Digestion of proteoglycans in porcine pancreatic elastase-induced emphysema in rats

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ABSTRACT: Pulmonary emphysema was induced in rats by a single intratracheal instillation of pancreatic elastase. The short-term effects of elastase instillation on basement membrane components were evaluated using immunohistochemical and biochemical methods.

Lung alveoli showed a decrease in heparan sulphate proteoglycan content (especially of its heparan sulphate chains) 3 h to 7 days after induction. Type IV collagen, laminin and fibronectin were not affected. The glycosaminoglycan content of the lung was decreased during the first 3 days after induction, while the glycosaminoglycan concentration in urine was increased during the first 4 days by an increase of heparan sulphate and dermatan sulphate. The increase in urinary glycosaminoglycan content was positively correlated with the extent of emphysema developed after 40 days.

We conclude that proteoglycans are target molecules for elastase, and may be involved in the pathogenesis of emphysema.

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The connective tissue of the lung parenchyma plays an important role in the functioning of the respiratory system. Alterations in the connective tissue skeleton are believed to be important in several chronic lung diseases, such as emphysema and fibrosis [1, 2]. Major components of lung extracellular matrix are elastin, collagen and proteoglycans (PGs). PGs consist of a core protein, to which one or more glycosaminoglycan (GAG) chains are covalently attached. GAGs are polysaccharides which are strongly negatively-charged due to an abundance of carboxylic and/or sulphate groups. In the alveolar wall, two types of PGs are predominantly present: decorin and perlecain. Decorin is a small dermatan sulphate (DS) proteoglycan which is associated with collagen fibrils [3-6], and perlecain is a heparan sulphate proteoglycan (HSPG) present in basement membranes [6-9].

The predominant theory on the pathogenesis of emphysema is the protease/antiprotease concept [10-12]. This concept emanates from a relative excess of proteases (elastases), due to an increase of proteases and/or a decrease of protease inhibitors. An excess of proteases leads to the degradation of the connective tissue skeleton and ultimately to emphysema. One has almost exclusively focused on elastin as the target molecule for proteases, in part because the most studied animal model for emphysema is elastase-induced emphysema. Elastase, however, is also capable of degrading a number of other proteins, including PGs, fibronectin and some types of collagen in vitro [13, 14]. Digestion of PGs may be of particular importance, since: 1) PGs are involved in fibrillogenesis of collagen and elastin, and can modulate their mechanical characteristics [3, 4]; 2) PGs interact with and modulate a number of molecules, including growth factors and extracellular matrix molecules [15-21]; and 3) GAGs can act as powerful protease inhibitors, e.g. for leucocyte elastase and cathepsin G [22-24]. In addition, the close association of PGs with structural elements in the alveolar wall (e.g. basement membranes, collagen fibrils and elastin fibres) indicates a protective role [6, 25-28].

In this respect, we studied the effects of an emphysema-inducing dose of pancreatic elastase in rats on perlecain (the core protein as well as the heparan sulphate (HS) side-chains) and on other extracellular matrix components of the alveolar wall by immunohistochemical and biochemical methods. Additionally, we investigated the excretion of GAG into the urine following elastase treatment.

Material and methods

1,9-Dimethylmethylene blue (80% pure) was purchased from Aldrich Chemical Co., Bornem, Belgium; Universal Gel/8 1% agarose gels from Ciba-Corning GmbH, Fernwald, Germany; Aquamount from BDH Ltd, Poole UK; halothane from APharmo, Arnhem, The Netherlands; pentobarbitol (Narcovet) from ICI-Farma, Rotterdam, The Netherlands; diethylaminoethyl (DEAE)-Sepharose Fast Flow from Pharmacia, Uppsala, Sweden; Azure A,
Tween-20, whale cartilage chondroitin 4-sulphate, bovine kidney HS, porcine pancreatic elastase, bovine serum albumin (BSA), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG), FITC-conjugated goat anti-mouse immunoglobulin M (IgM) and affinity purified rabbit anti-laminin IgG from Sigma Inc., St. Louis, MO, USA; tetra-methylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG from Dako, Glostrup, Denmark. Rabbit anti-basement membrane HS, porcine pancreatic elastase, bovine Tween-20, whale cartilage chondroitin 4-sulphate, bovine

**PROTEOGLYCANS IN ELASTASE-INDUCED EMPHYSEMA**

For immunofluorescence studies, lungs were dissected, and inflated through a polyethylene catheter inserted into the trachea with phosphate-buffered saline (PBS) at a pressure of 25 cm H₂O. PBS-filled lungs were frozen in liquid nitrogen and stored at -70°C. Cryosections (6 μm) were stored at -70°C. Cryosections were rehydrated for 10 min in PBS, containing 1% BSA. Antibodies were applied for 1.5 h in PBS containing 1% BSA. After each antibody, incubation sections were washed in PBS (3×5 min). Anti-HS antibodies and all secondary antibodies were used at a dilution of 1:100. All other antibodies were used at 1:50. Double staining was performed using fluorescein- and rhodamine-labelled secondary antibodies. Sections were embedded in aquamount and examined on a Zeiss axioskop microscope (Carl Zeiss, Oberkochen, Germany). Observations were made by two blinded observers. A "strong" decrease was defined by large areas in the section showing no specific antibody staining. "Weak" was defined by some small areas in the section which showed a reduced immunostaining. "Moderate" was defined as a moderate number of areas (between strong and weak) showing a clear, but not complete, absence of staining.

**Purification of urinary glycosaminoglycans**

Rat urine was collected one day before elastase instillation, then daily for 9 days and finally on Day 19 post instillation. GAGs were purified from urine by anion exchange chromatography. Urine was centrifuged at 2,000xg for 10 min (at 4°C), and 20 ml of the supernatant was diluted to 50 ml by adding 10 mmol·l⁻¹ Tris-HCl (pH 6.8). The diluted samples were loaded onto a column of 5x0.5 cm containing 0.5 ml DEAE-Sephadex Fast Flow. After an initial wash with 3 ml 0.2 mol·l⁻¹ NaCl/10 mmol·l⁻¹ Tris-HCl (pH 6.8), GAGs were eluted with 4 ml 2 mol·l⁻¹ NaCl/10 mmol·l⁻¹ Tris-HCl (pH 6.8). Recovery of GAGs was monitored by adding 9.10⁶ Bq [³⁵S]GAG to the urine, and was between 72–101%.

**Extraction of lung glycosaminoglycans**

GAGs were extracted according to a modified method of Hoffmann [32]. Pleura, large airways and blood vessels were removed. The remaining tissue was defatted in 20 vol. acetone at -20°C for 16 h, followed by extraction with 20 vol. ether/methanol (1:1) at 4°C for 24 h. The material was dried, pulverized with a glass rod and suspended in 40 vol. (w/v) of 0.75 mol·l⁻¹ NaOH/10 mmol·l⁻¹ NaBH₄. After 1 h at 73°C, the mixture was neutralized with 6 mol·l⁻¹ HCl and 100% (w/v) trichloroacetic acid was added to a final concentration of 6%. After 1 h at 4°C, and centrifugation (15 min, 2,000×g, 4°C), the pellet was dissolved in 0.1 mol·l⁻¹ NaOH. The protein concentration was determined according to Lowry et al. [33]. To the supernatant, 5 vol. of ethanol (100%) was added and after 16 h at -20°C the mixture was centrifuged (30 min, 15,000×g, 4°C). The precipitated GAGs were dried and dissolved in deionized water. GAGs were quantified using the 1,9-dimethylmethane blue (DMMB) assay (see below).

**Animal accommodation**

Animals were accommodated in groups of three and fed ad libitum. For urine collection, animals were accommodated separately in metabolic cages. To avoid stress factors, animals were habituated to these cages for 7 days before treatment.

**Induction of pulmonary emphysema**

Male Wistar rats (weight 200±10 g) were anaesthetized with 3% halothane, intubated and artificially ventilated according to Maudely [30]. Rats were hyperventilated, to stop intrinsic breathing for a few seconds, and were instilled during this time span with 0.2 IU pancreatic elastase-g⁻¹ weight in 0.5 ml saline. Control animals were instilled with 0.5 ml saline. After instillation, rats were ventilated until they awoke.

**Histology**

Animals were sacrificed by an overdose of pentobarbital injected i.p. For morphometric studies, lungs were dissected and fixed with 2% glutaraldehyde in 150 mmol·l⁻¹ phosphate buffer (pH 7.2), administered through a polyethylene catheter inserted into the trachea at a pressure of 25 cm H₂O. After 30 min, lungs were fixed for an additional 24 h in 2% glutaraldehyde, dehydrated and embedded in paraffin. Tissue sections (6 μm) were contrasted using the trichrome staining of Goldner [31]. To assess the degree of emphysema, the mean linear intercept was determined using a Mop-videoplan image analyser (Kontron GmbH, Eching, München, Germany). Images of tissue sections were projected on a digitization tablet on which a line raster was applied. Of each lung, a total of 400 intercepts, derived from two sections, were measured.
Quantification of sulphated glycosaminoglycans

The content of sulphated GAGs was determined using the DMMB assay of Farndale and co-workers [34, 35]. To 100 μl sample (urine or lung extract) 2.5 ml of DMMB reagent was added and the absorbance at 525 nm was measured directly. The DMMB reagent consists of 48 μmol/l DMMB (initially 48 μmol DMMB was dissolved in 5 ml 96% ethanol), 42 mmol/l glycine and 42 mmol/l NaCl, adjusted to pH 3.0 with 1 mol/l HCl. Chondroitin 4-sulphate was taken as a standard and included within each series of assays. For calculations we used second order regression analysis.

Quantification of urinary heparan sulphate content

Urinary HS content was determined by a nonequilibrium inhibition enzyme-linked immunosorbent assay (ELISA). To 100 μl sample 100 μl anti-HS (JM403) (dilution 1/40,000 in Tris-buffered saline (pH 7.2) containing 0.1% Tween-20 (TBST) and 1% BSA) was added. After 16 h at 4°C, 100 μl of this mixture was transferred to a HS-coated microtitre plate, and free anti-HS antibodies were allowed to bind to the coated HS for 2 h. The plate was washed with TBST and 100 μl alkaline phosphatase conjugated anti-mouse Ig, at a dilution of 1/2,000 in TBST/BSA was added. After 1 h, the plates were washed with TBST and alkaline phosphatase was detected using 1 mg.ml⁻¹ dinitrophenyl phosphate in 1 mol.l⁻¹ diethanolamine (pH 9.8) containing 50 mmol.l⁻¹ MgCl₂. Absorbance was measured at 405 nm. Bovine kidney HS was taken as a standard within each series of assays.

Characterization of urinary glycosaminoglycans

Purified urinary GAGs were separated on agarose gel using 0.1 mol.l⁻¹ barium acetate (pH 5.0) as electrophoresis buffer. GAGs were visualized using a combined azure A silver-staining [36].

Creatinine assay

Creatinine was measured using the alkaline picrate method, based on the Jaffé reaction (Sigma, procedure No. 555).

Statistical analysis

Intergroup comparison was calculated using Student's t-test. Correlation analysis was performed using Pearson's product moment correlation test [37]. All values are given as mean±SEM. Data were normally distributed.

Fig. 1. – Immunofluorescence of lung tissue stained with anti-heparan sulphate (HS) antibodies 3 h after treatment of rats with elastase: A) detail and C) overview; or saline: B) detail and D) overview. Note the abrupt changes in immunostaining (see arrows). (Internal scale bar = 100 μm).
Fig. 2. — Immunofluorescence double staining of rat lung tissue 1 day after elastase treatment. Panels A, C, E and G show staining for heparan sulphate (HS) and panels B, D, F and H staining for heparan sulphate proteoglycan (HSPG) core-protein, type IV collagen, laminin and fibronectin, respectively. Note that only staining for HS and HSPG is reduced or absent compared to lung of saline-treated rats (see fig. 1 for HS). (Internal scale bar = 100 μm).
Table 1. - Effects of elastase treatment on immunostaining of alveolar basement membrane components

<table>
<thead>
<tr>
<th>Time after elastase treatment</th>
<th>3 h</th>
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<th>2 d</th>
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<th>5 d</th>
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HS: heparan sulphate; HSPG: heparan sulphate proteoglycan; ND: not determined; -: no effect; ↓: weak decrease; ↓↓: moderate decrease; ↓↓↓: strong decrease.

Results

General histology

Three hours after intratracheal instillation of porcine pancreatic elastase, we observed an infiltration of inflammatory cells into the lung accompanied by haemorrhages and pulmonary oedema, indicating acute lung injury. This stage persisted for about 3 days. Lung morphology returned to normal after 5–7 days. Two weeks after elastase treatment the first emphysematous lesions could be observed. Forty days after elastase treatment the mean linear intercept was increased to 81.7±2.3 μm (n=17), compared to 58.6±1.2 μm (n=8) for controls.

Immunofluorescence studies of basement membrane components

In lung parenchyma, HS is linearly distributed in alveoli in accordance with its presence in basement membranes (fig. 1). Table 1 summarizes the effects of elastase on basement membrane components. Three hours after elastase treatment, large areas of the lung lacked HS staining (fig. 1A and C). In some cases, one alveolus was completely devoid of HS, whilst in the adjacent alveolus staining was normal (fig. 1A). At places where HS staining was reduced or absent, staining for the core protein of HSPG was also reduced, although to a lesser extent (fig. 2A and B). After 5 days, most HS staining had returned. At all times, staining for type IV collagen, laminin and fibronectin was normal, even at places where HS staining was absent (fig. 2C–H). Attempts to localize elastase using commercial antibodies were unsuccessful. The elastase used was devoid of HS digesting activity, as demonstrated by incubation of HS with elastase followed by assaying the molecular mass of HS by polyacrylamide gel electrophoresis (data not shown).

Lung and urinary GAG content after elastase instillation

During the first 3 days after elastase treatment, the content of GAGs in lung tissue was significantly decreased.
degradation of many proteins, including proteoglycans, collagen, fibronectin and laminin. Little is known about the in vivo effect of elastase on these molecules. In this study, we have shown that, in particular, proteoglycans are target molecules for instilled elastase in rat lungs. Fibronectin, type IV collagen and laminin were not affected, although small changes may have gone undetected using immunohistochemistry. These results are in accordance with an electron microscopical study on pancreatic elastase-induced emphysema showing no change in laminin or appearance of the basement membrane, but elimination of HS [39, 40]. The observation that in places where HS staining is absent staining for HSPG core protein is only reduced, can be explained by incomplete cleavage of the HSPG core protein. Since all three HS chains are attached to the N-terminus of the protein [41, 42], one cut at this end of the protein will release all HS chains, whereas a large part of the core protein, and thus most of the epitopes for the polyclonal anti-HSPG antibodies, remain present in the basement membrane.

It has been postulated that proteases can induce emphysematous lesions only if they possess elastolytic activity [43]. However, several animal models for emphysema exist where there is no elastin breakdown, including emphysema induced by CdCl$_2$ [44, 45], by 90% oxygen [46], or by collagenase [47], and genetic emphysema in the tight-skin mouse [48]. In addition, leucocyte elastase, implicated in emphysematogenesis in man, has been reported to lack elastolytic activity [49]. There is no consensus on elastin breakdown in human pulmonary emphysema [50–52]. We suggest, as an alternative possibility, that degradation of proteoglycans (e.g. by leucocyte elastase) is associated with the onset of the disease.

Proteoglycans are implicated in a number of functions important for the integrity of the extracellular matrix, which is compromised in emphysema. The GAGs are powerful inhibitors of proteases, including leucocyte elastase [20, 22, 53], and prevent leucocyte elastase-induced emphysema in mouse and rat [23, 24]. Ultrastructurally, proteoglycans are present around collagen fibrils, elastin fibres and in basement membranes [6, 25, 27, 28]. Removal of proteoglycans associated with elastin facilitates the digestion of the latter [54–56]. In addition, proteoglycans are involved in the fibrillogenesis of the major fibrillar components, collagen and elastin, and may influence their mechanical characteristics [3, 4, 57]. Proteoglycans, especially HSPGs, bind and modulate growth factors, including basic fibroblast growth factor (bFGF), interferon-γ, interleukin-8 and transforming growth factor-β [16–20]. In lung parenchyma, degradation of HS by heparitinase results in the release of bFGF [58]. Hence, removal of proteoglycans from the alveolar wall, e.g. by proteolysis, may result in a disturbance of the extracellular matrix, which may ultimately lead to emphysematous lesions. Intratracheal instillation of hyaluronidase (which digests some types of GAG) together with 60% oxygen results in airspace enlargement after 4 days [59].

Discussion

The predominant theory on the pathogenesis of emphysema emanates from a disturbance of the protease/antiprotease balance, favouring the proteases [10–12]. Elastin has been regarded as the target molecule in the extracellular matrix. In pancreatic elastase-induced emphysema, elastin breakdown occurs [38]. Elastase, however, is an indiscriminate enzyme, capable of the in vitro
The disappearance of HSPG in the lung is accompanied by an increase of GAGs in the urine. This increase, observed during the first 4 days after elastase instillation, is positively correlated with the increase of the mean linear intercept, an indicator for parenchymal destruction, 40 days after instillation. Increase of urinary GAGs may, therefore, be an indicator for tissue destruction, is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation. Increase of urinary GAGs is positively correlated with the increase of the content of tissue destruction, 40 days after instillation.

In conclusion, intratracheal instillation of pancreatic elastase in rats results in digestion of proteoglycans but not of other basement membrane components. The concomitant increase in urinary GAGs is positively correlated with the degree of emphysematosus lesions observed after 40 days. The vulnerability of PGs toward proteases may be of importance in the pathogenesis of emphysema.

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