Proteoglycan Expression during Transforming Growth Factor β-induced Keratocyte-Myofibroblast Transdifferentiation*

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Keratocytes of the corneal stroma secrete a unique population of proteoglycan molecules considered essential for corneal transparency. In healing corneal wounds, keratocytes exhibit a myofibroblastic phenotype in response to transforming growth factor β (TGF-β), characterized by expression of α-smooth muscle actin. This study examined proteoglycan and collagen expression by keratocytes in vitro during the TGF-β-induced keratocyte-myofibroblast transition. TGF-β-treated primary bovine keratocytes developed myofibroblastic features, including actin stress fibers anchored to paxillin-containing focal adhesions, cell-associated fibronectin, α5 integrin, and α-smooth muscle actin. Collagen I and III protein and mRNA increased in response to TGF-β. Secretion of [35S]sulfate-labeled keratan sulfate proteoglycans decreased markedly in response to TGF-β. Dermatan sulfate proteoglycans, however, increased in size and abundance. Protein and mRNA transcripts for normal stromal proteoglycans (lumican, keratocan, and mimecan), that in other tissues occur as nonsulfated glycoproteins (1–3), were increased in size and abundance in response to TGF-β, but protein expression and mRNA for biglycan, a proteoglycan present in fibrotic tissue, was markedly up-regulated. These results show that TGF-β in vitro induces a proteoglycan expression pattern similar to that of corneal scars in vivo. This altered proteoglycan expression occurred coordinately with transdifferentiation of keratocytes to the myofibroblastic phenotype, implicating these cells as the source of fibrotic tissue in nontransparent corneal scars.

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Transparency of the cornea to light is a function of the ultrastructure of the corneal stroma, a tissue composed of multiple lamellae of parallel, highly regular collagen fibrils. Trauma, inflammation, and several chronic pathological conditions lead to disruption of the stromal molecular architecture by the accumulation of a disorganized fibrotic extracellular matrix. This scarring is often associated with loss of corneal transparency and can contribute to permanent loss of vision. The essential role of corneal extracellular matrix in vision has led to an extensive characterization of the molecular components of the stroma of both normal and fibrotic corneas.

One specialized feature of the stroma is the composition of the hydrated matrix surrounding the collagen fibrils. This extracellular matrix consists of proteoglycans and glycoproteins and includes a unique class of keratan sulfate-containing proteoglycans. Keratan sulfate glycosaminoglycan chains in the stroma modify three proteins, lumican, keratocan, and mimecan, that in other tissues occur as nonsulfated glycoproteins (1–3). The unique abundance and tissue-specific nature of the corneal keratan sulfate proteoglycans (KSPGs)1 suggests their role in corneal transparency. This hypothesis is supported by the occurrence of corneal opacity in diseases in which corneal keratan sulfate is absent and also in mice lacking lumican expression in which corneal keratan sulfate is reduced (4, 5). The lumican knockout mice develop corneal haze at 3–6 months of age, the same period of time during which corneal keratan sulfate accumulates in normal mouse corneas (6). In corneal scars resulting from trauma or from chronic pathologies, keratan sulfate is reduced and dermatan sulfate-containing proteoglycans are elevated (7–11). This characteristic alteration in stromal proteoglycans appears likely to be an important factor in the loss of corneal transparency.

Keratocytes are quiescent, neural crest-derived cells that populate the corneal stroma and secrete the molecular components of the stromal extracellular matrix. In healing corneal wounds, keratocytes become activated, migrate to the wound site, and begin mitosis (12). In later stages of healing, keratocytes develop F-actin stress fibers containing the muscle protein α-smooth muscle actin (13). These contractile cells, known as myofibroblasts, are hypothesized to be involved in wound closure (14). Keratocytes in vitro develop myofibroblastic characteristics in response to exogenous or autocrine TGF-β (15, 16). In vivo, antibodies blocking TGF-β eliminate the presence of myofibroblasts in healing wounds (17). These observations suggest that TGF-β plays an important role in the biology of wound healing and may be involved in the secretion of fibrotic tissue.

We recently demonstrated that bovine keratocytes maintained in vitro without serum or in reduced mitogen-horse serum, maintain stable secretion of proteoglycans similar to those produced in vivo (18). Growth of the cells in fetal bovine serum, however, rapidly reduced the KSPG synthesis by these cultures in a manner similar to that of healing wounds (19). This earlier study also presented preliminary data suggesting that KSPGs were down-regulated by exposure of keratocytes to TGF-β (19). In the current study, we have identified conditions

1 The abbreviations used are: KSPG, keratan sulfate proteoglycan; TGF-β, transforming growth factor β; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.
under which bovine keratocytes develop myofibroblastic characteristics in response to TGF-β and examined the proteoglycans secreted during this transition. The results suggest that secretion of proteoglycans characteristic of fibrotic scars is coordinated with the development of a myofibroblastic phenotype as a response to TGF-β.

**MATERIALS AND METHODS**

**Cell Culture**—Primary cultures of bovine keratocytes were prepared and cultured at 4 × 10^6 cells/cm^2^ in Dulbecco's modified Eagle's medium-F-12 medium with antibiotics and 1% (v/v) platelet-poor horse serum (previously described (19)). All protocols involved 6-day culture. Recombinant human TGF-β1 (Sigma T7039) was added to a concentration of 1 ng/ml at different times during the 6-day culture to achieve the length of exposure noted in individual experiments.

**Proteoglycan Analysis**—Proteoglycans were metabolically labeled with [35S]sulfate (ICN Radiochemicals; catalog no. 64041), 100 μCi/ml or with a 35S-labeled mixture of methionine and cysteine (TransLabel; ICN Radiochemicals; catalog no. 51006) at 100 μCi/ml for 24 h before analysis. Proteoglycans were recovered from culture media by ion exchange chromatography on SPEC-NH₂ microcolumns (Ansys Diagnostics, Inc.), and dermantan sulfate and keratan sulfate-containing proteoglycans were separated by fractional precipitation with 50 and 70% ethanol as described previously (19). The purified, labeled proteoglycans were dissolved in 0.1M Tris acetate, 0.1% Triton X-100, pH 8, and radioactivity was determined by scintillation counting. The 70% (dermantan sulfate) precipitate was digested for 2 h at 37°C with 0.1 unit/ml affinity-pure chondroitinase ABC (Sigma) to remove residual dermantan sulfate. The intact proteoglycans were separated on 3–12% SDS-PAGE gels, transferred to Millipore Immobilon PVDF membranes, and detected by autoradiography using an Eastman Kodak Co. LE Transcreen and BIOMax MR film as previously described (19).

**Western Blotting**—Proteoglycan proteins were detected by immunoblotting after digestion of glycosaminoglycan chains from nonlabeled proteoglycans using a procedure similar to that described previously (20). Dermantan sulfate proteoglycans were treated with chondroitinase as described above, and keratan sulfate was digested with endo-β-galactosidase (Seikagaku Corp.; 4 millunits/ml for 2 h at 37°C). The core proteins were separated on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and detected with an affinity-purified antibody that detects all three KSPG proteins or with antisera against synthetic peptides of decorin (LF96) or biglycan (LF94) (10, 21, 22).

**Collagen Analysis**—Cultures exposed to TGF-β for different time periods were supplemented with sodium ascorbate (50 μg/ml) 24 h before harvesting and metabolically labeled with 50 μCi/ml [3H]proline for the final 18 h. The cell layer from each 35 mm dish was extracted in 0.5 ml of cold 0.5 M acetic acid, and cell debris was removed by centrifugation at 10,000 × g. Noncollagenous proteins were digested with 20 μg/ml pepsin at 4°C overnight followed by dialysis against 0.5 M acetic acid. Samples were lyophilized, proteins were separated on 12% SDS-PAGE gels and transferred to PVDF membranes, and proteins were detected by autoradiography or immunoblotting as described above using monoclonal antibody clone COL-I against collagen I and clone FH-7A against collagen III (Sigma).

**Northern Blotting**—Total RNA isolated from cultured keratocytes was separated on 1% agarose-formaldehyde gels by electrophoresis. The RNA was transferred under alkaline conditions to nitrocellulose membranes by downward flow for 2.5 h and the RNA was cross-linked by ultraviolet exposure. Proteoglycan transcripts were detected with [32P]dCTP-labeled DNA probes generated by random priming using oligo(dT) primers (Stratagene; La Jolla, CA) according to protocols provided by the manufacturer. Templates for keratan sulfate bovine proteins were generated by polymerase chain reaction from cDNA plasmids using the following primers: lumican, 508 base pairs, 5'-CATTC-GACCTCAGAATAAGC-3' (sense) and 5'-GCAATGGAAGAAGG-3' (antisense); keratan, 953 base pairs, 5'-TTCAGCATTCTGACGAACTCTG-3' (sense) and 5'-GGTATCATTTGTGTGTTCTACG-3' (antisense); decorin, 953 base pairs, 5'-CAGCAGCACTCTCACAGC-3' (sense) and 5'-CCAAGTGAGGAGGGAGG-3' (antisense). Other cDNA probes were amplified from cDNA reverse transcribed from cultured keratocyte RNA using Superscript II (Life Technologies, Inc.) primed with oligo(dT) according to protocols provided by the manufacturer. Biglycan cDNA was amplified using 5'-TTGTCGAGATCTGCAGAATAAGC-3' (sense) and 5'-AGGTCTCAAGCCGCGTTCTC-3' (antisense), and decorin cDNA was amplified with 5'-GGATGGACAGAGTGTCG-3' (sense), and TGACTTTATGATCGGCCAGC-3' (antisense). The amplified cDNA was cloned into a pEG-T plasmid (Promega), and the identity of the inserted DNA was confirmed by sequence analysis. Bovine collagen α(1)I cDNA was amplified using 5'-GGTGAATGGAAGAAGG-3' (sense) and 5'-AGGTTCCACCAGATCCG-3' (antisense), and collagen α(1)(III) cDNA was amplified using 5'-GGGTTGCTCTCAACAGG-3' (sense) and 5'-CCAGATGGTTTGGTCGACAA-3' (antisense). Sequence of the collagen templates was confirmed by direct analysis of the amplified products. Murine ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase cDNA templates were obtained from Ambion Inc. Hybridization was carried out in 5 ml of Ultrahyb buffer (Ambion) at 45°C overnight. The blots were washed twice with 2 × SSC, 0.1% SDS at room temperature and twice with 0.2 × SSC, 0.1% SDS at 45°C for 30 min. After autoradiography, blots were stripped of labeled probes using proprietary buffers obtained from Ambion Inc. Autoradiograms in each figure were generated from a single blot, repeatedly stripped, and reprobed. Each result was repeated at least two times.

**Immunohistology**—Primary keratocyte cultures in eight-well glass chamber slides (Nunc; catalog no. 177402) were rinsed briefly in room temperature PBS, fixed for 10–15 min in 3% paraformaldehyde in PBS at room temperature, rinsed in PBS, and stored at −4°C in 50% glycerol in PBS until staining. Nonspecific binding was blocked with 1% bovine serum albumin in PBS. Phalloidin was detected using monoclonal antibody clone PX-C10 (Sigma; catalog no. P1983) (1:400 dilution for 90 min, room temperature) and with Cy3-conjugated goat anti-mouse secondary (Accurate Chemical & Scientific; catalog no. JMG-165108) for 1 h. Filamentous actin was detected with fluorescein isothiocyanate-phalloidin (Sigma; catalog no. P5282) 2 μg/ml for 1 h. The double-labeled samples were photographed using a Zeiss LSM 410 confocal microscope with a 63× oil objective.

**RESULTS**

**Cytoskeleton-Matrix Interactions in Response to TGF-β**—We recently demonstrated that cultured primary bovine keratocytes maintained in low mitogen horse serum retain a morphological and proteoglycan expression characteristic of these cells in vitro (18, 19). Cultured rabbit keratocytes are also reported to maintain a stellate morphology in serum-free conditions and respond to TGF-β by reorganization of the actin cytoskeleton (15). In initial experiments we found that cultured primary bovine keratocytes exposed to TGF-β responded in a manner similar to that reported for rabbit keratocytes. Cultures maintained for 6 days in 1% platelet-poor horse serum exhibited a stellate morphology with extensive branched processes (Fig. 1A). Filamentous actin detected by phalloidin binding showed a

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**Fig. 1. Cytoskeletal reorganization in bovine keratocytes in response to TGF-β.** Primary keratocytes cultured 6 days in 1% platelet-poor horse serum (A) or cultured similarly with 1 ng/ml TGF-β (B) were fixed and stained with fluorescein isothiocyanate-phalloidin for 2 h and then with Cy3-conjugated goat anti-mouse secondary (green) to show focal adhesions as described under “Materials and Methods.”
limited cortical localization and was not organized into distinct stress fibers. Paxillin staining was diffuse. When cultured in the presence of 1 ng/ml TGF-β (Fig. 1B), the keratocytes showed marked alteration in morphology. Extensive actin stress fibers crossed the cell body and were anchored to the extracellular matrix via focal adhesions as demonstrated by basal accumulations of paxillin associated with the termini of the actin cables.

The time course of the alteration in cytoskeletal/matrix interactions was documented by the data in Fig. 2. Fibronectin, initially not associated with the cell layer, begins to accumulate rapidly after 2 days of exposure to TGF-β (Fig. 2A). α5 integrin, a component of the fibronectin receptor (α5β1), like fibronectin, is not observed in the differentiated keratocyte but accumulates in the cells after 1 day of exposure to TGF-β (Fig. 2B). The appearance of α-smooth muscle actin (Fig. 2C) is concurrent with fibronectin and α5 integrin. The presence of this muscle-specific protein marks acquisition of a contractile myofibroblastic phenotype in response to TGF-β.

Proteoglycans—In vivo keratocytes secrete keratan sulfate glycosaminoglycan chains modifying three proteins (lumican, keratocan, and mimecan) and dermatan sulfate chains primarily attached to decorin. Alteration of this proteoglycan secretion profile in response to TGF-β was examined by isolating keratan and dermatan sulfate-containing proteoglycans, metabolically labeled in the glycosaminoglycan moieties with [35S]sulfate or [35S]methionine (open symbols) in the final 18 h of culture. Proteoglycans isolated from culture media were separated into pools containing keratan sulfate (A) and dermatan sulfate (B) as described under “Materials and Methods.” C, the ratio of sulfate/methionine incorporation was calculated for KSPG (circles) and for dermatan sulfate proteoglycans (triangles). Data are expressed as a percentage of control cultures. Error bars show standard deviation among triplicate samples.

The primary dermatan sulfate-containing stromal proteoglycan is decorin (23), but biglycan, a similar proteoglycan that is not present in normal stroma, has been observed to accumulate in corneas with chronic pathological conditions (10). Biglycan protein contains two glycosaminoglycan attachment sites and in most tissues exhibits a higher molecular size than decorin (10, 24). Immunoprecipitation of the sulfate-labeled dermatan sulfate-containing proteoglycans from TGF-β-treated cultures with monospecific antibodies to decorin and biglycan (Fig. 5) showed the high molecular weight dermatan sulfate proteoglycan.
can accumulating in response to TGF-β to precipitate with the anti-biglycan antibody but not the antibody to decorin.

Detection by immunoblotting of core proteins released from nonlabeled secreted proteoglycans (Fig. 6) supported the conclusions of Fig. 3, suggesting that KSPG proteins undergo a modest overall decrease after 5–6 days of exposure to TGF-β. Secreted decorin also decreased somewhat in abundance over the 6-day treatment period. Biglycan, on the other hand, was not detected in untreated cultures but appeared in the pool of secreted proteoglycan within 2 days of TGF-β treatment and increased over several days.

Pools of mRNA for the KSPG proteins, detected by Northern blotting in Fig. 7, exhibited a pattern of alterations similar to that seen in the secreted proteoglycan proteins. Lumican transcripts underwent a moderate decrease, whereas those for mimecan exhibited a more marked reduction. Mimecan transcripts occur in several alternately spliced forms, and it appeared that TGF-β treatment shifted the ratio of these in addition to decreasing their overall abundance. Keratocan transcripts were present at the limit of detection and did not exhibit much apparent alteration during TGF-β treatment. RNA transcripts of decorin, similar to the protein levels, were reduced somewhat by TGF-β, and expression of biglycan mRNA, almost undetectable in untreated cultures, was rapidly up-regulated (Fig. 8).

Collagen—In most tissues, fibrosis is associated with marked accumulation of collagen. In cornea, type I and particularly type III collagens are more abundant in healing wounds and in fibrotic regions of chronic pathological corneas and mRNA pools for these proteins are elevated (25–31). We observed that [3H]proline incorporation into pepsin-resistant protein was dramatically increased in response to TGF-β (Fig. 9A). This pepsin-resistant protein band showed reactivity with both collagen types I and type III antibodies in Western blotting. Increased pools of collagen α2(I) and α1(III) mRNA were observed in the cells by Northern blotting (Fig. 9B). This up-regulation of fibrillar collagen expression in response to TGF-β correlates with biglycan expression implicating myofibroblasts in secretion of fibrotic tissue.

**DISCUSSION**

In this study, we found that bovine keratocytes respond to TGF-β by the development of extensive F-actin stress fibers terminating at paxillin-containing focal adhesions. The formation of new cytoskeletal elements correlates with accumulation of cell-associated fibronectin and its receptor, α5 integrin. Such a fibronectin matrix and associated cytoskeleton is not observed in keratocytes in quiescent cultures (Fig. 1A), nor is it characteristic of keratocytes in vivo (13). Expression of α5 and interaction with a fibronectin matrix, however, represents a classic marker of the fibroblastic phenotype and is exhibited by most adherent cells in culture as well as by keratocytes that populate healing corneal wounds (13, 15, 25). Stromal fibronectin accumulation is also observed in human corneas from several chronic pathological conditions as well as in acute healing wounds (29, 32).

α-Smooth muscle actin is a protein originally thought to be restricted to muscle cells, and its appearance in fibroblastic cells has given rise to the term “myofibroblast.” Corneal myofibroblasts are contractile and have been suggested to play a role in wound closure (14). Accumulation of α-smooth muscle actin in rabbit keratocytes has been shown to depend on interaction of the fibronectin receptor with its ligand (the amino acid sequence RGD) (33). Consequently, it is thought that the corneal myofibroblast represents a phenotype derived from fibroblasts, not directly from the differentiated keratocytes, which lack cell-associated fibronectin (34). Myofibroblastic cells are widely observed in normal and pathological tissue environments, and in lung and liver there is an association of myofi-
of sulfate into keratan sulfate on these proteoglycans, on the other hand, was rapidly and dramatically reduced by TGF-β, resulting in a change in the KS/protein ratio of 10-fold compared with untreated cells (Fig. 1). This result suggests a change in the size and/or sulfation of the KS chains modifying the KSPG proteins. The decrease in KSPG molecular size (Fig. 4) is also consistent with an alteration in the glycosylation of the KSPG proteins. Altered keratan sulfate glycoforms were identified in cultures of stromal fibroblasts (40). In these cells, lumican, keratocan, and mimecan were modified with oligolactosamine chains of low sulfation (40). These data suggest that reduction of keratan sulfate biosynthesis comes as a result of changes in glycosyl- or sulfotransferase activity in the keratocytes. A report by Nakazawa et al. (43) documented loss of N-acetylglucosamine-6-O-sulfotransferase activity in chicken keratocytes exposed to serum in vitro, providing a candidate enzyme for this modulation.

Dermatan sulfate-linked proteoglycans secreted by keratocytes respond in a reciprocal manner to the keratan sulfate proteoglycans (Fig. 9). Overall, the biosynthesis of dermal sulfate-linked proteins decreased in response to TGF-β, whereas the intact proteoglycans increased in size and sulfation. These results suggest an increase in the length and/or sulfation of the dermal sulfate chains modifying these proteins. Such alterations in corneal dermal sulfate have been observed in healing wounds and in human corneas undergoing rejection, suggesting that this response in vitro models fibrosis in vivo (8–11, 44, 45). The exact nature of the structural changes in the dermanian sulfate and the enzymes that effect these changes remain to be elucidated.

The most marked change in proteoglycan proteins secreted in response to TGF-β was in biglycan. Biglycan is not present in the normal stroma and neither biglycan mRNA nor biglycan protein secretion was detected in untreated cultured keratocytes. Within 24 h of TGF-β treatment, however, biglycan mRNA was detected and protein was detected in 48 h (Figs. 6 and 8). Biglycan transcripts and protein increased during several days of exposure to TGF-β. We previously found biglycan to be markedly increased in the stromas of human corneas with chronic pathological conditions (10). The large molecular size of the biglycan secreted by keratocytes in response to TGF-β is similar to a large molecular weight dermal sulfate proteoglycan observed in healing rabbit corneal wounds (11). Biglycan accumulation is also associated with fibrosis in lung and hepatic tissues (37, 46). These data together with our current observations suggest biglycan as a sensitive marker of fibrotic tissue deposition in the stroma.

The up-regulation of biglycan in the keratocyte appears to be transcriptional; i.e. biglycan mRNA was not detected in the untreated cells. The biglycan gene promoter is reported to contain TGF-β response elements; however, biglycan transcription does not necessarily respond directly to TGF-β (47). In the keratocyte system, we observed that biglycan expression in primary cultures was not altered by TGF-β in the complete absence of serum (data not shown). Consequently, the current experiments were carried out in 1% platelet-poor horse serum, a medium that maintains much of the keratocyte differentiated phenotype (19). Rabbit keratocytes do become myofibroblastic in response to TGF-β in serum-free conditions (15), a transition dependent on interaction with extracellular RGD-containing ligands (33). Thus, the response to TGF-β in both systems appears to require signals in addition to TGF-β.

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