Effects of Human Neutrophil Elastase (HNE) on Neutrophil Function In Vitro and in Inflamed Microvessels

By Richard C. Woodman, Paul H. Reinhardt, Samina Kanwar, Frances L. Johnston, and Paul Kubes

The primary objective of this study was to test the hypothesis that human neutrophil elastase (HNE) affects neutrophil infiltration (adhesion and emigration) into inflamed vessels. To determine whether HNE contributes to neutrophil adhesion in vivo, intravital microscopy was used to study neutrophil-endothelial cell interactions in single inflamed postcapillary venules. Superfusion of platelet-activating factor (PAF) (100 nmol/L) onto the mesentery caused an increase in neutrophil-neutrophil interactions, neutrophil adhesion to postcapillary venules, and cellular emigration out of the vasculature. Both L658 758 (an elastase-specific inhibitor) and Eglin C (an elastase and cathepsin G inhibitor) significantly attenuated all of these parameters in vivo. To further characterize the mechanism(s) involved, various in vitro parameters were assessed. HNE, but not trypsin, caused a dose-dependent (0.01 to 1.0 μg/ml) increase in the expression of the β subunit (CD18) of the CD11/CD18 adhesive glycoprotein complex on neutrophils. An HNE-dependent increase in CD11b expression was also observed; however, HNE did not affect the expression of other neutrophil adhesion molecules (L-selectin, superoxide production, or degranulation). PAF-enhanced CD18 expression on neutrophils and neutrophil migration were both abolished by L658 758 but PAF-induced neutrophil adhesion to endothelial monolayers was not affected by the antiprotease. The in vitro data suggest that the antiproteases do not directly prevent neutrophil adhesion in vivo but may be important in other CD18-dependent events such as neutrophil-neutrophil interaction or neutrophil infiltration (chemotaxis). These results translate into an important, rate-limiting role for elastase in the process of leukocyte infiltration and accumulation in inflamed microvessels.

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the role of elastase on neutrophil migration, respiratory burst activity, degranulation, adhesion, as well as CD11b, CD18, and L-selectin expression was examined.

MATERIALS AND METHODS

Chemicals. Human serum albumin, trypsin, PAF, bovine erythrocyte superoxide dismutase (SOD), cytochrome c (horse heart type VI), 4-methylumbellifluorone, phorbol myristate acetate (PMA), FMLP, propidium iodide, and Histopaque were obtained from Sigma Chemical Co; St Louis, MO. HNE was purchased from Calbiochem (La Jolla, CA). A murine anti-human CD18 MoAb (IG2-subclass; fluorescein isothiocyanate [FITC] conjugate), murine anti-human L-selectin MoAb (IG5b-subclass; FITC conjugate), and murine anti-human CD11b antibody (IG2b-subclass; Physocerythrin conjugate) were purchased from Becton Dickinson Systems Inc, Mountain View, CA. Appropriate isotype-specific negative control antibodies were also purchased from Becton Dickinson. Simply Cellular Microbeads were purchased from Flow Cytometry Systems Corp, Research Triangle Park, NC. L658 758 the elastase-specific inhibitor11 was obtained from Merck, Sharpe and Dohme Research Laboratory, Rahway, NJ, and Eglin C, the elastase and cathepsin G inhibitor was obtained from Ciba-Geigy, Basel, Switzerland. Other reagents were the best grade commercially available and were used without further purification.

Intravital microscopy experimentation. Fifteen male Wistar rats (180 to 250 g) were maintained on a purified laboratory diet and fasted for 24 hours prior to surgery. The animals were initially anesthetized with pentobarbital sodium (0.65 mg/kg body weight), a right carotid artery and jugular vein were cannulated to measure systemic arterial pressure (Statham P23A pressure transducer and a Grass physiologic recorder, Scarborough, Canada) and drug administration, respectively. A midline abdominal incision was made and the animals were placed in a supine position. A segment of the mesentery was suffused with warmed bicarbonate-buffered saline (pH 7.4). An intravital microscope (Nikon Optiphot-2, Japan) with a 25 objective lens (Leitz Wetzlar L25/0.35, Germany) and a Grass physiologic recorder, Scarborough, Canada) and drug administration, respectively. A midline abdominal incision was made and the animals were placed in a supine position. A segment of the mid-jejunum was exteriorized through the abdominal incision and all exposed tissue was covered with saline soaked gauze to minimize tissue dehydration. The mesentery was carefully placed over an optically clear viewing pedestal that allowed for transillumination of a 2 cm² segment of tissue. The temperature of the pedestal was maintained at 37°C with a constant temperature circulator (Fisher Scientific, model 80, Edmonton, Canada). Rectal and mesenteric temperatures were monitored using an electrothermometer. The mesentery was suffused with warmed bicarbonate-buffered saline (pH 7.4). An intravital microscope (Nikon Optiphot-2, Japan) with an ×25 objective lens (Leitz Wetzlar L25/0.35, Germany) and ×10 eyepiece was used to observe the mesenteric microcirculation as previously described.12,13,15 A video camera mounted on the microscope projected the image onto a color monitor and the images were recorded for playback analysis using a video cassette recorder. Single unbranched venules with diameters ranging between 25 and 40 μm were selected for study. Venular diameter was measured on line using a video calliper. The number of adherent and emigrated neutrophils was determined off-line during playback of videotaped images. A neutrophil was considered adherent to venular endothe-lium if it remained stationary for 30 seconds or more. Rolling neutrophils were defined as those leukocytes that moved at a velocity less than that of erythrocytes in the same vessel. Leukocyte rolling velocity was determined from the recorded frames. A leukocyte to traverse a given distance along the length of the vessel. Neutrophil-neutrophil interactions were also quantitated when a rolling neutrophil made contact with a second adherent or rolling neutrophil for an interval greater than 10 seconds.

Experimental protocol. After all hemodynamic parameters were in steady state, images from the mesentery were recorded for 5 minutes. The mesentery was then superfused for 60 minutes with 100 nmol/L PAF dissolved in bicarbonate-buffered saline. Measurements of aforementioned parameters were performed again at 30 and 60 minutes of PAF superfusion. In two experimental groups, animals were pretreated with either intravenous L658 758 or Eglin C and the mesenteric preparations were again expsured to PAF superfusion as described above. Both antiproteinases were given as 10 mg/kg initial dose followed by 0.05 mg/min continuous intravenous infusion.11,18

Isolation of neutrophils. Neutrophils from healthy donors were purified by dextran sedimentation followed by hypotonic lysis and Histopaque centrifugation as previously described.25 Except for the dextran sedimentation step, which was performed at room temperature, the cells were kept at 4°C throughout the isolation procedure. Cell preparations contained greater than 95% neutrophils with greater than 99% viability using Trypan Blue dye exclusion.25 After isolation, neutrophils were resuspended at a final concentration of 2 × 10⁶ cells/mL in phosphate buffered saline (PBS). Aliquots of cells were then incubated at 37°C for 20 minutes with PMA (final concentration 10 nmol/L) or varying concentrations of HNE or trypsin. In two experiments, HNE and polymyxin B (5 μg/mL) were incubated together with an aliquot of neutrophils. After washing, neutrophils were incubated in the dark at 4°C for 30 minutes with saturating concentrations of the conjugated murine anti-human CD18, L-selectin, CD11b, or the murine-negative control antibody. Immunofluorescence staining and fluorescence-activated cell sorter (FACS) analysis. Direct immunofluorescence as a measure of CD18 surface expression was determined by analysis on a FACScan (Becton Dickinson) using the channel number (log scale) representing the mean fluorescence intensity of 10,000 cells as previously described.24 The specific mean fluorescence intensity for cells stained by each antibody was calculated after subtracting the mean fluorescence intensity of the cells exposed to the negative control antibody. The number of murine anti-human CD18 antibodies bound per neutrophil was calculated with the Simply Cellular microbeads after establishing a fluorescence-intensity calibration curve. Nonviable cells were excluded using propidium iodide as previously described.23 In two additional experiments, HNE was heat inactivated (100°C for 20 minutes) and then exposed to neutrophils to determine whether the proteolytic activity was necessary for CD18 upregulation.

Adherence assay. Human umbilical vein endothelial cells (HUVEC) were grown to confluence and neutrophil adhesion to this biologic substratum was assessed. Briefly, umbilical cord veins were rinsed of formed blood elements with PBS containing antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Collagenase (2.5 mg/mL; 149 U/mg) was instilled into the vein and the cord incubated for 20 minutes at 37°C. The cords were gently massaged to ensure detachment of endothelial cells from the vessel wall. The digest was collected into centrifuge tubes, the collagenase inactivated with fetal calf serum, and centrifuged (400g for 10 minutes at 25°C). The pellet was resuspended in M199 containing 10% fetal calf serum and antibiotics plated in 25 cm² flasks. Cultures were incubated in 5% CO₂ at 37°C and 96% humidity, expanded by trypsinization, and grown to confluence in 48-well plates.

The PMN adherence assay was a modification26 of the method of Fehr and Dahinden.27 Briefly, neutrophils were radiolabeled by incubating purified neutrophils (2 × 10⁶ cells/mL) with 30 μCi/mL of Na¹⁵₁⁹⁴K at 37°C for 60 minutes. The cells were washed three times and resuspended in PBS. Neutrophils were allowed to adhere to the endothelial monolayers for 20 minutes under control conditions and in the presence of PAF (10⁻¹⁰ mol/L) or PMA (3 × 10⁻⁷ mol/L) in the absence or presence of L658 758 (250 μmol/L) or MoAb IB (20 μg/mL). We have previously reported that this concentration of MoAb IB, and L658 758 maximally blocked stimu-
lated neutrophil adhesion and fibronectin degradation, respectively. In additional experiments, neutrophils were allowed to adhere for 20 minutes to the endothelial monolayers in the presence of 0.01, 0.1, and 1.0 μg/mL HNE. In a final series of experiments, the neutrophils were preincubated with HNE for 20 minutes at the above concentrations and then exposed for 20 minutes to the endothelial monolayers. The supernatant of each well was then aspirated and the well was gently washed with 0.5 mL of NaOH (2N). The cell lysate was collected and the lysate and supernatant were assayed for 51Cr activity. Neutrophil adherence was calculated as the ratio of radioactivity in the cell lysate versus the radioactivity in the cell lysate plus supernatant.

Migration assay. To study neutrophil migration towards a concentration of PAF, collagen-treated transwell polycarbonate filters (Costar, Cambridge, MA) with 3.0 μm pores were used. Briefly, 51Cr-labelled neutrophils were added to the luminal side of the filter and incubated at 37°C with PAF (10^-6 mol/L) on the abluminal side of the filter in the presence or absence of L658.758 (250 μmol/L) or MoAb IB4 (20 μg/mL). The protease inhibitor and anti-CD18 antibody were added to both the luminal and abluminal side of the filter. After 1 hour, the fluid from the abluminal side of the filter was collected, the well was washed with NaOH, and the radioactivity of the lysate determined. This assay is a modified chemotaxis assay in which the neutrophils have to migrate through pores across the filters but do not have to degrade a matrix.

Superoxide production and degranulation assays. The rates of superoxide production by intact neutrophils in suspension were determined by measuring the kinetics of SOD-inhibitable reduction of ferricytochrome c using a previously published method. Neutrophil superoxide rates were measured after incubation with either HNE (0.1 to 1.0 μg/mL), PMA (3 × 10^-7 mol/L), fMLP (10^-5 mol/L) or PAF (5 × 10^-8 mol/L). Following a 20 minute incubation at 37°C in the presence and absence of HNE (1 μg/mL), superoxide production by neutrophils was also determined following stimulation with PMA, PAF, and fMLP. β-glucuronidase activity in supernatants was measured fluorimetrically as a marker of azurophilic granules using 4-methylumbelliferone as a substrate in the presence of the aforementioned activators. As a positive control for degranulation, some neutrophils were preincubated with dihydrocytochalasin B (DHCB; 10 μmol/L) at 37°C for 15 minutes followed by subsequent stimulation with fMLP (10^-8 mol/L). Glucose-6 phosphate dehydrogenase (G6PD) was used as a cytosolic marker for cell lysis. Enzyme activity (G6PD or β-glucuronidase) released into the surrounding medium during incubation was expressed as a percentage of total activity determined by sonication of an equal number of neutrophils.

Statistics. The data were analyzed using analysis of variance with the Scheffe’s (posthoc) test. All values are reported as mean ± standard error. Statistical significance was set at P <.05.

RESULTS

Intravital microscopy was used to study various neutrophil parameters including neutrophil rolling, neutrophil-neutrophil interactions, adhesion, and emigration in inflamed microvessels. Figure 1 is a set of photomicrographs showing a 35-μm venule before and after superfusion with PAF for 60 minutes. Following PAF exposure, many leukocytes (arrow) have infiltrated the vessel and are firmly adherent to the endothelium as well as other leukocytes (see aggregates). Previous work from our laboratory using cats suggests that more than 85% of these cells are neutrophils. No adhesion or rolling was observed in the arteriole. Table 1 summarizes vessel diameter, neutrophil rolling velocity, and neutrophil flux (number/min passing through the vessel). PAF superfusion at 100 nmol/L had no effect on vessel

Table 1. Vessel Diameter and Neutrophil Rolling Velocity and Flux Before and During PAF Superfusion

<table>
<thead>
<tr>
<th>Time of PAF Superfusion</th>
<th>Vessel diameter (μm)</th>
<th>Neutrophil rolling velocity (μm/s)</th>
<th>Neutrophil flux (no./min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>32.4 ± 1.0</td>
<td>22.3 ± 3.1</td>
<td>34.9 ± 9.6</td>
</tr>
<tr>
<td>30 min</td>
<td>30.7 ± 2.1</td>
<td>19.9 ± 2.9</td>
<td>35.0 ± 10.9</td>
</tr>
<tr>
<td>60 min</td>
<td>30.6 ± 2.6</td>
<td>21.7 ± 2.7</td>
<td>18.7 ± 6.5*</td>
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</table>

Average vessel diameter, neutrophil rolling velocity, and neutrophil flux in the untreated group (n = 5) over 1 hour of PAF superfusion. Data for the L658 758 and Eglin C are not included because they did not differ from the untreated group.

* P < .05 relative to time 0 min.
ELASTASE AND NEUTROPHIL FUNCTION

Figure 2. Neutrophil adhesion per 100 μm length of venule over 1 hour in the untreated group, the anti-elastase (L658 758) and anti-elastase/cathepsin G (Eglin C) pretreated group (n = 5 each). *P < .05 relative to control (0 min) value. tP < .05 relative to untreated group.

Figure 3. Neutrophil emigration per field of view over 1 hour in the untreated, L658 758 and Eglin C groups (n = 5 each). Emigration of neutrophils is generally observed between 30 and 60 minutes of PAF superfusion. *P < .05 relative to control (0 min) value. tP < .05 relative to untreated group.

diameter or neutrophil rolling velocity but reduced by 50% the flux of neutrophils through a given vessel over the 60 minutes exposure to PAF.

Figure 2 shows that superfusion of PAF onto the rat mesentery produced a significant sixfold increase in neutrophil adhesion that was time dependent. In the presence of Eglin C, PAF still caused a significant increase in neutrophil adhesion; however, the magnitude of this increase was reduced by more than 60%. L658 758, the specific elastase inhibitor, completely abolished the PAF-induced neutrophil adhesion. PAF superfusion of the mesentery caused approximately 60% of the adherent neutrophils to emigrate out of the microvasculature into the surrounding tissue within 60 minutes (Fig 3), which is consistent with previously published data. However, administration of either of the anti-proteinases essentially abolished neutrophil emigration associated with PAF superfusion. Neutrophil-neutrophil interactions were quantified during PAF superfusion and defined as those cells that made contact with each other for a period of 10 seconds or longer. Under control conditions 1.4 ± 0.6 interactions per minute were observed. This value increased more than fivefold following PAF administration (Table 2). L658 758 and Eglin C both significantly reduced PAF-induced neutrophil interactions (P < 0.05). This reduction in neutrophil-neutrophil interactions occurred independent of an effect on the number (flux) of rolling leukocytes (Table 2).

To elucidate the mechanism by which HNE affected neutrophil infiltration in vivo, the effect of varying doses of HNE on the neutrophil surface expression of the beta (CD18) subunit of the CD11/CD18 complex was tested. Neutrophils were treated with 0.01 to 1.0 μg/mL of HNE and then analyzed by flow cytometry (Fig 4). A dose of 0.1 μg/mL HNE caused a slight increase in CD18 expression in every experiment (n = 5). Moreover, 0.5 and 1.0 μg/mL HNE elicited a significant increase in CD18 expression. Polymyxin B had no effect on HNE-induced upregulation. In all experiments, more than 90% of the neutrophils were viable (as assessed by propidium iodide) and viability was not affected following HNE exposure. Trypsin did not induce CD18 upregulation suggesting that not all proteinases will induce this effect (Fig 4). Heat-inactivated HNE (proteolytic activity reduced by 95%) also did not promote CD18 upregulation suggesting that proteolytic activity is necessary for enhanced CD18 expression (Fig 4). The effects of L658 758 on HNE-, PAF-, and PMA-induced expression of CD18 binding sites are shown in Table 3. The HNE inhibitor prevented the increased expression of CD18 to HNE and PAF by more than 90% and 80%, respectively. L658 758 had no effect on PMA-induced increase in CD18 expression. In two additional experiments, an increase in CD11b expression was also observed with 1 μg/mL of HNE (data not shown).

Table 2. Effect of Elastase Inhibitors on PAF-Induced Neutrophil Interactions and Neutrophil Flux In Vivo

<table>
<thead>
<tr>
<th></th>
<th>Neutrophil-Neutrophil Interactions x Control†</th>
<th>Flux of Rolling Leukocytes (no. cells/min)</th>
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<tbody>
<tr>
<td>PAF</td>
<td>5.3 ± 0.8</td>
<td>18.7 ± 6.5</td>
</tr>
<tr>
<td>PAF + L658 758</td>
<td>2.6 ± 0.9†</td>
<td>12.1 ± 3.6</td>
</tr>
<tr>
<td>PAF + Eglin C</td>
<td>1.4 ± 0.4†</td>
<td>23.2 ± 8.2</td>
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</table>

* Represents mean ± 1 SE (n = 5).
† P < .05 relative to PAF value.
We investigated various CD18-dependent neutrophil functions in vitro including neutrophil migration and adhesion in an attempt to understand how HNE modified PAF-induced neutrophil infiltration in vivo. PAF significantly increased (approximately fourfold) neutrophil migration across collagen-treated filters after 60 minutes (Fig 5). The elastase inhibitor L658 758 essentially abolished (95%) the PAF-induced increase in neutrophil chemotaxis. In contrast, L658 758 did not significantly affect PAF-induced increase in neutrophil adhesion to HUVEC (Fig 5) suggesting that HNE may affect neutrophil migration but not adhesion per se. We also examined the direct effect of HNE on neutrophil migration and neutrophil adhesion to HUVEC. Figure 5 shows that HNE per se was not a chemotactic agent. Moreover, HNE caused a very subtle increase in neutrophil adhesion to HUVEC, an effect that was unlikely to be of any significant physiologic relevance. It should be noted that, when the endothelium was exposed to HNE along with the neutrophils, no increase in adhesion was observed. In each of the adhesion assays, PMA was used as a positive control to ensure that neutrophil adhesion could be enhanced. In every case, PMA caused more than 50% of the neutrophils to adhere to the endothelial monolayer (data not shown).

The effect of HNE on other neutrophil functions was also assessed. Table 4 summarizes the data for L-selectin expression, superoxide production (O₂⁻) and degranulation in the presence of increasing concentrations of elastase as well as PMA and PAF. HNE did not effect L-selectin expression, O₂⁻ production, or degranulation, whereas PMA and PAF both caused shedding of L-selectin and stimulated O₂⁻ production. Moreover, HNE did not enhance the superoxide production in response to PMA (122 ± 4 v 121 ± 8 nmol/min/10⁷ cells), PAF (47 ± 5 v 46 ± 3 nmol/min/10⁷ cells), or fMLP (56 ± 6 v 64 ± 10 nmol/min/10⁷ cells). These data are important inasmuch as they suggest that HNE does not directly affect the function of PAF or fMLP receptors on the surface of the neutrophil. It should also be noted that there was less than 2% neutrophil lysis (as determined by G6PD activity) under all conditions including the highest concentration of HNE.

TABLE 3. Effects of Elastase-Specific Inhibitor (L658, 758) on CD18 Expression

<table>
<thead>
<tr>
<th>CD18 Binding Sites (X10⁵/cell)*</th>
<th>-L658, 758</th>
<th>+L658, 758</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>2.35 ± 0.22</td>
<td>2.27 ± 0.33</td>
</tr>
<tr>
<td>HNE (1.0 mg/mL)</td>
<td>2.77 ± 0.24</td>
<td>2.37 ± 0.31</td>
</tr>
<tr>
<td>PAF (10⁻⁸ mol/L)</td>
<td>2.74 ± 0.45</td>
<td>2.45 ± 0.34</td>
</tr>
<tr>
<td>PMA (10⁻⁸ mol/L)</td>
<td>3.60 ± 0.45</td>
<td>3.58 ± 0.56</td>
</tr>
</tbody>
</table>

* CD18 binding sites were determined by Simply Cellular Microbeads as described in Materials and Methods (n = 4).

**DISCUSSION**

Intravital microscopy was used in this study to examine the role of elastase in PAF-induced neutrophil infiltration.
be achieved in vivo inasmuch as data in this study indicate that HNE directly increases the selective for CD 18 inasmuch as L-selectin levels remained activated. The HNE-associated changes appear to be unchanged. Moreover, the increase in CD 18 expression appears to be independent of degradation, superoxide production, or cell lysis. Although it is always possible that the stock of elastase was contaminated with endotoxin, this is unlikely because the elastase-induced increase in CD18 up-regulation could be attenuated using the specific elastase inhibitor L658 758 and was unaffected by polymyxin B. The elastase-mediated changes were also not attributable to direct injury to the neutrophils as viability was above 90% and lytic injury remained below 2% during the incubation periods. Finally, other proteinases including trypsin (this study) and chymotrypsin, as well as heat-inactivated HNE had no effect on CD18 expression.

CD11/CD18 receptors on neutrophils are known to mediate chemotaxis, neutrophil-neutrophil aggregation, neutrophil adhesion to the lining of vessels, and transvascular migration, events that are absent in patients lacking this glycoprotein (leukocyte adhesion deficiency, Type I). Our in vivo results show that elastase inhibitors significantly attenuated all of the aforementioned neutrophil functions whereas in vitro PAF-induced neutrophil chemotaxis (not neutrophil adhesion), was prevented. Moreover, purified elastase and PAF caused a significant upregulation of the CD11/CD18 glycoprotein complex, events that were prevented by the protease inhibitor L658,758. These data suggest that (1) elastase selectively enhances or regulates CD18-dependent chemotaxis, and (2) this CD18-associated biologic function may be a rate-limiting step for other neutrophil functions in vivo including adhesion and emigration. The HNE-induced increase in CD18 expression and neutrophil migration is interesting in light of the model proposed by Francis et al, whereby increased sequential expression of CD11/CD18 would contribute to the locomotion of adherent neutrophils. They showed that CD11/CD18 was upregulated at the lamellipodia and swept to the uropod as the cell migrates. They postulated that the newly expressed adhesion molecules were essential for cell chemotaxis. Interestingly, these same newly expressed adhesion molecules do not appear to contribute in a significant manner to neutrophil adhesion, also consistent with the lack of effect of HNE or L658 758 on neutrophil adhesion in vitro in our study. Although the molecular mechanisms contributing to chemotaxis rather than adhesion remains unclear, selective domains within the subunits appear to mediate the two distinct functions. Certain MoAbs directed against CD11b only prevent neutrophil binding to IC3b-coated surface, whereas others block neutrophil aggregation and chemotaxis. Although our study implicates a role for HNE in the CD18-dependent chemotaxis in vitro (not adhesion) and PAF-induced neutrophil accumulation in vivo, a potential mechanism of action for elastase in neutrophil locomotion remains to be elucidated.

Although only PAF was tested in this study, HNE also contributes to the function of other chemoattractants inasmuch as proteinase inhibitors have previously been reported to inhibit fMLP-induced polarization and locomotion of neutrophils in vitro. Moreover, L658 758 reduced neutrophil infiltration (adhesion and emigration) into postischemic microvessels in vivo, an event known to be mediated by both PAF and LTB4. Although Zimmerman and Granger suggested that limited release of elastase by

<table>
<thead>
<tr>
<th>Table 4. Effects of HNE on Neutrophil Function</th>
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<tr>
<td><strong>Superoxide</strong></td>
</tr>
<tr>
<td>Production (mmol/min/10^6 cells)</td>
</tr>
<tr>
<td>(n = 5)</td>
</tr>
<tr>
<td>Unstimulated</td>
</tr>
<tr>
<td>HNE (μg/mL)</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>PAFt</td>
</tr>
<tr>
<td>PMA</td>
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<td>fMLP</td>
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Abbreviation: MFV, mean fluorescence volume.

* Neutrophils were pretreated with dihydrocytochalasin B (final concentration 10 μmol/L) as described in Materials and Methods. Based on G6PD activity, there was less than 2% cell lysis under all conditions.

† Final PAF concentration for superoxide stimulation and L-selectin expression was 5 x 10^-8 mmol/L and 1 x 10^-7 mol/L, respectively.

‡ Final PMA concentration for superoxide stimulation and degranulation was 3 x 10^-7 mol/L/L. For L-Selectin, mol/L n = 2.

§ Final fMLP concentrations for superoxide production and degranulation were 1 x 10^-8 mol/L and 1 x 10^-6 mol/L, respectively.

... into inflamed postcapillary venules. This model has been studied and characterized by us and others. Briefly, neutrophil infiltration into PAF-inflamed vessels begins as a rolling movement along the length of the vessel wall. Within 20 minutes the number of rolling cells that become adherent increases and a proportion of the adherent cells migrate out of the vasculature and into the interstitium. The PAF-induced neutrophil infiltration, adhesion, and emigration is CD18-dependent because these events are prevented by MoAbs directed against the adhesive glycoprotein complex. Moreover, PAF causes microvascular dysfunction that is associated with neutrophil adhesion and subsequent release of both oxidants and proteases. Although HNE has been implicated as a mediator of tissue injury in a number of inflammatory models, a direct effect of elastase on neutrophil function has not been described to date. In this study, we present in vivo evidence that elastase significantly contributes to neutrophil infiltration, aggregation, adhesion, and emigration in inflammatory conditions inasmuch as the PAF-induced neutrophil infiltration was greatly attenuated by both L658 758 and Eقلین C.

To further understand the mechanism by which elastase inhibitors prevent neutrophil infiltration, the direct action of this proteinase on neutrophils was assessed in vitro. The data in this study indicate that HNE directly increases the upregulation of the subunit of the CD11/CD18 glycoprotein complex between approximately 0.01 and 1.0 μg/2 x 10^6 neutrophils. These concentrations of HNE are likely to be achieved in vivo inasmuch as 2 million neutrophils contain approximately 4 to 8 μg of elastase and release approximately 20% to 30% of their elastase content when activated. The HNE-associated changes appear to be selective for CD18 as L-selectin levels remained unchanged. Moreover, the increase in CD18 expression appeared to be independent of degradation, superoxide production, or cell lysis. Although it is always possible that the stock of elastase was contaminated with endotoxin, this is unlikely because the elastase-induced increase in CD18 up-regulation could be attenuated using the specific elastase inhibitor L658 758 and was unaffected by polymyxin B. The elastase-mediated changes were also not attributable to direct injury to the neutrophils as viability was above 90% and lytic injury remained below 2% during the incubation periods. Finally, other proteinases including trypsin (this study) and chymotrypsin, as well as heat-inactivated HNE had no effect on CD18 expression.

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neutrophils during diapedesis could effectively digest a route for transvascular emigration, our data raise the possibility that elastase may also significantly contribute to the localization of leukocytes into inflamed postcapillary venules, an event that precedes emigration of these cells.

It is also conceivable that in vivo elastase may affect other inflammatory cells, which would contribute to PAF-induced neutrophil infiltration. For example, binding of elastase to macrophages stimulates the production and release of the potent neutrophil chemoattractant leukotriene B4,9 which can also enhance neutrophil infiltration, adhesion, and transmigration.31,41 Alternatively, platelets can also be activated by neutrophil proteinases, and may in turn influence neutrophil function.42 Moreover, neutrophil-neutrophil interactions may also contribute to neutrophil infiltration in vivo. We (this study) and others (32) have observed that neutrophil-neutrophil interactions significantly increase in postcapillary vessels in the presence of PAF. Bjork et al23 reported that the aggregation was in fact an important component of the adhesion process. For example, during homotypic (neutrophil-neutrophil) adhesion, an adherent neutrophil may enhance the likelihood that a rolling neutrophil will adhere to it and subsequently to the endothelium. Although the importance of this mechanism to neutrophil accumulation remains to be elucidated, the PAF-induced neutrophil adhesion observed in this study occurred in aggregates (Fig 1), and these homotypic interactions were essentially abolished by the elastase inhibitors (Table 2).

It is well established that proinflammatory stimuli, including leukotrienes, cytokines, and PAF, can induce neutrophil rolling, chemotaxis, adhesion, and emigration in postcapillary venules via a direct effect on L-selectin and the CD11/CD18 glycoprotein complex.15,16 However, the concept that neutrophils may also regulate their own function has only recently been proposed. Hermanowski-Yosak et al25 have reported that an integrin modulating factor (IMF-1) produced by activated neutrophils appears to reversibly alter the avidity (not number) of the integrin CD11/CD18, transiently promoting neutrophil adhesion to the endothelium. Moreover, Jutila et al37 have shown that chymotrypsin-like proteinases regulate the homing receptor (L-selectin) on neutrophils, which appears to be essential for neutrophil rolling. In this study, we show that HNE increases the expression of CD11/CD18, an event that may be important in chemotactically stimulated neutrophil locomotion.37

It remains unclear as to how HNE might enhance neutrophil infiltration in light of the fact that plasma contains a series of potent anti-proteinases that can effectively inactivate extracellular HNE and prevent the protease from binding to neutrophils.24 In fact, it has been shown that 1 mL of plasma contains sufficient anti-proteolytic activity to inactivate 750 μg of elastase.3 However, given the finding that two structurally different elastase inhibitors greatly attenuated the PAF-induced neutrophil adhesion and emigration process, it is reasonable to assume that elastase can circumvent the entire antiproteinase shield and modulate neutrophil function in vivo. Moreover, once elastase binds to a cell receptor it may be protected against degradation from plasma antiproteinases, an event recently reported for cathepsin G binding to platelets.42 In contrast to plasma antiproteinases, the elastase inhibitors are very small molecules and insensitive to the site-specific attack of oxidants. Therefore, L658 758 (330 MW) and Eglin C (8,000 MW) are likely to penetrate the neutrophil-endothelial cell microenvironment (limited size restriction) and, because they are impervious to oxidants, they are likely to inactivate much of the released elastase and perhaps modulate neutrophil function. In support of this hypothesis is the observation from our laboratory that small, synthetic antiproteinases prevented fibronectin degradation by adherent leukocytes more effectively than plasma per se.28

Many questions remain regarding the mechanism leading to elastase-dependent CD18 expression. For example, the possibility exists that, rather than causing translocation of intracellular CD18 to the cell surface, HNE may proteolytically remove other membrane molecules thereby increasing access to constitutive CD18. Although this hypothesis cannot be dismissed, HNE did not affect L-selectin expression in vitro suggesting that elastase did not affect access to this particular surface antigen. Despite the fact that trypsin had no affect on CD18 expression, the role of other neutrophilic serine proteinases (cathepsin G and metalloproteinases) on CD18 expression warrants further investigation. Finally, these findings may be important in chronic inflammatory conditions where the antiproteinase barrier is decimated (evidenced by measurable elastase activity in exudate fluids and plasma) and free elastase might affect neutrophil function and contribute to uncontrolled inflammation.

NOTE ADDED IN PROOF

Leukosialin (CD43) is a major membrane sialoglycoprotein on neutrophils whose function remains unknown. In preliminary observations from our laboratory, both exogenous and endogenous HNE shed CD43 from the neutrophil membrane. This elastase-dependent shedding may influence: (1) access to constitutive CD18, and (2) leukocyte-endothelial cell interactions.

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