Production of Prostaglandin D Synthase as a Keratan Sulfate Proteoglycan by Cultured Bovine Keratocytes

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PURPOSE. To characterize the major proteoglycans produced and secreted by collagenase-isolated bovine keratocytes in culture.

METHODS. Freshly isolated keratocytes from mature bovine corneas were cultured in serum-free Dulbecco’s modified Eagle’s medium/F12. Secreted proteoglycans were radiolabeled with protein labeling mix (35S-Express; DuPont NEN Life Science Products, Boston, MA) and digested with chondroitinase ABC, keratanase, and endo-β-galactosidase to remove glycosaminoglycan chains, and core proteins were analyzed by autoradiography and Western blot analysis. An unidentiﬁed keratan sulfate proteoglycan (KSPG) was puriﬁed by gel ﬁltration (Superose 6; Amersham Pharmacia, Piscataway, NJ) and anion-exchange chromatography (Resource Q; Amersham Pharmacia) and subjected to amino acid sequencing.

RESULTS. Keratanase digestion of proteoglycans produced ~50 kDa core proteins that immunoreacted with antisera to lumican, keratocan, and osteoglycin/mimecan. Chondroitinase ABC digestion produced a ~55 kDa core protein that immunoreacted with antisera to decorin. A 28-kDa band generated by keratanase or endo-β-galactosidase digestion did not react with these antibodies. Chromatographic puriﬁcation and amino acid sequencing revealed that the protein was prostaglandin D synthase (PGDS). Identity was conﬁrmed by Western blot analysis using antisera to recombinant PGDS. PGDS isolated from corneal extracts was not keratanase sensitive but was susceptible to endo-β-galactosidase, suggesting that it contains unsulfated polylactosamine chains in native tissue and is therefore present as a glycoprotein.

CONCLUSIONS. These results indicate that bovine keratocytes, when cultured under serum-free conditions, produce the four previously identiﬁed corneal stroma proteoglycans: decorin, lumican, keratocan, and osteoglycin/mimecan and maintain a phenotype that is comparable to that of in situ keratocytes. Additionally, these cells produce PGDS, a known retinoid transporter, as a KSPG.


Keratocytes, the principal cell type of the adult corneal stroma, are responsible for producing the extensive and uniquely transparent extracellular matrix of the corneal stroma.1,2 The keratocytes in adult corneas are quiescent, but on stromal wounding are activated, proliferate, become ﬁbroblasts and myoﬁbroblasts, migrate to the wound site,3,4 and produce a disorganized extracellular matrix5 without keratan sulfate6—factors that probably contribute to the formation of an opaque scar in the cornea. Keratocytes that have been isolated from the stroma and cultured under standard conditions exhibit characteristics of the ﬁbroblast and myoﬁbroblast phenotypes, including cell shape, the presence of α-smooth muscle actin, low levels of keratan sulfate production, expression of the fibronectin receptor, and extensive cell proliferation.7-11 These cells are not useful for studying properties of the keratocytes that produce corneal transparency. Recent studies, however, indicate that collagenase-isolated keratocytes plated in media without fetal bovine serum do not become ﬁbroblasts or myoﬁbroblasts in culture. Collagenase-isolated rabbit keratocytes cultured in serum-free media do not proliferate, appear dendritic, and have no α-smooth muscle actin.12 Similarly, isolated and cultured bovine keratocytes do not proliferate, appear dendritic, and synthesize high levels of keratan sulfate proteoglycans (KSPGs).13 The dendritic appearance of these cultured cells is similar to the appearance of keratocytes in situ.12,14,15 This indicates that keratocytes cultured in vitro can retain their in situ phenotype when isolated by using collagenase and cultured in the absence of serum.

This new serum-free keratocyte culturing method provides an opportunity to more fully characterize the keratocyte phenotype and its transition to other phenotypes. In this report, we identify the major secreted proteoglycans of keratocytes in serum-free cell culture and ﬁnd that the keratocytes make all four previously identiﬁed corneal stroma proteoglycans: decorin, lumican, keratocan, and osteoglycin. In addition, they make a novel small KSPG that has been identiﬁed as prostaglandin D synthase (PGDS), a secreted product that has not been previously shown to be made by cultured keratocytes or made as a proteoglycan.

MATERIALS AND METHODS

Keratocyte Isolation and Culture

Bovine keratocytes were isolated using a modiﬁcation of a collagenase digestion method,13 using only two sequential digestions. Briefly, corneas were procured from twenty-four freshly harvested, adult bovine eyes (Pel-Freeze Biologicals, Rogers, AR), and three 8-mm disks were removed from the central region of each and processed as described before. Two equivalent groups of quartered discs were subjected to collagenase digestion for 30 to 45 minutes at 37°C with shaking at 142 rpm. Collagenase solution proceeded for 150 minutes under anaerobic conditions. Cells from the second digestion were pelleted by low-speed centrifugation and resuspended in Dulbecco’s modiﬁed Eagle medium/F12 (DMEM/F12, Gibco–Life Technologies, Grand Island, NY). Cell number and viability were determined using trypan blue exclusion. After a second low-speed centrifugation, cells were resuspended in DMEM/F12 supplemented with 1% platelet-poor horse serum (PPhS; Sigma, St. Louis, MO), plated into six-well tissue culture dishes (Costar, Cambridge, MA) at high density (400,000 cells/well) and allowed to attach overnight at 37°C in 5% CO2. Media (2 ml/well) were changed to DMEM/F12, and incubation proceeded until day 4.

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Biosynthetic Radiolabeling of Keratocytes

Cultures used for analysis of keratocyte protein and proteoglycan products were radiolabeled under serum-free conditions either on day 1 or 3 by addition of 100 μCi/ml protein labeling mix (FeS-Express; DuPont–NEN, Boston, MA) in DMEM:F-12 and subsequently incubated until day 4.

Autoradiographic Analysis of Keratocyte Proteoglycan Products

Media (12 ml) were removed from keratocytes after a 3-day labeling period, centrifuged at 800 rpm for 10 minutes to remove debris, and concentrated to 300 μl using 10-K centrifugal concentrators (Macrosep; USA Pall Filtron, Northborough, MA). Overnight dialysis at 4°C against 10 l of deionized water in 10-K cassettes (Slide-A-Lyzer; Pierce, Rockford, IL) was used to reduce unincorporated radioactivity and remove salts. Alternatively, media were lyophilized, reconstituted in 2 ml of 4 M guanidine–HCl, and fractionated on Sepharose columns (PD-10 Sephadex; Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in 4 M guanidine HCl to remove unincorporated radionuclides. Radiolabeled macromolecular fractions were dialyzed against water, lyophilized, and reconstituted in 100 μl of water. Ten-microliter portions were digested with chondroitinase ABC, endo-β-galactosidase or keratanase (Seikagaku America, Falmouth, MA) according to the manufacturer’s directions, and samples with and without digestion were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), as described before. Gels were fluorographically enhanced (Entensify; DuPont–NEN) and exposed to film (X-OMAT; Eastman Kodak, Rochester, NY) at −80°C for 16 to 48 hours.

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 was a gift from James Funderburgh (University of Pittsburgh). Rabbit antiserum to bovine lumican was prepared using an internal amino acid sequence (CPDFPQALYGRSC) as a peptide antigen. Rabbit antiserum to bovine decorin (LF-94) was a gift of Larry Fisher and cooker. Rabbit antiserum to bovine PGDS was a gift of Gary Killian.

Western Blot Analysis of Media from Keratocytes and Extracts from Whole Bovine Corneas

Media were collected from keratocyte cultures 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), according to the manufacturer’s specifications. Aliquots with and without digestion were applied to 10% bis-tris polyacrylamide gels (NuPage; Invitrogen, Carlsbad, CA) and electrophoresed and reduced with and without digestion were applied to 10% bis-tris polyacrylamide gels (NuPage; Invitrogen, Carlsbad, CA) and electrophoresed under reducing conditions. Proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) at room temperature, blocked with 5% milk in phosphate-buffered saline/Tween 20 (PBST), and incubated overnight at 4°C with either 1:1,000 monoclonal anti-β-galactosidase mAb, 1:1,000 rabbit anti-bovine decorin antigen, 1:1,000 rabbit anti-bovine keratanase antigen, or 1:20,000 affinity-purified rabbit anti-bovine recombinant PGDS. Membranes were rinsed in PBST, incubated in either horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse IgG (Amersham Pharmacia), and rinsed four times with PBST. Protein bands were developed using chemiluminescence detection (ECL; Amersham Pharmacia). A separate experiment, 2 g (wet weight) of frozen bovine corneas was extracted with 4 M guanidine HCl overnight, and the resultant material was initially dialyzed against water followed by one exchange into 6 M urea containing 0.15 M NaCl, 0.04 M tris (pH 8.0), and 0.05% 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS) and applied to a 2.5 × 5-cm column (Q Sepharose; Amersham) in the same solvent. The column was then eluted with a 0.15 to 1.15 M NaCl gradient in the urea solvent. Fractions were pooled based on UV absorption peaks. Each sample was dialyzed against water, lyophilized, and reconstituted in deionized water to a 1 mg/ml concentration. Ten-microgram portions were digested with endo-β-galactosidase and keratanase II and applied to 10% bis-tris acrylamide gels. Autoradiography was used to detect fractions containing radiolabeled macromolecules, and the fractions containing keratin sulfate proteoglycan X were pooled, adjusted to 0.1 M Tris (pH 8.0), applied to a 1 ml anion-exchange column (Resource Q; Amersham Pharmacia) and eluted with a 0.1 M NaCl gradient in the same buffer. Portions of resultant fractions were electrophoresed with and without keratanase digestion and analysis by autoradiography. The two fractions containing KS-PG-X (where X indicates an unknown proteoglycan) were sent to the W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, CT) for amino acid sequencing.

RESULTS

Culture media that had been radiolabeled were digested with chondroitinase ABC, keratanase, and a mixture of the two enzymes subjected to SDS-PAGE, and analyzed by autoradiogram to characterize in vitro keratocyte proteoglycans. Undigested material (Fig. 1, UD lane) contained a prominent band with an approximate molecular mass of 127 kDa, as well as a diffuse band located between 30 and 39 kDa. Chondroitinase ABC digestion affected only the 127-kDa band, shifting this to approximately 51 kDa (Fig. 1, U lane). Keratanase digestion, in contrast, did not shift the size of the 127-kDa band but produced faint broad bands near the 51-kDa marker as well as a shift of the prominent diffuse band between 30 and 39 kDa to a lower mass estimated at 28 and 30 kDa (Fig. 1, Case lane). Digestion of the material with both chondroitinase and keratanase served to increase the width of the band migrating around 51 kDa, indicating the presence of comigrating core proteins of chondroitin sulfate and keratan sulfate proteoglycans.

Media from cultures with and without radiolabel were examined by Western blot analysis to further confirm the identity of keratocyte protein products (Fig. 2). Antisera to decorin reacted with a broad band between the 191- and 64-kDa molecular weight markers (Fig. 2, UD lane). Digestion with chondroitinase (Fig. 2, Case lane) created a distinct broad band located between the 64- and 51-kDa markers, the same migration position of the radiolabeled band after chondroitinase digestion (Fig. 1, Case lane). Probing the blot with antiserum to decorin after keratanase digestion revealed no band shifts.
Antisera against lumican, keratocan, and osteoglycin were strongly immunoreactive to keratanase-digested material. Each antibody bound specifically to a band that migrated near the 51-kDa marker, which corresponds to the faint bands at \( \sim 51 \) kDa in the radiolabeled material (Fig. 2, Kase lane). None of the antisera reacted with the keratanase-generated 28- to 30-kDa band seen in the radiolabeled samples (Fig. 1).

Keratanase digestion produced core proteins with a broad range of molecular weights (Figs. 1, 2). Keratanase requires the presence of a sulfate ester on the polylactosamine chain for cleavage. It is possible that these keratan sulfate proteoglycans have a variable absence of sulfation on that part of the polylactosamine chain near the core protein. Consequently, to achieve complete digestion of polylactosamine chains from the core proteins, media from radiolabeled cultures were digested with endo-\( \beta \)-galactosidase, which cleaves an unsulfated polylactosamine and analyzed by autoradiogram (Fig. 3). Endo-\( \beta \)-galactosidase digestion produced a tighter core protein band than keratanase digestion for lumican, keratocan, and osteoglycin (the band just below the 51-kDa marker). In addition the diffuse 28- to 30-kDa band was now reduced to a sharper prominent band at 28 kDa by digestion with endo-\( \beta \)-galactosidase. This confirms the absence of sulfation on that region of the polylactosamine chains near the core protein of these proteoglycans. This also further confirms the presence of a

**Figure 1.** Autoradiogram of 24-hour radiolabeled keratocyte culture media. Aliquots of DMEM/F12 media were digested with chondroitinase ABC (Case), keratanase (Kase), or both enzymes or left undigested (UD) and were electrophoresed on polyacrylamide gels.

**Figure 2.** Western blot analysis of culture media, with and without chondroitinase (Case) and keratanase (Kase) digestion, using antibodies to known corneal proteoglycans. Keratocan and lumican both migrated to approximately 51 kDa. None of these antibodies cross-reacted with a 28-kDa band present on the autoradiogram in Figure 1. UD, undigested.

**Figure 3.** Autoradiogram of 24-hour radiolabeled keratocyte culture media digested with chondroitinase ABC (Case) or endo-\( \beta \)-galactosidase (EBG). Digestion with EBG produced enhanced resolution of the KSPG core proteins and generated a well-defined band at 28 kDa. UD, undigested.
novel KSPG in this culture system, which showed no reactivity to antisera to the three known KSPGs. The unknown proteoglycan was denoted KSPG-X.

Radiolabeled media from keratocytes were collected on day 4, and the components were chromatographed on a gel filtration column to begin purification of KSPG-X. Radiolabeled macromolecules eluted into three peaks (Fig. 4A). Fractions from these regions were analyzed on SDS-PAGE autoradiograms to determine the elution position of the 28-kDa KSPG-X (Fig. 4B). Fractions 20, 21, and 22 each contained a species that migrated to 28 kDa. These were pooled and chromatographed on an anion-exchange column eluted with a salt gradient for optimum resolution of KSPG-X. A prominent peak consisting of three fractions eluted late in the column run (Fig. 5A). Portions of each of these three fractions were electrophoresed, both with and without keratanase digestion, and were analyzed on an autoradiogram (Fig. 5B). Fractions 23 and 24 contained a broad, diffuse region that migrated between the 39- and 28-kDa molecular weight markers in the undigested lanes. Keratanase digestion shifted this region to a sharp band located at 28 kDa. The remainder of fraction 23 was digested with trypsin and the peptides separated by high-performance liquid chromatography (HPLC). A purified, trypsin-generated peptide from KSPG-X in fraction 23 was sequenced and found to be identical with residues 29-42 of prostaglandin D2 (PGD2) synthase (Table 1).

Keratocyte culture media with and without endo-β-galactosidase digestion were electrophoresed on acrylamide gels, subjected to Western blot analysis, and reacted with rabbit antisera to bovine PGDS to further confirm the identity of KSPG-X. Undigested material contained a diffuse band located between the 39- and 28-kDa molecular weight markers that reacted with this antibody (Fig. 6). Endo-β-galactosidase digestion shifted this region to a strongly immunoreactive band that migrated to 28 kDa.

To determine whether PGDS is present in corneal tissue, extracts from bovine corneas were applied to a Sepharose column and eluted with a NaCl gradient (Fig. 7). Fractions were pooled into five regions and each tested for immunoreactivity with the bovine anti-PGDS antibody by Western blot analysis with and without digestion by endo-β-galactosidase. Only the pass-through fraction (peak A) contained an immunoreactive band at 28 kDa generated by endo-β-galactosidase digestion but not by keratanase II (Fig. 8).

![Figure 4](https://example.com/fig4.png)

**Figure 4.** (A) Chromatography of proteoglycans synthesized by bovine keratocytes cultured in DMEM/F12. Cells were radiolabeled for 24 hours, media from these were collected and pooled with media from nonradiolabeled cell cultures, and proteoglycans were separated by gel filtration. (B) Autoradiogram analysis of SDS-PAGE separation of fractions resulting from chromatography. All fractions were assayed by SDS-PAGE. Fractions 20, 21, and 22 contained the 28-kDa proteoglycan core protein band.

![Figure 5](https://example.com/fig5.png)

**Figure 5.** (A) Separation of pooled gel filtration fractions on a Resource Q anion-exchange column. Three fractions that contained the 28-kDa proteoglycan core protein band were pooled and further separated on the basis of charge for optimum resolution of the molecule. (B) Autoradiogram analysis of Resource Q column fractions. Remaining material from fraction 23 contained a protein identified as PGDS through amino acid sequencing analysis.

![Table 1](https://example.com/table1.png)

**Table 1. Alignment of Bovine PGDS and a Peptide Generated from Tryptic Digest of a 28-kDa Protein Secreted into Bovine Keratocyte Culture Media**

<table>
<thead>
<tr>
<th>Region</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>Peptide from 28-kDa protein</td>
<td>ALQPNFEDKFLGR</td>
</tr>
<tr>
<td>Bovine PGDS*</td>
<td>29–42 ALQPNFEDKFLGR</td>
</tr>
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* NCBI accession number 002853.
DISCUSSION

Previous studies have shown that keratocytes cultured in serum-free media synthesize 44% of their proteoglycans as KSPG and 46% as CSPG, levels that closely mimic the percentage of KSPG found in corneas maintained in organ culture. In this study, we identified these CSPGs and KSPGs as the leucine-rich proteoglycans decorin, lumican, keratocan, and osteoglycin by analysis of radiolabel incorporation into core proteins of correct size and by subsequent confirmation of Western blot immunoreactivity with antibodies raised against these corneal proteoglycans. Autoradiographic and Western blot analysis of serum-free keratocyte culture media also revealed the presence of a 28-kDa protein containing keratan sulfate chains that was not immunoreactive with antisera to known corneal KSPGs. Sequence from the 28-kDa protein was found to be identical with bovine PGDS. This finding was confirmed by Western blot analysis using antibodies raised against recombinant PGDS. The sequence of PGDS contains a signal peptide at its N terminus, which accounts for its secretion into the media by the keratocytes.

Keratan sulfate found in the cornea is linked to the core protein through an N-linked oligosaccharide. There are two potential N-linked sites (NXT/S) in bovine PGDS that could serve as attachment points for keratan sulfate chains. PGDS isolated from keratocyte culture media contained keratan sulfate chains, as demonstrated by sensitivity to keratanase, an enzyme that requires the presence of sulfate esters on polyacatosamine for polymer degradation, and by the requirement of high salt for elution from Sepharose. However, the PGDS isolated from extracted bovine cornea did not bind to the Sepha-

**FIGURE 6.** Western blot analysis of keratocyte culture media with antibodies to PGDS. A polyclonal anti-PGDS antibody showed reactivity with a tight 28-kDa band generated by endo-β-galactosidase (EBG) digestion of culture media. UD, undigested.

**FIGURE 7.** Chromatograph of material extracted from frozen bovine corneas. Corneas were extracted with 4 M guanidine HCl and applied to a Sepharose column after dialysis in 6 M urea containing 0.15 M NaCl, 0.01 M Tris (pH 8.0), and 0.05% CHAPS. Bracketed regions A through E indicate fractions that were pooled based on their UV-absorption spectra. Dashed line: 1.0 M NaCl gradient used for proteoglycan elution. The pass-through fraction is denoted as region A.

**FIGURE 8.** Reactivity of Sepharose pass-through material from extracted bovine corneas with anti-PGDS on Western blot analysis. The polyclonal anti-PGDS antibody showed reactivity with a tight 28-kDa band generated by endo-β-galactosidase (EBG) digestion. Keratanase II (KII) digestion did not shift the core protein's position, indicating an absence of keratan sulfate chains on the molecule.
rose even under low-salt conditions, was not sensitive to kerat-
anase, and was sensitive to endo-β-galactosidase, an enzyme
that cleaves unsulfated glycosaminoglycans. This indicates
that PGDS in the bovine cornea is not sulfated, in contrast to
PGDS produced by keratocytes in vitro. The presence of sulfate
esters on PGDS produced by keratocytes in culture could be
a consequence of the collagenase isolation procedure or a result
of secretion of extracellular matrix molecules into the culture
media, rather than their accumulation around the cells. Despite
these differences in posttranslational modification, this report
demonstrates the production of PGDS by keratocytes in culture
and its presence in the cornea.

Two biochemically distinct types of PGDS have been
characterized: a glutathione-dependent, hematopoietic PGDS,
which is detected in spleen, thymus, and bone marrow, and
a glutathione-independent, lipocalin-type PGDS, first purified
in rat brain and said to be secreted into the cerebrospinal fluid
as β-trace. Lipocalin-type PGDS has been identified in bovine
semina plasma, rat brain and spinal cord, rat cochlea, human
prostate, human and rat epididymis and testes, and ocular tissues
but until this report, there has been no direct evidence that it is produced by keratocytes.

Lipocalin-type PGDS catalyzes the formation of PGD₂ from
its arachidonic acid-derived precursor prostaglandin H₂. PGD₂ is responsible for such diverse functions as regulation of
intraocular pressure, induction of non-rapid eye movement
sleep, prevention of platelet aggregation and induction of
vasodilation and bronchoconstriction, and thermoregula-
tion and modulation of odor response. Although PGD₂ is a
major prostanoid in ocular tissues, PGDS enzymatic activity is
negligible in the cornea, suggesting that PGDS may have another role at this site. PGDS isolated from rat brain binds
all-trans retinoic acid, cis-retinoic acid, and retinal with high
affinity, and it is quite possible that PGDS produced by
keratocytes in the cornea is involved in transporting retinoid
derivatives throughout the cornea for maintenance of a healthy
stoma. Vitamin A (retinol) is necessary for glycoprotein bio-
synthesis in the cornea, and a deficiency in this nutrient can result in such corneal diseases as xerosis, ulceration, or kera-
tomalacia. Persons with keratomalacia unusually experience a
complete dissolution or melting of the cornea, eventually leading
to loss of the eye. Although evidence indicates that a
stromal injury precedes onset of keratomalacia, investigators
have also found cases in which the corneal epithelium remained
intact, suggesting an underlying metabolic anomaly.

Retinol binding protein (RBP), a member of the lipocalin
family, is the major plasma transport protein for retinol. Point
mutations in the RBP gene have caused complete elimination
of circulating plasma RBP, yet target tissues such as the eye
were either mildly affected or completely unaffected, despite
the absence of circulating retinol. This strongly indicates an
alternate pathway of vitamin A utilization, such as tissue-spe-
cific retinoid-binding proteins that could compensate for the
absence of retinol by binding and transporting alternate retin-
oid forms. It is possible that PGDS plays such a role in cornea-
specific retinoid transport mechanisms.

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