Hypoxia Differentially Enhances the Effects of Transforming Growth Factor-β Isoforms on the Synthesis and Secretion of Glycosaminoglycans by Human Lung Fibroblasts

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ABSTRACT

Interstitial lung diseases associated with hypoxia, such as lung fibrosis, are characterized by enhanced production of transforming growth factor-β (TGF-β) and increased deposition of extracellular matrix (ECM) molecules, including glycosaminoglycans (GAGs). In this study, we investigated the effect of hypoxia (3% O2) on TGF-β-induced GAG synthesis by primary human pulmonary fibroblasts, established from lung biopsies. Total GAG synthesis was assessed by the incorporation of [3H]glucosamine into GAGs associated with the cell layer (cells and ECM) or secreted in the medium. GAGs were isolated and purified by gel filtration, fractionated by electrophoresis on cellulose acetate membranes, and characterized using GAG-degrading enzymes. GAG molecules identified in the cell layer and the medium were: hyaluronic acid, and chondroitin, dermatan, and heparan sulfates. All TGF-β isoforms time dependently induced [3H]glucosamine incorporation into GAGs of the cell layer or the medium. Characterization of individual GAG molecules indicated that this was attributed to dermatan and heparan sulfates in the cell layer and to hyaluronic acid and chondroitin and dermatan sulfates in the medium. Hypoxia enhanced the effect of all TGF-β isoforms, particularly that of TGF-β3, on the secretion of hyaluronic acid and chondroitin and dermatan sulfates. In the cell layer, hypoxia stimulated only the effect of TGF-β2-induced [3H]glucosamine incorporation into GAGs. Our data indicate that hypoxia differentially enhances the effect of TGF-β isoforms on the secretion and deposition of GAGs and may hasten ECM remodeling associated with the pathogenesis of lung fibrosis.

The extracellular matrix (ECM) of the human lung is mainly produced by pulmonary fibroblasts and comprises essentially molecules such as collagens, elastin, glycosaminoglycans (GAGs), and proteoglycans (Dunsmore and Rannels, 1996). Under physiological conditions, lung ECM is subjected to a continuous daily turnover of over 10% of its total mass (McAnulty and Laurent, 1995). This is achieved by a tightly controlled equilibrium between de novo synthesis and degradation of the pulmonary ECM, which is critical for the maintenance of the structural and functional integrity of the lungs (Dunsmore and Rannels, 1996). However, in interstitial lung diseases, such as pulmonary fibrosis, this dynamic equilibrium is disturbed, leading to excessive deposition of ECM macromolecules in the pulmonary interstitium, which is believed to be responsible for the ensuing vital deficiency of lung functions (McAnulty and Laurent, 1995).

The increased deposition and accumulation of ECM in the pulmonary interstitium during the pathogenesis of lung fibrosis involves the local over-expression of a variety of cytokines and/or growth factors; among these, the isoforms of transforming growth factor-β (TGF-β) are generally recognized as key mediators responsible for the accumulation of ECM during the development of lung fibrosis (McAnulty and Laurent, 1995; Liu and Brody, 2001). TGF-β isoforms are consistently over-expressed in biopsies from fibrotic lungs, especially in areas of active fibrosis (Coker et al., 1997). In vitro experiments confirm that TGF-β isoforms are associated with lung fibrosis by demonstrating that these growth factors up-regulate mRNA and protein levels of collagens, fibronectins, and laminins in a variety of cell types (Coker et al., 1997; Eickelberg et al., 1999).

The severe loss of lung function in patients suffering from pulmonary fibrosis is also associated with hypoxia, which is a usual consequence of interstitial lung diseases (Schutte et al., 1996). Hypoxia results in increased pulmonary arterial and interstitial pressure (Miserocchi et al., 2001) and eventually may lead to the development of secondary pulmonary hypertension, which is a disease characterized by hyperplasia of vascular smooth muscle cells (VSMC) and fibroblasts.

ABBREVIATIONS

ECM, extracellular matrix; GAG, glycosaminoglycans; TGF, transforming growth factor; PBS, phosphate-buffered saline; FCS, fetal calf serum; VSMC, vascular smooth muscle cell.
and enhanced deposition of ECM molecules, leading to extensive fibrosis (Kullmann et al., 1993).

As mentioned above, GAGs represent one of the major components of the pulmonary ECM. During neonatal lung growth (Schmid et al., 1982), acute lung injury (Cantor et al., 1980), and the development of pulmonary fibrosis (Cantor et al., 1983), GAGs undergo significant alterations in content, synthesis, and distribution, indicating that these macromolecules are essentially involved in the functional and structural organization of the lung in health and disease. Experiments using bovine pulmonary cell cultures indicated that hypoxia increases the content of certain GAGs (Karlinsky et al., 1992). Furthermore, biopsy specimens from the lungs of patients with pulmonary fibrosis show increased content of heparin (Sasaki et al., 2000).

We have recently started to investigate the combined effect of hypoxia and GAG synthesis on the composition of the lung ECM associated with interstitial lung diseases. Using cultures of primary human pulmonary VSMC and/or fibroblasts, we have shown that hypoxia modifies the effects of platelet-activating factor and/or platelet-derived growth factor on the production of interleukin-6 and -8 and cell proliferation (Tamm et al., 1998) and synthesis of GAGs (Papakonstantinou et al., 2000). The aim of the present study was to investigate the effect of hypoxia on TGF-β-induced synthesis, secretion, and deposition of GAGs by human lung fibroblasts. We found that hypoxia differentially enhanced the effect of TGF-β isoforms on the synthesis of specific GAG molecules associated with the cell layers (cells and ECM) or in the culture medium of human lung fibroblasts. Our data indicate that this combined effect of hypoxia and TGF-β may accelerate ECM remodeling associated with the progression of interstitial lung diseases, such as lung fibrosis.

Materials and Methods

Human Pulmonary Fibroblast Cultures. Cell cultures of primary pulmonary fibroblasts were grown out from sterile lung biopsies of normal tissue obtained after lung resection during surgical lung cancer therapy, under a protocol approved by the ethical committee of the Faculty of Medicine, University of Basel (Switzerland). The harvested biopsy samples were kept in sterile phosphate-buffered saline (PBS) (Seromed-Biochrom, Berlin, Germany) at 4°C overnight. Tissue samples were then cut into small pieces (1–5 mm³) and placed, in groups of 10, in cell culture dishes (Falcon, Basel, Switzerland) prewetted with 1 ml of culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum (FCS), 8 mM L-glutamine (all obtained from Seromed-Fakola, Basel, Switzerland), 20 mM HEPES, and 1× amino acid mix (both purchased from Invitrogen, Carlsbad, CA). The same medium was used for subsequent cultures of primary fibroblasts. Antibiotics or antimycotics were not added to the culture media at any time. Incubations were carried out at 37°C, under 21% O₂, 74% N₂, and 5% CO₂. Growth of cells was monitored by light microscopy every day during the first week and every second day thereafter. Spindle-like fibroblasts started growing out from tissue samples from days 2 to 3, whereas the vast majority of epithelial-like cells remained attached to the tissue samples. Outgrowth of fibroblasts took 1 to 2 weeks. Tissue samples were then removed by aspiration, and cells were allowed to reach confluence, at which point fibroblasts were overwhelming outgrowing epithelial cells. Fibroblasts at confluence were expanded by trypsinization and used thereafter between passages 2 and 6. Epithelial cells were insensitive to trypsin harvesting. The phenotype of fibroblasts was determined by immunohistochemical staining with monoclonal antibodies specific for smooth muscle cell actin, cytokeratin, fibronectin, laminin, or von Willebrand factor (Roche Applied Science, Indianapolis, IN; or Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cells were grown in Lab-Tek tissue culture chamber slides (Bayer Corporation, Elkhart, IN) until confluence and fixed in 4% paraformaldehyde. Nonspecific protein binding was blocked by incubating the cells in PBS (Seromed, Berlin, Germany) supplemented with 0.5% (v/v) bovine serum albumin (Fluka Chemie, Buchs, Switzerland) for 20 min. The slides were then incubated with one of the above-mentioned antibodies for 60 min, washed three times with PBS, and further incubated with a fluorescein- or rhodamine-linked anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Inc.). Preparations were washed three times with PBS, mounted with Fluoresave reagent (Calbiochem-Novabiochem, San Diego, CA), and analyzed using a microscope equipped with epi-illumination and specific filters (Axiopt; Carl Zeiss, Inc., Oberkochem, Germany). Nonspecific binding of the fluorescein- or rhodamine-linked antibody was excluded using the second antibody alone.

Cell Culture Conditions. Cells were seeded onto 24-well culture plates (Falcon, Basel, Switzerland) and cultivated to 80% confluence (approximately 1 × 10⁴ cells/ml/well). Before stimulation with TGF-β isoforms, subconfluent cell cultures were serum-deprived for 48 h with low serum medium (RPMI 1640, supplemented with 0.1% FCS and 20 mM HEPES). To avoid autostimulation of cells, low serum medium was exchanged every 12 h. Subconfluent quiescent cells were then stimulated with recombinant human activated forms of TGF-β isoforms (R & D Systems, Minneapolis, MN; catalogue number 240-B, TGF-β1; 302-B2, TGF-β2; and 243-B3, TGF-β3) and incubated under hypoxic or normoxic conditions for 12, 24, or 48 h. Normoxic culture conditions were defined as 21% O₂, 74% N₂, and 5% CO₂. For hypoxic culture conditions, the concentration of O₂ was reduced to 3% by replacement with N₂, keeping CO₂ constant at 5%.

Measurement of Total GAG Synthesis. Subconfluent primary lung fibroblasts were incubated under normoxic or hypoxic conditions for 12, 24, or 48 h, in the presence or absence of 0.1 to 2 ng/ml TGF-β isoforms. Routinely, 1 ng/ml TGF-β isoforms was used since dose-response experiments indicated that maximum effects were attained at this concentration. Also, this concentration has previously been shown as optimal for TGF-β-associated ECM deposition in cultures of pulmonary fibroblasts (Eickelberg et al., 1999). In all cases [³H]glucosamine (0.5 μCi/ml) (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) was added in the culture media. When the effect of hypoxia alone on GAG synthesis was tested, respective incubations under normoxia were used as controls. When the effect of hypoxia on TGF-β-induced synthesis of GAGs was tested, unstimulated cells, that is cells in the absence of growth factor, incubated either under normoxia or hypoxia in the presence of the radioligand alone were used as controls. Culture medium and the cell layer (cells together with the ECM) were collected separately and digested with 0.1 KU of pronase (Streptomyces griseus; Calbiochem, Lucerne, Switzerland). Total GAGs were precipitated by adding a mixture of ethanol (80% final concentration) containing 1.3% (w/v) sodium acetate. The samples were stored at −20°C overnight and then centrifuged at 10.000g. The pellets were dissolved in 0.5 M NaOH and total GAG synthesis was assessed by measuring the amount of [³H]glucosamine incorporated into GAGs. The results are expressed as a percentage of radioligand incorporated compared with their respective controls, which were set to 100%.

Isolation and Purification of GAGs. Total GAGs from cultures of primary human pulmonary fibroblasts, cultivated in cell culture flasks (Falcon) under the experimental conditions described above, were isolated and purified, as previously described (Papakonstantinou et al., 1995). In brief, supernatants (20 ml) were collected separately, and the cells with associated ECM (cell layer) were washed twice with 10 ml of ice-cold PBS and harvested by scraping. Total glycans were isolated and purified from the culture medium or the cell layer as follows. Lipids were extracted with 4 volumes of
the glycans were subjected to adjusted to between 10.0 and 11.0 by addition of 10 mM NaOH, and pronase and incubating the mixture at 60 °C.

Adjusted to 1 mM, and the reaction was stopped by adding 0.1 KU of dissolved in 100 mM Tris-HCl buffer, pH 7.0, containing 3 mM CaCl₂ for 16 h at 37 °C. At the end of the incubation period, the CaCl₂ concentration of the solution was adjusted to 150 mM NaCl and 10 mM MgCl₂, and DNA digestion was accomplished by adding 400 KU of DNase I (EC 3.1.2.1; Calbiochem) and incubating for 16 h at 37 °C. 6) Hyaluronidase: samples dissolved in 20 mM sodium acetate, buffered with acetic acid to pH 5.0, were incubated with 2 U of hyaluronidase (F. heparinum; Sigma-Aldrich) for 16 h at 37°C. 5) Keratanase: samples dissolved in 50 mM Tris-HCl buffer, pH 7.4, were incubated with 0.05 U of keratan-sulfate endo-β-D-galactosidase (EC 3.2.1.103; Pseudomonas species; Sigma-Aldrich) for 16 h at 37°C. 6) Hyaluronidase: samples dissolved in 20 mM sodium acetate, buffered with acetic acid to pH 5.0, were incubated with 4 U of hyaluronate lyase (EC 4.2.2.1; Streptomyces hyalurolyticus; Sigma-Aldrich) for 14 h at 60°C.

Electrophoresis on Cellulose Acetate Membranes. Two microliters of the GAG solution, containing about 4 μg of uronic acids, were placed at the origin (10 mm from the cathode side) of a cellulose acetate strip. Electrophoresis was carried out in 100 mM pyridine/470 mM formic acid, pH 3.0, using 7 mA constant current at room temperature for 70 min. After electrophoresis, the cellulose acetate strip was stained with 0.2% Alcian Blue (w/v) in 0.1% acetic acid (v/v) for 10 min and washed with 0.1% acetic acid (v/v) for 20 min (Papakonstantinou et al., 1998b). The intensity of the staining was quantified by the computer-assisted image analysis program of Kodak (Eastman Kodak, Rochester, NY).

Treatment of the Purified Glycans with GAG-Degrading Enzymes. Speed-dried GAGs (5 μg of uronic acids) were incubated in a final volume of 15 μl as follows. 1) Heparinase: samples were dissolved in 100 mM Tris-HCl buffer, pH 7.0, containing 3 mM CaCl₂ and incubated with 4 × 10⁻⁴ U of heparin lyase I (EC 4.2.2.7; Flavobacterium heparinum; Seikagaku, Tokyo, Japan) for 15 h at 30°C. 2) Heparitinase: samples dissolved as above were incubated with 4 × 10⁻⁴ U of heparan sulfate lyase (heparitinase: EC 4.2.2.8; F. heparinum; Seikagaku) for 16 h at 43°C. 3) Chondroitinase ABC: samples dissolved in 100 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium acetate were incubated with 2 × 10⁻⁴ U of chondroitin ABC lyase (EC 4.2.2.4; Proteus vulgaris; Sigma-Aldrich, St. Louis, MO) for 16 h at 37°C. 4) Chondroitinase B: samples dissolved in 100 mM Tris-HCl buffer, pH 7.4, were incubated with 0.1 U of chondroitin B lyase (F. heparinum; Sigma-Aldrich) for 16 h at 37°C. 5) Keratanase: samples dissolved in 50 mM Tris-HCl buffer, pH 7.4, were incubated with 0.05 U of keratan-sulfate endo-β-D-galactosidase (EC 3.2.1.103; Pseudomonas species; Sigma-Aldrich) for 16 h at 37°C.

Incubation times and enzyme concentrations used were those required for the complete degradation of their respective standard substrates, as estimated by a preliminary investigation. In this preliminary study, the standard GAGs (10 μg) chondroitin sulfate A (bovine trachea), chondroitin sulfate B (porcine skin), chondroitin sulfate C (shark cartilage), hyaluronic acid (bovine trachea), keratan-sulfate (bovine cornea), heparan sulfate (bovine intestinal mucosa), and heparin (all from Sigma-Aldrich) were treated individually with each of the above-mentioned GAG-degrading enzymes following appropriate incubation procedures. Substrates incubated separately with their respective buffers served as controls. Digestion
was evaluated by electrophoresis on cellulose acetate membranes and quantified by the computer-assisted image analysis program of Kodak (Papakonstantinou et al., 1998b).

Statistics. Means ± S.E. were calculated from results obtained from cultures of primary fibroblasts established from lung tissue biopsies from at least four different patients. Determinations were always made in triplicate. Statistical analysis was performed using analysis of variance.

Results

Characterization of Human Pulmonary Fibroblast Cultures

Cultures of primary human lung fibroblasts were established from sterile peripheral lung tissue biopsies. Over serial passages up to passage 6, all cells displayed typical spindle-shaped morphology under light microscopy and stained positive for fibronectin (Fig. 1a) and laminin (Fig. 1b) but were negative for immunostaining with monoclonal antibodies against von Willebrand factor (Fig. 1c), cytokeratin (Fig. 1d), smooth muscle cell actin, or Factor VIII (data not shown), indicating that there was no contamination with cells such as VSMC, epithelial cells, or endothelial cells in any of the fibroblast cell cultures used. All subsequent experiments were performed using subconfluent quiescent cultures of fibroblast cells between passages 2 and 6 to maintain comparability.

Effect of Hypoxia on the TGF-β-Induced GAG Synthesis by Human Lung Fibroblasts

Neither hypoxia (3% O₂) nor any of the TGF-β isoforms (0.1–2 ng/ml) affected the viability of human lung fibroblasts in culture as assessed by trypan blue exclusion staining (data not shown). Hypoxia alone did not significantly affect the incorporation of [³H]glucosamine into GAGs secreted by fibroblasts compared with the cell layer or secreted in the medium (Fig. 2). The effect of hypoxia on the TGF-β-induced GAG synthesis by human lung fibroblasts was as follows.

Cell Layer. Under normoxic conditions, all three TGF-β isoforms (1 ng/ml) induced the incorporation of [³H]glucosamine into GAGs synthesized by subconfluent primary human lung fibroblasts compared with unstimulated controls (set as 100%) (Fig. 3). This effect was time-dependent, but it became statistically significant after 24 h (p < 0.05) and 48 h (p < 0.02) of incubation. There were no significant differences between the three TGF-β isoforms on the above effect. Hypoxia did not significantly affect the normoxic effect of TGF-β1 (Fig. 3A) or TGF-β2 (Fig. 3C) on the synthesis of GAGs. However, hypoxia significantly stimulated the TGF-β2-induced incorporation of [³H]glucosamine into GAGs after 24 h (p < 0.05) and 48 h (p < 0.02) of incubation (Fig. 3B).

Culture Medium. Under normoxic conditions, all three TGF-β isoforms (1 ng/ml) induced the incorporation of [³H]glucosamine into GAGs secreted by fibroblasts compared with unstimulated controls (set as 100%) (Fig. 4). This effect was time-dependent; it became statistically significant after 24 h (p < 0.05) of incubation but was less evident compared

with the amount of GAGs associated with the cell layer. Hypoxia significantly stimulated the normoxic TGF-β-induced GAG secretion in the culture medium in a time-dependent manner from 12 to 48 h of incubation (Fig. 4). This effect of hypoxia was statistically significant at 24 h (p < 0.02) and 48 h (p < 0.01) of incubation, and it was most prominent for the TGF-β3 isoform (Fig. 4C). Hypoxia enhanced significantly the TGF-β3-induced secretion of GAGs even after 12 h. This effect was increased by almost 2-fold after 48 h compared with normoxia.

Isolation and Purification of GAGs

Total GAGs were isolated from the culture media and the cell layer of human lung fibroblasts after delipidation and

containing 0.5% bovine serum albumin for 20 min. The slides were then incubated for 30 min with monoclonal antibodies specific for fibronectin (a), laminin (b), von Willebrand factor (c), or cytokeratin (d), washed three times with blocking buffer, and further incubated for 30 min with either fluorescein- or rhodamine-coupled anti-rabbit IgG or anti-mouse IgG. After washing, the preparations were mounted with Fluorosave reagent and observed under a microscope.
Fractionation of Total GAGs Using Electrophoresis on Cellulose Acetate Membranes and Characterization by GAG-Degrading Enzymes

The isolated and purified total GAGs, corresponding to 4 µg of uronic acids, were fractionated according to charge using electrophoresis on cellulose acetate membranes and stained with Alcian Blue. Under normoxic conditions total GAGs isolated from the cell layer (Fig. 6A, lane A) or the culture medium (Fig. 6B, lane A) were fractionated in four distinct GAG populations (Fig. 6, arrows 1 to 4) which migrated with the same electrophoretic mobility as commercially available hyaluronic acid, heparan sulfate, dermatan sulfate, or chondroitin sulfate, respectively. Enzymatic treatment with GAG-degrading enzymes (Table 1) confirmed the conclusion drawn based on the electrophoretic mobility of GAG populations. The uppermost population (Fig. 6, A and B, arrow 1) was completely degraded only by hyaluronidase, indicating hyaluronic acid; the second GAG population (Fig. 6, A and B, arrow 2) was completely degraded only by heparitinase, indicating a heparan sulfate structure; the third GAG population (Fig. 6, A and B, arrow 3) was completely degraded only by chondroitinase ABC and chondroitinase B, corresponding to dermatan sulfate; the final GAG population (Fig. 6, A and B, arrow 4) was completely degraded only by chondroitinase ABC, indicating a structure of chondroitin sulfate A and/or chondroitin sulfate C.

Similar migration patterns of GAG populations were obtained for each TGF-β isoform. Figure 6, A and B, depicts typical sets of results for TGF-β2 and TGF-β3 isoforms, respectively, following 48 h of incubation. Hypoxia or treatment with any of the TGF-β isoforms did not affect the nature of individual GAG molecules, even after 48 h of incubation.

Cell Layer. Quantification of the intensity of the Alcian Blue staining by a computer-assisted image analysis program revealed that hypoxia did not affect the relative proportions of GAGs (Fig. 6A, lane C) compared with normoxia (Fig. 6A, lane A). Furthermore, under normoxic or hypoxic conditions, all TGF-β isoforms altered the relative ratio of individual GAG molecules after 48 h of incubation. This effect was most pronounced for TGF-β2, which, under hypoxia, induced the relative intensity of dermatan and heparan sulfates (Fig. 6A, lane D), compared with normoxia (Fig. 6A, lane B).

Culture Medium. Alcian Blue staining showed that hypoxia increased the relative intensity of heparan sulfate (Fig. 6B, lane C) compared with normoxia (Fig. 6B, lane A). Similar to the observation in the cell layer, all TGF-β isoforms altered the relative ratio of individual GAG molecules after 48 h of incubation, both under normoxia and hypoxia. This effect was most prominent for TGF-β3 which, under hypoxia, induced almost a 2-fold increase in the relative intensity of hyaluronic acid, dermatan sulfate, and chondroitin sulfate (Fig. 6B, lane D) compared with normoxia (Fig. 6B, lane B).

Discussion

In the present study, we provide evidence that hypoxia differentially enhanced the effects of TGF-β isoforms on the synthesis of specific GAG molecules associated with the cell layer, comprising cells and ECM, or secreted in the culture medium of primary human lung fibroblasts.
Morphological analysis of fibroblasts and determination of their phenotype indicated that there were no signs of cellular toxicity in response to hypoxic culture conditions employed (3% \( O_2 \)) or treatment with any of the TGF-\( \beta \) isoforms used. This is in agreement with previous results studying the effect of hypoxia or TGF-\( \beta \) isoforms on human pulmonary VSMC and/or fibroblasts (Tamm et al., 1998; Eickelberg et al., 1999; Papakonstantinou et al., 2000) and on human cardiac fibroblasts (Agocha et al., 1997).

Under normoxic conditions, all three TGF-\( \beta \) isoforms induced the synthesis of GAGs associated with the cell layer or GAGs secreted in the culture medium of human lung fibroblasts. Isolation, purification, fractionation, and enzymatic characterization of individual GAG molecules revealed the presence of hyaluronic acid, and heparan, chondroitin, and dermatan sulfates in both the cell layer and culture medium. These results are in agreement with reports demonstrating that TGF-\( \beta \) isoforms induce the production or expression of: 1) hyaluronic acid (Westergren-Thorsson et al., 1990); 2) membrane-bound dermatan and heparan sulfates, and hyaluronic acid and chondroitin and dermatan sulfates (Dubaybo and Thet, 1990) secreted by human lung fibroblasts; and 3) hyaluronic acid secreted by murine lung fibroblasts (Li et al., 2000).

With respect to the effects of hypoxia alone, it has been reported that hypoxia increases pulmonary fragmentation of chondroitin sulfate- and heparan sulfate-proteoglycans (Miserocchi et al., 2001) and heparan sulfate in bovine pulmonary artery endothelial cell cultures (Karlinsky et al., 1992). However, in human lung fibroblasts hypoxia did not significantly affect the incorporation of \[^3\text{H}]\text{glucosamine in total GAGs associated with the cell layer or secreted in the medium. Furthermore, hypoxia did not alter the nature of individual GAGs secreted or deposited, but it increased the relative amount of heparan sulfate secreted in the culture medium. In addition, hypoxia differentially enhanced the secretion of GAGs induced by individual TGF-\( \beta \) isoforms, in a time-dependent manner, apparently by increasing the secretion of hyaluronic acid, dermatan sulfate, and chondroitin sulfate. The order by which the TGF-\( \beta \)-induced GAG secretion was enhanced by hypoxia was TGF-\( \beta \_3 > \)TGF-\( \beta \_2 > \)TGF-\( \beta \_1 \). With respect to GAG deposition, hypoxia enhanced only the effect of TGF-\( \beta \_2 \) by inducing the synthesis mainly of dermatan and heparan sulfates.

Heparin was not identified among the GAGs secreted or deposited by human lung fibroblasts in response to hypoxia and/or TGF-\( \beta \) isoforms, even though it has been reported that the content of heparin increases in biopsy specimens from the lungs of patients with pulmonary fibrosis (Sasaki et al., 2000). It appears that pulmonary fibroblasts or VSMC, as we have previously shown (Papakonstantinou et al., 2000), are not responsible for the increased content of heparin in fibrotic lungs.

The increased secretion and/or deposition of dermatan and chondroitin sulfate subpopulations observed following hypoxia-TGF-\( \beta \) treatment may reflect the influence of these GAGs to different ends in fibrosis. In the ECM, dermatan sulfate bound to the small proteoglycan decorin is associated with collagen fibers (Scott, 1996), and it may provide additional strength by assisting in the orientation of these fibers. Chondroitin sulfate chains are more diverse in function. They constitute part of both small and large proteoglycans, such as biglycan and versican, respectively, that may be important in epithelial cell proliferation (Zimmermann et al., 1994) and in hyaluronic acid-medi-

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**Fig. 6.** Electrophoresis on cellulose acetate membranes of total GAGs isolated from the cell layer (A) or the culture medium (B) of cultures of primary human lung fibroblasts, which were cultivated in the absence (−) or presence (+) of TGF-\( \beta \_2 \) (A) or TGF-\( \beta \_3 \) (B) (1 ng/ml) under normoxia or hypoxia. Two microliters of the glycan solution containing about 4 µg of uronic acids were placed at the origin (10 mm from the cathode side) of a cellulose acetate strip. Electrophoresis was carried out in a mixture of 100 mM pyridine and 470 mM formic acid, pH 3.0, using 7 mA constant current, at room temperature, for 70 min. The cellulose acetate strips were then stained with 0.2% Alcian Blue (dissolved in 0.1% acetic acid) for 10 min and washed with 0.1% acetic acid for 20 min. Migration of commercially available markers (HA, hyaluronic acid; HS, heparan sulfate; DS, dermatan sulfate; CSC, chondroitin sulfate C) is indicated by arrows. GAG populations are indicated by arrows 1 to 4.
ated fibroblast aggregation and cell movement (Weber et al., 1996). It has also been reported that chondroitin sulfate increases cell proliferation (Terry and Clark, 1996) and may, thus, be associated with the pathophysiological manifestation of increased cell proliferation in lung fibrosis.

However, hyaluronic acid and dermatan sulfate may also have a protective role. We have previously shown that hyaluronic acid secreted by human lung VSMC (Papakonstantinou et al., 1995) acts as a negative regulator for VSMC proliferation and as a positive regulator for VSMC migration (Papakonstantinou et al., 1998a). Similarly, dermatan sulfate has been correlated with lowered rates of cell proliferation and cellular senescence (Passi et al., 1997). Heparin also exhibits antiproliferative activity on VSMC, suppresses vascular remodeling, and reduces the development of pulmonary hypertension in vivo (Thompson et al., 1994). Thus, it is possible that the deposition of molecules that inhibit lung cell proliferation, such as hyaluronic acid (Papakonstantinou et al., 1998a), dermatan sulfate (Passi et al., 1997), or heparin (Sasaki et al., 2000), may represent an autoregulatory mechanism by which lung cells counteract the effects of mitogenic stimuli associated with lung fibrosis (Moseley et al., 1986).

At the molecular level, hyaluronan may produce the above-described effects by inducing the production of growth factors, such as TGF-β itself. It has been reported that hyaluronan increases TGF-β1 mRNA levels in human dermal fibroblasts (Falanga et al., 1991), and mesothelial (Saed et al., 2000) and hepatoma cells (Patel et al., 1994), as well as TGF-β2 mRNA levels in human mesothelial cells (Saed et al., 2000). It has also been shown that hyaluronan increases TGF-β1 protein in human proximal tubular epithelial cells (Orphanides et al., 1997) and dermal fibroblasts (Falanga et al., 1991). However, caution is required in adopting this hypothesis for human lung fibroblast since the effect of hyaluronan on TGF-β production appears to be species- and tissue-specific. For instance, it has also been reported that hyaluronan did not significantly affect TGF-β1 mRNA expression in cultured fetal sheep and adult ewe dermal fibroblasts (Scheid et al., 2000) and TGF-β2 by human dermal fibroblast cultures (Falanga et al., 1991). Furthermore, in the normal or hypoxic human lung, it is mainly the bronchial epithelial cells and, to a lesser extent, alveolar macrophages and smooth muscle cells that are responsible for TGF-β production, whereas little (in the order of picograms) or no TGF-β is produced by other cell populations, including fibroblasts (Khalil and Greenberg, 1991; Magnan et al., 1994).

The molecular mechanism by which TGF-β isoforms induce the synthesis of GAGs may be attributed to interference with enzymes involved in their de novo synthesis and degradation, since it has been reported that TGF-β1 stimulation of lung fibroblasts and irradiation-evoked lung fibrosis in rats inhibit the activity of the rHYAL2 isoform of hyaluronidase and upregulate the activity of the rHAS2 isoform of hyaluronic acid synthase (Li et al., 2000). With respect to dermatan and chondroitin sulfates, it remains to be elucidated whether epimerases and sulfotransferases responsible for the differential synthesis of dermatan sulfate during the common biosynthetic pathway of dermatan and chondroitin sulfates (Coster et al., 1991) are affected by hyaluronic acid or TGF-β isoforms.

The rate of internalization of GAGs by fibroblasts is another feasible molecular target for hyaluronic acid or TGF-β isoforms, since it has been reported that there is increased internalization of hyaluronic acid by human lung fibroblasts (Sampson et al., 1992). Our findings indicate that this is not the case for hyaluronic acid, since the combination of hyaluronan and TGF-β isoforms did not increase its content associated with the cell layer. In contrast, it is possible that the increased secretion of dermatan and heparan sulfates in response to hyaluronic acid and TGF-β isoforms that we describe here is partially masked by the increased internalization of these GAG molecules by human lung fibroblasts.

Studies investigating the biological effects of different TGF-β isoforms demonstrated a considerable overlap of their activities. We have shown that all three TGF-β isoforms were almost equally potent in inducing the synthesis of GAGs by human lung fibroblast, in agreement with our previous report that TGF-β1 and TGF-β3 were equally effective in inducing collagen deposition by the same cell type (Eickelberg et al., 1999). However, the potency of TGF-β isoforms appears to be tissue-specific, since it has also been reported that TGF-β isoforms have distinct effects and/or potencies in vitro.

### Table 1

<table>
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<tr>
<th>Substrate</th>
<th>GAG Populationa</th>
<th>Chondroitinase ABC</th>
<th>Chondroitinase</th>
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<th>Heparinase</th>
<th>Heparitinase</th>
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a GAG populations are obtained following cellulose acetate electrophoresis, and numbers correspond to those of Fig. 4.

CSA, chondroitin sulfate A; DS, dermatan sulfate B; CSC, chondroitin sulfate C; H, heparin; HA, hyaluronic acid; HS, heparan sulfate; KS, keratan sulfate; (+), 100% degradation; (-), no detectable degradation.
In conclusion, characteristics of lung fibrosis include, among others, the activation of fibroblasts to synthesize and deposit ECM molecules in the lung interstitium, such as GAGs (Moseley et al., 1986; Sampson et al., 1992), the overexpression of TGF-β isoforms (Coker et al., 1997), and, inevitably, hypoxia (Schutte et al., 1996). The TGF-β-induced synthesis, secretion, and deposition of specific GAG molecules that we report here and the TGF-β-induced collagen deposition (Eickelberg et al., 1999) by cultures of primary human lung fibroblasts enlighten to possible differences of the fibrogenic potency among TGF-β isoforms. Furthermore, our results demonstrate that hypoxia augments the TGF-β-induced synthesis, secretion, and deposition of specific GAG molecules by human lung fibroblasts, reinforcing the concept that the manifestation of pathophysiological changes observed in interstitial lung diseases associated with hypoxia, such as lung fibrosis, is associated with changes in the content of certain GAGs. Thus, in lung fibrosis, hypoxia may hasten the development of the disease, by accelerating the accumulation of GAG molecules in the interstitium of injured lung. Our results also underline the possibility that TGF-β isoforms or the regulation of the homeostasis of specific GAG molecules may offer alternative pharmacological targets to prevent and/or treat the manifestation of ECM remodeling associated with interstitial lung diseases.

References


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