Characterization of Proteoglycans Synthesized by Cultured Corneal Fibroblasts in Response to Transforming Growth Factor β and Fetal Calf Serum*  

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A culture system was developed to analyze the relationship between proteoglycans and growth factors during corneal injury. Specifically, the effects of transforming growth factor β-1 (TGF-β1) and fetal calf serum on proteoglycan synthesis in corneal fibroblasts were examined. Glycosaminoglycan synthesis and sulfation were determined using selective polysaccharidases. Proteoglycan core proteins were analyzed using gel electrophoresis and Western blotting. Cells cultured in 10% dialyzed fetal calf serum exhibited decreased synthesis of more highly sulfated chondroitin sulfate and heparan sulfate compared with cells cultured in 1% dialyzed fetal calf serum. The amount and sulfation of the glycosaminoglycans was not significantly influenced by TGF-β1. The major proteoglycan species secreted into the media were decorin and perlecan. Decorin was glycosaminoglycans was not significantly influenced by TGF-β1 and serum induced substantial increases in perlecan bearing chondroitin sulfate and/or heparan sulfate chains. In contrast, after extended periods in culture, the amount of perlecan bearing heparan sulfate chains was unaffected by TGF-β1 and decreased by serum. The levels of perlecan bearing chondroitin sulfate chains were elevated with TGF-β1 treatment and were decreased with serum. Because both decorin and perlecan bind growth factors and are proposed to modulate their activity, changes in the expression of either of these proteoglycans could substantially affect the cellular response to injury.

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1 The abbreviations used are: ECM, extracellular matrix; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; FCS, fetal calf serum; dFCS, dialyzed FCS; DMEM, Dulbecco’s modified Eagle’s medium; GAG, glycosaminoglycan; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; KS, keratan sulfate; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TGF-β, transforming growth factor β.
neal fibroblasts in culture synthesize keratan sulfate proteoglycans with shorter KS chains and lower sulfation compared with those in normal corneas. The altered properties of KS and the increase in chondroitin sulfate proteoglycan (CSPG) and HSPG synthesis suggests that the conditions of cell culture may recapitulate some of the aspects of injury. To date, most studies of corneal proteoglycans produced in vitro have been based upon biochemical analysis of GAG chains, with only limited analysis of the protein cores. Additionally, each of these studies employed different culture techniques, making the results difficult to compare.

We developed a culture system to examine the regulation of proteoglycan synthesis by corneal fibroblasts during injury. The major proteoglycans synthesized by corneal fibroblasts were characterized and identified after culture in a defined environment. Specifically, we evaluated the effects of TGF-β1 and serum on the synthesis of specific GAGs and protein cores. We found that corneal fibroblasts synthesized predominantly CS and HS, with only trace amounts of an unsulfated form of keratan. The major proteoglycan species secreted into the medium were decorin and perlecan, and proteoglycan synthesis was mediated by TGF-β1 and serum. This model will allow us to systematically examine the relationship between specific growth factors and proteoglycan expression using a defined culture system.

**EXPERIMENTAL PROCEDURES**

Materials—Chondroitinase ABC (protease-free), keratanase II, chondroitin sulfate B, keratan sulfate, and the mouse monoclonal antibody 3G10 directed against unsaturated uronic acid residues arising from heparan sulfate depigmentation of heparan sulfate were purchased from Seikagaku America, Inc. (Ijamsville, MD). Endo-β-galactosidase was purchased from Boehringer Mannheim. Heparan sulfate, heparin, heparis III, phenylmethylsulfonyl fluoride, benzamidine, N-ethylmaleimide, and peroxidase-conjugated donkey anti-sheep IgG antibodies were from Sigma. Q-Sepharose came from Pharmacia Biotech Inc. (Uppsala, Sweden). Peroxidase-conjugated donkey anti-rat IgG antibodies were purchased from Amersham Pharmacia Biotech. Ultrapure urea, sodium chloride, Tween-20, Tris-HCl, bovine serum albumin, and bovine serum albumin were purchased from Amersham Pharmacia Biotech. Ultrapure (Uppsala, Sweden). Peroxidase-conjugated donkey anti-rat IgG antibodies were purchased from Amersham Pharmacia Biotech. Ultrapure urea, sodium chloride, Tween-20, Tris-HCl, bovine serum albumin, and EDTA were obtained from American Bioanalytical (Natick, MA). TGF-β1 was obtained from R & D Systems (Minneapolis, MN). Culture reagents were purchased from Life Technologies, Inc. The culture medium were supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum (FCS). Cell were plated in 75-mm vented tissue culture flasks, and cultures were maintained in DMEM supplemented with 10% fetal calf serum (FCS). Upon confluency, the cultures achieved confluency after 7–10 days, at which time cells were passaged 1:4 and cultured in 4% FCS for 3 days. All experiments were performed on confluent fibroblast cultures that had been passaged once.

**Cell Treatment and Metabolic Labeling**—The synthesis of sulfated glycosaminoglycans was followed by metabolically radiolabeling corneal fibroblasts with [³⁵S]cysteine/methionine (50 μCi/ml). Corneal fibroblasts in first passage were cultured until confluent (3 days) in 4% FCS. Upon confluence, corneal fibroblasts were treated as indicated in the figure legends. Radioisotopes were added immediately after the addition of TGF-β1. After the designated radiolabeling period, the medium was collected and immediately combined with 1.0 ml of TUE (8 M urea, 50 mM Tris-HCl, pH 7.5) and immediately combined with two volumes of 10 M sulfuric acid containing 50 mM Tris-Cl, 10 mM EDTA, pH 7.0. Cell monolayers were washed with phosphate buffered saline (pH 7.4) and ECM proteins were isolated by gently scraping cell monolayers in 1.0 ml urea, 50 mM Tris-HCl, 50 mM EDTA, pH 7.0. The resulting suspension was centrifuged (5500 × g) for 10 min, and the supernatant was collected and defined as the ECM fraction. The pellet was resuspended with 50 mM Tris-HCl, 0.1% Triton X-100, pH 7.0. The extract was clarified by centrifugation, and the supernatant was collected and defined as the cell fraction (30). Cell number was determined by measuring acid phosphatase activity on a replicate set of cultures (31). Total radiolabeled protein present in [³⁵S]cysteine/methionine labeled samples was determined by performing trichloroacetic acid precipitation on aliquots of medium and cell fractions prior to proteoglycan purification and quantitating the radioactivity in a liquid scintillation counter (27).

**Proteoglycan Purification**—Medium, cell, or ECM fractions were mixed with 1.0 ml of a 70% Q-Sepharose suspension and rocked for 45 min. The slurries were poured into 5.0-ml disposable minicolumns (Pierce), and the unbound fractions were discarded. The columns were washed with 25 column volumes of TUE (8 M urea, 50 mM Tris-HCl, 50 mM EDTA, pH 7.0) and subsequently washed with 25 column volumes of TUE containing 0.2 M NaCl. Columns were eluted with 7 column volumes of TUE containing 1.5 M NaCl. Salt fractions were exhaustively dialyzed against Milli-Q water, using membranes with a molecular weight cutoff of 25,000 (Spectrope, Laguna Hills, CA), and lyophilized. Samples were resuspended in 2 ml sodium phosphate, 0 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, pH 7.4.

Selective Polysaccharidase Treatment—Selective polysaccharidases were used to identify and quantitate GAGs and proteoglycan core proteins. Digestion conditions were optimized for time, temperature, concentration, and pH. Enzymes were routinely tested for activity and specificity using highly purified GAG standards (Seikagaku, Tokyo, Japan) and the dimethyl methylene blue assay (32). Purified proteoglycans were subjected to digestion for 3 h at 37°C in 40 mM Tris-HCl. The pH of the digest was adjusted to the optimum for each enzyme: chondroitinase ABC (1.0 unit/ml, pH 5.0), a mixture of heparinase I and heparinase III (10 and 20 units/ml respectively, pH 7.8), and a mixture of keratanase II and endo-β-galactosidase (both enzymes 0.1 unit/ml, pH 6.0).

**Glycosaminoglycans Analysis**—Specific GAGs were quantitated by measuring the low molecular weight digestion products released after polysaccharidase treatment. Purified GAGs co-radiolabeled with [³⁵S]glucosamine and [³⁵S]SO₄ were treated with chondroitinase ABC, a mixture of heparinase I and heparinase III, a mixture of keratanase II and endo-β-galactosidase, or control with buffer lacking enzyme. Digested residues resulting from chondroitinase ABC treatment was a generous gift from Dr. John Coughman (University of Alabama, Birmingham, AL).

**Corneal Fibroblast Isolation and Cell Culture**—Corneas were excised from whole rabbit eyes purchased from Pel Freeze (Rogers, AR), and the epithelium and endothelium were removed as described previously (29). The corneas were washed two times with Dulbecco’s modified Eagle’s medium (DMEM) containing 1000 units/ml penicillin, 1.0 mg/ml streptomyein sulfate, and 20 units/ml nystatin. The corneas were minced with a sterile razor blade and subsequently digested with collagenase A (1.5 mg/ml) in DMEM containing 200 units/ml penicillin, 200 μg/ml streptomyein, and 100 units/ml nystatin for 2-3 h with agitation at 37°C. The digests were centrifuged at 1800 × g for 10 min, and the cells were suspended in DME supplemented with 100 units/ml penicillin, 100 μg/ml streptomyein, 100 units/ml nystatin, nonessential amino acids, 10% fetal calf serum (FCS). Cell were plated in 75-mm vented tissue culture flasks, and cultures were maintained in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomyein, 100 units/ml nystatin, nonessential amino acids and 4% fetal calf serum. The cultures achieved confluency after 7–10 days, at which time cells were passaged 1:4 and cultured in 4% FCS for 3 days. All experiments were performed on confluent fibroblast cultures that had been passaged once.

**Cell Treatment and Metabolic Labeling**—The synthesis of sulfated glycosaminoglycans was followed by metabolically radiolabeling corneal fibroblasts with [³⁵S]cysteine/methionine (50 μCi/ml) and [³⁵S]sulfate (36 μCi/ml). Proteoglycan core proteins were metabolically labeled with [³⁵S]cysteine/methionine and [³⁵S]sulfate (36 μCi/ml). Corneal fibroblasts in first passage were cultured until confluent (3 days) in 4% FCS. Upon confluence, corneal fibroblasts were treated as indicated in the figure legends. Radioisotopes were added immediately after the addition of TGF-β1.

After the designated radiolabeling period, the medium was collected and immediately combined with 1.0 ml of TUE (8 M urea, 50 mM Tris-HCl, 10 mM EDTA, pH 7.0). Cell monolayers were washed with phosphate buffered saline (pH 7.4) and ECM proteins were isolated by gently scraping cell monolayers in 1.0 ml urea, 50 mM Tris-HCl, 50 mM EDTA, pH 7.0. The resulting suspension was centrifuged (5500 × g) for 10 min, and the supernatant was collected and defined as the ECM fraction. The pellet was resuspended with 50 mM Tris-HCl, 0.1% Triton X-100, pH 7.0. The extract was clarified by centrifugation, and the supernatant was collected and defined as the cell fraction (30). Cell number was determined by measuring acid phosphatase activity on a replicate set of cultures (31). Total radiolabeled protein present in [³⁵S]cysteine/methionine labeled samples was determined by performing trichloroacetic acid precipitation on aliquots of medium and cell fractions prior to proteoglycan purification and quantitating the radioactivity in a liquid scintillation counter (27).
Proteoglycans Synthesized by Cultured Corneal Fibroblasts

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TABLE I

|$^3$H|Glucosamine incorporation into GAGs by corneal fibroblasts cultured in 1 or 10% dFCS with or without TGF-β1 for 96 h

| Fraction          | Chondroitin/dermatan sulfate<sup>a</sup> | Keratan sulfate<sup>b</sup> | Heparan sulfate<sup>c</sup> |
|-------------------|----------------------------------------|--------------------------|---------------------------|
|                   | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 |
|                   | %       |        |        |        |        |        |        |        |
| Medium            | 1       | 14,259 ± 890 | 13,863 ± 493 | 129 ± 3 | 174 ± 4 | 3126 ± 180 | 3594 ± 166 |
|                   | 10      | 8062 ± 112 | 7479 ± 402 | 52 ± 9 | 57 ± 7 | 3029 ± 60 | 2519 ± 91 |
| Cell              | 1       | 3076 ± 167 | 2037 ± 584 | 27 ± 14 | 25 ± 14 | 1842 ± 159 | 1495 ± 362 |
|                   | 10      | 1234 ± 211 | 1233 ± 67 | 10 ± 2 | 4 ± 3 | 648 ± 110 | 661 ± 29 |
| ECM               | 1       | 486 ± 117 | 358 ± 37 | 2 ± 2 | 7 ± 1 | 426 ± 95 | 324 ± 37 |
|                   | 10      | 170 ± 12 | 186 ± 10 | 0 ± 1 | 1 ± 0 | 106 ± 14 | 118 ± 2 |

<sup>a</sup> Data obtained using chondroitinase ABC.
<sup>b</sup> Data obtained using a mixture of keratanase II and endo-β-galactosidase.
<sup>c</sup> Data obtained using a mixture of heparinases I and III.

Approximately 75–85% of the |H|GAGs synthesized during the 96-h incubation period were secreted into the medium with the remaining present in the cell (16–21%) and ECM (2–3%) fractions. Compositional analysis revealed that the majority of the |H|GAGs synthesized by corneal fibroblast in culture were CS (53–80%) and HS (18–47%), with a trace amount of a nonsulfated form of keratan (<1%). Corneal fibroblasts cultured in 10% dFCS showed an overall reduction in CS synthesis compared with cells cultured in 1% dFCS. This was evident from both |[35S]SO<sub>4</sub>| and |[3H]glucosamine incorporation.

RESULTS

Effects of TGF-β1 and Fetal Calf Serum on GAG Synthesis—Confluent corneal fibroblasts were cultured for 96 h in either 1 or 10% dialyzed fetal calf serum with or without daily treatments with TGF-β1 (1 ng/ml). Cellular viability studies were performed, and no significant differences were observed after 6 days of culture in 0, 1, or 10% FCS (data not shown). To evaluate GAG synthesis and sulfation, cells were metabolically labeled with |[35S]SO<sub>4</sub>| and |[3H]glucosamine. Glycosaminoglycans in medium, cell, and ECM fractions were purified using anion exchange chromatography on Q-Sepharose. The chromatographic conditions were optimized to separate highly charged proteoglycans from weakly charged glycoproteins and hyaluronic acid (16). Specific GAGs were quantitated by selective digestion with polysaccharidases. Chondroitin sulfate was determined using chondroitinase ABC. Glycosaminoglycans susceptible to chondroitinase ABC are referred to as CS because this enzyme does not distinguish between polymers containing idurionate and glucuronate (34). Keratan sulfate was determined using a mixture of keratanase II and endo-β-galactosidase, and HS was determined using a mixture of heparinases I and III. Sulfation was defined as the ratio of polysaccharide-sensitive |[35S]SO<sub>4</sub>| to polysaccharide-sensitive |[3H]glucosamine. Tables I and II summarize the data obtained by enzymatic analysis of purified GAGs.

Approximately 75–85% of the |H|GAGs synthesized during the 96-h incubation period were secreted into the medium with the remaining present in the cell (16–21%) and ECM (2–3%) fractions. Compositional analysis revealed that the majority of the |H|GAGs synthesized by corneal fibroblast in culture were CS (53–80%) and HS (18–47%), with a trace amount of a nonsulfated form of keratan (<1%). Corneal fibroblasts cultured in 10% dFCS showed an overall reduction in CS synthesis compared with cells cultured in 1% dFCS. This was evident from both |[35S]SO<sub>4</sub>| and |[3H]glucosamine incorporation. A similar decrease in HS was observed when cells were cultured in 10% dFCS compared with 1% dFCS. Treatment with TGF-β1 resulted in a reduction in the amount of |[35S]SO<sub>4</sub>| and |[3H]glucosamine-labeled CS recovered from cell and ECM fractions from cells cultured in 1% dFCS. Corneal fibroblasts grown in the presence of 10% dFCS showed increased sulfation of both CS and HS compared with 1% dFCS (Table III). Moreover, this increase in GAG sulfation must result from some nonspecific alteration in GAG metabolism, as both extracellular and cellular populations of CS and HS exhibited this response. TGF-β1 did not significantly influence the sulfation of GAGs synthesized by cells cultured in 1 or 10% dFCS.

The decrease in GAG synthesis observed with culture in 10% dFCS compared with 1% dFCS seems to be influenced by TGF-β1. Corneal fibroblasts exhibited smaller serum-associated decreases in |[3H]Cys| and |[3H]HS| isolated from cell and ECM fractions when TGF-β1 was present (Table IV). To evaluate the extent of this response, GAGs synthesized by corneal fibroblasts cultured in 1 or 10% dFCS with or without TGF-β1 (1 ng/ml) were monitored over a 96-h time course using |[35S]SO<sub>4</sub>| incorporation. Fig. 1 depicts the amount of GAGs synthesized by corneal fibroblasts cultured in 1% dFCS relative to 10% dFCS. Treatment with TGF-β1 attenuated the increase in |[35S]CS| synthesis observed when cells were cultured in medium containing 1% dFCS. This change was apparent in cell and medium fractions by 39 h and in ECM fractions after 96 h. TGF-β1 did not significantly alter the ability of serum to suppress synthesis of either cell-associated or secreted |[3H]HS|. The response to TGF-β1 was less pronounced when measuring |[35S]GAGs compared with |[3H]GAGs, presumably because of the increase in sulfation observed with culture in high serum.

Effects of TGF-β1 and Fetal Calf Serum on Protein Core Synthesis—Radiolabeled |([35S]Cys/Met)| proteoglycans were purified from the medium and cell fractions of confluent corneal fibroblast cultures treated with 1 or 10% dFCS with or without TGF-β1 for 24 or 96 h. Proteoglycan protein cores were detected using Western blotting and autoradiography, and band intensities were estimated using densitometry. Gel load-
Corneal fibroblasts were cultured for 96 h in 1 or 10% dFCS, and GAGs were labeled with [35S]SO4 and [3H]glucosamine. Purified GAGs were analyzed using selective polysaccharidases as described under “Experimental Procedures.” Data presented are the ratio of [35S]sulfate cpm to [3H]glucosamine cpm. Values were calculated from the results presented in Tables I and II.

| Fraction | dFCS | Chondroitin/dermatan sulfate | Keratan sulfate | Heparan sulfate |
|----------|------|------------------------------|-----------------|-----------------|
|          |      | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 |
| Medium   | 1    | 0.166  | 0.163  | 0.000  | 0.000  | 0.140  | 0.132  |
|          | 10   | 0.221  | 0.251  | 0.000  | 0.000  | 0.165  | 0.186  |
| Cell     | 1    | 0.136  | 0.119  | 0.000  | 0.000  | 0.091  | 0.091  |
|          | 10   | 0.197  | 0.204  | 0.000  | 0.000  | 0.123  | 0.131  |
| ECM      | 1    | 0.133  | 0.121  | 0.000  | 0.000  | 0.084  | 0.088  |
|          | 10   | 0.199  | 0.208  | 0.000  | 0.000  | 0.131  | 0.139  |

* Data obtained using chondroitinase ABC.
* Data obtained using a mixture of keratanase II and endo-β-galactosidase.
* Data obtained using a mixture of heparinases I and III.
* ND, not detected.

**Table III**

**Sulfation of GAGs synthesized by corneal fibroblasts cultured in 1 or 10% dFCS with or without TGF-β1 for 96 h**

Glycosaminoglycans synthesized by corneal fibroblasts were compared in cells treated with or without TGF-β1. Values are the fold differences in specific GAGs synthesized after cultured in 1% dFCS compared to specific GAGs synthesized after cultured in 10% dFCS.

| Fraction | dFCS | Chondroitin/dermatan sulfate | Keratan sulfate | Heparan sulfate |
|----------|------|------------------------------|-----------------|-----------------|
|          |      | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 |
| Medium   | 1    | 1.58   | 1.85   | 1.03  | 1.43  | 2.84  | 2.26  |
|          | 10   | 2.49   | 2.12   | 4.01  | 2.75  |

The influence of TGF-β1 on serum associated changes in GAG synthesis by corneal fibroblasts cultured for 96 h

**Table IV**

Glycosaminoglycans synthesized by corneal fibroblasts were compared in cells treated with or without TGF-β1. Values are the fold differences in specific GAGs synthesized after cultured in 1% dFCS compared to specific GAGs synthesized after cultured in 10% dFCS.

| Fraction | Chondroitin/dermatan sulfate | Heparan sulfate |
|----------|------------------------------|-----------------|
|          | −TGF-β1 | +TGF-β1 | −TGF-β1 | +TGF-β1 |
| Medium   | 1.58   | 1.85   | 1.03  | 1.43  |
| Cell     | 2.49   | 2.12   | 4.01  | 2.75  |
| ECM      | 2.85   | 1.92   | 2.84  | 2.26  |

Electrophoresis of proteoglycans from the medium on 10% SDS-PAGE gels without prior enzyme treatment resulted in poorly resolved smears migrating between Mr 140,000 and 200,000. After digestion with chondroitinase ABC, these smears were not longer apparent, and bands with Mr 60,000, and Mr 119,100 were present (Fig. 2.). A core protein (Mr 60, 600) was also released by heparinase treatment. Digestion with both chondroitinase ABC and heparinases I and III did not release any additional core proteins or substantially alter the electrophoretic mobility of the protein cores. A chondroitin/dermatan sulfate proteoglycan core (Mr 45,000) protein migrated as a doublet and was identified as decorin using Western blot analysis (Fig. 3.). After 24 h, decorin levels were 1.4-fold higher in cells cultured in 1% dFCS than in cells cultured in 10% dFCS. Cultures treated with TGF-β1 had decorin levels similar to controls. After 96 h, decorin was 2.1-fold higher in cells cultured in 1% dFCS than in cells cultured in 10% TGF-β1 decreased decorin by 32 and 19% in 1 and 10% dFCS respectively. The Mr 60,000 and Mr 119,100 core proteins detected after 96 h in culture migrated with Mr consistent with those of syndecan-1 and betaglycan (35, 36). Syndecan-1 belongs to the class of transmembrane proteins that undergo proteolytic cleavage and release their ectodomains into the extracellular environment (37, 38). Betaglycan has been reported to exist as a soluble form that is released by cells into the medium and is found in the extracellular matrices and serum (36, 39).

Analysis of lyase-treated proteoglycans from the medium on 5% SDS-PAGE gels revealed the presence of three high Mr proteoglycan core proteins (Mr 375,000, ~ 440,000, and ~ 480,000) (Fig. 4). The Mr 375,000 protein core was released by treatment with both chondroitinase ABC, heparinases I and III, or a mixture of both lyases, indicative of a proteoglycan bearing either CS, HS, or both CS and HS. The Mr 440,000 core protein could only be resolved after treatment with heparinases I and III or with both heparinases and chondroitinase ABC and was therefore synthesized either as an HSPG bearing only HS chains or a hybrid proteoglycan possessing both HS and CS chains. Both the Mr 375,000 and Mr 440,000 core proteins containing either HS and/or CS chains reacted with a monoclonal antibody directed against perlecain (Fig. 5). This heterogeneity observed in the size of the perlecain core protein is suggestive of alternative splicing, as has been reported in several species (40). Heparan sulfate proteoglycan and CSPG
forms of perlecan have been reported in the Engelbreth-Holm-Swarm tumor matrix (41). The Mr 480,000 protein core was derived from a CSPG, as it was only observed after treatment with chondroitinase ABC or both chondroitinase ABC and heparinase treatment. The Mr 480,000, core protein did not react with the antibody directed against perlecan. The smear between Mr 400,000 and 460,000 in both the heparinase-treated and untreated control lanes was not present after treatment with chondroitinase ABC and likely represents an intact CSPG.

To determine the effects of TGF-β1 and serum on the secreted high molecular weight core proteins, bands detected on autoradiographs after combined chondroitinase ABC and heparinases I and III treatment were compared by densitometry. A 1.6-fold increase in the amount of the Mr 375,000 perlecan secreted by TGF-β1-treated corneal fibroblasts cultured in 1% dFCS was observed after 24 h, and a 2.0-fold increase was observed after 96 h. This stimulatory effect of TGF-β1 did not depend on serum factors, as similar increases were observed when cells were cultured in 10% dFCS for 24 h. Further more, culture in 10% dFCS for 24 h increased the levels of the Mr 440,000 core by 2.3-fold with respect to 1% dFCS.

Polysaccharidase-treated proteoglycans purified from cell fractions were analyzed by Western blot analysis. Blots were probed with antibodies that recognize CS stubs remaining after chondroitinase ABC treatment (R36) or HS stubs remaining after heparinase treatment (mAb 3G10). Western blot analysis with R38 revealed the presence of a Mr 544,700 band that was released only with combined chondroitinase and heparinase treatment (Fig. 6A). Interestingly, this band did not react with mAb 3G10. It is conceivable that chondroitinase ABC may have modified the epitope for mAb 3G10. Heparinases I and III released a Mr 51,600 protein core that reacted with mAb 3G10 (Fig. 6B). This band was not detected after combined chondroitinase ABC and heparinase treatment. The presence of both CS and HS chains and the electrophoretic mobility

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**FIG. 1.** TGF-β1 modulates serum-associated changes in GAG synthesis. Corneal fibroblasts were metabolically radiolabeled with [35S]SO₄ and medium (A and B), cell (C and D), and ECM (E and F) fractions were dot blotted onto replicate pairs of cationic nylon filters. One filter was treated with nitrous acid and the other was not treated. [35S]GAG was determined by counting each filter using liquid scintillation and the values were normalized to cell number. Heparan sulfate was defined as [35S]GAG susceptible to nitrous acid, and CS content was defined as [35S]GAG resistant to nitrous acid. Serum associated changes in CS (A, C, and E) and HS (B, D, and F) synthesis after treatment with (●) or without TGF-β1 (○) was examined over a 96 h time course. The data are presented as the fold differences in specific GAGs synthesized after culture in 1% dFCS compared with cultured in 10% dFCS ± S.E. (n = 3).
Cultured for 96 h in 1 or 10% dFCS with [35S]SO_4 were analyzed on 5% SDS-PAGE gels. Bands although modestly elevated after treatment with 1 ng/ml TGF-β exhibited 5.7-fold decrease relative to control after treatment with either 5 or 10 ng/ml TGF-β. Both the synthesis decreased by as much as 61.6-fold relative to control. During this period, HS synthesis, exhibited broad size heterogeneity characteristic of proteoglycan migration on SDS-PAGE gels (Fig. 7). Chondroitin sulfate synthesis decreased by as much as 3.0-fold) (Fig. 7, C). From 24 to 48 h, the overall synthesis of of CS was not significantly affected by TGF-β. After 96 h, both the $M_r = 44,700$ and $M_r = 51,600$ protein cores were decreased in cells cultured in 10% dFCS compared with 1% dFCS but were unchanged by treatment with TGF-β1.

**Effects of TGF-β1 on Proteoglycan Synthesis**—To examine the effects of TGF-β1 in the absence of serum factors, corneal fibroblasts were cultured without dFCS for 18 h and subsequently treated with 0, 1, 5, or 10 ng/ml TGF-β1. Proteoglycans were radiolabeled with [35S]SO_4 for 0–24, 24–48, or 48–72 h, and medium fractions were collected after the radiolabeling periods. Aliquots of medium were treated with chondroitinase ABC or left untreated, and resistant proteoglycans were isolated on Q-Sepharose columns. Chondroitin sulfate was defined as GAG susceptible to chondroitinase ABC. Heparan sulfate was defined as GAG resistant to chondroitinase ABC. The TGF-β1-induced changes in secreteable core protein (median $M_r \approx 175,000$), a high $M_r$ proteoglycan (median $M_r \approx 420,000$), and a second high $M_r$ proteoglycan that barely entered the gel. To quantitate TGF-β1-induced changes in secreted proteoglycans, bands detected on autoradiographs were normalized to the amount total radioactive protein present in medium prior to anion exchange chromatography. Data are representative of at least three experiments.

**Fig. 2. Analysis of low $M_r$ core proteins.** Corneal fibroblasts were cultured for 96 h in 1 or 10% dFCS ± TGF-β1 (1 ng/ml), and proteins were radiolabeled with [35S]Cys/Met. Proteoglycans purified from the medium were digested with either chondroitinase ABC, both chondroitinase ABC and heparinases I and III, or buffer lacking enzyme. Digests were run under reducing conditions on 5% SDS-PAGE gels. Radioactive proteins were detected using autoradiography. Sample loading was normalized to the amount total radioactive protein present in 10% (trichloroacetic acid precipitation) prior to anion exchange chromatography. Data are representative of at least three experiments.

**Fig. 3. Analysis of decorin.** Corneal fibroblasts were cultured for 24 h in 1 or 10% dFCS ± TGF-β1 (1 ng/ml). Radiolabeled ([35S]Cys/Met) proteoglycans purified from the medium were digested with chondroitinase ABC or buffer lacking enzyme. Digests were run on 10% SDS-PAGE gels under reducing conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride. A, blots were probed with polyclonal antiserum raised against rabbit corneal decorin. B, after probing, radioactive proteins were detected using autoradiography. Sample loading was normalized to the amount total radioactive protein present in medium (trichloroacetic acid precipitation) prior to Q-Sepharose chromatography. Data are representative of at least three experiments.

**Fig. 4. Analysis of high $M_r$ core proteins.** Corneal fibroblasts were cultured for 96 h in 1 or 10% dFCS ± TGF-β1 (1 ng/ml), and proteins were radiolabeled with [35S]Cys/Met. Proteoglycans purified from the medium were digested with chondroitinase ABC, both chondroitinase ABC and heparinases I and III, or buffer lacking enzyme. Digests were run under reducing conditions on 5% SDS-PAGE gels. Sample loading was normalized to total radioactive protein present in medium (trichloroacetic acid precipitation) prior to purification. Radioactive proteins were detected using autoradiography. Data are representative of at least three experiments.
Compared by densitometry. The second high $M_r$ proteoglycan was not sufficiently resolved to be accurately quantitated. TGF-β1 treatment resulted in dose dependent increases in the high $M_r$ proteoglycans from 0 to 24 h (up to 4.7-fold) and from 24 to 48 h (up to 3.3-fold). In contrast, the low $M_r$ decreased as much as 47% during the 0–24 h labeling period and by as much as 70% during the 48–72 h labeling period. During the 48–72 h labeling period, TGF-β1 treatment resulted in substantial decreases in both the low and high $M_r$ proteoglycans. The low $M_r$ proteoglycan decreased by 76% in 1 ng/ml TGF-β1, relative to control, and was not detected at higher concentrations of TGF-β1. The high $M_r$ was not significantly changed by 1 ng/ml TGF-β1, and was not detected at higher concentrations of TGF-β1. These TGF-β1-dependent decreases in proteoglycan synthesis from 48 to 72 h after treatment were not the result of decreased cell viability as cells treated with TGF-β1 exhibited similar levels of $[^{3}H]$thymidine incorporation into DNA during this period.

**DISCUSSION**

The current study was initiated to identify the major core proteins and GAG chains synthesized by rabbit corneal fibroblasts in culture. A defined culture system will allow the systematic examination of the relationship between specific growth factors and proteoglycans within the injured cornea in vitro. This system should provide a useful model of corneal injury. Because TGF-β has been detected in the corneal stroma after injury, we examined the effects of TGF-β1 and serum on proteoglycan synthesis by corneal fibroblasts (16, 21). The results of these studies showed that the synthetic profile of proteoglycans produced by corneal fibroblasts in culture, although significantly different from those of normal corneas, were remarkably similar to those found in wounded corneas. Approximately 60% of the GAG in the normal corneal stroma is KS and 40% is chondroitin/dermatan sulfate (2–4). Wounded corneal stromas synthesize increased quantities of both CS and HS and have reduced KS content (13–17). Corneal fibroblasts, in our culture system, synthesize substantial quantities of CS and HS with negligible amounts of an unsulfated form of keratan. Hassell et al. (13) reported that wounded corneas synthesize unusually large CSPGs (13). We detected substantial quantities of large proteoglycans bearing CS and HS chains secreted into the culture medium. Several reports have documented increased sulfation of CS and HS and decreased sulfation of KS after wounding (14–15, 17). Keratan was not sulfated in our system, whereas the sulfation of CS and HS was significantly increased with increased serum. These results suggest that conventional cell culture and injury induce similar phenotypic changes and that altered proteoglycan expression is a reflection of these changes.

In addition to the GAG chains, we extended the analysis of proteoglycan production by characterizing and identifying core proteins secreted into the medium of cultured corneal fibroblasts. A defined culture system will allow the study of proteoglycan synthesis by corneal fibroblasts (16, 21). The results of these studies showed that the synthetic profile of proteoglycans produced by corneal fibroblasts in culture, although significantly different from those of normal corneas, were remarkably similar to those found in wounded corneas. Approximately 60% of the GAG in the normal corneal stroma is KS and 40% is chondroitin/dermatan sulfate (2–4). Wounded corneal stromas synthesize increased quantities of both CS and HS and have reduced KS content (13–17). Corneal fibroblasts, in our culture system, synthesize substantial quantities of CS and HS with negligible amounts of an unsulfated form of keratan. Hassell et al. (13) reported that wounded corneas synthesize unusually large CSPGs (13). We detected substantial quantities of large proteoglycans bearing CS and HS chains secreted into the culture medium. Several reports have documented increased sulfation of CS and HS and decreased sulfation of KS after wounding (14–15, 17). Keratan was not sulfated in our system, whereas the sulfation of CS and HS was significantly increased with increased serum. These results suggest that conventional cell culture and injury induce similar phenotypic changes and that altered proteoglycan expression is a reflection of these changes.

**Fig. 5. Analysis of perlecan.** Corneal fibroblasts were cultured for 24 h in 1 or 10% dFCS + TGF-β1 (1 ng/ml). Radiolabeled proteoglycans ($[^{35}S]$Cys/Met) purified from the medium were digested with chondroitinase ABC, heparinase I and III, or with both chondroitinase ABC and heparinase I and III. Digests were run under reducing conditions on 5% SDS-PAGE gels and electrophoretically transferred to polyvinylidene difluoride. Sample loading was normalized to total radioactive protein present in medium (trichloroacetic acid precipitation) prior to purification. An autoradiograph was developed, and the blots were probed with mAb A7L6. Data are representative of at least three experiments.

**Fig. 6. Analysis of cell-associated CSPGs and HSPGs.** Corneal fibroblasts were cultured for 96 h in 1 or 10% dFCS + TGF-β1 (1 ng/ml), and radiolabeled proteoglycans ($[^{35}S]$Cys/Met) were purified from the cell fraction on Q-Sepharose columns. An immunoblot analysis of proteoglycans digested with chondroitinase ABC, chondroitinase ABC and heparinase I and III, or a buffer lacking enzyme. Blots were probed with antibody R36 directed against the CS stub remaining after chondroitinase ABC digestion. A, immunoblot analysis of proteoglycans digested with chondroitinase ABC and heparinase I and III, or a buffer lacking enzyme. Blots were probed with mAb 3G10 directed against the HS stub remaining after heparinase treatment. Sample loading was normalized to total radioactive protein present (trichloroacetic acid precipitation) prior to purification. Data are representative of at least three experiments.
proteoglycans and a normal constituent of the corneal stroma (10). There is increasing evidence that decorin is an important regulator of a number of important physiological processes. Several studies suggest an inhibitory role for decorin on cell proliferation through TGF-β-dependent and TGF-β-independent mechanisms. Overexpression of decorin inhibits cell growth in a number of different cell types (42–45). Addition of exogenous recombinant decorin to cultures of several tumor cell lines suppresses cell growth (43). Decorin suppresses cell growth by activating the epidermal growth factor receptor and elevating cytosolic Ca\(^{2+}\) in A431 squamous carcinoma cells (46–47). In addition, Yamaguchi et al. (48) demonstrated that decorin specifically binds TGF-β and suppressed its growth stimulatory activity in Chinese hamster ovary cells. The ability of decorin to modulate the bioactivity of TGF-β1 has been demonstrated in a number of different systems (49–51). The effects of TGF-β on decorin expression vary widely among different fibroblast types. TGF-β down-regulates decorin expression in dermal (52) and gingival fibroblasts (53), whereas TGF-β up-regulates decorin expression in lung fibroblasts (54) and myocardial fibroblasts (55). TGF-β has been shown to stimulate proliferation of corneal fibroblasts through a mechanism that may involve the induction of basic fibroblast growth factor (56). In our model, both TGF-β treatment and culture in high serum decreased decorin production (Figs. 2 and 3). This is consistent with studies showing a substantial induction of decorin expression upon quiescence in a variety of fibroblasts (52, 57, 58). The observations that decorin is an inducer of quiescence and that decorin is induced upon quiescence are suggestive of an autocrine mechanism of cell growth control possibly involving TGF-β. It is conceivable that the substantial quantities of decorin in the normal corneal stroma limits TGF-β activity. In this manner, the proteolytic degradation of decorin likely to occur within a corneal wound might remove the restriction on TGF-β activity within this localized region.

In addition to decorin, corneal fibroblasts in our system synthesized two large core proteins (M\(_r\) \(\approx\) 375,000 and \(\approx\) 440,000) that reacted with a mAb directed against perlecan. The M\(_r\) \(\approx\) 440,000 isoform was synthesized as either an HSPG or a hybrid possessing both CS and HS. The M\(_r\) \(\approx\) 375,000 isoform was primarily glycanated with CS; however, forms bearing HS or potentially both CS and HS were detected. Perlecan, initially identified as an HSPG (59), has also been shown to be glycanated with CS or both CS and HS in a number of different tissues and cell types (26, 41, 60–64). Perlecan isolated from the culture media in our system has shown heterogeneity not only in GAG chain substitution but also in the size of the core proteins. Size variants of perlecan bearing HS chains have been detected in the Engelbreth-Holm-Swarm tumor matrix (41). Several studies suggest that perlecan variants may be generated by alternative splicing in human (65–66) and mouse (67). The significance of this heterogeneity of perlecan is not fully understood. However, when considering the potential importance of HS and its interactions with growth factors (68), it is conceivable that alternate glycanation could have a substantial impact on the biological activity of perlecan. We find that both TGF-β1 and serum induce substantial increases in perlecan bearing both CS and HS chains at the early time point (Fig. 5). In contrast, after extended periods in culture the amount of perlecan bearing HS chains in the medium was unaffected by TGF-β1, and decreased by serum. The levels of perlecan bearing CS chains were elevated with TGF-β1 treatment and were decreased with serum (Fig. 6). These apparent differences with respect to culture duration and perlecan expression are indicative of an indirect response. The fact that TGF-β can induce secondary effectors, such as growth factors
and matrix molecules, introduces further levels of complexity to the mechanism through which TGF-β may influence proteoglycan synthesis.

Corneal fibroblasts secreted an additional large core protein linked to CS (Mₚ ~ 480,000) that did not react with a mAb directed against perlecan. It is unlikely that the Mₚ ~ 480,000 protein core was a hybrid possessing both CS and HS chains, as the band intensities of the cores released with both chondroitinase ABC and heparinase did not significantly differ from those released with chondroitinase ABC alone. This core protein may be a perlecan variant lacking the epitope recognized by mAb 9AT6 or may represent a novel CSPG. Although the identity of this proteoglycan is unclear, it may be important during wound healing as it responds to both TGF-β1 and serum in culture.

Our results indicate that corneal fibroblasts in culture synthesize predominantly CS and HS with trace amounts of an unsulfated keratan. The major proteoglycan species secreted into the medium were decorin and perlecan. Our analysis indicates that TGF-β1 and serum modulate the GAG chains and protein cores of both of these proteoglycans. In light of the influence that both decorin and perlecan exert on growth factor activity, changes in the expression of either of these proteoglycans will affect the cooperation between proteoglycans and growth factors analyzed in detail. These studies could ultimately provide important insight into the mechanisms that control tissue remodeling after corneal stromal injury.

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