Proteoglycan involvement during development of lesional pulmonary edema

DANIELA NEGRINI,1 ALBERTO PASSI,2 GIANCARLO DE LUCA,2 AND GIUSEPPE MISEROCCHI1
1Istituto di Fisiologia Umana, Facoltà di Medicina e Chirurgia, Università degli Studi, 20133 Milan; and 2Dipartimento di Biochimica “A. Castellani,” Facoltà di Medicina e Chirurgia II, Università degli Studi, 27100 Pavia, Italy

Negrini, Daniela, Alberto Passi, Giancarlo De Luca, and Giuseppe Miserocchi. Proteoglycan involvement during development of lesional pulmonary edema. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L203–L211, 1998.—We evaluated the effect of pancreatic elastase (7 IU iv) on pulmonary interstitial pressure (P\text{Ip}) in situ rabbit lungs by a micropuncture technique through the intact parietal pleura. P\text{Ip} was \(-10.8 \pm 2.2\) (SD) cmH\(\text{2O}\) in the control condition, increased to \(+5.1 \pm 1.7\) cmH\(\text{2O}\) at \(~\sim\)60 min (condition referred to as mild edema [ME]), and subsequently decreased to \(-0.15 \pm 0.8\) cmH\(\text{2O}\), remaining steady from 80 up to 200 min with a marked increase in lung wet-to-dry weight ratio [condition referred to as severe edema (SE)], suggesting an increase in tissue compliance. We functionally correlated the measured P\text{Ip} to structural modifications of proteoglycans, the major interfibrillar component of the extracellular matrix (ECM). The strength of the noncovalent bonds linking proteoglycans to other ECM components decreased with increasing severity of edema, as indicated by the increased extractability of proteoglycans with guanidine hydrochloride. Total proteoglycan recovery (expressed as µg hexuronate/g dry tissue) increased from \(436.8 \pm 14\) in the control condition to \(495.3 \pm 23\) and \(547.0 \pm 10\) in ME and SE, respectively. Gel-filtration chromatography showed in ME a fragmentation of heparan sulfate proteoglycans, suggesting that elastase treatment first affected basement membrane integrity, whereas large chondroitin sulfate proteoglycans were degraded only in SE. Elastase caused a fragmentation only of the core protein of proteoglycans, the binding properties of which to collagens, fibronectin, and hyaluronic acid were markedly decreased, as indicated by a solid-phase binding assay. The sequential degradation of heparan sulfate and chondroitin sulfate proteoglycans may account for the initial increase in microvascular permeability, followed by a loss of the native architecture of the ECM, which may be responsible for the increase in tissue compliance.

heparan sulfate proteoglycans; chondroitin sulfate proteoglycans; pulmonary interstitial pressure; elastase

PULMONARY FUNCTION RELIES on the maintenance of a “dry” interstitial tissue as indicated by the fact that the interstitial pressure (P\text{Ip}) is much lower in the lung parenchyma (approximately \(-10\) cmH\(\text{2O}\); see Refs. 15, 16) compared with other tissues in control conditions. To minimize interstitial fluid volume, the lung requires 1) a selective endothelial barrier limiting capillary to interstitium fluid filtration by restricting plasma protein escape (31), 2) an efficient interstitial fluid drainage provided by pulmonary lymphatics, and 3) a very low tissue matrix compliance that depends mainly on the macromolecular organization of extracellular ma-

trix (ECM). These functions add to provide a large “tissue safety factor” that limits edema formation.

The ECM of lung alveoli consists of a meshwork of fibrous proteins (mainly collagen and elastin) that provides a mechanical scaffolding for proteoglycans, which are the major component of the nonfibrillar compartment of the interstitium. Proteoglycans include various families of multidomain core proteins, which are genetically unrelated and contain one or more covalently linked glycosaminoglycan (GAG) chains (8, 27). Proteoglycans are not only involved in the determination and maintenance of tissue mechanical properties but also participate in a number of dynamic biological processes that include regulation of cell adhesion, cell migration, and cell proliferation as well as modulation of the biological activities of matrix-bound growth factors and cytokines (8, 27). Moreover, proteoglycans may also act as protease inhibitors (12, 25, 37).

Different proteoglycan populations are present in lung alveoli. The large chondroitin sulfate (CS)-containing proteoglycan (versican) may form aggregates with hyaluronic acid in the interstitial matrix, whereas small dermatan sulfate (DS)-containing proteoglycans (mainly decorin) are associated with collagen fibrils (27). Heparan sulfate (HS)-containing proteoglycans include perlec of the epithelial basement membrane and syndecans of the cell surface (4, 27, 41). These molecules are organized in the ECM through noncovalent interactions, which are affected in various forms of lung injury such as saline-induced edema, where a partial breakdown of proteoglycans occurs (21).

The ECM is a dynamic structure, and proteoglycans turn over rapidly (3). Therefore, a balance must exist between the synthetic and degradative pathways to maintain a normal content of these molecules that are responsible for the structural integrity of the alveolar wall.

It is well known that elastase treatment induces lung injury in many animal models (5, 12, 22, 25, 33), causing emphysema. Elastase is an omnivorous proteolytic enzyme at neutral pH, with broad affinity for a variety of soluble and insoluble protein substrates, including all structural components of the lung ECM, as well as for proteoglycans (34).

In the present study, we evaluated the effect of an intravenous injection of elastase in rabbits on the mechanical properties of the lung interstitium and on the molecular structure of the ECM by focusing on proteoglycans because compliance of the lung ECM is related to proteoglycan structure.
METHODS

Adult New Zealand rabbits \( [n = 26; \text{body weight} = 2.23 \pm 0.17 (SD) \text{ kg}, \text{range} 2-2.5 \text{ kg}] \) were anesthetized with a cocktail of 2.5 ml/kg of 25% (wt/vol) urethan and 1.5 ml of pentobarbital sodium (60 mg/kg) injected into an ear vein. Additional anesthesia was given throughout the experiment as judged by arousal of ocular reflexes. The animals were tracheotomized, and the trachea was cannulated with an infant tube to allow spontaneous breathing. The carotid artery was cannulated via a saline-filled catheter, and the line was connected to a pressure transducer (model 4-3271, TransAmerica Delaval, Pasadena, CA) for continuous monitoring of systemic arterial pressure \((P_{\text{pa}})\), left atrial pressure \((P_{\text{la}})\), and cardiac output \((Q)\) measured in the control condition and up to ~200 min after intravenous infusion of 200 \( \mu \text{g} \) (7 IU) of porcine pancreatic elastase (Sigma Chemical, St. Louis, MO). \( P_{\text{pa}} \) and \( P_{\text{la}} \) were measured with a surgical procedure previously developed in our laboratory (18, 20) that allows for continuous monitoring of pulmonary vascular parameters in spontaneously breathing rabbits. The surgical procedure consisted of removing the skin and the external intercostal muscle insertions on the medial portion of the sternum. The latter was then widely opened by a midsternal incision running from the manubrium to the xiphoid process; after the soft mediastinal connective tissue and the thymus were moved, the pericardium was exposed and cut along a medial longitudinal line. A saline-filled catheter inserted into the right ventricular wall was driven into the pulmonary artery, and another catheter was placed in the left atrium. Both catheters were linked to pressure transducers placed at the same level as their tips. \( P_{\text{pa}} \) was measured via an ultrasonic flow probe (model T201, Transonic Systems, Ithaca, NY) secured around either the ascending aorta or the pulmonary artery. This approach, which is possible in rabbits because the pleural spaces are ventrally separated, allowed us to preserve the integrity of the pleural sacs.

Blood samples (~0.5 ml each) were withdrawn in the control condition and after elastase administration; after separation by centrifugation, the total plasma protein concentration was determined through an optical refractometer (model SPR-N, Atago). Measurements of \( P_{\text{pi}} \) and intercostal interstitial pressure \( P_{\text{ip}} \) and intercostal interstitial pressure were measured in eight animals in which the variables listed in General and hemodynamic variables were not measured to avoid a difficult surgical procedure. The animal breathed spontaneously through the tracheotomy while in the supine position, and the superficial thoracic tissues were removed on one side of the chest. A “pleural window” (surface area 1 cm\(^2\)) was prepared at approximately the same level as the heart by removing the internal intercostal muscles down to the endothoracic fascia. In three animals, two adjacent pleural windows were prepared in the sixth (midaxillary line) and seventh (dorsal) intercostal spaces at an ~2-cm distance from each other. The endothoracic fascia was carefully stripped with iridectomy forceps under stereomicroscopic view (SMZ-2T, Nikon) to expose ~0.2 cm\(^2\) of the parietal pleura. This preparation allowed us to obtain a clear view of the pulmonary structures (microvasculature, alveoli, and septa) through the intact parietal pleura when the lungs were expanded at a physiologically negative intrapleural pressure and zero alveolar pressure so that the integrity of the lung-chest wall coupling and of the entire pulmonary circulation were preserved.

To proceed with the micropunctures, respiratory movements were removed by paralyzing the animal with intravenous pancuronium bromide (0.2 ml/kg body weight). During micropuncture, the animals were oxygenated with humidified 50% \( \text{O}_2 \) delivered via an intratracheal catheter at an outflow pressure of ~1 cmH\(_2\)O. Between two consecutive micropunctures, the inspiratory line was connected to a mechanical ventilator (model 6025, Biological Research Apparatus, Basile, Comerio, Italy) that delivered room air at a tidal volume of 20 ml and a frequency of ~20~22 cycles/min.

\( P_{\text{ip}} \) was recorded via glass micropipettes with an ~200-\( \mu \text{m} \) taper and beveled down to tips of 2- to 4-\( \mu \text{m} \) diameter. The pipettes were filled with a 0.5 M NaCl solution stained with lissamine green that had been previously filtered through 0.2-\( \mu \text{m} \) Millipore filters. The pipettes were linked to a pressure transducer (Gould P23XL, Cernusco) motor driven by a servo-null controlling system (model 5A, Instrumentation for Physiology and Medicine, San Diego, CA).

Immediately before use, each pipette was calibrated to verify the linearity of the pressure response in the range of ~30 cmH\(_2\)O. Before each measurement, the electrical zero of the servo-null system was assessed by inserting the pipette into a small saline pool located at the same height as the pleural window and ground to the animal. The same control measurement was repeated at the end of all recordings to check the stability of the electrical zero. Pipette insertion was observed on a color monitor (model TM-150 PNS-K, Multisystem RGB, JVC) with a video camera (Panasonic F15) hooked to the stereomicroscope that could be set at a magnification of either ~60 or ~100. Final total magnification on the video screen was double microscopic size.

Micropuncture of the pulmonary interstitium was performed by advancing the pipette through the intact pleural window into the lung tissue at an angle of ~45° relative to the lung surface. We preferentially micropunctured the perimicrovascular interstitium, i.e., the interstitial space surrounding microvessels of ~30- to 60-\( \mu \text{m} \) diameter, running over the lung surface. The perimicrovascular interstitial thickness of 10-150 \( \mu \text{m} \) (35) allowed an easy insertion of the pipette tip and stable and repeated recordings over time from the same interstitial region.

Even when respiratory movements were abolished, extensive tissue movements occurred related to heart activity. To reduce this movement, micropuncture was preferentially done in the inferior right lung lobe, which is caudal and distant to the heart and is less affected by cardiac contraction.

The hydraulic pressure in the interstitial space lying between the internal intercostal muscles and the endothoracic fascia \( (P_{\text{pi}}) \) was recorded in the area surrounding the border of the pleural window and at the same height as the \( P_{\text{ip}} \) recordings.

The following general criteria were adopted to assess the validity of a micropuncture pressure recording: 1) the electrical zero must be unchanged after withdrawing the pipette from the tissue, 2) a stable tracer has to be recorded for at least 1 min, and 3) recordings from the same interstitial region on successive micropuncture attempts must provide values within ±2 cmH\(_2\)O of each other.
Experimental protocol. \( P_{ip} \) and \( P_{esl} \) were recorded for the control condition and at regular intervals (15–20 min) after intravenous administration of 200 µg (7 IU) of elastase. Animals receiving elastase were euthanized by an overdose of pentobarbital sodium and urethan between 100 and 160 min. After complete bleeding through the carotid artery, the chest wall was opened and the lungs were excised. Tissue water content was determined by measuring timed wet weight-to-dry weight ratio (W/D) of tissue specimens from the intercostal muscles and of lung tissue cleared of large blood vessels and main bronchi; each specimen was weighed immediately after sampling and after oven drying at 70°C for at least 24 h.

Structural properties of proteoglycans. A separate group of rabbits (n = 12) was used to evaluate the structure of pulmonary interstitial proteoglycans. The grouping of the animals was decided on the basis of the time course of \( P_{ip} \) and the corresponding lung W/D values. We therefore performed the biochemical study on proteoglycans in 1) control lungs (n = 4 animals); 2) at ~80 min after elastase administration (n = 4 animals), this time corresponding to the early phase of a marked change in \( P_{ip} \), and still with a low lung W/D; this condition will be referred to as "mild edema" (ME); and 3) at ~200 min after elastase administration (n = 4 animals), a time corresponding to the development of a severe lung edema characterized by minor changes in \( P_{ip} \), but a marked increase in the W/D; we refer to this condition as "severe edema" (SE). Animals in group 1 were euthanized with an overdose of pentobarbital sodium and urethan into an ear vein without receiving any surgical treatment. Animals in groups 2 and 3 were anesthetized and tracheotomized and were given 200 µg (7 IU) of elastase into the jugular vein. Then they breathed spontaneously through the intratracheal tube and were euthanized with an overdose of pentobarbital sodium and urethan at either 80 or 200 min, respectively. For each condition, lung samples were pooled, and the average W/D values were measured.

Proteoglycan extraction. Immediately after death and exhauster bleeding of the animals, the lungs were removed, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, and 1 mM KH2PO4, pH 7.4), and put to liquid N2 until used. Pools of lung samples (control condition, ME, and SE) were then cleaned, washed with PBS containing protease inhibitors (5 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na2-EDTA, and 100 mM 6-aminohexanoic acid), and cut into small slices, separating the pulmonary parenchyma from the terminal bronchioles. All procedures were carried out at 4°C. Tissue specimens were treated with guanidine hydrochloride (GuHCl), which allows proteoglycan extraction by breaking the intermolecular noncovalent bonds linking proteoglycans to other ECM components (9). Lung samples were suspended in 0.4 M GuHCl in 50 mM sodium acetate buffer (pH 6.0) containing protease inhibitors at a volume five times their weight and gently shaken at 4°C for 24 h. The extraction was repeated, and the extracts were pooled (0.4 M extract). Tissue residues were then suspended in 4 M GuHCl in 50 mM sodium acetate buffer (pH 6.0) containing protease inhibitors and 0.5% (vol/vol) Triton X-100 (buffer A). The detergent Triton X-100 was added to this extraction buffer to upset the hydrophobic interactions. Again, the extraction was repeated, and the extracts were pooled (4 M extract).

Proteoglycan isolation. After substitution of buffer A with 50 mM sodium acetate buffer (pH 5.8) containing 8 M urea, 0.15 M NaCl, 0.5% (vol/vol) Triton X-100, and protease inhibitors (buffer B), proteoglycans were isolated from the proteins and hyaluronic acid present in the extracts by ion-exchange chromatography with a DEAE-Sephalocolumn (4 × 30 cm, flow rate 30 ml/h) eluted with a step gradient of 0.15, 0.3, and 1.2 M NaCl in buffer B. Proteoglycans were fully recovered in the 1.2 M NaCl fraction. The fractions were then concentrated in an Amicon apparatus with a PM 10 membrane and kept frozen until used.

Proteoglycan characterization. After DEAE-Sephal chromatography, isolated proteoglycans were quantitated with a hexorunaxon assay (2) after precipitation with ethanol (21). Proteoglycans were then analyzed by agarose-polyacrylamide gel electrophoresis (A-PAGE) (10) before and after treatment with specific eliminases to determine the type of GAG chains linked to the core protein (see below). After electrophoresis, proteoglycans were detected by toluidine blue staining, and the relative content of the different proteoglycan populations was determined by densitometric analysis performed by a laser densitometric ictuscan (LKB).

Aliquots of isolated proteoglycans (50 µg as protein) were radiolabeled for 10 min with two iodo beads (Pierce Chemical) previously incubated for 5 min with 17 MBq125I (Dupont-NEN) in PBS following the manufacturer’s instructions. After elimination of nonincorporated iodine by chromatography on PD10 columns (Pharmacia) eluted in PBS, radiolabeled proteoglycans were gel filtered and associative conditions on a 4B columns (1 × 50 cm, flow rate 6 ml/h) eluted with 4 M GuHCl in 50 mM sodium acetate buffer (pH 6.0) containing protease inhibitors. Radiolabeled fractions were counted in a Cobra II gamma counter (Canberra-Packard).

The GAG composition of the proteoglycans was determined by digestion with chondroitinase (Chase) ABC for 24 h at 37°C in 50 mM tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (pH 8.0), which degrades galactosamine-containing GAGs such as CS and DS (40). The degradation of HS, which resists Chase ABC digestion, was done either by nitrous acid treatment at low pH (28) or by specific digestion with heparinase and heparitinase (23). Unsaturated disaccharides released by Chase ABC digestion from galactosamine-containing GAGs and by heparinase and heparitinase from HS chains were then fractionated and identified by capillary electrophoresis performed with a Biofocus 3000 apparatus (Bio-Rad) that was equipped with a diode-array detector set at 232 and 200 nm simultaneously. Separation was carried out on an uncoated fused-silica capillary tube (50-µm ID, 50-cm total length) at 20°C with 100 mM phosphate buffer (pH 2.5) (Bio-Rad). The identification of unsaturated disaccharides was performed with external standard mixtures of commercially available disaccharides (Sigma Chemical). The electropherogram was carried out at 15 kV, reversing the polarity of the system.

Molecular size distribution of native GAG chains was assessed after papain digestion of the core protein and removal of protease-resistant peptides by β-elimination (23). The analysis was performed in a high-performance liquid chromatography (HPLC) apparatus (System Gold, Beckman) by gel filtration on TSK gel G3000 SWXL and TSK gel G2000 SWXL columns (Toso Haas) in series (7.8 × 300 mm, flow rate 0.5 ml/min) eluted with water. The elution of GAGs was followed by monitoring absorbance at 206 nm. The absence of contaminating material was always checked by a diode-array HPLC spectrophotometer, scanning the recorded peaks from 190 to 340 nm.

Binding studies. Proteoglycan interactions with type I, type II, and type IV collagens (from human tissues), fibronectin (from human plasma), and hyaluronic acid (from human umbilical cord) were studied by a solid-phase binding assay (36). Wells were coated with 20 µl of ligand solution (100 µg/ml) and incubated overnight at 4°C. After nonspecific...
binding sites in the wells were blocked with 2% (wt/vol) albumin in PBS for 1 h, radiolabeled proteoglycans (10⁶ counts·min⁻¹·well⁻¹) were incubated for 4 h at 37°C in the presence of 0.1% (wt/vol) albumin. After exhaustive washing with PBS containing 0.1% (vol/vol) Brij, the bound proteoglycans were solubilized in PBS containing 2% (wt/vol) sodium dodecyl sulfate, and the radioactivity was measured in a gamma counter. The radioactivity in all the samples was corrected for background values corresponding to the radioactivity recovered in the wells coated with albumin alone and is expressed as the percent increase in proteoglycan binding to specific ligands relative to the nonspecific binding to albumin.

Analytic methods. Total hexuronate was assayed by the carbazole method (2). Protein content was determined according to Smith et al. (30).

Statistical analysis. Data are expressed as the arithmetic means ± SD. Comparison between mean values in the control condition and after elastase infusion was done by applying the paired t-test.

RESULTS

Hemodynamic parameters. $P_{es}$, $P_{sys}$, $P_{pa}$, $P_{la}$, and $Q$ in the control condition and after elastase administration are reported in Table 1. Over time, $P_{sys}$ remained essentially unchanged, whereas $P_{pa}$ and $P_{la}$ increased slightly. $Q$ tended to decrease, although not significantly, during edema development. As a result, the total flow resistance offered by the pulmonary vascular bed ($R_{tot}$), calculated as $R_{tot} = (P_{pa} - P_{la})/Q$, increased over time, although not significantly. No significant change in end-expiratory $P_{es}$ was observed.

Total plasma protein concentration ($C_p$) averaged $5.73 ± 0.38$ g/dl in the control condition and significantly decreased to $5.25 ± 0.23$ g/dl at 67 min, $4.55 ± 0.33$ g/dl at 131 min, and $4.75 ± 0.73$ g/dl at 207 min after elastase administration ($P > 0.05$); $C_p$ data can be fitted by the linear regression, $C_p = 5.79 - 0.0058 t$ ($r^2 = 0.67$), where $t$ is time in minutes. Plasma colloid osmotic pressure ($II$; calculated as $II = 4.64C_p + 0.0027C_p^2$, see Ref. 19) was $26.6$ cmH₂O in the control condition and significantly decreased by 2.3 and $4.6$ cmH₂O at 67 and 207 min, respectively ($P > 0.05$ by paired t-test).

Micropuncture measurements. In three rabbits (Fig. 1), we measured $P_{ip}$ after elastase administration (injection at $t = 0$) in two recording sites (sixth intercostal space on the midaxillary line and seventh intercostal space dorsal). Although the time course of $P_{ip}$ differs among animals, it appears similar for the two recording sites within each animal.

Figure 2A shows $P_{ip}$ in the control condition and at different times after elastase administration for all the animals studied; for the three animals shown in Fig. 1, average values from the two recording sites are shown. Despite an individual variability in $P_{ip}$ measurements, in all experiments, a similar trend occurred over time. After elastase administration, $P_{ip}$ increased from the control value (~$10.8 ± 2.2$ cmH₂O; $n = 5$ animals) to positive values, subsequently decreased to zero, and remained essentially unchanged. In Fig. 2B, the data of Fig. 2A were normalized by plotting the $P_{ip}$ values as a function of time, with zero time corresponding to the peak positive $P_{ip}$ value. On average, a positive peak pressure of $5.1 ± 1.7$ cmH₂O was attained at $56 ± 16$ min after elastase administration, followed by a pressure decline that was essentially zero at ~60 min after the peak positive $P_{ip}$ value. This trend is similar to that observed in a previous study during development of hydrostatic edema (Fig. 2B, dashed line; Ref. 21). $P_{el}$ was $-1.9 ± 0.8$ cmH₂O in the control condition and increased slightly after elastase administration according to the equation $P_{el} = -1.19 + 0.008 t$ ($r^2 = 0.37$).

Table 1. $P_{es}$, $P_{sys}$, $P_{pa}$, $P_{la}$, and $Q$ in control condition and at 30, 60, and 140 min after intravenous addition of 200 µg (7IU) of pancreatic elastase

<table>
<thead>
<tr>
<th>Time</th>
<th>$P_{es}$, cmH₂O</th>
<th>$P_{sys}$, mmHg</th>
<th>$P_{pa}$, cmH₂O</th>
<th>$P_{la}$, cmH₂O</th>
<th>$Q$, ml/min</th>
<th>$R_{tot}$, cmH₂O·min·ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$-2.2 ± 0.3$</td>
<td>$90 ± 14.1$</td>
<td>$17.6 ± 5.8$</td>
<td>$1.75 ± 2.47$</td>
<td>$165 ± 14.1$</td>
<td>$0.0957 ± 0.024$</td>
</tr>
<tr>
<td>30 min</td>
<td>$-3.2 ± 2.5$</td>
<td>$85 ± 21.2$</td>
<td>$19.6 ± 7.5$</td>
<td>$3.75 ± 0.35$</td>
<td>$155 ± 7.1$</td>
<td>$0.1019 ± 0.033$</td>
</tr>
<tr>
<td>60 min</td>
<td>$-2.8 ± 2.8$</td>
<td>$100 ± 14.1$</td>
<td>$20.2 ± 2.8$</td>
<td>$4.75 ± 1.1$</td>
<td>$112 ± 53$</td>
<td>$0.1360 ± 0.057$</td>
</tr>
<tr>
<td>140 min</td>
<td>$-1.5 ± 3.5$</td>
<td>$95 ± 7.1$</td>
<td>$21.5 ± 4.9$</td>
<td>$3 ± 1.4$</td>
<td>$122 ± 38$</td>
<td>$0.1516 ± 0.044$</td>
</tr>
</tbody>
</table>

Values are means ± SD. $P_{es}$, esophageal pressure; $P_{sys}$, systemic arterial pressure; $P_{pa}$, pulmonary arterial pressure; $P_{la}$, left atrial pressure; $Q$, cardiac output. Total pulmonary blood flow resistance ($R_{tot} = (P_{pa} - P_{la})/Q$). Over time, $R_{tot}$ increased, although not significantly, by ~60%.
size proteoglycans, contained mainly CS and DS chains because 73% of this band disappeared after Chase ABC digestion. Therefore, this large material is likely versican. The broad, fast-moving band, consisting of heterogeneous, lower molecular size proteoglycans, contained mainly HS chains because 78% of this material resisted Chase ABC digestion, disappearing completely after nitrous acid treatment. HS-containing proteoglycans extracted from the basement membrane are probably included in this material. Similar results were obtained in the analysis of the 4 M extracts.

125I-radiolabeled proteoglycans, isolated from the 0.4 and 4 M extracts, were gel filtered under dissociative conditions (in the presence of 4 M GuHCl) to prevent molecular interactions, which may affect molecular size analysis (9). Under these experimental conditions, radiolabeled proteoglycans isolated from the 0.4 M extracts were resolved into three main different populations (Fig. 3, top). Part of the radioactivity was eluted in the void volume of the column (peak L). It likely corresponds to the population of large-size proteoglycans, which shows slower electrophoretic mobility in A-PAGE and carries CS chains such as versican. This interpretation was confirmed by the marked sensitivity of this material to Chase ABC treatment; moreover, the unsaturated disaccharides released by this digestion were identified by capillary electrophoresis analysis as the typical constituents of galactosamine containing GAG chains: α-D-4,5-unsaturated hexuronic acid-[1→3]N-acetylgalactosamine 4-sulfate, α-D-4,5-unsaturated hexuronic acid-[1→3]N-acetylgalactosamine 6-sulfate, and α-D-4,5-unsaturated hexuronic acid-2-sulfate-[1→3]N-acetylgalactosamine 4-sulfate (40). Part of the radioactivity was included in the gel, consisting of a heterogeneous mixture of smaller size proteoglycans (peak I) that contained mainly HS chains as indicated by the lower sensitivity to Chase ABC digestion. Moreover, capillary electrophoresis analysis showed that treatment of this material with heparinase and heparitinase released unsaturated monosulfated disaccharides that contained glucosamine and represented typical constituents of HS chains (α-D-4,5-unsaturated hexuronic acid-[1→4]N-sulfate-glucosamine and α-D-4,5-unsaturated hexuronic acid-[1→4]N-acetylglucosamine 6-sulfate) (32). Finally, part of the radioactivity was eluted in the total volume of the column and consisted of low-molecular-size material (peak S).

The relative content of the different proteoglycan populations changed markedly with the development of elastase-induced edema (Fig. 3, ME and SE). The percentage of radioactivity recovered in peak S increased progressively from the control condition to ME and SE (10.7, 25.7, and 37.2%, respectively). Treatment of this material with Chase ABC and heparanase plus...
heparitinase released unsaturated disaccharides containing galactosamine and glucosamine, respectively, as indicated by capillary electrophoresis analysis. Therefore, peak S contained GAG chains, including both CS and HS, which are likely to represent degradation products of larger size proteoglycans.

In ME, the relative content of large-size proteoglycans (peak L) was unaffected, suggesting that the increase in fragmentation products in this phase might depend mainly on partial degradation of smaller size proteoglycans containing mostly HS chains. However, in SE, the relative content of large proteoglycans was greatly reduced, indicating that the further development of elastase-induced lung injury was coupled with a fragmentation of large CS-containing proteoglycans.

Proteoglycans eluted in both peak L and peak I from control lungs were degraded by further incubation with pancreatic elastase (90 mlU/ml in 0.1 M Tris-acetate buffer, pH 7.0, 24 h, 37°C). Gel-filtration chromatography of the elastase-treated material showed a complete disappearance of both peaks. This finding confirms in vitro that lung interstitial proteoglycans can become a substrate for pancreatic elastase.

Similar results were obtained in the study of radiolabeled proteoglycans isolated from the 4 M extracts.

Size-distribution analysis of native GAG chains freed of proteins was performed by gel-filtration HPLC. In all the samples, the chain size was dispersed within the same range of values, but material of very low molecular size was never detected, indicating that GAG chains were not subjected to extensive degradation during the development of elastase-induced edema. This finding is in agreement with the observation that pancreatic elastase is devoid of digesting activity on free HS chains (33). On this basis, the elastase-induced degradation of lung proteoglycans is likely to depend only on the digestion of the core protein.

The binding properties of total proteoglycans isolated from the 0.4 and 4 M extracts (Fig. 4), assayed for different ECM components by solid-phase binding assay, changed with increasing severity of lung edema. On average, the binding properties of proteoglycans extracted with 0.4 M GuHCl were always lower than those extracted with 4 M GuHCl. This result is in agreement with the ability of the lowest GuHCl concentration to extract from the tissue only the molecules that interact more weakly with the other ECM components. In the case of both the 0.4 and 4 M extracts, proteoglycan ability to bind the different ligands was always markedly reduced in SE in comparison to the
control of elastase-induced edema was coupled with both structural and functional modifications of proteoglycans. On the contrary, the pattern of proteoglycan interaction properties was less homogeneous in ME, and significant differences were not always found with respect to the control condition, in particular in the case of proteoglycans isolated from the 4 M extract.

**DISCUSSION**

Pulmonary edema was studied with an intravenous administration of elastase, a proteolytic enzyme that can be discharged into the intestinal lumen with other pancreatic enzymes. These enzymes may also be secreted into the blood after an injury affecting the permeability of the pancreatic cell membrane, as in acute pancreatitis. In this pathological condition, loss of the protective role of the ECM with respect to the control condition, indicating that the complete development of elastase-induced edema was coupled with both structural and functional modifications of proteoglycans. On the contrary, the pattern of proteoglycan interaction properties was less homogeneous in ME, and significant differences were not always found with respect to the control condition, in particular in the case of proteoglycans isolated from the 4 M extract.

A single dose of 200 µg (7 IU) of pancreatic elastase, which provides an average plasma concentration of ~90 mIU/ml, was used. At this plasma concentration, elastase is unlikely to affect the elastic properties of the lung as suggested by the finding that end-expiratory P_{es} remains essentially unchanged during the development of edema (Table 1). Over a longer time frame, elastase has been used at similar concentrations to damage the elastic lung scaffold and cause pulmonary emphysema (1, 24, 33).

In the present study, we followed the progression of lung edema in the intact lung on the basis of changes observed in P_{ip}. The similar pattern of P_{ip} changes at two different sites within the same animal (Fig. 1) suggests a homogeneous time course for the cascade of events leading to edema. The different time courses of P_{ip} among the animals (Fig. 2A) can reflect either individual differences in the diffusion rate of elastase and/or different individual availability of proteinase inhibitors such as α2-proteinase inhibitor (34). The initial phase of the increase in P_{ip} stems from an increased microvascular filtration rate, whereas the subsequent decrease in P_{ip} with a marked increase in lung WD suggests an increase in tissue compliance.

From the macromolecular standpoint, the development of the edema associated with elastase administration reveals a sequential susceptibility of different proteoglycans to elastase-induced degradation, which affected only their core protein. In fact, in ME, large-size CS proteoglycans were unaffected, whereas intermediate-size HS proteoglycans were markedly reduced (Fig. 3). Because elastase, due to its low molecular mass (25.9 kDa; see Ref. 29), diffuses from the luminal toward the abluminal endothelial side, it is likely that HS proteoglycans of the basement membrane are the first to be degraded by the enzyme in the subendothelial compartment; this process appears to worsen in SE (Fig. 3).

The nonsignificant decrease in proteoglycan binding properties to collagen type IV in ME (Fig. 4) can be explained on the basis of the following considerations. Basement membrane HS proteoglycan contains three HS chains, which are attached to the NH₂ terminus of the core protein and are peripherally oriented, that bind to collagen type IV (41). Therefore, any elastase-mediated cut of the core protein that does not affect the NH₂-terminal domain will preserve the ability of HS chains to bind to collagen type IV; however, the fragmentation of the core protein implies a consistent alteration of the native architecture of the basement membrane. The further proteolytic degradation occurring in SE might then lead to a complete separation of HS chains, thus ultimately affecting their optimal spatial arrangement, which is likely to be critical for binding to collagen type IV.

The early damage of HS proteoglycans is likely to cause an increase in endothelial permeability to solutes and water, leading to increased fluid filtration from capillary to interstitium. This interpretation is also supported by the data of Guretzky et al. (6), who showed that loss of HS proteoglycan integrity caused a threefold increase in in vitro endothelial monolayer permeability. The microvascular wall permeability does not depend only on the integrity of the basement membrane but also on the glycocalix covering the endothelial luminal side and on the endothelial pores (26). We showed degradation of HS proteoglycans of the basement membrane in ME, although we cannot exclude that the initial permeability increase is due to endothelial junction opening.

The structural modifications of proteoglycans associated with edema development markedly affected the strength of their interactions with other ECM components, as indicated by the progressive increase in proteoglycan extractability with GuHCl and by the decrease in proteoglycan binding properties in a solid-phase binding assay, which was particularly evident in SE (Fig. 4). In ME, this assay did not show significant modifications for most of the ligands, in particular in the case of the 4 M extract. This result might be explained by considering that proteoglycan degradation is only partial in ME and that the 4 M extract includes proteoglycans that interact strongly in the tissue and might have been poorly affected by the morbid process, thus maintaining their ability to interact with the specific ligands.

The damage of different proteoglycan populations found in ME and SE actually depended on elastase activity. Under our experimental conditions, all proteoglycan populations extracted from lung specimens were degraded by in vitro treatment with pancreatic elastase, which digested the core protein without affecting the size of the GAG chains. Pancreatic elastase is known to degrade the main components of the basal membrane and ECM (34), and its capability to digest proteoglycans has also been demonstrated in rat lung using immunohistochemical methods after intraacral instillation of the enzyme (33). Moreover, elastase can also degrade specific inhibitors of the endogenous
proteinases, such as α1-proteinase inhibitor, thus reducing the anti-proteolytic and anti-elastase defenses of the lung (34). However, we cannot exclude that, under our experimental conditions, proteoglycan degradation might also be performed by other proteinases proteolytically activated by elastase.

The large subatmospheric $P_{ip}$ measured in the intact lung suggests that a normal respiratory function can be achieved through a dry, low-compliant pulmonary interstitium. This condition is attained through the matching of transcapillary fluid filtration, mechanical properties of the ECM, and fluid and solute removal through the lymphatic system. ME seems to occur as a consequence of an increased capillary permeability, whereas the lymphatic system. ME seems to occur as a consequence of an increased capillary permeability, whereas the structural integrity of large CS proteoglycans in the interstitial ECM is still preserved. Increased fluid filtration into a compact, low-compliant ECM detriments the structural integrity of large CS proteoglycans in the interstitial ECM is still preserved. Increased fluid filtration into a compact, low-compliant ECM determines a consistent increase in Pip (as shown in Figs. 1 and 2), with a minor change in extravascular water content going from the control condition to ME. The attainment of positive $P_{ip}$ values counteracts further fluid filtration into the perimicrovascular interstitial space; therefore, the maintenance of large CS proteoglycan integrity seems to be critical for ECM architecture and might be regarded as a powerful tissue safety factor, preventing a higher degree of tissue hydration.

From the mechanical standpoint, the loss of macromolecular assembly alters tissue compliance, as suggested by the fact that $P_{ip}$ drops to atmospheric values and remains unaltered as edema progresses (Fig. 2). An increase in tissue compliance after exposure to elastase has also been reported for the vascular intima (11). The present data are similar to previous results from our laboratory (14, 21) on the development of hydraulic edema, showing that the low tissue compliance is critical to minimize interstitial fluid volume and is coupled with the maintenance of normal proteoglycan structure. This behavior was not observed in the interstitium of the intercostal muscles, a tissue that can stand large hydrations without functional impairment. The concept of tissue compliance as a safety factor against the development of edema was originally proposed for subcutaneous tissue by Guyton (7) and subsequently developed by Wiig and Reed (38).

It is a common clinical observation that the transition from mild interstitial edema, normally undetectable with common diagnostic procedure, to accelerated alveolar edema occurs very rapidly. In light of the present results, lesional elastase-induced edema develops as a consequence of a cascade of the following events: 1) an increase in capillary permeability (loss of the structural integrity of HS proteoglycans) and 2) a decrease in interstitial tissue compliance (fragmentation of large size CS proteoglycans) that abolishes the tissue safety factor.

Human type I collagen and human fibronectin were kindly provided by Profs. Ruggero Tenni and Pietro Speziale, respectively (Department of Biochemistry "A. Castellani," Università degli Studi, Pavia, Italy).

This research was supported by grants from the Italian Ministry of the University and of Scientific and Technological Research (MURST 40 and 60%) and Grant CT 96.03519 from the National Research Council.

Address for reprint requests: D. Negrini, Istituto di Fisiologia Umana, Via Mangiagalli 32, 20133 Milan, Italy.

Received 4 August 1997; accepted in final form 29 October 1997.