LIMITED PROTEOLYSIS BY MACROPHAGE ELASTASE INACTIVATES HUMAN $\alpha_1$-PROTEINASE INHIBITOR*

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Ever since the initial description of $\alpha_1$-proteinase inhibitor (1) in disease and in healthy physiology has been the subject of much investigation (2). $\alpha_1$PI 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 $\alpha_1$PI 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 $\alpha_1$PI and serine proteinases from granules of polymorphonuclear leukocytes. Because an imbalance in the ratio between $\alpha_1$PI 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 $\alpha_1$PI-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 $\alpha_1$PI 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 $\alpha_1$PI-proteinase balance. We have found that the purified form of macrophage elastase catalytically degrades and inactivates $\alpha_1$PI 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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$\alpha_1$PI-Proteinase inhibitor is also known as $\alpha_1$-trypsin inhibitor and $\alpha_1$-antitrypsin.

Abbreviations used in this paper: $\alpha_2$M, $\alpha_2$-macroglobulin; $\alpha_1$PI, $\alpha_1$-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 $\alpha$PI, 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, $\alpha$PI 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 $\alpha_2$-macroglobulin ($\alpha_2$M) was purified from fresh haptoglobin type 1-1 serum by Cibacron blue-Sepharose 4B affinity chromatography (18). The purified $\alpha_2$M gave a single line of identity against rabbit anti-human whole serum (Bio-Rad Laboratories, Richmond, Calif.) and rabbit anti-human $\alpha_2$M (Bio-Rad Laboratories). It was $>95\%$ active when calibrated by inhibition of active site-titrated trypsin (Worthington Biochemical Corp., Freehold, N. J.). $\alpha_2$M migrated as a single band 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 $\alpha_2$M protein. Another band at $M_r$ 85,000 was the result of interaction of proteinases with $\alpha_2$M.

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 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), $\beta$-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 [3H]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 [3H]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 $\alpha$PI. ME was incubated at 37°C with $\alpha$PI (ME:$\alpha$PI, 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 $\alpha_2$M: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 $\alpha$PI. HGE was incubated at 37°C with $\alpha$PI (HGE:$\alpha$PI, 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-$\alpha$PI reactions were stopped by the addition of SPCK (SPCK:HGE, 6:1 mol:mol), and the mixtures were analyzed on gels.

$\alpha$PI degraded by ME was assayed for residual inhibitory activity toward HGE. ME-$\alpha$PI reactions were stopped with 30 mM EDTA, and then aliquots (0.28 µg of $\alpha$PI) 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 [3H]elastin assay.
Incubation of ME with HGE and α1PI. α1PI was incubated with HGE (5:1, wt:wt) for 30 min at room temperature and then incubated with ME (α1PI: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 α1PI by ME. Reaction mixtures of ME-α1PI were examined by SDS-polyacrylamide gel electrophoresis (Fig. 1). Coomassie blue staining of the gels demonstrated that purified α1PI migrated as a band at Mr 58,000 even after a 6-h sham incubation. When ME and α1PI were incubated for 6 h at a 1:100 (wt:wt) ratio, the α1PI band migrated faster, at Mr 53,000–54,000. Staining of the reaction mixtures with fluorescamine revealed an Mr 4,000–5,000 fragment (data not shown) that was always associated with ME-degraded α1PI.

In the ME-α1PI 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 α1PI by ME from that attributed to some serine proteinases (23, 24). When α1PI was incubated with an excess of pancreatic elastase, a low percentage of α1PI was degraded only after the formation of a stable Mr 78,000 intermediate (23). However, in the present study, ME recognized α1PI as an acceptable substrate and degraded it catalytically.

Inhibition of Proteolysis of α1PI 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 α1PI (Fig. 1). The inhibition of ME by stoichiometric amounts of the endopeptidase inhibitor, α2M, also prevented degradation of α1PI. The result of the interaction of α2M with ME was the cleavage of an Mr 85,000...
fragment from α2M. 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 α1PI Degraded by ME. To determine whether α1PI retained inhibitory activity after being degraded by ME, aliquots of ME-α1PI reaction mixtures were incubated with HGE, and elastinolytic activity was determined by means of the 3H-elastin assay. Direct elastinolytic activity by ME was eliminated before mixing with HGE by the addition of EDTA to the ME-α1PI 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 α1PI 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 α1PI with ME.

Effect of ME on HGE-α1PI Complexes. In contrast to ME, HGE forms a typical proteinase-inhibitor complex with α1PI during incubation (Fig. 4). The HGE-α1PI inhibitor complex requires an unoccupied HGE active site. When excess SPCK, a

Fig. 2. Time-course of ME degradation of α1PI. Each lane was loaded with aliquots of ME:α1PI (1:100 wt:wt) reaction mixtures incubated in 18 mM Tris-HCl, 10 mM CaCl₂, 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, × 10⁻³) are indicated at the left.

Fig. 3. Inhibitory activity of ME-degraded α1PI. Reaction mixtures of ME:α1PI (1:100 wt:wt) were assayed for residual α1PI inhibitory activity against HGE, as described in Materials and Methods. The zero-time activity is that of unreacted α1PI normalized to 100%. The reaction mixtures were identical to those analyzed by electrophoresis in Fig. 2.
specific active site-directed inhibitor of granulocyte elastase (21), was preincubated with HGE before addition of α1PI, no proteinase-inhibitor complex was formed.

To test the effect of ME on HGE-α1PI complexes, HGE was incubated with excess α1PI before incubation with ME. During the course of incubation (Fig. 4), ME preferentially degraded the free α1PI rather than the HGE-α1PI complex. Only after apparently complete degradation of free α1PI was there any suggestion of ME cleavage of the HGE-α1PI complex. ME did not release any detectable HGE elastinolytic activity from the HGE-α1PI complex. It is therefore unlikely that ME will interfere with the fate of proteinase-α1PI complexes.

Macrophage elastase degraded α1PI even at stoichiometric ratios. Comparison of reaction mixtures of α1PI 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 α1PI. This mechanism of destruction of α1PI is independent of the oxidative attenuation of α1PI 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 α1PI 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 elastolysis 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 elastolysis.
Our data suggest another role for the active ME in connective tissue destruction. Because α1PI 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 α2M is not likely to abrogate the potential physiologic importance of these findings. Although α1PI is plentiful in the lung, α2M has been detected only in trace quantities in lung lavage fluids (26).

The inactivation of α1PI by other enzymes has been studied. Thiol proteinases such as cathepsin B (27) and papain (28) are not inhibited by α1PI. 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 α1PI, but because they degrade it (30).

Proteolytic inactivation of α1PI is not limited to thiol proteinases. The culture supernates from Pseudomonas aeruginosa have also been shown to inactivate α1PI (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 α1PI 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 α1PI.

Oxidation of reactive site methionyl residues in α1PI (8, 9) and proteolytic cleavage of α1PI by bacterial proteinases (31, 32) have significant potential to mediate the degradation of lung elastin. Curiously, the degradation of α1PI by cathepsin B occurs only at stoichiometric concentrations (30). If this observation is an accurate representation of cathepsin B-α1PI inactivation in vivo, cathepsin B is unlikely to be an initiator of α1PI 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 α1PI 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 α1PI 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 α1PI have substantial populations of macrophages, and thus may be exquisitely sensitive to an imbalance of the serine proteinase:α1PI ratio.

Summary

Inflammatory mouse peritoneal macrophages secrete a metalloproteinase that is not inhibited by α1-proteinase inhibitor. This proteinase, macrophage elastase, recognizes α1-proteinase inhibitor as a substrate and catalytically degrades it. The interaction of α1-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, α1-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-α1-proteinase inhibitor complexes or release active granulocyte elastase from these complexes. The ability of macrophage elastase to degrade α1-proteinase inhibitor is inhibited by EDTA and α2-macroglobulin.

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