JCI The Journal of Clinical Investigation

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J Clin Invest. 1993;92(2):632-637. https://doi.org/10.1172/JCI116631.

Research Article

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Altered Expression of Small Proteoglycans, Collagen, and Transforming Growth Factor-β_1 in Developing Bleomycin-induced Pulmonary Fibrosis in Rats

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Abstract

The development of bleomycin-induced pulmonary fibrosis in rats was studied over a period of 21 d after an intratracheal instillation of bleomycin. The expression of three small proteoglycans (biglycan, decorin, and fibromodulin), collagen III and TGF-β1 was studied by RNA-transfer blot analysis. The proteoglycans were also studied by SDS-polyacrylamide gel electrophoresis and Western blots.

TGF-β1 mRNA increased threefold already on day 3 and remained elevated until day 10. After the increase of TGF-β1 mRNA the messages for biglycan and collagen III steadily increased to reach a maximum 10 d after bleomycin instillation. The mRNA for biglycan increased maximally fourfold and that of collagen III 2.5-fold. Decorin mRNA, in contrast to biglycan decreased and reached 20% of control on day 10. The message for fibromodulin remained constant throughout the study period.

The amounts of biglycan and decorin in the tissue changed in accordance with the mRNA levels. The results corroborate and extend previous in vitro studies concerning the effect of TGF-β1 on the metabolism of small proteoglycans and show that these macromolecules are regulated differently also in vivo. The marked alterations of biglycan and decorin during the development of fibrosis suggests that these proteoglycans have a regulating role in this process. (*J. Clin. Invest.* 92:632–637.)
Key words: biglycan • decorin • fibromodulin • mRNA • cytokines

Introduction

Pulmonary fibrosis is the final pathway of many interstitial lung diseases and is characterized by a massive production of fibrous connective tissue around the alveoli (1). The mechanisms responsible for the inflammatory/immune processes and the ensuing alterations in connective tissue are not understood. Bleomycin-induced pulmonary injury/fibrosis is a frequently used animal model. It is characterized by an initial alveolitis phase with recruitment of inflammatory cells like macrophages, granulocytes, and lymphocytes (2, 3). These cells secrete factors that affect both cell proliferation (4, 5) and production of extracellular matrix components from fibroblasts in the alveolar interstitium (5). Studies of growth factors (e.g., TGF-β) have suggested a role in the development of fibrosis (6). In the early stage of fibrosis, TGF-β is found in macrophages dispersed in the alveolar interstitium and in organized clusters. Later in the course of the response, TGF-β is primarily associated with extracellular matrix in regions of increased cell number (7, 8). Bleomycin-induced fibrosis has been used to study the accumulation of several connective tissue components and potential markers of inflammation/fibrosis. Examples are collagen (9, 10), hyaluronan (11, 12), and fibronectin (9, 10, 12).

Other important matrix components with both potential structural roles and regulatory functions are biglycan (PG-S1), decorin (PG-S2), and fibromodulin. These proteoglycans are structurally related (13) but differ in at least some functions. Decorin and fibromodulin both influence the fibril formation of collagens I and II (14), whereas biglycan has no effect in this system. Decorin has also been shown to bind TGF-β (15). The production of these proteoglycans is differently regulated in vitro, where biglycan is stimulated by TGF-β (16–18). The factor also induces synthesis of proteoglycans with altered copolymeric structure of their glycosaminoglycan side chains (18).

To study the in vivo regulation of fibrous connective tissue, we have studied developing pulmonary fibrosis in the rat, with major alterations of matrix composition and properties. The expression of a regulating factor, TGF-β, has been correlated to the expression, as mRNA of collagen III, the three small proteoglycans, biglycan, decorin, and fibromodulin. In the case of the proteoglycans, also the abundance of the molecules in the tissue has been measured.

Methods

Materials. Male Sprague-Dawley rats weighing ~ 200–250 g were supplied by Möllegaard Breeding Centre Ltd. (Ejby, Denmark). Bleomycin sulfate was purchased from AB H. Lundbeck & Co. (Copenhagen, Denmark) Sephacryl S-500 HR and Mono Q HR were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and DEAE cellulose (DE 52) was from Whatman Chemical Separation Inc. (Clifton, NJ) Chondroitin ABC lyase (EC 4.2.2.4) and heparan sulfate lyases (EC 4.2.2.8) were products of Seikagaku Kogyo Co. (Tokyo, Japan). Other chemicals were obtained from sources previously listed (17, 18).

Treatment of animals. Rats were instilled with 2.5 mg/kg body wt of bleomycinin sterile 0.9% saline in a volume of 1 ml/kg body wt. Control animals received 0.9% saline only. Animals from the control group and from the bleomycin-treated group were killed on days 3, 7, 10, 14, and 21 ($n = 4$). The lungs were perfused with sterile phosphate-buffered saline containing 0.1% diethyl pyrocarbonate, excised, and weighed after removal of the bronchi.

For proteoglycan extraction, animals from the bleomycin-treated group were killed on days 7, 14, and 21 (one at each time point), as well as one control animal on day 3. Those lungs were not perfused as described above.

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Received for publication 28 September 1992 and in revised form 23 March 1993.

J. Clin. Invest.

0021-9738/93/08/0632/06 $2.00
Volume 92, August 1993, 632–637

Antibodies. In previous studies of bovine tissues, antibodies raised in rabbits against bovine decorin, biglycan, and fibromodulin have been successfully used. The cross reactivity of such rabbit antibodies between species is, however, rather restricted. Antibodies were therefore raised in egg-laying hens against bovine biglycan. Initial immunization was with 130 μg of the respective proteoglycan in Freund's complete adjuvant. Booster doses were given after 1 and 2 mo with 100 μg of proteoglycan each time. Immunoglobulins containing the antibodies were purified from the egg yolk using precipitation with a combination of dextran sulfate and ammonium sulfate (19). Antibody reactivity was determined by Western blotting and ELISA (E. Hedbom and D. Heinegård, unpublished observation). The antibody against decorin was prepared from bovine sources, as described by Heinegård et al. (20).

cDNA-probes. cDNA probes used were for collagen III described by Sandberg and Vuorio (21), for biglycan described by Fisher et al. (22) and for fibromodulin described by Oldberg et al. (13). A 1,050-bp Eco RI fragment of TGF-β1 cDNA (23) and a 900-bp Pst1 fragment of cDNA for glyceraldehyde phosphate dehydrogenase (GAPDH)[1] were also used. Decorin cDNA was cloned from a λgt11 cDNA library from neonatal rat calvaria (24). The identity of the cDNA was confirmed by partial sequence analysis (Westergren-Thorsson, G., unpublished observation). ^{32}P-labeled probes were prepared using a random priming cDNA labeling kit (Amersham International, Amersham, United Kingdom) as described (17).

Isolation and analysis of RNA. The tissue was homogenized in guanidinium isothiocyanate for 1 min at 35,000 rpm in a Bühler knife blade homogenizer. RNA was isolated by phenol/chloroform/isoamyl alcohol extraction (25). Total RNA (35–40 μg/well) was electrophoresed on 1% agarose gel containing formaldehyde and transferred to nitrocellulose filters. After electrophoresis, a part of the gel was stained with ethidium bromide to verify that equal amounts of RNA were applied in each well of the gel. The filters were hybridized with ^{32}P-labeled cDNA probes overnight in 50% formamide at 42°C (17) and then sequentially washed with 2× SSC (0.3 M sodium chloride, 30 mM sodium citrate pH 7.0) containing 0.1% sodium dodecyl sulfate at 42°C and then with 0.2× SSC, 0.1% sodium dodecyl sulfate at 50°C. Radioactivity was visualized (Hyperfilm™-MP; Amersham). The amount of radiolabel was quantified by scanning with a video densitometric system (Makab, Göteborg, Sweden).

Extraction and isolation of proteoglycans. The frozen lungs were powdered in a Retsch ball mill for 2 min at the highest frequency while cooled with liquid N_2. The proteoglycans were extracted for 12 h at 4°C with 10 vol of 4 M guanidinium chloride, 50 mM sodium acetate, pH 5.8, containing 1% Triton and the protease inhibitors 10 mM EDTA, 10 mM 6-amino hexanoic acid, 5 mM benzamidine hydrochloride, and 5 mM *N*-ethyl maleimide (26). The extract was also supplemented with diisopropyl fluorophosphate to a final concentration of 1 mM. After centrifugation at 15,000 rpm (r_{av} = 7 cm) in a centrifuge (model 101 M; Sigma Immunochemicals, St. Louis, MO) for 30 min, the pellets were reextracted for 16 h with 10 vol of extraction buffer. The supernatants were pooled and diluted with 20 vol of 6 M urea, 50 mM sodium acetate, pH 5.8, protease inhibitors, and 0.1% Mulgophene (26), and applied to columns (0.5 × 2.8 cm) of DE 52 equilibrated with the same buffer. The columns were eluted with 6 vol of 0.5 M sodium acetate buffer pH 5.8, containing 6 M urea, protease inhibitors, and 0.1% Mulgophene. Finally, proteoglycans were eluted with 3 bed vol of 4 M guanidinium chloride, 50 mM sodium acetate pH 5.8 containing 0.1% Mulgophene, and diisopropyl fluorophosphate was added to a final concentration of 1 mM. After dialysis against 7 M urea, 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl containing 0.1% Mulgophene, proteoglycans were further purified by ion exchange chromatography on a Mono Q HR 5/5 column connected to an LKB HPLC system. The column was eluted with a linear gradient of 0.1–1.2 M NaCl in 7 M urea, 10 mM Tris-HCl pH 8.0 containing 0.1% Mulgophene (27) at a flow rate of 0.5 ml/min. Proteoglycans, which were eluted between 0.6 and 1 M of NaCl, were pooled, diluted with 4 vol of 6 M urea, 0.05 M sodium acetate pH 5.8 containing 0.1% Mulgophene, and concentrated on a DE 52 ion exchanger eluted stepwise as described above. This material was subjected to gel chromatography on a column of Sephacryl S-500 (Pharmacia HR 16/50) connected to an LKB HPLC system. The column was eluted with 4 M guanidinium chloride, 50 mM sodium acetate pH 5.8 containing 0.1% Mulgophene at a flow rate of 0.25 ml/min. Pooled proteoglycan fractions were dialyzed against 6 M urea and 50 mM sodium acetate pH 5.8, containing protease inhibitors and 0.1% Mulgophene, concentrated by ion exchange chromatography on DE-52 with stepwise elution as described above, and finally identified by SDS-PAGE and immunoblotting. The recovery in each step was ~ 80%, in samples from both control and bleomycin-treated lungs.

Immunoblot of biglycan and decorin. Material from the initial DE 52 chromatography was digested with chondroitin ABC lyase and subjected to SDS-PAGE. The gels were then blotted to nitrocellulose filters and proteoglycan core proteins were detected with antibodies against biglycan and decorin. Before immunostaining, biglycan was added to the antiserum against decorin to abolish any cross reactivity. The components were visualized using an ECL™ kit (Amersham) and quantitated using a video densitometric system as described above.

Degradation methods. Digestion with chondroitin ABC lyase (20 mU/ml) was performed at 37°C for 4 h in 50 mM Tris/acetate buffer pH 7.3 with 2 μg/ml of ovomucoid added. Digestion with heparan sulfate lyase (1 mU/ml) was performed in 3 mM calcium acetate, 10 mM Hepes pH 7.0, 0.1% (vol/vol) Triton X-100, 10 mM Na_2EDTA, 10 mM *N*-ethyl malemide, and 2 μg/ml of ovomucoid at 37°C for 4 h. Before digestion the samples were precipitated with 9 vol of 95% (vol/vol) ethanol and dissolved in the appropriate digestion buffer.

Analytical methods. Sulfated glycosaminoglycans were quantified using 1,9-dimethylmethylene blue in microtiter plates (28). Samples eluted from Sephacryl S-500 were first precipitated with ethanol with 100 μg of dextran T-100 as carrier, before determining the amount of sulfated glycosaminoglycans as described above. SDS-PAGE (3–12% acrylamide) was performed according to Laemmli (29). The gels were stained with Coomassie brilliant blue and scanned as described above.

Statistical methods. Mean values±SEM were calculated. Student's *t* test was used to evaluate the differences of the means between groups. $P < 0.05$ were considered significant.

Results

Bleomycin treatment induced pronounced increases in weight and RNA content of the lung. On day 3, a 1.6-fold increase in weight was registered, which remained at this level throughout the experiment. Also the total RNA of the treated lung increased to a maximum of 1.6 times that of controls on day 10. It then declined to reach starting values on day 21. Northern blots show that the relative amount of TGF-β1 mRNA increased already on day 3 and remained elevated throughout the experiment (Fig. 1). To quantify mRNA the films were scanned with a video densitometric system. The message for TGF-β1 increased and reached a maximum (3.0±0.4-fold [n = 4]) 7 d after treatment (Fig. 2 *A*). The TGF-β1 mRNA then declined but did not return to control levels (1.4±0.1 [n = 4]) during the course of the experiment. The increase of TGF-β1 mRNA was followed by an increase of biglycan and, to a lesser degree, of collagen III mRNA (Fig. 1). Biglycan mRNA gradually increased to a maximum 3.7±0.8-fold (n = 4) 10 d after

1. *Abbreviation used in this paper:* GAPDH, glyceraldehyde phosphate dehydrogenase.

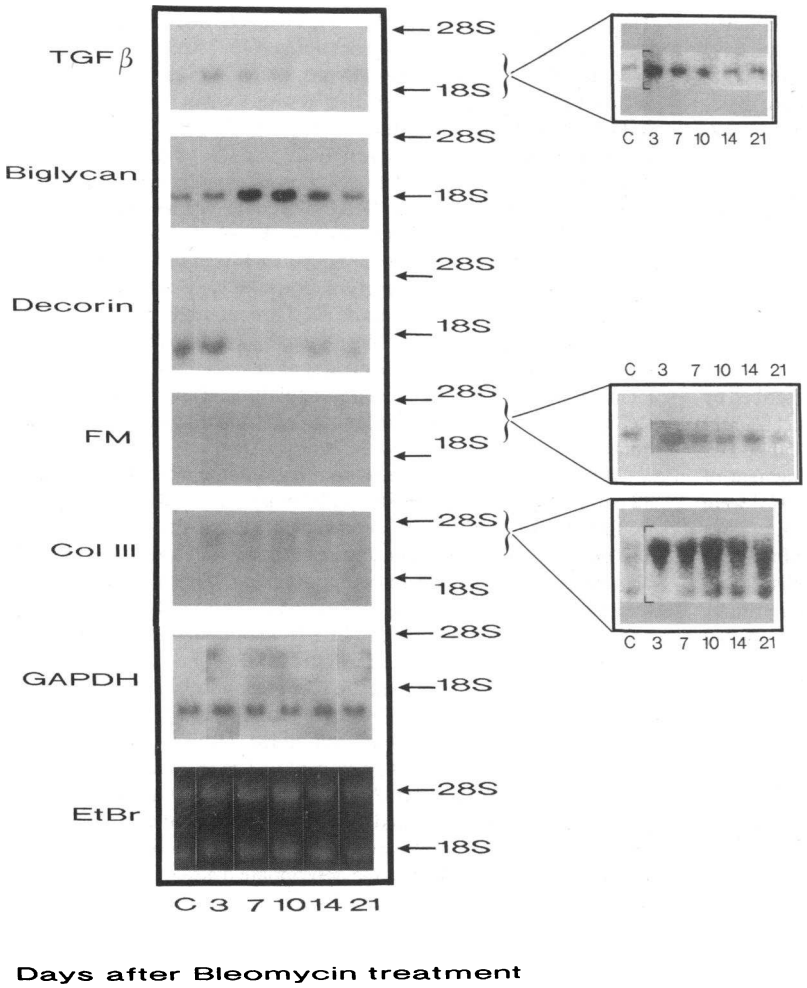

Figure 1. Northern blot hybridization with cDNA probes specific for TGF-β1, biglycan, decorin, fibromodulin, and collagen III of RNA isolated from lungs treated with bleomycin analyzed at increasing time interval after instillation. Total RNA was isolated and separated by electrophoresis on a 1% agarose gel (~ 35–40 μg RNA/well). RNA was then transferred to nitrocellulose and hybridized with ^{32}P-labeled cDNA probes. The migration of 18S and 28S rRNA is indicated. The ethidium bromide stain (*EtBr*) shows the ribosomal RNA and verifies that the same amount of RNA was applied in each well, also verified by the rather constant signal for GAPDH mRNA. Since no differences in the messages from the control lungs at the different times were noted, only those from day 3 are shown. (*C*, control). The inserts show amplified signals for TGF-β1, fibromodulin, and collagen III.

bleomycin instillation. 21 d after bleomycin instillation, the biglycan mRNA had returned to control values, 1.2±0.3 (n = 4) (Fig. 2 *B*). In a course similar to that of biglycan, the two messages for collagen III showed a maximum of 2.4 times those of the starting levels 10 d after bleomycin instillation (Fig. 2 *D*). Decorin, in contrast to biglycan mRNA decreased (Fig. 1) and reached 20% of the starting level 10 d after instillation, whereafter it slowly increased but did not return to starting values during the course of the experiment (Fig. 2 *C*). There was no detectable change of the level of fibromodulin mRNA (Fig. 1) or of mRNA for the house keeping protein GAPDH. Ethidium bromide staining verified that the same amount of RNA was applied in each well (Fig. 1).

To determine if the change of mRNA for proteoglycans also resulted in an changed abundance of these molecules in the tissue, proteoglycans were extracted from control and bleomycin-treated lungs were taken 7, 14, and 21 d after instillation. The proteoglycans were extracted and purified using stepwise ion exchange chromatography, gradient elution from a Mono Q ion exchanger and finally gel chromatography on Sephacryl S-500. The various fractions were subjected to SDS-PAGE followed by staining with Coomassie blue or by Western blots before and after chondroitin ABC and heparan sulphate lyase digestion. A large proteoglycan with both chondroitin sulphate and heparan sulphate side chains constituted < 15% of the total amount of proteoglycans (data not shown). Predominating were small proteoglycans consisting of biglycan, decorin and at least two heparan sulphate proteoglycans. The major components in this group migrated on electrophoresis as biglycan and decorin and were presented in roughly equal proportion (data not shown). To more conclusively follow the expression of biglycan and decorin, the DE 52 fractions containing these molecules were digested with chondroitinase to reduce the polydispersity and the core proteins released were analyzed by SDS-PAGE and Western blotting. The blots show that the biglycan protein mass gradually increased in the tissue (Fig. 3 *A*). A scanning of the immunoblot demonstrated that 14 d after instillation, biglycan reached a maximum of 1.5 times that of the starting value. Thus the maximum value of this proteoglycan expectedly appeared somewhat later than the peak value of its mRNA. At 21 d, the biglycan content of the lung had partially returned and was 1.2 times the initial value. Decorin, on the other hand, decreased and reached a minimum on day 14 (50% of the control value) (Fig. 3 *B*), also somewhat later than the observed minimum decorin mRNA level. The level of the core protein then gradually returned and had nearly reached the starting value on day 21. Similar changes in the relative abundance of proteoglycans were found in a different set of animals by isolation and characterization of proteoglycans in a semiquantitative way using gel and ion exchange chromatography followed by SDS-PAGE (data not shown).

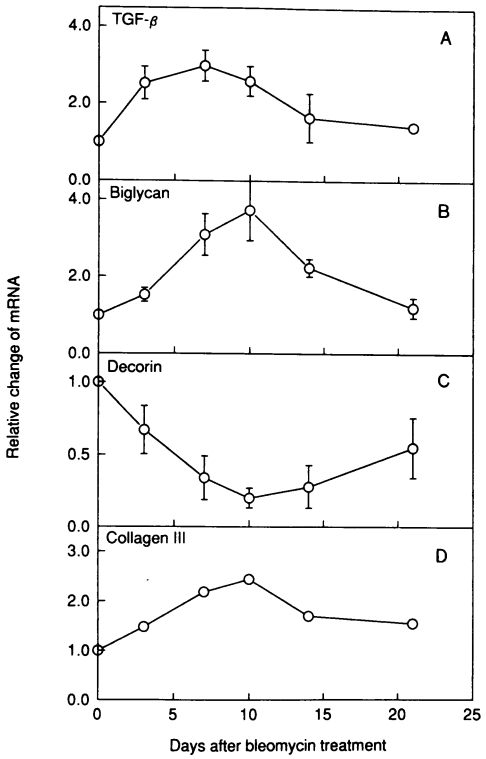

Figure 2. Relative amounts of mRNA for (*A*) TGF-β, (*B*) biglycan, (*C*) decorin, and (*D*) collagen III from bleomycin-treated lungs and control lungs. The Northern blots (Fig. 1) were scanned and the components were quantified. Each point represents mean values±SEM on analyses of lungs from four different animals except for collagen III, which represents analyzes from lungs of one animal. The sum of the two messages for collagen III is shown. Statistically significant differences were observed between the level of the message for TGF-β between day 10 and day 21 ($P = 0.038$), for biglycan both between day 10 and day 21 ($P = 0.024$), and day 3 and day 10 ($P = 0.035$).

Discussion

During the development of bleomycin-induced pulmonary fibrosis, total lung RNA transiently increased with a maximum on day 10 after bleomycin instillation. This is in accordance with previous studies in rats (9) and is probably a result of an influx of inflammatory and reparative cells. These cells are most likely important producers of various growth factors and cytokines, including TGF-β, which is increased over the entire time period, corroborating previous data (6, 7). The increased TGF-β mRNA precedes the changes of mRNA for collagen and proteoglycans possibly indicating an effect and effector relationship. The increased collagen III, message with a maximum on day 10, confirms data in earlier studies (6, 9). The increased message for biglycan (maximum on day 10) and a decreased message for decorin (minimum on day 10) and the ensuing correspondingly altered levels of the proteoglycans provides new important information on the process of fibrosis. One may speculate on mechanisms for these altered levels of proteoglycans and collagen mRNA and protein. It is likely that TGF-β represents one important factor in mediating these effects in vivo. In support, TGF-β injected into wound chambers (30), into incisions (31), or subcutaneously (32) induces influx of inflammatory cells and increase in fibroblast number, as well as enhanced collagen synthesis. Also in vitro TGF-β induces increased expression of several connective tissue components; e.g., collagen and fibronectin (33), hyaluronan (34), and proteoglycans (16, 17, 18). The in vivo model used in this study provides results corroborating in vitro studies; i.e., an increase in the expression of biglycan and no change of that of fibromodulin (17). Also of great interest are the lowered levels of decorin mRNA observed after bleomycin treatment. The same effect has been observed in vitro when fibroblasts were incubated with TGF-β for extended periods (16).

The altered synthesis of decorin and biglycan are sufficiently pronounced to alter the levels of the molecules in the whole lung, as shown by the quantitation of the proteoglycans from the tissue, where biglycan increases during the early phases of fibrosis, with a simultaneous decrease in decorin. It is likely that locally the levels may change even more strikingly, with major implications for tissue properties and function. It is less likely that different extractability of the proteoglycans decorin and biglycan explain the alterations observed, since the alterations in abundance of the proteins actually match alterations in their message levels.

Decorin and fibromodulin both appear to influence the fibril formation and properties of collagen (14). A decreased decorin concentration with a simultaneous increase in collagen

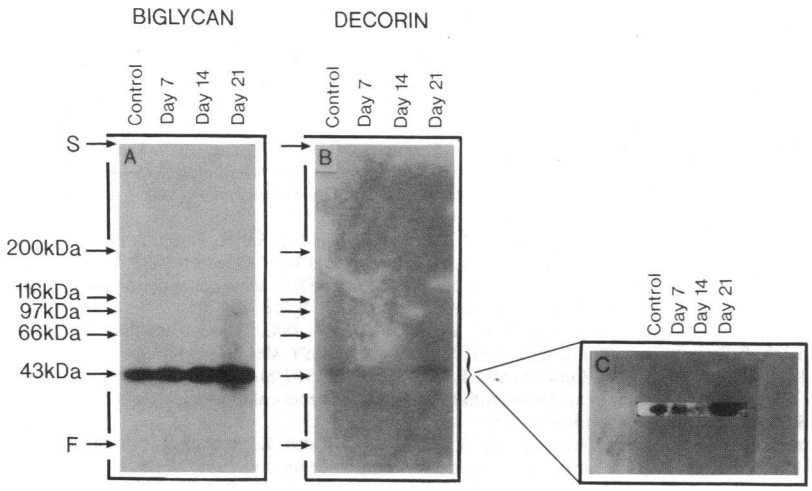

Figure 3. Western blots of control lungs and of bleomycin-treated lungs using antibodies against biglycan (*A*) and decorin (*B*). Proteoglycans were extracted from lungs at 7, 14, and 21 d after bleomycin instillation, as well as from a control non treated animal. Partial purification was achieved by ion exchange chromatography on DE 52. Proteoglycan material representing equal lung weight was precipitated with ethanol and digested with chondroitinase ABC. The digests were then subjected to SDS-PAGE followed by Western blotting using antibodies towards biglycan (*A*) and decorin (*B*). The migration positions of molecular mass standards are indicated on the left; *S*, start; *F*, front. (*C*) is the same as (*B*), but signal for decorin has been intensified by a video densitometric system (*see inset*). The absence of staining for intact proteoglycans indicate complete digestion.

should effect collagen fibril dimensions and properties. It has been proposed (5), that in a rapidly remodeling tissue, such as the fibrotic lung tissue or in other wound healing processes, it is possible that an altered collagen network is required for rapid reconstruction and penetration of inflammatory and reparative cells. The role of biglycan in fibrosis is an enigma. It is quite interesting that biglycan appears to interact selectively with collagen VI, while not with the fibril forming collagens (E. Hedbom and D. Heinegård, unpublished data). The contrasting regulation of biglycan compared with decorin, however, indicates rather distinct roles in the fibrotic process.

TGF-β induces a changed copolymeric structure of the side chains of decorin and biglycan in the direction of a lower iduronate content (18). Dermatan sulphate with a lower iduronate content has been shown to be less efficient in inhibiting cell proliferation (35). Thus increased cell proliferation may be expected in TGF-β induced fibrosis, in turn resulting in an increased production of matrix components, thus accelerating the fibrotic process.

The proteoglycans may be involved in the regulation of growth factor activity in other ways. The core proteins of decorin have been shown to bind TGF-β in vitro (15). Thus, a decreasing concentration of decorin may further enhance the effects of TGF-β via a lower degree of immobilization of the growth factor. The importance of this interaction for the development of glomerulosclerosis has been raised by Border (36), who showed that decorin neutralizes the inductive TGF-β -effect.

The transient changes noted in the bleomycin-induced fibrosis may occur also in other pathological conditions such as idiopathic pulmonary fibrosis, sarcoidosis and scleroderma. The functional significance of the altered proteoglycan pattern for TGF-β activity, for the organisation of the collagen fibres and for the enhancement of fibroblast proliferation during the development of fibrosis, however, remains to be established.

Acknowledgments

We are indebted to Dr. L. Fisher for providing us a cDNA probe for biglycan, to Dr. E. Vuorio for providing us a cDNA probe for collagen III, and to Dr. R. Derynck, Genentech Inc. (San Francisco, CA) for providing us a cDNA probe for TGF-β1. We thank Urszula Endresén for technical assistance and Birgitta Jönsson for art work.

The work was supported by grants from the Swedish Heart-Lung Foundation, AB Draco's Research Foundation, Johan and Greta Kocks Stiftelse, Trelleborg, Riksförbundet mot Reumatism, Alfred Österlunds Stiftelse, Gustaf V:s 80-årsfond, Axel and Margaret Axelsson Johnsons Stiftelse, The Swedish Society for Medical Research, the Swedish Cancer Society, the Medical Faculty of Lund University, and the Swedish Medical Research Council.

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