Hypoxia modifies the effect of PDGF on glycosaminoglycan synthesis by primary human lung cells

ELENI PAPAKONSTANTINOU,1 GEORGE KARAKIULAKIS,1 MICHAEL TAMM,2 ANDRÉ P. PERRUCHOUD,2 AND MICHAEL ROTH2
1Department of Pharmacology, School of Medicine, Aristotle University, 54006 Thessaloniki, Greece; and 2Departments of Research and Internal Medicine, University Hospital Basel, 4031 Basel, Switzerland

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Papakonstantinou, Eleni, George Karakiulakis, Michael Tamm, André P. Perruchoud, and Michael Roth. Hypoxia modifies the effect of PDGF on glycosaminoglycan synthesis by primary human lung cells. Am J Physiol Lung Cell Mol Physiol 279: L825–L834, 2000.—Hypoxia, a consequence of interstitial lung diseases, may lead to secondary pulmonary hypertension and pulmonary vascular remodeling. Hypoxia induces activation and proliferation of lung cells and enhances the deposition of extracellular matrix including glycosaminoglycans (GAGs). To elucidate the cell biological mechanisms underlying the development of secondary pulmonary hypertension, we studied the effect of hypoxia on GAG synthesis by human lung cells. GAG synthesis was measured by incorporation of [3H]glucosamine; GAGs were isolated, purified, and characterized with GAG-degrading enzymes. Fibroblasts and vascular smooth muscle cells (VSMCs) synthesized hyaluronic acid, heparan sulfate, and chondroitin sulfates, whereas dermatan sulfate was found only in fibroblasts. Hypoxia did not influence the size or charge of the individual GAGs. However, hypoxia inhibited platelet-derived growth factor-induced [3H]glucosamine incorporation in secreted GAGs, especially hyaluronic acid, in VSMCs. In contrast, it stimulated GAG secretion, specifically heparan sulfate, by fibroblasts. Our results indicate that hypoxia induces modifications in GAG synthesis by human lung VSMCs and fibroblasts that may be correlated to pathophysiological manifestations in lung diseases causing hypoxia.

platelet-derived growth factor; primary lung fibroblasts; primary lung vascular smooth muscle cells; pulmonary fibrosis; pulmonary hypertension

SECONDARY PULMONARY HYPERTENSION may result from hypoxia, which is often present in patients with interstitial lung diseases such as lung fibrosis. Secondary pulmonary hypertension is characterized by vascular remodeling of the bronchial arteriolar wall, which is attributed to abnormalities in vascular tone, hyperplasia of vascular smooth muscle cells (VSMCs) and fibroblasts, and enhanced deposition of extracellular matrix molecules and extensive fibrosis (13, 24). In pulmonary hypertension, the proliferation of VSMCs and the enhanced deposition of collagens result in structural changes of small vessels (24, 36). Hypoxia-mediated cell proliferation has also been demonstrated in cell cultures of different cell lines of rat and human origin and has been associated with the induction of several growth factors and cytokines, including platelet-derived growth factor (PDGF) (1, 8, 15, 31). Tamm et al. (31) have recently shown that PDGF is also involved in hypoxia-dependent expression of interleukin-6 and interleukin-8 in primary cultures of human lung VSMCs and fibroblasts, whereas neutralizing anti-PDGF antibodies significantly reduced the hypoxia-induced proliferation of fibroblasts and VSMCs. Thus it appears that PDGF plays a substantial role in the development of pulmonary hypertension and subsequent pulmonary vascular remodeling (6, 14).

The glycosaminoglycans (GAGs) represent one of the major components of the extracellular matrix. During neonatal lung growth (28), acute lung injury (3), and the development of pulmonary fibrosis (4), 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. It has also been shown that GAGs influence fibroblast migration and anchorage (9). Experiments with cell cultures of pulmonary origin reinforced the concept that the manifestation of pathophysiological changes observed in vascular remodeling in pulmonary diseases is associated with changes in the content of certain GAGs. For example, it has been demonstrated that cell cultures from bovine pulmonary artery endothelium exposed to hypoxia accumulated significantly less proteoglycans in the medium than cell cultures exposed to normoxia (10). Furthermore, the synthesis of heparan sulfate was reduced during exposure of endothelial cells obtained from bovine aorta to hypoxia (11). However, most of the relevant work on the effect of hypoxia on GAG synthesis performed so far has employed cell cultures of animal origin and was in the absence of cytokines. Papakonstantinou et al. (20) have recently...
shown that in primary human VSMC cultures, PDGF stimulates the synthesis of GAGs and, in particular, of a 340-kDa hyaluronic acid. This hyaluronic acid molecule inhibits the proliferation of VSMCs and enhances their migration (19).

Considering all the above evidence, we studied the effect of hypoxia on the PDGF-induced GAG synthesis by primary cultures of human lung VSMCs and fibroblasts to elucidate the mechanisms underlying the development of hypoxia-associated secondary pulmonary hypertension. We found that hypoxia reduced the relative amount of PDGF-induced hyaluronic acid secreted by VSMCs and increased the relative amount of PDGF-induced heparan sulfate secreted by fibroblasts.

MATERIALS AND METHODS

Human Pulmonary Artery VSMC Cultures

Primary cell cultures of VSMCs were established from pulmonary arteries obtained from patients undergoing a lobectomy or pneumonectomy for peripheral lung cancer. The arteries were kept overnight in Hanks' buffered salt solution (Seromed, Fakola, Basel, Switzerland) at 4°C before the intimal cell layer and residual adventitial tissue were stripped off with forceps. The remaining media of the vessels was cut into small pieces (3–5 mm) and transferred to prewetted cell culture flasks (Falcon, Inotech, Basel, Switzerland). The VSMCs were allowed to grow out by incubating the tissue for 1 wk in minimal essential medium (Seromed) supplemented with 5% fetal calf serum (FCS) and 20 mM HEPES buffer (boehringer Mannheim, Mannheim, Germany). In brief, the cells were grown in Lab-Tek tissue culture chamber slides (Miles Scientific Division, Naperville, IL) until confluence and fixed in 4% paraformaldehyde. Nonspecific protein binding was blocked by incubating the slides in PBS containing 0.5% BSA (Fluka Chemie, Buchs, Switzerland). The VSMCs were allowed to grow out by incubating the tissue for 1 wk in minimal essential medium (Seromed) supplemented with 5% fetal calf serum (FCS) and 20 mM HEPES buffer (both Seromed). No antibiotics or antifungamycotics were added to the culture medium at any time. The cells were subcultivated in the same medium after trypsin treatment, and experiments were performed between passages 2 and 6.

The VSMCs were characterized by immunohistochemical staining with monoclonal antibodies (MAbs) specific for either smooth muscle cell actin, keratin, fibronectin, laminin, or von Willebrand factor (all antibodies from Boehringer Mannheim, Mannheim, Germany). In brief, the cells were grown in Lab-Tek tissue culture chamber slides (Miles Scientific Division, Naperville, IL) until confluence and fixed in 4% paraformaldehyde. Nonspecific protein binding was blocked by incubating the cells in PBS (Seromed) supplemented with 0.5% BSA (Fluka Chemie, Buchs, Switzerland) for 20 min. The slides were then incubated with each of the above-described MAbs, washed three times with blocking buffer, and further incubated for 30 min with either fluorescein-coupled anti-rabbit IgG or anti-mouse IgG (Boehringer Mannheim). After being washed, the preparations were mounted with Fluorosave reagent and observed on a microscope as previously described (17).

Cell Culture Conditions

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% and replaced with N2, keeping CO2 constant at 5%. Before stimulation with PDGF, confluent cell cultures were serum deprived for 48 h with low-serum medium (RPMI 1640 medium supplemented with 0.1% FCS and 20 mM HEPES). To avoid autostimulation of the cells, low-serum medium was exchanged every 12 h. Subconfluent, quiescent cells were incubated under either hypoxic or normoxic conditions for 12, 24, or 48 h.

Incorporation of [3H]Glucosamine

Subconfluent primary pulmonary VSMCs or fibroblasts were incubated under normoxic or hypoxic conditions for 12, 24, or 48 h with 10 ng/ml of PDGF-BB in the presence of 0.5 μCi/ml of [3H]glucosamine (Amersham). Culture medium and cell layers (cells together with the extracellular matrix) were collected separately, and the incorporation of [3H]glucosamine was assessed as previously described (18).

Isolation and Purification of GAGs

Total glycans were isolated and purified from primary cultures of human pulmonary VSMCs and fibroblasts cultivated under normoxia or hypoxia 48 h after the addition of PDGF-BB as previously described (20). In brief, supernatants (25 ml) were collected separately, and cells with the associated extracellular matrix (cell layer) were washed twice with 10 ml of ice-cold PBS and harvested by scraping. Total glycans were isolated and purified from the cell layers or their respective supernatants as follows. Lipids were extracted with four volumes of chloroform-methanol (1:2 volume). Organic solvents were removed by centrifugation (3,200 g for 20 min at 4°C), and the pellet was washed with 10 ml of ethanol, centrifuged as described above, and dried at 40°C for 4 h. The pellet was resuspended in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM CaCl2 and subjected to protein digestion with 0.1 kilounits (KU) of Pronase (Streptomyces griseus; Calbiochem, Lucerne, Switzerland). The Pronase solution was preincubated for 30 min at 60°C to eliminate any glycosidase activity. Digestion was carried out for 72 h at 60°C by adding equal amounts of Pronase at 24-h intervals. The sample concentration was then adjusted to 150 mM NaCl and 10 mM MgCl2, and DNA digestion was accomplished by adding 400 KU of DNase I (EC 3.1.21.1; Calbiochem) and incubating for 16 h at 37°C. At the end of the incubation period, the CaCl2 concentration of the solution was adjusted to 1 mM, and the reaction was stopped by adding 0.1 KU of Pronase and incubating the mixture at 60°C for 24 h. The pH was then adjusted to 10.0–11.0 by the addition of 10 mM NaOH, and the glycans were subjected to β-elimination in the presence of 1 M NaBH4 for 16 h at 45°C. The samples were then neutralized with 50% (vol/vol) acetic acid. The glycans were separated from degradation products...
by gel filtration on a Sephadex G-25 column (0.6 × 25 cm) eluted with 10 mM pyridine acetate, pH 5.0. Fractions of 0.5 ml were collected and analyzed for their content of neutral hexoses (29) and uronic acids (2). Glyceran fractions were pooled, lyophilized, dissolved in double-distilled H2O, and stored at 4°C. Total glycans were also isolated and purified from unstimulated cell cultures and served as controls.

PAGE

Linear gradient (4–20%) polyacrylamide gels, 0.8 × 120 × 120 mm, were prepared as previously described (21). Gels were stained with a solution of 0.5% (wt/vol) Alcian blue (Fluka) that was dissolved in 25% (vol/vol) isopropyl alcohol and 1% (vol/vol) acetic acid for 12 h. The same solution without the dye was used for destaining. The intensity of the staining was quantified by scanning densitometry. Commercial GAGs, the molecular weight of which had been determined by analytical ultracentrifugation (21), were used as molecular weight markers.

Electrophoresis on Cellulose Acetate Membranes

Two microliters of the glycan solution containing ~3–4 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, under 7 mA constant current at room temperature for 70 min. After electrophoresis, the cellulose acetate strip was stained with 0.2% Alcian blue (wt/vol) in 0.1% acetic acid (vol/vol) for 10 min and washed with 0.1% acetic acid (vol/vol) for 20 min (22). The intensity of the staining was quantified by scanning densitometry.

Treatment of the Purified Glycans With GAG-Degrading Enzymes

Speed-dried glycans (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; Flavobacterium 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) for 6 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 (Flavobacterium heparinum; Sigma) for 6 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-β-n-galactosidase (EC 3.2.10.3; Pseudomonas species; Sigma) for 16 h at 37°C; and 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) for 14 h at 60°C.

The 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 (20 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) were treated individually with each of the above-mentioned GAG-degrading enzymes after appropriate incubation procedures. Substrates incubated separately with their respective buffers served as controls. Digestion was evaluated by PAGE and by electrophoresis on cellulose acetate membranes and quantified by scanning densitometry (22).

Statistical Analysis

Means ± SE were calculated from the results obtained from at least four different primary cultures of VSMCs and fibroblasts. Determinations were always made in triplicate. Statistical analysis was performed with ANOVA.

RESULTS

Characterization of Human Pulmonary Artery VSMC Cultures

Five primary cultures of VSMCs were established from human pulmonary arteries. Outgrowing cells could be observed after the second day of setting up the cultures surrounding several small pieces of the media of the vessels. After the first passage of the primary cell cultures, morphological analysis by light microscopy revealed exclusively VSMCs. Over serial passages up to passage 6, all cells stained positive for smooth muscle cell actin but were negative for immunostaining with MAbs against laminin, fibronectin, keratin, factor VIII, or von Willebrand factor (17) (data not shown). Therefore, no contamination with cells such as fibroblasts, epithelial cells, or endothelial cells was detected in any of the VSMC cultures used between passages 2 and 6 in the experiments performed.

Characterization of Human Pulmonary Fibroblast Cultures

Five primary cultures of human lung fibroblasts were established from small lung biopsies. First, outgrown cells surrounding a piece of solid lung tissue could be observed between days 2 and 6 after the cultures were set up. After the first passage of the primary cell cultures, morphological analysis by light microscopy revealed exclusively fibroblasts displaying typical spindle-shape morphology. Over serial passages up to passage 6, all spindle-shaped cells stained positive for laminin and fibronectin but were negative for immunostaining with MAbs against smooth muscle cell actin, keratin, factor VIII, or von Willebrand factor (17) (data not shown). Therefore, no contamination with cells such as VSMCs, epithelial cells, or endothelial cells was detected in any of the fibroblast cell cultures used between passages 2 and 6 in the experiments performed.

Effects of Hypoxia on the PDGF-Induced GAG Synthesis by VSMCs

Subconfluent, quiescent cells were incubated under either hypoxic or normoxic conditions for 12, 24, or 48 h. Morphological analysis of VSMCs with light microscopy and determination of their phenotype with immunostaining with specific MAbs indicated that
there were no signs of cellular toxicity in response to the hypoxic culture conditions employed (3% O₂).

Under normoxic conditions, PDGF-BB (10 ng/ml) significantly induced the incorporation of [³H]glucosamine into GAGs synthesized in the cell layer by subconfluent primary VSMCs obtained from human pulmonary arteries. Subconfluent cultures of VSMCs were cultivated under normoxic or hypoxic conditions for 12, 24, or 48 h in the presence and absence of 10 ng/ml of PDGF-BB. In all cases, 0.5 μCi/ml of [³H]glucosamine was included in the incubation medium. The amount of [³H]glucosamine incorporated into GAGs was measured in the cell layer (cells together with the extracellular matrix; A) and culture medium (B) as described in MATERIALS AND METHODS. Values are presented as percent of control (incubation in the absence of PDGF) and are means ± SE from duplicate determinations performed in 5 different cell lines. *P < 0.05. **P < 0.01 (both by ANOVA).

Under normoxic conditions, PDGF-BB (10 ng/ml) also significantly induced the incorporation of [³H]glucosamine into GAGs secreted in the culture medium of VSMCs after 12 h of incubation (P < 0.01; Fig. 1B). This effect of PDGF persisted up to 48 h of incubation. Hypoxic conditions inhibited the PDGF-induced GAG secretion to control levels after 12 and 24 h of incubation. However, after 48 h of incubation under hypoxia, the PDGF-induced glycan secretion recovered toward normoxic levels (Fig. 1B).

**Effects of Hypoxia on the PDGF-Induced GAG Synthesis by Fibroblasts**

Subconfluent, quiescent cells were incubated under either hypoxic or normoxic conditions for 12, 24, or 48 h. Morphological analysis of fibroblasts with light microscopy and determination of their phenotype with...
immunostaining with specific MAbs indicated that there were no signs of cellular toxicity in response to the hypoxic culture conditions employed (3% O₂).

Under normoxia, PDGF-BB (10 ng/ml) significantly induced the incorporation of [³H]glucosamine into GAGs synthesized in the cell layer by subconfluent primary fibroblasts obtained from human lungs in a time-dependent manner from 12 to 48 h of incubation ($P < 0.01$; Fig. 2A). Hypoxia did not significantly affect the normoxic PDGF-induced glycan synthesis in the cell layer of fibroblasts (Fig. 2A).

Under normoxia, PDGF-BB (10 ng/ml) also induced the incorporation of [³H]glucosamine into GAGs secreted in the culture medium of fibroblasts in a time-dependent manner from 12 to 48 h of incubation (Fig. 2B). However, this effect became significant only at 48 h of incubation ($P < 0.01$). Hypoxia significantly stimulated (~2.5-fold) the normoxic PDGF-induced GAG secretion in the culture medium of fibroblasts in a time-dependent manner from 12 to 48 h of incubation ($P < 0.01$; Fig. 2B).

**Isolation and Purification of GAGs From Human Lung Cells**

Total glycans were isolated from the cell layers and the cell culture medium of human lung VSMCs and fibroblasts after delipidation and sequential treatment with Pronase, DNase, and alkali borohydride. Purification of the total GAGs from the digestion products was achieved by gel filtration on a Sephadex G-25 column. Measurement of the uronic acid content of the fractions revealed that total GAGs were eluted as a single peak near the void volume of the column, with nearly 90% recovery. A typical set of results for total GAGs isolated and purified from the cell culture medium and the cell layers for both VSMCs and fibroblasts, respectively. The GAG elution profile was not significantly affected by the time of incubation. Fractions containing GAGs were pooled, lyophilized, dissolved in double-distilled H₂O, and stored at 4°C.

**Fractionation of Total GAGs by Electrophoresis on Cellulose Acetate Membranes and Characterization by GAG-Degrading Enzymes**

The isolated and purified total GAGs were further fractionated according to charge by electrophoresis on cellulose acetate membranes. Relative GAG ratios were monitored and compared by the intensity of the Alcian blue staining.

**VSMC cell layer.** Under normoxic conditions, cellulose acetate electrophoresis of 3 μg of uronic acids from the total GAGs isolated from the cell layers resulted in three distinct GAG populations that migrated with the same mobility as hyaluronic acid, heparan sulfate, and shown in Fig. 3. The same elution pattern was obtained for the total glycans isolated from the cell culture medium and the cell layers for both VSMCs and fibroblasts, respectively. The GAG elution profile was not significantly affected by the time of incubation. Fractions containing GAGs were pooled, lyophilized, dissolved in double-distilled H₂O, and stored at 4°C.

**Fig. 3.** Representative elution pattern of total GAGs isolated and purified from the culture medium of primary cultures of VSMCs obtained from human pulmonary arteries and cultivated for 48 h under hypoxic conditions on Sephadex G-25 column ($0.6 \times 25$ cm), eluted with 10 mM pyridine acetate, pH 5.0. Fractions of 0.5 ml were collected and analyzed for their content of uronic acids. Fractions 9–15 were pooled.

**Fig. 4.** Electrophoresis on cellulose acetate membranes of the total GAGs isolated from the cell layer (A) and culture medium (B) of primary cultures of VSMCs obtained from human pulmonary arteries and cultivated in the absence (−: lanes A and C) or presence (+: lanes B and D) of PDGF-BB under normoxia or hypoxia. Two microliters of the glycan solution containing ~3 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, under 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. HA, hyaluronic acid; HS, heparan sulfate; CSB, dermatan sulfate; CSC, chondroitin sulfate C. Arrowheads 1–3, GAG populations.
chondroitin sulfates (Fig. 4A, lane A). Enzymatic treatment with GAG-degrading enzymes (Table 1) confirmed that the uppermost GAG population (Fig. 4A, arrowhead 1) is hyaluronic acid because it was completely degraded only by hyaluronidase. The second GAG population (Fig. 4A, arrowhead 2) was completely degraded only by heparitinase, indicating heparan sulfate. The third GAG population (Fig. 4A, arrowhead 3) was completely susceptible only to chondroitinase ABC, indicating chondroitin sulfate A and/or chondroitin sulfate C. PDGF did not alter the nature or the relative ratio of GAGs (Fig. 4A, lane B) compared with the control value.

Hypoxia did not affect the control or the PDGF-induced relative GAG ratio (Fig. 4A, lanes C and D, respectively).

**VSMC culture medium.** The same migration pattern of GAGs as that described for the cell layer was also obtained for GAGs isolated from the culture medium of VSMCs incubated under normoxic or hypoxic conditions (Fig. 4B, lanes A and B, respectively). Enzymatic treatment with GAG-degrading enzymes (Table 1) indicated that GAGs secreted by VSMCs in the medium under normoxic or hypoxic conditions were hyaluronic acid, heparan sulfate, and chondroitin sulfates. However, PDGF increased the relative proportion of hyaluronic acid secreted in the medium of VSMCs under normoxic conditions (Fig. 4B, lane C). This effect of PDGF was not apparent under hypoxic conditions (Fig. 4B, lane D).

**Fibroblast cell layer.** Under normoxic conditions, 4 μg of uronic acids from the total GAGs isolated from the cell layer resulted in four distinct GAG populations that migrated with the same mobility as hyaluronic acid, heparan sulfate, dermatan sulfate, and chondroitin sulfates (Fig. 5A, lane A). Enzymatic treatment with GAG-degrading enzymes (Table 1) confirmed that the uppermost population (Fig. 5A, arrowhead 1) was hyaluronic acid because it was completely degraded only by hyaluronidase. The second GAG population (Fig. 5A, arrowhead 2) was completely degraded only by heparitinase, indicating a heparan sulfate structure. The third GAG population (Fig. 5A, arrowhead 3) corresponds to dermatan sulfate because it was completely degraded only by chondroitinase ABC and chondroitinase B. Finally, the fourth GAG population (Fig. 5A, arrowhead 4) was completely degraded only by chondroitinase ABC, indicating a structure of chondroitin sulfate A and/or chondroitin sulfate C. Under normoxic conditions, PDGF did not alter the nature or the relative ratio of GAGs (Fig. 5A, lane B) compared with the control value.

Hypoxia did not affect the relative proportions of GAGs obtained under normoxic conditions (Fig. 5A, lane C), but it considerably increased the PDGF-induced heparan sulfate synthesis (Fig. 5A, lane D).

**Fibroblast culture medium.** A similar migration pattern of GAGs as that described for the cell layer was also obtained for GAGs isolated from the culture medium of human lung fibroblasts incubated under normoxic conditions.
moxia or hypoxia (Fig. 5B, lanes A and C, respectively). Enzymatic treatment with GAG-degrading enzymes (Table 1) revealed that fibroblasts also secrete hyaluronic acid, heparan sulfate, dermatan sulfate, and chondroitin sulfates. PDGF under normoxia (Fig. 5B, lane B) or hypoxia (Fig. 5B, lane D) increased the secretion of hyaluronic acid in the culture medium compared with that in unstimulated cells. Furthermore, the relative amount of heparan sulfate secreted was increased under hypoxic conditions in both control and PDGF-stimulated cells (Fig. 5B, lanes C and D, respectively).

**PAGE**

The effect of hypoxia on the electrophoretic mobility of the total GAGs isolated and purified from human lung VSMCs and fibroblasts was investigated with PAGE (Fig. 6). The nature of the individual GAG populations was established with GAG-degrading enzymes, and the relative GAG ratios were monitored by the intensity of the Alcian blue staining.

VSMCs. PAGE analysis of total GAGs (15 μg of uronic acids) isolated from the culture medium or the cell layer under normoxic conditions resulted in three broad but distinct GAG populations with average molecular masses of 340, 65, and 25 kDa (Fig. 6A, lanes A and E). Enzymatic treatment with GAG-degrading enzymes revealed that the three glycan populations correspond to hyaluronic acid, chondroitin sulfates, and heparan sulfate (data not shown). PDGF or a hypoxic condition did not alter the electrophoretic mobility of the three GAG species (Fig. 6A). However, PDGF under normoxic conditions increased the relative proportion of hyaluronic acid that was secreted in the culture medium (VSMCs, Fig. 6B, lane C). Enzymatic analysis of the Alcian blue staining indicated that this increase was in the range of 25%, which is in good agreement with the metabolic labeling results (Fig. 1B). Hypoxia inhibited the above-described PDGF-induced effect (Fig. 6A, lane D).

**Fibroblasts.** PAGE analysis of total GAGs (20 μg of uronic acids) isolated from the culture medium or the cell layer under normoxic conditions resulted in three broad but distinct GAG populations with average molecular masses 340, 65, and 25 kDa (Fig. 6B, lanes A and E). Enzymatic treatment with GAG-degrading enzymes revealed that the 340- and 65-kDa glycan populations correspond to hyaluronic acid and chondroitin sulfates, respectively, whereas the 25-kDa glycan population corresponds to a mixture of heparan sulfate and dermatan sulfate (data not shown). The relative amount of heparan sulfate in the cell layer was higher compared with that in the culture medium. PDGF or hypoxia did not alter the electrophoretic mobility of the identified glycan species. However, PDGF increased the secretion of hyaluronic acid in the culture medium under both normoxia and hypoxia (Fig. 6B, lanes B and D) compared with that in unstimulated cells.

**DISCUSSION**

In the present study, we provide evidence on the effects of hypoxia on the PDGF-induced synthesis of GAGs by primary cultures of human pulmonary fibroblasts and VSMCs and report that hypoxia differentially affects the PDGF-induced GAG synthesis by these types of human lung cells. The use of VSMCs and fibroblasts that were obtained from surgically removed lung tissue from the same individual precludes the influence of parameters such as age, drug treatment, or disease state, which may otherwise interfere with the interpretation of the results. We found that under normoxic conditions PDGF induced the synthesis of GAGs associated with the cell layer of human lung primary VSMCs as well as the secretion of GAGs in the culture medium. Hypoxia did not significantly affect the normoxic effect of PDGF on the synthesis of GAGs associated with the cell layer, but it considerably delayed the normoxic effect of PDGF on the secretion of GAGs. Analysis of the total GAGs associated with the cell layer or secreted in the culture medium by electrophoresis on cellulose acetate membranes and poly-
acrylamide gels and characterization by GAG-degrading enzymes revealed hyaluronic acid, heparan sulfate, and chondroitin sulfates. Hypoxia did not influence the size or charge of the individual GAGs. However, hypoxia reduced the normoxic PDGF-induced relative amount of hyaluronic acid.

Papakonstantinou and colleagues have previously shown that PDGF stimulates the secretion by human lung VSMCs of a hyaluronic acid molecule (20), which acts as a negative regulator for VSMC proliferation and as a positive regulator for VSMC migration (19). Heparin, a GAG that also exhibits antiproliferative activity on VSMCs, inhibits vascular remodeling and reduces the development of pulmonary hypertension in vivo (7, 30), with an efficiency directly related to the antiproliferative potency of heparin preparations (34). Thus it is possible that deposition by lung cells of molecules that inhibit lung VSMC proliferation, such as hyaluronic acid (19), may represent an autoregulatory mechanism by which VSMCs downregulate the effects of mitogenic stimuli. Hypoxia-associated pulmonary hypertension is characterized by, among other things, expression of cytokines and growth factors such as PDGF (1, 8, 31) that enhance the proliferation of VSMCs in small vessels (24, 36) and eventually lead to concentric media hypertrophy (12). The enhanced VSMC proliferation consequent to the hypoxia-induced expression of growth factors such as PDGF correlates with the hypoxia-induced reduction in the synthesis of antiproliferative matrix molecules such as hyaluronic acid.

The molecular parameters for this hypoxia-induced effect on hyaluronic acid synthesis by VSMCs could be attributed to increased internalization and/or degradation of hyaluronic acid. It remains to be clarified whether human lung VSMCs can internalize hyaluronic acid as has been reported to be the case for alveolar macrophages (32, 35) and fibroblasts (27). We are currently investigating the effect of hypoxia on intracellular or secreted hyaluronidase activity in lung cells. Furthermore, the hypoxia-induced effect on GAG synthesis by VSMCs and fibroblasts should be extended to include myofibroblasts obtained from fibrotic lung biopsies as well as the recently reported phenotypically heterogeneous subpopulations of VSMCs of pulmonary arteries, which may contribute differentially to the pathogenesis of pulmonary hypertension (5) or other lung diseases.

In fibroblasts incubated under normoxic conditions, PDGF also induced the synthesis of GAGs in a time-dependent manner. Hypoxia did not significantly affect the normoxic effect of PDGF on GAG synthesis associated with the cell layers. However, hypoxia significantly stimulated, in a time-dependent manner, the normoxic PDGF-induced GAG secretion in the culture medium. Characterization of the total GAGs secreted revealed hyaluronic acid, heparan sulfate, chondroitin sulfates, and dermatan sulfate. Hypoxia did not influence the size or charge of the individual GAGs. In contrast, hypoxia increased the normoxic PDGF-induced relative amount of heparan sulfate. Some of the characteristics of fibrotic lung injury include the pro-

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Fig. 6. PAGE on 4–20% linear gradient gels of total GAGs isolated and purified from primary cultures of VSMCs (15 μg of uronic acids aliquots; A) or fibroblasts (20 μg of uronic acids aliquots; B) that were cultivated in the absence (lanes A, C, E, and G) and presence (lanes B, D, F, and H) of PDGF under normoxic or hypoxic conditions. After electrophoresis, gels were stained with Alcian blue. H, heparin. Nos. at left, molecular mass.
liferation (16) and activation (12, 16, 27, 33) of fibroblasts and deposition of GAGs in the lung interstitium. Thus hypoxia-induced expression of PDGF, cell proliferation, and secretion of GAGs, mainly of heparan sulfate, may contribute to the development of secondary pulmonary hypertension. Dermatan sulfate may also be important in hypoxia-associated tissue remodeling because it has been reported to induce fibroblast proliferation (23).

The results presented in this study indicate that hypoxia-induced alterations in the synthesis of GAGs by human lung cells may be associated with the lung tissue remodeling observed in hypoxia-induced pulmonary hypertension. Furthermore, hypoxia-induced alterations in the synthesis of GAGs may be responsible for initiating a vicious circle by exacerbating preexisting interstitial lung diseases such as fibrosis that lead to hypoxia. It remains to be clarified whether regulation of the homeostasis of specific GAG molecules may be used as an additional therapeutic strategy to prevent and/or treat the manifestation of vascular remodeling associated with pulmonary hypertension.

REFERENCES


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