Cleavage and Inactivation of \( \alpha_1 \)-Antitrypsin by Metalloproteinases Released from Neutrophils

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Abstract

Human neutrophils, when stimulated with phorbol myristate acetate or fMet-Leu-Phe in the presence or absence of cytochalasin B, released metalloproteinases that catalytically inactivated the plasma serine proteinase inhibitor, \( \alpha_1 \)-antitrypsin. Inactivation, measured as loss of elastase inhibitory capacity, was accompanied by cleavage of a \( M_r \) 4,000 peptide from the COOH-terminus. Cleavage of \( \alpha_1 \)-antitrypsin by cell supernatants was inhibited by EDTA, \( \alpha \)-phenanthroline, and DTT, but not by inhibitors of serine or thiol proteinases. Gelatinase and collagenase were separated from the medium of stimulated neutrophils. Both preparations cleaved and inactivated \( \alpha_1 \)-antitrypsin, with cleavage occurring close to the reactive center, at the Phe-Leu bond between positions P2 and P1. Cleavage by purified gelatinase was very slow and could account for only a minor fraction of the activity of neutrophil supernatants. The collagenase preparation was more active. However, the unusual cleavage site, and the ability of fMet-Leu-Phe-stimulated neutrophils to cleave \( \alpha_1 \)-antitrypsin without releasing collagenase, suggests that collagenase is not responsible for cleavage by the cells, which, by implication, is due to an as yet uncharacterized metalloenzyme. Our results demonstrate that by releasing metalloproteinases, neutrophils could proteolytically inactivate \( \alpha_1 \)-antitrypsin at sites of inflammation. This provides an alternative to the previously documented mechanism of inactivation by neutrophil-derived oxidants.

Introduction

PMN (neutrophils) release the serine proteinases elastase and cathepsin G and a variety of other hydrolytic enzymes (1), which can degrade both invading pathogens and host tissues in inflammation (2, 3). At physiological concentrations, the plasma protein \( \alpha_1 \)-antitrypsin (\( \alpha_1 \)-proteinase inhibitor) inhibits both elastase and cathepsin G (4), and is generally thought to be a major influence in limiting tissue damage.

The elastase inhibitory activity of \( \alpha_1 \)-antitrypsin can be impaired by neutrophil oxidants, which react with the methionine at the reactive center (5–7). Once oxidized, \( \alpha_1 \)-antitrypsin is no longer able to prevent neutrophil-mediated digestion of extracellular matrix proteins (8, 9). In this paper, we describe a new mechanism of inactivation of \( \alpha_1 \)-antitrypsin by stimulated neutrophils, involving proteolytic cleavage by metalloproteinases released by the cells. We show that supernatants from stimulated neutrophils cleave \( \alpha_1 \)-antitrypsin, and we examine the ability of the two known metalloproteinases, collagenase and gelatinase (10) to cause this cleavage.

Methods

Materials

Ficoll 400 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. [3H]Sodium borohydride (200–500 mCi/mmoll) was from Amersham Corp., Amersham, England. Other chemicals were obtained from either Sigma Chemical Co., St. Louis, MO, or British Drug Houses, Poole, England.

Preparation of neutrophils

Human neutrophils were prepared from the peripheral blood of healthy donors by centrifugation through Ficoll-Hypaque, dextran sedimentation, and hypotonic lysis of contaminating red cells (11). The cell suspensions contained 95–97% neutrophils and 3–5% eosinophils, and were > 98% viable as assessed by trypan blue exclusion. The cells were suspended at \( 10^7 \) ml in PBS (pH 7.4), supplemented with 1 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), and 1 mg/ml glucose.

Neutrophils were stimulated at 37°C with either 0.1 \( \mu \)g/ml phorbol myristate acetate (PMA) or \( 10^{-7} \) M fMet-Leu-Phe. When appropriate, 5 \( \mu \)g/ml cytochalasin B was added 2 min before stimulation. After 10 min, the neutrophils were pelleted by centrifugation (1,000 g) for 10 min and the supernatant either assayed immediately or stored at \(-20^\circ\)C.

Purification of gelatinase and collagenase

Neutrophils separated from 1.5 liter blood were stimulated with PMA, and the gelatinase and collagenase purified by the method of Hibbs et al. (12), with the following modifications. The cell supernatant, containing 2 mM PMSF, was applied to a DEAE-Sephadex A50 column (2.5 \( \times \) 25 cm) and three A\(_{280}\) peaks were eluted with the starting buffer. The first showed collagenase activity which was further purified by gel filtration on a Sephadex G150 column with dimensions 2 \( \times \) 75 cm, calibrated with standard proteins in the \( M_r \) range 14,000–240,000. Collagenase activity eluted as a single peak with an apparent \( M_r \) 66,000–68,000. Murphy et al. (13) obtained a similar \( M_r \) by gel filtration, and Hasty et al. (14) observed two active collagenase bands at 66 and 52 kD. SDS-PAGE of the 68 kD peak (pooled and concentrated by ultrafiltration) showed two major bands with apparent \( M_r \) \approx 90,000 and 30,000 and several other minor bands. Although this pattern is difficult to relate to the gel filtration profile, there are similarities to the SDS-PAGE results of Hasty et al. (14). Their 66-kD band on gel filtration ran between 66 and 92 kD SDS-PAGE, and they also observed an inactive band, probably a degradation product, with \( M_r \) 22,000. Our observations, therefore, are consistent with this fraction containing neutrophil collagenase, although not completely pure.

Gelatinase, which remained bound to the DEAE-Sephadex column, was eluted and further purified by affinity chromatography on
gelatin-Sepharose (12). Fractions containing gelatinase activity were pooled and concentrated. SDS-PAGE of the purified gelatinase activity gave a single band with an apparent Mr of 92,000 (Fig. 1) in agreement with Sopata (15) and Hibbs et al. (12). The total protein content of the two enzyme preparations was measured by the Lowry method (16).

Cleavage and inactivation of α1-antitrypsin

α1-Antitrypsin was purified from human plasma by thiol-disulphide interchange (17). Preparations were >90% active as assessed by inhibition of elastase. Elastase inhibitory capacity was measured by incubating with excess porcine pancreatic elastase for 5 min, then measuring the residual elastase activity with succinyl-Ala-Ala-Ala-p-nitroanilide (18). To measure cleavage and inactivation, 50 μg α1-antitrypsin was incubated in 200 μl of 80 mM Tris/HCl, pH 7.6, with 5 mM CaCl₂ and 0.02% NaN₃ and either neutrophil supernatant or purified collagenase or gelatinase. Aliquots were removed at intervals between 30 min and 48 h and analyzed (a) for elastase inhibitory capacity and (b) for proteolytic cleavage of α1-antitrypsin by SDS-PAGE on 7.5% gels with mercaptoethanol (19).

Determination of site of cleavage by collagenase-containing fraction and gelatinase

α1-Antitrypsin (2.5 mg in 1 ml) was incubated as above with sufficient collagenase or gelatinase until it had lost ~50% of its elastase inhibitory capacity. After addition of SDS, the cleaved Mr 4,000 peptide was isolated from the remainder of the protein by ethanol precipitation (20). The peptide was recovered from the supernatant by precipitation with acetone containing 1% HCl, and sequenced by manual Edman degradation (21) incorporating the modifications of Brennan and Carrell (20). PTH amino acids were identified by reverse phase HPLC using a Nova pac column (Waters Associates, Millipore Corp., Milford, MA).

Enzyme assays

Collagenase. Type I collagen (calf skin; Sigma Chemical Co.) was dissolved at 2 mg/ml in 0.2 M acetic acid and 10 μl was added to each sample to be assayed (10 μl) and 80 μl of 0.1 M Tris/HCl, pH 7.6, containing 5 mM CaCl₂, 0.02% NaN₃, and 4 mM PMSF. When required, 2 mM phenylmercuric acetate was added to activate latent collagenase. After 18 h at 25°C, the solution was heated with SDS and mercaptoethanol at 90°C for 3 min, and the peptides were separated by SDS-PAGE on 6% gels (12). Collagenase activity was assessed as specific cleavage of the α, β, and γ subunits.

Gelatinase. Type I collagen, labeled with [³H]NaBH₄ (22) was diluted to 100,000 cpm/ml with unlabeled collagen in 0.1 M Tris/HCl pH 7.6, containing 5 mM CaCl₂, 0.02% NaN₃, and 4 mM PMSF, and denatured by incubating at 65°C for 15 min. The heat-denatured collagen (gelatin) (200 μg in 200 μl of the above buffer) was incubated at 37°C with up to 25 μl of neutrophil supernatant or purified enzyme. When required, 2 mM phenylmercuric acetate was included to activate latent gelatinase. After 60 min, an equal volume of 20% (w/v) ethanol TCA was added. The tubes were cooled to 4°C for 20 min and centrifuged at 15,000 g for 2 min and 100 μl of each supernatant was assayed for solubilized [³H]gelatin.

Results

Inactivation of α1-antitrypsin by media from stimulated neutrophils

PMA-stimulated neutrophils. On incubation with the supernatant from PMA-stimulated cells, α1-antitrypsin underwent progressive cleavage, as demonstrated by SDS-PAGE (Fig. 2). The change in electrophoretic mobility is consistent with a molecular mass decrease of ~4,000 D. The appearance of the lower molecular weight form was paralleled by a decrease in elastase inhibitory capacity of the α1-antitrypsin (Fig. 2).

Oxidants were not involved in this inactivation, since neither catalase (500 U/ml), when present during stimulation of the neutrophils, nor 5 mM methionine added to the supernatant, prevented the loss of elastase inhibitory capacity or cleavage of α1-antitrypsin. These procedures prevent the formation of, or scavenge, long-lived oxidants such as chloramines. α1-Antitrypsin treated with N-chlorosuccinimide, which oxidizes the active site methionine (7), was relatively resistant to cleavage (Fig. 2).

As shown in Fig. 3, cleavage of α1-antitrypsin was prevented by the metalloproteinase inhibitors EDTA and o-phenanthroline, but not by N-ethylmaleimide, iodoacetic acid, or PMSF. DTT, another inhibitor of metalloproteinases, also prevented cleavage (not shown). Phenylmercury acetate was added to each assay as a metalloproteinase activator, since gelatinase and collagenase are both present in neutrophil granules in latent form (9). However, α1-antitrypsin cleavage was seen in the absence of phenylmercury acetate (Fig. 3, lane 8), indicating that the relevant metalloproteinase in the PMA supernatant was already activated. Cleavage was always paralleled by loss of elastase-inhibitory capacity (not shown).

βMet-Leu-Phe-stimulated neutrophils. Supernatants were prepared from neutrophils stimulated in the presence and absence of cytochalasin B. Both supernatants, in the presence of phenylmercury acetate and PMSF, cleaved α1-antitrypsin and concomitantly destroyed its elastase inhibitory capacity. There was two to three times more cleavage for an equivalent number of cells when cytochalasin B was present during stimula-
Figure 3. SDS-PAGE showing the effect of proteinase inhibitors on cleavage of α₁-antitrypsin by the supernatant from PMA-stimulated neutrophils. Lane 1 shows α₁-antitrypsin. The other lanes show α₁-antitrypsin after incubation of 50 μg for 18 h with supernatant from 2 × 10⁷ neutrophils plus (except for lane 8) 2 mM phenylmercury acetate. Other additions: lane 2, none; lane 3, 4 mM PMSF; lane 4, 2 mM N-ethylmaleimide; lane 5, 10 mM EDTA; lane 6, 2 mM iodoacetic acid; lane 7, 2 mM o-phenanthroline; lane 8, no phenylmercury acetate. Arrows denote the positions of the markers shown in Fig. 1.

of the two known neutrophil metalloproteinases, gelatinase is released from the cells by fMet-Leu-Phe alone, whereas in the presence of cytochalasin B, both collagenase and gelatinase are released (23). In agreement with these observations, we detected collagenase activity, measured as specific cleavage of the α, β, and γ bands seen on SDS-PAGE to smaller α', β', and γ' bands, only in the presence of cytochalasin B (Fig. 5). Phenylmercury acetate was present to activate any latent collagenase. Gelatinase was detected in both supernatants, but its activity (measured in the presence of phenylmercury acetate) was 2.2 to three times higher (range of six assays on different neutrophils) in the presence of cytochalasin B. Gelatin degradation was measured in the presence of PMSF and was inhibited by EDTA, o-phenanthroline, and DTT. The gelatinase released from cells stimulated by fMet-Leu-Phe alone was almost entirely latent, and activated by phenylmercury acetate. In comparison, gelatinase released from cells stimulated in the presence of cytochalasin B was about two-thirds active. This is in accordance with our observation (24) that gelatinase is activated by elastase, which is also released in the presence of cytochalasin B.

Inactivation of α₁-antitrypsin by collagenase-containing fraction and gelatinase. Since the extent of α₁-antitrypsin inactivation by the neutrophil supernatants broadly followed the pattern of gelatinase release, the above results would be compatible with gelatinase being responsible for the cleavage. To examine more directly the activities of neutrophil collagenase and gelatinase against α₁-antitrypsin, the two enzymes were isolated from the medium of stimulated cells. The gelatinase fraction ran as one band on SDS-PAGE (Fig. 1) and degraded gelatin (Table I). Gelatin degradation was observed in the presence of PMSF and phenylmercury acetate, and was inhibited by EDTA, o-phenanthroline, and DTT, but not by N-ethylmaleimide. The preparation contained no detectable specific collagenase activity against native collagen (Fig. 5). The collagenase preparation specifically cleaved native collagen in the presence and absence of phenylmercury acetate, as shown by the SDS-PAGE profile (Fig. 5). Activity was abolished by EDTA. The preparation was not completely pure (see Methods), but it contained no detectable active or latent gelatinase (Table I).

Table I. Gelatinase Activity in Enzyme Preparations

<table>
<thead>
<tr>
<th>Additions</th>
<th>Gelatinase</th>
<th>Collagenase</th>
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<tbody>
<tr>
<td>None</td>
<td>82</td>
<td>2.0</td>
</tr>
<tr>
<td>EDTA (10 mM)</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>o-Phenanthroline (2 mM)</td>
<td>3.7</td>
<td>ND</td>
</tr>
<tr>
<td>DTT (1 mM)</td>
<td>21</td>
<td>ND</td>
</tr>
</tbody>
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Each assay contained 24,300 cpm [³H]gelatin, 0.1 μg enzyme, 4 mM PMSF and 2 mM phenylmercury acetate. A background of 1,700 TCA-soluble cpm in the absence of enzyme have been subtracted before calculating percentages of gelatin solubilized.
The preparation containing collagenase readily destroyed the elastase inhibitory capacity of α1-antitrypsin (Fig. 6). Gelatinase also inactivated α1-antitrypsin, but the reaction was very slow. Purified enzyme with gelatin-degrading ability equivalent to 10⁶ Met-Leu-Phe/cytochalasin B-stimulated neutrophils gave 22±7% (n = 4) inactivation of 120 μg α1-antitrypsin in 18 h, and even after 66 h under these conditions, gave only a small amount of cleavage (Fig. 7). For comparison, the fMet-Leu-Phe/cytochalasin B supernatant from 5 × 10⁵ cells gave ~55% inactivation in 18 h, i.e., gelatinase could account for no more than 20% of the α1-antitrypsin inactivation by the cell supernatant. With both enzyme preparations, loss of α1-antitrypsin antielastase activity was accompanied by an Mᵦ decrease of 4,000 D, and cleavage was inhibited by EDTA, o-phenanthroline, and DTT, but not PMSF, iodoacetic acid, or N-ethylmaleimide (Fig. 7).

Site of α₁-antitrypsin cleavage by collagenase and gelatinase. The small peptide cleaved by the collagenase-containing fraction was isolated by ethanol extraction and acetone precipitation in essentially pure form (Fig. 8). Both were shown to have the NH₂-terminal sequence Leu-Glu-Ala-Ile-Pro-Met. This sequence corresponds to the reactive centre residues 353–358 of α₁-antitrypsin and indicates that cleavage occurred at the Phe-Leu bond between positions 352 and 353 (Pᵦ and Pᵦ). Cleavage close to the reactive center would be expected to inactivate the inhibitor. A 41-residue peptide would be released, which is consistent with the observed 4,000-D decrease in molecular mass.

Discussion

α₁-Antitrypsin, the major plasma inhibitor of serine proteinases, inhibits neutrophil elastase and cathepsin G, and is considered to limit tissue degradation when these enzymes are released from the cells. We have demonstrated that human neutrophils, stimulated either with PMA or fMet-Leu-Phe, are capable of inactivating α₁-antitrypsin. Inactivation is associated with cleavage and a 4,000-D decrease in molecular mass. Studies with inhibitors showed that this decrease is due to the action of metalloproteinases released from the cells.

Two metalloproteinases have been described (10). Gelatinase, present in C particles, is released when the cells are stimulated with fMet-Leu-Phe alone (23). Collagenase is present in specific granules and is released along with gelatinase when cytochalasin B is present, or the cells are stimulated with PMA (23). We observed α₁-antitrypsin inactivation by all three supernatants, suggesting that gelatinase could be the metalloproteinase responsible. Gelatinase, purified to apparent homogeneity and free of collagenase activity, did cleave α₁-antitrypsin. However, its activity was low, and could account for only a minor portion of the cleavage by neutrophil supernatants. The collagenase preparation was considerably more active. Although not completely pure, this preparation specifically cleaved type I collagen, and it lacked activity against gelatin. It also had the characteristic Mᵦ on gel filtration reported for neutrophil collagenase (13, 14).

However, two aspects of our study strongly suggest that gelatinase is not the enzyme responsible for cleavage. First, we found that cleavage occurred at the Phe-Leu bond. In view of the specificity of collagenases for selected Gly-Leu or Gly-Ileu bonds in the triple helices of collagen molecules, it seems unlikely that this enzyme would cleave a Phe-Leu bond rapidly, if at all. Secondly, there was appreciable α₁-antitrypsin inactivation and cleavage by supernatants from fMet-Leu-Phe-stimulated cells that contain no detectable collagenase. Gelatinase

Figure 7. Effect of inhibitors on cleavage of α₁-antitrypsin by gelatinase and collagenase-containing fraction. SDS-PAGE of α₁-antitrypsin (lane 1) or 120 μg α₁-antitrypsin incubated for 66 h with purified gelatinase, activity equivalent to 2 × 10⁶ neutrophils (lanes 2–8), or for 18 h with collagenase fraction as for Fig. 6, except with no PMSF added (lanes 9–15). Phenylmercury acetate was present in all samples. Lanes 2 and 9, no further additions; lanes 3 and 10, plus 4 mM PMSF; lanes 4 and 11, plus 10 mM EDTA; lanes 5 and 12, plus 2 mM o-phenanthroline; lanes 6 and 13, plus 2 mM DTT; lanes 7 and 14, plus 2 mM N-ethylmaleimide; lanes 8 and 15, plus 2 mM iodoacetic acid. The double bands in each lane are an artefact of this particular run.

Figure 8. SDS-PAGE of small peptide purified from α₁-antitrypsin cleaved by gelatinase and collagenase-containing fraction. SDS-PAGE carried out on a 10–20% gradient gel. Lane 1, α₁-antitrypsin; lane 2, treated with gelatinase; lane 3, treated with gelatinase and purified; lane 4, treated with collagenase fraction; lane 5, treated with collagenase fraction and purified.
activity is apparently much too low to account for the amount of inactivation observed. Hence, we must consider the possibility that neutrophils release another hitherto uncharacterized metalloproteinase, which copurifies with collagenase and is able to cleave and inactivate $\alpha_1$-antitrypsin. Since this activity was released from fMet-Leu-Phe- and PMA-stimulated cells, it seems likely that the enzyme is localized in C particles. We are currently investigating the possibility of a new metalloenzyme present in neutrophils.

Cleavage of $\alpha_1$-antitrypsin by a variety of thiol and metalloproteinases (25–27), including macrophage elastase (28, 29) has been reported. The site of cleavage by the neutrophil metalloproteinase activity (between residues P$_{7}$ and P$_{8}$) is in an exposed loop that contains the reactive center (30). This sequence is particularly susceptible to proteolysis, with papain and Staphylococcus aureus metalloproteinase also cleaving at the P$_{7}$–P$_{8}$ position (27, 30, 31). We found that oxidation of $\alpha_1$-antitrypsin by N-chlorosuccinimide decreased its susceptibility to cleavage. As oxidation affects Met P$_{8}$ and Met P$_{9}$, this observation is consistent with a decrease in the cleavage rate.

Our findings demonstrate a new physiological mechanism by which $\alpha_1$-antitrypsin could be inactivated in the vicinity of stimulated neutrophils, by the action of metalloproteinases(s) released from the cells. Such inactivation could upset the balance between $\alpha_1$-antitrypsin and neutrophil elastase, allowing elastolytic degradation of connective tissue proteins. This mechanism has been proposed for oxidative inactivation of $\alpha_1$-antitrypsin, caused either by cigarette smoke components or oxidants produced by phagocytic cells (5–7, 32). However, although myeloperoxidase-derived hypochlorous acid readily inactivates $\alpha_1$-antitrypsin, it is not very discriminatory, and is likely to be scavenged by many plasma constituents (33). Proteolytic inactivation of $\alpha_1$-antitrypsin could be more selective and, therefore, more significant than oxidative inactivation. A similar suggestion has been made by Banda et al. (29) for inactivation of $\alpha_1$-antitrypsin by macrophage elastase. In contrast to oxidative inactivation that is reversible (34), proteolytic inactivation is irreversible and complete. Further, the metalloproteinase activity can be released by neutrophils at concentrations of stimulants that are chemotactic but induce little, if any, oxidant production (23).

$\alpha_1$-Antitrypsin is a member of a family of structurally related serine proteinase inhibitors or serpins (30), including $\alpha_1$-antichymotrypsin and C1-esterase inhibitor. Bacterial metalloproteinases inactivate all these inhibitors by cleavage (26, 27), and there is a strong possibility that they will also be susceptible to the neutrophil metalloenzymes. If so, this would provide a mechanism for the neutrophil to regulate the local activity of these inflammatory mediators.

Acknowledgments

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References


