Journal of Cellular Biochemistry 77:298–309 (2000)

Alteration of Proteoglycan Synthesis in Human Lung Fibroblasts Induced by Interleukin-1β and Tumor Necrosis Factor-α

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Abstract Important constituents of extracellular matrix are collagen, fibronectin, hyaluronan, and various types of proteoglycans, such as versican, perlecan, decorin, and biglycan. Remodeling of extracellular matrix occurs continuously and is affected by various cytokines. The aim of this study was to investigate how interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), separately and in combination, alter fibroblast proliferation, as well as the production of extracellular matrix molecules produced by human fibroblasts from lung. Fibroblast proliferation was inhibited significantly by all treatments, by 12% with IL-1β and by 16% with TNF-α. The combination of IL-1β and TNF-α increased the inhibition further, by 27%. Hyaluronan production was increased by all treatments: 1.7-fold by IL-1β and 1.5-fold by TNF-α. The combination of the two gave a further increase (2.5-fold). Similarly, the production of total proteoglycans was increased. The small proteoglycans, decorin, and biglycan, were regulated differently. Decorin production was inhibited by about 34% by all treatments, while biglycan was upregulated 1.3-fold by TNF-α. Versican was upregulated by IL-1β (1.7-fold), whereas TNF-α was without effect. Perlecan was mostly unaffected. The changes in protein production of the various proteoglycans were due to increased transcription, as mRNA levels were also changed to the same extent. Synthesis of mRNA for collagen type I was inhibited by up to 75% with the IL-1β/TNF-α combination. The separate cytokines also decreased the level of collagen type I mRNA, but to a lesser extent: 60% with IL-1β and 40% with TNF-α. In summary, our study indicates that these proinflammatory cytokines affect the regulation of extracellular matrix production, which is of importance for the inflammatory process. J. Cell. Biochem. 77:298–309, 2000.

Key words: fibroblast; hyaluronan; interleukin-1β; proteoglycan; tumor necrosis factor-α

Extracellular matrix is composed of a collection of macromolecules surrounding the cells and comprising a substrate to which cells may be attached. The extracellular matrix molecules are produced mainly by fibroblasts and interact with resident and invading cells, playing an important role in modulation of the connective tissue. In addition to fibrous proteins, such as collagen and fibronectin, hyaluronan and proteoglycans are important constituents of the extracellular matrix. The large proteoglycans produced by lung fibroblasts are mainly versican and perlecan [Tiedemann et al., 1997]. Also produced are decorin and biglycan, which belong to the family of small proteoglycans [Iozzo, 1997]. The proteoglycans interact with many components in the extracellular matrix, forming a network. Decorin is known to bind to collagen types I, III, and VI, while biglycan interacts only with collagen VI [Hedbom and Heinegård, 1993]. Variation in the relative composition of the different proteoglycans thus influences the organization and thereby the properties of the matrix.

Abbreviations used: CTGF, connective tissue growth factor; EGF, epidermal growth factor; GAG, glycosaminoglycan; GAPDH, glyceraldehyde phosphate dehydrogenase; GuHCl, guanidinium hydrogen chloride; HIC, hydrophobic interaction chromatography; HSPG, heparan sulfate proteoglycan; IL-1β, interleukin-1β; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

Grant sponsor: Swedish Medical Research Council; Grant number: 11550; Grant sponsor: Swedish Cancer Fund; Grant sponsor: Swedish Society for Medical Research; Grant sponsor: J.A. Persson Foundation; Grant sponsor: G. Nilsson Foundation; Grant sponsor: Greta and John Kock Foundation; Grant sponsor: A. Österlund Foundation; Grant sponsor: Anna-Greta Crafoord Foundation; Grant sponsor: Swedish Rheumatism Association; Grant sponsor: Gustaf V 80 Years Fund; Grant sponsor: Heart-Lung Foundation; Grant sponsor: Medical Faculty, University of Lund.

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Received 30 June 1999; Accepted 8 November 1999

Remodeling of the extracellular matrix occurs continuously as part of physiological processes such as development, growth, and aging. It also occurs in pathophysiological processes such as wound healing, inflammation [Westergren-Thorsson et al., 1993a], and fibrosis [Bensadoun et al., 1996]. Factors involved in the regulation of extracellular matrix remodeling include cytokines, such as transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF), which are known to be potent regulators of the inflammatory process. Other cytokines that are also of importance are the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).

IL-1β and TNF-α have overlapping biological functions and often work synergistically. They also induce production of each other [Dinarello et al., 1986]. It has previously been shown that they alter the synthesis of extracellular matrix molecules, the effects varying, depending on the type of cells involved. It is well established that mainly IL-1β, but also TNF-α, have inhibitory effects on the production of extracellular matrix molecules in cartilage; a decrease in hyaluronan and proteoglycan [Yaron et al., 1989], versican [Qwarnström et al., 1993], decorin, and biglycan [von den Hoff et al., 1995], as well as an increase in protease activity [Hardingham et al., 1992]. By contrast, little is known about the effects of IL-1β and TNF-α on lung fibroblasts, but these cytokines seem to have more stimulatory effects on the production of extracellular matrix molecules. Effects observed in lung fibroblasts are increases in hyaluronan synthetase activity, hyaluronan synthesis [Sampson et al., 1992] and proliferation [Elias, 1988]. These cytokines also elevate the level of type VII collagen mRNA [Chen et al., 1994]. However, both IL-1β and TNF-α decrease the level of type I and III collagen [Mauviel et al., 1994], as well as collagenase gene expression.

We can conclude that IL-1β and TNF-α have different effects on hyaluronan and proteoglycan synthesis in different cells, and even in different tissues. The aim of this study was to investigate how IL-1β and TNF-α, separately and in combination, affect the synthesis of different types of proteoglycans, as well as hyaluronan, in human lung fibroblasts in culture. We also wanted to study how these cytokines affect fibroblast proliferation, and thereby indirectly the production of proteoglycans.

MATERIALS AND METHODS

Materials

Recombinant human IL-1β and TNF-α were purchased from Novakemi AB (Enskede, Sweden). Human embryonic lung fibroblast cells, phenotype stable up to passage number 25, were prepared as described [Malmström et al., 1975]. Cell culture medium was obtained from Gibco (Paisley, UK). [^{35}S]-sulfate and [^{3}H]-glucosamine were purchased from ARC (St. Louis, MO) and ICN (Irvine, CA), respectively. The ion-exchange resin DEAE-52 was purchased from Whatman (Maidstone, UK). Sephacryl-500 and octyl-Sepharose CL-4B were from Pharmacia (Uppsala, Sweden) and Ready Safe from Beckman Instruments (Fullerton, CA).

cDNA probes of versican, perlecan, biglycan, decorin, collagen I, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used. The versican cDNA probe was a 400-bp *Sal*I-*Xho*I fragment [Dours-Zimmermann and Zimmermann, 1994]; the perlecan cDNA probe was an insert of 1,500-bp *Eco*RI [Kallunki and Tryggvason, 1992]. The biglycan cDNA probe was a 603-bp *Xba*I-*Stu*I fragment [Fisher et al., 1989], the decorin cDNA probe was an insert of a 1,162-bp *Eco*RI [Day et al., 1987], the collagen I cDNA probe was a 372-bp *Pvu*II-*Pst*I fragment [Sandberg and Vuorio, 1987], and the GAPDH cDNA probe was a 900-bp *Pst*I fragment. The [^{32}P]-labeled probes, with a specific activity of about 8×10^8 cpm/μg DNA, were prepared using a DNA labeling kit (Pharmacia). Chondroitin ABC lyase (E4.2.2.4) was a product of Seikagaku Kogyo (Tokyo, Japan). Alcian blue was produced by Fluka (Neu-Ulm, Switzerland).

The versican antibody was raised against versican from bovine aorta [Heinegård et al., 1985]; monoclonal mouse anti-perlecan (HSPG2) was purchased from Zymed Laboratories (San Francisco, CA). Polyclonal antibodies against human decorin (LF-30) and biglycan (LF-51) were raised in rabbits [Fisher et al., 1983]. PVDF-P Immobilon membranes for blotting were purchased from Millipore (Bedford, MA).

Cell Proliferation Assay

Human lung fibroblast cultures of passage number 7–10 were used for the experiments.

The cells were allowed to grow in a 96-well plate (5000 cells/well) in serum-free F12-IT (10 μg/ml insulin and 25 μg/ml transferrin) medium, supplemented with 10 ng/ml epidermal growth factor (EGF). Proliferation studies were made in the more nutritious F12-IT medium to enhance proliferation. IL-1β, TNF-α, or IL-1β and TNF-α were added in doses of 1, 10 and 100 ng/ml. Incubation was completed after 24, 48, and 96 h. After incubation the amount of cells was determined with a crystal violet method [Westergren-Thorsson et al., 1993b].

Cell Culture: Proteoglycan Analysis

Human lung fibroblast cultures, of passages 11–18, were used for the experiments. The cells were allowed to grow in a 75-cm^2 flask in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). Confluent cells were used to minimize further cell proliferation, which was confirmed by DNA analysis. The medium was changed to sulfate-poor Dulbecco's MEM supplemented with 0.4% FBS and with cytokines; IL-1β, TNF-α or IL-1β + TNF-α at doses of 1, 10, and 100 ng/ml. After 3 h of pre-incubation with various cytokines, the cells were labeled with 25 μCi/ml [^{3}H]-glucosamine (for measurements of hyaluronan) and 50 μCi/ml [^{35}S]-sulfate (for measurements of proteoglycan). Incubation was complete after 24, 48, and 96 h. After incubation, the cell medium was decanted, and diisopropylfluorophosphate (Merck, Darmstadt, Germany) was added to a final concentration of 1 mM. The cell layer was extracted with 4 M guanidinium hydrogen chloride (GuHCl) buffer containing 50 mM sodium acetate, pH 5.8, 0.1 M EDTA, 1% Triton X-100, and 5 μg/ml ovalbumin at 4°C overnight. Cell medium and cell extract (after dilution with equilibration buffer) were applied on DEAE-52 columns.

Chromatographic Procedure

Ion-exchange chromatography on DEAE-52. Hyaluronan and proteoglycans from both the medium and the cell layer were purified by passage over columns (0.7 × 4 cm) of DEAE-52 cellulose (1 ml) equilibrated with 6 M urea, 50 mM sodium acetate, 5 mM N-ethylmaleimide (NEM), 1 mM EDTA, and 5 μg/ml ovalbumin, pH 5.8 [Schmidtchen et al., 1990]. Unincorporated [^{3}H]-glucosamine and [^{35}S]-sulfate were washed out with equilibration buffer. Hyaluronan was eluted by increasing the acetate concentration to 0.5 M. This material was sensitive to *Streptomyces* hyaluronidase digestion. Proteoglycans were eluted with the use of 4 M GuHCl, 50 mM acetate, and 5μg/ml ovalbumin, pH 5.8.

Gel chromatography on Sephacryl-500 HR. The proteoglycans were further separated on a column of Sephacryl-500, using 4 M GuHCl, 50 mM acetate, pH 5.8, at a flow rate of 0.25 ml/min (average recovery: 82%). The fractions representing large and small proteoglycans were pooled for further analyses.

Hydrophobic interaction chromatography (HIC). Pooled fractions of small proteoglycans obtained after Sephacryl-500 chromatography were diluted to 2 M of GuHCl and applied to an octyl-Sepharose CL-4B column (0.5 × 10-cm Omnifit column) [Choi et al., 1989; Westergren-Thorsson et al., 1991], using a high-performance liquid chromatography (HPLC) system (LKB, Bromma, Sweden). The column was pre-equilibrated with 2 M GuHCl, 50 mM acetic acid, at pH 5.8, and eluted with a linear gradient (2–6 M GuHCl) at a flow rate of 0.1 ml/min (average recovery: 73%).

Superose 6. To study the length of the glycosaminoglycan (GAG) chains, alkali eliminated proteoglycan chains (see below) were applied on a Superose 6 column, using 4 M GuHCl, 50 mM acetate, pH 5.8, as eluent at a flow rate of 0.25 ml/min (average recovery: 83%).

Degradative Methods

Purified proteoglycans were precipitated with a threefold excess of 95% ethanol and 0.4% acetate and recovered by centrifugation at 12,000*g* for 20 min in a Biofuge A centrifuge. Pellets were dried in a Speed Vac and solubilized in appropriate digestion buffer. Digestions with chondroitin ABC were performed at room temperature, 4 h. Heparan sulfate was degraded by HNO_2 [Westergren-Thorsson et al., 1992].

Alkali elimination was performed to separate the glycosaminoglycan chains from the proteoglycan core protein. After precipitation, as above, the pellets were solubilized in 0.5 M LiOH and the eliminations were performed at 4°C overnight and then neutralized with acetate.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Large and small proteoglycans separated by gel chromatography on Sephacryl S-500 HR were further identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After ethanol precipitation and digestion, electrophoresis was performed on 3–12% polyacrylamide gradient gel (T/C = 30/0.8), using a 3% stacking gel and the buffer system of Laemmli [1970]. The gels were analyzed using a Fuji BAS 2000 image analyzer.

Analytical Methods

Radioactivity was measured by a LKB Wallac liquid scintillation counter with a dpm correction. Hyaluronan was also quantitated using iodinated hyaluronan binding protein obtained from Pharmacia. This method was used to exclude that the observed changes on hyaluronan production measured as [^{3}H]-glucosamine could be due to changes of the specific activity of the UDP-N-acetylhexosamine pool [Eriksson et al., 1984]. The samples were first purified using ion-exchange chromatography (DEAE-52), to exclude the possibility that the samples contained hyaluronan-binding proteins, which could interfere in this assay.

DNA Assay

DNA in the cell layer was measured by the fluorescent dye Hoechst 33528 (2-(2-(4-hydroxyphenyl)-6-benzimidazolyl)-6-(1-methyl-4-piperazyl)benzimidazole 3HCl) in a spectrofluorometer. An excitation maximum of 356 nm and an emission maximum of 458 nm were employed [Labarca and Paigen, 1980].

Agarose Electrophoresis and Western Blot

The proteoglycans were also identified by Western blot after precipitation with Alcian blue and by separation on a 2% agarose gel operated in a discontinuous buffer. After electrophoresis the proteoglycans were transferred to PVDF-P membranes. The filters were digested with chondroitin ABC lyase and HNO_2 (to facilitate the binding of the antibodies) and were treated with antibodies against perlecan, versican, decorin, and biglycan, respectively. Finally, the blots of versican, decorin, and biglycan were visualized using a second peroxidase-conjugated swine-anti-rabbit antibody. Perlecan was visualized using a second peroxidase-conjugated rabbit-anti-mouse antibody [Björnsson, 1995].

Isolation and Analysis of RNA

RNA was isolated by extraction with guanidine isothiocyanate [Chomczynski and Sacchi, 1986]. Equal amounts of RNA (15 μg) were separated by electrophoresis, transferred to nylon filters, and hybridized by [^{32}P]-labeled probes overnight in 50% formamide at 42°C [Westergren-Thorsson et al., 1991]. The filters were washed sequentially with 2 × saline sodium citrate (SSC) (0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0) 0.1% SDS at room temperature and with 0.2 × SSC 0.1% SDS at 50–60°C. The filters were analyzed using a Fuji BAS 2000 bio-image plate analyzer.

Statistical Methods

Mean values ±standard errors of the mean (SEM) were calculated. Analysis of variance was followed by Student's *t*-test.

RESULTS

Effect on Cell Proliferation

The effect was investigated of IL-1β, TNF-α, or IL-1β and TNF-α in combination, on the proliferation of human embryonic lung fibroblasts. Both IL-1β and TNF-α cause a dose-dependent decrease in fibroblast proliferation (data not shown). At 96 h, proliferation in the presence of IL-1β or TNF-α (10 ng/ml) was inhibited by 12% and 16%, respectively ($P < 0.001$), and the combination caused an inhibition of 27% of growth compared with control ($P < 0.001$) (Fig. 1). No significant effects of the growth factors were seen after 24- or 48-h incubation.

Effect on Hyaluronan Production

Hyaluronan secretion, measured as [^{3}H]-glucosamine incorporation, was increased by both IL-1β and TNF-α; the effect of the combination was additive. Significant changes were noted even at 48-h incubation, but maximum effects were seen after 96 h. Separately, IL-1β and TNF-α, respectively, induced a 1.5-fold and 1.7-fold increase and the combination a 2.5-fold increase (at 10 ng/ml and 96 h) compared with control ($P < 0.001$) (Fig. 2A). The dose-response assay showed that 10 ng/ml had the largest effect (data not shown). Only small amounts of hyaluronan (approximately 4%)

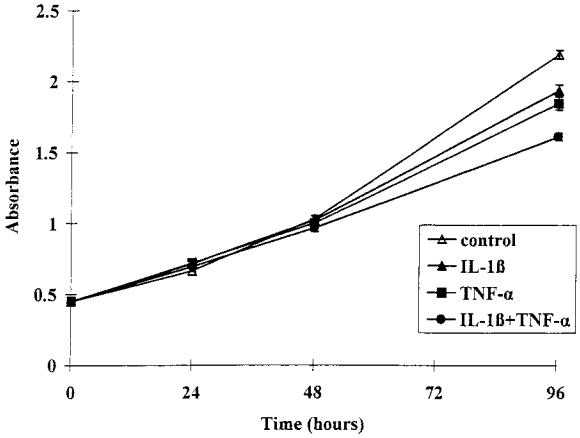

Fig. 1. Effect of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and of both in combination, on proliferation of human lung fibroblasts. Serum-starved embryonic fibroblasts, at a density of 5,000 cells/well, were incubated with IL-1β, TNF-α, or IL-1β and TNF-α in combination, at a dose of 10 ng/ml, in F12-IT medium for 24, 48, or 96 h. Number of cells was estimated by the crystal violet method. The values represent the mean ±SEM (n = 5).

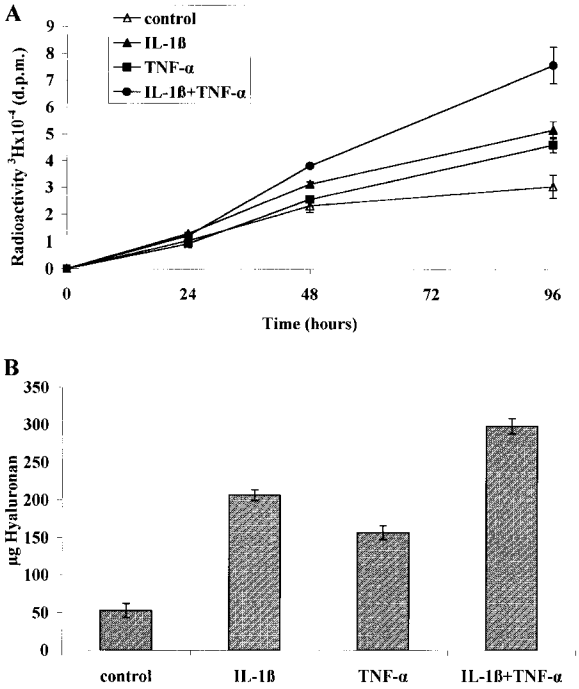

Fig. 2. Effect of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and of both in combination, on hyaluronan production in human lung fibroblasts. Hyaluronan accumulation in cell medium was measured by [^{3}H]-glucosamine incorporation (**A**). Confluent human embryonic lung fibroblasts were incubated with IL-1β, TNF-α, or IL-1β and TNF-α in combination (10 ng/ml), for 24, 48, and 96 h. Labeling with 25 μCi/ml [^{3}H]-glucosamine and 50 μCi/ml [^{35}S]-sulfate was started after 3 h of preincubation with various cytokines, the experiment continued for a further 21, 45, and 93 h, respectively. Hyaluronan was then purified by ion-exchange chromatography on a DEAE-52 column (see under Materials and Methods). Values are given as means ±SEM, n = 5–7. **B:** In a control experiment, hyaluronan in cell medium after 96-h incubation was also measured using a radioimmunoassay (RIA)-based method. Values are given as means ±SEM, n = 2.

were retained in the cell layer (at 10 ng/ml and 96 h); the same changes were noted here as in the culture medium. To confirm above data, hyaluronan identification was also performed (Fig. 2B) using an iodinated hyaluronan binding protein assay. The results obtained from the [^{3}H]-glucosamine incorporation and the RIA method both indicated increases (at 96 h). Confluent cell cultures were used and DNA analysis confirmed that there were no changes in cell proliferation depending on cytokines added (data not shown).

Effect on Proteoglycan Production

Both IL-1β and TNF-α induced a significant increase in proteoglycan accumulation, measured as [^{35}S]-sulfate incorporation (Fig. 3), an established method used to measure proteoglycan content. As seen with the effect on hyaluronan production, the combination has an additive effect, with a 1.4-fold increase compared with control ($P < 0.001$). The dose-response curve showed that the largest effects were seen at 10 ng/ml (Fig. 3, inset). Using this concentration, significant effects were seen at 24 h, increased effects at 48 h; finally, the largest amount of proteoglycan was secreted into the medium after 96 h incubation. In the cell layer the cytokines, separate and in combination, induce the same effects as recorded in the medium. No further analyses were done on the cell layer, as only a low amount of proteoglycan (15% at 96 h) was retained in this compartment.

Identification of Proteoglycans

Proteoglycans from the cell medium were further separated into large and small proteoglycans by gel chromatography on a Sephacryl-500 column (Fig. 4A). The pooled fractions of large and small proteoglycans were further characterized by SDS-PAGE and by agarose electrophoresis followed by Western blotting. SDS-PAGE analysis of large proteoglycans indicated versican or perlecan types, the latter resistant to chondroitin ABC lyase digestion

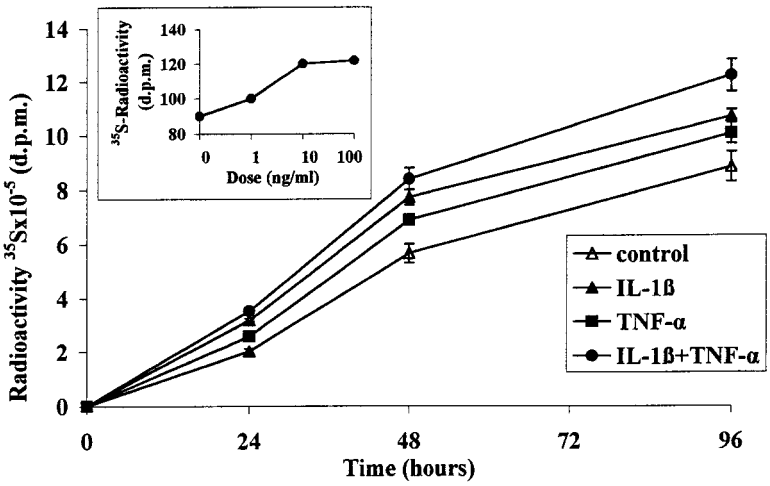

Fig. 3. Effect of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and of both in combination, on proteoglycan production in human lung fibroblasts. Accumulation of [^{35}S]-labeled proteoglycan in cell medium. Confluent human embryonic lung fibroblasts were incubated with IL-1β, TNF-α, or IL-1β and TNF-α in combination (10 ng/ml), for 24, 48, or 96 h. Labeling with 25 μCi/ml [^{3}H]-glucosamine and 50 μCi/ml [^{35}S]-sulfate was started after 3-h preincubation with various cytokines, with the experiment continued for a further 21, 45, and 93 h, respectively. Proteoglycans were purified by ion-exchange chromatography on a DEAE-52 column (see under Materials and Methods). Values are given as means ±SEM, n = 5. **Inset:** Representative dose-response curve after 96-h incubation with the combination of IL-1β and TNF-α in doses of 1, 10, and 100 ng/ml.

(Fig. 4B). Positive identification by Western blotting confirmed that the two components were versican and perlecan (Fig. 4C). Pooled fractions of the small proteoglycans were similarly characterized. SDS-PAGE showed that the small proteoglycans consisted of decorin, biglycan, and small heparan sulfate proteoglycans, the latter being resistant to chondroitin ABC lyase digestion (Fig. 4D). Decorin and biglycan were identified by agarose electrophoresis, followed by Western blot analysis (Fig. 4E). Versican and perlecan are the major large proteoglycans (other types found in this pool are less than 5%), whereas biglycan and decorin are the major small proteoglycans, secreted from lung fibroblasts [for detailed characterization, see Westergren-Thorsson et al., 1992].

Effect on Production of Large Proteoglycans

After chondroitin ABC lyase digestion of the large proteoglycans, the amounts of versican and perlecan, measured as [^{35}S]-sulfate incorporation, were calculated. IL-1β induced a 1.7-fold increase of versican compared with control ($P < 0.005$), 96 h after cytokine exposure (Fig. 5A). A similar increase (1.5-fold; $P < 0.01$) was also seen with the combination of IL-1β and TNF-α. TNF-α by itself did not significantly affect the level of versican. The levels of mRNA for versican, as shown by Northern blots, were increased by 1.5-fold ($P < 0.05$) at 48 h after IL-1β exposure (Fig. 5B). Effects on the mRNA levels peaked at 48-h incubation, whereas the secreted amounts of proteoglycan were most affected 96 h after incubation. Perlecan was not significantly affected, though a tendency to a decrease could be seen 96 h after IL-1β exposure (Fig. 5A). The mRNA level of perlecan at 48 h after cytokine exposure followed the same tendency (Fig. 5B), but not significantly.

Effect on Production of Small Proteoglycans

The pooled fractions of small proteoglycans, measured as [^{35}S]-sulfate incorporation, were further separated by HIC. This permitted separation of the small proteoglycans into three components, one that was not bound, and two that were retarded. The first peak consisted of heparan sulfate proteoglycans and small amounts of glycosaminoglycan chains, the second peak was composed of decorin and the third of biglycan [Tiedemann et al., 1997; Westergren-Thorsson et al., 1992](Fig. 6, inset). The level of decorin was decreased by about 34% ($P < 0.05$) compared with control by all treatments; IL-1β and TNF-α, separately and in combination (Fig. 6A). This finding was in accordance with the level of decorin mRNA, which was decreased by about 39% compared with control by all treatments (Fig. 6B). By contrast, TNF-α treatment increased the level of biglycan by 1.3-fold ($P < 0.005$) compared with control (Fig. 6A) and the level of biglycan mRNA by 1.5-fold (Fig. 6B). No significant effects were seen with IL-1β itself. However, while TNF-α in combination with IL-1α induced the same increase in mRNA level of biglycan as TNF-α separately (Fig. 6B), the combination had no effect on the level of biglycan (Fig. 6A). The length of the GAG chains of

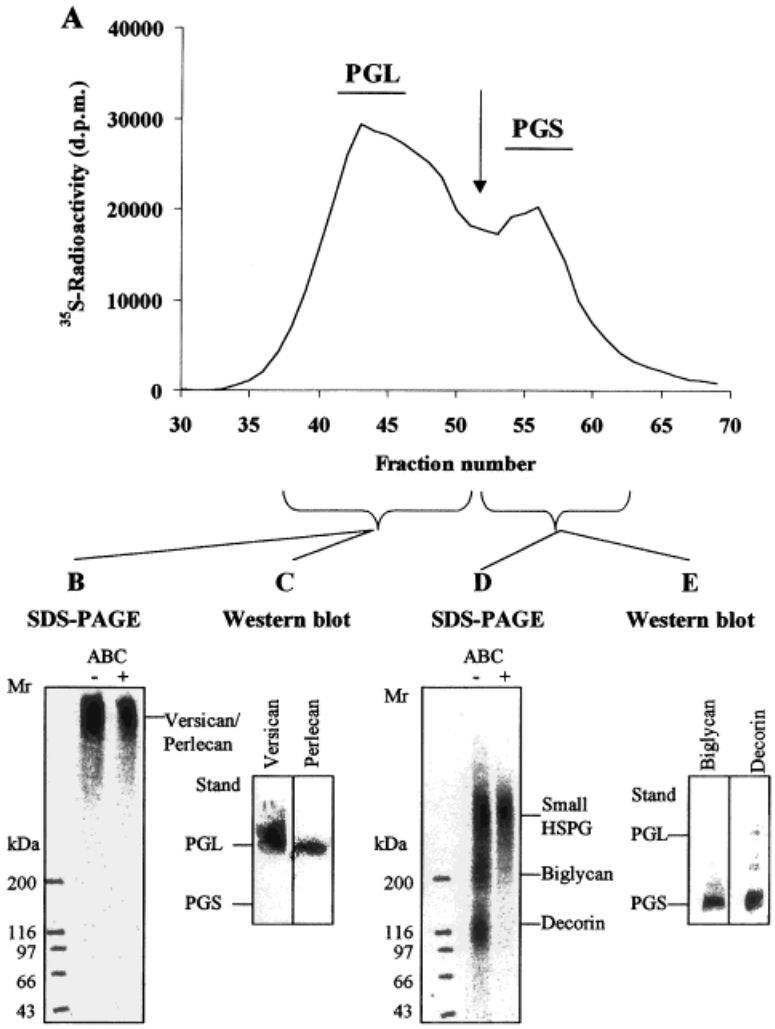

Fig. 4. Separation by gel chromatography of large and small proteoglycans from human fibroblasts cultured with interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and of both in combination. A representative chromatogram of Sephacryl-500 is shown. Proteoglycans secreted into culture medium were purified by ion-exchange chromatography and then subjected to gel chromatography, resulting in separation of large (PGL) and small (PGS) proteoglycans (**A**). The fractions were pooled as indicated by the arrow. PGL consists of versican and perlecan, while PGS consists of small heparan sulfate proteoglycans (HSPG), biglycan, and decorin. **B:** Large proteoglycans were further identified by SDS-PAGE with (+) or without (−) digestion with chondroitin ABC lyase. **C:** The large proteoglycans versican and perlecan were also identified by agarose electrophoresis followed by Western blots using respective antibodies. **D:** Small proteoglycans were identified by SDS-PAGE with (+) or without (−) digestion with chondroitin ABC lyase and also by (**E**) agarose electrophoresis followed by Western blots identifying biglycan and decorin, using respective antibodies. Standards (Stand) used in Western blots were PGL and PGS, prepared from bovine sclera [Cöster and Fransson, 1981]. M_r, standard marker protein.

decorin and biglycan was not affected by any cytokine treatment (data not shown).

Effect on Collagen Type I mRNA Expression

The level of collagen type I mRNA was decreased by TNF-α as well as by IL-1β, and the effect of the combination was additive. The maximal inhibition induced by the combination was 75% (Fig. 7). Separately, the cytokines also induced inhibition, but to a lesser extent, 60% with IL-1β and 40% with TNF-α.

DISCUSSION

It is well established that cytokines affect a variety of cellular responses. Cytokines such as IL-1β and TNF-α affect both the expression of matrix components and cell proliferation. In cell culture, the production of both proteoglycan and hyaluronan was affected by IL-1β and TNF-α given separately or in combination. A significant change in the relative composition of the different proteoglycans was seen: IL-1β increased the production of versican and TNF-α that of biglycan. Both cytokines also decreased the production of decorin and the level of collagen I mRNA. Generally, the combination of IL-1β and TNF-α showed additive effects on matrix production, and no synergistic effects were obtained. Probably IL-1β and TNF-α bind to different receptors with different modifying actions on cell behavior. The fact that TNF-α increases the production of biglycan, but both TNF-α and IL-1β decrease the level of decorin, indicates that these two very similar proteoglycans are regulated independently. These differences are possibly due to differences in promoter regions of biglycan and decorin. Both promoters have a TNF-α-

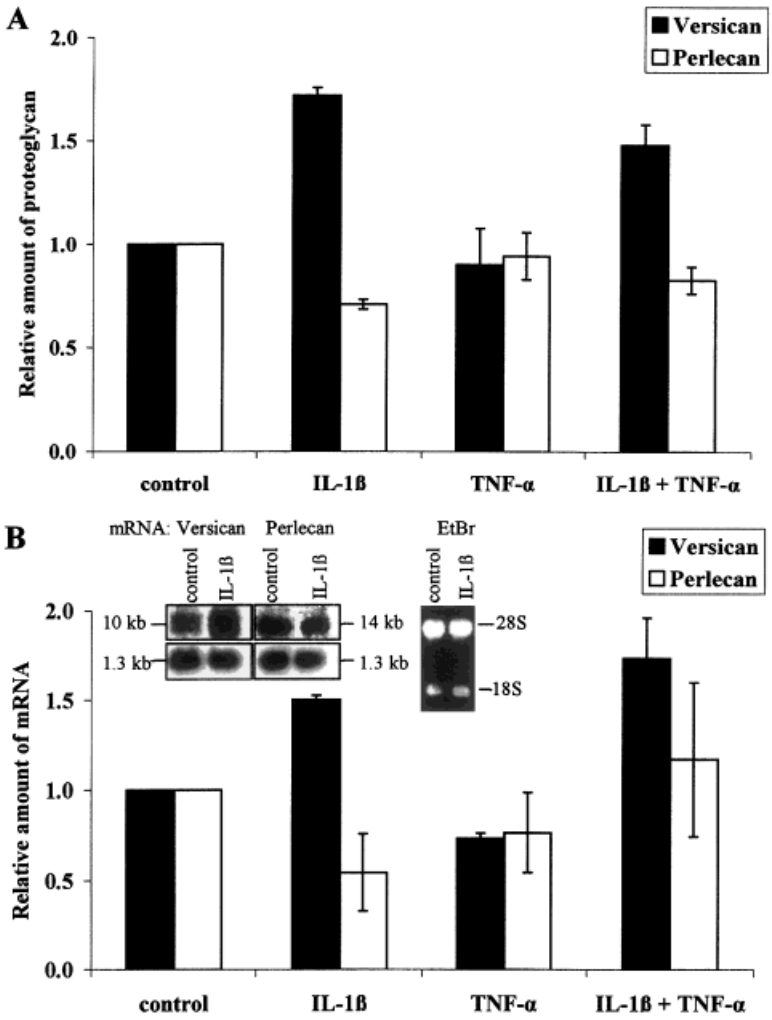

Fig. 5. Effect of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and of both in combination, on large proteoglycan production in human lung fibroblasts, as pooled fractions after gel chromatography (**A**) and as mRNA using Northern blot (**B**). The eluents of Sephacryl-500 columns were pooled, as indicated by the arrow (Fig. 4). **A:** The relative amount of [^{35}S]-sulfate incorporated into the large proteoglycans versican and perlecan was further determined (96-h incubation) after chondroitin ABC lyase digestion and SDS-PAGE. **B:** mRNA level of versican and perlecan in the control and cytokine-treated cultures (48-h incubation). **Inset:** Representative Northern blots with a 10-kb transcript of versican, a 14-kb transcript of perlecan and the respective 1.3-kb transcripts of GAPDH. A gel stained with EtBr is shown to check that the same amount of RNA was applied to the gel and that the RNA was intact. SDS-PAGE and Northern blots were scanned by the Fuji Image Analyze system. The mRNA was quantified in relation to GAPDH. Relative amounts of production and mRNA levels are shown. Values are given as means ±SEM, n = 4–5 in protein and n = 2 in mRNA measurements.

responsive element and are known to be regulated transcriptionally by TNF-α and other inflammatory cytokines, like TGF-β [Mauviel et el., 1995; Ugenfroren and Krull, 1996].

Because proteins are secreted into the medium and deposited there, 96-h incubation was chosen to measure protein levels. The mRNA levels were most pronounced at 48-h incubation. The changes in protein are in most cases in accordance with changes in mRNA, suggesting that these extracellular matrix proteins are regulated mainly at the level of transcription. However, the combination of IL-1β and TNF-α did increase the mRNA level of biglycan, which was not followed by an increase of its protein level. This could be due to effects on degradatory pathways, through increased expression of proteases [Hardingham et al., 1992], or to uptake of biglycan through receptor-mediated endocytosis [Hausser et al., 1992].

Incorporation of [^{35}S]-sulfate is considered a general and reliable method for measurements of proteoglycan content as the only major influx of sulfate into the intracellular sulfate pool is from the culture medium. This exchange is rapid, and a constant specific activity is reached within minutes, both in the sulfate and in the 3′-phosphoadenosine-5′-phosphosulfate pool [Eriksson et al., 1984]. In this study, no effects on the ratio of [^{35}S]-sulfate/[^{3}H]-glucosamine were observed, indicating that there is no effect of the specific activity of the precursor and that there is no change in the degree of sulfation of the GAG chains. Furthermore, no change of specific activity of hyaluronan is noted, as [^{3}H]-glucosamine incorporation as well as measurement with RIA obtained the same relative results.

As fibroblasts are the main producers of extracellular matrix components, induction and

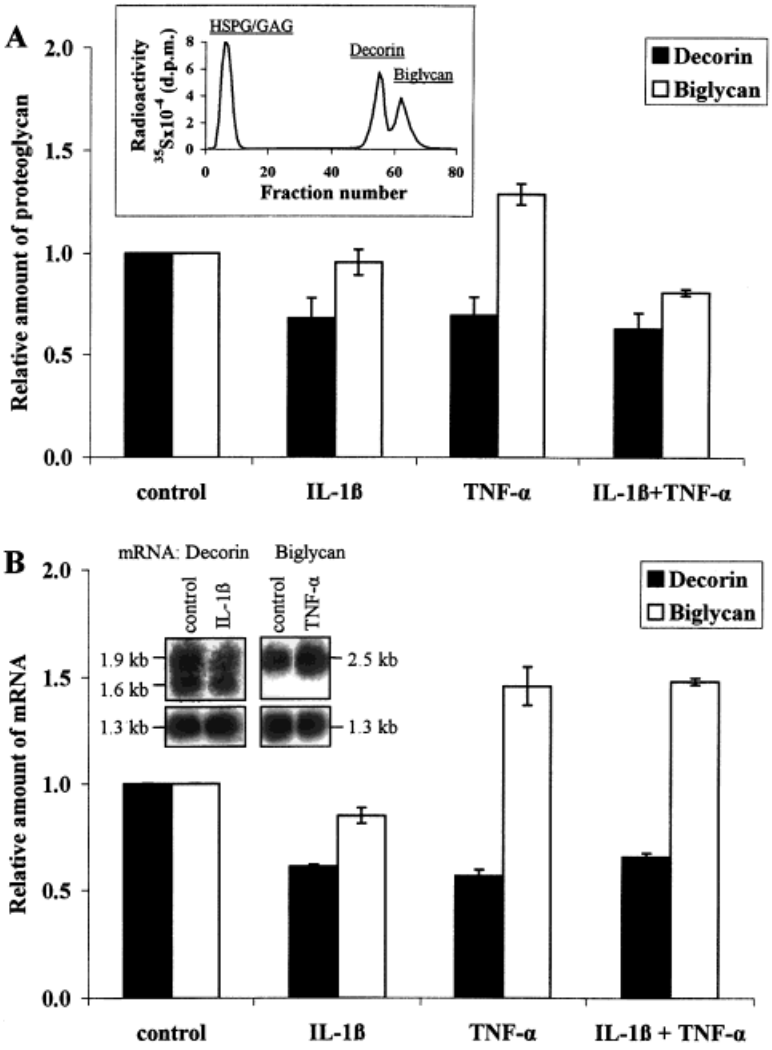

Fig. 6. Effect of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and of both in combination, on decorin and biglycan production in human lung fibroblasts, as pooled fractions after hydrophobic interaction chromatography (**A**) and as mRNA using Northern blot (**B**). Small proteoglycans (PGS) were separated from the culture medium of untreated and cytokine treated cells by ion-exchange chromatography on DEAE-52 and gel chromatography on Sephacryl-500. Small proteoglycans were pooled as indicated by the arrow (see Fig. 4) and finally separated on an octyl-Sepharose column into small heparan sulfate proteoglycans (HSPG)/glycosaminoglycans (GAG), decorin, and biglycan. **Inset, A**: Representative HIC chromatogram of the control. Relative amount of [^{35}S]-sulfate incorporated into decorin and biglycan after 96-h incubation was determined (**A**), as well as the mRNA level of decorin and biglycan after 48-h incubation (**B**). Northern blots were scanned by the Fuji Image Analyze system. The decorin and biglycan mRNA were quantified in relation to GAPDH. **Inset, B**: Northern blots with transcripts of decorin at 1.9 and 1.6 kb, a 2.5-kb transcript of biglycan and the respective 1.3-kb transcripts of GAPDH. Relative amounts of production and mRNA levels are shown. Values are given as means ±SEM, n = 4 in protein and n = 2 in mRNA measurements.

inhibition of fibroblast proliferation are important in the remodeling of connective tissue. Previous studies have shown that both IL-1β and TNF-α stimulate [Elias, 1988] or inhibit [Jordana et al., 1987] the proliferation of lung fibroblasts. Our results show that these cytokines inhibit proliferation of lung fibroblasts. These effects may be due to changes of the synthesis of prostaglandins [Kelley, 1990] or the amount of platelet-derived growth factor-β (PDGF-β)-receptors [Battegay et al., 1995], both factors that downregulate fibroblast proliferation.

If the observed changes on matrix production and composition also occur in vivo, different cytokines, separately or in combination, would influence the properties of the connective tissue. In proteoglycans, both the protein core, and especially the GAG side chains, possess biological properties, which result in changes in tissue characteristics. Surrounding the cells there are fibers of collagen, mostly types I and III, which are organized by decorin, fibromodulin, and FACIT collagens. The proinflammatory cytokines IL-1β and TNF-α downregulate decorin and collagen I, changing the composition of the fiber network and producing a looser type of tissue. This is promoted by the stimulatory effect of IL-1β and TNF-α on collagenase synthesis [Chen et al., 1994]. Biglycan, which is upregulated by TNF-α, does not bind to the fibrillar collagens, but it may influence the collagen matrix by modifying the decorin interaction by association through the GAG chains [Fransson, 1987]. Another important property of biglycan is its stimulatory effect on cell migration that can enhance the recruitment of fibroblasts [Kinsella et al., 1997]. Both biglycan

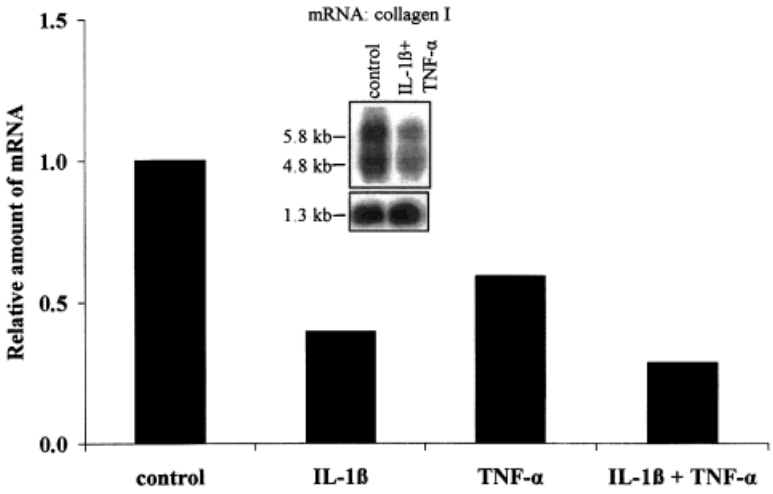

Fig. 7. Effect of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and of both in combination, on the level of collagen type I mRNA in human lung fibroblasts, using Northern blot. In one separate experiment, collagen type I mRNA was measured to compare our system with others [Mauviel et al., 1994]. mRNA from the control and the cells treated with various cytokines (48-h incubation) were hybridized with cDNA of collagen type I. Northern blots were scanned by the Fuji Image Analyze system. The mRNA of collagen type I was quantified in relation to GAPDH. **Inset:** Transcripts of collagen type I at 5.8 and 4.8 kb, and respective 1.3-kb transcripts of GAPDH. Relative amounts of production and mRNA levels are shown.

and versican are proteoglycans situated between the collagen fibrils and tend to fill up the space between the fibrils, rather than disperse them. Versican, which is upregulated by IL-1β, has the ability to form large aggregates with hyaluronan. As a result of its high water-binding capacity, an increase in these aggregates leads to retention of water and tissue swelling, facilitating cell infiltration. In our study we have also shown that IL-1β and TNF-α increase the production of hyaluronan, in accordance with other studies conducted with fibroblasts [Yaron et al., 1989; Sampson, 1992]. The effects were most prominent at 96-h incubation, indicating that the cells are still producing hyaluronan in response to the cytokines added. Only a low percentage of hyaluronan is seen in the cell layer. This is because hyaluronan is produced in the cells and is immediately secreted from the cells into the matrix. However, hyaluronan present in the cell layer may be retained either in the pericellular matrix due to interaction with versican or at the cell surface by hyaluronan receptors.

It has been shown that biglycan is a proteoglycan formed in the early phase of inflammation [Westergren-Thorsson et al., 1993a; Tremblay et al., 1998], while decorin is a marker for the later phase, when fibrosis is already established [Westergren-Thorsson et al., 1996]. This study shows that the extracellular matrix obtained after treatment with the proinflammatory cytokines IL-1β and TNF-α, may not be of typical fibrous type, but rather, as expected, of the inflammatory type found in early inflammation. In vivo, this may result in a less dense tissue of the lung, where inflammatory cells can infiltrate more easily.

ACKNOWLEDGMENTS

We are grateful to Larry Fisher for providing us with antibodies. We thank Urszula Endresén for expert technical assistance, and Lena Åberg and Susanne Persson for assistance concerning the cell culturing.

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