Fetal Lung Fibroblasts Selectively Down-regulate Proteoglycan Synthesis in Response to Elevated Oxygen*

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Cell proliferation is in part regulated by extracellular matrix. Therefore, it is possible that elevated O₂ may indirectly affect lung fibroblast growth via modulation of extracellular matrix. In the present study, we investigated the effect of elevated O₂ on the synthesis of glycosaminoglycans (GAGs) and proteoglycans (PGs) by fetal lung fibroblasts. A 48-h exposure to 50% O₂ reduced the incorporation of [³H]glucosamine and [³⁵S]sulfate into GAGs by fetal lung fibroblasts. The relative proportion of the individual GAG molecules was not altered by elevated O₂. Fibroblasts exposed to 50% O₂ secreted less [³⁵S]proteoglycans into the medium than controls. Specifically, the synthesis of the small soluble PG, biglycan, was decreased by exposure to 50% O₂. Fetal lung fibroblasts did not synthesize the small chondroitin/dermatan sulfate PG, decorin. Elevated O₂ concentrations also reduced the synthesis of membrane- and matrix-associated PGs. Furthermore, exposure of fetal lung fibroblasts to 50% O₂ resulted in a decreased mRNA expression for biglycan and versican core protein sequences. In contrast, elevated O₂ increased the message for type I collagen and fibronectin without affecting that of β-actin. The inhibitory effect of elevated O₂ on biglycan mRNA and protein expression was overcome by incubating the cells in 3% O₂ after the 48-h exposure to 50% O₂. The latter treatment also reversed the increased mRNA expression of type I collagen associated with elevated O₂ but not that of fibronectin. These results demonstrate that fetal lung fibroblasts, in response to elevated oxygen concentrations, selectively down-regulate their GAG and PG synthesis and that this O₂ effect is reversible.

Disruption of cell-matrix interactions during lung development may induce cellular responses, which can result in scar formation. Administration of high concentrations of oxygen has been shown to induce changes in extracellular matrix (1). Glycosaminoglycans (GAGs)

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1The abbreviations used are: GAG, glycosaminoglycan; PG, proteoglycan; TGF, transforming growth factor; kb, kilobase(s); HPLC, high performance liquid chromatography; HBSS, ( ), Hanks’ buffered salt solution without calcium or magnesium; MEM, minimal essential medium; PBS, phosphate-buffered saline.
O2 Effect on PG Synthesis

EXPERIMENTAL PROCEDURES

Materials—Female (200–250 g) and male (250–300 g) Wistar rats were purchased from Charles River (St. Constant, Quebec) and bred in our animal facilities. The sources of all cell culture material have been described elsewhere (17). Na235SO4 ([35S]dCTP, and Hybond N membranes were from Amersham Canada. Hyaluronidase, chondroitinase ABC, and heparitinase were from Seikagaku America (Rockville, MD). Human biglycan (1.7-kb) and decorin (1.6-kb) cDNAs were generous gifts from Dr. L. W. Fisher, (National Institutes of Health, Bethesda, MD). Human versican cDNA (1.3 kb) was from Telios (San Diego, CA). Procollagen (α1 type I) (1.8-kb) cDNA was provided by Dr. F. Ramirez (University of New Jersey, Piscataway, NJ) and the 0.5-kb rat fibronectin cDNA fragment by Dr. R. Hynes (Center for Cancer Research, Cambridge, MA). Rat β-actin cDNA (0.7 kb) was generated by reverse transcription-polymerase chain reaction cloning using rat β-actin primers (Clontech, Palo Alto, CA). Other materials were from Sigma unless otherwise stated. High performance liquid chromatography (HPLC) was performed on a Rainin (Woburn, MA) HPLC system with a 7.5 × 7.5-mm DEAE-S-PW column (Anachem, Luton, UK).

Cell Culture—Rats were sacrificed by diethylthiobis (on day 19 of gestation (term = 22 days). The fetuses were aseptically removed from the mothers, and the fetal lungs were dissected out in cold Hank’s balanced salt solution without calcium or magnesium (HBSS) (−) and cleared of major airways and vessels. The lungs were washed twice in HBSS (−), minced, and suspended in HBSS (−). Fibroblasts were isolated from the fetal lungs as described in detail previously (17) except that cells were grown in a gas phase of 3% O2, 5% CO2, 92% N2 to mimic the normal fetal arterial oxygen concentration (~20 mm Hg observed in vivo). The purity of the fibroblast cultures were comparable with previously published data (17).

Effect of O2 on GAG Synthesis—To investigate the effect of O2 on GAG synthesis, cells grown in 75-cm2 tissue culture flasks were trypsinized and diluited in Eagle’s minimal essential medium (MEM) with 10% (v/v) fetal bovine serum in 3% O2 and were then maintained in serum-free MEM containing either 5% CO2, 92% N2, or 0% O2. GAG synthesis by fetal rat lung fibroblasts. The samples were dissolved in 150 mM Tris, pH 8.3, 10 mM Na2EDTA, 3,500) in the presence of 1 mM phenylmethylsulfonyl fluoride. The remaining extracellular matrix fraction was rinsed with PBS and treated with either chondroitinase ABC or heparitinase to detect glycosaminoglycan linkage. The solutions were then neutralized (pH 6.8) with 2 N HCl. A aliquots of 1 µl of labeled media samples, cell membrane fraction, and extracellular matrix fraction were then incubated in PBS with or without chondroitinase ABC (1000 units/ml) or heparitinase (200 units/ml) at 37°C. After a 24-h incubation, samples were boiled in SDS-sample buffer and fractionated on 5% (w/v) SDS-polyacrylamide gel. Gels were fixed in 10% (v/v) acetic acid, prepared for fluorography by soaking in En3Hance (DuPont NEN), dried, and exposed to Kodak X-AR-5 film using Dupont Cronex intensifying screens. The films were quantified using an Ultrascan XL laser densitometer (Pharmacia Biotech Inc.).

Northern Analyses—Total cellular RNA was isolated from the fibroblast cultures by lysing the cells in 4 mL guanidinium thiocyanate followed by centrifugation on a 5.7 M cesium chloride cushion to pellet RNA. This total RNA (15 µg) was size-fractionated on a 1% (w/v) agarose gel in 1.5× TBE buffer. The RNA bands were transferred onto nylon membranes and immobilized by UV cross-linking. The cDNA probes were labeled with [35S]dCTP using random primers. Prehybridization and hybridization were performed in 50% (v/v) formamide, 5 × SSPE, 0.5% (v/v) SDS, 5 × Denhardt’s solution, and 100 µg/ml denatured salmon sperm DNA at 42°C. Following hybridization, the blots were washed with 0.5 × SSC containing 0.2% (w/v) SDS at 65°C and autoradiographed with Kodak XAR-5 film overnight at −70°C. Blots were then stripped and, for normalization, prehybridized with radiolabeled rat β-actin cDNA. The autoradiographs were quantified using an Ultrascan XL laser densitometer (Pharmacia).

Statistical Analysis—Statistical significance (p < 0.05) was determined by analysis of variance followed by assessment of differences using Dunnet’s two-sided test (22) or Duncan’s multiple range test (23).

RESULTS

Effect of O2 on GAG Synthesis—Exposure of fetal lung fibroblasts to 50% O2 for 48 h reduced the incorporation of [3H]glucosamine into total GAGs by ~40% without changing the number of cells adhered to the plastic (Fig. 1). A 95% O2 exposure reduced both cell number and GAG synthesis. However, GAG synthesis of cells exposed to 95% O2 was not further reduced from that of fibroblasts exposed to 50% O2. The relative distribution of GAGs between medium and cell-matrix layer was not.
Effect of elevated O2 concentrations on synthesis of individual GAG molecules by fetal rat lung fibroblasts. Glucosamine-labeled GAGs were separated by HPLC using a DEAE column. HA, hyaluronan; HS, heparan sulfate; CS, chondroitin sulfate. Values are means ± S.E., n = 3 independent experiments each carried out in triplicate.

O2 Effect on PG Synthesis

Effect of O2 on PG Synthesis—Fetal lung fibroblasts, grown in 3% O2, synthesized and deposited hyaluronan, heparan sulfate, and chondroitin/dermatan sulfate in the cell-matrix compartment. Heparan sulfate was the main GAG component secreted into the medium. The effect of elevated O2 on the individual GAG molecules produced by the fibroblasts is shown in Fig. 2. Although total GAG synthesis decreased, the composition of GAG molecules in the medium and in the cell-matrix layer was not significantly changed by exposing the cells to either 50% or 95% O2.

Effect of O2 on Soluble PG Synthesis—As exposure to 95% O2 affected cell number (Fig. 1) and cell viability (41), we carried out all further experiments at 50% O2. Confluent fibroblast cultures were incubated with [35S]SO4 to label the PGs, and the conditioned medium was analyzed by SDS-polyacrylamide gel electrophoresis to identify the soluble PGs. The results demonstrated that fetal lung fibroblasts synthesized [35S]sulfate-labeled macromolecules with high Mw values that did not enter into the gel and remained on top of the separating gel (Fig. 3). In addition, the fibroblasts synthesized [35S]sulfate-labeled macromolecules with relatively small molecular weights (Fig. 3). The approximate Mw ranges from 190,000 to 250,000, which is in the same range as that of biglycan (24–26). The [35S]sulfate-labeled macromolecules in this band were sensitive to chondroitinase ABC lyase digestion, suggesting that the PGs in this band belong to the chondroitin/dermatan sulfate type. The [35S]sulfate-labeled macromolecules that remain on top of the gel were partially degraded by chondroitinase ABC lyase. Heparitinase treatment reduced the intensity of the large Mw band but not that of the biglycan band. Thus, it is likely that this band represents the large PGs, versican and heparan sulfate PGs. No sulfated band with a molecular mass of approximately 100–150 kDa, representing the small chondroitin/dermatan sulfate proteoglycan decorin (24–26), was synthesized by the fibroblasts. Exposure of fetal lung fibroblasts to 50% O2 decreased the incorporation of [35SO4] into both biglycan and larger PGs by 3.4 ± 0.6- and 3.3 ± 0.7-fold (mean ± S.E., n = 3 separate experiments), respectively (Fig. 3). To evaluate reversibility, fetal lung fibroblasts were first exposed for 48 h to 50% O2 and then for 48 h to 3% O2. As can been seen in Fig. 4, the incorporation of [35SO4] into biglycan in these cells was similar to that of cells exposed to 3% O2, suggesting that the effect of elevated O2 on PG synthesis is reversible.

Effect of O2 on PGs in Matrix and Cell Membrane—To examine the effect of O2 on PGs associated with the cell membrane or extracellular matrix, fibroblasts were incubated in 3 or 50% O2 and labeled with [35SO4]. The membrane fraction of fetal lung fibroblasts was extracted by 2% (v/v) Triton X-100, and the remaining fraction was the extracellular matrix fraction. The cell-associated (membrane) fraction also contained [35S]biglycan and [35S]sulfate-labeled large PGs (Fig. 5). A major portion of the large macromolecules was resistant to chondroitinase ABC but not to heparitinase digestion, suggesting that cell-associated (membrane) PGs are mainly heparan sulfate PGs. Exposure of fibroblasts to 50% O2 decreased the synthesis of cell-associated (membrane) PGs. Besides [35S]biglycan, the cell-matrix fraction was also found to contain PGs with high Mw values (Fig. 5). The large PGs were partly degraded by chondroitinase ABC but completely by heparitinase, suggesting that extracellular matrix PGs consist of large chondroitin sulfate as well as heparan sulfate PGs. Again, fibroblasts exposed to 50% O2 incorporated less [35SO4] into PGs associated with the matrix fraction. The O2 effect on membrane and extracellular matrix PGs was also reversed by incubating the cells in 3% O2 after the 50% O2 exposure (not shown).

Effect of O2 on PG Gene Expression—To determine whether the alterations noted in [35S]PG synthesis by elevated O2 concentrations might be accompanied by similar changes in mRNA expression for PGs, total mRNA was extracted from fetal lung fibroblasts and hybridized with cDNAs coding for three different PG core proteins (Fig. 6). The biglycan cDNA detected a single mRNA transcript of 2.5 kb, consistent with biglycan mRNA transcript sizes reported for other cell types (26, 27). Expression of biglycan did not change with time in culture. Exposure of fibroblasts to 50% O2 for 24 h resulted in a 4-fold decrease in biglycan mRNA levels (Fig. 7). Longer exposures to 50% O2 (48–72 h) or exposures to higher concentrations of O2 (90%) did not result in a further decrease in biglycan mRNA expression. In contrast to biglycan probe, a cDNA for decorin did not hybridize to mRNA of fetal lung fibroblasts (Fig. 6). The decorin cDNA detected, however, a transcript of 1.9 kb in fetal fibroblasts. Exposure of fetal lung fibroblasts to 50% O2 for 24 h resulted in a 4-fold decrease in decorin mRNA levels (Fig. 8). Longer exposures to 50% O2 (48–72 h) or exposures to higher concentrations of O2 (90%) did not result in a further decrease in decorin mRNA expression.
skin fibroblasts. This finding and the observation that fetal lung fibroblasts did not incorporate detectable amounts of $^{35}$SO$_4$ into chondroitinase ABC-sensitive macromolecules with $M_r$ of 120,000–160,000 suggest that fetal lung fibroblasts do not synthesize decorin. The effect of O$_2$ on the expression of the large fibroblast proteoglycan, versican, core protein gene was also examined in the same mRNA preparations by Northern hybridizations. The versican cDNA hybridized to a 10-kb mRNA transcript (Fig. 6), a size identical to that of versican mRNA from several other cell types previously described (28). The versican mRNA expression in fetal lung fibroblasts did not change with time in culture. However, a 2-fold decrease in versican mRNA levels was noted in fibroblasts cultured for 24 h in a gas phase of 50% O$_2$ (Fig. 7). Rehybridization of the filters with $\beta$-actin cDNA did not reveal any significant differences in $\beta$-actin mRNA levels (Figs. 6 and 7). To examine whether the decrease of PG mRNA by elevated O$_2$ was a general metabolic effect, total RNA from fibroblasts exposed for 48 h to 3 or 50% O$_2$ was also hybridized with cDNAs coding for procollagen type I and fibronectin. Again, exposure to 50% O$_2$ reduced biglycan mRNA expression (Fig. 8). In contrast, procollagen type I and fibronectin mRNA expression were up-regulated by elevated O$_2$ (Fig. 8). As mentioned previously, reversibility was tested by exposing fetal lung fibroblasts first to 50% O$_2$ and then to 3% O$_2$. In these cells, message levels for biglycan and type I collagen returned to that of control. However, fibronectin gene expression increased even further (Fig. 8). $\beta$-Actin gene expression was not affected by these treat-
these first weeks or months of life. Chronic exposure to ele-
ments (Fig. 8). These findings suggest that elevated O₂ selec-
tively down-regulates PG mRNA expression in fetal lung fibro-
basts and that the O₂ effect on PG synthesis is reversible.

**DISCUSSION**

Increasing evidence suggests that cell-matrix interactions play an important role in lung morphogenesis. Several studies suggest that matrix molecules can alter the growth of cells, with some components increasing and others decreasing pro-
fibronectin (44–46). To our knowledge, no studies have re-
declined by the action of xanthine oxidase on hypoxanthine, has
We have previously reported growth inhibition of cultured fet-
algrowth and to cause pulmonary fibrosis in these infants (30, 31).

In the present study, we report that elevated O₂ concentra-
tions decrease the synthesis of GAGs and PGs by fetal lung fibro-
basts. Using metabolic labeling with Na₂³⁵SO₄, we
showed that biglycan was the most abundant proteoglycan
secreted into the medium by fetal lung fibroblasts. In contrast
to several different types of cells, including human skin and
gingival lung fibroblasts (32), fetal rat lung fibroblasts did not
synthesize the small soluble proteoglycan, decorin. Cultured
bovine aortic endothelial cells (27), human umbilical vein endo-
theal cells (27), and rat pleural mesothelial cells (33) have also
been found to express biglycan but not decorin. The lack of
decorin synthesis may be due to the use of primary cell cul-
tures, because the human embryonic lung fibroblast cell line,
HFL-1, has been shown to synthesize decorin (34). Although
cell culture findings should be interpreted with caution, it is
likely that the absence of decorin synthesis has a functional
role in late fetal lung development. It should be noted that
under similar experimental conditions as used for fetal rat lung
fibroblasts, fetal rat skin fibroblasts expressed decorin. Thus, it
is unlikely that the decorin gene is silenced by methylation of
the control regions in isolated fetal lung fibroblasts. The exact
role of soluble PGs is unknown, but they have been implicated in
elastin and fibronectin (44–46). To our knowledge, no studies have re-
present temporal relationship of PG and collagen in an O₂
toxicity model of pulmonary fibrosis. In the present study, we
found that fetal lung fibroblasts increased type I collagen and
fibronectin mRNA expression in response to elevated concen-
trations of O₂, suggesting that fetal lung fibroblasts selectively
up-regulate and down-regulate gene expression of individual
extracellular matrix molecules. The observation that the effect
of elevated O₂ concentrations on PG and type I collagen gene
expression was reversible but that on fibronectin mRNA ex-
pression was not strongly supports such selective regulation of
extracellular matrix expression by fetal lung fibroblasts in
response to O₂.

The exact mechanism by which oxygen may modulate GAG
and PG synthesis of fetal lung fibroblasts is not known. The
inhibition of GAG and PG synthesis by 50% O₂ is not due to the
loss of cell viability, and the cell number remained constant.
Furthermore, we have previously demonstrated that fetal lung
fibroblasts developed tolerance to 50% O₂, as measured by LDH
release (37). Similarly, an alteration of ³⁵SO₄ incorporation
induced by elevated oxygen is not due to the changes in sulfo-
transferase activities because [³⁵S]glucosamine incorporation
into GAGs was also altered. The toxic effects of oxygen are
believed to be initiated through increased intracellular gener-
ation of partially reduced oxygen species. Superoxide, gener-
ated by the action of xanthine oxidase on hypoxanthine, has
been reported to decrease the synthesis of PGs in cultured
bovine chondrocytes (16), bovine articular cartilage explants
(47), and isolated perfused rat kidneys (48). Superoxide also
damaged PG molecules at the level of the core protein, while
GAG side chains were resistant to free radical attack (16). In
the present study, we did not investigate whether elevated
concentrations of O₂ damaged intact PGs synthesized by fetal
lung fibroblasts. However, the parallel decreases in PG mRNA and 35SO4 incorporation into PGs make it unlikely that the observed decrease in PGs is due to such an action by reactive oxygen species. Consistent with our findings, superoxide has been shown to increase collagen synthesis in the human fetal fibroblast cell line, 1MR-90 (49).

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