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Research Article

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Proteolytic Inactivation of Alpha-1-Proteinase Inhibitor by a Neutrophil Metalloproteinase

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Abstract

Human neutrophils triggered with phorbol myristate acetate or opsonized zymosan particles released a metalloproteinase (MP) capable of cleaving and inactivating alpha-1-proteinase inhibitor (α-1-PI). Sequence analysis of the amino acids in proteolyzed, native α-1-PI revealed a unique single cleavage site between Phe-352 and Leu-353. An analysis of the process regulating the enzyme's activity revealed that the neutrophil MP was released from cells in a latent form whose activation was tightly linked to the generation of hypochlorous acid. These results indicate that human neutrophils use chlorinated oxidants to activate a latent MP that is capable of proteolytically inactivating α-1-PI by cleaving the antiproteinase at a unique point in its inhibitory site region.

Introduction

Human neutrophils can exert powerful proinflammatory effects by releasing elastase, a serine proteinase capable of altering vascular permeability, mediating cytotoxicity, and digesting a wide variety of connective tissue components (1, 2). In vivo, neutrophil elastase released into the extracellular milieu is normally regulated by alpha-1-proteinase inhibitor (α-1-PI),[1] a plasma antiproteinase that irreversibly inactivates the proteinase by forming a 1:1 complex (3). Nonetheless, triggered neutrophils can penetrate the antiproteinase shield in vitro by using the H_2O_2-myeloperoxidase-Cl^- system to generate chlorinated oxidants capable of attacking a critical methionine at α-1-PI's inhibitory site (1–3). The in vivo pathologic significance of such a process has been strengthened by the fact that oxidized α-1-PI and free neutrophil elastase activity have been detected in fluids recovered from inflamed sites (4). However, proteolyzed α-1-PI has also been identified in these samples (4), and this has led to the suggestion that the proteolytic inactivation of the antiproteinase may also play an important role in promoting neutrophil elastase-dependent damage (5). To date, only two mammalian enzymes, both of macrophage origin, are known to express α-1-PI-cleaving activity, cathepsin L, and macrophage elastase (5–7). The role of these enzymes in human pathophysiology is unclear, however because cathepsin L is a lysosomal cysteine proteinase with a pH optimum of ~ 5.0 (6) and only animal macrophage elastases have been shown to cleave human α-1-PI under physiological conditions (5, 7). In this study, we demonstrate that human neutrophils themselves have the unexpected ability to release and oxidatively activate a latent metalloproteinase (MP) that destroys native α-1-PI activity by cleaving the antiproteinase at a unique point in its reactive site region.

Methods

Cell preparation. Neutrophils were isolated from the peripheral venous blood of either normal donors or a patient with chronic granulomatous disease (CGD) by Ficoll-Hypaque density centrifugation followed by dextran sedimentation (2). Cells were suspended in HBSS (pH 7.4; Gibco Laboratories, Grand Island, NY).

Incubation conditions. Cells (10^7/ml) were incubated alone or with 50 ng/ml phorbol myristate acetate (PMA) or 2.5 mg/ml zymosan particles opsonized with autologous serum (8) for 15 min at 37°C. Other additions to the incubation mixture included SOD (2,800 U/mg), glucose oxidase (1,400 U/ml), catalase (65,000 U/mg) (Cooper Biomedical, Inc., Malvern, PA), azide or L-methionine. The cells were then pelleted (1,000 *g*, 5 min), and 0.1-ml aliquots of the supernatant were removed and incubated alone or with either 1 mM PMSF, 0.1 mM *N*-ethyl-maleimide, 0.3 mM E-64, 1 mM 4-aminophenylmercuric acetate, 10 mM EDTA, 1 mM *o*-phenanthroline, or purified human fibroblast tissue inhibitor of metalloproteinases (TIMP; prepared and kindly provided by W. A. Galloway, G. D. Searle, High Wycombe, United Kingdom) for 30 min. All samples were then treated with 100 nmol methionine to reduce *N*-chloroamines in the cell-free supernatants (8) before varying amounts of either purified human α-1-PI (obtained commercially from Calbiochem-Behring Corp., La Jolla, CA, or from N. Matheson and J. Travis, University of Georgia) or oxidized α-1-PI (prepared as described in reference 2) were added. The final mixtures (0.3 ml) were then incubated for indicated periods of time at 37°C. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

Analyses of α-1-PI. After incubation of native or oxidized α-1-PI alone or with cell-free supernatants, the antiproteinase was analyzed by

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1. *Abbreviations used in this paper:* α-1-PI, alpha-1-proteinase inhibitor; CGD, chronic granulomatous disease; MP, metalloproteinase; PMA, phorbol myristate acetate; TIMP, tissue inhibitor of metalloproteinases.

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SDS-PAGE, by NH_2-terminal sequence analysis or for elastase inhibitory capacity.

Equivalent amounts of native, oxidized, or proteolyzed α-1-PI (15 μg final) were analyzed by SDS-PAGE directly or after incubation with purified neutrophil elastase (Elastin Products Co., Inc., Pacific, MO) using a 3% polyacrylamide stacking gel and a 7.5% polyacrylamide resolving gel as described (2). Protein bands were visualized with Coomassie Brilliant Blue. Under these conditions, native and oxidized α-1-PI comigrated with an apparent M_r of ~ 58,000 as reported previously (2, 5).

For sequence analysis, samples of native, oxidized, or proteolyzed α-1-PI were dialyzed against water, lyophilized, and sequenced according to the method of Tarr (9).

The neutrophil elastase inhibitory capacity of native or proteolyzed α-1-PI was determined as previously described (2), except that human neutrophil elastase was used in place of porcine pancreatic elastase and methoxysuccinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide (Calbiochem-Behring Corp.) was used as the elastase substrate (2).

Results

Proteolysis of α-1-PI by triggered neutrophils. As shown in Fig. 1, α-1-PI incubated alone (lane *1*) or with supernatants recovered from resting cells (lane *2*) migrated as a single major band (M_r = 58,000). However, if supernatants were obtained from cells that were incubated with PMA (lane *3*) or opsonized zymosan (lane *4*), the antiproteinase was cleaved to yield a major fragment (M_r = 54,000). Supernatants obtained from triggered monocytes or eosinophils did not cleave α-1-PI (data not shown).

If cell-free supernatants obtained from PMA- or zymosan-triggered neutrophils were first treated with the serine proteinase inhibitor, PMSF, and then incubated with α-1-PI, proteolysis was unchanged (lanes *5* and *6*). Likewise, the addition of the thiol proteinase inhibitors, *N*-ethylmaleimide or E-64, had no effect on proteolysis (data not shown). If, however supernatants were first treated with either EDTA or *o*-phenanthroline, proteolysis was completely inhibited (lanes *7* and *8*). Because these results are consistent for the involvement of a neutral MP, supernatants were incubated with TIMP, a 28.5-kD glycoprotein known to inhibit a variety of MPs (10), including those released by the human neutrophil (11). As shown in lane *9*, 1.0 μg TIMP completely blocked α-1-PI proteolysis. Together, these results indicate that a neutrophil-derived MP is responsible for α-1-PI cleavage.

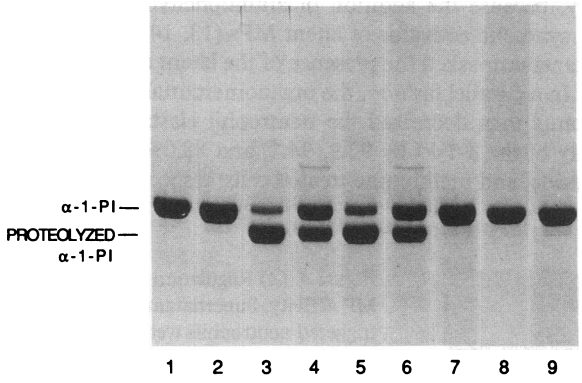

Figure 1. Proteolytic cleavage of α-1-PI by neutrophil MP. 0.1-ml supernatants from resting or triggered neutrophils were treated as described, incubated with 100 μg of α-1-PI for 20 h at 37°C and analyzed by SDS-PAGE. The α-1-PI was incubated alone (lane *1*), with supernatants from resting neutrophils (lane *2*), or neutrophils stimulated with PMA (lane *3*) or opsonized zymosan (lane *4*). Supernatants from neutrophils triggered with PMA (lane *5*) or opsonized zymosan (lane *6*) were also preincubated with 1 mM PMSF before α-1-PI was added. In lanes *7–9*, supernatants from PMA-triggered cells were treated with either 10 mM EDTA, 1 mM *o*-phenanthroline, or 1 μg TIMP, respectively.

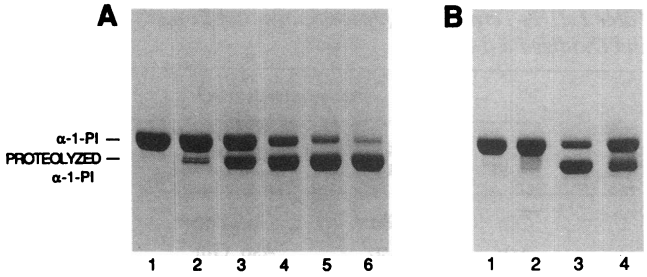

Figure 2. (*A*) Time course of proteolytic cleavage of α-1-PI. Supernatants from PMA-triggered neutrophils were incubated with 100 μg of α-1-PI for 0, 1, 5, 10, 20, and 40 h (lanes *1–6*, respectively) at 37°C and analyzed by SDS-PAGE. (*B*) Proteolysis of native vs. oxidized α-1-PI. 100 μg native or oxidized α-1-PI was incubated alone (lanes *1* and *2*, respectively) or with PMSF-treated supernatants from PMA-triggered neutrophils (lanes *3* and *4*, respectively) for 20 h at 37°C and analyzed by SDS-PAGE.

An analysis of the time course of proteolysis (Fig. 2 *A*) revealed that detectable cleavage occurs at 1 h with almost complete degradation occurring by 40 h. These kinetics are similar, if not identical, to those recently reported for the mouse macrophage elastase (7). In contrast to the macrophage elastase, however, which recognized oxidized α-1-PI only as a poor substrate (7), the neutrophil MP readily cleaved the oxidized antiproteinase, albeit at a slower rate than that observed with native α-1-PI (Fig. 2 *B*).

Inactivation of α-1-PI by the neutrophil MP. To determine if the cleaved α-1-PI had also lost its elastase inhibitory capac-

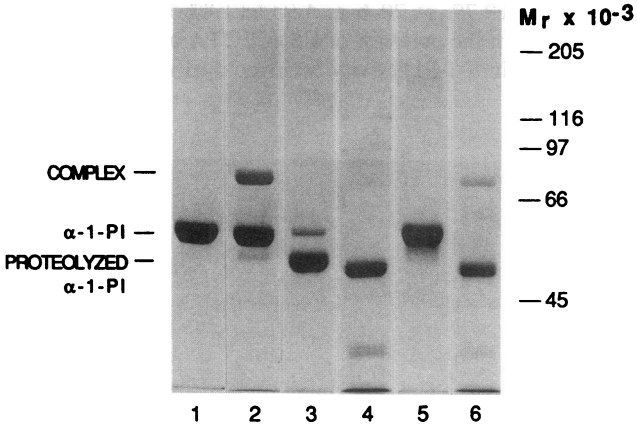

Figure 3. Inactivation of α-1-PI by proteolytic cleavage. 30 μg of native α-1-PI (lane *1*), α-1-PI cleaved by the neutrophil MP for 20 h (lane *3*), or oxidized α-1-PI (lane *5*) were incubated alone or with 7.5 μg purified neutrophil elastase (lanes *2*, *4*, and *6*, respectively) for 2 h at 37°C and analyzed by SDS-PAGE.

Table I. NH_2-Terminal Sequence Analysis of Proteolyzed Native and Oxidized α-1-PI

Cycle	Amino acids detected‡			
	Native α-1-PI	Oxidized α-1-PI	Native α-1-PI + MP	Oxidized α-1-PI + MP
1	Glu	Glu	Glu, Leu	Glu, Leu
2	Asp	Asp	Asp, Glu	Asp, Glu
3	Pro	Pro	Pro, Ala	Pro, Ala
4	Gln	Gln	Gln, Ile	Gln, Ile
5	Gly	Gly	Gly, Pro	Gly, Pro
6	Asp	Asp	Asp, Met	Asp, Met
7	Ala	Ala	Ala, Ser	Ala, Ser
8	Ala	Ala	Ala, Ile	Ala, Ile
9	Gln	Gln	Gln, Pro	Gln, Pro
10	Lys	Lys	Lys, Pro	Lys, Pro

* Results are from a single representative analysis of two performed.
‡ Repetitive efficiency was >93%.

ity, proteolyzed α-1-PI was incubated with purified neutrophil elastase. As shown in Fig. 3, native α-1-PI (lane *1*) formed the expected complex with neutrophil elastase (lane *2*). However, when neutrophil elastase was incubated with α-1-PI that had been almost completely cleaved by the neutrophil MP (lane *3*), no complex could be detected and the M_r = 54,000 band was further degraded (lane *4*). Identical results were obtained with porcine pancreatic elastase (data not shown). This final product comigrated with the major fragment generated after incubation of oxidized α-1-PI (lane *5*) with neutrophil elastase in which small amounts of the oxidized α-1-PI–neutrophil elastase complex can be detected along with M_r = 53,000–54,000 fragment (lane *6*) (3). In the presence of free neutrophil elastase, the cleaved or oxidized α-1-PI thus were further proteolyzed and migrated as indistinguishable products.

Quantitative analyses revealed that 0.1 ml supernatant obtained from PMA-triggered neutrophils (10^7/ml) decreased the neutrophil elastase inhibitory capacity of α-1-PI (100 μg/0.3 ml) by 77.9±9.7% at 20 h and 93.6±1.8% at 40 h (n = 4, mean±SD). In the presence of either EDTA or *o*-phenanthroline, no loss in α-1-PI activity occurred during the 20-h incubation with the triggered neutrophil supernatant (n = 2). Finally, when the α-1-PI concentration was increased to the physiologic range (i.e., 450 μg/0.3 ml or ~ 1.5 mg/ml), the MP degraded 428±24 μg (n = 4) of the antiproteinase at 20 h.

Identification of the α-1-PI cleavage site. Human α-1-PI contains an amino acid sequence in a stressed loop configuration between residues 350 and 358 (12). Cleavage by a variety of enzymes, including bacterial, plant, and reptilian as well as mammalian proteinases within this sequence leads to an irreversible loss of antiproteinase activity (5–7). To determine the site of antiproteinase cleavage by the neutrophil MP, proteolyzed native and oxidized α-1-PI were examined by NH_2-terminal sequence analysis. As shown in Table I, sequence analyses identified two NH_2-terminals for the MP-inactivated native α-1-PI and MP-cleaved oxidized α-1-PI. In both cases, the cleavage site was identified as occurring between Phe-352 and Leu-353. Cleavage at this site would yield an α-1-PI fragment whose molecular weight would be decreased by 4.5×10^3, a result consistent with results obtained by SDS-PAGE.

Regulation of neutrophil MP activity. Recently, we demonstrated that neutrophils are able to activate the endogenous latent MPs, collagenase and gelatinase, by a process linked to their ability to generate hypochlorous acid (HOCl) via the H_2O_2-myeloperoxidase-Cl^- system (13, 14). To determine whether the α-1-PI–cleaving MP is regulated by a similar process, neutrophils were triggered in the presence of SOD, which dismutates O_2^- to H_2O_2; catalase, which reduces H_2O_2; azide, which inhibits myeloperoxidase; or methionine, which scavenges HOCl. As shown in Fig. 4 *A*, supernatants recovered from cells triggered in the absence or presence of SOD, readily cleaved α-1-PI (lanes *2* and *3*). However, when cells were triggered in the presence of native (but not heat-inactivated) catalase, azide, or methionine, proteolysis was almost completely inhibited (lanes *4–6*) and no loss in the neutrophil elastase inhibitory capacity of the α-1-PI could be detected (compared with a loss of 77.4% for the PMA-triggered cells; n = 2). None of these agents interfered with the release of the MP or its activity, because the addition of aminophenylmercuric acetate, a synthetic activator of latent MPs (13, 14), to these supernatants unmasked the presence of the latent enzyme (lanes *7–10*). In a parallel fashion, the organomercurial-activated supernatants then decreased the neutrophil elastase inhibitory capacity of the α-1-PI by 92.9, 94.1, and 88.0% for the catalase-, azide, and methionine-treated cells, respectively (n = 2).

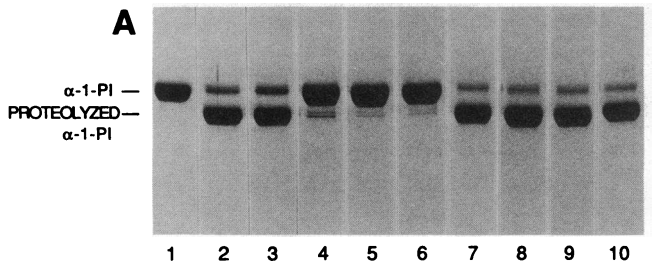

Figure 4. (*A*) Regulation of neutrophil MP activity. Supernatants from PMA-triggered neutrophils were incubated with 100 μg α-1-PI for 20 h at 37°C and analyzed by SDS-PAGE. α-1-PI incubated alone (lane *1*) or with supernatants from neutrophils incubated with PMA (lane *2*), PMA and 10 μg/ml SOD (lane *3*), PMA and 25 μg/ml catalase (lane *4*), PMA and 1 mM azide (lane *5*), or PMA and 5 mM methionine (lane *6*). Supernatants from neutrophils triggered with PMA alone, PMA and catalase, PMA and azide, or PMA and methionine were also incubated with aminophenylmercuric acetate for 30 min before α-1-PI was added (lanes *7–10*, respectively). (*B*) Proteolysis of α-1-PI by CGD neutrophils. CGD neutrophils were triggered with PMA alone (lane *1*), triggered with PMA and the supernatant (0.1 ml) treated with aminophenylmercuric acetate (lane *2*), or triggered with PMA in the presence of 15 mU/ml glucose oxidase, treated with catalase and then incubated with 100 μg α-1-PI for 20 h at 37°C (lane *3*).

Finally, the neutrophil's reliance on the oxidative pathway for MP activation was independently confirmed by the inability of PMA-triggered CGD neutrophils to cleave α-1-PI unless the supernatant was treated with aminophenylmercuric acetate or the cells were triggered in the presence of an exogenous source of H_2O_2 (Fig. 4 *B*).

Discussion

We have demonstrated that human neutrophils are able to inactivate α-1-PI by releasing an MP that cleaves α-1-PI in its proteinase-sensitive region (i.e., residues 350–358). Although we are aware of no other enzyme that attacks native α-1-PI at this site, the MP is qualitatively and quantitatively similar in action to murine or rabbit macrophage elastase (5, 7). The macrophage metalloenzyme has recently been reported to cleave native, human α-1-PI near its inhibitory site between Pro-357 and Met-358 (7). In contrast, oxidized α-1-PI was cleaved between Phe-352 and Leu-353 (7), which is at the same site at which the neutrophil MP attacked both the native and oxidized antiproteinase. Thus, two different inflammatory cell-derived metalloenzymes have now been described that can inactivate native α-1-PI. Note, however, that (*a*) macrophage elastase was isolated only from animal cells, whereas the α-1-PI substrate was of human origin (5, 7) and (*b*) in contrast to animal cells, human macrophages secrete only trace quantities of elastase and the human MP has not been shown to cleave α-1-PI (15). Banda et al. have suggested that macrophages could play a unique, cooperative role in tissue-destructive events by proteolytically depleting the α-1-PI shield and allowing neutrophil elastase to degrade substrates in an unmolested manner (5, 7). However, our results indicate that neutrophils themselves can catalyze an almost identical proteolytic inactivation of α-1-PI independently of any accessory cell population.

Based on the fact that two better characterized neutrophil MPs, collagenase and gelatinase, are stored in latent forms and activated by a process linked to HOCl generation (13, 14), we sought to determine whether a similar process applied to the regulation of the α-1-PI–cleaving MP. Indeed, if neutrophils were triggered under conditions that either prevented HOCl generation or intercepted HOCl, the MP was released, but not activated. A key role for HOCl in the activation process was independently confirmed with CGD neutrophils that failed to activate their released MP unless an exogenous source of H_2O_2 was provided. Thus, HOCl not only regulates the collagenolytic and gelatinolytic activity of the neutrophil, but its α-1-PI–cleaving activity as well. Together, these findings reinforce our contention that neutrophils are able to transform the short-acting and nonspecific effects mediated by reactive oxygen metabolites into long-acting and specific effects by using oxidants to regulate a complex array of endogenous proteolytic activities.

The identity of the MP responsible for α-1-PI proteolysis remains unknown, but neither collagenase nor gelatinase has been demonstrated to cleave between Phe-Leu sequences (16). Although we cannot rule out the possibility that either of these MPs possesses unexpected activities, we have found that neutrophils incubated with FMLP and cytochalasin released both collagenase and gelatinase, but only gelatinase was substantially activated and the supernatants expressed only small amounts of α-1-PI-cleaving activity (unpublished observation). Significant collagenolytic or α-1-PI–cleaving activity could only be detected in these supernatants if aminophenylmercuric acetate was added (unpublished observation). These findings serve to link the activation of collagenase, rather than gelatinase, more closely to the activity of α-1-PI inactivator. Whether this relationship reflects the dual activities of a single MP or the coordinate regulation of two different MPs is the focus of ongoing investigations.

In combination with earlier reports from our group as well as others (1–3), the results presented in this report clearly demonstrate that neutrophils can use chlorinated oxidants to inactivate α-1-PI by two distinct mechanisms: (*a*) by directly oxidizing Met-358 at the inhibitory site of α-1-PI, or (*b*) by indirectly activating a latent MP that cleaves between Phe-352 and Leu-353. α-1-PI thus has two regions susceptible to neutrophil attack. Although one might predict that multiple forms of inactive α-1-PI might be detected in vivo (4), it should be noted that either oxidized or MP-cleaved α-1-PI can be degraded by neutrophil elastase to yield a fragment that migrates similarly on SDS-PAGE (Fig. 3). In addition, our findings suggest that α-1-PI mutants that are specifically bioengineered to resist only direct oxidative inactivation (e.g., Met-358 → Val) may be less effective in attenuating neutrophil-mediated damage in vivo than originally predicted (17). Understandably, no studies have yet examined biological fluids for the α-1-PI–cleaving MP. However, recent reports have demonstrated that active myeloperoxidase and neutrophil collagenase can be detected in lavage fluids recovered from inflamed lungs (18, 19). Together, these findings strongly suggest that in disease states an environment can be created that is conducive to the oxidative activation of neutrophil MPs as well as the attendant pathological consequences.

Acknowledgments

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Note added in proof. A preliminary report by Vissers et al. has also described an α-1-PI-cleaving MP released from neutrophils (20).

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