THE INHIBITORY COMPLEX OF HUMAN $\alpha_1$-PROTEINASE INHIBITOR AND HUMAN LEUKOCYTE ELASTASE IS A NEUTROPHIL CHEMOATTRACTANT

BY MICHAEL J. BANDA,* AUDIE G. RICE,* GAIL L. GRIFFIN,† AND ROBERT M. SENIOR‡

From the *Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California 94143; and the †Respiratory and Critical Care Division, Department of Medicine, Jewish Hospital at Washington University Medical Center, St. Louis, Missouri 63110

$\alpha_1$-Proteinase inhibitor ($\alpha_1$-PI) is a member of the serpin group of serine proteinase inhibitors (1, 2). Based on inhibitory kinetics, the principal target of $\alpha_1$-PI is human leukocyte elastase (HLE) (3). The metalloproteinase elastase secreted by mouse macrophages inactivates $\alpha_1$-PI (4) by hydrolyzing the Pro357-Met358 bond (5), which results in $M_r$ 47,800 and $M_r$ 4,200 fragments. We recently observed that $\alpha_1$-PI proteolytically inactivated by mouse macrophage elastase becomes a potent chemotactic factor for human neutrophils (6). After proteolysis, the 47,800 and 4,200 fragments of $\alpha_1$-PI remain associated with each other in a bimolecular complex, with the chemotactic activity residing in a newly exposed region of the 4,200 fragment (6).

When HLE is inactivated by $\alpha_1$-PI, the Met358-Ser359 bond is hydrolyzed, resulting in a COOH-terminal cleavage fragment very similar to that generated by mouse macrophage elastase. In contrast to mouse macrophage elastase, which does not form an inhibitory complex with $\alpha_1$-PI, HLE remains tightly associated with $\alpha_1$-PI. Therefore, the HLE--$\alpha_1$-PI complex actually consists of three molecules: HLE and two fragments of $\alpha_1$-PI. Because it is likely that the orientation of the two fragments of $\alpha_1$-PI in the HLE--$\alpha_1$-PI complex is similar to that of the two $\alpha_1$-PI fragments generated after proteolysis by mouse macrophage elastase, we considered the possibility that the HLE--$\alpha_1$-PI complex possesses chemotactic activity similar to that of the proteolytically inactivated $\alpha_1$-PI. In the study reported here, using homologous reagents, we examined the ability of complexes of HLE and $\alpha_1$-PI to stimulate directed migration of human neutrophils.

Materials and Methods

Materials. Chemicals were purchased from the following sources: human $\alpha_1$-PI from CalBiochem-Behring Corp., La Jolla, CA; human leukocyte elastase from Elastin Prod-

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†Abbreviations used in this paper: $\alpha_1$-PI, $\alpha_1$-proteinase inhibitor; HLE, human leukocyte elastase (also known as human neutrophil elastase and human granulocyte elastase).

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ucts Co., Pacific, MO; rabbit anti-α1-PI IgG and anti-α2-macroglobulin IgG from Sigma Chemical Co., St. Louis, MO; pre-stained protein standards for SDS-PAGE from Bethesda Research Laboratories, Gaithersburg, MD; and all reagents for electrophoresis from Bio-Rad Laboratories, Richmond, CA. Sheep anti-HLE IgG was a gift of D. Burnett and R. Stockley, The General Hospital, Birmingham, England.

**HLE-α1-PI Complexes.** Reaction mixtures containing HLE and α1-PI (1:1, mol/mol) were incubated at 37°C in a 10 mM phosphate buffer, pH 6.8, for 15 min. The reaction was stopped either by addition of PMSF (≤1 mM) or by injection onto an anion-exchange column.

**Electrophoresis.** SDS-PAGE was performed according to the method of Laemmli (7) using 12% resolving gels on a Mini-slab apparatus from Idea Scientific (Corvallis, OR). After electrophoresis the protein bands were stained for 15 min with 0.5% (vol/vol) Coomassie Blue R250 in acetic acid/isopropyl alcohol/water (1:3:6), destained with 50% methyl alcohol, and silver stained according to Wray et al. (8).

**Cells.** Neutrophils were separated on Ficoll-Paque gradients from peripheral blood obtained from healthy volunteers (9).

**Chemotaxis.** Chemotaxis was determined in modified Boyden chambers, as previously described (10, 11). Briefly, 1.2 × 10^6 neutrophils/ml were placed in the upper compartment and separated from chemoattractants in the lower compartment by a 2-μm pore size filter (Nucleopore Corp., Pleasanton, CA) overlaying a 0.45-μm pore size filter (Millipore Corp., Bedford, MA). After 1 h the chambers were disassembled and the membranes were stained with hematoxylin. Chemotaxis was quantified by counting at high dry magnification (× 400) the number of cells that migrated to the interface between the membranes (expressed as cells per high power grid). Five fields were counted for each experiment, and the results were corrected for the number of cells migrating in control chambers, which contained cells in the upper compartment and medium alone in the lower compartment. Experiments were performed in triplicate.

**Purification of HLE-α1-PI Complexes.** Chromatography was performed on a liquid chromatograph (model 1090; Hewlett-Packard Co., Palo Alto, CA) equipped with an HP1040 photodiode array detector. Elutions were monitored at 280 nm. Samples of HLE-α1-PI reaction mixtures were injected onto an AX-300 anion-exchange column (4.6 × 250 mm) (Brownlee Labs, Santa Clara, CA) equilibrated with 5 mM Tris-HCl, pH 8.0 (solvent A), and developed with a gradient to 0.8 M NaCl in solvent A (solvent B). The gradient was delivered at 0.5 ml/min, as follows: 0–5 min, 0% B; 5–10 min, 0–20% B; 10–20 min, 20–25% B; 20–25 min, 25% B; 25–65 min, 25–65% B.

**α1-PI Fragment.** The M, 4,200 fragment of α1-PI was generated by hydrolysis of the Pro-357-Met-358 bond by mouse macrophage elastase and purified as described previously (6).

**Antibodies.** Purified HLE-α1-PI complex (100 μl, 10^-6 M) was incubated with 14 μl of stock anti-α1-PI IgG, 2 μl of anti-HLE IgG, or 24 μl of anti-α2-macroglobulin IgG before use in a chemotactic assay. Controls were HLE-α1-PI complex incubated in assay medium alone and FMLP incubated in assay medium alone or with anti-α1-PI IgG. All incubations were at ambient temperature for 60 min.

**Preexposure to Competitive Ligands.** Neutrophils were incubated in 10 nM purified HLE-α1-PI complex or 10 nM purified M, 4,200 fragment of α1-PI for 30 min at ambient temperature, then washed three times with medium. The cells were then assayed for a chemotactic response to 1 nM HLE-α1-PI complex, 1 nM 4,200 fragment, or 10 nM FMLP.

**Results**

Equal molar amounts of HLE and human α1-PI were incubated together at 37°C for 15 min, resulting in the formation of a stable HLE-α1-PI inhibitory complex. Dilutions of this reaction mixture were assayed for their ability to stimulate migration of human neutrophils in modified Boyden chambers. Migration
CHEMOTAXIS TO PROTEINASE- INHIBITOR COMPLEXES

FIGURE 1. Stimulation of neutrophil chemotaxis by reaction mixtures containing HLE-α1-PI complexes. α1-PI was incubated for 15 min with HLE, 1:1 mol/mol (○); HLE, 1:0.25 mol/mol (△); or HLE (1:1 mol/mol) that had been inactivated with PMSF before incubation (□). Dilutions of the reaction mixtures were then assayed for their ability to stimulate chemotaxis of human neutrophils. The points represent the average of triplicate determinations, and the error bars represent the standard error of the mean. h.p.g., high power grid. Migration stimulated by 10⁻⁶ M FMLP (the positive control) was 153 ± 4.2 cells.

was equivalent to that stimulated by 10 nM FMLP (the positive control). Maximal stimulation of migration was achieved at a dilution equivalent to 1 nM α1-PI initially added to the reaction mixture (Fig. 1). Chemotaxis was reduced when the proportion of HLE in the reaction mixture was reduced four-fold, suggesting that the chemotactic activity was proportional to the HLE-α1-PI complex in the reaction mixture. Neither native α1-PI nor HLE alone stimulated migration of neutrophils.

In our previous study (6), proteolytically modified α1-PI was shown to be chemotactic for neutrophils. In that study the metalloproteinase macrophage elastase was used to degrade α1-PI because it would not form an inhibitory complex (4, 5). In the present study, a stable inhibitory complex was formed between

FIGURE 2. Purification of HLE-α1-PI complex. α1-PI was incubated with an excess of HLE, then applied to an AX-500 anion-exchange column equilibrated with 5 mM Tris-HCl, pH 8.0. Absorbance was monitored at 280 nm and expressed as milliabsorbance units (mAU). The column was developed with a gradient of NaCl as described in Materials and Methods. (Inset) Silver-stained SDS-polyacrylamide electrophoretic gel containing native α1-PI (N), HLE-α1-PI reaction mixture (R), and an aliquot of a fraction corresponding to peak C containing HLE-α1-PI complex (C). Molecular weight markers (M, X 10⁻⁷) are shown at the left.
HLE and $\alpha_{1}$-PI. When HLE and $\alpha_{1}$-PI were incubated under conditions of excess HLE, proteolytically modified complex and $\alpha_{1}$-PI were detected in addition to the classical inhibitory complex. To determine if the inhibitory complex itself stimulated migration, the complex was purified. Equal molar amounts of HLE and $\alpha_{1}$-PI were incubated together to form the inhibitory complex. The reaction mixture was then fractionated on an AX-300 anion-exchange column, and peak fractions were analyzed by electrophoresis. The HLE-$\alpha_{1}$-PI complex eluted as a single band with an apparent $M_{r}$ of 80,300 (Fig. 2). Various concentrations of the purified complex were assayed for their ability to stimulate migration of neutrophils. Again, migration was equivalent to that stimulated by the FMLP positive control (Fig. 3). The optimal concentration of purified HLE-$\alpha_{1}$-PI complex was 1 nM, the same concentration that stimulated maximal migration when the crude reaction mixture was used (Fig. 1).

To distinguish chemotactic migration from chemokinetic migration, various concentrations of purified HLE-$\alpha_{1}$-PI complex were placed in the upper and lower compartments of modified Boyden chambers during the migration assay. A "checkerboard" analysis verified that HLE-$\alpha_{1}$-PI complex stimulated chemotaxis, because cells migrated only when there was a concentration gradient (Table I).

| "Checkerboard" Analysis of Chemotaxis to HLE-$\alpha_{1}$-PI Complex |
|---------------------------------------------------------------|
| **HLE-$\alpha_{1}$-PI (M) in upper compartment** | **HLE-$\alpha_{1}$-PI (M) in lower compartment** |
| $10^{-11}$ | 34* | 12 | 2 | 12 |
| $10^{-10}$ | 74 | 14 | 8 | 17 |
| $10^{-9}$ | 168 | 102 | 64 | 26 |
| Data are expressed as mean number of cells ± SE per high power grid. |
CHEMOTAXIS TO PROTEINASE-INHIBITOR COMPLEXES

Because the HLE-α1-PI complex contains elastase as well as α1-PI, it was possible that the chemotactic signal was associated with the elastase portion of the complex. To test this possibility, purified HLE-α1-PI complex was separately incubated with anti-α1-PI, anti-HLE, and anti-α2-macroglobulin antibodies before use in the migration assay. The antibody against α1-PI inhibited chemotaxis, whereas antibody against HLE did not (Fig. 4). Therefore, the chemotactic signal was associated with α1-PI and not the elastase portion of the complex. The lack of inhibition by anti-α2-macroglobulin antibody (data not shown) demonstrated that the inhibition of chemotaxis by anti-α1-PI antibody was not due to nonspecific protein binding. Moreover, because antibody to α1-PI did not inhibit chemotaxis stimulated by FMLP, it was evident that inclusion of anti-α1-PI antibody in the reaction mixture did not generally interfere with the ability of neutrophils to migrate.

The chemotactic activity of proteolytically inactivated α1-PI resides in the M, 4,200 fragment (6), which is the result of hydrolysis of the Pro-357-Met-358 peptide bond (5). When HLE forms an inhibitory complex with α1-PI, the Met-358-Ser-359 bond is broken, resulting in a similar fragment. In both cases the cleavage fragment is not released as a free molecule but remains associated with the remainder of the α1-PI molecule in a rearranged position (6, 12). To deter...
mine if the source of the activity of the HLE-α₁-PI inhibitory complex could be localized to the region of the 4,200 fragment, the following experiments were carried out. Neutrophils were incubated with 10 nM 4,200 fragment isolated from proteolytically inactivated α₁-PI and then washed free of unbound fragment before they were tested for their chemotactic response to 1 nM HLE-α₁-PI complex. This pretreatment eliminated chemotaxis (Fig. 5). Similarly, when neutrophils were preincubated with 10 nM HLE-α₁-PI complex, migration toward 1 nM 4,200 fragment was eliminated. Therefore, the source of the chemotactic activity of the HLE-α₁-PI complex was the same as that of the 4,200 fragment. Preincubation with either 4,200 fragment or HLE-α₁-PI complex had no effect on migration toward 10 nM FMLP, indicating that the preincubations did not produce a generalized decrease in the chemotactic responses of these cells.

Discussion

The function of α₁-PI is usually considered solely in light of its ability to inactivate serine proteinases, especially HLE. The present study suggests that a consequence of α₁-PI-mediated inhibition of HLE is the generation of a chemotactic signal for neutrophils. The inhibitory complex therefore becomes an inflammatory mediator. This newly described characteristic of α₁-PI is not limited to proteinase–inhibitor complexes, because proteolytically inactivated α₁-PI also seems to act as an inflammatory mediator by a similar mechanism (6). The data suggest that an identical site with chemotactic potential is exposed in both HLE-α₁-PI complex and α₁-PI proteolytically inactivated by hydrolysis of the Pro-357-Met-358 bond.

The local regulation of α₁-PI–mediated chemotaxis may be quite complicated. α₁-PI from the circulation may diffuse into tissue and form inhibitory complexes or become proteolytically inactivated. In addition, α₁-PI may be made locally by human monocytes (13) and macrophages (14). It is unclear whether human monocytes or macrophages secrete metalloproteinases that could proteolytically inactivate α₁-PI. However, neutrophil collagenase can proteolytically inactivate α₁-PI (15). Human monocytes have been reported to produce a serine elastase similar to HLE (16) and may generate HLE-α₁-PI complexes (14). Therefore, local production of α₁-PI, HLE, and α₁-PI–degrading proteinases may initiate or alter the inflammatory response.

Evidence presented in this study suggests that a single neutrophil surface receptor binds both proteolytically inactivated α₁-PI and the α₁-PI in the inhibitory complex. The site responsible for recognition appears to reside in a domain carboxyl to the Met-358-Ser-359 peptide bond; exposure of this area is probably the result of the severe rearrangement of modified α₁-PI (12). Saturation of the receptor by preincubation with HLE-α₁-PI complex prevents further chemotaxis toward modified α₁-PI but does not interfere with chemotaxis toward an unrelated molecule, FMLP. Therefore, this as yet uncharacterized receptor must be different from the FMLP receptor. Whether it is a receptor for other known neutrophil chemotactic stimulants, such as C5a or LTB4, remains to be determined.

Little is known about the fate of HLE-α₁-PI complexes. α₂-Macroglobulin—
proteinase complexes are cleared by a macrophage cell surface receptor (17, 18). Although it has been suggested that HLE is transferred from \( \alpha_1 \)-PI to \( \alpha_2 \)-macroglobulin (19), this is unlikely because of the nature of the HLE–\( \alpha_1 \)-PI bond. Furthermore, because binding of HLE–\( \alpha_1 \)-PI to neutrophils is directly mediated by \( \alpha_1 \)-PI, transfer to \( \alpha_2 \)-macroglobulin and subsequent binding to the \( \alpha_2 \)-macroglobulin receptor does not appear likely. The putative receptor for modified \( \alpha_1 \)-PI may provide a clearance mechanism for HLE–\( \alpha_1 \)-PI complexes as well as for proteolytically inactivated \( \alpha_1 \)-PI. If so, turnover of native \( \alpha_1 \)-PI would also involve this receptor.

### Summary

An inhibitor–proteinase complex consisting of human \( \alpha_1 \)-PI and human leukocyte elastase is chemotactic for human neutrophils. The chemotactic activity is optimal at 1 nM and is associated only with the \( \alpha_1 \)-PI portion of the complex. Neither HLE in the complex, free HLE, nor native \( \alpha_1 \)-PI possesses chemotactic activity for human neutrophils. \( \alpha_1 \)-PI in complex is hydrolyzed at the Met-358-Ser-359 bond. The chemotactic activity is associated with the \( M_4,200 \) fragment of \( \alpha_1 \)-PI that has Ser-359 as its NH\(_2\) terminus. The region of the HLE–\( \alpha_1 \)-PI complex that stimulates chemotaxis appears to be the same as that of the \( M_4,200 \) fragment generated by hydrolysis of the Pro-357-Met-358 bond during proteolytic inactivation of \( \alpha_1 \)-PI. The data suggest the presence of a neutrophil surface receptor bound by \( \alpha_1 \)-PI after the formation of a complex with HLE or after proteolytic degradation. This receptor may play a role in clearance of these modified \( \alpha_1 \)-PI molecules.

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