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Research Article

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Cleavage and Inactivation of α_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, α_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 α_1-antitrypsin by cell supernatants was inhibited by EDTA, o-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 α_1-antitrypsin, with cleavage occurring close to the reactive center, at the Phe-Leu bond between positions P_7 and P_6. 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 α_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 α_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 α_1-antitrypsin (α_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 α_1-antitrypsin can be impaired by neutrophil oxidants, which react with the methionine at the reactive center (5–7). Once oxidized, α_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 α_1-antitrypsin by stimulated neutrophils, involving proteolytic cleavage by metalloproteinases released by the cells. We show that supernatants from stimulated neutrophils cleave α_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/mmol) 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 μg/ml phorbol myristate acetate (PMA) or 10^{-7} M fMet-Leu-Phe. When appropriate, 5 μg/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°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 × 25 m) 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 × 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 $M_r \sim 90,000$ 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
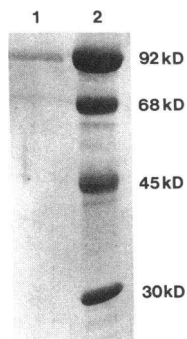

Figure 1. SDS-PAGE of purified gelatinase. A 7.5% gel with mercaptoethanol, showing lane 1, gelatinase; lane 2, molecular mass markers in descending order, phosphorylase B (92 kD), BSA (68 kD), ovalbumin (45 kD), and carbonic anhydrase (30 kD).

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 M_r 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 α_1-antitrypsin

α_1-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 α_1-antitrypsin was incubated in 200 μl of 80 mM Tris/HCl, pH 7.6, with 5 mM $CaCl_2$ and 0.02% NaN_3 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 α_1-antitrypsin by SDS-PAGE on 7.5% gels with mercaptoethanol (19).

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

α_1-Antitrypsin (2.5 mg in 1 ml) was incubated as above with sufficient collagenase or gelatinase until it had lost $\sim$ 50% of its elastase inhibitory capacity. After addition of SDS, the cleaved M_r 4,000 peptide was isolated from the remainder of the protein by ethanol extraction (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 $CaCl_2$, 0.02% NaN_3, and 4 mM PMSF. When required, 2 mM phenylmercury 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 [^{3}H]NaBH$_4$ (22) was diluted to 100,000 cpm/mg with unlabeled collagen in 0.1 M Tris/HCl, pH 7.6, containing 5 mM $CaCl_2$, 0.02% NaN_3, and 4 mM PMSF, and denatured by incubating at 60°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 phenylmercury acetate was included to activate latent gelatinase. After 60 min, an equal volume of 20% (wt/vol) 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 [^{3}H]gelatin.

Results

Inactivation of α_1-antitrypsin by media from stimulated neutrophils

PMA-stimulated neutrophils. On incubation with the supernatant from PMA-stimulated cells, α_1-antitrypsin underwent progressive cleavage, as demonstrated by SDS-PAGE (Fig. 2). The change in electrophoretic mobility is consistent with a molecular mass decrease of $\sim$ 4,000 D. The appearance of the lower molecular weight form was paralleled by a decrease in elastase inhibitory capacity of the α_1-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 α_1-antitrypsin. These procedures prevent the formation of, or scavenge, long-lived oxidants such as chloramines. α_1-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 α_1-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, α_1-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).

fMet-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 α_1-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-
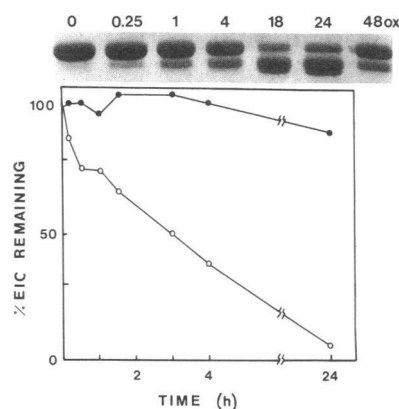

Figure 2. Inactivation and cleavage of α_1-antitrypsin by the supernatant from PMA-stimulated neutrophils. 50 μg α_1-antitrypsin was incubated in the presence (o) or absence (•) of supernatant from 2×10^5 neutrophils as described in Methods. Aliquots were removed at intervals, assayed for elastase inhibitory capacity (EIC), and analyzed by SDS-PAGE. Shown are the profiles obtained from active α_1-antitrypsin incubated with neutrophil supernatant for 0, 0.25, 1, 4, 18, and 24 h or oxidized α_1-antitrypsin for 48 h. The α_1-antitrypsin band (M_r 52,000) ran between the 68 kD and 45 kD markers, as in Fig. 3. Results shown are from one experiment. The same trends were observed on other occasions, although actual rates of inactivation varied for different cell preparations.
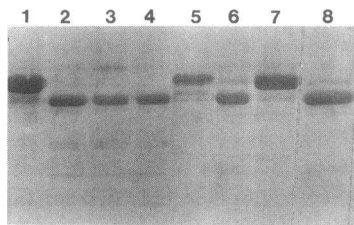
Figure 3. SDS-PAGE showing the effect of proteinase inhibitors on cleavage of α_1-antitrypsin by the supernatant from PMA-stimulated neutrophils. Lane *1* shows α_1-antitrypsin. The other lanes show α_1-antitrypsin after incubation of 50 μg for 18 h with supernatant from 2×10^5 neutrophils plus (except 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.

tion, and EDTA, *o*-phenanthroline, and DTT were inhibitory (Fig. 4). Compared with the two fMet-Leu-Phe supernatants, the supernatant from PMA-stimulated neutrophils gave an intermediate amount of cleavage (not shown).

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^A, β^A, and γ^A 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 α_1-antitrypsin by collagenase-containing fraction and gelatinase. Since the extent of α_1-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
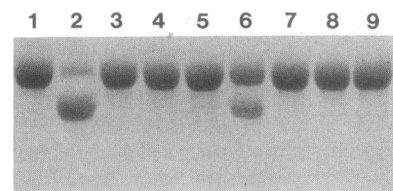

Figure 4. Cleavage of α_1-antitrypsin by the supernatant of neutrophils stimulated with fMet-Leu-Phe. α_1-Antitrypsin (180 μg), untreated (lane *1*), or incubated for 15 h with supernatant from 5×10^5 neutrophils stimulated with fMet-Leu-Phe in the presence (lanes *2-5*) or absence (lanes *6-9*) of cytochalasin B. Phenylmercury acetate was present in all samples. Lanes *2* and *6*, no further additions; lanes *3* and *7*, plus 10 mM EDTA; lanes *4* and *8*, plus 2 mM *o*-phenanthroline; lanes *5* and *9*, plus 2 mM DTT.
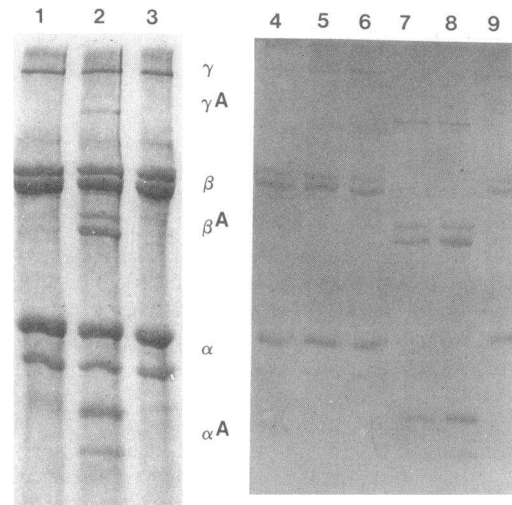

Figure 5. Specific cleavage of type I collagen. Collagen was incubated either for 18 h with supernatant from 2×10^5 neutrophils stimulated with fMet-Leu-Phe, or for 48 h with 0.1 μg purified gelatinase or collagenase, as shown in Methods. Native collagen bands are designated α, β, and γ, and the specific cleavage products α^A, β^A, and γ^A. Lanes *1* and *6* show untreated collagen. Lane *2*, incubated with fMet-Leu-Phe plus cytochalasin B supernatant plus phenylmercury acetate; lane *3*, fMet-Leu-Phe supernatant plus phenylmercury acetate; lane *4*, gelatinase; lane *5*, gelatinase plus phenylmercury acetate; lane *7*, collagenase; lane *8*, collagenase plus phenylmercury acetate; lane *9*, collagenase plus phenylmercury acetate plus EDTA.

and gelatinase against α_1-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

Additions	[^{3}H]Gelatin solubilized in 1 h (% of total counts)	
	Gelatinase	Collagenase
None	82	2.0
EDTA (*10 mM*)	2.1	1.8
o-Phenanthroline (*2 mM*)	3.7	ND
DTT (*1 mM*)	21	ND

Each assay contained 24,300 cpm [^{3}H]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 α_1-antitrypsin (Fig. 6). Gelatinase also inactivated α_1-antitrypsin, but the reaction was very slow. Purified enzyme with gelatin-degrading ability equivalent to 10^6 fMet-Leu-Phe/cytochalasin B-stimulated neutrophils gave $22\pm7\%$ ($n = 4$) inactivation of 120 μg α_1-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 5×10^5 cells gave $\sim 55\%$ inactivation in 18 h, i.e., gelatinase could account for no more than 20% of the α_1-antitrypsin inactivation by the cell supernatant. With both enzyme preparations, loss of α_1-antitrypsin antielastase activity was accompanied by an M_r 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 α_1-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 NH$_2$-terminal sequence Leu-Glu-Ala-Ile-Pro-Met. This sequence corresponds to the reactive centre residues 353–358 of α_1-antitrypsin and indicates that cleavage occurred at the Phe-Leu bond between positions 352 and 353 (P$_7$ and P$_6$). Cleavage this 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

α_1-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 α_1-antitrypsin. Inactivation is asso-
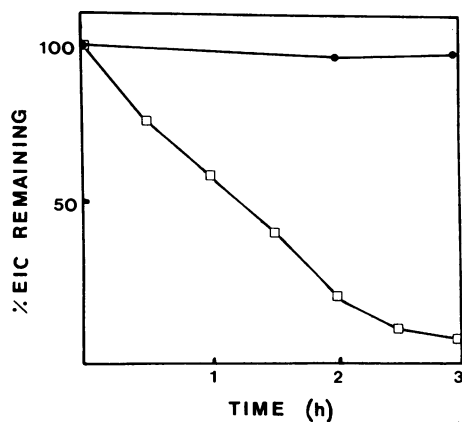

Figure 7. Effect of inhibitors on cleavage of α_1-antitrypsin by gelatinase and collagenase-containing fraction. SDS-PAGE of α_1-antitrypsin (lane *1*) or 120 μg α_1-antitrypsin incubated for 66 h with purified gelatinase, activity equivalent to 2×10^5 neutrophils (lanes 2–8), or for 18 h with collagenase 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.

ciated 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 neutrophil 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 α_1-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 α_1-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 M_r on gel filtration reported for neutrophil collagenase (13, 14).

However, two aspects of our study strongly suggest that collagenase is not the enzyme responsible for cleavage. First, we found that cleavage occurred at a 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 α_1-antitrypsin inactivation and cleavage by supernatants from fMet-Leu-Phe stimulated cells that contain no detectable collagenase. Gelatinase

Figure 6. Loss of elastase inhibitory capacity (EIC) of α_1-antitrypsin on incubation with collagenase-containing fraction. α_1-Antitrypsin (50 μg) was incubated with 4 mM PMSF and 2 mM phenylmercury acetate with (□) and without (●) 0.1 μg protein from the collagenase-containing fraction. Elastase activity was determined on samples diluted by the assay reagents, at which dilutions PMSF did not affect the elastase activity.
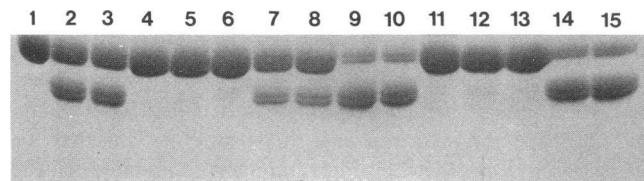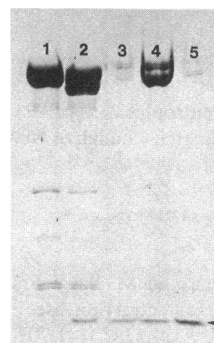

Figure 8. SDS-PAGE of small peptide purified from α_1-antitrypsin cleaved by gelatinase and collagenase-containing fraction. SDS-PAGE carried out on a 10–20% gradient gel. Lane *1*, α_1-antitrypsin; lane *2*, treated with gelatinase; lane *3*, treated with gelatinase and purified; lane *4*, treated with 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 α_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 α_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_6) 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_6 position (27, 30, 31). We found that oxidation of α_1-antitrypsin by N-chlorosuccinimide decreased its susceptibility to cleavage. As oxidation affects Met P_8 and Met P_1, this observation is consistent with a decrease in the cleavage rate.

Our findings demonstrate a new physiological mechanism by which α_1-antitrypsin could be inactivated in the vicinity of stimulated neutrophils, by the action of metalloproteinase(s) released from the cells. Such inactivation could upset the balance between α_1-antitrypsin and neutrophil elastase, allowing elastolytic degradation of connective tissue proteins. This mechanism has been proposed for oxidative inactivation of α_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 α_1-antitrypsin, it is not very discriminatory, and is likely to be scavenged by many plasma constituents (33). Proteolytic inactivation of α_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 α_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).

α_1-Antitrypsin is a member of a family of structurally related serine proteinase inhibitors or serpins (30), including α_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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