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 (alpha-1-PI) rendering it, in turn, impotent as an anti-elastase. This suggested an analogous scenario for pulmonary vascular damage: namely, undefended 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 "neutrophils." The latter are elastase-free cytoplasmic blebs derived from PMN. When activated, both PMN and neutrophils generate similar amounts of toxic oxygen species; yet neutrophils caused insignificant endothelial damage, measured as "lift-off" from anchoring matrix (PMN = 24.3% ± 1.8% vs neutrophil = 1.2% ± 0.4%; p < 0.001). Adding pure elastase back to neutrophils increased endothelial cell lift-off (7% ± 0.2%). Although the prototypic serine protease inhibitor phenyl methylisulfonylfluoride (PMSF) protected endothelium from PMNs, pure alpha-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 neutrophils, we demonstrated that MPO-dependent oxidants, probably N-chloramines, are critical inactivators of alpha-1-PI. This was further confirmed since added free methionine, a scavenger of chloramine, protected alpha-1-PI from inactivation by reagent chloramine or that produced by rearmed neutrophils or PMN. Concomitantly, free methionine inhibited PMN-provoked endothelial cell lift-off in a dose-dependent fashion while conjointly defending anti-elastase activity of alpha-1-PI. We conclude that stimulated neutrophils can injure vascular tissue by combining elastase secretion with oxidative inactivation of the anti-elastase, alpha-1-PI. (Arteriosclerosis 6:332–340, May/June 1986)

The initial event leading to atherosclerosis is probably injury to blood vessel endothelium.1 Although much interest has centered on the monocyte-macrophage as an effector of endothelial damage,2 we have suggested that granulocytes might be particularly important in initiating atherosclerosis.3 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.4,5 We and others have also demonstrated that granulocytes damage endothelium by generating toxic oxygen species as well as by releasing potent proteolytic enzymes.6–10 Granulocyte-oxidant damage can be modulated by exogenously-added scavengers of O2, such as catalase and superoxide dismutase,7 as well as by intracellular reductants of the endothelial cells themselves including glutathione.11 Defense
against protease-mediated damage is primarily extracellular, and serum protease inhibitors such as alpha-1-protease inhibitor (alpha-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 potent capacity of alpha-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 alpha-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. Suspicion that a similar combination may be germane to endothelial damage has been raised by the studies of Weiss and Regiani,12 who showed that alpha-1-PI can prevent granulocyte-mediated degradation of endothelial matrix proteins in vitro; however, this protection is abrogated if the alpha-1-PI is altered by exposure to granulocyte-derived oxidants. Furthermore, Weiss suggested that the critical granulocyte oxidant of alpha-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 injury. 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 alpha-1-PI is inactivated by MPO-derived oxidants, including the N-chloramines; when so oxidized, we found that alpha-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 alpha-1-PI. Indeed, we showed that the addition of free methionine to our system of stimulated granulocytes, endothelial cells, and alpha-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.

Reagents

Alpha-1-antitrypsin (alpha-1-PI) (chromatographically prepared from human plasma), methionine, methionine sulfide, 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), phosphol myristate acetate (PMA), porcine pancreatic elastase (type I) (15 U/mg protein), phenylmethylsulfonylfluoride (PMSF), Dithionitrobenzoic acid (DTNB), cytochrome C type III, and methoxybutaryl-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 hydroxyethyl starch (Hesperian, 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 collected and 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 10^7/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.16 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 uM. This suspension was layered on a discontinuous gradient of 16% Ficoll and 25% Ficoll 70 solution (Pharmacia, Piscataway, New Jersey). The cell 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 × 10^7/ml in HBSS. As demonstrated in our previous study,16 neutroplasts are virtually granule-free by electron microscopic examination and release virtually no (<1%) beta glucuronidase,16 elastase,20 myeloperoxidase,21 or lactoferrin upon stimulation with 10^{-7} M 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 minutes23) as 2.5 × 10^6 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.24 The endothelial cell monolayers were washed twice with sterile HBSS at 37°C, were labeled with 2.5 µCi Na_2^{51}CrO_4 (Amersham, Arlington Heights, Illinois) for 3 hours, and were then washed four times with HBSS. PMNs (2.5 × 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 ^{51}Cr was determined from the decrease in absorbance by using the extinction coefficient:

\[ E_{412} = 1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \] (3)

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.25

**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 × 10^6/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 (2.5 × 10^6) or neutroplasts (10^7/ml) 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.13
Results

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

Previous studies by others\(^\text{12}\) 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) \(^{51}\text{Cr} \) release from endothelial cells (4.2% ± 0.7% and 3.0% ± 0.4% respectively). This specific \(^{51}\text{Cr} \) 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 ineffectual 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.

\[\text{PMSF} \quad \text{a-1-PI (mg/ml)}\]

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 \(^{51}\text{Cr} \) 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 α-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 α-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 α-1-PI. Indeed, as shown in Figure 3, we found that stimulated granulocytes (2.5 × 10⁹) 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 × 10⁹) generated hardly any (4.0 ± 3.4 nmol/90 minutes) as measured by TNB oxidation (see Methods); when 10⁷ 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 α-1-PI as shown in Tables 1 and 2. For instance, unstimulated granulocytes caused no change in the EIC of α-1-PI, while PMA-stimulated granulocytes decreased EIC from 94.6% to 2.8% (Table 1). PMA-stimulated chronic granulomatous disease PMNs (2.5 × 10⁹) did not significantly decrease the EIC of α-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 α-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 α-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 α-1-PI (96.5% and 87.0%, respectively). However, when myeloperoxidase was added back to stimulated neutrophils, chloramines were produced, and the EIC of α-1-PI was reduced to 3.0% ± 2.2%. The inactivation of α-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 H₂O₂ 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 α-1-PI, while the scavenging of superoxide with superoxide dismutase did not (Table 2).

Table 1. Effect of Granulocytes on Elastase Inhibitory Capacity of α-1-PI

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Elastase inhibitory capacity (%)</th>
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<tbody>
<tr>
<td>α-1-PI alone (n = 21)</td>
<td>94.6 ± 0.7</td>
</tr>
<tr>
<td>+ PMN (n = 10)</td>
<td>94.1 ± 1.0</td>
</tr>
<tr>
<td>+ PMN + PMA (n = 10)</td>
<td>2.8 ± 2.0</td>
</tr>
<tr>
<td>+ PMN + PMA + methionine (n = 10)</td>
<td>88.1 ± 2.4</td>
</tr>
<tr>
<td>+ PMN + PMA + methionine sulfoxide (n = 6)</td>
<td>4.2 ± 2.9</td>
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The elastase inhibitory capacity (EIC) of the supernatant of each incubation mixture against purified human granulocyte elastase (10 μg) was measured as described in Methods. The concentrations used were as follows: PMN (10⁶/ml), PMA (30 ng/ml), α-1-PI (0.5 mg/ml), methionine (1.0 mM), and methionine-sulfoxide (1.0 mM).

The elastase inhibitory capacity was calculated as follows:

EIC = activity (elastase) – activity (elastase + incubation mixture) / activity (elastase) × 100%

The results are expressed as the mean percentage ± sem. Numbers in parentheses signify experiments performed.
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>
</tr>
</thead>
<tbody>
<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 (n = 9)</td>
<td>96.2 ± 0.8</td>
</tr>
<tr>
<td>+ Neutrophils + PMA + MPO + SOD (n = 9)</td>
<td>3.7 ± 2.5</td>
</tr>
<tr>
<td>+ Neutrophils + PMA + MPO + catalase (n = 8)</td>
<td>96.2 ± 0.7</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>
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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 × 10⁶/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 α-1-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 α-1-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 α-1-PI from granulocyte-derived oxidants with its ability to protect endothelium from granulocyte assault is shown in Figure 5. Despite the addition of physiologic levels of α-1-PI to endothelium exposed to PMA-stimulated granulocytes, significant endothelial detachment occurred in 3 hours. However, excellent and parallel prevention of both α-1-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 α-1-PI in 5% serum incubated with PMA-stimulated PMNs was 7.9% ± 4.6%, but if methionine was added, the EIC increased to 96.2% ± 0.7% (p < 0.01).

Figure 4. N-chloramine inactivates α-1-PI anti-elastase activity, but not in the presence of free methionine. α-1-PI (0.5 mg/ml) was added to varying concentrations of reagent N-chloramine ± methionine (1 mM) and incubated for 60 minutes at 37°C as described in Methods. The ability of α-1-PI to inhibit human granulocyte (PMN) elastase (10 μg/ml) was measured, and the elastase inhibitory capacity (EIC) was calculated as described in the tables and in Methods. Values represent the mean ± se of five experiments done in duplicate.

Figure 5. The effect of varying concentrations of methionine on granulocyte (PMN)-mediated endothelial cell damage (solid line) and α-1-PI inactivation (dashed line). Left (axis). Endothelial cell monolayers were incubated with PMN (2.5 × 10⁶/ml), α-1-PI (1.0 mg/ml), methionine, and phorbol myristate acetate (PMA) (100 ng/ml) for 3 hours at 37°C. Detachment was measured by 51Cr release. Values, expressed as percent inhibition of detachment caused by PMA-stimulated PMNs, represent the mean ± se of four experiments done in duplicate. Right (axis). α-1-PI (0.5 mg/ml) was incubated with PMN (1 × 10⁶), PMA (30 ng/ml), and methionine for 60 minutes at 37°C. Elastase inhibitory capacity (EIC) is expressed as the mean ± se of four experiments done in duplicate.
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 inhibitory 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. Harvey Sharp, University of Minnesota) was used in our detachment 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 detachment 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 detaching endothelial cells from their underlying matrix. Thus, neutrophils, which efficiently produce superoxide and hydrogen 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 confirmation 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 others, 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 detach endothelial 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 endothelium.⁸,⁹ Our studies have shown that the adherence of stimulated neutrophils (approximated to endothelium with a force of 1 g) was somewhat less than that of stimulated PMN. However neutrophil adhesion does increase threefold after stimulation by PMA, indicating that neutrophils can significantly increase their adhesivity to endothelium. Furthermore, in our endothelial cell detachment studies, neutrophils and PMN were brought into endothelial contact with a force of 60 g to insure appropriate adhesion. With such techniques, PMA-stimulated neutrophils have been shown in the present studies and those 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 neutrophils 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 “reconstituted,” 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 congruent with the findings of Campbell et al.³⁰ 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-workers⁴ have favored a primary role for PMN-derived neutral proteases in causing endothelial ⁵¹Cr release in culture. Their studies, using techniques that measure mainly endothelial cell lift-off from culture vessels, showed serine protease inhibitors and serum, but not oxygen radical scavengers, diminished PMN-mediated endothelial release. Our own previous studies⁷ have focused on the toxic oxygen species derived from stimulated neutrophils as directly injurious to cultured endothelium. Our results have been further confirmed 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 labeled 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 can be provoked by a toxic oxygen species, such as H₂O₂, that are released by stimulated PMNs; 2) in contrast, endothelial lift-off, assayed by release of radiolabeled cells from anchoring matrices is fostered by PMN proteases, particularly elastase, but other proteases such as collagenase and cathepsin D may also be involved; however, this “injury” is also critically assisted by toxic oxygen species of stimulated PMNs which inactivate the otherwise potent physiologic anti-elastase, α-1-PI.

We believe that the mechanism of oxidant inactivation of α-1-PI that has been worked out for inflammatory pulmonary disease by Beatty et al.¹¹ 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 = 10^8 vs 10^9 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-workers 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. We have extended their studies to the cellular level by demonstrating that free methionine will conjointly 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. 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. We and others have emphasized the role of complement-activated PMNs in ARDS, a syndrome characterized by microvascular damage leading to plasma leakage into pulmonary interstitial tissues. Recently, Cochran and co-workers have demonstrated that bronchoalveolar lavage fluids from ARDS patients contain α-1-PI that is relatively inef-fectual 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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References

2. Faggiotto A, Ross R, Harker L. Studies of hypercholesterol-


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