Neutrophil Oxidants Inactivate Alpha-1-Protease Inhibitor and Promote PMN-Mediated Detachment of Cultured Endothelium

Protection by Free Methionine

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Activated granulocytes have been implicated in mediating pulmonary endothelial damage in the Adult Respiratory Distress Syndrome. In another lung disease, emphysema, pulmonary granulocytes (PMNs) are thought to be doubly responsible for lung dissolution: they release potent proteolytic enzymes including elastase, and they generate reactive oxygen species that oxidize a reactive site methionine group in alpha-1-protease inhibitor (α-1-PI) rendering it, in turn, impotent as an anti- elastase. This suggested an analogous scenario for pulmonary vascular damage: namely, undetected PMN elastase might also mediate endothelial injury. Our strategy to prove this notion used chromium-labeled human endothelial cells exposed to intact PMN or to enucleate “neutroplasts.” The latter are elastase-free cytoplasmic blebs derived from PMN. When activated, both PMN and neutroplasts generate similar amounts of toxic oxygen species; yet neutroplasts caused insignificant endothelial damage, measured as Cr "lift-off" from anchoring matrix (PMN = 24.3% ± 1.8% vs neutro- plast = 1.2% ± 0.4%; p < 0.001). Adding pure elastase back to neutroplasts increased endothelial cell lift-off (7% ± 0.2%). Although the prototypic serine protease inhibitor phenyl methylsulfonylfluoride (PMSF) protected endothelium from PMNs, pure α-1-PI (also a potent anti-elastase) when added in physiologic amounts did not protect endothelial cells from PMN assault, suggesting that PMN oxidants might inactivate it. By adding exogenous myeloperoxidase (MPO) to MPO-deficient neutroplasts, we demonstrated that MPO-dependent oxidants, probably N-chloramines, are critical inactivators of α-1-PI. This was further confirmed since added free methionine, a scavenger of chloramine, protected α-1-PI from inactivation by reagent chloramine or that produced by rearmed neutroplasts or PMN. Concomitantly, free methionine inhibited PMN-provoked endothelial cell lift-off in a dose-dependent fashion while jointly defending anti-elastase activity of α-1-PI. We conclude that stimulated neutrophils can injure vascular tissue by combining elastase secretion with oxidative inactivation of the anti-elastase, α-1-PI. (Arteriosclerosis 6:332–340, May/June 1986)

The initial event leading to atherosclerosis is probably injury to blood vessel endothelium. Although much interest has centered on the monocyte-macrophage as an effector of endothelial damage, we have suggested that granulocytes might be particularly important in initiating atherosclerosis. We have shown that stimulated granulocytes, which normally adhere to vascular endothelium before diapedeses, can directly damage vascular endothelium in various clinical syndromes such as in hemodialysis, in the adult respiratory distress syndrome, and in the cholesterol embolization syndrome. We and others have also demonstrated that granulocytes damage endothelium by generating toxic oxygen species as well as by releasing potent proteolytic enzymes. Granulocyte-oxidant damage can be modulated by exogenously-added scavengers of O₂⁻ species, such as catalase and superoxide dismutase, as well as by intracellular reductants of the endothelial cells themselves including glutathione.
against protease-mediated damage is primarily extracellular, and serum protease inhibitors such as alpha-1 protease inhibitor (α-1-PI) are capable of dampening granulocyte-mediated proteolysis of endothelial matrix.12

That both oxidants and proteases can act synergistically in destroying tissue has been suggested by studies into the pathogenesis of emphysema; that is, granulocytes can damage lung tissue by releasing neutral proteases, particularly elastase. Additional insult occurs when the potential capacity of α-1-PI to inhibit elastase is blocked after its oxidation by granulocyte oxidants: specifically, products of the granulocyte myeloperoxidase system have been shown to oxidize the active-site methionine of α-1-PI, rendering it inactive against neutrophil elastase.13 These data have been synthesized into a scenario in which granulocytes damage lung tissue through the direct action of their proteases, and simultaneously enhance that damage by oxidatively inactivating a critical protease (elastase) inhibitor. Suspicions that a similar combination may be germane to endothelial damage has been raised by the studies of Weiss and Regiani,12 who showed that α-1-PI can prevent granulocyte-mediated degradation of endothelial matrix proteins in vitro; however, this protection is abrogated if the α-1-PI is altered by exposure to granulocyte-derived oxidants. Furthermore, Weiss suggested that the critical granulocyte oxidant of α-1-PI was hypochlorous acid or its amino adducts, the N-chloramines, which is consistent with his and other previous studies14 that myeloperoxidase-derived hypohalous acids are probably the most potent oxidants generated by stimulated granulocytes.

In the present studies we have utilized a novel technique first described by Roos et al.15 to produce enucleated and granule-depleted neutrophils in order to further explore how PMN oxidants and proteases may interact to provoke granulocyte-mediated endothelial damage. These enucleated cytoplasts, or neutroplasts, are virtually devoid of granule contents including myeloperoxidase and elastase; yet upon stimulation they produce superoxide and hydrogen peroxide in amounts similar to those of intact granulocytes.15, 16 We have found that by adding to neutroplasts one or another granule product, it is possible to dissect the role of a specific lysosomal constituent in granulocyte-mediated injury. Using this system, we confirmed a critical role for granulocyte elastase in promoting the detachment of endothelial cells from their anchoring matrix in vitro. Moreover, by adding exogenous myeloperoxidase (MPO) to neutroplasts, we demonstrated that α-1-PI is inactivated by MPO-derived oxidants, including the N-chloramines; when so oxidized, we found that α-1-PI is impotent in preventing granulocyte-mediated endothelial cell detachment. We reasoned that fluid-phase methionine might serve as bait for oxidants and might thereby preserve the active-site methionine of α-1-PI. Indeed, we showed that the addition of free methionine to our system of stimulated granulocytes, endothelial cells, and α-1-PI preserved the anti-elastase activity of the latter and protected the endothelium from granulocyte assault. Our results, which have been published in preliminary form elsewhere,17 indicate that, as in their role in the pathogenesis of emphysema, granulocytes utilize both oxidants and proteases in consort to provoke endothelial cell damage.

**Methods**

**Reagents**

Alpha-1-antitrypsin (α-1-PI) (chromatographically prepared from human plasma), methionine, methionine sulfide, tauine, catalase (bovine liver, 11,000 U/mg protein, thymol-free), N-formyl-methionine-leucine-phenylalanine (FMLP), superoxide dismutase (type I, bovine blood, 2750 U/mg protein), phorbol myristate acetate (PMA), porcine pancreatic elastase (type I) (15 U/mg protein), phenylmethylsulfonylfluoride (PMSF), Dithionitrobenzoic acid (DTNB), cytochrome C type III, and methoxysuccinyl-alanyl-alanyl-prolyl-valine-nitroanilide (MeO-Suc-Ala-Ala-Pro-Val-nitroanilide) were obtained from the Sigma Chemical Company, St. Louis Missouri. Sodium azide, sodium cyanide, and sodium hydroxide were obtained from the J.T. Baker Chemical Company, Phillipsburg New Jersey. Purified human neutrophil myeloperoxidase (MPO) was a gift from Dr. Robert Nelson (Department of Surgery, University of Minnesota, Minneapolis Minnesota). Cytochalasin B was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin.

**Preparation of Granulocytes**

Human venous blood was obtained from normal volunteers after informed consent. The blood (40 ml) was drawn into a plastic syringe containing 20 ml of hydroxy-ethyl-starch (Hespan, American Hospital Supply Corporation, Irvine, California) and 200 U of preservative-free heparin. The mixture was allowed to sediment for 30 minutes at room temperature; the supernatant was centrifuged at 400 g for 5 minutes at 4°C; and the pellet was resuspended in 0.2 ml Hanks' buffered salt solution (HBSS) (Gibco Laboratories, Chagrin Falls, Ohio). Residual erythrocytes were lysed in 15 ml of ice-cold distilled water, and after 25 seconds, isotonicity was reconstituted by the addition of 5 ml of 3.6% NaCl. This suspension was centrifuged at 400 g for 5 minutes; the pellet was resuspended in 5 ml of HBSS and was carefully layered on top of 8 ml of Percoll (Pharmacia, Piscataway, New Jersey) made up to a density of 1.075. This was then centrifuged at 19,000 g for 30 minutes at 4°C. The resulting PMNs (>95%) were washed once and suspended at 107/ml in HBSS. In some experiments, we used PMNs from a patient with chronic granulomatous disease who was referred by Dr. Paul Quie at the University of Minnesota.

**Preparation of Enucleated Cytoplasts of Granulocytes**

Enucleated cytoplasts of PMNs (neutroplasts) were prepared according to the method of Roos et al.15 and Korchak et al.18 In brief, PMNs prepared as above were suspended in a 12.5% (wt/vol) Ficoll 70 solution (Pharmacia, Piscataway, New Jersey) containing cytochalasin B at 5 μg/ml. This suspension was layered on a discontinuous gradient of 16% Ficoll and 25% Ficoll and centrifuged at 100,000 g for 30 minutes at 37°C. The band of neutroplasts was harvested from the interface of the 12.5%/16% Ficoll layers and was washed three times in HBSS. The
neutroplasts were counted by hemocytometry, as well as with a Coulter counter (Coulter, Hialeah, Florida), and were suspended at a concentration of 2 x 10^7/ml in HBSS. As demonstrated in our previous study, neutroplasts are virtually granule-free by electron microscopic examination and release virtually no (<1%) beta glucuronidase, elastase, myeloperoxidase, or lactoferrin upon stimulation with 10^{-7} FMLP and 5 μg/ml cytochalasin B, compared to intact stimulated PMNs. In our experiments, 10^7 neutroplasts produced similar quantities of superoxide (measured by cytochrome C reduction in 15 minutes) as 2.5 x 10^8 PMNs.

**Endothelial Cell Detachment Assay**

Human umbilical vein endothelial cells were grown to confluence in 24-well 2 cm^2 dishes (Costar, Cambridge, Massachusetts), and contained Factor VIII antigen by immunofluorescence assay. The endothelial cell monolayers were washed twice with sterile HBSS at 37° C, were labeled with 2.5 μCi Na^31CrO_4 (Amersham, Arlington Heights, Illinois) for 3 hours, and were then washed four times with HBSS. PMNs (2.5 x 10^6) or neutroplasts (10^7) were added to each well and were stimulated with 100 ng/ml PMA in final volume of 1 ml. PMN or neutroplast contact with endothelial cell monolayers was ensured by 60 g centrifugation for 3 minutes at room temperature of the plates which were then incubated for 3 hours at 37° C. Thereafter the reaction solution (1 ml) was removed, the well pellet was washed twice with 1 ml HBSS, and the combined washes from each well were centrifuged at 500 g for 10 minutes. The amount of ^51Cr was determined in the supernatant of the endothelial cell monolayers. The remaining adherent endothelial cells were quantitated after first detaching them by incubating the washed monolayers with 1.0 ml of 1 N NaOH for 10 minutes. The fraction of cells detached was calculated with the formula:

$$\frac{B}{A + B + C}$$

A represents the cpm in the supernatant of the endothelial cell wash; B represents the cpm of the wash pellet; C represents the cpm of the cells remaining adherent to wells after washing. Specific detachment was calculated by subtracting the fraction of cells detached in a well containing buffer (HBSS) alone, which was always less than 3%. The specific free ^51Cr release (endothelial cell "lysis") at 3 hours was calculated with the formula:

$$\frac{A}{A + B + C} - \text{ spontaneous } ^{51}\text{Cr release}$$

with HBSS alone

PMA-stimulated PMNs released 4.2% ± 0.7% ^51Cr, while PMA-stimulated neutroplasts released 3.0% ± 0.4% ^51Cr.

**Granulocyte and Cytoplast Adhesion to Endothelium**

Neutroplast and PMN adhesion to endothelium was measured by ^111In labeling of neutroplasts and PMNs as described by Goldstein. Indium-labeled neutroplast (2.0 x 10^6) or PMNs (0.5 x 10^6) were added to endothelial cell monolayers in microtiter wells and were incubated at 1 g for 30 minutes at 37° C. The wells were then washed twice with HBSS, and adherent neutroplasts or PMNs were counted in a gamma counter. Resting neutroplast adherence was 7.2% ± 1.9% while PMA-stimulated adherence increased to 22.1% ± 3.2%. Resting PMN adherence to endothelium was 24.1 ± 2.2 while PMA-stimulated adhesion was 79.8 ± 2.6.

**N-Chloramine Assay**

N-Chloramines were assayed by their ability to oxidize thionitrobenzoic acid (TNB) to dithionitrobenzoic acid (DTNB) as described by Weiss et al. Briefly, PMN (2.5 x 10^6/ml) or neutroplasts (10^7/ml) were stimulated with PMA (30 ng/ml) and were incubated in a shaking water bath at 37° C for 90 minutes. At the end of the incubation, catalase (100 μg/ml) and TNB (100 μM) were added to each sample, which was centrifuged at 500 g for 10 minutes, and the amount of TNB oxidized was measured spectrophotometrically at 412 nm. The quantity of TNB oxidized was calculated from the decrease in absorbance by using the extinction coefficient:

$$E_{412} = 1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$

Further characterization of the N-chloramines produced by PMA-stimulated PMNs and neutroplasts were obtained by measuring the ultraviolet absorption maximum at 252 nm of stimulated cell supernatants as described.

**Assay of the Elastase Inhibitory Capacity of Alpha-1-Protease**

To examine the effect on α-1-PI of cell-derived oxidants, α-1-PI (partially purified from human plasma, Sigma A6150) (0.5 mg/ml) was added to 10^7/ml PMNs or neutroplasts (4 x 10^9/ml) or to reagent taurine chloramine prepared by adding 5% NaOCl to a fivefold excess of taurine, and correcting the pH to 7.4 with 1.0 N HCl. The neutroplasts or PMNs were stimulated with PMA (30 ng/ml) for 60 minutes at 37° C, the cells were removed by centrifugation, and the anti-elastase capacity of the α-1-PI solutions was tested as follows: Both purified PMN elastase (a gift from Dr. Steven McGowan, University of Minnesota) or PMN supernatant were used as sources of elastase; supernatant was made by stimulating PMNs (10^{-7}M) with FMLP (10^{-7}M) and cytochalasin B (5 μg/ml) for 60 minutes at 37° C in a shaking water bath and harvesting the elastase-rich PMN supernatant after centrifugation. The α-1-PI solution (0.1 ml) was added to elastase-rich PMN supernatant or purified elastase (0.3 ml) and an elastase substrate, MeO-Suc-Ala-Ala-Pro-Val-nitroanilide, in 1% DMSO in PBS at pH 7.4 to make a final volume of 1.0 ml. The change in absorbance was measured spectrophotometrically at 410 nm over 15 minutes at 37° C. The amount of elastase activity present in each sample is proportionate to the amount of nitroanilide split from this tetrapeptide as measured spectrophotometrically (E_{410} = 8800 M^{-1} cm^{-1}). The elastase inhibitory capacity (EIC) of the α-1-PI samples was calculated as previously described.
Results

Opposing Role of Elastase and Serum Protease Inhibitors In Endothelial Cell Lift-Off by Granulocytes

Previous studies by others have indicated that granulocyte-released elastase can digest matrix proteins that are synthesized by cultured endothelial cells; moreover, α-1-PI, if protected from oxidation, can abrogate this degradation. Studies shown in Figures 1 and 2 extend these results to the cellular level. That is, intact granulocytes, when stimulated with PMA, can detach 24.3% ± 1.8% of cultured human umbilical vein endothelial cells from their anchoring matrix (left bar, Figure 1). PMN alone and PMA alone caused 0.8% ± 2% and 0.8% ± 3% lift-off, respectively. A critical role of a lysosomal constituent in this detachment is validated in that lysosome-free neutroplasts, even when used in numbers to produce similar amounts of assayable superoxide as granulocytes (39.8 ± 3.4 vs 42.3 ± 3.1 nmol/15 minutes), are virtually incapable of causing endothelial lift-off (center bar, Figure 1), but both activated granulocytes and neutroplasts caused specific (soluble) 51Cr release from endothelial cells (4.2% ± 0.7% and 3.0% ± 0.4% respectively). This specific 51Cr release was 89.0% ± 3.3% inhibitable by the addition of catalase 100 μg/ml and superoxide dismutase (SOD) 10 μg/ml to either PMNs or neutroplasts. However, these oxidant inhibitors failed to block PMN-mediated lift-off (catalase 100 μg/ml 32.6% ± 3.7% and SOD 10 μg/ml 25.4% ± 2.8% detachment, respectively). The neutroplast's ability to detach endothelium is significantly rejuvenated by the addition of exogenous, purified elastase (10 μg/ml) (3rd bar, Figure 1) — to a level about twice that noted when elastase is added alone (3.7% ± 0.5% detachment).

As might be predicted from the above, elastase inhibitors reduce granulocyte-mediated endothelial detachment; the prototype serine protease inhibitor, PMSF (1 mM), is efficient in this regard (Figure 2, bar). Lower concentrations of PMSF (0.1 and 0.01 mM) inhibited detachment inefficiently however (1.2% ± 1.3% and 0.5% ± 0.9%), respectively. However, the more physiologically-relevant, serine protease inhibitor, α-1-PI, is relatively ineffective in protecting endothelium from granulocyte assault. At several concentrations (0.05 to 0.09 mM), α-1-PI was unable to prevent endothelial detachment by granulocytes, and only at concentrations higher than normally found in serum is α-1-PI significantly inhibitory (Figure 2). Furthermore, PMA-stimulated PMNs from a patient with chronic granulomatous disease (CGD) readily detached endothelial cells 30.3% ± 4.8%, but α-1-PI at concentrations of 1 mg/ml and 2.5 mg/ml inhibited this detachment by 65.6% and 53.7%, respectively. These data suggest that α-1-PI can be inactivated by PMN-derived oxidants since the oxidant from CGD cells allowed the α-1-PI to prevent EIC detachment.

Figure 1. Effect of activated granulocytes (PMNs) and neutroplasts on endothelial cell monolayers. PMN (2.5 × 10⁶/ml), neutroplasts (10⁶/ml) and neutroplasts (10⁷/ml) plus elastase (10 μg) were stimulated with phorbol myristate acetate (PMA) (100 ng/ml) at 37°C for 3 hours; endothelial cell detachment was measured by ⁵¹Cr release as described in Methods. Values represent the means ± se of at least eight experiments done in duplicate. PMN vs neutroplast, p < 0.001; neutroplasts vs neutroplasts plus elastase, p < 0.01. The addition of elastase to unstimulated neutroplasts causes 3.5% ± 0.8% lift-off (p < 0.01) which was significantly less (p < 0.05) than the lift-off caused by the addition of elastase to stimulated neutroplasts.

Figure 2. Inhibition of endothelial cell detachment by protease inhibitors. Granulocytes (PMNs) (2.5 × 10⁶/ml), phorbol myristate acetate (PMA) (100 ng/ml), and varying amounts of α-1-PI (left) or phenyl methylsulfonylfluoride (PMSF) (1 mM) (right) were added to endothelial cell monolayers and incubated at 37°C for 3 hours. Detachment was measured by ⁵¹Cr release and is expressed as a percent inhibition of detachment caused by PMA-stimulated PMNs without protease inhibitors. Values represent the means ± SE of at least five experiments done in duplicate.
Inactivation of \( \alpha-1 \)-Protease Inhibitor by Granulocyte-Derived Chloramines and Its Protection by Methionine

These results suggest that the endothelial-protective role of \( \alpha-1 \)-PI is rendered incompetent by stimulated granulocytes. In fact, Weiss et al. have reported that granulocytes use a myeloperoxidase-dependent process to produce long-acting oxidants, the N-chloramines, which are capable of oxidatively inactivating \( \alpha-1 \)-PI. We confirmed this using our neutrophil system as follows: since neutrophils contain no detectable myeloperoxidase, we postulated that they would be unable to produce significant quantities of chloramines, unless rearmed with exogenously added MPO; as a corollary, "naked" and MPO-rearmed neutrophils should be useful in investigating the role of chloramines in the inactivation of \( \alpha-1 \)-PI. Indeed, as shown in Figure 3, we found that stimulated granulocytes (2.5 \( \times \) \( 10^6 \)) produced ample amounts of chloramine (42.3 ± 2.9 nmol/90 min), while similarly stimulated neutrophils in numbers chosen to produce identical amounts of superoxide as granulocytes (1 \( \times \) \( 10^6 \)) generated hardly any (4.0 ± 3.4 nmol/90 minutes), as measured by TNB oxidation (see Methods); when \( 10^7 \) neutrophils were rearmed with MPO, however, they increased their chloramine production to normal levels (33.2 ± 3.4 nmol/90 min). TNB oxidation mainly reflects N-chloramine production since the stimulated cell supernatants had a characteristic chloramine ultraviolet absorption maximum at 252 nm. As also shown in Figure 3, methionine, previously reported to be a scavenger of both hypochlorous acid and N-chloramines, completely blocked the ability of granulocytes and MPO-armed neutrophils to generate assayable chloramines (2.1 ± 0.7 and 3.0 ± 1.3 nmol TNB oxidized/90 minutes, respectively). Conversely, in ancillary studies we added pre-oxidized methionine, methionine sulfoxide, to PMA-stimulated granulocytes and found it much less effective in inhibiting chloramine production (14.1 ± 2.2 nmol/90 min).

This ability of granulocytes and neutrophils with added MPO to produce chloramines closely parallels their capacity to inactivate \( \alpha-1 \)-PI as shown in Tables 1 and 2. For instance, unstimulated granulocytes caused no change in the EIC of \( \alpha-1 \)-PI, while PMA-stimulated granulocytes decreased EIC from 94.6% to 2.8% (Table 1). PMA-stimulated chronic granulomatous disease PMNs (2.5 \( \times \) \( 10^6 \)) did not significantly decrease the EIC of \( \alpha-1 \)-PI (98%), nor did they produce chloramines (1.6 ± 0.6 nmol/90 min). Addition of the chloramine scavenger, methionine, to stimulated granulocytes resulted in almost complete restitution of the EIC of \( \alpha-1 \)-PI (88.1% ± 2.4%), while the oxidized methionine sulfoxide was not restitutive (Table 1). To determine which granulocyte constituents are required to inactivate \( \alpha-1 \)-PI, we utilized neutrophils and added back various components of interest. As seen in Table 2, neutrophils alone, or even when stimulated with PMA, had no effect on the EIC activity of \( \alpha-1 \)-PI (96.5% and 87.0%, respectively). However, when myeloperoxidase was added back to stimulated neutrophils, chloramines were produced, and the EIC of \( \alpha-1 \)-PI was reduced to 3.0% ± 2.2%. The inactivation of \( \alpha-1 \)-PI by MPO-rearmed neutrophils can be blocked by the addition of MPO inhibitors such as azide or cyanide; moreover, since chloramine production requires \( \mathrm{H}_2\mathrm{O}_2 \) to generate hypohalous acids, it is not surprising that the hydrogen peroxide scavenger, catalase, and the chloramine scavenger, methionine, also inhibited MPO-rearmed neutrophil inactivation of \( \alpha-1 \)-PI, while the scavenging of superoxide with superoxide dismutase did not (Table 2).

![Figure 3. Neutrophils and neutrophils re-armed with myeloperoxidase produce N-chloramines. Granulocytes (PMNs) (2.5 \( \times \) \( 10^6 \)) or neutrophils (2.5 \( \times \) \( 10^6 \)) were stimulated with phorbol myristate acetate (PMA) (30 ng/ml) for 90 minutes at 37°C. Methionine (1 mM) or myeloperoxidase (MPO) (10 \( \mu \)M) were added to the incubates as indicated. The results are expressed as the mean nmol ± SE thionitrobenzoic acid (TNB) oxidized per 90 minutes measured as described in Methods. The results represent six experiments done in duplicate.](image-url)
Table 2. Effect of Neutrophils on Elastase Inhibitory Capacity of Alpha-1-Protease

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Elastase inhibitory capacity (%)</th>
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<tr>
<td>α-1-PI alone (n = 24)</td>
<td>94.6 ± 0.7</td>
</tr>
<tr>
<td>+ Neutrophils (n = 4)</td>
<td>96.5 ± 1.0</td>
</tr>
<tr>
<td>+ Neutrophils + PMA (n = 13)</td>
<td>87.0 ± 4.1</td>
</tr>
<tr>
<td>+ Neutrophils + PMA + MPO (n = 11)</td>
<td>3.0 ± 2.2</td>
</tr>
<tr>
<td>+ Neutrophils + PMA + MPO + methionine</td>
<td>96.2 ± 0.8</td>
</tr>
<tr>
<td>+ Neutrophils + PMA + MPO + SOD (n = 8)</td>
<td>3.7 ± 2.5</td>
</tr>
<tr>
<td>+ Neutrophils + PMA + MPO + azide (n = 9)</td>
<td>95.4 ± 1.0</td>
</tr>
<tr>
<td>+ Neutrophils + PMA + MPO + cyanide (n = 9)</td>
<td>96.0 ± 0.7</td>
</tr>
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</table>

The elastase inhibitory capacity (EIC) was calculated as in Table 1 and Methods. Neutrophils, with or without added phorbol myristate acetate (PMA), demonstrated no intrinsic elastase inhibitory capacity. The concentrations used were as follows: neutrophils (4 x 10^6/ml), PMA (30 ng/ml), α-1-PI (0.5 mg/ml), methionine and taurine (1.0 mM), myeloperoxidase (MPO) (10 μM/ml), superoxide dismutase (SOD) (10 μg/ml), azide (1.0 mM), cyanide (1.0 mM), and catalase (100 μg/ml).

The results are expressed as the mean percentage ± SEM.

A more direct validation that granulocyte-derived chloramines are important inhibitors of anti-elastase activity of α1 PI is shown in Figure 4. We prepared purified taurine chloramine from hypochlorous acid and taurine and found that it inhibited α1-PI in a dose-dependent fashion (solid line, Figure 4); as in our granulocyte/neutrophil studies, the addition of excess free methionine to this system completely prevented chloramine’s inactivation of α1-PI (dashed line, Figure 4).

In a final synthesis of these results, a close association between the ability of methionine to protect the anti-elastase activity of α1-PI and its ability to protect endothelium from granulocyte assault is shown in Figure 5. Despite the addition of physiologic levels of α1-PI to endothelium exposed to PMA-stimulated granulocytes, significant endothelial detachment occurred in 3 hours. However, excellent and parallel prevention of both α1-PI inactivation and endothelial detachment followed the addition of as little as 0.2 mM methionine. The addition of methionine 1.0 mM alone to PMA-stimulated PMNs failed to inhibit lift-off. In fact, detachment was increased by 6.5% ± 3.7% (NS).

To determine if serum proteins could inhibit lift-off, we added serum to PMA-stimulated PMNs. Although fresh serum at a final concentration of 5% decreased lift-off by only 13.7% ± 3.6% (p = NS), the addition of 1.0 mM methionine and 5% serum resulted in the inhibition of lift-off by 44.5% ± 4.9% (p < 0.01). Analogous results were found when the serum’s anti-elastase activity was measured. The EIC of the α1-PI in 5% serum incubated with PMA-stimulated PMNs was 7.9% ± 4.6%, but if methio-
nine (1.0 mM) was added, the EIC increased to 77.6 ± 6.3
(p < 0.01). The addition of higher concentrations of serum
(20%) decreased lift-off by 53.0% ± 6.3% and added
methionine (1.0 mM) failed to further enhance the inhibi-
tory effect (57.0% ± 8.3%). Serum at a concentration of
20% incubated with PMA-stimulated PMNs had an EIC of
66.2 ± 3.8 which increased only slightly to 73.4 ± 9.0
when 1.0 mM methionine was added. Serum from a patient
homozygous ZZ for α-1-PI deficiency (a gift from Dr. Har-
vey Sharp, University of Minnesota) was used in our de-
tachment assay. The α-1-PI-deficient sera (0.4 mg/ml) at
5% did not inhibit (1% inhibition) detachment but at 20% it
inhibited by 59%. Methionine (1mM) added to the 5% α-1-
PI deficient sera increased its inhibitory effect on detach-
ment to 16.3% but did not enhance the 20% deficient sera-
inhibiting capacity.

Discussion

We used a novel technique in the present studies to
produce lysosome-free neutrophils to demonstrate that
PMN lysosomal enzymes are critically involved in delami-
nating endothelial cells from their underlying matrix. Thus,
neutrophils, which efficiently produce superoxide and hy-
drogen peroxide but which contain virtually no lysosomal
enzymes, cannot detach endothelial cells unless there is
exogenously added elastase in amounts similar to that
released by 2.5 × 10⁶ PMNs. By way of further confirma-
tion of elastase's critical role in endothelial detachment, we
observed that the elastase inhibitor, PMSF, inhibits PMN-
mediated lift-off. α-1-PI was relatively inert in protecting
endothelium from elastase assault of normal PMNs. The
oxidant-poor CGD PMNs detached endothelium which, in
contrast to normal PMNs, was inhibited by α-1-PI. The
reasonable hypothesis, provoked by previous work of oth-
ers, was that α-1-PI might be particularly vulnerable to
inactivation by PMN-derived oxidants; this was confirmed —
again using rearmed neutrophils. In this case we used
neutrophils with added exogenous myeloperoxidase to
demonstrate that MPO-catalysed oxidants inactivate α-1-
PI. We believed that the N-chloramines are particularly
attractive candidates as critical α-1-PI inactivators in that
their assayable levels after stimulation of intact PMNs or
neutrophils with MPO correlate well with the degree of α-
1-PI inactivation. Moreover, if PMN/neutrophil generation
of chloramine is inhibited with azide, cyanide, or catalase,
or if it is scavenged by methionine, α-1-PI is completely
protected from inactivation (Tables 1 and 2).

Although it is a reasonable hypothesis, we believe that
the inability of stimulated neutrophils to delaminate endo-
thelial cells is not simply due to their decreased endothelial
adhesion. Harlan et al.⁷ have shown that stimulated
PMNs will not disrupt endothelial cell monolayers unless
the PMNs are in close contact with the endothelium. In
elegant studies, they showed that activated PMNs that are
genetically devoid of a membrane glycoprotein, GP 150,
which is necessary for PMN adhesion, cannot detach en-
dothelium.⁸, ⁹ Our studies have shown that the adher-
ence of stimulated neutrophils (approximated to endothe-
lum with a force of 1 g) was somewhat less than that of
stimulated PMN. However neutrophil adhesion does in-
crease threefold after stimulation by PMA, indicating that
neutrophils can significantly increase their adhesiveity to
endothelium. Furthermore, in our endothelial cell detach-
ment studies, neutrophils and PMN were brought into
endothelial contact with a force of 60 g to insure appro-
priate adhesion. With such techniques, PMA-stimulated neu-
traphils have been shown in the present studies and
these recently published¹⁰ to be adhesive enough to cause
significant ⁵¹Cr release from red blood cells. Finally, since
SOD and catalase failed to inhibit lift-off induced by intact
PMNs, it is doubtful that the oxidants produced by neutro-
phils would be sufficient to cause lift-off even if their ability
to adhere to endothelium could be improved.

The important role of PMN adhesion in the detachment
of endothelial cells may, however, explain the inability of
elastase-reconstituted neutrophils to detach endothelial
cells to the same extent as stimulated PMNs. Although the
amount of elastase added to the neutrophils was equal to
that expected to be found in the number of PMN used in the
assay, the elastase was merely added to the wells with
neutrophils. Since the neutrophils were not truly "recon-
stituted," they would be unable to deliver elastase directly
to endothelium as stimulated PMNs can and thus might be
less efficient in detaching endothelial cells. This is congru-
ent with the findings of Campbell et al.¹⁰ that demonstrate
the need for close contact between PMNs and substrate
for efficient proteolysis.

Our studies also help resolve the seemingly conflicting
conclusions in the literature concerning PMN-mediated
endothelial injury in vitro. Harlan and co-workers¹¹ have
favorized a primary role for PMN-derived neutral proteases
in causing endothelial ⁵¹Cr release in culture. Their stud-
ies, using techniques that measure mainly endothelial cell
lift-off from culture vessels, showed serine protease inhibi-
tors and serum, but not oxygen radical scavengers, dimin-
ished PMN-mediated endothelial release. Our own pre-
vious studies⁷ have focused on the toxic oxygen species
derived from stimulated neutrophils as directly injurious to
cultured endothelium. Our results have been further con-
firmed by two other groups⁸, ⁹ who found that PMN-derived
hydrogen peroxide was particularly relevant to endothelial
damage. These latter studies, as well as our own, have
assayed the release of soluble radioactive chromium from
cultured endothelium, but not particularly the detachment
of intact endothelial cells from their underlying matrix. We
suggest that a reasonable resolution and synthesis of
these past studies with the present ones might be: 1) direct
endothelial cell damage with release of soluble radiolabel
that can be provoked by a toxic oxygen species, such as H₂O₂,
that are released by stimulated PMNs; 2) in contrast, endo-
thelial lift-off, assayed by release of radiolabeled cells from
anchoring matrices is fostered by PMN proteases, particu-
larly elastase, but other proteases such as collagenase and
cathepsin D may also be involved; however, this "in-
jury" is also critically assisted by toxic oxygen species of
stimulated PMNs which inactivate the otherwise potent
topological anti-elastase, α-1-PI.

We believe that the mechanism of oxidant inactivation of
α-1-PI that has been worked out for inflammatory pulmo-
nary disease by Beatty et al.¹² is germane in our investiga-
tions of PMN-mediated endothelial injury. They demonstrated that PMNs can oxidize an active site methionine of α-1-PI, rendering it a far less potent anti-elastase (Ka = 10^3 vs 10^4 when oxidized). Using our rearmed neutrophil system, we have now showed that this oxidation requires PMN myeloperoxidase. Moreover, we added data showing that the simple addition of free methionine can protect α-1-PI from PMN-mediated oxidation, and we presume it does so by acting as an irrelevant bait preserving the active site methionine.

These results validate those of Weiss and co-workers14 who alternatively suggested that free methionine acts as a chloramine scavenger. They further showed that α-1-PI, protected by methionine, inhibits PMN-induced proteolysis of matrix proteins that are deposited by cultured endothelial cells.15 We have extended their studies to the cellular level by demonstrating that free methionine will conjoinly protect α-1-PI and prevent PMN-stimulated endothelial cell delamination from culture dishes (Figure 5).

We have also shown that serum can protect α-1-PI from oxidative inactivation. The α-1-PI in 20% serum remained active after being exposed to stimulated PMNs and was able to prevent lift-off of endothelium by stimulated PMNs. Since the amount of methionine expected to be found in 20% serum (less than 50 μM) would provide very little protection of α-1-PI itself, other substances in serum may also be scavenging chloramines. Test et al.32 have shown that albumin can react with chloramine.-Our results suggest that serum protects the α-1-PI well from oxidative inactivation. However, in cases of marked local inflammation, massive systemic inflammation, or chronic inflammation, the oxidative inactivation of α-1-PI could become important.

Insights into the mechanism of vascular damage in clinical syndromes namely, the Adult Respiratory Distress Syndrome (ARDS), are suggested by this work. We8 and others have emphasized the role of complement-activated PMNs in ARDS, a syndrome characterized by microvascular damage leading to pulmonary and interstitial tissues. Recently, Cochrane and co-workers32 have demonstrated that bronchoalveolar lavage fluids from ARDS patients contain α-1-PI that is relatively ineffecual as an anti-elastase because its active site methionine is oxidized to the sulfoxide. They surmise, reasonably we believe, that oxidant products generated by the markedly increased numbers of PMNs that accumulate in ARDS pulmonary microvasculature are the inactivators of α-1-PI.

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