

Compromised inhibition of human lung lavage cell elastases

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Abstract Increased elastolytic activity has been correlated with the degree of lung damage occurring in a variety of lung diseases including cystic fibrosis; serine proteinase inhibitors are currently on trial for the treatment of some lung disorders. However, human lung lavage cells also secrete metallo-dependent elastases. Here we show, for the first time, that whilst these are readily inhibited by EDTA, inhibition of serine elastases using serpins (serine proteinase inhibitors) is not always possible. This may reflect inactivation of serpins by uninhibited metalloproteinases and oxidants in a low protein milieu. Thus, the therapeutic inhibition of excessive elastolytic activity may require a combination of inhibitors to work efficiently.

Key words: Bronchoalveolar lavage; Neutrophil elastase; α -1-Proteinase inhibitor; Matrix metalloproteinases; Alveolar macrophage

1. Introduction

An increase in the proteolytic activity of respiratory tract secretions, in particular an excess of elastolytic activity, has been implicated in the pathology of a number of pulmonary conditions including cystic fibrosis and acute lung injury, as well as in diseases of tobacco smokers such as bronchitis and emphysema [1–5].

Elastolytic enzymes are released by the polymorphonuclear neutrophil, which produces at least two serine-dependent elastases, neutrophil elastase (EC 3.4.21.37) and proteinase 3 (EC 3.4.21.76), and the alveolar macrophage which can synthesize cysteinyl- and metalloenzymes with elastolytic activity (reviewed in [6]). The first elastolytic enzyme to be identified was neutrophil elastase, an enzyme with a broad spectrum of pro-inflammatory activity; in addition to its elastolytic properties, it can act as a secretagogue, stimulate GM-CSF release from alveolar macrophages and act as a neutrophil chemoattractant by activating C5a and stimulating IL-8 production by epithelial cells. Recently, attention has focused on the matrix metalloproteinases (MMPs). Alveolar macrophages constitutively produce MMPs-2, 3, 9, 10 and 12 and, thus, can degrade a variety of connective tissue components, including elastin.

The relative importance of the extracellular release of these different classes of enzyme into the airspaces is unclear since they have differing activities on a molar basis [6] and because

epithelial secretions contain inhibitors for all three classes of enzyme. Furthermore, the enzymes of one class may inactivate the inhibitors and activate the proenzymes of another class; either action could potentially amplify the elastolytic burden of the lung. For example, neutrophil elastase can both activate metalloelastase (MMP-12; EC 3.4.24) and cleave its inhibitors; conversely, MMP-12 can inactivate at least one serine proteinase inhibitor (or serpin), α -1-proteinase inhibitor [7]. As alveolar macrophages and neutrophils are often found in close proximity in the respiratory tract, particularly in pathological states, for example cystic fibrosis, such interactions may amplify the total elastase burden, so increasing the severity of tissue damage.

To evaluate the elastolytic potential of proteases released by mixed populations of bronchoalveolar lavage cells (BAL cells), we have quantified the relative and total activities of serine elastases and matrix metalloproteinases released by BAL cells from patients. In addition, in order to establish the overall effect of inhibition of one class of enzyme (as might occur during the therapeutic use of α -1-proteinase inhibitor), we have observed their interactions in the presence of the serine- and metalloproteinase inhibitors, α -1-proteinase inhibitor and EDTA, and the highly specific inhibitor of neutrophil elastase, *N*^ε-(1-adamantanesulphonyl)-*N*^ε-(4-carboxybenzoyl)-L-lysyl-L-alanyl-L-valinal (Ro 31-3537, also known as 'Compound 3' [8,9]).

2. Materials and methods

2.1. Collection of bronchoalveolar lavage from patients

Bronchoalveolar lavage was collected from 14 sequential patients (25–66 years, median 54 years) undergoing routine fiberoptic bronchoscopy with lavage as part of their diagnostic work-up; most were undergoing investigation for suspected bronchial carcinoma. Seven were current smokers and seven ex- or non-smokers. Ex-smokers had not smoked for at least 2 years prior to the bronchoscopy. Bronchoalveolar lavage, performed as previously described [4], was used to collect material from a region of the lung which was radiographically normal and normal on visual inspection. The recovered fluid and cells were processed as described previously [4]. No sample had evidence of haemorrhage or severe inflammation (taken as 1×10^5 neutrophils/ml).

2.2. Bronchoalveolar lavage cell culture

Mixed BAL cells were plated at a concentration of 10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) or low protein hybridoma medium (LPHM; Gibco, UK) in 24 well plates. After 1 h, the conditioned medium and any non-adherent cells were removed and cells and medium separated by centrifugation [10]. The acellular conditioned media were stored at -20°C until analysis. The adherent cells were cultured for a further 3 h in the equivalent volume of fresh medium, then the conditioned media harvested and stored as before.

2.3. Elastolytic activity in conditioned media; effect of native and synthetic inhibitors

Samples of conditioned media were preincubated for 30 min at 37°C with an equal volume of 0.1 mol/l Tris-HCl, pH 7.0 (to measure

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Abbreviations: MMP, matrix metalloproteinase; BAL, bronchoalveolar lavage; Ro 31-3537, *N*^ε-(1-adamantanesulphonyl)-*N*^ε-(4-carboxybenzoyl)-L-lysyl-L-alanyl-L-valinal; DMEM, Dulbecco's modified Eagle's medium; LPHM, low protein hybridoma medium; PI, α -1-proteinase inhibitor

total elastase activity) or buffer containing elastase inhibitors: α -1-proteinase inhibitor (192.5 nmol/l; Sigma, UK), EDTA (100 μ mol/l; Sigma, UK) or Ro 31-3537 (1 mmol/l), in the final concentrations shown in parentheses. Uninhibited elastase activity was then measured by quantifying the release of soluble elastin peptides from an insoluble [3 H]elastin substrate (Elastin Products, MI, USA) as previously described [4]. Human neutrophil elastase (Elastin Products) of known activity [11], made up in DMEM or LPHM as appropriate, was used as a standard. Sample, inhibitor and reagent blanks in DMEM and LPHM were assayed as described above.

2.4. Effect of incubation time on the inhibitory capacity of α -1-proteinase inhibitor against elastinolytic activity

Samples of DMEM conditioned by exposure to cells ($n = 6$ subjects) were preincubated with α -1-proteinase inhibitor for 30 min at 37°C as described above. They were then incubated with [3 H]elastin for 24 h as described previously [4] or for 4 h only, after which uninhibited elastase activity was measured as described above.

2.5. Investigation of elastase: α -1-proteinase inhibitor mixtures for modified α -1-proteinase inhibitor

Samples of α -1-proteinase inhibitor (70 μ g) which had been preincubated for 30 min at 37°C with neutrophil elastase (0.025–25 μ g in DMEM) or conditioned DMEM, were concentrated by centrifugation at 5000 $\times g$ for 1.5 h at 4°C through Centricon filters with a nominal molecular mass cut-off of 10 kDa (Amicon, UK). Concentrates were Western blotted [10] and stained for α -1-proteinase inhibitor with a specific polyclonal antibody (DAKO, UK).

2.6. Statistics

The Wilcoxon rank sum test for unpaired data or the Wilcoxon signed rank test for paired data was used as appropriate, taking the level of significance as $P < 0.05$ in a two-tailed test.

3. Results

3.1. Bronchoalveolar lavage data

Fluid recoveries and BAL cell numbers and profiles are shown in Table 1. There were no significant differences in differential cell counts (mainly alveolar macrophages) between the BAL cells cultured in DMEM and those cultured in LPHM. Approximately 50% of the cells adhered after 1 h in culture irrespective of the medium used.

3.2. Initial elastinolytic activities in conditioned media in the absence of inhibitors

Elastinolytic activity in media conditioned by the mixed BAL cell population was significantly greater than that in media conditioned by adherent cells alone, even when allowance was made for cell number (not shown); there was no significant difference in elastinolytic activity between the two types of culture medium (Table 1).

Table 1

Fluid and cell recovery from bronchoalveolar lavage; profile of cells cultured in LPHM and DMEM and elastinolytic activity released by total and adherent BAL cells

	LPHM ($n = 6$)		DMEM ($n = 8$)	
Fluid (% instilled)	53	(27–71)	39	(31–55)
BAL cells ($\times 10^6$)	28.1	(16.8–38.7)	48.6	(7.2–86.2)
Alveolar macrophages (%)	89	(78–97)	88	(60–97)
Neutrophils (%)	3	(0–15)	4	(0–10)
Lymphocytes (%)	5	(1–19)	7	(1–30)
<i>Media conditioned by total cell population</i>				
Elastinolytic activity (ng/ml medium/h)	25	(7–83)	38	(16–783)
<i>Media conditioned by adherent cells</i>				
Elastinolytic activity (ng/ml medium/h)	7	(2–30)*	15	(3–59)*

The elastinolytic activities of conditioned media are expressed in neutrophil elastase equivalents.

*Less than in media conditioned by total cell population, $P < 0.01$.

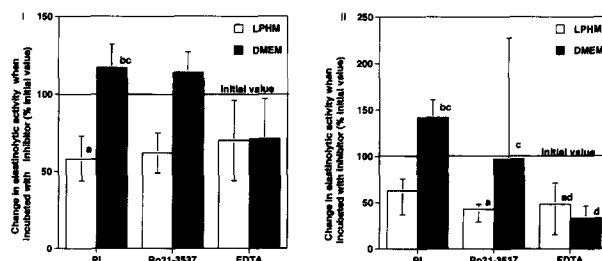


Fig. 1. Inhibition of extracellular elastinolytic activity from cultured BAL cells. Inhibition of extracellular elastinolytic activity from total BAL cell populations (panel i) and adherent BAL cells alone (panel ii). Activities in the presence of inhibitors are expressed as percentages of the initial values (in the absence of any added inhibitor). Median values with the interquartile ranges. a < initial elastinolytic activity, b > initial elastinolytic activity, c > LPHM, d < media conditioned by total cell population; $P < 0.05$.

3.3. Elastinolytic activities in conditioned media in the presence of inhibitors

Neither α -1-proteinase inhibitor nor Ro 31-3537 inhibited elastinolytic activity released in to DMEM, although both consistently inhibited activity released into LPHM (Fig. 1). Choice of medium had no effect on the inhibitory capacity of EDTA, which consistently inhibited 30% of the elastinolytic activity in media conditioned by total BAL cell populations and approximately 60% of that in media conditioned by adherent BAL cells (Fig. 1).

3.4. Effect of incubation time on inhibition of elastinolytic activity in DMEM by α -1-proteinase inhibitor

After a 4 h incubation with [3 H]elastin, α -1-proteinase inhibitor reduced the initial elastinolytic activity of paired media from three subjects and enhanced that from the other three pairs (Fig. 2). When the time of incubation with elastin was increased to 24 h, α -1-proteinase inhibitor enhance the elastinolytic activity of all the conditioned media tested (Fig. 2).

3.5. Western blotting

Western blotting showed evidence of α -1-proteinase inhibitor complex formation in the presence of both conditioned media and pure neutrophil elastase; this could be increased using increasing amounts of neutrophil elastase relative to α -1-proteinase inhibitor (Fig. 3). However, there was no evidence of α -1-proteinase inhibitor fragmentation by conditioned media or neutrophil elastase at the concentrations used in the experiments described above (Fig. 3).

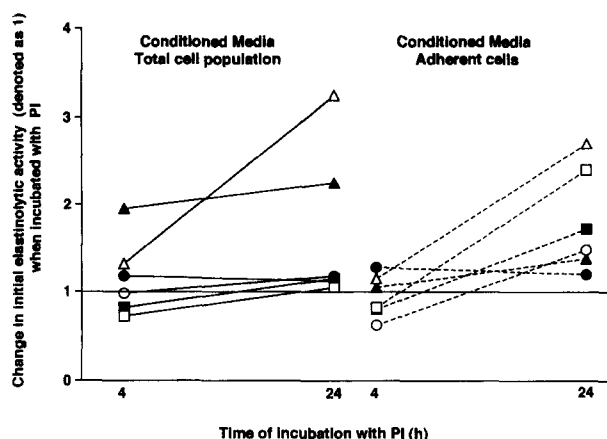


Fig. 2. Effect of incubation time on inhibition of elastolytic activity in conditioned media by α -1-proteinase inhibitor. Each symbol represents DMEM conditioned by cells from the same subject. The initial elastolytic activity (in the absence of inhibitors) varied considerably between subjects (see Table 1). This initial activity is expressed as 1 for all subjects in order to demonstrate whether incubation with α -1-proteinase inhibitor decreased or increased the activity and for clarity of illustration. In five out of six paired samples, increasing the time of incubation with α -1-proteinase inhibitor increased the measurable elastolytic activity.

4 Discussion

Since unrestrained elastolytic activity from inflammatory cells is believed to contribute to the pathogenesis of a variety of lung diseases, there is considerable interest in using elastase inhibitors therapeutically. Hence, it is necessary to characterise the enzymes which contribute to the elastolytic burden in the human lung in order to use effective inhibitors; cultured BAL cells provide us with a means of doing this relatively non-invasively. In the current study, we detected elastolytic activity in media conditioned by patients' BAL cells (Table 1), confirming that these cells possess and release elastolytic enzymes. Interestingly, when DMEM was used as a culture medium, we were able to inhibit only metallo-dependent elastolytic activity; furthermore, elastolytic activity was higher rather than lower when serine-elastase inhibitors were present (Fig. 1). In contrast, if LPHM was used, serine-dependent enzymes could be inhibited as expected.

A possible explanation for these observations is that oxidants produced by the cells in culture remain reactive in DMEM, but not in LPHM and, thus, can inactivate α -1-proteinase inhibitor when it is subsequently added to conditioned DMEM, but not conditioned LPHM. The latter is a supplemented medium relatively rich in proteins, which are known to scavenge cell-derived oxidants. In contrast, DMEM is not supplemented; in the absence of complex molecules, cell-derived oxidants might interact with amino acids in DMEM, but remain able to undergo subsequent reactions with added proteins, in this case α -1-proteinase inhibitor, by which they would finally be consumed. (For example, specific amino acids have previously been shown to potentiate the toxicity of oxidants [12].) α -1-Proteinase inhibitor is particularly susceptible to inactivation by oxidation, since it has a methionine residue at its active site [13,14]. In contrast, neutrophil elastase, which has no methionine at its active site, is relatively less affected by oxidants [15]. Since pro-matrix metalloproteinases can be activated both by serine proteinases such as neutrophil elas-

tase and by cell-derived oxidants [16], such metalloproteinases could inactivate α -1-proteinase inhibitor [17] and enable continued neutrophil elastase activity. Thus, overall, elastinolysis would be amplified. We found no evidence of α -1-proteinase inhibitor degradation and only minimal complex formation of α -1-proteinase inhibitor with neutrophil elastase in conditioned medium (Fig. 3), suggesting that the α -1-proteinase inhibitor was inactivated either by enzymatic nicking of the reactive site loop or by oxidation of the reactive site methionine, neither of which would be detected by Western blotting. Western blotting confirmed that our findings were not caused

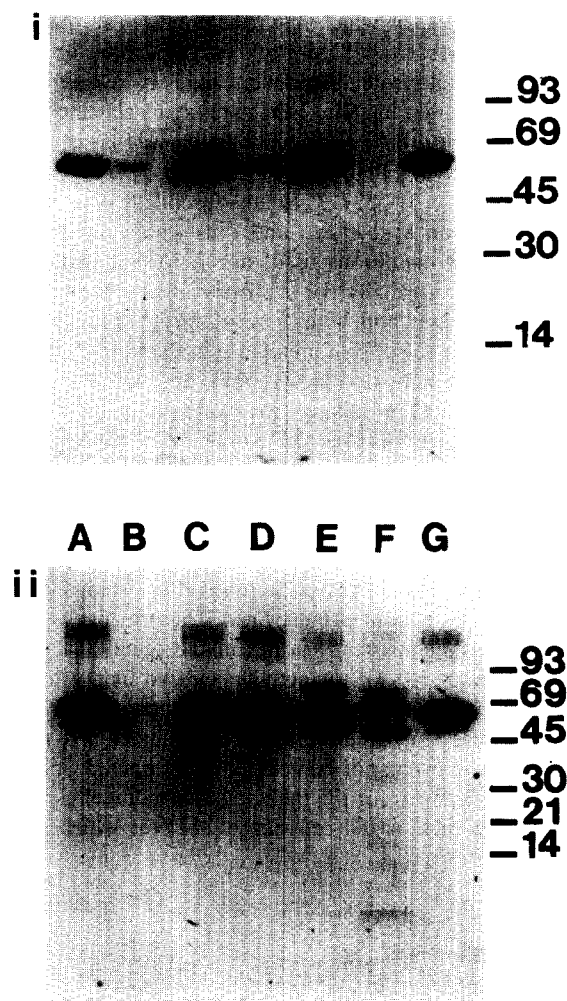


Fig. 3. Immunoblot showing the effect of pure neutrophil elastase and conditioned media on the format of α -1-proteinase inhibitor. Panel i: Lane A, pure α -1-proteinase inhibitor; lane B, pure neutrophil elastase; lane C, α -1-proteinase inhibitor following preincubation with neutrophil elastase in the molar ratio 400:1; lanes D and F, samples of conditioned DMEM; lanes E and G, exogenous α -1-proteinase inhibitor following preincubation with the same conditioned media. The molecular weight of α -1-proteinase inhibitor, 54 kDa, was unaffected by any treatment. Panel ii: Lanes A and G, pure α -1-proteinase inhibitor; lane B, pure neutrophil elastase; the remaining lanes contain α -1-proteinase inhibitor following preincubation with neutrophil elastase in the molar ratios: lane C, 400:1; lane D, 40:1; lane E, 4:1; lane F, 0.4:1. Native α -1-proteinase inhibitor had a molecular weight of 55 kDa. Inhibitor:enzyme complexes (72 kDa) were formed at low molar ratios (lanes D, E and F). At ratios unlikely to occur in vivo (lanes E and F), partially degraded α -1-proteinase inhibitor (49 and 62 kDa) was observed.

by contamination of the α -1-proteinase inhibitor by neutrophil elastase.

At the concentrations used in this study, EDTA does not inhibit neutrophil elastase (data not shown). Approximately 30% and 60% of the elastolytic activity released by total and adherent BAL cell populations respectively is metallo-dependent. This activity is likely to be from alveolar macrophages, which predominate in the adherent cell population, although there were a few adherent neutrophils which may have released some of the EDTA-susceptible elastases. The enzymes most likely to have contributed this activity are MMP-9 (gelatinase B; EC 3.4.24.35), which is released by alveolar macrophages and neutrophils, and MMP-12, a metalloelastase from alveolar macrophages which may be implicated in the pathogenesis of emphysema [6]; these two enzymes make up 50% of the matrix metalloproteinase elastase activity released by alveolar macrophages [6]. The EDTA-resistant elastolytic activity released into the conditioned media was largely serine-dependent, since in LPHM it was inhibited by α -1-proteinase inhibitor and Ro 31-3537. Cysteine-dependent elastases (EC 3.4.22) require an acid milieu and thus would not be active under the assay conditions used, nor following extracellular release *in vivo*. Normally the synthesis and release of serine-dependent elastases and matrix metalloproteinases is strictly regulated; where there is an imbalance in the normal BAL cell profile, so that activated neutrophils and alveolar macrophages are in close proximity, for example, in smokers and patients with cystic fibrosis, the co-release of proteolytic enzymes may amplify the overall elastolytic burden by the processes described above.

In summary, mixed populations of BAL cells contain, and may release, a variety of elastolytic enzymes, the diversity of which may prolong increased activity, for example by the proteolytic activation of pro-enzymes. The presence of metalloenzymes was a consistent feature; matrix metalloproteinases contribute about 30% of the activity released by the total cell population, largely from the alveolar macrophage. Importantly, the elastolytic enzymes released by mixed populations of BAL cells cannot be controlled by a single inhibitor. This has implications for the therapeutic use of serine-proteinase inhibitors such as α -1-proteinase inhibitor which may be

inactivated by non-serine proteinases. Finally, we have demonstrated the importance of a high protein environment to 'buffer' α -1-proteinase inhibitor against inactivation by metalloproteinases and oxidants; this may be of particular importance in smokers, who often have less protein in their epithelial lining fluid than non-smokers [4].

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