Proteoglycan Synthesis by Bovine Keratocytes and Corneal Fibroblasts: Maintenance of the Keratocyte Phenotype in Culture

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PURPOSE. To determine the effect of serum on morphology, growth, and proteoglycan synthesis by primary cultures of collagenase-isolated bovine keratocytes.

METHODS. Keratocytes were isolated from bovine corneas using sequential collagenase digestion and cultured in Dulbecco’s modified Eagle’s medium (DMEM), with and without fetal bovine serum (FBS). Proteoglycans synthesized by the cells in culture and by keratocytes in intact cornea culture were metabolically radiolabeled with 35SO4. The proteoglycans were characterized by their sensitivity to keratanase, chondroitinase ABC, and heparitinase, and by their size on Superose 6 HR. Cell number was determined by measuring DNA content of the culture dishes.

RESULTS. Keratocytes cultured in 10% FBS proliferated, appeared fibroblastic, and synthesized only 9% of the total glycosaminoglycan as keratan sulfate (KS), whereas cells in serum-free media were quiescent, appeared dendritic, and synthesized 47% KS, a value similar to the 45% KS for corneas radiolabeled overnight in organ culture. This increased proportion of KS synthesis in serum-free media was caused by a moderate increase in KS synthesis combined with a substantial decrease in chondroitin sulfate (CS) synthesis. Fractionation on Superose 6 High Resolution showed the size and relative amounts of the CS- and KS-containing proteoglycans synthesized by keratocytes in serum-free media also more closely resembled that of keratocytes in corneas in organ culture than keratocytes in media containing serum.

CONCLUSIONS. A comparison of proteoglycan synthesis and cell morphology between keratocytes in corneas in organ culture and in cell culture indicates that keratocytes maintain a more native biosynthetic phenotype and appearance when cultured in serum-free media. These results also suggest that culturing in the presence of serum fundamentally alters the keratocyte phenotype to an activated cell, mimicking certain changes observed during wound healing. (Invest Ophthalmol Vis Sci. 1999;40:1658–1665)

The corneal stroma matrix consists primarily of collagen I, V, VI, and XII1–4; the proteoglycan decorin,5 which contains a single dermatan sulfate side chain; and the proteoglycans lumican,6 keratan,7 and osteoglycin/mimecan,8 which contain keratan sulfate (KS) side chains. There are several lines of evidence that indicate that KS proteoglycans play an important role in providing corneal transparency. Keratan sulfate content increases substantially during the acquisition of transparency in development,9 it is absent in opaque corneal scars,10 and it is absent in corneas of patients with macular corneal dystrophy type I.11,12 Furthermore, the absence of lumican, the major corneal KS proteoglycan, from the corneal stroma of mice with a lumican null mutation results in the development of corneal clouding, whereas the absence of decorin does not adversely affect corneal transparency.13,14

The keratocyte produces the collagens and proteoglycans that comprise the stromal extracellular matrix. Studies using freshly isolated monkey corneas have shown that proteoglycans synthesized overnight in organ culture are similar, if not identical, in structure and relative amounts with that present in the stromal matrix, and this has been extended to studies in chicken15 and human corneas11,12,17 as well. Keratan sulfate can be as much as 47% of the total glycosaminoglycan (GAG) synthesized by corneas in organ culture.16 In contrast, keratocytes grown from human, rabbit, and bovine corneas and cultured under standard conditions generally produce little (∼3%) or no KS,17–19 although a recent study reported 15% KS.20 Serum-cultured keratocytes also express the fibronectin receptor α5β1, which is not expressed by keratocytes in situ; have a predominantly fibroblastic morphology; and show an actin cytoskeletal organization more characteristic of corneal myofibroblasts.21–23 Furthermore, when passed at low cell density, keratocyte cultures become entirely myofibroblastic and show changes in cadherin expression.22,24 These observations suggest that standard cell culture conditions alter the keratocyte from its in situ phenotype.

Standard culture conditions for keratocytes include using fetal bovine serum in the media. A recent study showed,
however, that primary cultures of collagenase-isolated rabbit keratocytes cultured in serum-free media exhibited a dendritic morphology with multiple and extensive interconnective cellular processes. The appearance of these dendritic cells is similar to the morphology of keratocytes in situ and is distinctly different from the fibroblastic or myofibroblastic appearance of keratocytes grown in serum-containing medium. In this study we used collagenase-isolated bovine keratocytes and serum-containing or serum-free media to culture the keratocytes. The results show that collagenase-isolated bovine keratocytes cultured in serum-free media are quiescent, maintain a dendritic morphology and synthesize high levels of KS—all phenotypic characteristics similar to those of in situ keratocytes or keratocytes in cornes cultured in organ culture.

**MATERIALS AND METHODS**

**Keratocyte Isolation**

Bovine keratocytes were isolated using a modification of a sequential collagenase digestion method for embryonic chick cornes. Fourteen freshly harvested, adult bovine eyes were obtained from Pel-Freeze Biologicals (Rogers, AR) by overnight shipment on wet ice. The corneas with a scleral rim were removed using a scalpel and placed epithelium side up on the round end of a rubber centrifuge adapter (model 8441; Corning, Corning, NY). Three disks were removed from the central region of the cornea using an 8-mm disposable biopsy punch (Tru-Punch 961130; Sklar, West Chester, PA). The disks were placed in Dulbecco’s modified Eagle’s medium (DMEM 12400-65; Gibco, Gaithersburg, MD) containing antibiotics and cut into quarters, using two single-edged razor blades drawn across each other on a rubber stopper. The quartered disks were divided into two groups and each placed in a 50-ml conical centrifuge tube with 21 ml DMEM containing 3.3 mg/ml collagenase (type L.C8170; Sigma, St. Louis, MO) and incubated at 37°C with shaking (126 rpm) for 30 minutes. The tubes were then vortexed for 30 seconds and the tissue pieces separated from the collagenase solution using a cell strainer (98-771-2; Gibco, Gaithersburg, MD) containing antibiotics and cut into quarters, using two single-edged razor blades drawn across each other on a rubber stopper. The quartered disks were divided into two groups and each placed in a 50-ml conical centrifuge tube with 21 ml DMEM containing 3.3 mg/ml collagenase (type L.C8170; Sigma, St. Louis, MO) and incubated at 37°C with shaking (126 rpm) for 30 minutes. The tubes were then vortexed for 30 seconds and the tissue pieces separated from the collagenase solution using a cell strainer (98-771-2; Falcon, Oxnard, CA). Digestion was continued with a second 21-ml aliquot of collagenase for 60 minutes and (after separating the collagenase from the tissue pieces as described) with a third 21-ml aliquot of collagenase for 180 minutes, followed by a repeat of the separation procedure. The cells in each of the three collagenase digestions were collected by low-speed centrifugation (1400 rpm 30 minutes) at the end of each digestion period and the cells resuspended in 10 ml DMEM. The viability and appearance of the cells was determined by trypan blue (Sigma) exclusion, using an inverted microscope and counting the number of the cells by hemocytometer. In initial experiments, cells from the second and third collagenase digestion were resuspended in DMEM containing 10% fetal bovine serum (FBS; 26140-076; Gibco), plated in tissue culture dishes and visually inspected for attachment and spreading.

**Keratocyte and Cornea Organ Culture**

Only cells obtained from the third digestion were used for cell culture experiments. These cells were resuspended at 150,000 to 175,000 cells/ml in DMEM containing 10% FBS, DMEM containing 1% platelet-poor horse serum (PPHS; P552; Sigma), or DMEM alone. The cell suspensions were plated at 2 ml/35 mm dish. Cells in DMEM-10% FBS or DMEM-1% PPHS were plated in six-well tissue culture dishes (model 3516; Costar, Cambridge, MA), whereas cells in DMEM alone were plated in six-well Primaria (3846; Falcon) dishes. The cells were allowed to attach overnight and the media changed the next day (day 1). Some of the cells plated in DMEM containing 1% PPHS were changed to DMEM on day 1. Media were changed every 2 to 3 days. Cell cultures were radiolabeled for 2 days by the addition of media containing 50 μCi/ml Na35SO4 (Nex-041; Dupont-New England Nuclear, Boston, MA) on day 2. Intact cornes with a scleral rim were also incubated overnight in 2.5 ml DMEM/cornea containing 50 μCi/ml Na35SO4 in a 35-mm dish, as previously described.

**Proteoglycan Isolation and Characterization**

The media were removed from each culture dish at the end of the radiolabeling period and applied directly to a Spec 3 ml NH2 column (Anysync, Irvine, CA) prepared according to the manufacturer’s directions. The column was washed three times with 3 ml 6 M urea containing 0.15 M NaCl and 0.02 M Tris (pH 7.4) to remove free 35SO4, and the bound proteoglycans were eluted from the NH2 column with 1 ml 4 M guanidine-HCl. The proteoglycan fraction was then dialyzed against distilled water.

Intact cornes were removed from the media at the end of the radiolabeling period and the scleral rim removed. Each cornea was extracted with 2 ml 8 M guanidine-HCl, and the extract was dialyzed against 6 M urea containing 0.15 M NaCl and 0.02M Tris (pH 7.4) and applied to a 0.4-ml column of diethylaminoethyl Sepharose (Pharmacia, Piscataway, NJ). After washing with 6 M urea, the proteoglycans were eluted with 4 M guanidine HCl and collected.

Aliquots of the proteoglycan fraction from 35SO4 radiolabeled cultures were digested with either chondroitinase ABC, keratanase, or heparinase (Seikagaku America, Rockville, MD) for 3 hours, according to the manufacturer’s directions. The enzymes were inactivated by boiling, 0.5 mg carrier chondroitin sulfate (Sigma) added, and the undigested GAGs precipitated by the addition of 3 volumes of 95% ethanol containing 0.1% potassium acetate at 4°C. Insoluble (undigested) material was pelleted by centrifugation, an aliquot of the supernatant (which contained the digested GAGs) was removed, and the radioactivity was measured by liquid scintillation spectrophotometry. The percentage digested by each enzyme was calculated from the total digested by all three enzymes.

The proteoglycan fraction from the cell cultures and the intact cornes were fractionated on a column (1 x 30 cm) of Superose 6 HR in 4 M guanidine HCl. Fractions of 0.5 ml were collected, and a portion of each fraction was measured for radioactivity. Fractions were pooled and dialyzed against distilled water, and the GAG type were determined as described.

**DNA Content**

After removal of the media, 0.15 ml 0.1% sodium dodecyl sulfate (SDS) was added to each culture dish for five minutes to lyse the cells. Proteinase K (1.35 ml; 500 μg/ml 0.002 M EDTA, 0.1 M NaPO4 [pH 6.5]) was then added to each dish, the contents of the dish scraped into the proteinase K solution. The solution was placed in screw-capped test tubes and incubated at 60°C overnight. The DNA content in the proteinase K digest was determined by fluorometry, using the Hoechst 33258 dye (Hoefer Scientific, San Francisco, CA).
RESULTS

The viability of cells isolated from each of the three collagenase digestions was determined using trypan blue exclusion. Most of the cells from the first digestion appeared epithelial and were not investigated further. Cells from the second digestion were only 50% to 75% viable and did not readily attach and spread when plated overnight in DMEM containing 10% FBS. Cells from the third digestion were 92% to 98% viable and readily attached and spread in DMEM containing 10% FBS. Only cells from the third digestion were used for cell culture experiments.

After 4 days of culture, cells in 10% FBS achieved a fibroblastic appearance (Fig. 1, 10% FBS) whereas cells cultured continuously with 1% PPHS appeared dendritic with numerous, randomly branching, processes (Fig. 1, 1% PPHS). Cells initially plated in 1% PPHS but switched to DMEM alone on the following day appeared similar to those cultured continuously in 1% PPHS, although the central cell body appeared smaller and less spread out (Fig. 1, 1%/DMEM). By comparison, cells cultured in DMEM alone with Primaria plates were also dendritic (Fig. 1, DMEM), but the keratocytes had relatively small cell bodies with markedly fewer cell processes. Furthermore, under these conditions, cell processes appeared to extend directly, contacting to adjacent cells without substantial branching.

An equal number of cells were plated in the four culture conditions and the DNA content measured after 4 days of culture (Fig. 2). Cells cultured in DMEM with 10% FBS contained 94% more DNA than cells in DMEM with 1% PPHS and 170% more DNA than cells plated on Primaria plates in DMEM alone. Although plating efficiencies and rates of proliferation were not measured in these cultures, daily visual inspection indicated that cells in 10% FBS were proliferating much more rapidly than cells in the other media and had grown to confluence by the end of the 4-day culture period.

Cells cultured in the four different conditions were also radiolabeled with 35SO4 from days 2 to 4 of culture, the proteoglycans isolated from the media, and the amount degraded by chondroitinase ABC, keratanase, and heparatinase determined. Only small amounts (1%–3%) of incorporated 35SO4 were found to be degraded by heparatinase. Consequently, only the keratanase- and chondroitinase-digested materials are reported as a percentage of the total GAG. Cells cultured in...
The proteoglycans in the media of cells cultured under these different conditions and from intact corneas radiolabeled overnight in DMEM were chromatographed on a column of Superose 6 HR (Fig. 4) to evaluate proteoglycan size. The proteoglycans synthesized by cells in DMEM containing 10% FBS eluted primarily in a sharp peak early in the chromatogram (Fig. 4, 10% FBS, area 1) with several minor peaks eluting later (area 2). The proteoglycans from the early-eluting peak (area 1) were 96% CS, whereas the proteoglycans in area 2 were 53% CS (Table 1). A similar profile was obtained for proteoglycans from cells cultured continuously in 1% PPHS (Fig. 4, 1% PPHS), but proteoglycans in area 2 (Table 1) contained only 11% CS. Cells plated in DMEM containing 1% PPHS and switched to DMEM (Fig. 4, 1%/DMEM) and cells plated on Primaria plates in DMEM alone (Fig. 4, DMEM) also synthesized proteoglycans that eluted in a prominent peak (area 1), early in the chromatogram, that contained almost exclusively CS (Table 1), although it eluted three tubes later (indicating a smaller size) than the corresponding peak of cells cultured in 10% FBS. Cells in these cultures (Fig. 4, 1%/DMEM and DMEM), however, also synthesized proteoglycans, eluting in a prominent second peak that contained almost exclusively KS (Table 1, area 2). Radiolabeled proteoglycans from overnight cultures of intact corneas (Fig. 4, intact cornea) also fractionated into two prominent peaks: an early- (area 1) and a late-eluting peak (area 2) that contained primarily CS and KS, respectively (Table 1). Keratan sulfate synthesis by the cornea in organ culture was determined to be 45% of the total KS and CS combined: a value similar to that of keratocytes in DMEM alone. The results of these analyses indicate that CS-containing proteoglycans were larger than the KS-containing proteoglycans and that the profile of cells plated in 1% PPHS and switched to DMEM and the profiles of cells in DMEM alone appeared more similar to the profiles of the intact cornea than the profiles of cells cultured in 10% FBS or in 1% PPHS continuously. The results also show that CS-containing proteoglycans made by keratocytes in 10% FBS were larger than the CS-containing proteoglycans made by keratocytes cultured in serum-free media.

**DISCUSSION**

The results of this study show that the presence of serum in the media alters the synthesis of proteoglycans by keratocytes in culture. Keratocytes cultured in DMEM containing 10% FBS synthesized proteoglycans containing only 9% KS, but keratocytes cultured in DMEM alone on Primaria plates synthesized 477% KS, a value similar to the 45% KS synthesis by keratocytes in overnight organ culture. Furthermore, the size and GAG type of the proteoglycans synthesized by keratocytes in DMEM alone were more similar to that of the proteoglycans produced by keratocytes in intact corneas in overnight culture than were the proteoglycans produced by keratocytes cultured in 10% FBS or in 1% PPHS (Fig. 4). These observations on proteoglycan synthesis indicate that keratocytes isolated by collagenase digestion and cultured in serum-free media appear to retain their native phenotype.

Previous studies have evaluated proteoglycan synthesis by primary cultures of chick keratocytes isolated by collagenase, although they were cultured in media containing FBS. Chick keratocytes plated on plastic dishes in 10% FBS and then switched to 0.3% FBS synthesized as much as 17% KS, a value...
between those achieved in our studies using 10% FBS (9% KS) and 1% PPHS (29% KS). The results of our studies indicate that FBS acts primarily to stimulate CS synthesis and has only a relatively small effect on KS synthesis. It is possible that there are growth factors and cytokines in FBS that are potent stimulators of CS synthesis and that these are not present in PPHS or are present in reduced amounts. Alternatively, FBS could be inducing the fibroblastic phenotype, and this phenotype produces a large CS-containing proteoglycan as one of its characteristics. Whatever the mechanism, these results indicate that even very low levels of serum significantly stimulate CS synthesis by keratocytes.

The sequential digestion of stromal tissue used in this study may be helpful in isolating keratocytes in their native phenotype. We removed corneal epithelium using a brief (30-minute) collagenase digestion rather than by scraping, as is done in other procedures, which would induce keratocyte apoptosis. The second collagenase digestion yielded cells with poor viability and attachment properties. These may represent the damaged keratocytes on the cut surfaces of the stroma that would be the first to be released by collagenase digestion. The third collagenase digestion released cells with excellent viability and attachment properties. These were probably cells that were not on the cut surfaces but deeper in the stromal pieces.

Culturing keratocytes in 10% FBS also resulted in a nearly threefold increase in the number of cells over that with DMEM alone on Primaria plates. This difference in cell number was probably caused by proliferation of cells in 10% FBS, whereas cells in DMEM remained quiescent. Proliferation of keratocytes cultured in FBS has been considered similar to the activation of keratocytes in situ after corneal injury. In contrast to native keratocytes in situ, serum-cultured keratocytes and in situ corneal myofibroblasts or activated keratocytes proliferate, exhibit prominent microfilament bundles, focal adhesions, and enhanced expression of α5β1 integrin and fibronectin. Interestingly, our current studies also show that keratocytes cultured in 10% FBS synthesized more than 90% CS, a value that is similar to the 95% CS synthesized by corneal myofibroblasts in 2-week-old corneal scars. Furthermore, the CS-containing proteoglycans made by the myofibroblasts in scars were larger than the CS proteoglycans made by keratocytes in situ, and we found that CS proteoglycans made by keratocytes cultured in 10% FBS were larger than the CS proteoglycans made by keratocytes cultured in serum-free media. These findings further support the view that culture of normal keratocytes in the presence of serum mimics the wound-healing response, whereas the culture of keratocytes in the absence of serum mimics the behavior of cells in situ.

**FIGURE 4.** Chromatography of proteoglycans synthesized by collagenase-isolated keratocytes cultured under the different conditions described in Figure 1. Cells were radiolabeled with $^{35}$SO$_4$ from days 2 to 4, and the proteoglycans were purified from the media and chromatographed on a column of Superose 6 HR. Proteoglycans from an intact cornea that was radiolabeled overnight in DMEM were also purified and chromatographed on the same column (Intact Cornea). Tubes under the bars labeled 1 and 2 were pooled separately, and the sensitivity of the GAG to keratanase and chondroitin ABC was determined for each area (Table 1). Two separate chromatographic runs were made for each condition. The results were similar, and therefore only one set is shown.
TABLE 1. Glycosaminoglycan Composition of Synthesized Proteoglycans

<table>
<thead>
<tr>
<th>Glycosaminoglycan Composition</th>
<th>10% FBS</th>
<th>1% PPHS</th>
<th>1%/DMEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CS</td>
<td>96</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>% KS</td>
<td>4</td>
<td>11</td>
<td>73</td>
</tr>
</tbody>
</table>

* From Superose 6 HR. 1 and 2 indicate areas 1 and 2 designated in Figure 4.

Acknowledgments

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References