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Comparison of live human neutrophil and alveolar macrophage elastolytic activity in vitro. Relative resistance of macrophage elastolytic activity to serum and alveolar proteinase inhibitors.

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Research Article

Elastin is an extracellular matrix protein critical to the normal structure and function of human lung. Recently reported data indicate that live human alveolar macrophages can degrade purified elastin in vitro. In this study, we directly compared the elastolytic activity of alveolar macrophages with that of human neutrophils. In the absence of proteinase inhibitors, human neutrophils degrade much more elastin than do human alveolar macrophages. However, macrophages cultured in 10% human serum and in contact with purified 3H-elastin degraded 4.7 micrograms elastin/10(6) cells per 24 h, as compared to less than 1 microgram/10(6) cells/24 h for neutrophils. We observed a similar pattern when the two cells were cultured in human alveolar fluid. We determined that the relative resistance of macrophage elastolytic activity to serum or alveolar proteinase inhibitors was not simply due to phagocytosis of substrate by the larger macrophages. Live macrophages as well as neutrophils degrade 125I-elastin coupled to noningestible sepharose beads. Again in serum-free media, neutrophils degraded eight-fold more elastin than macrophages but only macrophages degraded sepharose-coupled elastin in the presence of 10% serum. Because of these findings, we compared the enzymatic mechanisms of elastin breakdown by macrophages with that of neutrophils. Macrophage elastolytic activity is largely (65-80%) due to a cysteine proteinase(s), at least part of which is Cathepsin B. Approximately half of the cysteine proteinase activity […]

Comparison of Live Human Neutrophil and Alveolar Macrophage Elastolytic Activity in Vitro

Relative Resistance of Macrophage Elastolytic Activity to Serum and Alveolar Proteinase Inhibitors

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Abstract. Elastin is an extracellular matrix protein critical to the normal structure and function of human lung. Recently reported data indicate that live human alveolar macrophages can degrade purified elastin in vitro. In this study, we directly compared the elastolytic activity of alveolar macrophages with that of human neutrophils. In the absence of proteinase inhibitors, human neutrophils degrade much more elastin than do human alveolar macrophages. However, macrophages cultured in 10% human serum and in contact with purified ^{3}H-elastin degraded 4.7 μg elastin/10^6 cells per 24 h, as compared to <1 μg/10^6 cells/24 h for neutrophils. We observed a similar pattern when the two cells were cultured in human alveolar fluid. We determined that the relative resistance of macrophage elastolytic activity to serum or alveolar proteinase inhibitors was not simply due to phagocytosis of substrate by the larger macrophages. Live macrophages as well as neutrophils degrade ^{125}I-elastin coupled to noningestible sepharose beads. Again in serum-free media, neutrophils degraded eightfold more elastin than macrophages but only macrophages degraded sepharose-coupled elastin in the presence of 10% serum. Because of these findings, we compared the enzymatic mechanisms of elastin breakdown by macrophages with that of neutrophils. Macrophage elastolytic activity is largely (65–80%) due to a cysteine proteinase(s), at least part of which is Cathepsin B. Approximately half of the cysteine proteinase activity appeared to be expressed at or near the cell surface. These experiments defined two enzymatically distinct pathways of elastin breakdown by human inflammatory cells: the classic, neutrophil derived soluble elastase(s) that is sensitive to serum and alveolar proteinase inhibitors, and a macrophage-mediated pathway that is largely cell associated and relatively resistant to inhibitors. The function of the two pathways depends on the relative excess or deficiency of soluble inhibitors. At inflammatory sites rich in proteinase inhibitors, tissue macrophages may degrade more extracellular matrix elastin than neutrophils. In smokers without antiproteinase deficiency, pulmonary macrophages, which are known to be increased in number, may be the more important cause of elastin breakdown and emphysema.

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Introduction

Elastin is a normal component of many tissues and is especially important to the normal architecture of lung. Destruction of lung elastin in experimental animals by purified elastase can reproduce the histological and mechanical features of emphysema (1–5). Although alveolar macrophages are the major inflammatory cell in the histopathology of smoking individuals, neutrophils are currently considered the major source of elastase activity likely to lead to disruption of elastin and progressive emphysema. This hypothesis is based partly on the well known susceptibility of individuals, particularly smokers, with genetic deficiency in α_1-antiproteinase to develop emphysema and the fact that α_1-antiproteinase blocks neutrophil but not macrophage-derived elastase (6). Moreover, studies of culture supernates and cell lysates of human alveolar macrophages indicate that most of the observed elastolytic activity can be explained by neutrophil elastase in the preparations (7). Smoking individuals without genetic α_1-antiproteinase deficiency are also at risk for emphysema. Recently reported data indicate that the alveolar surfaces of most such individuals may not be deficient in functionally active α_1-antiproteinase

which makes the susceptibility of these subjects to emphysema more difficult to explain (8).

We recently observed that live human alveolar macrophages can degrade elastin if the cells contact the substrate (9). In this study, we directly compare the elastolytic activities of live human neutrophils and alveolar macrophages co-cultured with a purified, insoluble ^{3}H-elastin substrate. We find neutrophil elastolytic activity to be completely blocked by 1–10% human serum, whereas macrophage activity is inhibited only 20–40%. The difference in susceptibility of the two cells to macromolecular inhibitors is explained by fundamentally different degradative mechanisms. The findings suggest that in vivo, at sites of inflammation rich in proteinase inhibitors but where cells can contact substrate, e.g., elastin, macrophages degrade more matrix protein than neutrophils.

Methods

Cell preparation and cell culture. Healthy community volunteers underwent fiberoptic bronchoscopy as previously described (9). Eight subjects were nonsmokers and seven subjects smoked at least one pack per day. The mean numbers of total cells±1 SD in 100 ml of recovered alveolar lavage fluid from the nonsmoking and smoking groups were 11±4.3 and 60±37 million cells, respectively. Differential cell counting revealed mean values of 94 and 97% macrophages, 4.8 and 1.3% lymphocytes, and 0.5 and 1% neutrophils in the nonsmoking and smoking groups, respectively. The basic tissue culture medium used in all of the experiments in this study was Dulbecco's modified Eagle's medium (DMEM)[1] supplemented with 10 mM Hepes, streptomycin (100 μg/ml), and penicillin (100 U/ml). The pooled cellular pellets were counted by hemocytometer and washed in DMEM. The cells were then immediately used for experiments.

Human neutrophils were purified from heparinized blood that was obtained from separate normal volunteers. Leukocyte-rich plasma was recovered after spontaneous erythrocyte sedimentation at 37°C for 2 h. Neutrophils were then isolated by centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). The neutrophil preparations consisted of >95% neutrophils, <1% esterase-positive cells, and a few contaminating erythrocytes.

Preparations of alveolar fluid. Recovered alveolar fluid after bronchoalveolar lavage was dialyzed (3,200 mol wt cut off, A. H. Thomas) against water and lyophilized. The concentrate was reconstituted in phosphate-buffered saline (PBS) and total protein determined. The concentrate was then adjusted to 1 mg total protein per milliliter and dialyzed against tissue culture medium (DMEM) before use. 1% (vol/vol) human serum as used in these experiments had a protein concentration of 0.9 mg/ml.

Reagents. Human serum from nonsmoking individuals was prepared and stored as previously described (9). Bovine ligamentum elastin was obtained from Elastin Products, Inc. (St. Louis, MO) and tritiated by reductive alkylation with ^{3}H-borohydride (New England Nuclear, Boston, MA) as described by Gordon et al. (10). The radiolabeled elastin was washed until the washings contained <1% of the total radioactivity, were aliquoted, and stored at −70°C. Soluble bovine ligamentum elastin (Elastin Products, Inc.) was iodinated by a chloramine-T method (11). The specific activity of the preparation was 1×10^4 cpm/μg protein. The ^{125}I-elastin was then coupled to cyanogen bromide activated sepharose beads (Pharmacia Fine Chemicals). After extensive washing, the elastin-coated sepharose consisted of ~6–7 μg ^{125}I-elastin per 5 μl packed beads. The beads were rewashed before each experiment. Benzyloxycarbonyl-phenylalanyl-phenylalanyl-diazomethylketone (Z-phe-phe-CHN_2), Z-phenylalanyl-propyl-diazomethylketone (Z-phe-pro-CHN_2), benzyloxycarbonylphenylalanylalanyl-diazomethylketone (Z-phe-ala-CHN_2), and benzyloxycarbonyl-alanyl-arginyl-arginyl-4aminomethylcoumarin7-amide (Z-ala-arg-arg-AMC) were obtained from Enzyme Systems Products (Torrence, CA) and dissolved in dimethyl sulfoxide (DMSO) at 1,000 times the highest concentration tested. Phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) was also dissolved in DMSO at 1,000 times the highest concentration tested. All other reagents were of the highest grade commercially available.

Enzyme assays. Elastase activity was first assayed by solubilization of ^{3}H-elastin (bovine ligamentum nuchae) in the absence of detergent as previously described (9). ^{3}H-elastin suspended in water was sonicated and then dried in 16-mm culture wells (Costar, Cambridge, MA) at 47°C. Although sonicated, the elastin appeared mostly particulate by phase and scanning electron microscopy. Each well contained ~240,000 cpm releasable by porcine pancreatic elastase (Sigma Chemical Co., type III). The chambers were washed in PBS and intact alveolar macrophages (10^6 cells/well) or blood neutrophils (10^5–10^6 cells/well) adhered directly to the elastin-coated culture wells. The cells appeared by phase microscopy to adhere both to the elastin and to exposed plastic surfaces. The cells in duplicate were incubated in 500 μl of DMEM that contained various additives for 4–24 h at 37°C. After the culture period, 400 μl of culture media was removed, spun at 10,000 *g* in a microfuge, and 100-μl aliquots were assayed for degraded elastin by β-scintillation. Elastase activity is expressed as micrograms elastin solubilized in duplicate determinations per 24 h. Under the culture conditions described, porcine pancreatic elastase (0.1 μg/ml) released 32 μg and 0.01 μg/ml released 2 μg of elastin in 24 h at 37°C.

Elastase activity was also assayed using ^{125}I-elastin-sepharose beads as substrate (12). These experiments were done in Falcon microtiter chambers (Becton-Dickinson & Co., Cockeysville, MD). Alveolar macrophages (10^5/well) or blood neutrophils (10^5/well) were adhered to microtiter wells. Washed ^{125}I–elastin-sepharose beads (5 μl) were then allowed to settle over the cells (6–7 μg elastin/well). Preliminary data indicated that 5 μl of beads were nontoxic to the cells as judged by morphology and trypan blue staining, but completely covered the cell monolayers. Initial experiments also indicated that solubilization of elastin from the beads was proportional to cell number from 10^4–10^5 cells. Subsequently, 10^5 cells were used in all experiments. The elastin-sepharose and cells were co-cultured for 20 h at 37°C in 100 μl DMEM with or without supplemental serum and other additives as indicated below. After the incubation period, 50 μl of medium was removed, centrifuged in a microfuge, and assayed for radioactivity by β-scintillation. Data are expressed as nanograms elastin solubilized after substraction of nonspecific release of cpm from beads that were cultured without cells. Background release of cpm was generally ~5% of total radioactivity added to the wells.

Cathepsin B activity was assayed according to Barrett and Kirschke (13). After 20–24 h incubation at 37°C in DMEM with or without

1. *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfonylfluoride; Z-ala-arg-arg-AMC, benzyloxycarbonyl-alanyl-arginyl-arginyl-4aminomethylcoumarin7-amide; Z-phe-ala-CHN_2, benzyloxycarbonyl-phenylalanyl-alanyl-diazomethylketone; Z-phe-phe-CHN_2, benzyloxycarbonyl-phenylalanyl-phenylalanyl-diazomethylketone; Z-phe-pro-CHN_2, Z-phenylalanyl-prolyl-diazomethylketone.

possible inhibitors of lysosomal enzymes, the cells were washed extensively to remove inhibitors. One million macrophages were then lysed in 200 μl 88 mM KH_2PO_4-12 mM Na_2HPO_4 with 1.3 mM EDTA (pH 6.0) and 2.7 mM cysteine and 0.2% Triton X-100. Lysates were then diluted 1:25–1:100 in the same buffer without detergent. Z-ala-arg-arg AMC, 0.1 mM final concentration, was used as substrate in a total reaction volume of 1 ml. After 60 min at 37°C, enzyme activity was measured in arbitrary fluorescence units in a Perkin-Elmer spectrofluorometer. Preliminary data showed that fluorescence was proportional to enzyme concentration over the dilutions tested and that >95% of the fluorescence was blocked by preincubation of cellular lysates with the specific cysteine proteinase inhibitor, Z-phe-ala-CHN_2 (10^{-5} M). Each experiment tested 1:25, 1:50, and 1:100 dilutions of the cell lysate and the cysteine proteinase nature of the fluorescence was routinely confirmed by adding 10^{-5} M Z-phe-ala-CN_2 to an aliquot of the reaction mixture.

Protein determination. Protein concentration was determined by the method of Lowry using a bovine albumin standard (14).

Results

Degradation of elastin by macrophages and neutrophils. The data in Fig. 1 *A* indicate that the extent of elastin degradation

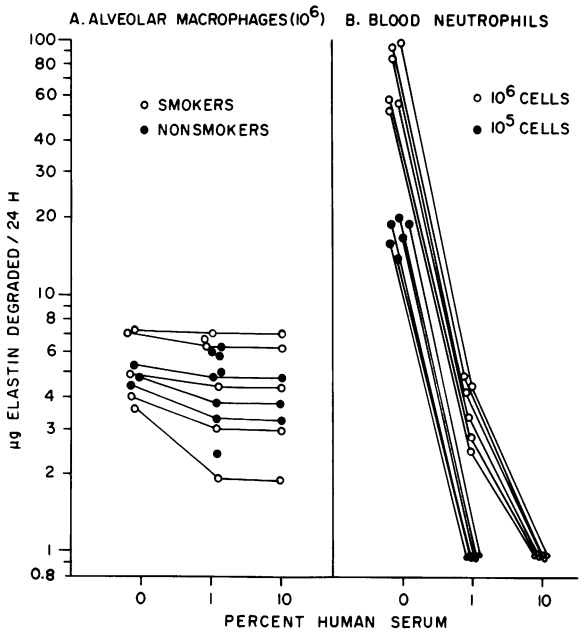

Figure 1. (*A*) Elastin degradation by live human alveolar macrophages. Lavage cells (>90% macrophages, <3% neutrophils) from smoking (•) or nonsmoking (o) volunteers were adhered to 16-mm culture chambers that were previously coated with ^{3}H-elastin (10^6 cells/well). Cells were cultured in the chambers for 24 h at 37°C in either DMEM or DMEM containing 1–10% (vol/vol) unheated human serum. Aliquots of medium were then centrifuged and assayed for degraded, soluble elastin by β-scintillation. Each data point represents mean microgram elastin degraded in duplicate determinations for each subject's cells. (*B*) Human neutrophils, 10^5 cells (•) or 10^6 (o) cells per well, purified from blood, were assayed for elastolytic activity identically to macrophages.

by live alveolar macrophages was dependent on the culture medium. Alveolar macrophages cultured in serum-free medium (DMEM) degraded a mean of 5.1 μg/10^6 cells per 24 h. These same cells, when cultured in 1% human serum, degraded 4.3 μg elastin ($P < 0.01$, paired t test, reference 15). The mean elastin degraded by cells from a larger group of 14 subjects (six smokers, eight nonsmokers) cultured in 1% human serum was 4.7 μg/24 h. Increasing the percentage unheated human serum from 1 to 10% did not further decrease elastin degradation in any of the subjects tested. As the data in Fig. 1 *A* also show, there was no difference in mean microgram elastin degraded on a per cell basis between smoking and nonsmoking individuals.

Data in Fig. 1 *B* show elastin degradation by blood neutrophils for comparison with macrophages. Neutrophils were cultured under identical conditions to that of macrophages and elastin degradation measured at both 4 and 24 h of culture. We made measurements at both 4 and 24 h because at 4 h, all cells were alive as judged by absence of trypan blue staining. At 24 h, most of the neutrophils, while intact, stained with trypan blue. Data in Fig. 1 *B* show the elastin degradation by 10^5 neutrophils per assay measured after 24 h culture. We show these data for comparison with alveolar macrophages because this cell number represents a higher proportion of neutrophils relative to 10^6 alveolar macrophages than was recovered by bronchoalveolar lavage from these smoking subjects or from smokers in previously reported data (16, 17). Neutrophils cultured in DMEM hydrolyzed a mean of 19 μg elastin, whereas cells cultured in DMEM containing 1% human serum hydrolyzed <1 μg elastin. The ratio of elastin degraded by 10^6 alveolar macrophages to that of 10^5 neutrophils when both cells were cultured in 1% serum was approximately six. The data also show that 10^6 neutrophils degrade at least an order of magnitude more elastin than 10^6 macrophages in a 24-h assay. Again, neutrophil-mediated elastinolysis was very sensitive to human serum in the assay. Increasing the percentage of human serum supplementation to 10% totally blocked elastin degradation by either 10^5 or 10^6 neutrophils. Thus, in medium that contained 10% serum, only live macrophages measurably degraded elastin. Measurements of neutrophil degradation at 4 h showed proportionately less elastin solubilized, but the percentage inhibition by human serum was identical (not shown).

We considered the possibility that the relative resistance of macrophages to serum proteinase inhibitors was due to phagocytosis of particulate elastin by the larger macrophages. Therefore, we performed similar experiments with iodinated, soluble elastin that was covalently coupled to sepharose beads. The beads were too large to be phagocytized but were not toxic to the cells, as judged by cellular morphology and trypan blue staining. 5 μl of packed beads that contained ^{125}I-elastin (6–7 μg) were layered on top of 10^5 live alveolar macrophages or 10^5 neutrophils in microtiter chambers. Elastin degradation was again measured as solubilized radioactivity after 18–20 h

Table I. Elastolytic Activity of Human Alveolar Macrophages and Blood Neutrophils toward ^{125}I-Elastin-Sepharose

Additives to basic culture medium	Elastin degraded			
	Alveolar macrophages		Neutrophils	
	ng/24 h	% Inhibition	ng/24 h	% Inhibition
None	473	0	3,825	0
1% Human serum	439	7	104	99
10% Human serum	288	39	0	100
1% Human serum plus Z-phe-ala-CHN_2	302	36	ND	ND

Alveolar macrophages or blood neutrophils, 10^5 cells, were adhered to microtiter wells. ^{125}I–elastin-Sepharose beads, 5 μl, were added and co-cultured with cells 24 h at 37°C in various media, as shown in the table. Aliquots of the medium were removed, centrifuged, and assayed for solubilized radioactivity. Data are expressed as nanogram elastin degraded/24 h and percentage inhibition of activity in serum-free media by media containing serum or serum with 10 μM Z-phe-ala-CHN_2. Data represent mean values from five separate experiments using cells from five subjects. Assays with blood neutrophils were done concurrently. ND, not determined.

at 37°C. The data in Table I show that in serum-free DMEM 10^5 neutrophils degraded an average of eight times more elastin (mean 3.8 μg/24 h) than 10^5 alveolar macrophages in five separate experiments. However, in DMEM that contained 1% human serum, these macrophages degraded four times the elastin (0.44 μg/10^5 cells per 24 h) as neutrophils. In 10% serum, macrophages still had 61% of the elastolytic activity measured in serum-free DMEM, whereas neutrophils did not measurably degrade the elastin. These findings confirm that even with a nonphagocytizable substrate, in the presence of macromolecular inhibitors, alveolar macrophages degrade severalfold more elastin than neutrophils.

Effect of concentrated alveolar fluid on macrophage and neutrophil elastolytic activities. We next determined whether the relative resistance of macrophage elastolytic activity to serum inhibitors applied also to proteinase inhibitors of alveolar fluid. Previous studies have compared the protein constituents of serum and alveolar fluid (18, 19). Albumin is the predominant protein in both fluids, and accounts for ~55% of the total protein. Notably, $\alpha_1$antiproteinase concentrations, determined by immunodiffusion, are also virtually identical in serum and alveolar fluid when expressed as a percentage of total protein (18). To compare serum with alveolar fluid, we adjusted the alveolar fluid obtained from each of six individuals (males who smoked at least 1 pack/d) to the equivalent total protein concentration of 1% (vol/vol) human serum. Data in Table II compare the inhibitory effect of these alveolar fluid preparations with serum. The macrophages and neutrophils were obtained from separate normal volunteers. As with serum, the alveolar fluid inhibited neutrophil elstolytic activity to a greater degree than macrophages (95 vs. 49%). In the presence of alveolar fluid, macrophages (10^6) degraded 12-fold

Table II. Comparison of Effects of Alveolar Fluid and Human Serum on Macrophage and Neutrophil Elastolytic Activities

	Tissue culture medium only	Human serum	Alveolar fluid
A % Inhibition of elastolytic activity			
10^6 Macrophages	0	23±10	49±8
Neutrophils			
10^5 Cells	0	99±.7	98±.7
10^6 Cells	0	95±.9	93±2.2
B Ratio elastin degraded, macrophages/ neutrophils			
$10^6/10^5$	0.35	15.0	12.0
$10^6/10^6$	0.11	1.5	0.6

Alveolar macrophages, 10^6 cells, or blood neutrophils, 10^5–10^6 cells, were added to ^{3}H-elastin-coated culture wells. Adherent cells were cultured in DMEM with or without 1% (vol/vol) human serum or alveolar fluid, 1 mg/ml, that was dialyzed against DMEM. Aliquots of the medium were assayed for degraded elastin after 24 h at 37°C. Data in A are expressed as mean percentage inhibition, ±SEM, of cellular elastolytic activity by either serum or alveolar fluid. In B, data are expressed as ratio of macrophage/neutrophil elastin degradation under the various culture conditions. The mean values represent results of six experiments using cells and alveolar fluid from separate subjects. The alveolar fluid in these experiments were obtained from six smoking individuals. No obvious differences in inhibiting effect (percentage inhibition) were observed between the fluid from these smoking subjects and that of two nonsmoking subjects in subsequent experiments (not shown).

more elastin than 10^5 neutrophils, even though in DMEM alone 10^5 neutrophils degraded much more elastin. These findings confirm that macrophages are relatively resistant to both blood and tissue proteinase inhibitors when compared to neutrophils. Interestingly, the alveolar fluid preparations consistently inhibited macrophage elastolytic activity to a greater extent than human serum (49 vs 23% inhibition, respectively). These differences were statistically significant ($P < 0.01$), based on two-tailed t test (15).

Comparison of the elastolytic mechanisms of alveolar macrophages and blood neutrophils. Because of the differences in susceptibility of the two cell types to serum and alveolar protease inhibitors, we compared the degradative mechanisms of live human neutrophil and alveolar macrophages. We recently reported that live alveolar macrophage elastolytic activity is a cooperative process involving several proteases but requiring cell contact with elastin (9). Data in Table II extend our previous observations with respect to macrophages, and compare the degradative properties of macrophages to that of neutrophils with respect to elastin degradation. Data are shown as percentage of neutrophil or macrophage elastin degradation when cells are cultured in ^{3}H–elastin-coated chambers without the indicated enzyme inhibitors. Several clear differences between the two cell types were apparent. First, soybean trypsin inhibitor markedly suppressed neutrophil activity but consistently slightly enhanced macrophage activity. PMSF, an inhib-

itor of serine proteinases, similarly inhibited the bulk of neutrophil elastolytic activity but <15% of macrophage elastolytic activity. This indicated that alveolar macrophage elastolytic activity could not be accounted for by neutrophils in the bronchoalveolar lavage or by release of neutrophil elastase from macrophages. Second, although both neutrophils and macrophages are phagocytic cells, inhibitors of lysosomal enzymes such as chloroquine or blockage of cellular phagocytosis with cytochalasin B (10 μM) partially suppressed only macrophage elastolytic activity. The increase over base-line elastolysis for neutrophils cultured in the presence of chloroquine is consistent with known stimulation of lysosomal hydrolase release from cultured cells by chloroquine (20, 21).

A third distinction between neutrophil and macrophage elastolytic activity was indicated by the diazomethylketone, Z-phe-ala-CN_2. This specific cysteine proteinase inhibitor (22) had no effect on neutrophil elastin degradation but consistently inhibited 65–80% of intact macrophage elastin degradation. We also observed leupeptin (5 μg/ml) to similarly block macrophage but not neutrophil degradation in three experiments (not shown). The major cellular proteinase likely to be capable of elastin degradation as well as to be inhibitable by Z-phe-ala-CHN_2 is the lysosomal proteinase, Cathepsin B. Recent studies show that rabbit and human alveolar macrophages contain a Cathepsin-B-like proteinase (23, 24). We measured the effect of varying concentrations of the diazomethylketone on both cellular Cathepsin B as well as elastolytic activity. For these experiments, we used phe-phe instead of phe-ala diazomethylketone. This compound was comparable elastolytic inhibitory activity with phe-ala-CHN_2 but was more available. Data in Fig. 2 show the effect of 1–10 μM Z-phe-phe-CHN_2 on both Cathepsin B and elastolytic activities. We observed a dose-dependent increase in percent inhibition of elastolytic activity. At the highest concentration shown (10 μM), macrophages were 95% viable at the end of the 24-h culture medium as judged by trypan blue uptake. The intracellular Cathepsin B activity after incubation of cells in 1–10 μM Z-phe-phe-CHN_2 also showed a progressive decline. Notably, concentrations of Z-phe-phe-CHN_2 which virtually completely blocked Cathepsin B (5 μM) still only submaximally inhibited elastolytic activity. Further increases in the diazomethylketone to 10 μM resulted in a greater inhibition of elastolytic activity. Concentrations of Z-phe-phe-CHN_2 above 10 μM did not further inhibit macrophage elastolysis. That these inhibitory effects are dependent on the amino acid components of the diazomethylketone is indicated by the fact that a separate diazomethylketone, Z-phe-pro-CHN_2 (10 μM), inhibited neither the elastolytic activity of live cells nor the amidolytic activity of cell lysates toward the cathepsin B substrate (not shown). Lysates of neutrophils had no measurable Cathepsin B activity which was consistent with the lack of effect of diazomethylketones on neutrophil elastolytic activity (not shown).

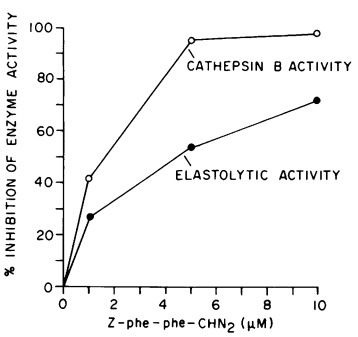

Figure 2. Concurrent inhibition of intact elastolytic and intracellular Cathepsin B activities by the diazomethylketone, Z-phe-phe-CHN_2. Alveolar macrophages from a single donor were cultured in either ^3H-elastin-coated wells or in uncoated chambers. The cells were cultured in medium that contained 1% human serum and Z-phe-phe-CHN_2, 1–10 μM as shown in the figure. The inhibitory effect of Z-phe-phe-CHN_2 on elastin degradation was assayed directly as described in the text. After the culture period, cells in uncoated chambers were extensively washed with PBS, lysed, and assayed for Cathepsin B activity at various dilutions of the lysate by fluorometry. Data are expressed as percent inhibition of elastolytic (•) or cathepsin (○) activity by various micromolar concentrations of Z-phe-phe-CHN_2 and represent mean values of three separate experiments.

We attempted to further distinguish between the neutrophil and macrophage elastolytic mechanisms by other classes of enzyme inhibitors. EDTA, a divalent cation chelator, did not distinguish the catalytic mechanisms. EDTA markedly inhibited live neutrophil activity (Table III). However, EDTA did not inhibit the elastase activity of neutrophils lysed in 0.2% Triton X-100 phosphate saline, pH 7.4 (not shown), which suggested that EDTA inhibits secretion of elastase by neutrophils and not the enzyme itself. Divalent cations are known to be critical to lysosomal enzyme secretion by neutrophils (25). Although human monocyte-derived macrophages reportedly contain an elastase that is a metalloproteinase (26), the observed effect of EDTA on live macrophage elastolytic activity may not be simply inhibition of a metalloproteinase. The chloromethylketone, succinyl-alanyl-alanyl-prolyl-valylchloromethylketone (Enzyme System Products) also did not distinguish the catalytic mechanisms. Although a known inhibitor of serine proteinases (27), and totally blocking neutrophil elastolytic activity at 0.1 mM, this agent also inhibits cysteine proteinases (21). We observed 0.1-mM concentrations of the chloromethylketone to totally block intracellular Cathepsin B activity in experiments identical to those shown in Fig. 2. Thus, the inhibitory effect of chloromethylketones can be explained by inhibition of the cysteine proteinase(s). The inhibition of neutrophil elastolytic activity by the chloromethylketone and PMSF, but not by the diazomethylketone, is consistent with previous reports that neutrophil elastases are serine proteinases (28, 29).

We also attempted to combine inhibitors of separate enzyme classes, i.e., PMSF and diazomethylketone, but resultant toxicity to the cells as judged morphologically precluded interpretation of these experiments. The toxicity probably relates to the observation that concentrations of solvents used to solubilize these reagents, DMSOs, above 0.1% (vol/vol) suppressed elastolytic activity.

Table III. Comparison of Intact Neutrophil and Alveolar Macrophage Elastolytic Activities

Inhibitor	Final concentration	Elastolytic activity: Blood neutrophil 4-h assay	Elastolytic activity: Blood neutrophil 24-h assay	Elastolytic activity: Alveolar macrophage 24-h assay
	mM			
None	—	100	100	100
Soybean trypsin inhibitor	0.02	13	10	105
PMSF	1.0	33	30	88
EDTA	0.1	50	20	56
Z-phe-ala- CHN_2	0.01	100	100	22
Chloroquine	0.05	165	200	60
Cytochalasin B	0.005	100	100	70
	0.01	ND	100	55
Cycloheximide	0.009	100	100	71

Alveolar macrophages (10^6) or blood neutrophils (10^6) were incubated in ^3H-elastin-coated chambers for 4 or 24 h as indicated in the table. The elastolytic activity (microgram elastin degraded/10^6 cells) of cells cultured in DMEM alone is given a value of 100. The activity of cells cultured in DMEM containing the various inhibitors is expressed as a percentage of control and represents a mean of at least four experiments for each inhibitor tested. All reagents were added to serum-free DMEM in the neutrophil assays. Chloroquine, cytochalasin B, cycloheximide, and Z-phe-ala-CHN_2 were added to DMEM that contained 1% human serum for macrophage assays and, in these cases, the activity of cells cultured in 1% serum alone was given a value of 100. 1% serum was added because the reagents were observed to be toxic to the macrophages in a 24-h assay in serum-free medium. Nevertheless, a similar pattern of inhibition was observed when macrophages were incubated in serum-free medium with Z-phe-ala-CN_2, chloroquine, or cytochalasin B (not shown).

Discussion

Previous studies have defined the elastolytic activity of human neutrophils. These cells elaborate a serine proteinase active at neutral pH with marked elastinolytic capability. The enzyme has been purified and characterized (28, 29). Moreover, Cathepsin G, also a serine proteinase, is released by neutrophils and cooperates with the elastase in elastin degradation (30). Our data are compatible with this mechanism of elastin degradation by intact neutrophils as well. Neutrophil elastolytic activity was entirely inhibitable by a chloromethylketone but not diazomethylketones. Human macrophage elastolytic mechanisms are fundamentally different from those of neutrophils. Macrophage activity is largely explained by a cysteine proteinase(s). Since the fractional inhibition of macrophage elastolytic activity by the diazomethylketone—an active site inhibitor (78%)—is greater than that achieved by blocking intracellular proteolysis with basic amines (40%), the results also suggest that part of the cysteine proteinase activity is expressed at the cell surface or in the immediate microenvironment. This is confirmed by the finding that Z-phe-ala-CHN_2 (10^{-5} M) also blocked 30–40% of elastin degradation by macrophages from three subjects who were co-incubated with ^{125}I-elastin coated sepharose beads (Table I). Measurements at the cell surface of adherent macrophages indicate a pH of 5–6 which allow considerable cathepsin-like activity (31). Overall, the data indicate that ~35–45% of the elastin degradation by live alveolar macrophages occurs intracellularly and is mediated by a cysteine proteinase(s). Another 30–35% of total activity is mediated by this mechanism but occurs at the cell surface or in the immediate cellular microenvironment. The enzymatic mechanism of the remaining 20–25% of total degradation is unclear, but may be mediated by a metalloproteinase with elastolytic activity previously described in human alveolar macrophages (26).

Differences in the mechanisms of elastin breakdown by macrophages and neutrophils correlate with functional differences in elastinolysis by live cells. Data reported here and previously (9) indicate that live human alveolar macrophages can degrade elastin even in the presence of serum proteinase inhibitors. This is not because macrophage elastolytic enzymes are intrinsically resistant to serum proteinase inhibitors (32). Rather, the close association of intact cells with substrate minimizes the effect of inhibitors. This appears to be particularly important for macrophage-mediated elastin degradation. Similar considerations apply to the resorption of bone matrix by osteoclasts and of extracellular matrix proteins by live murine peritoneal macrophages (33, 34). Neutrophils, in contrast, at least as judged by their interaction with purified elastin, are dependent on secretion of soluble enzymes that are more easily inhibited by soluble proteinase inhibitors. This dependence on a soluble pathway of enzymatic degradation could reflect less intimate contact with the substrate by neutrophils as compared to macrophages. If so, this pattern of interaction is not peculiar to purified elastin. Neutrophil-mediated degradation of extracellular matrix elastin (smooth muscle cell derived) or of fibrin is equally sensitive to macromolecular inhibitors (Chapman, H. A., and C. L. Allen, unpublished observations). In addition, endothelial cell detachment by stimulated neutrophils, a protease-dependent process, also is reportedly very sensitive to soluble proteinase inhibitors (35, 36). Our current studies, together with these previous observations, indicate that there are at least two major pathways of elastin degradation by human inflammatory cells: a neutrophil pathway involving released soluble elastases that are sensitive to serum and alveolar proteinase inhibitors; and macrophage-mediated elastolysis that functions as a largely cell-associated pathway relatively resistant to serum and alveolar inhibitors. Although neutrophils have much higher elastolytic potential, under conditions of proteinase inhibitor excess, macrophages degrade more elastin than neutrophils (Fig. 1). These observations may relate to connective tissue turnover at inflammatory sites. Direct analysis of proteinase inhibitor activity in human arthritic joints, for example, indicate an excess of inhibitors with little or no free proteolytic activity in the joint fluid (37, 38). Similarly, recent measurements of proteinase inhibitor

activity of alveolar lavage fluid of both nonsmoking and smoking subjects suggest an excess of inhibitors, including α_1-antiproteinase at the alveolar surface (8). Such evidence suggests that these cells usually function at inflammatory sites in a proteinase inhibitor-rich milieu. However, loss of effective soluble proteinase inhibitors could occur oxidatively (39, 40), genetically in α_1-antiproteinase deficiency (5), or potentially by intense local neutrophil accumulation. These situations would allow a much greater role for neutrophils and accelerated protein degradation at inflammatory sites.

Compared with nonsmokers, smoking subjects have a chronic macrophage bronchiolitis and alveolitis (41). This likely results from increased trafficking of blood monocytes or monocyte precursors into the lung, where they differentiate in the alveolar interstitium to macrophages and migrate through the interstitium onto the alveolar surfaces. The exact signals for such an inflammatory response are not defined. Emphysema generally develops in a subpopulation of these smoking subjects. Our data do not show a clear difference in elastin degradation between smoking (●) and nonsmoking (○) subjects on a per cell basis (Fig. 1). Rather, in that cellular movement is closely correlated with proteolytic activity (9, 42), it is likely that the increased trafficking of macrophages through the lung results in accelerated degradation of elastin that is known to be in the interstitium (43). The extent of injury could reflect in part the intensity and duration of the inflammatory response. However, we have not compared alveolar macrophages from smoking individuals with and without structural damage, i.e., emphysema. It remains possible that differences in elastolytic activity could exist between cells from these groups.

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