Partial Restoration of the Keratocyte Phenotype to Bovine Keratocytes Made Fibroblastic by Serum

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PURPOSE. To determine whether keratocytes made fibroblastic in vitro by addition of fetal bovine serum to the medium regain the keratocyte phenotype after culture in serum-free medium.

METHODS. Collagenase-isolated keratocytes from bovine corneas were plated in DMEM/F-12 containing 1% horse plasma, to allow cell attachment, and then cultured until day 4 in either DMEM/F-12 alone, to retain the keratocyte phenotype, or in DMEM containing 10% fetal bovine serum, to cause the keratocytes to become fibroblastic. Medium for the fibroblastic cells was replaced on day 4 with serum-free medium, and cells were cultured until day 12. Cell phenotypes were determined on days 4 to 5 and 11 to 12 of culture as follows: (1) by the morphologic appearance on phase-contrast microscopy; (2) by the levels of aldehyde dehydrogenase in the cells, determined by SDS-PAGE and Coomassie blue staining; (3) by the relative synthesis of collagen types I and V, determined by 14C-proline radiolabeling; (4) by pepsin digestion and analysis of collagen types by SDS-PAGE autoradiography; (5) by relative synthesis of cornea-specific proteoglycan core proteins determined by analysis of chondroitinase- and endo-β-galactosidase–generated radiolabeled core proteins by SDS-PAGE autoradiography; and (6) by the relative synthesis of keratan sulfate and chondroitin sulfate determined by 35SO4 radiolabeling and measuring the sensitivity to endo-β-galactosidase and chondroitinase ABC.

RESULTS. Keratocytes cultured in serum-free medium appeared dendritic and became fibroblastic in appearance when exposed to medium containing serum. Keratocytes and fibroblasts synthesized a similar proportion of collagen types I and V. However, compared with the keratocytes, the fibroblasts possessed no aldehyde dehydrogenase and synthesized significantly higher levels of decorin and significantly lower levels of prostaglandin D synthase (PGDS) and keratan sulfate. Subsequent culture of the fibroblasts in serum-free medium did not restore aldehyde dehydrogenase to keratocyte levels but did restore the cell morphology to a more dendritic appearance and returned the synthesis of decorin, PGDS, and keratan sulfate to keratocyte levels.

CONCLUSIONS. The results of these studies indicate that primary cultures of keratocytes made fibroblastic by exposure to serum can return to their keratocyte phenotype in synthesizing extracellular matrix. These results also indicate that the differences in the organization of the collagenous matrix produced by keratocytes and fibroblasts may be related more to the different proteoglycan types than to the collagen types produced. (Invest Ophtalmol Vis Sci. 2002;43:3416–3421)

The stroma is the major structural element in the cornea, consisting of a highly organized, uniquely transparent extracellular matrix that provides both the refractive shape and the tensile strength of the cornea. The extracellular matrix of the stroma is a dense network of collagen fibrils and proteoglycans. The stroma is initially formed during early development by invading neural crest cells. These cells then become keratocytes, which proliferate and produce the uniquely transparent collagenous matrix of the stroma. After corneal development is complete, the keratocytes become quiescent but remain interspersed throughout the stroma. When the integrity of the corneal stroma is disrupted, the quiescent keratocytes become fibroblasts and/or myofibroblasts, which can then proliferate and migrate to the site of injury, where they repair the stromal tissue. The repair, however, is flawed, because the extracellular matrix produced by the fibroblasts and myofibroblasts is disorganized and opaque, which results in a loss of corneal transparency.

The fibroblast and myofibroblast derivatives of keratocytes differ from keratocytes by a number of parameters including cell shape, proliferation rate, keratan sulfate production and the expression levels of gene products such as α-smooth muscle actin, aldehyde dehydrogenase (ALDH), and the fibronectin receptor. Such biochemical changes indicate that keratocytes undergo a phenotypic transition when they become fibroblastic. The properties of keratocytes and their transition to fibroblasts and myofibroblasts can now be readily studied in vitro. Recent studies using collagenase-isolated keratocytes from both rabbit and bovine corneas demonstrate that keratocytes can retain their in situ phenotype by culture in serum-free medium. This culture system has led to the discovery of prostaglandin D synthase (PGDS) as a secreted product of the keratocyte. It has also provided an opportunity to determine the causes of the keratocyte transition to fibroblasts and myofibroblasts and the acquisition of competency to activate nuclear factor (NF)-κB.

The ability of corneal myofibroblasts to return to their fibroblast phenotype has been readily demonstrated in cell culture. The critical issue for the restoration of corneal transparency, however, is the potential for these fibroblastic derivatives of keratocytes to return to the keratocyte phenotype. Corneal scar tissue usually remains opaque, but in some instances, the disorganized collagenous matrix becomes more organized again and some transparency is restored. This observation suggests that the fibroblastic cells may be capable of returning to their keratocyte phenotype and remodeling the flawed matrix. In this report, we characterize the changes in expression of phenotype markers during the fetal bovine serum–mediated keratocyte to fibroblast transition and then use...
the markers to determine whether the keratocyte phenotype can be restored when serum is removed from the medium.

**METHODS**

**Keratocyte Isolation and Culture**

Keratocytes were isolated from freshly harvested adult bovine eyes (Pel-Freeze Biological, Rogers, AR) by collagenase digestion, as previously described.10 The isolated keratocytes were suspended in Dulbecco’s modified Eagle’s medium F-12 (DMEM/F-12; Gibco-Life Technologies, Grand Island, NY) containing 1% horse plasma (platelet-poor horse serum; Sigma, St. Louis, MO), which served as a cell-attachment factor, and plated into six-well tissue culture dishes (Costar, Cambridge, MA) at either high density (400,000 cells/well) or low density (100,000 cells/well). The cells were allowed to attach overnight at 5% CO₂. The medium was changed the next morning (day 1). Cells plated at high density were changed to DMEM/F-12 to retain the keratocyte phenotype. Low-density cultures were changed to DMEM/F-12 containing 10% fetal bovine serum (FBS) to induce the fibroblast phenotype. The lower initial plating density for the keratocytes induced to become fibroblastic provided sufficient surface area for cell spreading and proliferation. Incubation proceeded until day 4 when the medium in the fibroblast cultures was changed to DMEM/F-12 to promote the keratocyte phenotype. Medium (2 mL/well) was replenished every 4 days, and incubation was continued until day 12.

**Antibodies**

Rabbit antiserum to bovine decorin (LF-94) was a gift of Larry Fisher (National Institute of Dental Research, Bethesda, MD). A mouse monoclonal antibody to bovine osteoglycin (mimecan) was a gift from James Funderburg (University of Pittsburgh, Pittsburgh, PA). Rabbit antiserum to bovine lumican was prepared with the N-terminal amino acid sequence (TYPDYVEYDFPQALYGRSC) used as a peptide antigen. Rabbit antiserum to bovine keratocan was prepared with a synthetic amino acid sequence (CPSTPTTLVPEDSFYGPHL) used as a peptide antigen. Rabbit antiserum to bovine prostat glandin D synthase was a gift of Gary Killian (Penn State University, State College, PA).

**Analysis of Water-Soluble Cell Proteins**

Medium was removed from six wells, and the cell layers were rinsed three times in phosphate-buffered saline before scraping and harvesting in 0.5 mL distilled water per well. The harvested cell layers were frozen and thawed twice, vortexed, and centrifuged at 14,000 rpm (Eppendorf, Fremont, CA) for 15 minutes to pellet insoluble proteins. The protein content of the supernatant was determined (DC Protein Assay, Bio-Rad, Hercules, CA), and equal amounts of protein from the different culture conditions were applied to 10% bis-tris polyacrylamide gels (Nupage; Invitrogen) and electrophoresed under reducing conditions, and stained with Coomassie blue. The region of gel containing the most prominent band, migrating at approximately 37°C in 5% CO₂. The medium was changed the next morning (day 1). Cells plated at high density were changed to DMEM/F-12 to retain the keratocyte phenotype. The lower initial plating density for the keratocytes induced to become fibroblastic provided sufficient surface area for cell spreading and proliferation. Incubation proceeded until day 4 when the medium in the fibroblast cultures was changed to DMEM/F-12 to promote the keratocyte phenotype. Medium (2 mL/well) was replenished every 4 days, and incubation was continued until day 12.

**Western Blot Analysis of Medium from Keratocytes and Extracts from Whole Bovine Corneas**

Media from six wells were combined on day 4, centrifuged to remove cell debris, dialyzed overnight against water, and lyophilized. Samples were reconstituted in 200 μL water, and 10-μL portions were digested with keratanase, chondroitinase ABC, or endo-β-galactosidase (Seikagaku America, Falmouth, MA), according to the manufacturer’s specifications. Aliquots with and without digestion were applied to 10% bis-tris polyacrylamide gels (Nupage; Invitrogen) and electrophoresed under reducing conditions. Proteins were transferred to nitrocellulose membrane (Bio-Rad) at room temperature, blocked with 5% milk in phosphate-buffered saline and 0.3% Tween-20 (PBS-T), and incubated overnight at 4°C with either 1:1000 monoclonal anti-bovine lumican antisera, 1:1000 rabbit anti-bovine recombinant PGDS, 1:1000 rabbit anti-bovine decorin, 1:1000 rabbit anti-bovine keratan, or 1:1000 rabbit anti-bovine osteoglycin. Membranes were rinsed in PBS-T, incubated in either horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse IgG (Pharmacia Biosciences, Piscataway, NJ), and rinsed four times with PBS-T. Protein bands were developed using chemiluminescence detection (ECL; Pharmacia Biosciences).

**Biosynthetic Radiolabeling**

Cultures used for the analysis of collagen synthesis were radiolabeled for 72 hours beginning on day 1 by the addition of 5 μCi [14C]proline (NIC-285E; Perkin Elmer Life Sciences, Wellesley, MA) per milliliter of medium supplemented with 50 μg/mL ascorbic acid. Cultures used for analysis of proteoglycan core protein synthesis were radiolabeled for 24 hours beginning on either day 4 or 11 by the addition of 100 μCi/mL of a mixture of 35S methionine and 35S cysteine (35S-Express; Perkin Elmer Life Sciences) in DMEM/F-12 medium. Cultures used for the analysis of glycosaminoglycan types were radiolabeled for 24 hours beginning on either day 4 or 11 by the addition of 80 μCi/mL 35SO₄²⁻ (Perkin Elmer Life Sciences) in DMEM/F-12.

**Analysis of Collagen Types Produced by Keratocytes and Fibroblasts**

Medium and cell layer samples with equivalent radiolabel incorporation as determined by scintillation counts were dried and resolved in nonreducing SDS-PAGE sample buffer. Samples were electrophoresed on 5% polyacrylamide gels (Mini Trans Blot; Bio-Rad) until the dye front reached the bottom of the gel. Gels were dried and exposed to film (Bio-Max MR; Eastman Kodak, Rochester, NY) for 1 to 14 days at ~80°C. The α1(V) and α2(I) bands were cut out of the dried gel using the autoradiogram as a reference. The dried bands were hydrated and solubilized at 37°C for 30 minutes. The solubilized proteins were counted (ScintiSafe ZZ23-5; Fisher Scientific, Suwanee, GA) using a scintillation counter with an efficiency of 95.5% (Packard 1900 CA; Perkin Elmer Life Sciences).

**Proteoglycan Core Protein Analysis**

Radiolabeled media removed from six wells were combined, centrifuged at 800 rpm to remove any debris, and concentrated to 0.5 mL with 10,000 MWCO (molecular weight cutoff) centrifugal concentrators (Macrosep; USA Pall Filtron, Northborough, MA). The medium was adjusted to 4 M guanidine-HCl by the addition of an equal volume of 8 M guanidine-HCl containing 0.1 M sodium acetate buffer (pH 6.0) and fractionated on Sepharose columns (PD-10 Sephadex; Amersham Pharmacia Biotech) equilibrated in 4 M guanidine-HCl to remove unincorporated radionucleotides. The radiolabeled macromolecular fractions were dialyzed against water, lyophilized, and reconstituted in 100 μL of water. Ten-microliter portions were digested with chondroitinase ABC or endo-β-galactosidase (Seikagaku America), according to the manufacturer’s directions, and samples with and without digestion were separated by SDS-PAGE as described earlier. Gels were fluorographically enhanced (Enstensity; Perkin Elmer Life Sciences) and exposed to film (X-OMAT; Eastman Kodak) at ~80°C for 16 to 48 hours. The pixel density of core protein bands in the linear range of film exposure was obtained by computer (Quantity One software; Bio-Rad).
Glycosaminoglycan Analysis

The media from $^{35}$SO$_4$ radiolabeled cultures were processed as described earlier for "proteoglycan core protein analysis," except that after lyophilization the material was reconstituted in 1 mL of distilled water. One-hundred-microliter portions were digested with either chondroitinase ABC or endo-$eta$-galactosidase. Samples with and without digestion were boiled to inactivate the enzyme and precipitated with ethanol in the presence of carrier chondroitin sulfate. The resultant supernatant, containing the digested glycosaminoglycan, was measured for radioactivity by liquid scintillation counting.

RESULTS

Keratocytes cultured in serum-free medium appeared dendritic on days 4 and 11 of culture (Fig. 1, K). Keratocytes cultured in medium containing 10% fetal bovine serum (FBS) appeared fibroblastic on day 4 (Fig. 1, F), but changing the medium to serum-free medium on day 4 caused the cells to return to a more dendritic appearance by day 11 of culture (Fig. 1, R). This indicates a partial restoration of cell morphology by removal of serum.

Analysis of the soluble proteins extracted from the cell layer of the cultured cells by SDS/PAGE and Coomassie blue staining showed that the extracts of the 4- and 11-day keratocyte cultures (Fig. 2, K lanes) contained a prominent band that migrated with an estimated molecular weight of 51 kDa. This band was excised from the gel, the protein in the gel digested with trypsin and the resultant peptide isolated and sequenced. The sequence obtained was M-I-A-E-T-S-S-G-G-V-T-A-N-D-V-V, which exactly matched the sequence in bovine ALDH-3 (GenBank protein accession no. P50907; GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/Genbank). Intensity of staining for the ALDH band was substantially reduced or the band was absent in keratocytes cultured in 10% FBS (Fig. 2, F lane), and switching to serum-free medium on day 4 did not cause the ALDH band to reappear (Fig. 2, R lane).

The production of collagen types I and V by the day 4 keratocyte cultures (Fig. 3, K) was determined and compared with day 4 cultures made fibroblastic by culture in serum (Fig. 3, F). Keratocyte and fibroblast cell layers and medium radiolabeled with $^{14}$C-proline were treated with pepsin to solubilize and convert procollagen to collagen. Collagen types were analyzed autoradiographically after SDS-PAGE to separate the collagen type I and V chains. The individual bands for collagen types I and V were excised from the gel and the radioactivity determined by liquid scintillation counting. The results showed that the keratocytes and fibroblasts make collagen types I and V in similar proportions, which indicates that there is no difference between keratocytes and fibroblasts in relative synthesis of these two collagen types.

The SDS-PAGE migration positions for the core proteins of the one chondroitin sulfate and the four keratan sulfate proteoglycans secreted by keratocytes into the medium were established by digestion with either chondroitinase ABC to remove chondroitin sulfate-dermatan sulfate chains or with endo-$eta$-galactosidase to remove the keratan sulfate chains, followed by Western blot with antiserum specific to each proteoglycan core protein (Fig. 4). Antiserum to decorin detected the decorin proteoglycan in the undigested lane (Fig. 4, Decorin, UD) as a broad band between the 191- and 64-kDa markers. Chondroitinase digestion produced the decorin core protein as a smear migrating just below the 51-kDa marker (Fig. 4, Decorin, C). Endo-$eta$-galactosidase digestion produced a sharp band of lesser intensity in the same migration position (Fig. 4, Decorin, E), which suggests that a portion of the decorin gene products are made without chondroitin sulfate chains, but with keratan sulfate chains instead. Digestion with endo-$eta$-galactosidase produced the lumican core protein migrating just below the 51-kDa marker (Fig. 4, Lumican, E), the osteoglycin (mimecan) core protein migrating midway between the 51- and 39-kDa markers (Fig. 4, osteoglycin, E), the keratocan core protein migrating just below the 51-kDa marker (Fig. 4, keratocan, E) and the prostaglandin D synthase core protein migrating at the 28-kDa marker (Fig. 4, PGDS, E). These results show that decorin has a core protein similar in size to that of lumican and keratocan, but the bulk of decorin core protein is produced by chondroitinase digestion, whereas lumican and keratocan core proteins are generated by endo-$eta$-galactosidase digestion. Osteoglycin and PGDS core proteins are also gener-

![Figure 1](https://example.com/image1)

**Figure 1.** Phase-contrast images of cultured bovine keratocytes. Keratocytes cultured in DMEM appeared dendritic on days 4 (Day 4/5, K) and 11 (Day 11/12, K). Keratocytes cultured in DMEM containing 10% fetal bovine serum appeared fibroblastic on day 4 (Day 4/5, F) but subsequent culture for 8 days in serum-free medium restored the cell morphology to a more dendritic appearance (Day 11/12, R).

![Figure 2](https://example.com/image2)

**Figure 2.** Analysis of water-soluble proteins extracted from cultured keratocytes by SDS-PAGE followed by Coomassie blue staining. Keratocytes cultured in DMEM/F-12 contained a 51-kDa protein, shown by amino acid sequencing to be ALDH, as a major band (Day 4/5 and Day 11/12, lane K). The ALDH band was substantially reduced in extracts of cells made fibroblastic by culture in DMEM/F-12 containing 10% fetal bovine serum (Day 4/5, lane F) and did not reappear after 8 days of culture in serum-free medium (Day 11/12, lane R).
ated by endo-β-galactosidase, but their core proteins differ in size from each other and from lumican and keratocan.

We measured core protein synthesis by biosynthetically radiolabeling the core proteins of the proteoglycans with a mixture of 35S methionine and 35S cysteine (35S-Express; Perkin Elmer Life Sciences), harvesting the medium containing the proteoglycans, digesting the contents of the medium with either chondroitinase ABC or endo-β-galactosidase, and detecting the core proteins by autoradiography after SDS-PAGE. This can be readily accomplished because of the limited number of products secreted into the medium by keratocytes and the absence of any major radiolabeled protein bands in the migration position of the core proteins.10 Because lumican and keratocan both have the same size core proteins generated by the same enzyme, their individual contributions are combined in this analysis. Results showed that day 4 keratocytes synthesized 51% of their total core protein as decorin (Fig. 5, Day 4/5, Dec) and 30% as PGDS (Fig. 5, Day 4/5, PGDS). Alternatively, keratocytes made fibroblastic by exposure to serum made only 20% of the total glycosaminoglycan as keratan sulfate (Fig. 6, Day 4/5), a significantly (P = 0.0003) lower amount. Removal of the serum from the fibroblastic cells and subsequent culture in serum-free medium restored keratan sulfate synthesis similar to keratocyte levels (Fig. 6, day 11/12).

**DISCUSSION**

The results of these studies indicate that primary cultures of bovine keratocytes made fibroblastic by culture in medium containing fetal bovine serum can regain certain characteristics of keratocytes by subsequent culture in medium without fetal bovine serum. The fibroblasts, cultured in medium without serum for 8 days, regained a more dendritic morphology resembling keratocytes, reduced the proportion of decorin synthesis to keratocyte levels, and increased both PGDS and keratocan synthesis as compared to the fibroblasts. This indicates that the relative synthesis of these two proteoglycans by the formerly fibroblastic cells has been restored to those levels seen in keratocytes.

Glycosaminoglycan synthesis was also evaluated in medium harvested from cells radiolabeled with 35SO4. The keratocytes, at the day 4 time point, incorporated 58% of the 35SO4 into endo-β-galactosidase-sensitive glycosaminoglycan, which would be keratan sulfate (Fig. 6, Day 4/5). In contrast, keratocytes made fibroblastic by serum made only 20% of the total glycosaminoglycan as keratan sulfate (Fig. 6, Day 4/5), a significantly (P = 0.0003) lower amount. Removal of the serum from the fibroblastic cells and subsequent culture in serum-free medium restored keratan sulfate synthesis similar to keratocyte levels (Fig. 6, day 11/12).
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Cross-linked collagen fibrils provide the tensile strength of the
cornea, and the uniform size and spacing of the fibrils is
one of the critical elements needed for transparency of the
stromal extracellular matrix. Collagen fibrils are composed of
at least two different fibrillar collagen types, and previous work
has shown the proportion of collagen types I and V can mod-
ulate fibril diameter.19,20 The core proteins of proteoglycans in
the stroma can also regulate fibril diameter, and two of the
proteoglycans, decorin and lumican, have been shown to have
different effects on collagen fibril diameter growth in vitro,21,22
This has been confirmed by comparing collagen fibrils in cor-
neas of lumican null mice.23,24 The interactions of collagen
types I and V with each other are proposed to be essential in
the initial stages of fibrillogenesis,20,25 whereas the core pro-
teins of the corneal proteoglycans play a role in the later stages
of fibrillogenesis.26 The similarities in collagen synthesis by
keratocytes and fibroblasts and the differences in core protein
synthesis observed in this report suggest that the disorganized
matrix present in corneal scars is due to differences in proteo-
glycan synthesis and their impact on fibril formation and ma-
trix assembly.

The fibroblastic phenotype has been defined primarily by the
shape of the cell and the myofibroblast phenotype by the
shape of the cell and the presence of α-smooth muscle actin in
the cytoplasm. Both rabbit and bovine keratocytes become

fibroblastic in vitro when cultured in serum-containing me-
dium and become myofibroblastic when TGF-β is added to the
medium as well.7-9,14,15 Rabbit keratocytes, however, can also
be made to become fibroblastic in vitro by TGF-β in the
absence of serum.8 Although studies indicate it is TGF-β that
causes keratocytes in vivo to become myofibroblastic,27 it has
not yet been demonstrated that it is serum or the factor in
serum that causes the keratocyte in vivo to become fibroblas-
tic. Nevertheless, the addition of serum to the medium of
keratocytes in vitro provides a useful and important model
system to study the keratocyte–fibroblast transition.

**Acknowledgments**

The authors thank Ewa Kolanko for her technical assistance in per-
forming the studies shown in Figure 2.

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