ELASTASE-MEDIATED FIBRINOGENOLYSIS BY CHEMOATTRACTANT-STIMULATED NEUTROPHILS OCCURS IN THE PRESENCE OF PHYSIOLOGIC CONCENTRATIONS OF ANTIPROTEINASES

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Human neutrophil elastase (HNE)[1] has been implicated in the pathogenesis of a wide variety of human diseases (1). In the past, it was difficult to measure HNE activity in vivo because the enzyme rapidly interacts with its major plasma inhibitor, α_1 proteinase–inhibitor (2), which complexes and inactivates the free enzyme with an estimated rate constant of association of $6.5 \times 10^7/M \cdot s$ (3). Recently, Weitz et al. (4) developed a sensitive assay to measure in vivo HNE activity. This assay makes use of the capacity of HNE to cleave the Val (Aα21)-Glu (Aα22) bond at the NH_2-terminal region of the Aα chain of fibrinogen, thus releasing the fibrinopeptide A-containing fragment Aα1–21. This peptide can be measured in plasma by RIA and its level reflects in vivo HNE activity (4). Using this assay, higher Aα1–21 levels were found in plasma of cigarette smokers than in plasma of nonsmokers (5). Further, individuals with congenital deficiency of α_1 proteinase–inhibitor had values of plasma Aα1–21 considerably higher than those in smokers (4). Increased Aα1–21 levels in patients lacking antiproteinase were expected. However, the presence of circulating Aα1–21 in individuals with normal plasma concentrations of antiproteinase was puzzling given the rapidity of the interaction between HNE and α_1 proteinase-inhibitor.

In vitro studies of proteolysis by polymorphonuclear leukocytes (PMN) suggest an explanation for the presence of Aα1–21 in the plasma of normal individuals. These studies show that enzymes released from stimulated PMN can degrade a variety of susceptible macromolecular substrates in the presence of antiproteinases (6–9), thereby raising the possibility that cell-associated proteinases are more resistant to inhibition than are the free enzymes. To examine this hypothesis, we

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[1] *Abbreviations used in this paper:* HNE, human neutrophil elastase; PMN, polymorphonuclear leukocytes; TBS, Tris-buffered saline; TIFPA, thrombin increasable fibronopeptide A immunoreactivity.

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studied PMN-mediated fibrinogenolysis in the presence and absence of antiproteinases. We report here that stimulated human PMN, migrating through fibrinogen-coated filters, demonstrate significant HNE-mediated fibrinogenolytic activity in the presence of proteinase inhibitors as small as 20,000 mol wt. In contrast, these same inhibitors completely block the fibrinogenolytic activity of soluble purified HNE of the cell-free secretory material of stimulated PMN. Our studies differ from previous ones in that: (*a*) we used PMN that had been stimulated by a specific chemoattractant, FMLP; (*b*) we measured the activity of a specific PMN enzyme (HNE) on a specific substrate (fibrinogen). This is in contrast to previous workers who measured nonspecific substrate proteolysis that may have resulted from the action of a variety of proteinases; and (*c*) we examined the inhibitory effects of undiluted serum or plasma and excess concentrations of antiproteinases on HNE activity.

Materials and Methods

Reagents. Purified HNE (10) and a specific polyclonal rabbit antibody to this enzyme (11) were generous gifts of Drs. M. Brower and P. Harpel, Cornell Medical Center, New York. Specific peptide chloromethyl ketone inhibitors of HNE and cathepsin G, MeO-Suc-Ala_2-Pro-ValCH_2Cl and Z-Gly-Leu-PheCH_2Cl, respectively, were from Enzyme Systems Products, Livermore, CA. FMLP was from Peninsula Laboratories, Inc., Belmont, CA; α_1 proteinase–inhibitor was from Calbiochem-Behring Corp., San Diego, CA; and soybean trypsin–inhibitor was from Sigma Chemical Co., St. Louis, MO. Poly-L-lysine (mol wt, ~70,000) was from Miles Laboratories Inc., Elkhart, IN; while Ficoll-400 and Hypaque sodium were from Pharmacia Fine Chemicals, Piscataway, NJ.

Preparation of Fibrinogen Substrate. Human fibrinogen (grade L; Kabi Diagnostica, Stockholm, Sweden) was rendered plasminogen-free by lysine Sepharose 4B affinity chromatography in the presence of aprotinin (100 Kallikrein inhibition units, KIU/ml). The fibrinogen was then further purified as previously described (12, 13). The clotting ability of the purified material was 96%. The absence of plasminogen was confirmed by incubating the fibrinogen for 48 h at 37°C with streptokinase (2 U/mg fibrinogen). Electrophoresis of 50 μg of unreduced sample on a 7.5% polyacrylamide gel revealed no evidence of degradation.

For some experiments, fibrinogen was trace-labeled with ^{125}I by the solid-phase lactoperoxidase–glucose oxidase procedure (14) to a specific activity of 3.1×10^8 cpm/μg. Unlabeled fibrinogen was mixed with the labeled material and the fibrinogen concentration was determined by measuring absorbance at 280 nm, using an extinction coefficient of 1.55.

Sartorius cellulose nitrate filters (Vanguard International Inc., Neptune, NJ) of 13-mm diameter and 3-μm pore size were placed in 16-mm wells of tissue culture plates (Costar, Cambridge, MA). The filters were incubated with 500 μl of 0.1 M NaCl buffered with 0.05 M Tris-HCl, pH 7.4 (TBS), containing 50 μg/ml poly-L-lysine for 30 min at 23°C with constant agitation. After washing with 500 μl of TBS three times, the poly-L-lysine–coated filters were incubated with 0.5 mg of fibrinogen suspended in 500 μl of TBS for 18 h at 23°C with constant agitation. The filters were then washed with 500 μl of TBS three times, air dried for 1 h, mounted on modified chemotactic chambers (Adaps Inc., Dedham, MA), and used within 24 h. Each filter was coated with ~95 μg of fibrinogen as determined from the amount of radiolabeled fibrinogen bound per filter and the specific activity of the tracer. There was little variation in amount of fibrinogen bound per filter (95 ± 4 μg) among different batches of filters.

PMN Lysates and Supernatants. PMN were isolated from heparinized venous blood obtained from normal volunteers by discontinuous Ficoll-Hypaque gradient (15). Unless otherwise specified, all experiments were carried out in HBSS containing 1 mg/ml BSA. For some studies however, the cells were directly suspended in undiluted human citrated

plasma (harvested from blood anticoagulated with 9:1 vol/vol, 3.8% trisodium citrate or in plasma-derived serum (prepared by $CaCl_2$ addition to citrated plasma at a final calcium concentration of 30 mM).

Cell-free lysates were prepared by freezing and thawing PMN (6.7×10^5 cells/ml HBSS) eight times and removing the cellular debris by centrifugation at 3,000 *g* for 15 min at 4°C. Supernatants of FMLP-stimulated PMN were obtained by incubating PMN (6.7×10^5/ml HBSS) with FMLP (10^{-7} M) for 60 min at 37°C, removing the cells by centrifugation at 3,000 *g* for 15 min at 4°C, and carefully collecting the supernatant and storing it at 4°C until used in an experiment later the same day.

Rabbit anti-HNE IgG or control IgG was isolated and coupled to activated CH-Sepharose 4B (Pharmacia Fine Chemicals) as previously described (4). Sepharose 4B (1 ml of a 50% suspension) bearing control or anti-HNE IgG and PMN lysates (0.5 ml) or supernatants of FMLP-stimulated PMN (0.5 ml) were mixed for 30 min at room temperature. After centrifugation, the HNE concentration in each supernatant was determined by measuring hydrolysis of MeO-Suc-Ala_2-Pro-ValAFC (Enzyme Systems Products), a synthetic HNE substrate, and comparing this with that produced by known concentrations of the purified enzyme. The HNE concentration in the lysates was 1.8 μg/ml, while that in the supernatants of FMLP-stimulated PMN was 0.3 μg/ml both before and after absorption with control IgG. In contrast, after immunoabsorption with anti-HNE IgG there was no measurable enzyme activity (<2 ng/ml) in either solution.

Assessment of Fibrinogenolysis. Chemotactic chambers fitted with fibrinogen-coated filters were suspended in 16-mm tissue culture wells. In cell-free experiments, one of the following substances was added to both the compartment above (0.27 ml) and below (1.23 ml) the filters and the chambers were then incubated at 37°C in a humidified 5% CO_2 incubator for 60 min: buffer; purified HNE (1–90 nM); PMN lysates; FMLP-stimulated PMN supernatants; HNE-immunodepleted PMN lysates; or HNE-immunodepleted, FMLP-stimulated PMN supernatants. In parallel experiments, the above assays were performed with one of the following additions: 1.0% plasma, 1.0% plasma-derived serum, α_1 proteinase–inhibitor (0.03 mM), soybean trypsin–inhibitor (0.3 mM), MeO-Suc-Ala_2-Pro-ValCH_2Cl (0.01 mM) or Z-Gly-Leu-PheCH_2Cl (0.1 mM). In every case, 1.5 ml of lysate from 10^6 PMN or 1.5 ml of supernatant of 10^6 FMLP-treated PMN was incubated with each filter.

HNE activity was quantified by measuring Aα1–21 release from the fibrinogen-coated filters. 50-μl aliquots were removed from both the upper and lower wells of each chamber at 0, 30, and 60 min intervals from the time at which the test solution was added to the filters. In some instances, an additional 5-μl aliquot was sampled as just described to determine the release of ^{125}I-labeled fibrinogen degradation products. Samples (50 μl) for Aα1–21 analysis were incubated with 150 μl ethanol on ice for 30 min, followed by rapid centrifugation (15,000 *g* for 3 min) to precipitate any fibrinogen or high molecular weight degradation products. The ethanol supernatants were collected, evaporated to dryness, and reconstituted to their original volume with distilled water as previously described (4). Release of ^{125}I-labeled fibrinogen degradation products was determined by counting the aliquots for 1 min on a Rackgamma counter (model 1270; LKB Instruments, Inc., Gaithersburg, MD). Aα1–21 concentrations were determined by RIA of thrombin increasable fibrinopeptide A immunoreactivity (TIFPA) as previously described (4). That TIFPA immunoreactivity reflects free Aα1–21 was confirmed by HPLC analysis of selected samples and the demonstration that immunoreactive fractions coeluted with synthetic Aα1–21 (4).

Experiments with intact neutrophils were performed as above but with the following differences: 10^6 PMN suspended in 0.27 ml of buffer were added above the fibrinogen-coated filters and 10^{-7} M FMLP was used below the filter as a chemoattractant. PMN were allowed to migrate through the filters for 60 min at 37°C. α_1 proteinase–inhibitor, soybean trypsin–inhibitor, MeO-Suc-Ala_2-Pro-ValCH_2Cl, and Z-Gly-Leu-PheCH_2Cl were added to the solutions on both sides of the filter as described above. In other experiments, the PMN and FMLP were suspended in 100% plasma or 100% plasma-derived serum to determine the effects of these substances on HNE activity. As controls, fibrinogen-coated

filters were incubated with unstimulated PMN or with 10^{-7} M FMLP alone. All experiments were performed in triplicate.

Chemotaxis Assays. Filters were removed from the chemotaxis chambers at the end of the assay and stained as described by Wilkinson (16). PMN chemotaxis was assessed by the leading front method (17). For each filter, the farthest distance at which two or more cells had migrated was measured in five × 400 fields in triplicate samples and the mean was determined.

Results

HNE-mediated Fibrinogenolysis. In evaluating the system used in this study, a number of preliminary experiments were performed. The sensitivity of the assay system was investigated by incubating the fibrinogen-coated filters with increasing concentrations of HNE. This resulted in dose-dependent release of Aα1–21 from fibrinogen (Fig. 1). When the molar ratio of HNE to fibrinogen was ≥0.25, all of the available peptide (2 mol/mol fibrinogen) was released. Similarly, HNE produced concentration-dependent release of ^{125}I-labeled fibrinogen degradation products (Fig. 2). Release of ^{125}I-labeled fibrinogen degradation products correlated with Aα1–21 release, but was not used as a specific measure of HNE activity.

Although quantitative release of Aα1–21 from fibrinogen coating the filters was effected by molar ratios of enzyme to substrate exceeding 0.25, quantitative peptide release from fibrinogen free in solution occurs at molar ratios of 0.025 or less (4). This discrepancy may be due to the relative inaccessibility of the Aα21–22 bond to HNE when the fibrinogen is immobilized on the filters. Alternatively, the negatively charged cellulose nitrate filters may adsorb the strongly cationic enzyme, thereby reducing the amount of HNE that is free to degrade fibrinogen.

There was negligible Aα1–21 release when the fibrinogen-coated filters were incubated with buffer alone or with FMLP (Table I). Unstimulated PMN in contact with fibrinogen-coated filters released 30.3 pmol of Aα1–21, suggesting that interaction of the cells with the substrate is in itself a stimulus for some HNE release. When PMN above the fibrinogen-coated filters were stimulated to migrate through the filter by 10^{-7} M FMLP on the opposite side, however,

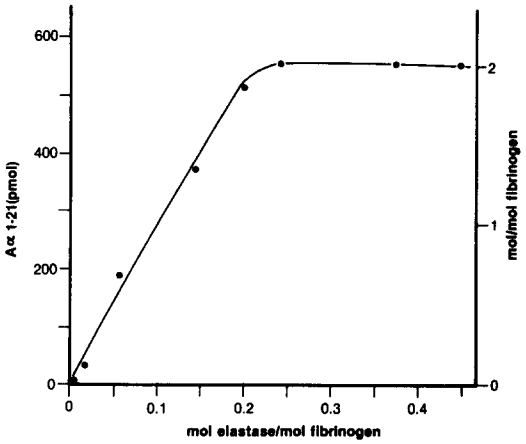

FIGURE 1. HNE-mediated Aα1–21 release from fibrinogen-coated filters. Fibrinogen-coated filters were incubated with increasing concentrations of purified HNE (1–90 nM) for 60 min at 37°C and Aα1–21 release was measured by RIA. The results shown are the mean of two separate experiments.

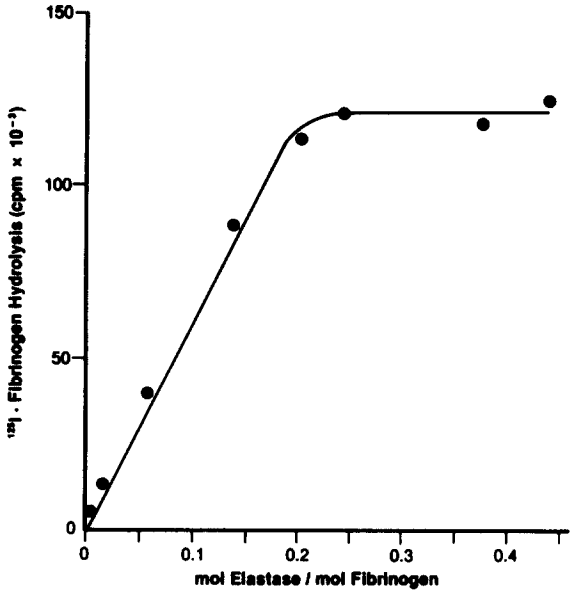

FIGURE 2. HNE-mediated ^{125}I-fibrinogen hydrolysis. Filters coated with ^{125}I-fibrinogen were incubated with increasing concentrations of purified HNE (1–90 nM) for 60 min at 37°C and release of ^{125}I-labeled fibrinogen degradation products was determined. A maximum of 59% of the ^{125}I-fibrinogen coating the filters was hydrolyzed by HNE. The results shown are the mean of two separate experiments.

TABLE I

Aα1-21 Release from Fibrinogen-coated Filters

Addition	Aα1-21 release at 60 min incubation
	pmol
Buffer alone	0.2 ± 0.01
FMLP alone	0.1 ± 0.01
PMN alone	30.3 ± 8.6
PMN + FMLP	161.3 ± 18.1

The results shown are the means ± SD from three separate experiments.

peptide release was increased more than fivefold, consistent with enhanced HNE release after chemoattractant stimulation of the cells. Thus, unstimulated PMN in contact with the fibrinogen-coated filters produced the same Aα1–21 release as did 2 nM purified HNE (Fig. 1), whereas peptide release by FMLP-stimulated PMN was equivalent to that produced by 11 nM HNE.

Proteinase Inhibitors Block Fibrinogenolysis by Supernatants of FMLP-stimulated PMN. We compared the relative ability of a variety of antiproteinases (including α_1 proteinase–inhibitor, soybean trypsin–inhibitor, MeO-Suc-Ala_2-Pro-Val-CH_2Cl, and *Z*-Gly-Leu-PheCH_2Cl) to inhibit the fibrinogenolytic activity of the enzymes present in the supernatants of FMLP-stimulated PMN with the ability of these agents to regulate fibrinogenolysis by intact PMN. The inhibitory effect of plasma and plasma-derived serum also was investigated. Plasma-derived serum was used to examine the possibility that the fibrinogen contained in plasma was providing additional substrate for the PMN proteinases. First we established that HNE was secreted by FMLP-stimulated PMN and confirmed its role in producing Aα1–21 release. Supernatants from FMLP-stimulated PMN incubated with fibrinogen-coated filters for 60 min released 151.0 pmol of Aα1–21 (Table II).

TABLE II
Effect of Inhibitors on Aα1-21 Release by Supernatants of FMLP-stimulated PMN

Inhibitor	Aα1-21 release at 60 min incubation
	pmol
None	151.0 ± 10.6
Z-Gly-Leu-PheCH$_2$Cl (0.1 mM)	162.3 ± 15.2
Plasma (1%)	0.1 ± 0.01
Serum (1%)	1.5 ± 0.9
α_1 Proteinase–inhibitor (0.03 mM)	0.1 ± 0.01
Soybean trypsin–inhibitor (0.3 mM)	0.9 ± 0.1
MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl (0.01 mM)	0.1 ± 0.01

The results shown are the means ± SD from two separate experiments.

The release of peptide by the free enzyme recovered in the supernatants of FMLP-stimulated cells was inhibited >99% by α_1 proteinase–inhibitor, soybean trypsin–inhibitor, 1% plasma and 1% plasma-derived serum (Table II). When fibrinogenolysis was assayed by measuring release of ^{125}I-labeled fibrinogen degradation products, similar results were obtained. Once again, release was completely blocked by α_1 proteinase–inhibitor, soybean trypsin–inhibitor, plasma, and plasma-derived serum (data not shown). Aα1–21 release was abolished by MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl, a specific peptide chloromethyl ketone inhibitor of HNE (18), whereas Z-Gly-Leu-PheCH$_2$Cl, a cathepsin G inhibitor (18), had no effect. Further confirmation that HNE was the enzyme responsible for the Aα1–21 release came from the demonstration that supernatants immunodepleted of HNE produced minimal peptide release (0.1 pmol). Thus, HNE was the enzyme in the supernatants of FMLP-stimulated PMN that promoted Aα1–21 release and free HNE in these solutions was readily inhibited by several antiproteinases.

HNE Is the Major Fibrinogenolytic Enzyme Contained in PMN. To determine whether the proteinases released from PMN after FMLP stimulation are representative of the major fibrinogenolytic enzymes contained within these cells, the experiments were repeated using PMN lysates (Table III). Release of Aα1–21 by the lysates was completely inhibited by plasma, plasma-derived serum, α_1 proteinase–inhibitor, and soybean trypsin–inhibitor. HNE was the enzyme responsible for peptide release since Aα1–21 release was completely inhibited by MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl, and lysates immunodepleted of HNE produced negligible peptide release (0.9 pmol). In contrast, the cathepsin G inhibitor, Z-Gly-Leu-PheCH$_2$Cl, had no effect.

When HNE concentration was measured by amidolytic activity, the lysates contained considerably more enzyme than did the supernatants of FMLP-treated PMN, and therefore, exerted greater fibrinogenolytic activity. These findings confirm previous reports that FMLP is a weak stimulus for HNE release from PMN (19).

All the inhibitors (1% plasma, 1% plasma-derived serum, α_1 proteinase–inhibitor, soybean trypsin–inhibitor, and MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl) blocked 99% of the activity of free HNE in the FMLP-stimulated PMN supernatants

TABLE III

Effect of Inhibitors on Aα1-21 Release from Fibrinogen-coated Filters Produced by PMN Lysates

Inhibitor	Aα1-21 release at 60 min incubation
	pmol
None	550.9 ± 18.6
Z-Gly-Leu-PheCH_2Cl (0.1 mM)	561.3 ± 20.2
Plasma (1%)	0.9 ± 0.1
Serum (1%)	1.1 ± 0.2
α_1 Proteinase–inhibitor (0.03 mM)	0.1 ± 0.01
Soybean trypsin–inhibitor (0.3 mM)	2.1 ± 0.3
MeO-Suc-Ala_2-Pro-ValCH_2Cl (0.01 mM)	0.1 ± 0.01

Inhibitors at the concentrations indicated were added to lysates from 10^6 PMN and incubated with fibrinogen-coated filters for 60 min at 37°C. Aα1-21 was assayed as described in Materials and Methods. 0.03 mM α_1-proteinase inhibitor (1.35 mg/100 ml) is within the physiologic range of this inhibitor in normal human plasma. The results shown are the means ± SD from two separate experiments.

TABLE IV

Effect of Inhibitors on Aα1-21 Release by FMLP-stimulated PMN

Inhibitor	Aα1-21 release at 60 min incubation	Inhibition
	pmol	%
None	161.3 ± 18.1	—
Plasma (100%)	24.6 ± 8.4	85 ± 3
Serum (100%)	30.9 ± 2.0	81 ± 1
α_1 Proteinase–inhibitor (0.03 mM)	19.3 ± 10.2	88 ± 4
Soybean trypsin–inhibitor (0.3 mM)	17.2 ± 5.8	89 ± 1
MeO-Suc-Ala_2-Pro-ValCH_2Cl (0.01 mM)	0.5 ± 0.2	>99

Inhibitors at the concentrations indicated were present in both the upper and lower chambers of chemotaxis apparatus. 10^6 PMN were used in each assay. Note that plasma, and plasma derived serum were used in 100 fold excess of the amounts required to inhibit 99.5% of all the elastase activity secreted by FMLP stimulated PMN (Table II), or present in lysates of 10^6 PMN (Table III). The results shown are the means and S.D. from three separate experiments.

(Table II) and >99.5% of the activity in the PMN lysates (Table III). This confirms that the inhibitors were used in sufficient concentrations to block the entire HNE content of all the PMN used in the assay.

PMN-mediated Fibrinogenolysis is Incompletely Blocked by High Molecular Weight Elastase Inhibitors. Given the >99% inhibition of soluble HNE by 1% plasma, 1% plasma-derived serum, α_1 proteinase–inhibitor, and soybean trypsin–inhibitor, we tested the ability of these agents to regulate PMN-mediated fibrinogenolysis. In contrast to their effects on soluble HNE, these inhibitors were significantly less potent in blocking the fibrinogenolytic activity of PMN that were stimulated by FMLP to migrate across fibrinogen-coated filters. Even in the presence of undiluted plasma or plasma-derived serum there was incomplete inhibition (85 and 81%, respectively) of Aα1–21 release (Table IV). Further, α_1 proteinase–inhibitor and soybean trypsin–inhibitor, at concentrations that inhib-

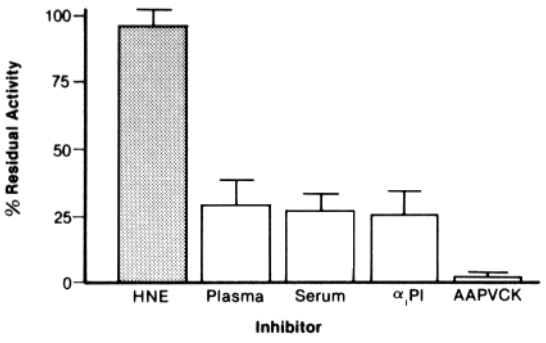

FIGURE 3. Inhibition of PMN-mediated ^{125}I-fibrinogen hydrolysis by antiproteinases. PMN (10^6/filter) were stimulated by FMLP (10^{-7} M) to migrate across ^{125}I-fibrinogen–coated filters in the presence and absence of antiproteinases. In buffer alone, the FMLP-stimulated PMN produced the same ^{125}I-fibrinogen hydrolysis as did 11 nM purified HNE, and this is shown for comparison. The percent residual activity after the addition of α_1 proteinase–inhibitor (α_1PI) (0.03 mM), plasma or serum (100%), and AAPVCK (MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl, 0.01 mM) is indicated. The bars represent the mean of three separate experiments (each done in triplicate), while the lines above the bars represent the standard deviations.

ited 99% of the HNE activity in lysates of all the PMN added to the assays (Table III), did not completely block Aα1–21 release (88 and 89%, respectively) by the migrating cells. In contrast, peptide release by migrating PMN was completely abolished by MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl.

Quantitatively similar results were obtained when fibrinogenolytic activity was monitored by measuring release of ^{125}I-labeled fibrinogen degradation products (Fig. 3). Again, the activity was incompletely blocked by 100% plasma, 100% plasma-derived serum, or α_1 proteinase inhibitor, but incompletely blocked by MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl.

The data given in Fig. 3 permit a quantitative evaluation of the ratio of proteinase inhibitors to HNE in the studies reported here. 11 pmol/ml of purified HNE released as much ^{125}I–fibrinogen degradation product from the filters as 10^6 chemotaxing PMN. The concentrations of α_1 proteinase– and soybean trypsin–inhibitors used in this experiment and in those reported in Table IV were in 3,000- and 30,000-fold molar excess of the amount of HNE required to give the fibrinogenolysis observed. Despite this vast excess of inhibitors they effected incomplete inhibition of PMN-mediated fibrinogenolysis. In contrast, MeO-Suc-Ala$_2$-Pro-ValCH$_2$Cl, which was present in only 1,000-fold molar excess of HNE (Fig. 3 and Table 4), gave >99% inhibition of PMN-mediated fibrinogenolysis.

Inactivation of α_1 proteinase–inhibitor by PMN-derived oxidants is one mechanism by which HNE released from the migrating PMN could remain active in the presence of plasma or serum containing this antiproteinase (20–23). However, this mechanism does not explain the incomplete inhibition of fibrinogenolytic activity by soybean trypsin–inhibitor, which is resistant to oxidant inactivation. Furthermore, chemoattractants such as FMLP are weak stimulators of oxidant release from PMN (24). Therefore, oxidative inactivation of plasma proteinase inhibitors is unlikely to be the mechanism by which HNE escapes regulation in this experimental system. These studies thus indicate that PMN-mediated fibrinogenolysis can occur in the presence of physiologic concentrations of antiproteinases and that only the low molecular weight chloromethyl ketone–inhibitor of HNE can totally inhibit PMN-mediated fibrinogenolysis.

TABLE V
Effect of Antiproteinases on PMN Migration through Fibrinogen-coated Filters

Inhibitor	PMN migration
	μm
None	123 ± 8
Plasma (100%)	128 ± 17
Serum (100%)	144 ± 8
α_1 Proteinase–inhibitor (0.03 mM)	144 ± 8
MeO-Suc-Ala_2-Pro-ValCH_2Cl (0.01 mM)	127 ± 6

Each result represents the mean ± SD of five separate fields measured in three replicate filters.

To investigate whether the mechanism by which MeO-Suc-Ala_2-Pro-ValCH_2Cl inhibited PMN-mediated fibrinogenolysis was through uptake and inhibition of intracellular HNE, we performed the following experiments. First, lysates were prepared from 10^6 PMNs that had been preincubated with 0.01 mM MeO-Suc-Ala_2-Pro-ValCH_2Cl for 60 min at 37°C and then washed extensively before preparation of the lysates. Incubation of these lysates with fibrinogen-coated filters resulted in similar Aα1–21 release as produced by PMN lysates prepared from cells not previously exposed to the HNE inhibitor (558.2 pmol and 550.9 pmol, respectively). Second, FMLP stimulation of PMN that had been preincubated for 60 min with 0.01 mM MeO-Suc-Ala_2-Pro-ValCH_2Cl resulted in similar Aα1–21 release from fibrinogen-coated filters as occurred with PMN not previously exposed to the peptide chloromethyl ketone–inhibitor (148.2 pmol and 161.2 pmol, respectively). These experiments thus indicate that MeO-Suc-Ala_2-Pro-ValCH_2Cl does not significantly inhibit intracellular HNE.

Inhibitors of PMN Fibrinogenolysis Do Not Alter PMN Chemotaxis. None of the inhibitors (including serum or plasma) altered PMN chemotaxis in response to FMLP (Table V), thus excluding the possibility that these agents exert their effects by impairing PMN migration. Since MeO-Suc-Ala_2-Pro-ValCH_2Cl completely inhibited HNE activity (as measured by Aα1–21 release) without affecting PMN chemotaxis, HNE activity is not a requirement for PMN chemotaxis in this system.

Discussion

Adherence of macrophages to a ligand-coated surface results in such close apposition of the cells to the surface that molecules as small as 50,000 mol wt cannot penetrate into this space. This led Wright and Silverstein (25) to propose that macrophage secretory products (such as proteolytic enzymes or H_2O_2) released at the cell-surface interface would be protected from the effects of inhibitors such as α_2 macroglobulin, α_1 proteinase–inhibitor, or catalase. The work of Baron et al. (26) suggests that a similar situation prevails when osteoclasts degrade bone. The osteoclasts form a tight seal against the bone, acidify the space between their membrane and the bone, and release matrix-degrading enzymes into this space. The net effect is bone resorption that is strictly confined

to the area directly beneath the osteoclast, and is unaffected by the neutral pH and proteinase inhibitors of the extracellular fluids.

In the studies reported here we have tested whether one PMN secretory product, HNE, also is protected from inhibition by plasma antiproteinases when PMN bind to, and migrate on, a fibrinogen-coated surface. Our results show that very low concentrations (1%) of plasma and serum or macromolecular proteinase inhibitors (α_1 proteinase inhibitor and soybean trypsin–inhibitor) completely blocked fibrinogenolysis mediated by soluble HNE present in PMN lysates or secreted by PMN stimulated with a chemoattractant (Tables II and III), but that the considerably higher concentrations of antiproteinases present in undiluted serum or plasma incompletely blocked (81–85%) fibrinogenolysis mediated by PMN migrating in response to a chemoattractant. It is important to note that the total amount of Aαcl-21 released by the lysates of 10^6 PMN was 3.4-fold greater than that produced by FMLP-stimulated PMN (550 pmol [Table III, line 1] vs. 151–161 pmol [Tables II and IV, line 1] Aα1–21 released, respectively); and that the total amount of enzyme in 10^6 PMN was inhibited >99% by the proteinase inhibitors present in 1% plasma or 1% plasma-derived serum. Thus, at minimum, the amount of HNE inhibitor in the 100% plasma or 100% plasma-derived serum used to block HNE activity of chemotaxing PMN (Fig. 3 and Table IV) was 340-fold in excess of the amount required to inhibit the total content of the HNE in the PMN used in these experiments. As indicated in Results (Fig. 3), we estimate that the α_1 proteinase– and soybean trypsin–inhibitors were in 3,000- and 30,000-fold molar excess, respectively, of the amount of HNE released by 10^6 chemotaxing HNE. Thus, it is evident that the macromolecular inhibitors used were present in sufficient concentrations and amounts to block all the HNE contained in the PMN used in these experiments.

In contrast, MeO-Suc-Ala_2-Pro-ValCH_2Cl, a specific low molecular weight inhibitor of HNE (18), used at a concentration that was only 1,000-fold in molar excess of the HNE secreted by chemotaxing PMN, blocked >99% of the fibrinogenolysis mediated by the PMN. MeO-Suc-Ala_2-Pro-ValCH_2Cl did not affect intracellular HNE, HNE secretion from the PMN, or PMN migration in response to the chemoattractant (Table V). These findings provide circumstantial evidence in support of the hypothesis that PMN migrating on fibrinogen-coated substrate occlude a significant portion of their zone of contact with the substrate from macromolecular proteinase inhibitors in plasma.

Casual inspection suggests that the findings of Chapman and Stone (9) are in conflict with those reported here. They demonstrated that elastin proteolysis by PMN is completely inhibited in the presence of 10% serum. However, the PMN used in their studies were not stimulated by chemoattractants and hence would differ from those used in our investigations in two important ways. First, since FMLP is a secretagogue for PMN, unstimulated PMN release less HNE than do stimulated cells. Second, and perhaps more importantly, FMLP causes quantitative and qualitative alterations in the expression and function of several PMN membrane receptors (27, 28).

Stimulation of PMN with FMLP or C5a produces a five- to sixfold increase in receptors that bind the C3b and C3bi cleavage products of the third component of complement (27). In addition, chemoattractant-stimulated PMN exhibit a

qualitative change in the activity of their fibronectin receptors (28). When PMN are stimulated by either chemoattractants or fibronectin they bind complement-coated particles via their C3b or C3bi receptors but do not ingest them. When stimulated by both chemoattractants and fibronectin, however, PMN bind and phagocytose C3b and C3bi coated particles (Pommier, C. G., and E. Brown, personal communication). Thus, ligation of receptors for chemoattractants functionally "activates" PMN fibronectin receptors and ligation of fibronectin receptors on these PMN functionally activates their complement receptors. Both fibronectin (29, 30) and C3bi (31, 32) contain the Arg-Gly-Asp (RGD) amino acid sequence. The RGD sequences in these molecules are ligands for the corresponding receptors on leukocytes (32, 33). Fibrinogen also contains RGD sequences and these mediate fibrinogen binding to platelets (34, 35) and perhaps to leukocytes (36). It is possible, therefore, that the RGD sequences on substrate-adherent fibrinogen may act as ligands for PMN receptors, e.g., the fibronectin and C3bi receptors (CR3). In the presence of a chemoattractant, ligand binding may result in receptor activation, thereby leading to close apposition of cells to substrate. This would result in the formation of a protected microenvironment that excludes macromolecular proteinase inhibitors similar to that which develops when macrophages interact with a C3bi-coated surface (25).

The results of these studies are consistent with the hypothesis that HNE escapes regulation in vivo because close contact between migrating PMN and fibrinogen prevents antiproteinase access to the enzyme. Further, these findings may provide an important link in understanding why Aα1–21 is found in the plasma of normal individuals and why elevated levels of this peptide are found in the plasma of cigarette smokers. Each day ~10^9 PMN (37) emigrate from the vascular compartment into the extravascular tissues. PMN move across endothelial cell monolayers only when stimulated by a chemotactic gradient (38). Such gradients may be generated under physiological conditions by bacteria in the intestinal tract and by the secretion of LTB_4 by macrophages (39) in response to a variety of stimuli. For the reason outlined above, a low level of fibrinogenolysis may thus be an inescapable consequence of PMN emigration from the vasculature in response to many environmental and microbial stimuli.

Cigarette smoking may amplify PMN emigration from the vasculature into the lungs. The peripheral leukocyte count is elevated in cigarette smokers (40), cigarette smoking delays PMN transit through the pulmonary circulation (41), and increased numbers of PMN are recovered in the bronchial lavage fluid of smokers (42). The mechanism for PMN attraction into the lungs is unknown but macrophage-derived chemoattractants (43, 44) and secretory components (45), nicotine (46), or complement components (47) have been implicated. The elevated plasma Aα1–21 levels in cigarette smokers may reflect the increase in PMN traffic through the lungs. In addition to fibrinogen proteolysis, proteinases released from PMN migrating through the lungs also may degrade components of the pulmonary interstitium, thereby explaining why cigarette smoking is the major risk factor for the development of chronic lung disease (48).

Summary

Plasma levels of the HNE-derived fibrinopeptide Aα1–21 reflect in vivo enzyme activity. To provide a possible explanation for the presence of circulating Aα1–21 in individuals with normal plasma antiproteinase concentrations we investigated whether PMN-associated HNE is more resistant to inhibition than the free enzyme. PMN were stimulated to migrate across ^{125}I-fibrinogen–coated nitrocellulose filters in response to 10^{-7} M FMLP, and the extent of fibrinogenolysis was determined by measuring release of Aα1–21 and ^{125}I-labeled fibrinogen degradation products. The fibrinogenolytic activity of migrating PMN was then compared with that of free HNE present in PMN lysates or secreted by PMN stimulated with FMLP. Whereas the fibrinogenolytic activity of soluble HNE was completely inhibited by low concentrations (1%) of plasma or serum and macromolecular antiproteinase (α_1 proteinase–inhibitor and soybean trypsin–inhibitor), even in the presence of undiluted plasma or serum the activity of the migrating PMN was incompletely blocked (81–85%). Further, concentrations of α_1 proteinase–inhibitor and soybean trypsin–inhibitor that totally inhibited free HNE activity also incompletely blocked (88–89%) the fibrinogenolytic activity of migrating PMN, indicating that FMLP-stimulated PMN demonstrate significant fibrinogenolytic activity in the presence of antiproteinases as small as 20,000 mol wt. A specific low molecular weight HNE inhibitor (MeO-Suc-Ala$_2$-Pro-ValCH_2Cl), however, totally blocked PMN-mediated fibrinogenolysis without affecting intracellular HNE activity, HNE secretion from PMN, or PMN migration in response to FMLP. These findings support the hypothesis that PMN migrating on a fibrinogen-coated surface form zones of close contact with fibrinogen, thus preventing access of plasma antiproteinases to HNE released at the cell-substrate interface. The occurrence of this phenomenon in vivo would explain the presence of circulating Aα1–21 in individuals with normal antiproteinase concentrations.

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