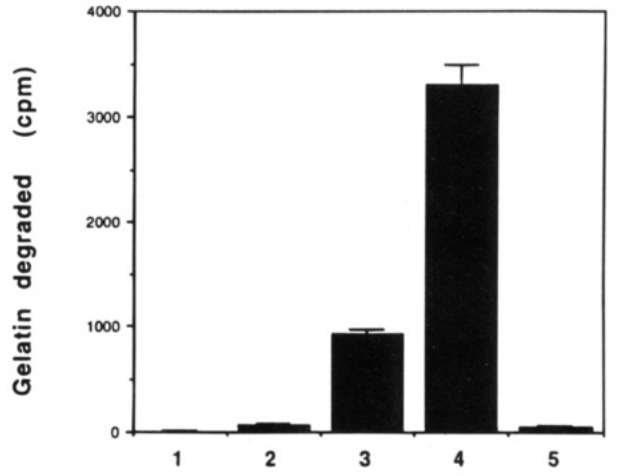

FIGURE 5: Modification of gelatinase A by elastase results in increased gelatinolytic activity. Gelatin degradation assays were performed with the following samples: 1, 72-kDa progelatinase A·TIMP-2 complexes; 2, 72-kDa progelatinase A·TIMP-2 complexes treated with elastase; 3, APMA-activated 62-kDa gelatinase A·TIMP-2 complexes; 4, APMA-activated 62-kDa gelatinase A·TIMP-2 complexes treated with elastase; 5, 72-kDa progelatinase A·TIMP-2 complexes treated with PMSF-inhibited neutrophil elastase as a control. Gelatinase A and elastase concentrations were 50 and 10 nM, respectively. Bars, SD. Values represent the average of five determinations.

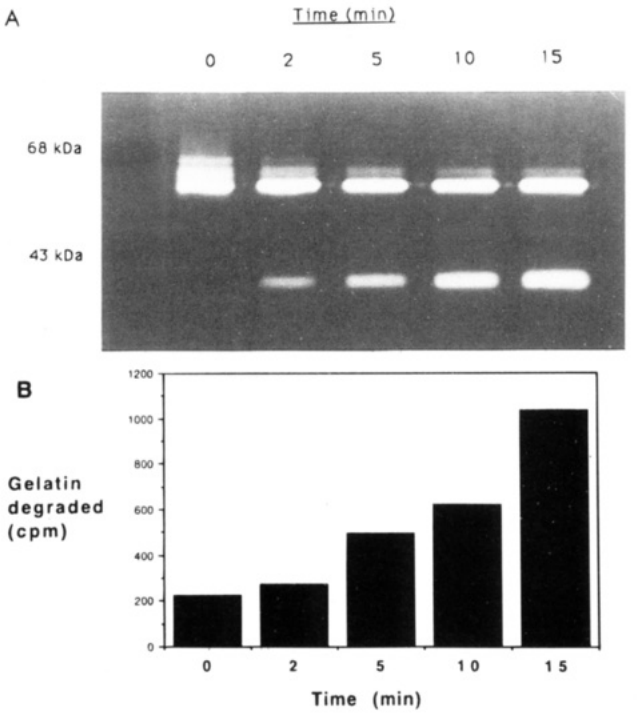

FIGURE 6: Increased gelatinolytic activity correlates with the production of the 40-kDa gelatinase. Progelatinase A·TIMP-2 complexes (10 nmol) were activated with 1 mM APMA for 1 h, followed by the addition of 2 nmol of neutrophil elastase and gelatin to give a final concentration of 0.5 mg/mL gelatin in the reaction mixture. At the designated times, samples were treated with 1 mM PMSF to inhibit the elastase and then were analyzed by gelatin zymography (panel A) or gelatin degradation assay (panel B). Molecular mass markers are the same as in Figure 2.

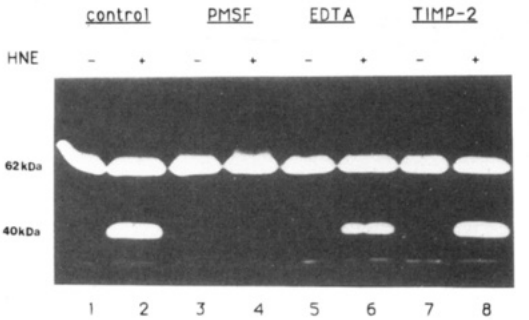

FIGURE 7: Formation of truncated gelatinases is the direct result of cleavage by neutrophil elastase. APMA-activated gelatinase A·TIMP-2 complexes (0.8 pmol) were incubated for 1 h at 37 °C with the following proteinase inhibitors: 1 mM PMSF (lanes 3, 4); 1 mM EDTA (lanes 5, 6); 2 pmol of TIMP-2 (lanes 7, 8). Lanes 1 and 2, control (no treatment with inhibitors). Samples in lanes 2, 4, 6, and 8 were then incubated with 2 pmol of neutrophil elastase (HNE) for 2 h at 37 °C and analyzed by gelatin zymography on 10% gels. Plus and minus indicate incubation with neutrophil elastase or without neutrophil elastase, respectively. All reaction mixtures contained 1 mg/mL gelatin. Gelatinase A migrated at 62 kDa, and the truncated gelatinase migrated at 40 kDa.

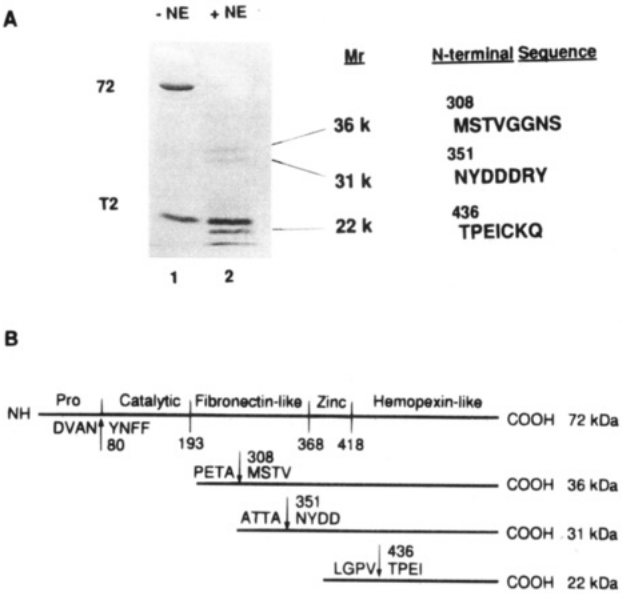

FIGURE 8: Elastase cleaves gelatinase A at three sites. Panel A: progelatinase A·TIMP-2 complexes were incubated with or without 3% (w/w) neutrophil elastase (−NE/+NE) in the absence of gelatin. Samples were analyzed on 10% acrylamide gels followed by electrophoretic transfer to a PVDF membrane in 10 mM CAPS buffer, pH 11.0. The blot was stained with Coomassie blue, destained, and subjected to amino acid analysis. The apparent molecular weight (M_r) and the amino-terminal sequence of the fragments are shown. Progelatinase is indicated by 72, and TIMP-2 is T2. Panel B: domain structure based on the primary sequence of progelatinase A (not drawn to scale). The three major products that result from elastase digestion of progelatinase A are shown below as black lines with their corresponding molecular masses, as determined by SDS−polyacrylamide electrophoresis. Arrows indicate the point of cleavage resulting from APMA treatment (a.a. residue 80) and elastase treatment (a.a. residues 308, 351, and 436).

least three peptide bonds of 72-kDa progelatinase A. Amino acid sequence analysis established that two bands at 36 and 31 kDa (Figure 8A) were the carboxyl-terminal fragments produced from cleavages made between Ala^{307}-Met^{308} and Ala^{350}-Asn^{351}, respectively, which are sites within the fibronectin-like gelatin-binding domain of gelatinase A. Cleavage at these sites led to a loss of activity, because the zinc-binding domain had been separated from the catalytic domain (Figure 8B). Another cleavage was made in the hemopexin-like domain between Val^{435} and Thr^{436}, resulting in the 22-kDa carboxyl-terminal fragment that migrated in front of TIMP-2 (Figure 8A).

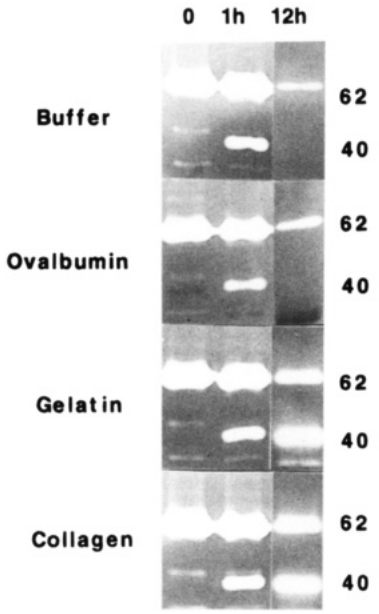

FIGURE 9: Gelatin protects gelatinase A from destructive degradation by elastase. APMA-activated gelatinase A·TIMP-2 complexes (10 pmol) were incubated with one of the following: ovalbumin (1 mg/mL); gelatin (porcine skin, 1 mg/mL); collagen (rat tail type 1, 1 mg/mL); or buffer control (20 mM HEPES, pH 7.4, 0.2 M NaCl, 1 mM $CaCl_2$) for 1 h at 37 °C. Neutrophil elastase (2 pmol) was then added, and samples were analyzed by gelatin substrate zymography at 1 and 12 h. Gelatinase (62 kDa) is indicated by 62, and the truncated gelatinase (40 kDa) is 40.

The Role of Matrix in the Interaction of Gelatinase A and Neutrophil Elastase. Because neutrophil elastase cleaves gelatinase A in its fibronectin-like gelatin-binding domain, the association of gelatinase A with 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 took place when either high neutrophil elastase concentrations or long digestion times were used. However, the presence of gelatin or collagen in the digestion mixture decreased the rate of destructive neutrophil elastase-mediated degradation of 62-kDa gelatinase A·TIMP-2 complexes (Figure 9). When the 62-kDa gelatinase A·TIMP-2 complexes were digested by neutrophil elastase in buffer or in the presence of ovalbumin, which can be degraded by elastase, the 40-kDa gelatinase A was detected at 1 h, but by 12 h it had been completely degraded. The 62-kDa gelatinase A was also almost completely degraded by 12 h, as reported by others (Okada & Nakanishi, 1989). However, when gelatin or collagen was included in the digestion mixture, both the 40-kDa fragment and the 62-kDa gelatinase A were still detected at 12 h. These data are consistent with the idea that neutrophil elastase cleaves gelatinase A at either of the two sites within the fibronectin-like gelatin-binding domain. The addition of gelatin or collagen to the digestion protects the gelatinase A from degradation and reduces the amounts of the 36-kDa and 31-kDa inactive fragments.

Neutrophils Produce Truncated 40-kDa Gelatinase A in Culture. To determine whether neutrophils could proteolytically modify gelatinase A, we incubated freshly isolated neutrophils with purified gelatinase A·TIMP-2 complexes. The truncated 40-kDa gelatinase was produced by this incubation (Figure 10). We also measured the amount of elastase released by the neutrophils. Unstimulated cells (i.e., with no additional stimulation besides the effect of separation from blood) released 0.36 μg of elastase per 10^6 cells. After stimulation by either 10 nM *N*-formyl-Met-Leu-Phe or phorbol ester, 1.5 μg of elastase per 10^6 cells was released (data not shown). This figure is in agreement with the estimated total amount of elastase per 10^6 cells, approximately 2 μg (Travis et al., 1991).

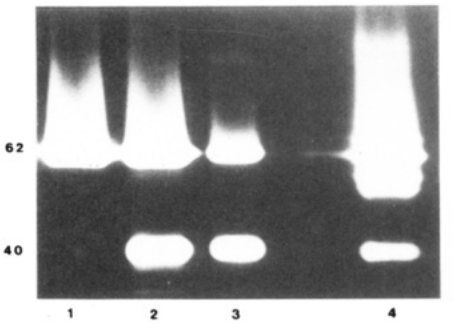

FIGURE 10: Neutrophils can modify gelatinase A in a manner similar to that of purified elastase. APMA-activated gelatinase A·TIMP-2 complex (lane 1) was treated with purified elastase (1:1, mol/mol, gelatinase:elastase ratio) for 2 h at ambient temperature in 0.05 M HEPES, pH 7.4, containing 0.15 M NaCl and 1 mg/mL gelatin and analyzed by gelatin substrate zymography (lane 2). A sample identical to that in lane 2 was diluted (1:2) before analysis (lane 3). In lane 4, APMA-activated gelatinase A·TIMP-2 complexes were added to 100 μL of a suspension of freshly isolated neutrophils (5.0×10^6 cells/mL in 0.05 M HEPES, pH 7.4, containing 0.15 M NaCl and 1 mg/mL gelatin) for 2 h at ambient temperature. The digestion mixture was centrifuged, and the supernatants were analyzed by gelatin substrate zymography. 62, gelatinase A; 40, truncated gelatinase.

DISCUSSION

We have shown how an inflammatory serine proteinase, neutrophil elastase, proteolytically modifies gelatinase A. Neutrophil elastase can cleave at a site within the hemopexin-like carboxyl-terminal domain of the gelatinase in either the 72-kDa progelatinase A·TIMP-2 complex or the APMA-activated complex, producing a 50-kDa or 40-kDa gelatinase, respectively. The completely activated 40-kDa form of gelatinase A was produced by APMA activation of the 72-kDa progelatinase A·TIMP-2 complex followed by digestion by neutrophil elastase or, conversely, by digestion of the 72-kDa progelatinase A·TIMP-2 complex with neutrophil elastase-followed by treatment with APMA. Because of the high gelatinolytic activity, the 40-kDa form of gelatinase appeared to have a higher specific activity than that of the APMA-activated gelatinase A·TIMP-2 complex. The truncated gelatinases no longer had the hemopexin-like carboxyl-terminal domain and were no longer associated with TIMP-2.

An early stage in inflammation 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. For gelatinase A, the nature of these modifications depends on whether the gelatinase A is free in solution or bound to gelatin. Free gelatinase A is destructively degraded by elastase. However, when the gelatinase A is bound to a matrix component, elastase cleaves preferentially at a site that generates a truncated yet more

active gelatinase. Although these studies were performed with purified components in vitro, interactions between elastase and gelatinase A are likely to occur at sites of inflammation in vivo. During the migration of neutrophils out of the blood and into the tissue, there are numerous encounters with cells that produce gelatinase A, such as endothelial cells and fibroblasts in the stroma underlying basement membranes. Neutrophils are found in granulation tissue, which is richly vascularized and potentially contains gelatinase A bound to connective tissue proteins. Neutrophils respond to an implanting blastocyst, surrounding it and possibly affecting the matrix around the implantation site (Finn & Pope, 1991). Mesangial cells have also been shown to secrete progelatinase A (Lovett et al., 1992). Inflammation of the kidney, which 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, we carried out experiments with freshly isolated neutrophils that were incubated with purified gelatinase A·TIMP-2 complexes. The amount of elastase released by the neutrophils was sufficient to produce the truncated 40-kDa gelatinase.

The presence of matrix components affects the modifications that neutrophil elastase can make on gelatinase A. In the absence of gelatin or collagen, elastase hydrolyzed gelatinase A at two sites within its fibronectin-like gelatin-binding domain. Because this fibronectin-like domain is between the catalytic domain and the zinc-binding domain, cleavage at these sites resulted in loss of activity of the enzyme. These findings are consistent with the observation that neutrophil elastase does not activate gelatinase A in the absence of gelatin but rather degrades it (Okada et al., 1988). However, when gelatin is bound to the fibronectin-like domain, these potential cleavage sites become more resistant to proteolysis by neutrophil elastase, 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 the proteolytic degradation of inhibitors. Neutrophil elastase has been reported to degrade TIMP-1 (Okada et al., 1988), a metalloproteinase inhibitor related in structure and function to TIMP-2. The enhanced activation of 72-kDa progelatinase A·TIMP-2 complexes by neutrophil elastase may be due to an autolytic event subsequent to TIMP-2 degradation that is similar to that seen with autoactivated 72-kDa progelatinase A (Howard et al., 1991a). Neutrophil elastase degrades TIMP-2, but more slowly than it degrades TIMP-1. When neutrophil elastase was incubated with 72-kDa progelatinase A·TIMP-2 complexes for a time sufficient for the complete degradation of progelatinase A, the TIMP-2 remained intact. Furthermore, the 40-kDa gelatinase A obtained from neutrophil elastase digestion of APMA-treated gelatinase A·TIMP-2 complexes was formed even when metalloproteinase inhibitors were present, suggesting that elastase directly generates the 40-kDa form of gelatinase A.

An additional consideration concerning the modifications of complexes by neutrophil elastase 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., 1987) and the seminal plasma protein PDC-109 (Banyai et al., 1990). Each type II structure is approximately 40 amino acids in length and includes 4 cysteines that, in fibronectin and PDC-109, are found to be completely oxidized, with 2 disulfide bonds connecting cysteine residue pairs 1−3 and 2−4 (Constantine et al., 1991). The cleavage of gelatinase A by neutrophil elastase 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 site recognized by elastase. The carboxyl-terminal products of these cleavages are the inactive 36-kDa and 31-kDa fragments that were detected in both reduced and unreduced samples. These data suggest that, unlike fibronectin and PDC-109, gelatinase A has no disulfide bond between the second and fourth cysteine residues of the third type II repeat. Because this region is adjacent to the zinc-binding domain, structural constraints may limit the proximity of cysteine residues and make it impossible for disulfide bonds to form between them.

The fate of secreted progelatinase A·TIMP-2 complexes is not known. 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 that these complexes become inactive, thereby limiting the extent of proteolytic degradation of the matrix. The present study indicates that neutrophil elastase can modify substrate-bound gelatinase A·TIMP-2 complexes. During an acute inflammatory response, these modifications, which increase the proteolytic potential of gelatinase A, may facilitate a more complete restructuring of the ECM, limited only by the availability of collagenous substrates. When the substrates have been degraded, free gelatinase A·TIMP-2 complexes may be quickly degraded into inactive fragments by neutrophil elastase. The carboxyl terminus of gelatinase A may be necessary for cell-associated activation (Murphy et al., 1992). Whether the 50-kDa progelatinase A that results from digestion of progelatinase A·TIMP-2 complexes by neutrophil elastase can be activated by this cell-associated mechanism is not known.

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