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Induction of Emphysematous Lesions in Rat Lung by β-D-xyloside, an Inhibitor of Proteoglycan Synthesis

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The possible involvement of proteoglycans in the pathogenesis of emphysema was studied in rats by a single intratracheal instillation of p-nitrophenyl-β-D-xylopyranoside (β-D-xyloside), an inhibitor of proteoglycan synthesis. The first 3 days after instillation are characterized by mild hemorrhages, some infiltration of inflammatory cells, and edema. After 1 wk, lung morphology is normal again. Forty days after instillation, considerable parenchymal destruction has occurred as determined by the mean linear intercept (81 ± 12 μm versus 57 ± 5 μm for control [P < 0.001]). Pulmonary fibrosis is not observed. Instillation with p-nitrophenyl-α-D-xylopyranoside and p-nitrophenol do not induce parenchymal destruction, indicating the specificity of β-D-xyloside action. Urinary glycosaminoglycan (GAG) content of the β-D-xyloside-treated rats is increased 15-fold during the first day after instillation, mainly due to elevated levels of chondroitin sulfate and dermatan sulfate. The increase is correlated to the extent of parenchymal destruction after 40 days (r = 0.68; P < 0.002). At day 2 and thereafter, levels are normal again. A short-term increase in dermatan and chondroitin sulfate content is also observed in serum, bronchoalveolar lavage (BAL) fluid, and lung tissue. Heparan sulfate content is decreased in BAL fluid and lung tissue. Instillation with p-nitrophenyl-α-D-xylopyranoside and p-nitrophenol do not induce elevated GAG concentration in urine. We suggest that a disturbance in proteoglycan synthesis accompanied by an increase of (β-D-xyloside-primed) free GAGs results in loss of stability and integrity of the alveolar wall, leading to parenchymal destruction and emphysematous lesions. β-D-xyloside treatment may be an alternative experimental method for inducing emphysema.


The predominant theory on the pathogenesis of emphysema is contained in the protease/antiprotease concept (1–3). A relative surplus of proteases causes degradation of the alveolar elastic fiber matrix, leading to a destruction of the alveolar walls. Elastin degradation is believed to be pivotal in this respect. However, several animal models have been described in which air space enlargement/emphysema is not accompanied by elastin degradation. These include emphysema induced by CdCl₂ (4, 5), by 90% oxygen (6), and by collagenase (7). Thus the causes of alveolar destruction appear to be complex and other macromolecules of the alveolar wall should be considered. A major component in the nonfibrillar part of the extracellular matrix of alveoli is proteoglycans. Proteoglycans consist of a core protein to which one or more strongly negatively charged glycosaminoglycan (GAG) side chains are covalently attached. In the alveolar wall several proteoglycans are present including heparan sulfate proteoglycans (e.g., the basement membrane-associated perlecan and the cell membrane-associated syndecans) and dermatan/chondroitin sulfate proteoglycans (e.g., the collagen fibril-associated decorin, and biglycan) (8).

Several characteristics of proteoglycans make them likely candidates for playing a key role in the maintenance of the structural integrity of the alveolar wall. They interact with and modulate several classes of molecules, including growth factors and extracellular matrix molecules (9–13); act as powerful protease inhibitors, e.g., for leukocyte elastase and cathepsin G (14–16); are involved in fibrillogenesis of collagen and elastin and can modulate their mechanical characteristics (17–19); offer protection against proteolysis of collagen and elastin (20–22); and have a high water-binding capacity, providing resilience to the alveolar wall (23). In ad-
dition, the close association of proteoglycans with structural elements in the alveolar wall (e.g., basement membranes, collagen fibrils, and elastic fibers) indicates a structural role.

Since the structural integrity of alveoli is compromised in emphysema, alterations in the proteoglycan composition should be considered as a factor contributing to the pathogenesis of emphysema. Proteoglycans have been almost entirely neglected in studies on emphysema (24). Previously we showed that proteoglycans are target molecules for pancreatic elastase instilled into rat lung (25). Now, to evaluate the importance of proteoglycans in alveolar wall stability and in the pathogenesis of emphysema, we studied the effect of a single intratracheal instillation of p-nitrophenyl-β-D-xlyopyranoside (β-D-xyloside), which is an inhibitor of proteoglycan synthesis (26). As control agents we applied p-nitrophenyl-α-D-xyloside (α-D-xyloside) and p-nitrophenol. The α-D-xyloside does not change proteoglycan synthesis via the false acceptor pathway, in contrast to the β-D-xyloside, and can be used to discern between the proteoglycan synthesis-disturbing effect of β-D-xyloside and other effects (27).

Materials and Methods

1.9-Dimethylamylene blue (DMMB) (80% pure) was purchased from Aldrich Chem. Co. (Bornem, Belgium); Universal Gel/8% agarose gels from Ciba-Corning GMBH (Fernwald, Germany); halothane from Apharomo (Arnhem, the Netherlands); pentobarbital (Narcovet) from ICI-Farma (Rotterdam, the Netherlands); diethyleniminethyl (DEAE)-Sepharose Fast Flow from Pharmacia (Uppsala, Sweden); p-nitrophenol, aquamount, and cetyl-trimethyl-ammoniumbromide from BDH Ltd. (Poole, UK); and Azure A, Tween-20, whale cartilage chondroitin 4-sulfate, bovine kidney heparan sulfate, porcine pancreatic elastase, bovine serum bromide from BDH Ltd. (Rotterdam, the Netherlands); diethylaminoethyl (DEAE)-Sepharose Fast Flow from Pharmacia (Uppsala, Sweden); p-nitrophenol, aquamount, and cetyl-trimethyl-ammoniumbromide from BDH Ltd. (Poole, UK); and Azure A, Tween-20, whale cartilage chondroitin 4-sulfate, bovine kidney heparan sulfate, porcine pancreatic elastase, bovine serum albumin (BSA), β-D-xlyopyranoside, α-D-xlyopyranoside, N-methylsuccinyl-ala-alapro-val7-amino-4-methyl-coumarin, fluoresceiniothiocyanate-conjugated goat antimouse IgM, and mouse antichondroitin sulfate (clone CS-56) from Sigma Inc. (St. Louis, MO). Mouse monoclonal antiheparan sulfate (JM403) antibody was obtained and characterized as described (28).

[35S]GAGs (specific activity 6 × 106 Bq/µg GAG) were obtained from rats injected twice i.p. with 37,000 Bq Na35SO4/g body weight with a 4-h time span between injections. Four hours after the second injection the rats were killed and the soft tissues (i.e., liver, kidney, intestine, etc.) removed. GAGs were extracted by alkaline borohydride (29).

Animal Accommodation

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

Intratracheal Instillation

Male Wistar rats (200 ± 10 g) were anesthetized with 3% halothane, intubated, and artificially respiration according to Mauderly (30). Rats were hyperventilated to stop breathing for a few seconds, and were instilled during this time span with either 0.5 ml 200 mM β-D-xyloside (100 µmol) in 10 vol % dimethyl sulfoxide (DMSO) in 0.14 M NaCl; 0.5 ml 40 mM β-D-xyloside (20 µmol) in 0.14 M NaCl; 0.5 ml 50 mM α-D-xyloside (25 µmol) in 10 vol % DMSO in 0.14 M NaCl; 0.5 ml 100 mM p-nitrophenol (50 µmol) in 10 vol % DMSO in 0.14% NaCl; 0.5 ml porcine pancreatic elastase (0.2 IU/g body weight) in 0.14 M NaCl; 0.5 ml 10% DMSO in 0.14 M NaCl; or 0.5 ml 0.14 M NaCl. Maximal doses of α-D-xyloside and p-nitrophenol were applied (25 and 50 µmol, respectively); higher doses were lethal. All solutions were administered at 37°C. After instillation, rats were resired until they awoke.

Histology

Animals were killed by an overdose of pentobarbital injected i.p. For morphometric studies, lungs were dissected and fixed with 2% formaldehyde in 150 mM 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 4% formaldehyde, dehydrated, and embedded in paraffin. Tissue sections (6 µm) were contrasted using the trichrome staining method of Goldner (31). To assess the degree of air-space enlargement, the mean linear intercept (MLI) was determined using a Mop-vidoeran image analyser (Kontron GMBH, Eching, München, Germany).

For immunofluorescence and biochemical studies, lungs were dissected and inflated through the trachea with 5 ml phosphate-buffered saline (PBS) (pH 7.2). PBS-filled lungs were frozen in liquid nitrogen and stored at −70°C. Cryosections (6 µm) 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 times for 5 min). Antiheparan sulfate antibodies and all secondary antibodies were used at a dilution of 1:100. All other antibodies were used at 1:50. Sections were embedded in aquamount and examined on a Zeiss axioskop photomicroscope (Carl Zeiss, Oberkochen, Germany).

Isolation of GAGs from Urine and BAL Fluid

Bronchoalveolar lavage (BAL) fluid was obtained from the left lung by washing the dissected lung once with 4 ml and 4 times with 2 ml PBS (4°C). Cells were removed from BAL fluid by centrifugation (1,000 × g, 10 min, 4°C). The total yield of BAL fluid was 10 ml.

Rat urines were collected 1 day before to 7 days after instillation (each day). Urine was centrifuged at 2,000 × g for 10 min (4°C), and 20 ml of the supernatant was diluted to 50 ml by adding 10 mM Tris-HCl (pH 6.8).

GAGs were purified from urine and BAL fluid by anion exchange chromatography as described (25). Urine samples (50 ml) or BAL fluids (7 ml) were loaded onto a column of 5 × 0.5 cm containing 0.5 ml DEAE-Sepharose Fast Flow. After an initial wash with 3 ml 0.2 M NaCl/10 mM Tris-HCl (pH 6.8), GAGs were eluted with 1 ml 2 M NaCl/10 mM Tris-HCl (pH 6.8) followed by 3 ml 10 mM/Tris-HCl (pH 6.8). Recovery of urinary GAGs was monitored by adding 9 × 106 Bq [35S]GAG to the urine sample and was between 72 and 101%.

Isolation of GAGs from Serum

To 100 µl serum, 8 µl trichloroacetic acid solution (1 g/ml)
was added. After centrifugation for 20 min at 10,000 × g, the supernatant was removed and stored on ice. The protein-bound GAGs were liberated from the pellet by β-elimination as follows. The pellet was suspended in 100 µl 0.75 M NaOH/50 mM NaBH₄ and incubated for 1 h at 73°C. After 1 h the mixture was cooled on ice and neutralized with 6 M HCl. The remaining proteins were removed by addition of 9 µl trichloroacetic acid solution (1 g/ml) and centrifugation (20 min, 10,000 × g). The supernatants of both precipitations were pooled and 5 vol of 100% ethanol were added. After 16 h at −20°C the mixture was centrifuged for 30 min at 15,000 × g (0°C), and the precipitated GAGs were dried, dissolved in 50 µl 50 mM barium acetate (pH 5.0)/40% glycerol/0.01% bromophenol blue, and subjected to agarose gel electrophoresis (see below).

Isolation of GAGs from Parenchymal Lung Tissue

GAGs were extracted according to a modified method of Hoffman (29). Pleura, large airways and blood vessels were removed from 30-µm-thick cryosections. The remaining tissue was lyophilized, weighed, and suspended in 200 µl 0.75 M NaOH/10 mM NaBH₄. After 1 h at 73°C, the mixture was neutralized with 6 M HCl and trichloroacetic acid was added to a final concentration of 60 mg/ml. After 1 h at 4°C and centrifugation for 15 min at 2,000 × g (4°C), the pellet was dissolved in 100 µl 100 mM NaOH and the protein concentration determined according to Lowry and colleagues (32). To the GAGs-containing supernatant, 5 vol of 100% ethanol were 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 demineralized water.

Quantification of GAGs by Gel Electrophoresis

GAG samples from urine, BAL fluid, serum, and lung tissue were separated on agarose gel using 0.05 M barium acetate (pH 5.0) as electrophoresis buffer. GAGs were visualized using a combined azure A/silver staining as described, and quantified by densitometric analysis (33). The nature of the GAGs was established by specific degradation procedures: nitrous acid for heparan sulfate, chondroitinase ABC digestion for chondroitin sulfate and dermatan sulfate, and chondroitinase AC digestion for chondroitin sulfate (33).

Quantification of Sulfated GAGs by a Spectrophotometric Assay

The content of sulfated GAGs was determined using the DMMB assay of Farndale and associates (34). To a 100-µl GAG sample, 2.5 ml of DMMB-reagent was added and the absorbance at 525 nm was measured directly. The DMMB-reagent consists of 48 µM DMMB (initially 48 µmol DMMB was dissolved in 5 ml 96% ethanol), 42 mM glycine, and 42 mM NaCl, adjusted to pH 3.0 with 1 M HC1. Chondroitin 4-sulfate was taken as a standard and included within each series of assays.

Determination of Elastase in BAL Fluid

Elastase activity was determined using the fluorogenic substrate N-methylsuccinyl-ala-ala-pro-val-7-amino-4-methylcoumarin according to Castillo and colleagues (35), with some modifications. Briefly, to 400 µl BAL fluid, 100 µl 4 M NaCl/500 mM Tris (pH 8.5)/0.4% cetyl-trimethyl-ammonium-bromide was added. After 5 min preincubation at 37°C, 5 µl substrate solution (10 mM N-methylsuccinyl-ala-ala-pro-val-7-amino-4-methyl-coumarin dissolved in DMSO) was added. Fluorescence was monitored on a Shimadzu RF-5000 spectrofluorometer at excitation and emission wavelengths of 375 and 440 nm, respectively. Human leukocyte elastase, a generous gift of Dr. J. Schalkwijk (Department of Dermatology, University of Nijmegen), was taken as a standard.

Statistical Analysis

Intergroup comparison was calculated using Student’s t test. Correlation analysis was performed using Pearson’s product moment correlation test (36). All values are given as mean ± SD.

Results

General Morphology

Three hours after intratracheal instillation of 100 µmol β-D-xyloside, mild hemorrhages and some infiltration of inflammatory cells are observed in the lung. These phenomena subside 3 days after instillation and lung morphology returns to normal after 1 wk. The extent of hemorrhages and infiltration of inflammatory cells is much less pronounced compared to elastase-treated animals (Figure 1). Animals instilled with α-D-xyloside, p-nitrophenol, 10% DMSO, or physiologic salt display less hemorrhages and inflammation than β-D-xyloside-treated rats. Edema is observed in bronchioli, blood vessels, and alveolar walls until day 5 after β-D-xyloside instillation, whereas other animals display only some edema in bronchioli and large blood vessels during the first 2 days. Forty days after treatment, parenchymal destruction is observed in β-D-xyloside-treated animals (Figure 2), as determined by an increase of the MLI to 81 ± 12 µm (n = 11) (Figure 3). The MLI is the average distance between two alveolar septa and is a parameter of air-space enlargement. Occasionally very severe parenchymal destruction is present (Figure 2c). Fibrosis is not observed. The extent of β-D-xyloside-induced parenchymal destruction is comparable to that induced by pancreatic elastase (MLI: 82 ± 11 µm [n = 12] versus 59 ± 4 µm [n = 8] for control, Figure 3). With a lower dose of β-D-xyloside (20 µmol), only about 30% of the treated animals developed emphysematous lesions (MLI > 70 µm) (Figure 3). α-D-xyloside and p-nitrophenol instillation do not induce an increase in MLI (56 ± 4 µm [n = 4], 54 ± 3 µm [n = 4], respectively).

Immunofluorescence Studies

Chondroitin sulfate staining is only occasionally present in normal rat lung (Figure 4a). Three h and 1 day after β-D-xyloside treatment, however, a diffuse chondroitin sulfate staining is observed in the alveolar wall (Figure 4b), which changes to a more distinct staining pattern 3 to 7 days after treatment (Figure 4c). Phase contrast microscopy indicates a colocalization of chondroitin sulfate staining with fiber-like structures, presumably elastic fibers (Figures 4e and 4d). Colocalization of chondroitin sulfate staining with these structures can also be observed in alveoli 2 to 14 days after pancreatic elastase treatment (data not shown).
Figure 1. Lung parenchyma 1 day after intratracheal instillation of: (a) 100 µmol β-D-xyloside in 10% DMSO; (b) 10% DMSO (control); (c) 40 IU pancreatic elastase in physiologic salt; (d) physiologic salt (control). In comparison with elastase instillation, β-D-xyloside-treated lungs show little hemorrhages and infiltration of inflammatory cells. Some perivascular edema (large arrows) and alveolar transudate (small arrows) can be observed. Bar: 100 µm.

Heparan sulfate staining was found linearly distributed in the alveolar wall, in accordance with its presence in basement membranes. No obvious alterations in heparan sulfate staining could be detected in β-D-xyloside-treated rats (Figure 5). The distance between the two opposite alveolar basement membranes in the alveolar wall, visualized by heparan sulfate staining, is increased (Figure 5). This indicates the presence of edema in the alveolar wall (Figure 5).

GAGs in Urine, Serum, BAL Fluid, and Lung
Since β-D-xyloside acts as an artificial initiator of GAG synthesis and could cause an increase in the synthesis of free GAGs (26), we examined the amount and composition of GAGs in BAL fluid, serum, urine, and lung tissue.

In the urine of rats instilled with 100 µmol β-xyloside, a 15-fold increase in GAG content is observed at day 1, whereas in rats instilled with 20 µmol a 13-fold increase is
noted (Table 1). \(\alpha\)-D-xyloside and \(p\)-nitrophenol-treated rats do not show an increase of GAG excretion (Table 1). At day 2 and thereafter, urinary GAG levels become normal again in \(\beta\)-D-xyloside-treated rats. The increase in urinary GAG can be allotted mainly to an increase in dermatan sulfate and chondroitin sulfate, as was determined by agarose gel electrophoresis (Figure 6). A significant correlation is found between the increase of urinary GAG content the first day after treatment, and the extent of air-space enlargement (MLI) developed after 40 days (Figure 7).

In serum, dermatan sulfate and chondroitin sulfate are increased during the first two days after treatment (Table 2). Heparan sulfate, which constitutes only 0.3% of serum GAGs (32), was not analyzed.

BAL fluid shows the most complex alterations in GAG content and composition (Table 3). The major GAG in BAL fluid from control animals is heparan sulfate, whereas chondroitin sulfate and dermatan sulfate are minor components. One hour after \(\beta\)-D-xyloside treatment, dermatan sulfate and chondroitin sulfate contents are dramatically increased, which is even more evident after 3 h. One day after treatment chondroitin sulfate migrates as a narrow band, indicating less heterogeneity in charge and/or size, while dermatan sulfate is separated into two distinct bands. Heparan sulfate content is significantly decreased at day 1 and is further reduced 2 days after treatment. Chondroitin sulfate has returned to control values at day 2. The nature of the GAGs, which was determined by agarose gel electrophoresis, was confirmed by enzymatic digestion (data not shown).

Analysis on cryosections of parenchymal lung tissue showed that dermatan sulfate and chondroitin sulfate are increased at 3 h and 1 day, and at 1 day after treatment, respectively, while the heparan sulfate content is significantly de-
Figure 4. Chondroitin sulfate immunostaining of rat lung after instillation of 100 µmol β-D-xyloside in 10% DMSO. (a) 3 h after instillation of 10% DMSO (control); (b) 3 h, and (c) 7 days after instillation of β-D-xyloside; (d) phase contrast micrograph of the same section as (c). Note the increase in chondroitin sulfate staining after β-D-xyloside treatment. At day 7, staining is focal and associated with elastin fibers (arrows in [c] and [d]). Bar: 20 µm.

Figure 5. Heparan sulfate immunostaining of rat lung 3 days after instillation of: (a) 10% DMSO; (b) 100 µmol β-D-xyloside in 10% DMSO. Heparan sulfate is linearly distributed in alveoli in accordance with its presence in basement membranes. In β-D-xyloside-treated rats the distance between the two (heparan sulfate-stained) basement membranes is increased, indicating edema (see arrow). Bar: 100 µm.
TABLE 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Control (10% DMSO)</th>
<th>β-D-xyloside (100 μmol)</th>
<th>β-D-xyloside (20 μmol)</th>
<th>α-D-xyloside (25 μmol)</th>
<th>p-nitrophenol (50 μmol)</th>
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<tr>
<td>0</td>
<td>36.4 ± 2.0 (5)</td>
<td>37.1 ± 6.1 (10)</td>
<td>40.7 ± 4.5 (11)</td>
<td>36.5 ± 8.0 (9)</td>
<td>32.8 ± 6.1 (7)</td>
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<td>1</td>
<td>38.9 ± 7.3 (5)</td>
<td>575.5 ± 175.2* (10)</td>
<td>490.7 ± 125.9* (11)</td>
<td>41.2 ± 8.9 (7)</td>
<td>40.6 ± 14.8 (6)</td>
</tr>
<tr>
<td>2</td>
<td>42.9 ± 2.4 (5)</td>
<td>45.9 ± 6.9 (8)</td>
<td>32.4 ± 9.3 (11)</td>
<td>45.7 ± 10.4 (4)</td>
<td>36.5 ± 15.6 (4)</td>
</tr>
<tr>
<td>3</td>
<td>38.3 ± 6.1 (5)</td>
<td>38.9 ± 7.4 (9)</td>
<td>33.5 ± 5.9 (11)</td>
<td>39.8 ± 17.7 (4)</td>
<td>28.6 ± 6.0 (4)</td>
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<tr>
<td>4</td>
<td>53.9 ± 8.1 (5)</td>
<td>57.5 ± 20.7 (9)</td>
<td>25.9 ± 6.4 (11)</td>
<td>34.1 ± 7.2 (4)</td>
<td>36.1 ± 12.3 (4)</td>
</tr>
<tr>
<td>5</td>
<td>36.2 ± 5.0 (5)</td>
<td>43.5 ± 8.1 (9)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>39.6 ± 4.0 (5)</td>
<td>41.5 ± 6.0 (9)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>40.9 ± 6.4 (5)</td>
<td>42.9 ± 5.9 (9)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Values are expressed as μg GAG/mg creatinine. The number of rats is indicated within parentheses. n.d.: not determined.
* P < 0.001 compared to all other values.

Elastase Activity in BAL Fluid

In β-D-xyloside-treated rats, BAL fluid shows a modest increase in elastase activity one day after treatment (3.2 ± 0.6 [n = 3] versus 2.1 ± 0.4 ng/ml BAL fluid for control [10% DMSO, n = 6]). Levels are normal the second day.

Discussion

In this study we show that intratracheal instillation of 100 μmol β-D-xyloside in rats induces the development of emphysematous lesions. After treatment with 20 μmol β-D-xyloside only about 30% of the animals developed emphysematous lesions, indicating a dose-dependent effect of the drug. α-D-xyloside and the aglycon p-nitrophenol do not induce emphysematous lesions, indicating that the effect is specific for β-D-xyloside. β-D-xyloside mimics the serine-xyloside GAG initiation site of the core protein of proteoglycans, and competes as a false acceptor for GAG synthesis (26). Administration of β-D-xyloside, but not α-D-xyloside or p-nitrophenol, increases GAG synthesis, which is particularly evident from the 15-fold increase of GAG content in the urine of treated rats. The increase comes mainly to the account of chondroitin and dermatan sulfates, which is typical for the β-D-xyloside preparation we used (p-nitrophenyl-β-D-xylopyranoside) (37). The strong increase of urinary GAG content cannot be attributed solely to an increase in the synthesis of GAGs in the lung. Other organs, which possibly receive a dose of β-D-xyloside indirectly through the blood, may also contribute. This especially applies to the kidney, since the GAG content in serum showed only a 2-fold increase compared to the 15-fold increase in urine. In an organ perfusion system, 2.5 mM β-D-xyloside stimulated the synthesis of chondroitin sulfate and dermatan sulfate in glomeruli (38). The amount of increase in urinary GAG content 1 day after treatment is positively correlated to the extent of paren-

Figure 6. Agarose gel electrophoresis of GAGs isolated from urine after instillation of 100 μmol β-D-xyloside. The nature of the different GAGs was established using specific enzymes (32). The amount of GAGs applied corresponds to 1 μg creatinine, except for lane D which corresponds to 0.08 μg creatinine. (A) day 0 (before instillation); (B) and (D) 1 day after instillation; (C) 2 days after instillation. M: reference GAGs (HS: heparan sulfate [6 ng]; DS: dermatan sulfate [6 ng]; CS: chondroitin 4-sulfate [6 ng]).

Figure 7. The relation between the mean linear intercept 40 days after instillation of various agents, and the content of urinary GAGs 1 day after instillation. Rats were instilled with 0.5 ml containing ■: 100 μmol β-D-xyloside; ●: 25 μmol α-D-xyloside; ○: 50 μmol p-nitrophenol. Controls received ●: 10% DMSO; O: no treatment. There is a significant correlation between the increase of urinary GAG content and the MLI. (r = 0.68, P < 0.002.)

chymal destruction observed at day 40. This indicates that the degree of disturbance in the lung during the initial phase of the insult determines the degree of parenchymal destruction in a later phase.

Although the mechanism of the parenchymal destruction due to β-D-xyloside treatment is not clear, it is likely mediated by the increase in synthesis of free GAGs and the concomitant decrease in proteoglycan synthesis. In BAL fluid and parenchymal lung tissue we found an increase in total GAG content due to an increase in dermatan and chondroitin sulfates, but heparan sulfate was decreased. We did not observe a clear decrease in heparan sulfate proteoglycans of the alveolar basement membrane using immunofluorescence. Others, however, found a 25% decrease in basement membrane proteoglycans (predominantly heparan sulfate proteoglycans) with ruthenium red staining in mouse submandibular salivary glands (39). A 43% decrease in heparan sulfate synthesis, together with a 4-fold increase in chondroitin sulfate synthesis was observed after β-D-xyloside treatment of the basement membrane producing EHS (Engelbreth-Holm-Swarm) tumor in vitro (40). Basement membrane heparan sulfate proteoglycan is believed to function as a charge barrier for proteins. A probable loss of heparan sulfate proteoglycans together with an increase in total GAGs, which possess high water-binding properties, may account for the observed edema in the treated rat lungs.

The observed increase in dermatan and chondroitin sulfates content may destabilize collagen fibrils and reduce their tensile strength. β-D-xyloside treatment of developing avian corneal stroma, which leads to a reduced synthesis of dermatan sulfate proteoglycans but an increase in free dermatan sulfate, resulted in a reduced collagen fibril packing and a disruption of lamellar organization (41). The situation may be somewhat analogous to cervical dilatation at the end of pregnancy, a process which is accompanied by a dramatic increase in collagen-associated dermatan sulfate (42, 43). An increase of chondroitin sulfate content can impair elastic fiber assembly by inhibition of the binding of the 67 kD elastin-binding protein to elastic fibers and the cell membrane (44). GAGs may further disrupt repair of the alveolar matrix since they are potent inhibitors of lysyl oxidase (45), an enzyme crucial for the cross-linking of collagen and elastin. Ex vivo treatment of mouse embryos with β-D-xyloside inhibited branching morphogenesis in the lung (46). Availability of growth factors may also be affected by the increase in GAGs; β-D-xyloside-treated GAGs are able to bind growth factors, as do GAGs in proteoglycans (47). All these effects will compromise the delicate balance of alveolar wall homeostasis and prevent an orderly repair, finally resulting in parenchymal destruction and emphysematous lesions.

In β-D-xyloside-treated rats only mild hemorrhages and inflammation were observed in the lung, in contrast to pancreatic elastase-treated animals (Figure 1) (25). This could be an effect of DMSO which was used as a solvent for β-D-xyloside. DMSO acts as an anti-inflammatory agent and is a scavenger of free radicals (48). The increase in free elastase activity measured in the BAL fluid is only moderate. Apart from the mild infiltration of inflammatory cells, this may also be an effect of the increase in GAGs which are potent inhibitors of neutrophil elastase (14, 15). Hemorrhages and inflammation may not play an important role in alveolar tissue degradation in β-D-xyloside-treated rats. The total free elastase activity in BAL fluid is 64 ng/rat, which stands small compared to the amount of neutrophil elastase necessary to induce pulmonary emphysema by intratracheal instillation (about 300 μg neutrophil elastase [49]). However, some damage to the alveolar wall due to inflammation and neutrophil elastase cannot be excluded.

The importance of proteoglycans in the stability of the lung parenchyma has recently been demonstrated. Intratracheal instillation of testicular hyaluronidase (which degrades hyaluronate and dermatan sulfate and chondroitin sulfate) followed by exposure to a nontoxic concentration of oxygen (60%) results in a significant air-space enlargement in hamster lungs (50). Hyaluronidase instillation also aug-

**TABLE 2**

<table>
<thead>
<tr>
<th>Time after Instillation</th>
<th>CS (μg/ml)</th>
<th>DS (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1 h)</td>
<td>0.24 ± 0.06</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>1 h</td>
<td>0.19 ± 0.02</td>
<td>0.68 ± 0.05*</td>
</tr>
<tr>
<td>3 h</td>
<td>0.14 ± 0.06</td>
<td>1.14 ± 0.21*</td>
</tr>
<tr>
<td>1 day</td>
<td>0.09 ± 0.02*</td>
<td>0.29 ± 0.06*</td>
</tr>
<tr>
<td>2 days</td>
<td>0.02 ± 0.01*</td>
<td>0.08 ± 0.04</td>
</tr>
</tbody>
</table>

Values are expressed as μg/ml BAL fluid and are mean ± SD of four animals.

* P < 0.01 versus control.

**TABLE 3**

<table>
<thead>
<tr>
<th>Time after Instillation</th>
<th>HS (μg/ml)</th>
<th>CS (μg/ml)</th>
<th>DS (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1 h)</td>
<td>3.54 ± 0.36</td>
<td>2.21 ± 0.19</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>1 h</td>
<td>5.55 ± 0.98*</td>
<td>5.48 ± 0.56*</td>
<td>0.71 ± 0.12*</td>
</tr>
<tr>
<td>3 h</td>
<td>6.87 ± 0.32*</td>
<td>5.09 ± 0.43*</td>
<td>0.80 ± 0.20*</td>
</tr>
<tr>
<td>1 day</td>
<td>8.33 ± 1.04*</td>
<td>5.12 ± 0.51*</td>
<td>0.94 ± 0.13*</td>
</tr>
<tr>
<td>3 days</td>
<td>4.38 ± 0.34</td>
<td>3.84 ± 0.41</td>
<td>0.12 ± 0.04*</td>
</tr>
<tr>
<td>7 days</td>
<td>3.17 ± 0.12</td>
<td>2.88 ± 0.44</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

Values are expressed as μg/ml serum and are mean ± SD of four animals.

* P < 0.05 versus control.
ments pancreatic elastase-induced parenchymal destruction (51). We showed an increase of urinary GAGs and a decrease of lung GAGs and heparan sulfate proteoglycans shortly after pancreatic elastase instillation into rat lungs (25). The urinary GAG content correlated also to the extent of emphysema developed after 40 days.

In conclusion, a single intratracheal instillation of a high dose of β-D-xyloside results in the development of emphysematous lesions. This effect is likely mediated by the short-term increase of free GAG chains (and possibly the decrease of proteoglycan content). It underscores the importance of proteoglycans in alveolar wall integrity and indicates their involvement in the pathogenesis of emphysema. β-D-xyloside treatment may be an alternative experimental way to induce emphysema. The detrimental effect of β-D-xyloside on the lungs should be taken into consideration when evaluating β-D-xyloside preparations as antithrombotic drugs (52).

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References

Table 4

<table>
<thead>
<tr>
<th>Time after Instillation</th>
<th>HS</th>
<th>CS</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1 h)</td>
<td>2.05 ± 0.66 (11)</td>
<td>0.67 ± 0.24 (13)</td>
<td>2.51 ± 0.56 (13)</td>
</tr>
<tr>
<td>1 h</td>
<td>2.91 (1)</td>
<td>0.74 (1)</td>
<td>3.46 (1)</td>
</tr>
<tr>
<td>3 h</td>
<td>1.63 ± 0.43 (3)</td>
<td>0.84 ± 0.21 (4)</td>
<td>3.44 ± 0.41 (4)</td>
</tr>
<tr>
<td>1 day</td>
<td>1.72 ± 0.22 (4)</td>
<td>2.06 ± 0.60 (4)</td>
<td>4.50 ± 0.48 (4)</td>
</tr>
<tr>
<td>2 days</td>
<td>1.13 ± 0.10 (3)</td>
<td>0.50 ± 0.08 (3)</td>
<td>3.16 ± 0.98 (3)</td>
</tr>
<tr>
<td>7 days</td>
<td>2.00 ± 0.63 (3)</td>
<td>0.58 ± 0.10 (3)</td>
<td>2.44 ± 0.35 (3)</td>
</tr>
</tbody>
</table>

Values are expressed as μg GAG/mg protein and are mean ± SD of the number of analyzed animals (shown in parentheses).

* P < 0.05.
† P < 0.01 versus control.

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