Is neutrophil elastase associated with elastic tissue in emphysema?

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SUMMARY To test the role of elastase in the pathogenesis of emphysema human neutrophil elastase (HNE) was localised by electron microscopy using an immunogold staining technique. Specific localisation of HNE to elastic tissue in emphysema did not occur, but non-specific binding of immunoglobulin G (IgG) to elastic tissue in emphysematous and normal lung tissue, which was completely blocked by the non-immune serum that was homologous to the gold labelled second antibody, was found. HNE was also present, however, in the granules of neutrophils in the same sections.

Non-specific labelling associated with elastin was probably due to binding of IgG to the high numbers of hydrophobic and charged regions known to be present in this molecule, and it is concluded that our findings do not support the existence of high concentrations of elastase in association with elastin in emphysematous lung tissue.

It is widely believed that increased elastolytic activity in the interstitium of the lung is the major cause of emphysema in man^{1,2} and that neutrophil elastase has an important role in this process. Although there is unequivocal evidence that neutrophil elastase can produce emphysema in experimental animals,³ the evidence for its role in emphysema in man has mainly been circumstantial. Most of those with the disease are smokers. There is an increase in neutrophils in the peripheral blood in smokers⁴ and of neutrophil elastase and neutrophils in bronchoalveolar lavage fluid in smokers both with and without emphysema.^{5,6}

Although we have an indication of what is happening to elastase in the capillaries and at the surface of the lung, until recently there was no evidence of what changes were taking place within the interstitium of the lung. Damiano *et al*⁷ showed an association between increased neutrophil elastase and elastic tissue in the interstitium of emphysematous lung at the electron microscopic level that was related to the severity of the emphysema present. This seemed to us such an important observation that we undertook a similar study using immunogold localisation of human neutrophil elastase (HNE) at the electron microscopic level in normal and emphysematous human lung tissue.

Material and methods

Pieces of fresh human lung tissue were taken from lobectomy specimens removed for carcinoma of the lung (three men, two women, aged between 55 and 84), bronchiectasis (one man aged 20) and for paraseptal emphysema (one man aged 30). All the patients were smokers apart from the man with bronchiectasis. The lung tissue was inflated with modified Karnovsky fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer) at a pressure of 25 cm of fixative and fixed overnight. After fixation pieces of lung tissue (1 mm)³ were sectioned, washed in sodium cacodylate-sucrose, and secondarily fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, followed by washing in distilled water. The tissue was block stained with 2% uranyl acetate and dehydrated to absolute alcohol. The tissue was embedded in liquid TAAB epoxy resin using propylene oxide as a link reagent and polymerised at 55°C for 48 hours. Polymerised blocks were cut at 1 µm and stained with 1% toluidine blue in 5% borax. These sections were assessed as normal (n = 5) and emphysematous (n = 6) using a computerised image analysis system. Selected areas were cut with a diamond knife to give a light golden interference colour, mounted on 400 mesh nickel grids, and allowed to dry overnight.

Lung tissue was also prepared for comparison using the method described by Damiano *et al*.⁷

Polymorphonuclear neutrophils were obtained from patients with chronic myeloid leukaemia undergoing leucopheresis, and neutrophil elastase was prepared by the method of Schmidt and Havemann.⁸

Antibodies to HNE were raised in 3 month old New Zealand white rabbits, which had been bled via the marginal ear vein, to obtain non-immune serum. HNE (125 µg) in 0.5 ml of 0.15 M sodium chloride was emulsified in 0.5 ml Freund's complete adjuvant and injected subcutaneously into four sites along the flanks of each animal. Antibody titres were boosted in each animal one month later with 125 µg HNE in 0.15 M sodium chloride emulsified in Freund's incomplete adjuvant (1/1, v/v), and again a month later with 75 µg HNE in 0.15 M sodium chloride. Test bleeds were made and booster doses of 20 µg HNE (in 0.15 M sodium chloride) were administered on alternate weeks until the antibody titre, which had been high, began to fall. The animals were then bled via cardiac puncture. All serum samples were tested for the presence of antibodies to HNE using Ouchterlony immunodiffusion⁹ and the immunoglobulin fraction of the positive and pre-immune sera which had been purified by previously described methods.⁹

ASSESSMENT OF EMPHYSEMA

The images of the 1 µm toluidine blue sections were back-projected on to a translucent glass digitiser tablet of a computerised image analysis system at a screen magnification of ×300, calibrated from a stage micrometer. A series of parallel lines, drawn on a sheet of clear acetate, were laid over the projected image. The digitiser tablet pen was used to trace the length of those lines which fell across air spaces (excluding bronchi) from epithelium to epithelium. Each of these lines was defined as a linear transect. All the linear transects within the whole section were measured.

IMMUNOGOLD STAINING

Grids were floated on distilled water for five minutes and immersed in normal goat serum diluted 1 in 5 with phosphate buffered saline (PBS), at pH 7.2, containing bovine serum albumin (BSA) and Tween 20 (100 ml PBS, 0.1 g BSA, 0.05 ml Tween 20 = PBST) for 25 minutes. Parallel grids were immersed in 1% ovalbumin (OA) in PBST. The excess blocking agent was then drained off with filter paper, the grids immersed in rabbit antihuman neutrophil elastase at a dilution of 1 in 1000 with PBST, and then incubated at 4°C for 72 hours. The grids were washed in several changes of PBST for 10 minutes at room temperature and then in several changes of Tris-buffered saline (TBS, pH 8.2) containing BSA and Tween 20 in similar concentrations to PBST for 10 minutes at room temperature. Gold conjugated, goat antirabbit IgG (Janssen Life Sciences Products, Wantage, United Kingdom)

(10 nm in diameter) was diluted in 1 in 10 with TBST and centrifuged at 2000 g for 20 minutes. The grids were immersed in the supernatant which contained immunogold for one hour at room temperature and then well washed with TBS and distilled water. Sections were contrasted with Reynold's lead citrate. The immunogold labelling method described by Damiano *et al.*,⁷ in which BSA was used as the sole blocking agent of non-specific binding sites, was adhered to as much as possible.

The controls used are listed in table 1. The controls used for the method of Damiano *et al.*⁷ were (i) neutrophils present in the alveoli and capillaries and (ii) replacement of anti-HNE by PBST. Non-immune goat serum was also used as a blocking agent instead of BSA.

Results

The lung tissue sections were classed as normal or emphysematous on the basis of mean transect lengths,

Table 1 Control conditions for examination of immunogold labelling of HNE in lung tissue

Control conditions	Reasons for controls
(a) Neutrophils in alveoli, interstitium and capillaries	Positive controls for HNE in the granules
(b) Pre-immune rabbit serum diluted 1 in 1000 in place of rabbit anti-HNE	To confirm the specificity of the antibody
(c) Rabbit anti-HNE preadsorbed with excess HNE as confirmed by lack of precipitate on double immunodiffusion	To show that after adsorption of the antibody to the antigen the reaction becomes negative
(d) Rabbit anti-HNE replaced by PBST	To detect any non-specific binding of the second antibody
(e) Rabbit anti-HNE replaced by rabbit anti-human factor VIII	To determine whether an entirely irrelevant antibody binds to elastic tissue
(f) Intratracheal instillation of: (i) 10 µg HNE in 0.5 ml 0.15 M sodium chloride, and (ii) 0.15 M sodium chloride into rat lungs for four minutes, then asphyxiation with carbon dioxide	To establish whether our technique could localise HNE in lung tissue
(g) Incubation of: (i) 2.5 µg HNE (ii) 10 µg HNE, and (iii) buffer with 1 mg human lung elastin (Elastin Products, Pacific, Minnesota, USA) in 100 µl 100 mM Tris-hydrochloric acid, 5 mM CaCl ₂ , pH 7.0 for two hours at 37°C, terminated by 1000 g centrifugation at 4°C for 15 minutes, then washed three times with buffer	To examine binding of HNE to elastin in vitro

Fig 1 Human lung tissue (a) normal (b) emphysematous of sections from which electron microscopic sections were taken. (Toluidine blue).

which for normal tissue ranged from 82–108 μm , and for those with emphysema was 117–177 μm (Wilcoxon rank sum test, $p < 0.01$) (fig 1).

The results are summarised in table 2. HNE was confined to granules of neutrophils (fig 2). There was no association between HNE and elastic tissue in normal or emphysematous lung when non-immune goat serum was used as the blocking agent (fig 3). In contrast, when OA/BSA was used to block non-specific binding (table 1), both neutrophil granules and elastic tissue were labelled. The granules in the neutrophils were not labelled when pre-immune serum, PBST or antihuman factor VIII were used in place of the primary rabbit HNE. Immunogold labelled HNE was present in the lung of the rats, within neutrophils in the

alveoli, and within the interstitium, but was not particularly related to elastic tissue. There was no HNE associated with elastin following incubation with elastin in vitro (fig 4). There was some residual background labelling even after blocking with goat serum mainly over nuclei, red cells, and connective tissue, but this was very slight.

When the method of Damiano *et al*⁷ was used there was apparent selective labelling of HNE in association with elastic tissue in normal and emphysematous lung. When non-immune goat serum was used to block non-specific binding, however, the results were different: there was no HNE in association with elastic tissue even though it was present in neutrophil granules in the same sections. The non-specific localisation of

Table 2 Comparison of OA/BSA and goat serum/BSA as blocking agents prior to immunogold labelling of HNE in lung tissue

Treatment	Blocking agent			
	OA/BSA		Goat serum/BSA	
	Polymorphonuclear leucocytes	Elastin	Polymorphonuclear leucocytes	Elastin
Anti-HNE	+	+	+	—
Pre-immune serum	—	+	—	—
HNE adsorbed anti-HNE	—	+	—	—
Anti-factor VIII (figs 6a and b)	—	+	—	—

Fig 2 Immunogold labelled HNE in azurophilic granules of human neutrophil in lung capillary. Bar mark = 250 nm.

Fig 3 Elastic tissue in human lung after labelling for HNE using goat serum/BSA block. No label present. Bar mark = 500 nm.

Fig 4 Human lung elastin after incubation with HNE in vitro (a) using goat serum/BSA block—no label present—(b) using OA/BSA block—immunogold label present. Bar mark = 500 nm.

Fig 5 Human lung elastic tissue after labelling for HNE using OA/BSA. Label confined to amorphous elastic tissue and not microfibrillar component (arrow). Bar mark = 500 nm.

immunogold was related to the amorphous part of the elastic tissue and not the fibrillar component (fig 5).

Discussion

We were unable to confirm the findings of Damiano *et al*⁷ of specific localisation of HNE to elastic tissue in emphysematous human lung tissue by immunogold labelling and electron microscopy. That our method was capable of showing specific localisation of HNE was confirmed by the presence of HNE within granules of neutrophils in the same sections of human lung tissue, and within rat lungs after intratracheal instillation of HNE. The apparent specific localisation of HNE to elastic tissue, when OA (this study) and BSA (Damiano *et al*⁷) were used as blocking agents, was probably due to the non-specific binding of rabbit IgG. We have shown that this effect could be blocked by the non-immune goat sera that was homologous to the second antibody. This was confirmed when we also found that elastic tissue in the lung was capable of binding gold conjugated antirabbit serum when used without any primary antiserum.

The binding of the primary antibody to elastic tissue was probably due to the existence of hydrophobic or electrostatic forces, or a combination,¹⁰ which are normally blocked by the addition of foreign protein or the serum from the animal in which the second antibody is made—in this case the goat. BSA was incapable of completely blocking hydrophobic regions

Fig 6 Human lung elastic tissue after labelling for factor VIII (a) with goat serum/BSA block showing background labelling alone (b) with OA/BSA block. Pronounced labelling of elastic tissue. Bar mark = 500 nm.

of the elastic molecule; these regions contain large amounts of glycine, proline, and other hydrophobic residues.^{11,12} The *in vitro* study described here clearly shows the inability of OA/BSA to block non-specific binding of IgG to elastin (fig 4). The fact that Damiano *et al* did not use goat serum as a blocking agent would account for their apparent identification of HNE in relation to elastic tissue.

The increased amounts of non-specific labelling found by Damiano *et al* with increasing severity of emphysema can also be explained thus. In emphysematous areas there may have been a change in the structure of elastic tissue with an increase in the hydrophobic regions.

Emphysema is a disease that develops slowly over many years and thus we were not surprised that we did not show residual increased concentrations of HNE associated with elastic in the advanced stages. The effect of mechanical forces and exercise for many years during breathing on elastic tissue that has already been damaged by elastase may well be responsible for the creation of the large air spaces characteristic of emphysema. That there is continued elastolysis of elastic tissue throughout life still remains to be established.

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