Inhibition of Neutrophil Elastase by α1-Protease Inhibitor at the Surface of Human Polymorphonuclear Neutrophils

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Inhibition of Neutrophil Elastase by α1-Protease Inhibitor at the Surface of Human Polymorphonuclear Neutrophils

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The uncontrolled proteolytic activity in lung secretions during lung inflammatory diseases might be due to the resistance of membrane-bound proteases to inhibition. We have used a new fluorogenic neutrophil elastase substrate to measure the activity of free and membrane-bound human neutrophil elastase (HNE) in the presence of α1-protease inhibitor (α1-Pi), the main physiological inhibitor of neutrophil serine proteases in lung secretions. Fixed and unfixed neutrophils bore the same amounts of active HNE at their surface. However, the HNE bound to the surface of unfixed neutrophils was fully inhibited by stoichiometric amounts of α1-Pi, unlike that of fixed neutrophils. The rate of inhibition of HNE bound to the surface of unfixed neutrophils was the same as that of free HNE. In the presence of α1-Pi, membrane-bound elastase is almost entirely removed from the unfixed neutrophil membrane to form soluble irreversible complexes. This was confirmed by flow cytometry using an anti-HNE mAb. HNE activity rapidly reappeared at the surface of HNE-depleted cells when they were triggered with the calcium ionophore A23187, and this activity was fully inhibited by stoichiometric amounts of α1-Pi. HNE was not released from the cell surface by oxidized, inactive α1-Pi, showing that active inhibitor is required to interact with active protease from the cell surface. We conclude that HNE activity at the surface of human neutrophils is fully controlled by α1-Pi when the cells are in suspension. Pericellular proteolysis could be limited to zones of contact between neutrophils and subjacent protease substrates where natural inhibitors cannot penetrate. The Journal of Immunology, 2005, 175: 3329–3338.

H uman neutrophil elastase (HNE)3 plays a pivotal role in the pathophysiology of acute lung injury. Its concentration is increased in clinical and animal models of the disease, and typical symptoms of lung injury are produced by the administration of HNE, which are reduced by inhibiting HNE (reviewed in Refs. 1–3). HNE is released from neutrophils at inflammatory sites when they are stimulated by a variety of compounds, including cytokines and chemoattractants such as TNF-α, IL-8, and fMLP (4). There is now a growing body of evidence that HNE, like related serine proteases from primary granules, remains in large part bound to the external surface of the plasma membrane, where it cleaves biologically relevant and synthetic substrates (5). Released, free proteases become pre-eminently upon further activation and subsequent cell lysis, as in the expectorations of cystic fibrosis patients and those suffering from severe acute lung injury such as acute respiratory distress syndrome (6). However, the regulation of proteolytic activity at inflammatory sites under conditions of mild neutrophil-dependent inflammation mainly concerns membrane-bound serine proteases that are exposed at the cell surface after mobilization of their primary granules. The extracellular milieu contains excess concentrations of high affinity protease inhibitors under these conditions, and these should normally control proteolytic activity in the pericellular environment. Several mechanisms have been proposed to explain the prolonged proteolytic activity that remains in the presence of inhibitors, including the proteolytic or oxidative inactivation of inhibitors (7), the adherence of cells and proteases to physiological substrates (8–11), and the resistance of membrane-bound proteases to naturally occurring protein inhibitors (4, 5, 12). Whereas it has been clearly shown that protease inhibitors, and especially α1-protease inhibitor (α1-Pi), confine proteolytic activity to the pericellular environment and have little effect on the breakdown of proteins that are in close contact with neutrophils (9), the persistence of proteolytic activity at the cell surface, because the access of proteinase inhibitors is restricted by steric hindrance, is more controversial. Although it has been shown that large inhibitors inhibit proteolytic activity at the cell surface less efficiently than do those of lower M, (4), membrane-bound proteases are fully active on protein substrates of high M, (13–15). Most experiments designed to measure proteolytic activity at the surface of purified neutrophils have used fixed activated cells to increase the HNE concentration at the cell surface, as this improves the detection of active protease and avoids the leakage of protease from the cell (5). New fluorogenic HNE substrates that are more specific and more sensitive than those previously used have been recently prepared (16–18). These are more suitable for quantifying HNE and studying the regulation of its activity by inhibitors at the surface of quiescent and triggered neutrophils. This is particularly important for developing anti-inflammatory treatment for lung inflammatory diseases using inhibitors designed to target both free and membrane-bound forms of active proteases at inflammatory sites (19–21). Several attempts
have been made to treat patients with cystic fibrosis with aerosolized or inhaled inhibitors, but the results have not been conclusive partly due to their limited access to plugged areas (22–24). The partition of free and membrane-associated proteases in lung inflammatory secretions and their relative sensitivities to inhibitors appears to be an important factor influencing treatment design. We have compared the activities of free HNE and membrane-bound HNE (mHNE); studied the interaction of α1-Pi, the main serine protease inhibitor in lung fluids, with freshly purified, quiescent, and triggered human neutrophils; and examined the fate of α1-Pi–HNE complexes after cell-inhibitor interaction.

Materials and Methods

Human neutrophil elastase (EC 3.4.21.37) and α1-Pi were obtained from Athens Research & Technology, Tripsyn (EC 3.4.21.4), A23187, and peroxidase-conjugated goat polyclonal anti-rabbit IgG Abs and anti-mouse F(ab’), FITC goat Abs and IMLP were from Sigma-Aldrich. Secretory leukocyte protease inhibitor (SLPI) and TNF-α were from R&D Systems. The specific elastase inhibitor EPI-hNE4 was a kind gift of F. Saudubray (Debiopharm, Lausanne, Switzerland). N,N-dimethylformamide was from Merck; N-chlorosuccinimide was from Valence Pharmaceuticals. Polymorphonuclear leukocytes and Lymphoprep were from Nycomed. Fluorescein-5-maleimide was from Beckman Coulter. Anti-HNE mAbs were from Biogenesis. All other reagents were of analytical grade.

Isolation of blood polymorphonuclear leukocytes (PMNs) and bronchoalveolar lavage (BAL) fluid (BALF) collection

Human PMNs were purified from 8-mL samples of peripheral blood collected from healthy volunteers into EDTA-containing tubes essentially as previously reported (25). The PMN pellet recovered after lysing the erythrocyte was washed with PBS and rinsed with PBS containing 4 mM EGTA and leukocyte protease inhibitor (SLPI) and TNF-α. Approximately 1.5 × 10⁶ PMNs in 200 μl of mAbs (Biogenesis clone 39A) diluted 1/50, for 30 min at 4°C, followed by two washes in PBS and incubation with FITC-conjugated F(ab’). the titer of which had been determined using bovine trypsinfitted with p-nitrophenyl-guandino-benzoate (29). Tripsyn was prepared as a 2 × 10⁻⁶ M stock solution in 100 mM Tris-HCl buffer, pH 8, 50 mM CaCl₂, then used in the same buffer as HNE. Because there is no significant α1-Pi inactivation via the substrate pathway upon interaction with HNE (30), a 1:1 stoichiometry was assumed. mHNE activity was quantified by comparing the rate of hydrolysis of its specific substrate (ortho-aminobenzenesulfonic acid (Abs)-APEE-RQ-EDDnp (2,4-dinitrophenyl) ethylenediamine (EDDnp) (18)) with that of titrated HNE under the same experimental conditions. The concentration of Abz-APEEMRQQ-EDDnp was determined by measuring the absorbance at 365 nm, using E₆₅₀nm = 17,300 M⁻¹ cm⁻¹ for EDDnp.

Scanning electron microscopy

Approximately 1.5 × 10⁶ cells were washed in PBS, deposited on polylysine-coated glass slides, and left to adhere for a few minutes. They were fixed with 1% glutaraldehyde (v/v) and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, postfixed in 2% osmium tetroxide, dehydrated in a graded acetone series, dried to the critical point using carbon dioxide, and sputter coated with platinum. Fixed cells were examined in a Gemini 982 Leo scanning electron microscope.

Enzyme assays

The activities of free HNE and mHNE were measured in PBS. Free HNE was titrated with α1-Pi, the titer of which had been determined using bovine trypsin titrated with p-nitrophenyl-guandino-benzoate (29). Tripsyn was prepared as a 2 × 10⁻⁶ M stock solution in 100 mM Tris-HCl buffer, pH 8, 50 mM CaCl₂, then used in the same buffer as HNE. Because there is no significant α1-Pi inactivation via the substrate pathway upon interaction with HNE (30), a 1:1 stoichiometry was assumed. mHNE activity was quantified by comparing the rate of hydrolysis of its specific substrate (ortho-aminobenzenesulfonic acid (Abs)-APEE-RQ-EDDnp (2,4-dinitrophenyl) ethylenediamine (EDDnp) (18)) with that of titrated HNE under the same experimental conditions. The concentration of Abz-APEEMRQQ-EDDnp was determined by measuring the absorbance at 365 nm, using E₆₅₀nm = 17,300 M⁻¹ cm⁻¹ for EDDnp.

Unactivated and activated, unfixed or fixed PMNs (1 × 10⁶ to 5 × 10⁶ cells), or purified proteases used as controls, were incubated with 20 μM Abz-APEEMRQQ-EDDnp in polypropylene microplate wells selected for their low binding properties (Hard-Shell Thin-Wall Microplates; MJ Research) at room temperature in activity buffer (10 mM PBS, pH 7.4). The fluorescence was measured at λₑₓ = 320 nm and λₑₓ = 420 nm using a microplate fluorescence reader (Spectra Max Gemini; Molecular Devices) under continuous stirring.

Release of membrane-bound proteases

The release of HNE into PBS/EGTA was checked by incubating unfixed and fixed cells (3 × 10⁵ cells/ml) in buffer for up to 1 h and measuring the peptidase activities in supernatants cleared of cells by centrifugation for 5 min at 500 × g. Cell pellets were suspended in the same buffer, and membrane-bound activity was measured using the same procedure. The procedure was repeated using the same buffer supplemented with 1.5 M NaCl. PMNs were kept for 1 h at room temperature or 24 h at 37°C under gentle stirring, collected by centrifugation at 500 × g for 15 min, and resuspended in PBS containing 1.5 M NaCl (final). The HNE activity in the supernatants of unfixed and fixed cells and on suspended fixed cells was measured and compared with the activity on unfractionated treated cells.

Inhibition of mHNE by α1-Pi, SLPI, and EPI-hNE4

Unactivated and activated, unfixed or fixed PMNs (1 × 10⁵ to 5 × 10⁶ cells), corresponding to 0.5–1.5 × 10⁻⁹ M active purified HNE, were incubated with equimolar concentrations and up to a 1000-fold molar excess of α1-Pi for 15–40 min under stirring. Residual HNE activity was then measured by adding Abz-APEEMRQQ-EDDnp (20 μM final). Progress curves for inhibition were recorded using equimolar amounts of α1-Pi and mHNE (0.1–10⁻⁹ M final). Inhibitor and substrate were added simultaneously to quiescent or activated PMNs. Fluorescence was recorded with the Gemini 70-970.
fluorometer at room temperature. These experiments were repeated using equimolar concentrations of mHNE and the potent, specific low M r elastase inhibitor EPI-hNE4, whereas a molar excess of SLPI was used to take into account its 0.1 nM Ki toward HNE (31), and its putative inhibition of cathepsin G.

Oxidation of α1-Pi

The α1-Pi (40 μM) was oxidized by a 20-fold molar excess of N-chlorosuccinimide (32), and excess oxidant was removed by ultrafiltration. The inhibitory capacity of the oxidized α1-Pi was compared with that of native inhibitor.

Activation of PMNs by the calcium ionophore during enzyme assay

The HNE activity at the surface of quiescent, unfixed cells was totally inhibited by α1-Pi. The cells were resuspended in PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ in the microplate wells, then activated with the calcium ionophore A23187. The reappearance of HNE activity was monitored continuously at room temperature, and further inhibition experiments with α1-Pi were conducted as above.

Electrophoresis and Western blotting

PMNs (70,000 cells/40 μl) corresponding to an HNE concentration of ~5 × 10⁻⁸ M and similar concentrations of free HNE were incubated with α1-Pi (5 × 10⁻⁸ M final) in PBS and 4 mM EGTA, for 2 min with gentle stirring, at room temperature. Cell mixtures were then centrifuged at 500 × g for 5 min. The supernatants were recovered, and the formation of HNE-α1-Pi complexes was analyzed by SDS-PAGE on 12% acrylamide/bisacrylamide gels. The resolved proteins were transferred to nitrocellulose membranes, and the complexes were detected by incubation with rabbit polyclonal anti-α1-Pi Abs (diluted 1/1,000), followed by peroxidase-coupled goat polyclonal anti-rabbit IgG Abs (diluted 1/15,000), using the Renaissance Plus kit (Valeant Pharmaceuticals).

Results

Elastase activity in suspensions of unfixed and fixed PMNs

HNE activity has been detected at the surface of activated neutrophils that were fixed to improve stability and avoid intracellular proteases leaking out during the time cells were reacted with substrates and/or inhibitors (5). Analysis of unactivated and activated neutrophils by flow cytometry before and after fixation indicated that anti-CD16b Abs reacted with their surface Ag at the surface of unfixed cells, but not at that of fixed cells (Fig. 1), while anti-CD63 Abs, used as a marker of neutrophil activation, reacted less efficiently with fixed activated neutrophils (Fig. 1). This suggests that the accessibility of epitopes at the cell surface is altered by fixation. The morphology of the surface of fixed neutrophils is significantly different from that of unfixed neutrophils (Fig. 1). We therefore measured the activities of HNE at the surface of fixed and unfixed neutrophils (2–4 × 10⁵ cells in 150 μl final) before and after activation by the calcium ionophore A23187 or by fMLP using a specific, sensitive elastase substrate (Abz-APEIMRQR-EDDnp) that allows the measurement of HNE at the cell surface even in the presence of the closely related protease 3 (18). Fixation did not alter the HNE activity of either quiescent or activated neutrophils. Unfixed and fixed neutrophils activated with the ionophore had 3–5 times greater activity than unactivated cells, while the activity of fMLP-activated cells was 1.5–2 times greater. The recovery of fluorescence after the total hydrolysis of the substrate by free HNE and mHNE was the same. Because an oxidized Met in the substrate sequence prevents further cleavage by HNE (16), there can have been no significant oxidation of the Met residue in the substrate by oxidants released from activated neutrophils. In agreement with these data, flow cytometry indicated that 10–30% of unactivated, unfixed PMNs bore HNE Ag at their surface, whereas all of the activated PMNs were labeled with the anti-HNE mAbs (Fig. 2B). This correlates well with the 3- to 5-fold higher HNE activity using activated cells. The fluorescence at the surface of fixed cells differed from that of unfixed cells, but the fluorescence with activated cells was still greater (Fig. 2C).

FIGURE 1. Flow cytometry analysis of quiescent (A and B) and triggered (C and D) purified human PMNs before (A and C) and after (B and D) fixation with glutaraldehyde/formaldehyde, as revealed with anti-CD16b and anti-CD63 Abs. The CD16b on quiescent, unfixed PMNs are labeled (A), but not those on fixed cells (B). Triggered, unfixed PMNs are labeled with both anti-CD16b and anti-CD63 Abs (C), whereas only some triggered, fixed cells are labeled by anti-CD63 Abs (D). The typical morphologies of quiescent and triggered PMNs before and after fixation by scanning electron microscopy are shown in insets for each analysis.
HNE stability at the surface of unfixed neutrophils

We checked the stability of the HNE at the surface of unfixed neutrophils by recording activities in supernatants and suspended cells. Approximately 20% of the mHNE activity was found in supernatants of quiescent PMNs in PBS/EGTA. This fraction remained stable for at least 1 h (Fig. 3A), suggesting that mHNE and free HNE are in equilibrium under these conditions. This is supported by our finding that there was no increase in the percentage of free HNE using unfixed, activated PMNs despite the increased HNE. Less than 10% of total HNE was found in the supernatant of fixed, quiescent, or triggered PMNs treated in the same way. Incubating unfixed cells in the PBS/EGTA buffer supplemented with 1.5 M NaCl for 60 min released all of the active mHNE (Fig. 3A), whereas only 50% of the HNE was released from the surface of fixed neutrophils under the same conditions (Fig. 3B). The total HNE activities in supernatants and suspended fixed cells (unfixed cells cannot be resuspended because they form aggregates after salt treatment and centrifugation) were the same as that initially found at the cell surface (Fig. 3B). Thus, enzymatically active HNE is bound mostly via electrostatic bonds to the surface of unfixed cells, and its activity toward synthetic substrates is not altered by its binding to the cell membrane. The percentage of HNE released from fixed PMNs by salt treatment remained unchanged after incubation overnight at 37°C (Fig. 3B), suggesting different modes of protease binding to cell surfaces that do not alter catalytic activity. We used the rate of hydrolysis of the fluorogenic substrate by free, titrated HNE to calculate that the concentration of active HNE on quiescent, fixed, or unfixed purified PMNs was \(<1 \text{nM}\) when \(10^6\) cells were incubated in 1 ml of final.

Inhibition of mHNE activity of unfixed and fixed neutrophils

Identical amounts of active HNE were found at the surfaces of unfixed and fixed neutrophils, suggesting that cell fixation does not...
alter the surface properties and function of the membrane. This supports data obtained using fixed, activated cells to measure membrane-bound proteolytic activities (5), but it disagrees with our flow cytometry observation that Abs reacted differently with their cognate Ags at the surface of fixed cells. This raises the question of the access of large Mₙ components such as Abs and protein inhibitors to the surfaces of fixed and unfixed neutrophils. The pericellular proteolysis in the vicinity of triggered neutrophils in the presence of an excess of inhibitors has been explained in part by the inability of α₁-Pi to reach the active site of mHNE (5, 12).

We measured the inhibition of mHNE by purified α₁-Pi using ~5 × 10⁵ quiescent PMNs and 10⁶ triggered PMNs to take into account the higher HNE expression at the surface of triggered cells. In agreement with others (5), we found that huge amounts of inhibitor were needed to inhibit HNE at the surface of fixed neutrophils (Fig. 4). In contrast, the elastase at the surface of unfixed quiescent and fMLP- or A23187-activated cells was rapidly inhibited by stoichiometric amounts of inhibitor (Fig. 4). Identical amounts of α₁-Pi were needed to fully inhibit both mHNE and the corresponding amount of free HNE that hydrolyzed the HNE substrate at the same rate (Fig. 5). This means that there is no significant α₁-Pi binding to any other protease target at the neutrophil surface (i.e., protease 3 (Pr3) and cathepsin G) under the conditions used. Therefore, active mHNE can be titrated by α₁-Pi. The inhibition progress curves for free and membrane-bound elastase were recorded using identical HNE and inhibitor concentrations in the nanomolar range (Fig. 5). The α₁-Pi inhibited free HNE and mHNE at the surface of unfixed, quiescent, and activated cells, at the same rate (Fig. 5), confirming that mHNE and free HNE have identical kinetic properties toward a synthetic low Mₙ substrate and toward its natural inhibitor. Under the same experimental conditions, mHNE at the surface of fixed cells was not inhibited (data not shown). A kₐ of at least 10⁷ M⁻¹ s⁻¹ was calculated with the equation: t₁/₂ = 1/kₐ I₀ (I₀ = initial inhibitor concentration) from the t₁/₂ of association obtained in three experiments using HNE concentrations of 0.3–1 nM. This is a minimal value because the initial substrate concentration (S₀) was in the same concentration range as the Km, which means that there may have been competition between substrate and inhibitor. However, this kₐ value agrees with those reported by others (33, 34). This also confirms that almost all α₁-Pi was bound to HNE under these experimental conditions, but not to Pr3 and cathepsin G, whose rate constants for association with α₁-Pi are lower than that for HNE (35, 36). Using a low Mₙ inhibitor specific for HNE such as EPI-hnE4 (Mₙ = 6,200) under the same experimental conditions, the HNE on unfixed cells was totally inhibited with stoichiometric amounts of inhibitor, whereas excess inhibitor was needed for significant inhibition at the surface of fixed cells (data not shown). The mHNE on unfixed cells was also totally inhibited by SLPI, but a 10-fold molar excess of the inhibitor was required because the Ki was not low enough for the protease-inhibitor complex to be entirely formed at that mHNE concentration (31).

The biological relevance of this process was tested using BALF from patients with acute respiratory distress syndrome. No HNE activity was detected in crude BALF, suggesting that both free HNE and mHNE are controlled by endogenous inhibitors, as previously shown by Western blotting (26). However, the BALF neutrophils were morphologically identical with activated blood neutrophils, and flow cytometry showed that they bore CD63 on their surface (i.e., protease 3 (Pr3) and cathepsin G) under the conditions used. Therefore, active mHNE can be titrated by α₁-Pi. The inhibition progress curves for free and membrane-bound elastase were recorded using identical HNE and inhibitor concentrations in the nanomolar range (Fig. 5). The α₁-Pi inhibited free HNE and mHNE at the surface of unfixed, quiescent, and activated cells, at the same rate (Fig. 5), confirming that mHNE and free HNE have identical kinetic properties toward a synthetic low Mₙ substrate and toward its natural inhibitor. Under the same experimental conditions, mHNE at the surface of fixed cells was not inhibited (data not shown). A kₐ of at least 10⁷ M⁻¹ s⁻¹ was calculated with the equation: t₁/₂ = 1/kₐ I₀ (I₀ = initial inhibitor concentration) from the t₁/₂ of association obtained in three experiments using HNE concentrations of 0.3–1 nM. This is a minimal value because the initial substrate concentration (S₀) was in the same concentration range as the Km, which means that there may have been competition between substrate and inhibitor. However, this kₐ value agrees with those reported by others (33, 34). This also confirms that almost all α₁-Pi was bound to HNE under these experimental conditions, but not to Pr3 and cathepsin G, whose rate constants for association with α₁-Pi are lower than that for HNE (35, 36). Using a low Mₙ inhibitor specific for HNE such as EPI-hnE4 (Mₙ = 6,200) under the same experimental conditions, the HNE on unfixed cells was totally inhibited with stoichiometric amounts of inhibitor, whereas excess inhibitor was needed for significant inhibition at the surface of fixed cells (data not shown). The mHNE on unfixed cells was also totally inhibited by SLPI, but a 10-fold molar excess of the inhibitor was required because the Ki was not low enough for the protease-inhibitor complex to be entirely formed at that mHNE concentration (31).
cell surface (Fig. 6), HNE activity was detected in cells that had been centrifuged and equilibrated in PBS, which indicates that new HNE molecules were exposed at the cell surface of BALF PMNs. The concentration of newly exposed HNE was similar (∼1 nM using 1-1.5 × 10^6 cells/ml) to that of the mHNE at the surface of purified blood PMNs. The mHNE in the buffered cell fraction of BALF was totally inhibited by similar or slightly higher concentrations of α1-Pi, confirming the biological relevance of this process.

The fate of α1-Pi-HNE complexes

The formation of the serpin-protease complex is complex, involving pole-to-pole displacement of the protease in the complex due to the cleavage of the reactive-center loop and its full insertion into the A-sheet of the serpin (37, 38). Thus, α1-Pi might remain or not at the cell surface after complex formation. We used Western blotting to look for α1-Pi-HNE complexes in the supernatants of cells incubated with α1-Pi, and flow cytometry to detect fluorescently labeled α1-Pi at the neutrophil surface. Mixtures of neutrophils and α1-Pi were prepared containing the amount of α1-Pi required to fully inhibit mHNE activity. They were centrifuged, and the supernatants were assayed for α1-Pi-protease complexes by SDS-PAGE, followed by Western blotting using anti-α1-Pi Abs. Most of the α1-Pi initially present in the neutrophil suspension had formed soluble complexes (Fig. 7A), which suggests that α1-Pi removed the bound protease from the cell surface during the formation of the irreversible complex. Oxidized α1-Pi, which inhibits free HNE very poorly, did not release HNE from the cell surface. In keeping with these data, flow cytometry showed no significant fluorescence on unfixed neutrophils incubated with fluorescently labeled α1-Pi, which demonstrates that no active protease remains at the cell surface (Fig. 7B). We also observed a nonspecific binding of fluorescent α1-Pi at the surface of fixed cells that does not result in any significant inhibition of mHNE and confirms that no significant amount of HNE is released by α1-Pi from the surface of fixed cells (Fig. 7B).

Because fluorescent α1-Pi only reacts with active HNE, we used anti-HNE mAbs to investigate the fate of total HNE Ag at the cell surface by flow cytometry after treating cells with α1-Pi, followed by anti-HNE mAbs. After treatment with α1-Pi, most of fluorescence disappeared from the surface of unfixed, but not of fixed, activated neutrophils, indicating that mHNE is present essentially (∼80%) as an active protease at the surface of unfixed activated PMNs (Fig. 8).

As incubation with α1-Pi removed all active HNE from the cell surface, we looked at the time-dependent recovery of elastase activity on suspended HNE-depleted cells. No significant HNE activity was recovered at the surface of unstimulated cells incubated with the specific HNE substrate for 1 h at 25°C (Fig. 9A). However, activity reappeared at the surface of HNE-depleted cells that had been activated with the calcium ionophore A23187 (Fig. 9Bb), it was comparable to that at the surface of control, triggered cells.

The concentration of newly exposed HNE was similar (∼1 nM using 1-1.5 × 10^6 cells/ml) to that of the mHNE at the surface of purified blood PMNs. The mHNE in the buffered cell fraction of BALF was totally inhibited by similar or slightly higher concentrations of α1-Pi, confirming the biological relevance of this process.

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that had not been incubated with $\alpha_1$-Pi (Fig. 9B, a and b). The new activity at the surface of depleted then activated cells was stoichiometrically inhibited by adding further $\alpha_1$-Pi (Fig. 9B, c and d).

There was a substrate-independent increase in fluorescence (Fig. 9Be), probably due to the in situ activation of neutrophils that release significant amounts of fluorescent NADPH, as previously reported when cells were activated by phorbol myristate or by the chemotactic peptide fMLP (39).

**Discussion**

Inflammatory lung diseases are due, at least in part, to the impaired regulation of protease activities by endogenous inhibitors at inflammatory sites. The local binding capacity of these inhibitors may be overwhelmed by the proteases brought by infiltrating neutrophils and other inflammatory cells including bacteria (40) and/or by the oxidative inactivation of critical Met residues by halogenated oxidants (41) and by proteolysis (42). However, uncontrolled proteolytic activity is not due solely to the overwhelming of the inhibitory capacity at inflammatory sites. Neutrophil proteases preserve their catalytic activity in the presence of protease inhibitors in several ways (reviewed in Ref. 4), such as the tight binding of neutrophil proteases to their target substrate, adherence of neutrophils to the extracellular matrix with the formation of an area of preserved proteolytic activity (43), and the resistance of membrane-bound proteases to inhibition by endogenous inhibitors (5). A more complete understanding of these mechanisms for preserving protease activity in the pericellular environment is necessary before protease inhibitors can be used as therapeutic tools to modulate the tissue destruction associated with acute inflammatory diseases. Whereas it has been clearly demonstrated that the access of protease inhibitors to the zone of contact between adherent neutrophils and subjacent substrates is impaired (43), their access to membrane-bound proteases in an environment replete with biologically active inhibitors is more controversial. A negative correlation was reported between the size of the inhibitor and its effectiveness against membrane-bound serine proteases, but these results were obtained with fixed, activated neutrophils (5, 44) or with purified membranes (12). Fixed and unfixed activated neutrophils retain their cell surface protease activity on synthetic low $M_r$ substrates and on protein substrates (5, 13, 15), but fixation can alter their accessibility and/or conformation. We have shown a difference in the surface morphologies of fixed and unfixed neutrophils by scanning electron microscopy, and different reactivities of Abs to membrane Ags by flow cytometry. This agrees with findings demonstrating a change in surface membrane and protein charge by chemical fixation (45).
We therefore compared the HNE activity at the surface of quiescent, unfixed neutrophils with that on fixed neutrophils before or after activation by a calcium ionophore or by fMLP. This was possible because we used the specific fluorogenic HNE substrate Abz-APEEMRRQ-EDDnp that is not cleaved by Pr3 and is sensitive enough to detect rapidly subnanomolar concentrations of free HNE and mHNE (18). HNE activity is resistant to inhibition at the surface of fixed cells, as reported (5), but mHNE is fully inhibited by stoichiometric amounts of α1-Pi when quiescent or activated unfixed cells are kept in suspension. The same result was obtained using activated neutrophils isolated from BALF from patients with acute respiratory distress syndrome that were morphologically similar to the activated blood neutrophils.

We also found that the inhibition rate of mHNE is similar to that measured with free HNE used at the same concentration, and that the HNE-α1-Pi complexes are all found in the soluble fraction. This probably favors their rapid elimination via the receptors to serpin-protease complexes (see Ref. 46 for review). But the mechanism of interaction between α1-Pi and mHNE is not yet understood. The α1-Pi could either bind to HNE at the cell surface and extract the membrane-bound protease to form the irreversible complex, or it could displace an equilibrium between free and membrane-bound protease by reacting only with the free enzyme. The peculiar way that serpins react with their target proteases (38) might explain that complexes do not remain at the cell surface and are found in the soluble fraction, which gives support to the former hypothesis, but the spontaneous release of mHNE from purified neutrophils after they have been centrifuged and equilibrated in buffer agrees with the latter.

Our finding that stoichiometric amounts of α1-Pi almost completely inhibit mHNE agrees with our recent finding that the inhibitor binds essentially to HNE, and not to Pr3 or cathepsin G, although these potential targets for this inhibitor are also present at the cell surface (26). This is explained, at least in part, by the different rate constants for association and by the two-step mechanism of Pr3 inhibition that could favor HNE binding when competition occurs (47).

Incubating purified neutrophils with 1.5 M NaCl releases all mHNE activity from the surface of quiescent and triggered, unfixed neutrophils. This agrees with the results obtained by Kolk-enbrock et al. (14) using membranes of activated neutrophils. But only part of the mHNE can be released from fixed neutrophils under the same conditions, further demonstrating that fixation alters cell surface properties. Exactly how HNE binds to the neutrophil membrane is not yet elucidated, but previously reported data suggest that it differs from that of related serine proteases from neutrophil primary granules (48). Nevertheless, all mHNE that is enzymatically active on peptide substrates can be inhibited by α1-Pi; however, it is bound. Our flow cytometry studies indicate that a major part of the fluorescence revealed by anti-HNE mAbs is removed from the cell surface after α1-Pi treatment. The minor part of HNE that remains at the cell surface after α1-Pi treatment is inactive or has an inaccessible active site so that it cannot be removed by its inhibitor. At least part of mHNE could be bound to CR3, the major adhesion protein of PMNs, through a mechanism that is dependent on its enzymatic activity (49) and could result in the inactivation of the protease (50).

Oxidation of inhibitors by reactive oxygen species released from activated neutrophils may also preserve membrane-bound proteolytic activity by inactivating lung protease inhibitors that have an oxidation-sensitive Met residue at their inhibitory site. We show in this study that α1-Pi inhibits free HNE and mHNE stoichiometrically at the same rate. This suggests that the inhibitor is not inactivated, even in the immediate cell environment, because oxidized α1-Pi inhibits HNE with a 2000-fold lower Kᵯ (51). Similarly, the hydrolysis of the HNE-specific substrate Abz-APEIMDRQ-EDDnp, which also contains a critical Met residue at its P1’ site and is no longer hydrolyzed once it is oxidized, remains unaltered (16). This agrees with the results of Chamba et al. (11), showing that oxidants have little influence on extracellular proteolysis by neutrophils. The absence of Met oxidation from both the substrate and inhibitor may be because the neutrophils were transferred to the reaction mixture after they had been activated and washed. The neutrophils were fluorescent when they were activated directly in the reaction mixture (Fig. 9), indicating the release of NADPH, an essential factor for the production of reactive oxygen species (52). However, there was no significant inactivation of α1-Pi or the HNE substrate, under our experimental conditions, during the time needed for total inhibition of mHNE.

The HNE activity of neutrophils whose mHNE has been completely removed by α1-Pi may rapidly reappear after they are triggered. Newly exposed HNE molecules that remain bound to the cells as well as secreted HNE can be immediately inhibited by any α1-Pi in the cell suspension. Hence, the proteolytic activity of neutrophil serine proteases is essentially due to adherent neutrophils that generate a protected pericellular environment, where they contact the underlying physiological substrate, as long as active inhibitors, especially α1-Pi, are present in the pericellular environment. In contrast, the activity at the surface of nonadherent cells is controlled by the inhibitors in the fluid phase. This is confirmed by our observation that there is no HNE activity in BALF supernatants of patients with moderate inflammation, but that this activity appears with time after cells have been centrifuged and suspended in an inhibitor-free medium (our unpublished data). Therefore, any HNE activity at the surface of neutrophils circulating in blood must be immediately inhibited by circulating α1-Pi. This also means that the low concentrations of HNE and related proteases that we and others find at the surface of unstimulated human PMNs by measuring their enzymatic activity or by immunofluorescence and immunogold staining (5, 18, 48, 53) probably appeared during their purification, once the cells were separated from plasma replete with inhibitors. HNE may be permanently present at the surface of activated neutrophils and its activity tightly controlled by endogenous inhibitors, especially α1-Pi, which is the most abundant HNE inhibitor in plasma and in BALF (54–56). But this control may be hampered once triggered neutrophils adhere and define a zone of restricted accessibility for inhibitors at the cell-subjacent substrate interface. The recent demonstration that neutrophil-bound HNE cleaves vascular endothelium cadherin, involved in the maintenance of endothelium integrity, during the process of transmigration supports this hypothesis (57). HNE activity should thus be limited to the zone of contact between adherent neutrophil and vascular endothelium, as suggested by previous studies, because of the rapid inhibition of mHNE by α1-Pi at the surface of nonadherent cells (9, 58). The HNE activity at the surface of nonadherent PMNs is therefore fully controlled by α1-Pi and related physiological inhibitors as long as they are present in excess in the microenvironment, and activated cells have not interacted with opsonized surfaces either during transendothelial migration or when they bind to insoluble matrix components. The permanent clearance of newly exposed HNE molecules from the cell surface therefore participates in the rapid consumption of active inhibitor molecules present locally, thus contributing to the disruption of the protease-inhibitor balance, helping to make inflammatory diseases involving PMN recruitment chronic.
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Disclosures

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