

Mechanisms of neutrophil damage to human alveolar extracellular matrix: The role of serine and metalloproteases

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*Many syndromes of lung injury are associated with accumulation of neutrophils within the pulmonary parenchyma. These neutrophils have the capacity to produce lung injury by products including proteases and reactive oxygen species (ROS). We examined the ability of activated neutrophils to solubilize human alveolar extracellular matrix (ECM), and by use of scavengers and inhibitors, evaluated the role of ROS and proteases in this process. Supernatants of phorbol myristate acetate-activated neutrophils routinely solubilized $10.2\% \pm 0.8\%$ ($n = 30$) of collagen in human alveolar ECM, as measured by hydroxyproline release. Scavengers of ROS had no significant effect on ECM solubilization. Inhibitors of metalloproteases partially inhibited ECM solubilization ($38.5\% \pm 4.6\%$ inhibition by ethylenediaminetetraacetic acid [$n = 6$], and $37.0\% \pm 14.7\%$ by 1,10-phenanthroline [$n = 6$]; $p < 0.05$). Inhibitors of the neutrophil serine proteases, elastase and cathepsin G, markedly inhibited ECM solubilization ($100.9\% \pm 3.7\%$ by α_1 -protease inhibitor [α_1 -PI] [$n = 6$] and $81.9\% \pm 0.1\%$ by soybean trypsin inhibitor [$n = 6$]; $p < 0.01$). Since α_1 -PI completely inhibited solubilization, metalloprotease activity appeared to be related to serine protease activity. This finding was confirmed by the observation that addition of a metalloenzyme activator, *p*-aminophenylmercuric acetate, in the presence of α_1 -PI, restored solubilization to the same level as that inhibited by metal chelators. We conclude that human neutrophil metalloproteases and serine proteases directly solubilize human alveolar ECM. Furthermore, neutrophil serine proteases activate latent metalloproteases. However, ROS were not demonstrated to play a major role in ECM solubilization in our system. (J ALLERGY CIN IMMUNOL 1992;89:905-15.)*

Key words: Extracellular matrix, neutrophil proteases, reactive oxygen species, lung-injury mechanisms

Infiltration of polymorphonuclear leukocytes (neutrophils) into the lung parenchyma occurs in a variety of pulmonary disorders, including syndromes of acute lung injury, such as the adult respiratory syndrome.¹ Bronchoalveolar lavage fluid from patients with syn-

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Abbreviations used

EDTA:	Ethylenediaminetetraacetic acid
ROS:	Reactive oxygen species
ECM:	Extracellular matrix
HBSS:	Hanks' balanced salt solution
PMA:	Phorbol myristate acetate
APMA:	<i>p</i> -Aminophenylmercuric acetate
1,10-P:	1,10-phenanthroline
SOD:	Superoxide dismutase
GBM:	Glomerular basement membrane
HP:	Hydroxyproline
HEPES:	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
α_1 -PI:	α_1 -Protease inhibitor
RT:	Room temperature
SBTI:	Soybean trypsin inhibitor

dromes of lung injury often contain elevated numbers of neutrophils and neutrophil products.^{2,3} The role of neutrophils in pulmonary damage has been demon-

strated in animal models by neutrophil depletion and the administration of inhibitors or scavengers of neutrophil products (proteases or ROS).⁴⁻⁶

During the inflammatory response to injury, neutrophils infiltrate sites of injury, are activated, and release proteases and ROS. These neutrophil products are capable of damaging various components of the ECM, including the collagen, fibronectin, and elastin exposed after endothelial or epithelial cell injury.⁷⁻⁹ Since ECM is essential for proper growth and repair of injured tissue, damage or disruption of ECM structure may interfere with the repair process.

A few studies have attempted to determine the role of neutrophil products in degradation of ECM by monitoring release of amino acids from ECM proteins. There are data to suggest that ROS are the predominant effectors of ECM protein damage, whereas other investigators report that serine proteases, not ROS, are responsible for this damage.¹⁰ Studies with GBM as substrates have also noted that metalloproteases play a role in ECM damage.^{8, 10} In this study, we examined the relative abilities of neutrophil ROS and proteases to damage human alveolar ECM.

MATERIAL AND METHODS

Material

Drugs and reagents used were supplied by the following companies: Triton X-100 from Schwartz-Mann Biotechnologies, Cleveland, Ohio; peroxide-free Triton X-100 from Pierce Chemical Co., Rockford, Ill.; HBSS, RPMI 1640, penicillin, streptomycin, and fungizone mixture from Irvine Scientific, Santa Ana, Calif.; Ficoll-Hypaque and Macrodex-6% Dextran 70 solution from Pharmacia Fine Chemicals, Piscataway, N. J.; PMA from Consolidated Midland Corp., and later L. C. Services Corp., Brewster, N. Y.; α -PI from Calbiochem-Behring Corp., La Jolla, Calif.; bovine SOD from Diagnostic Data, Inc., Mountain View, Calif.; L-hydroxyproline, chloramine-T, toluene, and 2-methoxyethanol from Aldrich Chemical Co., Milwaukee, Wis. (all solvents were high-performance liquid chromatography or spectral grade); sodium azide, type IV deoxyribonuclease, human thrombin, trypan blue, HEPES, SBTI, EDTA, 1,10-P, thymol-free catalase, methionine, taurine, p-dimethyl-aminobenzaldehyde, p-APMA, and type IV bacterial collagenase from Sigma Chemical Co., St. Louis, Mo.

Isolation of human alveolar ECM

Human lungs were obtained from organ donors, rinsed with phosphate-buffered saline, and stored frozen at -70°C . The lungs were thawed slowly at 4°C overnight. In preliminary experiments, the isolation procedure for alveolar ECM was modified from that previously used in studies of human GBM.⁸ This procedure included pressing pulmonary parenchyma through a sieve, washing the resulting suspension with various concentrations of salt solutions, and sonicating the suspension. This treatment, al-

though it is sufficient for glomeruli, failed to remove cellular debris trapped on the surface of the sticky alveolar ECM. To resolve this problem, a procedure in which detergents lysed and removed cellular debris was evaluated, modified, and used for isolation of human alveolar ECM. Human alveolar ECM was prepared by modification of a procedure for the isolation of rat alveolar wall and basement membrane developed by Zibrak et al.¹¹ Major airways were removed with scissors and scalpels. The remaining parenchyma was layered between crushed ice in a food processor and chopped, and the resulting suspension was pressed through a number 60 sieve ($250\text{ }\mu\text{m}$ openings) with more crushed ice. The volume was adjusted with cold distilled water to approximately 2 L/300 gm of processed lung. Sodium azide (0.5 gm/L) was added, and the suspension was stirred gently at RT for 2 hours before being centrifuged. The pellet was resuspended in 3 L of a 0.1% Triton X-100 solution and stirred overnight at 4°C . The suspension was then centrifuged, and the pellet was washed twice with distilled water to remove the Triton X-100 and any residual sodium azide. The pellet was washed three times with, and resuspended in, 1 mol/L of NaCl at 37°C . Deoxyribonuclease (100 mg/300 gm of tissue) was added, and the pellet was incubated at 37°C for 3 hours. After centrifugation, the pellet was washed once with distilled water, resuspended in 3% Triton X-100, and stirred for 2 hours at RT. The preparation was centrifuged and washed repeatedly with distilled water. The pellet was then resuspended in HBSS with $100\text{ }\mu\text{m}$ of penicillin, 100 mg of streptomycin, and 250 ng of fungizone/100 ml and incubated overnight in a 37°C CO_2 incubator. The suspension was centrifuged, washed three times with sterile distilled water, resuspended in a small amount of HBSS, and stored at -20°C .

Alternative preparation of human alveolar ECM with peroxide-free detergent

During the course of these experiments, investigators who prepared ECM by sonication⁸ observed that ROS participated in ECM damage. However, investigators who isolated ECM with detergent methods found that serine proteases, not ROS, were important in ECM damage.^{6, 9, 12} Since Triton X-100 contains varying concentrations of peroxides, dependent on the vendor,¹³ we postulated that peroxide-free Triton X-100 would provide an unoxidized substrate that could then be damaged by neutrophil ROS.

To examine the possibility that these peroxides oxidized the ECM during its preparation, a portion of minced lung tissue was treated exactly as previously described except that *peroxide-free* Triton X-100 was substituted for Triton X-100 that had not been treated to remove peroxides. The remainder of the lung was prepared with Triton X-100 as previously described.

Microscopic evaluation of human alveolar ECM

Freshly prepared human alveolar ECM was stained with methylene blue and examined with a light microscope for cellular debris and nuclei. For transmission electron mi-

croscopy, the ECM was prepared by staining with uranyl acetate and lead citrate, or with ruthenium red, or with both. For samples stained only with uranyl acetate and lead citrate, the ECM was fixed in 4% glutaraldehyde buffered with 0.067 mol/L of sodium cacodylate, pH 7.3, post fixed in 1.67% osmium tetroxide buffered with 0.06 mol/L of sodium cacodylate, pH 7.3, dehydrated in graded alcohols, and embedded in Epon resin. For samples stained with ruthenium red, the ECM was fixed in 1.2% glutaraldehyde buffered with 0.067 mol/L of sodium cacodylate, pH 7.3, containing ruthenium red (0.001 gm/ml). The samples were dehydrated in graded alcohols and embedded in Epon resin. Ultrathin sections were mounted on uncoated grids. Some sections with or without ruthenium red were stained with uranyl acetate and lead citrate, whereas other sections previously stained with ruthenium red were left unstained to observe the ruthenium red reaction with glycosaminoglycans. All samples were viewed in an electron microscope at an accelerating voltage of 80 keV.

Characterization of collagen by thrombin digestion

To determine what percentage of the collagen in alveolar ECM had been degraded during its preparation, ECM was incubated with thrombin that has been reported previously to solubilize denatured collagen but not native collagen.¹⁴ Six units of human thrombin was incubated for 3 hours with human alveolar ECM or with denatured human alveolar ECM (denatured by heating in an 85° C water bath for 30 minutes).^{14, 15} The suspensions were centrifuged, separated into pellet and supernatant, acid hydrolyzed, and analyzed for HP content, which will be described in detail below. The percent HP in the supernatants represents the percentage of denatured collagen solubilized by thrombin.

Estimation of available collagen

The maximum amount of collagen available as substrate for collagenolytic enzymes was determined by incubating alveolar ECM with a potent bacterial collagenase (type IV collagenase capable of digesting most mammalian and human collagens, including types I, III, and IV). Alveolar ECM, suspended in 500 μ l of HEPES (25 mmol/L) and containing approximately 10 to 20 μ g of HP, was incubated for 1 to 4 hours in a CO₂ incubator with 10 to 40 units of bacterial type IV collagenase. At hourly intervals, replicates were removed from the incubator and centrifuged in a microfuge at 10,000 rpm for 10 minutes. Supernatants were carefully removed from the pellets. Pellets were resuspended in 6 N HCl; supernatants received 0.5 ml of 12 N HCl to yield a final concentration of 6 N HCl. Both pellets and supernatants were acid hydrolyzed overnight in an oven at 100° C. HP concentrations of pellets and supernatants were determined as described below. The amount of HP in the supernatant was divided by the total amount of HP in both the supernatant and pellet and multiplied by 100 to determine the percent of HP released. The percent of HP released represents the maximum proportion of collagen that was available for enzymatic degradation.

Solubilization of human alveolar ECM by neutrophils: Isolation of human neutrophils

Sixty milliliters of peripheral blood was drawn from healthy volunteers. Neutrophils were isolated by discontinuous centrifugation on Ficoll-Hypaque gradients followed by dextran sedimentation and distilled water hypotonic lysis of any remaining red blood cells, as previously described.¹⁶ Viability was determined by trypan blue exclusion to assure viability >95%. Differential counts indicated that this technique consistently provided $\geq$ 99% neutrophils.

Chemiluminescence

Production of ROS in our system was verified by measuring chemiluminescence generated by neutrophils on exposure to PMA.¹⁷ The reaction mixture contained 10⁶ neutrophils and 1 μ g of PMA per ml of HBSS. The mixture was placed in a dark-adapted scintillation vial in a scintillation counter set out of coincidence in which the counts were monitored for 1 minute. Counts per minute were recorded before addition of PMA, immediately after addition, and at intervals thereafter for 9 minutes. The reaction mixture was maintained in the dark in a 37° C water bath when chemiluminescence was not being measured.

Preparation of neutrophil supernatants

The remaining neutrophils were resuspended in HBSS plus HEPES (25 mmol/L) buffer solution at a concentration of 10⁷ cells per milliliter, a concentration previously determined to solubilize 8% to 12% of the substrate.⁸ Suspensions were incubated for 1 hour in a 37° C, 5% CO₂ incubator with or without PMA (1 μ g/ml) to produce activated and unactivated neutrophils. After incubation, cells were removed by low-speed centrifugation. Neutrophil supernatants were combined immediately with human alveolar ECM, as described below.

In experiments designed to inhibit the actions of ROS, various agents were added directly to neutrophil suspensions along with PMA, incubated, and centrifuged to produce neutrophil supernatants. These agents included enzymes that decompose hydrogen peroxide (catalase, 25 μ g/ml) and scavenge superoxide (superoxide dismutase, 100 μ g/ml), an inhibitor of myeloperoxidase (sodium azide, 1 mmol/L), and scavengers of hypochlorous acid (methionine and taurine, 5 mmol/L). Concentrations were those previously reported.⁸ Other agents inhibiting neutrophil proteases were not added to neutrophil suspensions before incubation. Instead, those agents were added to the ECM suspension before addition of neutrophil supernatants.

Incubation of neutrophil supernatants with human alveolar ECM and inhibitory compounds

The procedure for incubating the human alveolar ECM with neutrophil supernatants and quantitating the solubilization of collagen was modified from previous studies.^{7, 8} Supernatants of neutrophils were used rather than whole cells so that inhibitor would have free access to the neutro-

phil proteases and would not be able to restrict diffusion by close adhesion to the substrate. Briefly, the incubation system consisted of human alveolar ECM suspended in HBSS plus HEPES (HBSS with calcium and magnesium and 25 mmol/L of HEPES at pH 7.4), a volume of neutrophil supernatant equivalent to 10^7 cells per milliliter, and enough HBSS plus HEPES to bring the final volume up to 500 μ l. Duplicate mixtures were incubated in a 5% CO₂ incubator at 37° C for 24 hours. The serine protease inhibitors, α_1 -PI (250 μ g/ml) and SBTI (250 μ g/ml), were added directly to the human alveolar ECM before addition of the neutrophil supernatant. In experiments in which the inhibitors of metalloproteases, EDTA (10 mmol/L) and 1,10-P (10 mmol/L) were used, they also were added to the human alveolar ECM before addition of the neutrophil supernatants. Concentrations of protease inhibitors and inhibitors/scavengers of ROS used in these experiments were chosen on the basis of previous data in which complete inactivation of the respective proteinases and ROS occurred in a similar system.⁸ In experiments in which metalloproteases were to be activated by APMA (0.5 mmol/L) in the presence of α_1 -PI, the APMA and α_1 -PI were added to human alveolar ECM before addition of neutrophil supernatants.

Negative controls contained only ECM suspended in HBSS plus HEPES. Unactivated controls contained ECM and supernatants prepared from neutrophils without PMA.

Suspensions of human alveolar ECM were incubated with inhibitory compounds and neutrophil supernatants in a 37° C, 5% CO₂ incubator for 18 to 24 hours and were then centrifuged. An exception to this procedure occurred when the role of ROS was investigated. In those experiments, inhibitory compounds were added during the time that supernatants were generated. To prevent further solubilization, supernatants were immediately transferred to glass screw-top tubes, and 0.5 ml of concentrated HCl was added to each tube. Pellets were left in the microfuge tubes in which they were incubated, and 1.0 ml of 6 N HCl was immediately added to each tube. Both supernatants and pellets were hydrolyzed overnight in an oven at 100° C.

Soluble-type IV collagen as substrate for neutrophil supernatants

Soluble, purified, type IV collagen (Collaborative Research, Inc., Bedford, Mass.) from murine EHS tumor cells was suspended in HBSS plus HEPES, and the pH was adjusted to 7.4 with sterile 0.5 N NaOH. Supernatants from the equivalent of 10^7 cells per milliliter, either stimulated with PMA or unstimulated, were added to type IV collagen solution and incubated for 24 hours in a CO₂ incubator at 37° C. Some tubes contained only type IV collagen (negative controls). In other tubes, 20 units of bacterial type IV collagenase was incubated with type IV collagen for 24 hours at 37° C (maximal degradation, positive controls). After incubation, the alveolar ECM could be centrifuged with the resulting pellets and supernatants separated, acid hydrolyzed, and analyzed for HP content. Since type IV collagen was dissolved in the medium and thus could not be centri-

fuged as such, the microfuge tubes containing type IV collagen were placed in an ice bath after the 24-hour incubation. Sodium chloride was gradually added during the course of 2 to 3 hours until the solution contained 4 mol/L of NaCl. The tubes were refrigerated overnight to facilitate complete precipitation of any undigested type IV collagen. The collagen precipitate was centrifuged at 50,000 g at 4° C, after which the supernatant was carefully removed from the pellet of undigested collagen. From this point on, the pellets and supernatants were acid hydrolyzed and analyzed for HP content as previously described for human alveolar ECM.

Incubation of human alveolar ECM with whole neutrophils

For some experiments, intact neutrophils rather than neutrophil supernatants provided neutrophil proteases and ROS. The incubation system contained alveolar ECM suspended in HBSS plus HEPES with or without PMA (1 mg/ml for the activated treatment), and with or without inhibitors/scavengers of ROS and proteases before the addition of 10^7 neutrophils per milliliter. The final volume was 500 μ l. Incubation, acid hydrolysis, and HP determination were performed as described for the incubation systems containing supernatants of neutrophils.

Incubation of peroxide-free detergent preparation of human alveolar ECM with whole neutrophils

In some experiments, human alveolar ECM, prepared with peroxide-free Triton X-100 or that prepared with regular Triton X-100, was incubated with 5×10^6 neutrophils in 500 μ l, centrifuged, and acid hydrolyzed, and the HP content was determined or the respective preparations as previously described.

HP determinations

After acid hydrolysis, the extent to which collagen in human alveolar ECM was solubilized was determined by a quantitative, colorimetric assay for HP, an amino acid characteristically high in collagen.^{8,18} Briefly, the pH was adjusted to neutral with sodium hydroxide and hydrochloric acid before the addition of borate buffer at 8.7 and chloramine-T (0.5 mmol/L). The oxidation process was stopped after 25 minutes with sodium thiosulfate (5 mol/L). To the resulting aqueous solution, KCl was added until approximately ¼ inch accumulated on the bottom of each tube. Interfering compounds were extracted with toluene and discarded. The mixture was boiled for 30 minutes before extracting HP with another portion of toluene. An aliquot of the toluene was combined with Ehrlich's reagent and incubated at RT for 30 minutes, and its absorbance was read at 560 nm on a Beckman spectrophotometer (Beckman Instruments, Inc., Irvine, Calif.).

Data analysis

Absorbance values were converted to concentrations of HP by use of a standard curve calculated by linear regression daily from known concentrations of pure HP.¹⁹ The per-

centage of HP solubilized from each sample of human alveolar ECM was calculated by dividing the micrograms of HP in the supernatant by the total amount of HP in the starting material (micrograms of HP in supernatant plus micrograms of HP in the pellet) and multiplying the quotient by 100, as presented in the formula below:

$$\% \text{ HP released} = \frac{\mu\text{g of HP in supernatant}}{(\mu\text{g of HP in supernatant} + \mu\text{g of HP in pellet})} \times 100$$

The percentages of HP released in the absence of neutrophil supernatants (the two negative control values) were determined for the experiments of each day, averaged, and subtracted from each data point obtained on the same day. Calculations were made on an IBM-compatible computer with the Statistix II program (NH Analytical Software, Roseville, Minn.). Differences between experimental groups were determined with analysis of variance from Statistix II and Tukey's paired comparison procedures²⁰ with the Statpro Statistical package (Electronic Publishing Co., Boston, Mass.) on an Apple IIe (Apple Computer, Inc., Cupertino, Calif.). Data different at the $p < 0.05$ level were considered significant (two-tailed tests). Values were reported as the mean $\pm$ standard error of the mean.

RESULTS

Characterization of human alveolar ECM

ECM prepared from human alveolar wall contained a predominance of intact basement membrane with some interstitial collagen and small amounts of elastin. The preparation was free of endothelial cells and their fragments and was morphologically similar to intact alveolar basement membrane (Fig. 1).²¹

Incubation of ECM preparations with thrombin, an enzyme that solubilizes denatured collagens, consistently demonstrated that 99.5% or more of the collagen was in the native state (data not presented). Preparations that were denatured by boiling were solubilized by thrombin in an enzyme concentration-dependent fashion.

To determine the maximum amount of ECM collagen available to neutrophil proteases in our system, ECM was incubated with up to 40 U/ml of potent, purified, bacterial collagenase. The collagenase completely solubilized the collagen ($98\% \pm 1.2\%$; $n = 6$) in our incubation system.

Chemiluminescence

Neutrophil activation by, and release of, ROS from neutrophils was confirmed by measurement of chemiluminescence. In our system, neutrophils consistently generated a respiratory burst resulting in a rapid increase in counts per minute compared to the background counts per minute. Peak counts per minute values were recorded between 3 and 5 minutes after addition of PMA and averaged $350,000 \pm 73,000$

($n = 11$) after subtraction of background counts per minute.

Solubilization of human alveolar ECM by neutrophil supernatants

Solubilization of human alveolar ECM collagen was studied under three experimental conditions. When ECM was incubated in HBSS plus HEPES alone, the mean percent HP ranged from 0.9% to 2.9% ($n = 12$). The mean percent HP released when ECM was incubated with supernatants from unactivated neutrophils ranged from 2.3% to 4.1% ($n = 12$). Thus, the net release of HP by the unactivated neutrophils was from 1% to 1.4%. Supernatants from neutrophils activated with PMA released from a mean of 9.4% to 11.6% HP ($n = 12$) after subtraction of the net release of HP by unactivated neutrophils. Within each data set, data were compared statistically to the PMA-activated values.

Solubilization of human alveolar ECM by serine and metalloproteases

Elastase and cathepsin G are the neutrophil serine proteases that have been reported to degrade collagen.²² When the serine protease inhibitors, α_1 -PI and SBTI, were incubated with ECM and neutrophil supernatants, α_1 -PI completely inhibited ($100.9\% \pm 3.7\%$ inhibition; $n = 6$) ECM solubilization, and SBTI inhibited $81.0\% \pm 9.1\%$ ($n = 6$) of the solubilization (Fig. 2).

The neutrophil metalloproteases also have been reported to degrade collagen.²³⁻²⁵ Therefore, the calcium and magnesium chelator, EDTA, and the iron and zinc chelator, 1,10-P, were added to the incubation system to inhibit the activities of metalloproteases. Solubilization of ECM was inhibited $38.5\% \pm 4.6\%$ ($n = 6$) (Fig. 3). Since some metalloproteases are latent when they are released from human neutrophils, a metalloprotease activator, APMA, was added to the human alveolar ECM along with α_1 -PI with some experiments. The amount of ECM solubilization restored by APMA was $42.3\% \pm 5.8\%$ ($n = 6$), a value not statistically different from the inhibition produced by the metal chelators, EDTA and 1,10-P.

Solubilization of human alveolar ECM by ROS

Since ROS are reportedly involved in collagen degradation, either directly or indirectly via activation of metalloproteases,^{8, 26} we examined the effect of SOD, a scavenger of superoxide anion, and catalase that destroys hydrogen peroxide, on ECM solubilization by activated neutrophils. Neither SOD nor catalase had any significant effect on ECM solubilization (Figs. 4

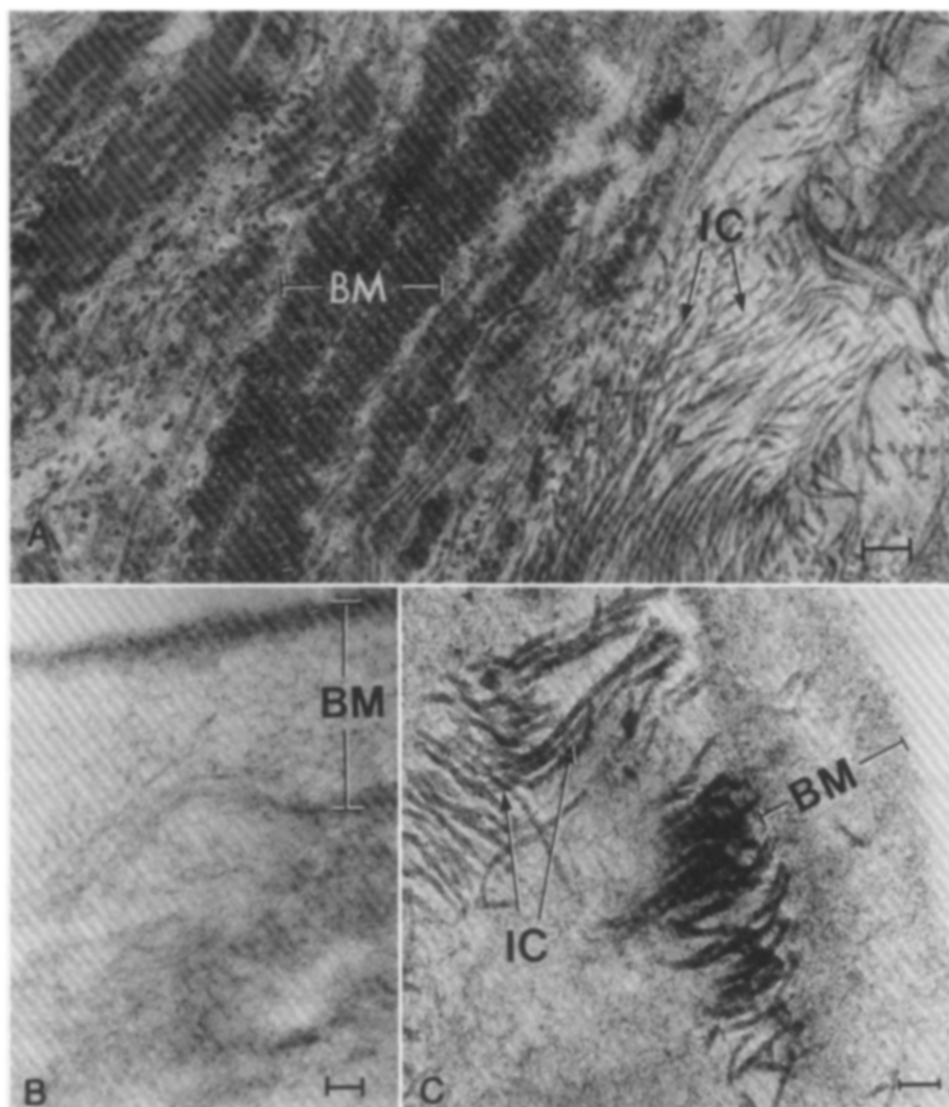


FIG. 1. Human alveolar ECM; transmission electron micrographs of ECM substrate for these studies. **A**, Human alveolar ECM preparation contains typical long, granular, electron-dense, double strands of basement membrane (*BM*) and striated interstitial collagen (*IC*) (Epon embedded, stained with uranyl acetate and lead citrate; bar measures 0.1 μ m). **B**, Glycosaminoglycans in human alveolar ECM outline network of type IV in basement membrane (*BM*) (Epon embedded, stained with ruthenium red; bar measures 0.1 μ m). **C**, Interstitial collagen (*IC*) is visible via counterstain, whereas glycosaminoglycans in basement membrane (*BM*) are masked (Epon embedded, stained in ruthenium red, uranyl acetate, and lead citrate; bar measures 0.1 μ m).

and 5). Likewise, when sodium azide was added to the neutrophil supernatant, no difference in ECM solubilization was noted (Fig. 6). In other experiments, methionine or taurine, scavengers of hypochlorous acid, also did not inhibit ECM solubilization.

Soluble type IV collagen as substrate

Purified type IV collagen proved highly susceptible to digestion by neutrophil products and bacterial col-

lagenase in six experiments. PMA-activated neutrophil supernatants and bacterial collagenase digested $75.8\% \pm 3.2\%$ and $93.1\% \pm 3.8\%$ of the type IV collagen, respectively. Unactivated neutrophil supernatants digested $27.0\% \pm 4.0\%$ of type IV collagen.

Solubilization of ECM by whole neutrophils

In previous experiments, supernatants from either PMA-activated or unactivated neutrophils were in-

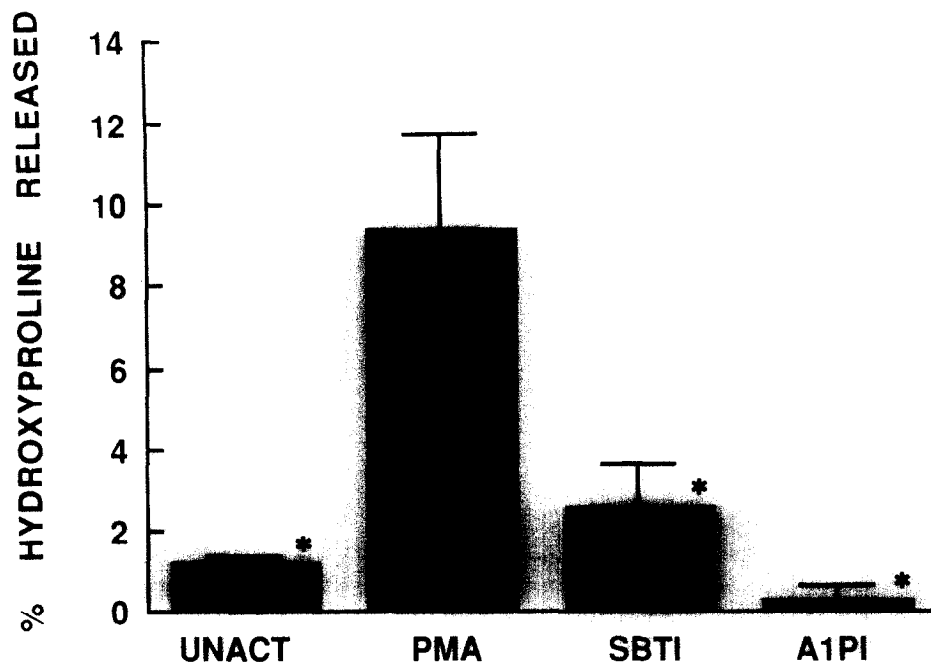


FIG. 2. Effects of serine protease inhibitors. *UNACT*, percentage of HP released by neutrophil supernatants that were not activated; *PMA*, percentage of HP released by neutrophil supernatants in which neutrophils were activated with PMA with 1 $\mu\text{g/ml}$; *A1PI* and *SBTI*, percentage of HP released by PMA-activated neutrophil supernatants in presence of $\alpha_1\text{-PI}$ (250 $\mu\text{g/ml}$) or *SBTI* (250 $\mu\text{g/ml}$), respectively. HP release was decreased by 81% with *SBTI* and by 100% with $\alpha_1\text{-PI}$. Values with asterisks are statistically different from PMA value ($p < 0.05$; $n = 6$).

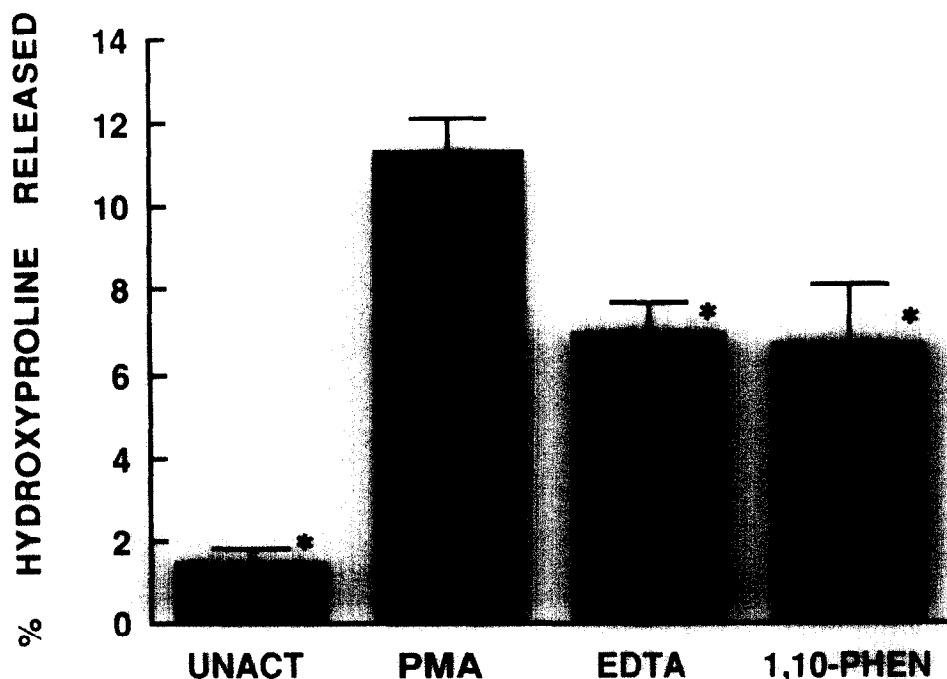


FIG. 3. Effects of metalloprotease inhibitors. *UNACT*, percentage of HP released by neutrophil supernatants that were not activated with PMA; *PMA*, amount of HP released by neutrophil supernatants activated with PMA (1 $\mu\text{g/ml}$); *EDTA* and 1,10-P, amount of HP released by PMA-activated neutrophil supernatants in presence of either EDTA (10 mmol/L) or 1,10-P (10 mmol/L). HP release was reduced by 37% of value with EDTA and by 38% with 1,10-P. Values with asterisks are statistically different from PMA value ($p < 0.05$; $n = 6$).

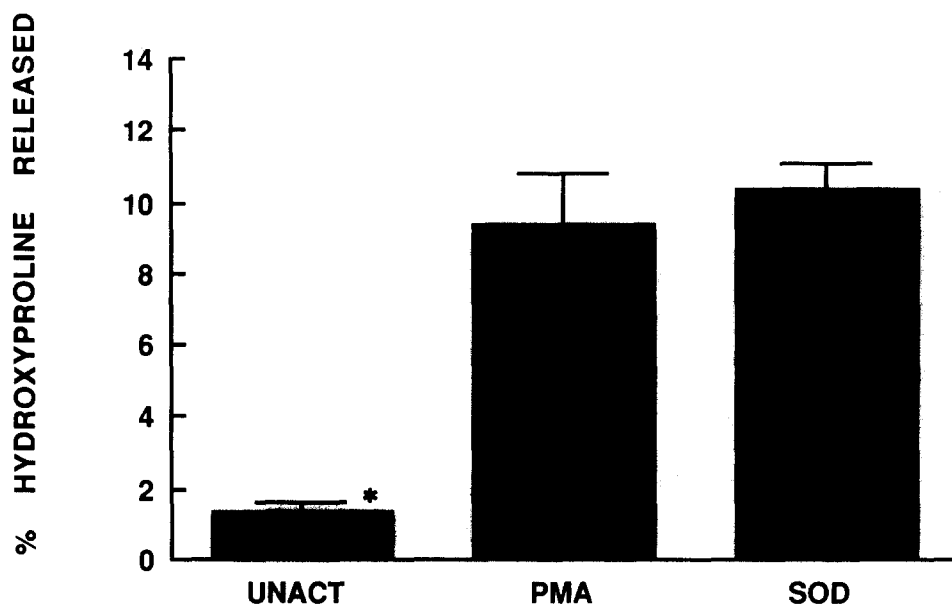


FIG. 4. Effects of SOD. *UNACT*, amount of HP released by neutrophil supernatants that were not activated with PMA; *PMA*, amount of HP released by neutrophil supernatants activated with 1 $\mu\text{g/ml}$; *SOD*, amount of HP released by PMA-activated neutrophil supernatants with SOD (100/ $\mu\text{g/ml}$). HP release was not reduced by SOD. Value with *asterisk* is statistically different from PMA value ($p < 0.05$; $n = 6$).

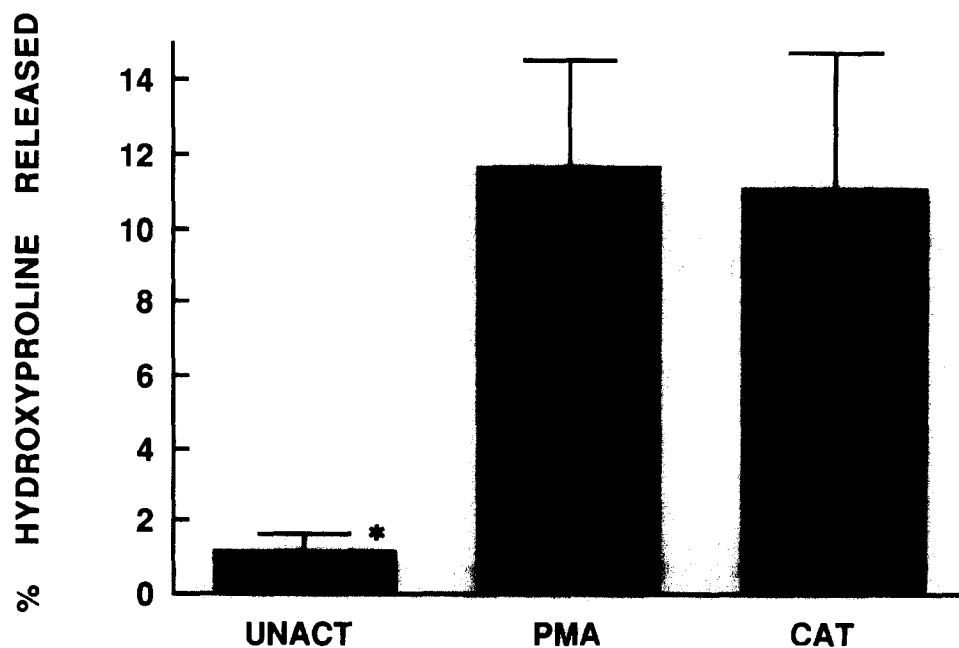


FIG. 5. Effects of catalase. *UNACT*, percentage of HP released by neutrophil supernatants that were not activated by PMA. The bar labeled *CAT* represents the amount of HP released by neutrophil supernatants in presence of catalase (25 $\mu\text{g/ml}$). HP release was not reduced by catalase. Value with *asterisk* is statistically different from PMA value ($p = 0.05$; $n = 6$).

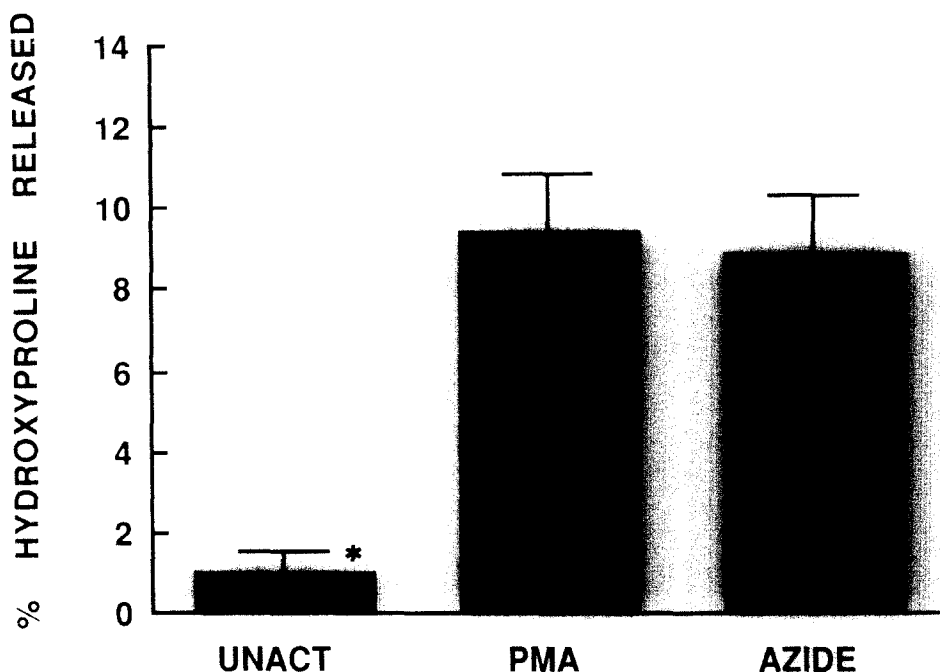


FIG. 6. Effects of myeloperoxidase inhibitor. *UNACT*, percentage of HP released by neutrophil supernatants that were not activated with PMA compared with the amount of HP released by neutrophil supernatants activated with PMA (1 $\mu\text{g}/\text{ml}$); *AZIDE*, amount of HP released by PMA-activated neutrophil supernatants in presence of sodium azide (1 mmol/L). HP release was not reduced by sodium azide. Value with *asterisk* is statistically different from PMA value ($p < 0.05$; $n = 6$).

cubated with human alveolar ECM or purified, soluble type IV collagen. In this set of six experiments, whole neutrophils were incubated with human alveolar ECM. Neutrophils activated with PMA released significantly more HP ($26.80\% \pm 1.8\%$) than did unactivated neutrophils ($6.3\% \pm 0.7\%$) or supernatants of PMA-activated neutrophils ($10.2\% \pm 0.8\%$).

Degradation of ECM prepared with peroxide-free Triton X-100

Previous studies contain conflicting data regarding the role of oxygen metabolites in production of damage to ECM by neutrophils. One difference in these studies appears to be the methods used to prepare the ECM. Those investigators reporting no role for oxygen metabolites used detergent to remove cells from ECM. Since peroxides are present in detergent, we postulated that they may have oxidized the ECM during its preparation. However, we detected no differences in the amount of HP released by whole neutrophils from ECM prepared with peroxide-containing detergent and that prepared with peroxide-free detergent (data not presented). Additionally, an inhibitor of the myelo-

peroxidase system, azide, that was previously reported to decrease HP release,⁸ failed to decrease the HP released from ECM prepared with either peroxide-free or peroxide-containing detergent (data not presented).

DISCUSSION

Our results demonstrate that the neutrophil proteases of both the serine and metalloprotease classes can participate in solubilization of alveolar ECM. These data are in agreement with previous data that demonstrate that neutrophil proteases degrade collagen in ECM from other organs.^{7, 22, 26} Serine proteases have failed to solubilize glomerular ECM and/or GBM in previous studies.⁸ The reason for the differences between our study results and those results are unclear. Differences in the susceptibility to neutrophil products with sonication of ECM could be an explanation.

Metalloproteases participate in the solubilization of collagen from GBM.^{8, 27, 28} Metalloproteases are often released as inactive (latent) enzymes^{8, 28, 29} that require activation by ROS or serine proteases. Thus, inhibitors or scavengers of ROS have been reported to pre-

vent activation of latent metalloproteases.^{8, 28} However, in another system, ROS could activate a maximum of only 30% of latent neutrophil gelatinase,²⁸ a finding that suggested to us that another mechanism must account for the remaining activation. In contrast, serine proteases, such as elastase, activated the latent neutrophil metalloproteases, whereas ROS did not.^{30, 31} Previous studies in which amnionic basement membrane was incubated with PMA-stimulated neutrophils indicated that neither SOD nor catalase prevented ECM degradation or solubilization of fibronectin, another protein constituent of ECM.⁹ Additionally, inhibitors of neutrophil elastase did diminish solubilization of fibronectin in that system. The investigators concluded that elastase rather than oxygen radicals solubilized fibronectin from the ECM. The latter mechanism for activation of human neutrophil metalloproteases (serine proteases) was operative in our study with human neutrophils and alveolar ECM.

In our study, α_1 -PI, an inhibitor of serine proteases, completely inhibited proteolytic activity of neutrophil supernatants. An artificial activator of latent metalloproteases, APMA, partially restored solubilization in the presence of α_1 -PI, indicating that α_1 -PI prevented activation of latent metalloproteases by serine proteases. These findings indicate therefore that in the absence of α_1 -PI, latent metalloproteases were activated by serine proteases.

Several specific proteases have been proposed as those most likely to directly solubilize ECM. The neutrophil serine proteases, elastase and cathepsin G, have collagenolytic activity *in vitro*.^{7, 22, 26, 27} The metalloprotease, gelatinase, solubilizes GBM collagen.⁸ Since latent gelatinase is only partially activated by ROS,²⁸ other mechanisms for its activation must operate in addition to ROS. Since elastase and possibly cathepsin G have been reported to be responsible for activation of latent metalloproteases,^{30, 32} we postulate that elastase may have both direct collagenolytic activity and indirect action by activating latent collagenolytic metalloproteases. Additional studies with specific enzyme inhibitors and purified serine proteases are required to confirm our hypothesis that elastase, cathepsin G, and gelatinase all contribute to human alveolar ECM solubilization.

Neutrophil supernatants degraded purified, murine EHS type IV soluble collagen better than collagen in human alveolar ECM. One possible explanation for the finding could be due to the loss of the 7S domain during acid treatment with our isolation procedure.³³ The soluble form of type IV collagen is a procollagen-like molecule consisting of disulfide-linked polypeptide chains intermediate between collagen and

chains.³¹ It appears to differ from acid-insoluble EHS tumor collagen by the lack of disulfide-linked chains. Removal of the 7S domain may result in greater susceptibility of collagen to attack by enzymes. Another factor could involve the loss of glycoproteins. The glycoproteins usually complexed with type IV collagen are also removed during the purification of type IV collagen. Removal of glycoproteins from intact, cultured, smooth muscle ECM increased elastin degradation by macrophages from undetectable to 39%.³⁴ This dramatic enhancement of elastin degradation was attributed to "uncovering" of the elastin. A similar "uncovering" of type IV collagen during purification could contribute significantly toward the ease with which neutrophil supernatants degraded purified type IV collagen.

A much greater release of HP from human alveolar ECM was noted when intact neutrophils were incubated with alveolar ECM than with supernatants of neutrophils. Thus, proteolysis may be enhanced by direct contact of regions of the neutrophil membrane in which locally high concentrations of proteases are maintained and protease inhibitors are excluded from the matrix substrate.^{35, 36} Even though quantities of supernatant equivalent to the number of whole neutrophils were incubated with ECM, supernatants contained evenly diluted enzymes, whereas whole neutrophils could release concentrated quantities of enzymes close to the substrate.

Animal studies have suggested that the use of inhibitors or scavengers of neutrophil products may have therapeutic use in the prevention or treatment of syndromes of human lung injury. Our data suggest that serine protease inhibitors are likely to have useful clinical effects. Moreover, the system described appears to be an extremely versatile model for testing compounds with therapeutic potential.

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REFERENCES

1. Rinaldo JE, Rogers RM. Adult respiratory distress syndrome. *New Engl J Med* 1982;306:900-9.
2. Christner P, Fein A, Goldberg S, Lippmann M, Abrams W, Weinbaum G. Collagenase in the lower respiratory tract of patients with adult respiratory distress syndrome. *Am Rev Respir Dis* 1985;131:690-5.
3. Goldstein W, Doring G. Lysosomal enzymes from polymorphonuclear leukocytes and protease inhibitors in patients with cystic fibrosis. *Am Rev Respir Dis* 1986;134:49-56.
4. Fligel SEG, Ward PA, Johnson KJ, Till GO. Evidence for a role of hydroxyl radical in immune-complex-induced vasculitis. *Am J Pathol* 1984;115:375-82.

5. Till GO, Hatherill JR, Tourtellotte WW, Lutz MJ, Ward PA. Lipid peroxidation and acute lung injury after thermal trauma to the skin: evidence of a role for hydroxyl radical. *Am J Pathol* 1985;199:376-84.
6. Freeman BA, Turrens JF, Mirza Z, Crapo JD, Young SL. Modulation of oxidant lung injury by using liposome-entrapped superoxide dismutase and catalase. *Fed Proc* 1985;44:2591-5.
7. Mainardi CL, Dixit SN, Kang AH. Degradation of type IV (basement membrane) collagen by a protease isolated from human polymorphonuclear leukocyte. *J Biol Chem* 1980;255:5435-41.
8. Shah SV, Baricos WH, Basci A. Degradation of human glomerular basement membrane by stimulated neutrophils: activation of a metalloprotease(s) by reactive oxygen metabolites. *J Clin Invest* 1987;79:25-31.
9. Sibille Y, Lwebuga-Mukasa, Jamson S, et al. An in vitro model for polymorphonuclear-leukocyte-induced injury to an extracellular matrix. *Am Rev Respir Dis* 1986;134:134-40.
10. Vissers MCM, Winterbourne CC. Myeloperoxidase-dependent oxidative inactivation of neutrophil neutral proteases and microbicidal enzymes. *Biochem J* 1987;245:277-80.
11. Zibrak JD, Farris B, Varccaro CA, Brody JS. Isolation and characterization of a rat lung fraction enriched in alveolar wall basement membranes. *Exp Lung Res* 1985;8:15-28.
12. Vissers MCM, Winterbourne CC. The effect of oxidants on neutrophil-mediated degradation of glomerular basement membrane collagen. *Biochem Biophys Acta* 1986;1986:277-86.
13. Ashani Y, Catravas GN. Highly reactive impurities in Triton X-100 and Brij 35: partial characterization and removal. *Anal Biochem* 1980;109:55-62.
14. Liotta LA, Goldfarb RH, Terranova VP. Cleavage of laminin by thrombin and plasmin: alpha thrombin selectively cleaves the beta chain of laminin. *Thromb Res* 1981;21:663-73.
15. Kalebic T, Garbisa S, Glaser B, Liotta LA. Basement membrane collagen: degradation by migrating endothelial cells. *Science* 1983;221:281-3.
16. Stossel TP. Phagocytosis. In: Rose NR, Friedman H, eds. *Manual of clinical immunology*. 2nd ed. Washington, D.C.: American Society for Microbiology, 1980:309-15.
17. deShazo RD, Hase T, Wright DG, Diem JD. Evidence for histamine-mediated inhibition of monocyte chemotaxis in atopic dermatitis. *J ALLERGY CLIN IMMUNOL* 1982;69:429-34.
18. Switzer BR, Sumner GR. Improved method for hydroxyproline analysis in tissue hydrolizates. *Annal Biochem* 1971;39:487-91.
19. Morrison D. *Multivariate statistical methods*. New York: McGraw-Hill, 1976.
20. Box G, Hunter W, Hunter J. *Statistics for experimenters*. New York: Wiley & Sons, 1978.
21. van Kuppevelt THMSM, Cremers FPM, Domen JGW, van Beuningen HM, van den Brule AJC, Kuyper CMA. Ultrastructural localization and characterization of proteoglycans in human lung alveoli. *Eur J Cell Biol* 1985;36:74-80.
22. Davies M, Barrett AJ, Travis J, Sanders E, Coles GA. The degradation of human glomerular basement membrane with purified lysosomal proteases: evidence for the pathogenic role of the polymorphonuclear leukocyte in glomerulonephritis. *Clin Sci Mol Med* 1978;54:233-40.
23. Lazarus GS, Daniels JR, Brown RS, Bladen HA, Fullmer HM. Degradation of collagen by a human granulocyte collagenolytic system. *J Clin Invest* 1968;47:2622-9.
24. Hibbs MS, Hasty KA, Kang AH, Mainardi CL. Secretion of collagenolytic enzymes by human polymorphonuclear leukocytes. *Coll Relat Res* 1984;4:467-77.
25. Hibbs MS, Hasty KA, Seyer JM, Kang AH, Mainardi CL. Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J Biol Chem* 1985;260:2493-500.
26. Weiss SJ, Curnutte KT, Regiani S. Neutrophil-mediated solubilization of the subendothelial matrix: oxidative and non-oxidative mechanisms of proteolysis used by normal and chronic granulomatous disease phagocytes. *J Immunol* 1986;136:636-41.
27. Vissers MCM, Winterbourne CC, Hunt JS. Degradation of glomerular basement membrane by human neutrophils in vitro. *Biochem Biophys Acta* 1984;804:154-60.
28. Peppin GJ, Weiss SJ. Activation of the endogenous metalloprotease, gelatinase, by triggered human neutrophils. *Proc Natl Acad Sci USA* 1986;83:4322-6.
29. Weiss SJ, Peppin G, Ortiz X, Ragsdale C, Test ST. Oxidative autoactivation of latent collagenase by human neutrophils. *Science* 1985;227:747-9.
30. Vissers MCM, Winterbourne CC. Activation of human gelatinase by endogenous serine proteases. *Biochem J* 1988a;249:327-31.
31. Vissers MCM, Winterbourne CC. Gelatinase contributes to the degradation of glomerular basement membrane collagen by human neutrophils. *Coll Relat Res* 1988b;8:113-22.
32. Timpl R, Wiedemann H, Van Delden V, Furthmayr H, Kuhn K. A network model for the organization of type IV collagen molecules in basement membranes. *Eur J Biochem* 1981;120:203-11.
33. Timpl R, Bruckner P, Fietzek P. Characterization of pepsin fragments of basement membrane collagen obtained from a mouse tumor. *Eur J Biochem* 1979;95:255-63.
34. Chapman HA Jr, Reilly JJ Jr, Kobzik L. Role of plasminogen activator in degradation of extracellular matrix protein by live human alveolar macrophages. *Am Rev Respir Dis* 1988;137:412-9.
35. Weitz JI, Huang AJ, Landman SL, Nicholson SC, Silverstein SC. Elastase-mediated fibrogenolysis by chemoattractant-stimulated neutrophils occurs in the presence of physiologic concentrations of antiproteases. *J Exp Med* 1987;166:1836-50.
36. Campbell EJ, Campbell MA. Pericellular proteolysis by neutrophils in the presence of proteinase inhibitors: effects of substrate opsonization. *J Cell Biol* 1988;106:677-6.