LIMITED PROTEOLYSIS BY MACROPHAGE ELASTASE INACTIVATES HUMAN α_1-PROTEINASE INHIBITOR*

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Ever since the initial description of α_1-proteinase inhibitor[1] (α_1PI),[2] the role of this plasma glycoprotein and its allelic polymorphism (1) in disease and in healthy physiology has been the subject of much investigation (2). α_1PI inactivates a number of serine proteinases, including granulocyte elastase (3), and thus affords protection from the connective tissue degradation mediated by this class of proteinases. The genetic deficiency of α_1PI in individuals with a Pi ZZ phenotype is associated with premature development of pulmonary emphysema (4, 5) and other disorders, such as childhood cirrhosis (6). Lung and liver are exposed to both circulating α_1PI and serine proteinases from granules of polymorphonuclear leukocytes. Because an imbalance in the ratio between α_1PI and proteinase may contribute to the development of destructive lung diseases, proteinases have been implicated in the pathogenesis of pulmonary emphysema (7).

Both macrophages and polymorphonuclear leukocytes have been implicated in disruption of the α_1PI-proteinase balance. The production of reactive oxygen species (8, 9) by phagocytosing cells (10) and exposure to cigarette smoke (11) has been suggested as a mechanism for inactivating α_1PI and inducing lung damage. In addition to their phagocytic activity, stimulated macrophages actively secrete a number of proteinases, including the metalloproteinases collagenase (12) and elastase (13–15).

In this report we demonstrate a new mechanism for alteration of the α_1PI-proteinase balance. We have found that the purified form of macrophage elastase catalytically degrades and inactivates α_1PI so that it no longer inhibits the elastinolytic activity of granulocyte elastase.

Materials and Methods

Enzymes. Macrophage elastase (ME) was purified from medium conditioned by thioglycollate-elicited mouse peritoneal macrophages as previously described (15). The preparation was purified 3,900-fold and had a 5,400 U/mg sp act, where 1 U of elastase degraded 1 μg of insoluble elastin/h at 37°C. ME migrated as a single band at an apparent molecular weight (M_r) of 22,000 on both reducing and nonreducing sodium dodecyl sulfate (SDS)-polyacrylamide electrophoretic gels.

Human granulocyte elastase (HGE) (16), a gift of A. J. Barrett (Strangeways Research

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[1] α_1-Proteinase inhibitor is also known as α_1-trypsin inhibitor and α_1-antitrypsin.

[2] *Abbreviations used in this paper:* α_2M, α_2-macroglobulin; α_1PI, α_1-proteinase inhibitor; HGE, human granulocyte elastase; M_r, apparent molecular weight(s); ME, macrophage elastase; SDS, sodium dodecyl sulfate; SPCK, methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone.

Laboratory, Cambridge, England), had a 35,000 U/mg sp act. On reducing SDS-polyacrylamide gels, the major band of HGE migrated at M_r 27,000.

Inhibitors. Human α_1PI, a gift of C. Glaser (Institutes of Medical Sciences, San Francisco, Calif.), was purified from human Pi MM plasma by a modification of the method of Glaser et al. (17); Cibacron blue (Ciba-Geigy Corp., Ardsley, N. Y.)-Sepharose 4B was used instead of concanavalin A for the removal of albumin. On reducing SDS-polyacrylamide gels, α_1PI migrated at M_r 58,000 either as a single band or as a double band (an artifact of Laemmli system gels). It exhibited >95% inhibitory activity against trypsin.

Human α_2-macroglobulin (α_2M) was purified from fresh haptoglobin type 1-1 serum by Cibacron blue-Sepharose 4B affinity chromatography (18). The purified α_2M gave a single line of identity against rabbit anti-human whole serum (Bio-Rad Laboratories, Richmond, Calif.) and rabbit anti-human α_2M (Bio-Rad Laboratories). It was >95% active when calibrated by inhibition of active site-titrated trypsin (Worthington Biochemical Corp., Freehold, N. J.). α_2M migrated as a single band at M_r 725,000 on pore-limit polyacrylamide gels (19). On reducing SDS-polyacrylamide gels, α_2M migrated as a major band at M_r 185,000 and a series of minor bands at M_r 139,000 and 64,000–68,000. These minor bands were heat-cleavage products (19, 20) and consisted of <1% of the total α_2M protein. Another band at M_r 85,000 was the result of interaction of proteinases with α_2M.

Methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone (SPCK), a specific active site-directed inhibitor of granulocyte elastase (21), was a gift of J. Powers (Georgia Institute of Technology, Athens, Ga.).

Electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out according to a modification of the method of Laemmli (22). The samples were resolved on a linear 7–18% polyacrylamide gradient and reduced by boiling in 0.5% 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.). After electrophoresis, gels were fixed in 45% trichloroacetic acid for 30 min at 4°C, then stained in 0.05% Coomassie blue R250 (Sigma). The addition of 0.02% fluorescamine (5 mg/ml in acetone) (Fluram; Roche Diagnostics Div., Hoffman La Roche Inc., Nutley, N. J.) to some of the electrophoresis samples after boiling allowed protein bands to be observed by their fluorescence when irradiated with 366-nm ultraviolet light during and after electrophoresis. Molecular weight markers were included in all gels. The marker mixture (Bio-Rad Laboratories) was composed of myosin (M_r 200,000), β-galactosidase (M_r 130,000), phosphorylase B (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 21,000), and lysozyme (M_r 14,300). Some gels included oxidized insulin B chain (M_r 3,600) (Sigma Chemical Co.).

Elastase Assay. Elastinolytic activity was measured by determining the amount of soluble radioactivity released from insoluble [^{3}H]elastin in the absence of SDS (14). Reaction mixtures were stabilized with 20 μg bovine serum albumin (Sigma Chemical Co.) and were incubated with 200 μg [^{3}H]elastin in 100 mM Tris-HCl, pH 8.0, which contained 5 mM $CaCl_2$ in a final volume of 300 μl. At the end of incubation, the reaction mixtures were centrifuged for 3 min in a Beckman Microfuge B (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and the radioactivity in 200 μl of supernate was determined by liquid scintillation spectrometry.

Incubation of ME with α_1PI. ME was incubated at 37°C with α_1PI (ME:α_1PI, 1:100 or 1:3.7 wt:wt), 18 mM Tris-HCl, pH 8.0, which contained 10 mM $CaCl_2$ and 30 mM NaCl, for times varying from 30 s to 8 h. When proteinase inhibitors were included (either α_2M:ME, 1:1 mol:mol; or 30 mM EDTA) they were preincubated with ME for 30 min at room temperature. The ME-dependent reactions were stopped by the addition of EDTA to a final concentration of 30 mM.

Incubation of HGE with α_1PI. HGE was incubated at 37°C with α_1PI (HGE:α_1PI, 1:5 wt:wt or 1:2.6 mol:mol) in 18 mM Tris-HCl, pH 8.0, which contained 10 mM $CaCl_2$ and 30 mM NaCl. When an inhibitor of HGE was included (SPCK:HGE, 6:1 mol:mol) it was preincubated with HGE for 30 min at room temperature. HGE-α_1PI reactions were stopped by the addition of SPCK (SPCK:HGE, 6:1 mol:mol), and the mixtures were analyzed on gels.

α_1PI degraded by ME was assayed for residual inhibitory activity toward HGE. ME-α_1PI reactions were stopped with 30 mM EDTA, and then aliquots (0.28 μg of α_1PI) were incubated with HGE (0.15 μg) for 30 min at room temperature and the elastinolytic activity of the mixture was determined by means of the ^{3}H-elastin assay.

Incubation of ME with HGE and α_1PI. α_1PI was incubated with HGE (5:1, wt:wt) for 30 min at room temperature and then incubated with ME (α_1PI:HGE:ME, 5:1:0.01 wt:wt:wt). Reactions were stopped by the addition of EDTA at a final concentration of 30 mM.

Results

Limited Proteolysis of α_1PI by ME. Reaction mixtures of ME-α_1PI were examined by SDS-polyacrylamide gel electrophoresis (Fig. 1). Coomassie blue staining of the gels demonstrated that purified α_1PI migrated as a band at M_r 58,000 even after a 6-h sham incubation. When ME and α_1PI were incubated for 6 h at a 1:100 (wt:wt) ratio, the α_1PI band migrated faster, at M_r 53,000–54,000. Staining of the reaction mixtures with fluorescamine revealed an M_r 4,000–5,000 fragment (data not shown) that was always associated with ME-degraded α_1PI.

In the ME-α_1PI reaction mixtures, there was no indication of the formation of a higher molecular weight proteinase-inhibitor complex stable in SDS. This distinguishes the degradation of α_1PI by ME from that attributed to some serine proteinases (23, 24). When α_1PI was incubated with an excess of pancreatic elastase, a low percentage of α_1PI was degraded only after the formation of a stable M_r 78,000 intermediate (23). However, in the present study, ME recognized α_1PI as an acceptable substrate and degraded it catalytically.

Inhibition of Proteolysis of α_1PI by ME. ME is a metalloproteinase that is inhibited by chelators of divalent cations, such as EDTA. Preincubation of ME with EDTA prevented degradation of α_1PI (Fig. 1). The inhibition of ME by stoichiometric amounts of the endopeptidase inhibitor, α_2M, also prevented degradation of α_1PI. The result of the interaction of α_2M with ME was the cleavage of an M_r 85,000

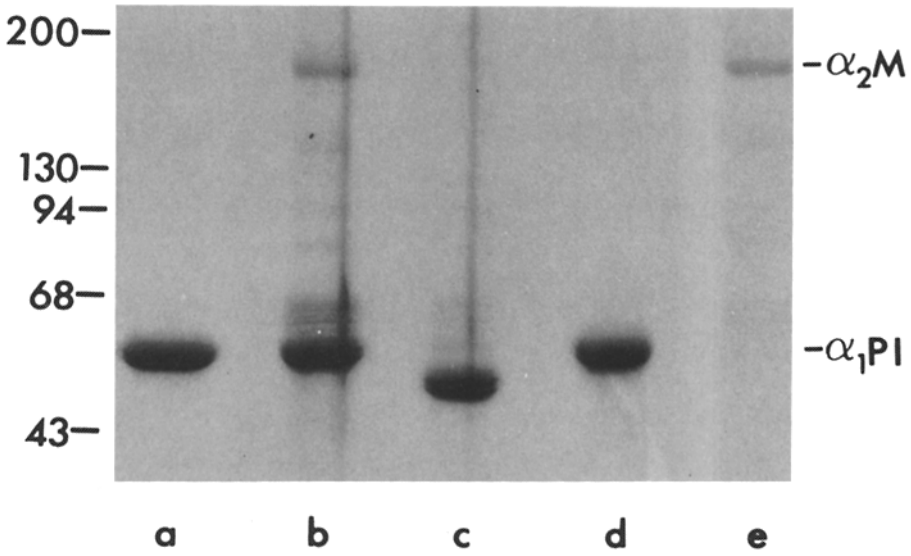

FIG. 1. Degradation of α_1PI by ME and the effect of EDTA and α_2M on the reaction. The samples in each lane were incubated at pH 8.0 for 6 h at 37°C in 18 mM Tris-HCl, 10 mM $CaCl_2$, and 30 mM NaCl that contained (a) 3 μg α_1PI, 0.03 μg ME, and 30 mM EDTA; (b) 3 μg α_1PI, 1 μg α_2M, and 0.03 μg ME (~1:1 mol:mol ME:α_2M); (c) 3 μg α_1PI and 0.03 μg ME; (d) 3 μg α_1PI; or (e) 1 μg α_2M. Samples were run on an SDS-polyacrylamide gradient gel (7–18%) under reducing conditions and stained with Coomassie blue. Molecular weight standards ($M_r \times 10^{-3}$) are indicated at the left.

fragment from α_2M. The effect of these inhibitors was consistent with the inhibitor profile of purified ME when elastin is used as a substrate.

Loss of Inhibitory Activity of α_1PI Degraded by ME. To determine whether α_1PI retained inhibitory activity after being degraded by ME, aliquots of ME-α_1PI reaction mixtures were incubated with HGE, and elastinolytic activity was determined by means of the ^{3}H-elastin assay. Direct elastinolytic activity by ME was eliminated before mixing with HGE by the addition of EDTA to the ME-α_1PI reaction mixtures at the end of the incubation times indicated in Fig. 2. This inhibited the metalloproteinase activity of ME but did not inhibit the serine proteinase activity of HGE. Fig. 3 indicates that only the intact α_1PI was inhibitory and that the loss of inhibitory capacity was proportional to the degree of degradation by ME. Degradation and the loss of inhibitory capacity were evident after only a 30-s incubation of α_1PI with ME.

Effect of ME on HGE-α_1PI Complexes. In contrast to ME, HGE forms a typical proteinase-inhibitor complex with α_1PI during incubation (Fig. 4). The HGE-α_1PI inhibitor complex requires an unoccupied HGE active site. When excess SPCK, a

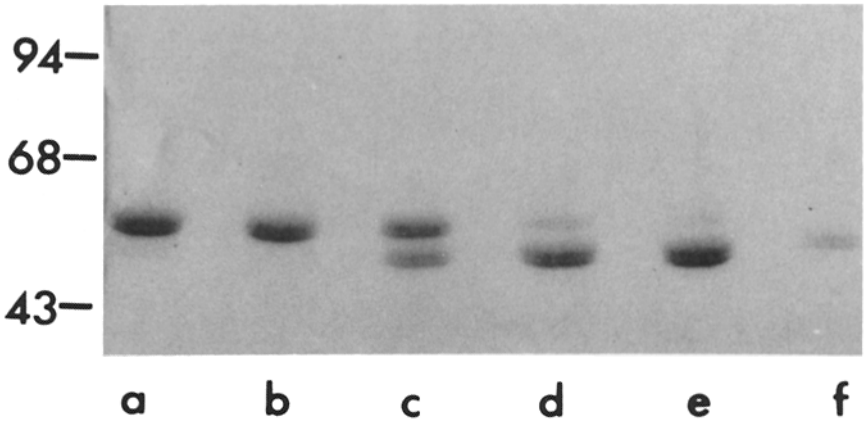

FIG. 2. Time-course of ME degradation of α_1PI. Each lane was loaded with aliquots of ME:α_1PI (1:100 wt:wt) reaction mixtures incubated in 18 mM Tris-HCl, 10 mM $CaCl_2$, and 20 mM NaCl, pH 8.0, at 37°C for: (a) 0 s; (b) 30 s; (c) 0.5 h; (d) 2 h; (e) 4 h; or (f) 8 h (lane f contained less protein than lanes a–e). Electrophoresis conditions were identical to those in Fig. 1. Molecular weight standards ($M_r \times 10^{-3}$) are indicated at the left.

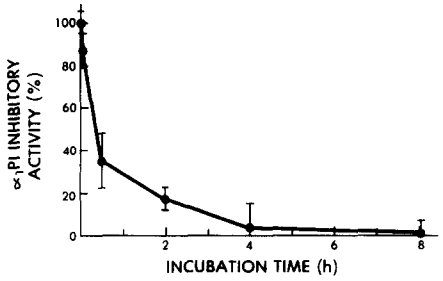

FIG. 3. Inhibitory activity of ME-degraded α_1PI. Reaction mixtures of ME:α_1PI (1:100 wt:wt) were assayed for residual α_1PI inhibitory activity against HGE, as described in Materials and Methods. The zero-time activity is that of unreacted α_1PI normalized to 100%. The reaction mixtures were identical to those analyzed by electrophoresis in Fig. 2.

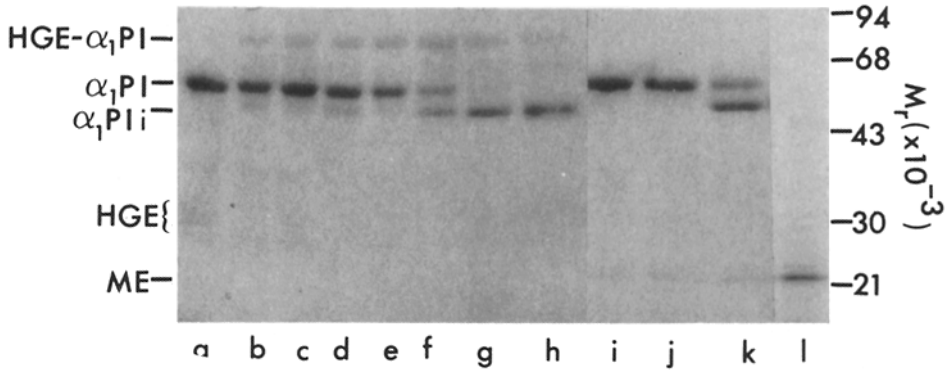

FIG. 4. Interaction of HGE with α_1PI and the effect of ME on the HGE-α_1PI complex. All reactions were carried out in 18 mM Tris-HCl, 10 mM $CaCl_2$, 30 mM NaCl, pH 8.0, at 37°C. Experimental details are described in Materials and Methods. Lane (a) contained 0.5 μg HGE (inactivated with 0.2 μg SPCK) and 1.75 μg α_1PI incubated for 15 h. Lanes (b–d) contained 0.5 μg HGE and 1.75 μg α_1PI incubated for (b) 0 h; (c) 0.5 h; or (d) 15 h. Lanes (e–h) contained 0.5 μg HGE and 1.75 μg α_1PI incubated at room temperature for 0.5 h before the addition of 0.018 μg ME and further incubation at 37°C for (e) 10 s; (f) 2 h; (g) 5 h; or (h) 15 h. Lanes (i–k) contained stoichiometric concentrations of ME (0.46 μg) and α_1PI (1.75 μg) incubated for (i) 15 h with 30 mM EDTA, or incubated alone for (j) 0 h or (k) 0.5 h. Lane (l) contained ME alone (2 μg). Electrophoresis conditions were identical to those in Fig. 1. HGE-α_1PI indicates the position of the stable proteinase-inhibitor complex. α_1PI_i indicates the position of the degraded α_1PI.

specific active site-directed inhibitor of granulocyte elastase (21), was preincubated with HGE before addition of α_1PI, no proteinase-inhibitor complex was formed.

To test the effect of ME on HGE-α_1PI complexes, HGE was incubated with excess α_1PI before incubation with ME. During the course of incubation (Fig. 4), ME preferentially degraded the free α_1PI rather than the HGE-α_1PI complex. Only after apparently complete degradation of free α_1PI was there any suggestion of ME cleavage of the HGE-α_1PI complex. ME did not release any detectable HGE elastinolytic activity from the HGE-α_1PI complex. It is therefore unlikely that ME will interfere with the fate of proteinase-α_1PI complexes.

Macrophage elastase degraded α_1PI even at stoichiometric ratios. Comparison of reaction mixtures of α_1PI and ME at catalytic ratios (Fig. 2) and at stoichiometric ratios (Fig. 4) shows similar degradation without the formation of a proteinase-inhibitor complex.

Discussion

The data presented here establish that a metalloproteinase secreted by inflammatory mouse macrophages catalytically inactivates α_1PI. This mechanism of destruction of α_1PI is independent of the oxidative attenuation of α_1PI by cigarette smoke, *N*-chlorosuccinimide, chloramine T, or the reactive oxygen species produced by phagocytes in which the metabolic burst has been stimulated (8–11). The ability of ME to inactivate α_1PI may be an important consideration when evaluating the role of inflammatory macrophages in chronic diseases such as pulmonary emphysema. Because the ratio of ME activity to granulocyte elastase activity in the lung is believed to be very low (25), the direct involvement of ME in elastinolysis during emphysema has been questioned. However, ~85% of mouse ME is secreted in an inactive form (14, 15), and the activation of elastase would increase its involvement in elastinolysis.

Our data suggest another role for the active ME in connective tissue destruction. Because α_1PI inactivated by ME would no longer protect lung elastin from proteolytic degradation by granulocyte elastase, macrophages and active ME may play a pivotal role in lung pathology.

The inhibition of ME by α_2M is not likely to abrogate the potential physiologic importance of these findings. Although α_1PI is plentiful in the lung, α_2M has been detected only in trace quantities in lung lavage fluids (26).

The inactivation of α_1PI by other enzymes has been studied. Thiol proteinases such as cathepsin B (27) and papain (28) are not inhibited by α_1PI. This characteristic of papain is likely to have been responsible for its favored use in animal model systems designed to study the development of emphysema (7, 29). More recently, it has been shown that thiol proteinases mediate lung damage not only because they are resistant to α_1PI, but because they degrade it (30).

Proteolytic inactivation of α_1PI is not limited to thiol proteinases. The culture supernates from *Pseudomonas aeruginosa* have also been shown to inactivate α_1PI (31). This activity has been attributed to the pseudomonal elastase (32), which is a neutral metalloproteinase. Other prokaryotic metalloproteinases have similarly been shown to inactivate α_1PI by degradation (32). It has also been demonstrated that eukaryotic metalloproteinases, such as those found in the venoms of Crotalidae, Viperidae, and Colubridae (33, 34) can degrade and inactivate α_1PI.

Oxidation of reactive site methionyl residues in α_1PI (8, 9) and proteolytic cleavage of α_1PI by bacterial proteinases (31, 32) have significant potential to mediate the degradation of lung elastin. Curiously, the degradation of α_1PI by cathepsin B occurs only at stoichiometric concentrations (30). If this observation is an accurate representation of cathepsin B-α_1PI inactivation in vivo, cathepsin B is unlikely to be an initiator of α_1PI inactivation.

The ability to mediate lung degradation by both direct and indirect elastinolysis is not unique to the elastase secreted by inflammatory macrophages. Indeed, the elastinolysis resulting from a *P. aeruginosa* pulmonary infection may be the result of both the pseudomonal elastase activity and the unchecked activity of endogenous granulocyte proteinases (31, 32). The inactivation of α_1PI by the metalloproteinases of that organism would disrupt the critical proteinase-inhibitor balance in the lungs and permit contributive elastinolysis by endogenous granulocyte elastase (9, 10). Because ME recognizes α_1PI as a substrate for proteolysis, inflammatory macrophages may also disrupt the proteinase-inhibitor balance, resulting in connective tissue destruction. The two organs, liver and lung, with pathology associated with an insufficiency of α_1PI have substantial populations of macrophages, and thus may be exquisitely sensitive to an imbalance of the serine proteinase:α_1PI ratio.

Summary

Inflammatory mouse peritoneal macrophages secrete a metalloproteinase that is not inhibited by α_1-proteinase inhibitor. This proteinase, macrophage elastase, recognizes α_1-proteinase inhibitor as a substrate and catalytically degrades it. The interaction of α_1-proteinase inhibitor with macrophage elastase does not involve a stable proteinase-inhibitor complex and results in the proteolytic removal of a peptide of apparent molecular weight 4,000–5,000 from the inhibitor. After degradation by macrophage elastase, α_1-proteinase inhibitor is no longer able to inhibit human

granulocyte elastase, a serine proteinase implicated in the pathogenesis of emphysema. Macrophage elastase apparently does not degrade human granulocyte elastase-α_1-proteinase inhibitor complexes or release active granulocyte elastase from these complexes. The ability of macrophage elastase to degrade α_1-proteinase inhibitor is inhibited by EDTA and α_2-macroglobulin.

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