Biosynthesis of Stromal Matrix Proteoglycans and Basement Membrane Components by Human Corneal Fibroblasts

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The proteoglycans produced by intact human corneas and corneal cells in culture were compared by characterizing the biosynthetically radiolabeled proteoglycans and by using antibodies to detect their core proteins. Organ cultures of corneas primarily produce a keratan sulfate proteoglycan (KSPG) and a chondroitin and dermatan sulfate proteoglycan (decorin). Immunostaining with antibodies specific for the core proteins of KSPG and decorin showed that these proteoglycans are localized to the corneal stroma. The stroma also contained trace amounts of matrix that stained with antibodies to basement membrane hepanan sulfate proteoglycan (perlecan) and laminin. Corneal fibroblasts in culture produced decorin, but the synthesis of KSPG appeared to be blocked at the level of core protein synthesis. Corneal fibroblasts in culture, however, produced perlecan in greater amounts than they did in organ cultures, and they synthesized both perlecan and laminin in greater amounts than did corneal epithelial cells in culture. These results indicate that the synthesis of proteoglycans by human corneal fibroblasts in culture is altered, resulting in increased production of basement membrane-associated proteoglycans and decreased synthesis of corneal stroma-associated proteoglycans. Invest Ophthalmol Vis Sci 33:547-557, 1992

The cornea consists of an extensive stromal matrix bordered by epithelial and endothelial cell layers on its outer and inner surfaces, respectively. The corneal stroma contains primarily proteoglycans and collagens with fibroblasts interspersed in this matrix. There are at least two proteoglycans in the stromal matrix; one with chondroitin and dermatan sulfate side chains and the other with keratan sulfate side chains (KSPG).1-8 By comparison with proteoglycans in other tissues, such as cartilage and basement membrane, both corneal proteoglycans are small. The proteoglycan bearing chondroitin and dermatan sulfate side chains (decorin) has been cloned and is present in most fibrillar extracellular matrixes.9,10 The major proteoglycan of the corneal stroma is KSPG. Immunologic studies, using antibodies directed against its core protein, suggest that it is a distinct gene product from the core protein of decorin11 and that, in contrast to decorin, it is present in other fibrillar matrixes in small amounts.12

Corneal transparency is dependent on normal corneal hydration, normal thickness, and the ordered arrangement of its stromal collagen fibrils.13 The corneal endothelium is essential for the maintenance of normal corneal hydration and thickness.14,15 In addition, KSPG is believed to play an important role in corneal transparency. It appears during the acquisition of transparency during development16,17 and is associated closely with corneal collagen fibrils.18 Furthermore, KSPG is absent or reduced in opaque corneal scars and reappears during restoration of transparency in corneal wound healing.19,20 In an inherited eye disease, macular corneal dystrophy, the sulfate esters are absent from the side chains11,21 and, as a result, produce corneal opacity and blindness that can only be restored by transplantation.

Cell culture has been used to help characterize matrix components and study regulation of their biosynthesis. We analyzed cultures of human corneal fibroblasts to determine the production of the two corneal...
stromal proteoglycans, using methods that identify them by their core protein and their glycosaminoglycan side chains. We detected the synthesis of decorin but only trace levels of KSPG. However, we found that corneal fibroblasts synthesize substantial amounts of two basement membrane components: (1) laminin, an abundant glycoprotein, and (2) perlecan, a basement membrane proteoglycan.

Materials and Methods

Proteoglycan Purifications

We purified KSPG and decorin from human corneas obtained from donor eyes, and perlecan was purified from a murine basement membrane-producing tumor (EHS tumor) by standard methods published elsewhere.2-3,22,23 Briefly, this purification involved (1) extracting the proteoglycans from the tissue with 4 M guanidine HC1 containing protease inhibitors and (2) purifying the proteoglycans in the extract by cesium chloride density-gradient centrifugation in 4 M guanidine HC1, chromatography on DEAE Sepharose (Pharmacia, Piscataway, NJ) in 6 M urea using a NaCl gradient elution followed by chromatography on Sepharose CL-4B in 4 M guanidine HC1. The elution positions of the proteoglycans were monitored by their core protein and their glycosaminoglycan side chains with either keratanase, chondroitinase ABC, or heparitinase, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining for core protein with Coomassie blue.2,24 The purity of the final preparation was determined by removing the glycosaminoglycan side chains with either keratanase, chondroitinase ABC, or heparatinase, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining for core protein with Coomassie blue.25,26

Antibody Production

Antibodies to KSPG and decorin, isolated from human corneas, and to perlecan and laminin, isolated from the EHS tumor, were raised in rabbits using MPL + TDM + CWS emulsion (RIBI Immunocchemicals, Hamilton, MT), according to the manufacturer’s directions. The titers of the antisera were determined by an enzyme-linked immunosorbent assay. The rabbits were maintained and used in accordance with National Institutes of Health guidelines and the ARVO Resolution on the Use of Animals in Research.

Antibody Specificity

Specificity of the antisera was shown by western blot analysis.25,26 In brief, proteoglycans purified by DEAE chromatography were digested with chondroitinase ABC or keratanase (Seikagaku America Inc., Rockville, MD) in the presence of protease inhibitors.2 The digested and undigested proteoglycans separated by SDS-PAGE, were transferred to nitrocellulose, and incubated first with 1:250 dilution of antisera and then with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. Color was produced with 4-chloro-1-naphtol.

Organ and Cell Culture

Human corneas were obtained from the Medical Eye Bank of Western Pennsylvania. The corneas used in organ culture were removed from donor eyes by cutting 2–3 mm outside the limbus. To label the proteoglycans synthesized in organ culture biosynthetically, the explants were submerged in Dulbecco’s modified Eagle’s medium (DMEM) with 15% fetal bovine serum containing 100 μCi/ml of 35SO₄ and incubated for 16 hr at 37°C in a tissue culture incubator in 5% CO₂ and 95% air. The explants then were removed from the medium, the sclera rims were trimmed off, the corneas cut into pieces, and the pieces extracted twice overnight in ten volumes of 4 M guanidine HC1 containing protease inhibitors at 4°C.3

Corneal fibroblasts were grown from stromal explants of human donor corneas in DMEM with 15% fetal bovine serum.27 Epithelial cells were grown from limbal explants in 35-mm dishes using modified medium consisting of DMEM and Ham’s F-12 (1:1) supplemented with mouse epidermal growth factor (10 ng/ml), insulin (5 μg/ml), cholera toxin (0.1 μg/ml), glutamine (1 μg/ml), dimethyl sulfoxide (0.5%), and 15% fetal bovine serum. Both stromal and epithelial cultures were grown at 37°C in 5% CO₂ and 95% air. The stromal and epithelial explants were removed, usually in 2–4 weeks, after extensive growth of cells from the explant had occurred. The cultures were allowed to become confluent, which took 4–6 weeks, before radiolabeling. Fibroblast cultures in passages 2–3 were used in some cases. No differences were observed between primary and passaged cultures.

The proteoglycans produced by the cultured corneal fibroblasts were radiolabeled biosynthetically for 16 hr by replacing the medium containing 100 μCi/ml of 35SO₄. The medium then was removed and adjusted to contain 4 M guanidine HC1 by adding 0.5 g/ml of guanidine HC1. The cell layer was extracted overnight at 4°C with 2 ml of 4 M guanidine HC1 containing protease inhibitors.

The proteins produced by the fibroblasts and the epithelial cell cultures were radiolabeled biosynthetically with 35S-methionine for use in subsequent immunoprecipitations to identify the precursor proteins to the stromal and basement membrane proteoglycan core proteins. This radiolabeling procedure, described
in detail elsewhere, consisted of incubating the cells in serum-free methionine-free medium for 30 min, removing the medium, and pulsing the cells for 30 min in fresh serum-free methionine-free medium containing 500 µCi/ml of 35S-methionine. The medium then was removed, and the cell layer was harvested in lysing buffer. Alternatively, for pulse–chase experiments, the medium was replaced with serum-free medium containing methionine. The culture was incubated for an additional 2 hr before the medium and cell layer were harvested separately. Material immunoprecipitated from the medium was digested with heparitinase or chondroitinase ABC in the presence of proteolytic inhibitors.

Analysis of 35SO4-Labeled Proteoglycans

The incorporated isotope in the guanidine HCl extracts of the cornea and cell cultures was isolated by chromatography on Sephadex G-50 (Pharmacia) packed in a 10-ml pipette. The macromolecular material was dialyzed against Tris-buffered urea (6 M urea with 0.15 M NaCl, 0.1% (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate), and 0.05 M Tris HCl, pH 6.8) and then applied to a column (1 x 3 cm) of DEAE Sepharose equilibrated and eluted with the same solvent. The pass-through material was discarded, and the proteoglycan was eluted with Tris-buffered urea containing 1.15 M NaCl. The proteoglycan fraction was applied to a Superose 6 column (HR 10/30; Pharmacia) equilibrated, and eluted with 4 M guanidine HCl containing 0.02 M Tris HCl, pH 6.8. Aliquots of each fraction were measured for radioactivity in a liquid scintillator using Ready Safe cocktail (Beckman Instruments, Inc., Fullerton, CA). Tubes containing peak fractions were pooled, dialyzed, and lyophilized.

The glycosaminoglycan types in each fraction were determined by their sensitivity to nitrous acid for heparan sulfate, chondroitinase ABC for chondroitin and dermatan sulfate, and keratanase for keratan sulfate, using methods previously described. This method uses molecular-sieve chromatography on Sephadex G-50 to separate the resistant glycosaminoglycans from those degraded by the treatment. The glycosaminoglycans first are digested with nitrous acid and then undergo chromatography on Sephadex G-50. The amount of heparan sulfate is determined by the percent of radioactivity shifting to a lower molecular weight. The undegraded glycosaminoglycan then is digested with chondroitinase ABC and the chromatography is repeated to determine the percent of chondroitin and dermatan sulfate. The remaining undegraded glycosaminoglycan then is digested with keratanase, and the chromatography is repeated for the last time to determine the percent of keratan sulfate. Any remaining undigested glycosaminoglycan is considered resistant because excess reagent or enzyme is used for each digestion.

Detection of 35S-Methionine Radiolabeled Precursor Protein

Immunoprecipitation and identification of precursor proteins to proteoglycan core proteins were done as described previously. In brief, a portion of the lysate from 35S-methionine-pulsed cells or the media from pulse–chased cells was incubated with protein A-Sepharose beads previously charged with antisera or preimmune sera. The beads then were washed, the bound immunoglobulin G and radiolabeled proteins were released by boiling in SDS and dithiothreitol, and then released material was separated by SDS-PAGE. The acrylamide gel was embedded with Fluoro-hance (Research Products International Corp., Mt. Prospect, IL), dried, and exposed to X-Omat film (Eastman Kodak Co., Rochester, NY) at −70° for 1 day to several weeks, depending on the amount of radiolabeled material. In all cases, immunoprecipitations in a group were done from equivalent amounts of incorporated radiolabel to allow direct comparison of precursor protein synthesis. The amount of incorporated radiolabel in the lysate or media was determined after precipitating proteins in an aliquot of the lysate or media with 5% trichloroacetic acid and counting the radioactivity in the precipitate.

Immunostaining

The corneas were embedded in O.C.T. compound (Miles, Elkhart, IN) and frozen. Cryostat sections (7 mm) of the corneas were transferred to gelatin-coated slides and immunoreacted with affinity-purified specific antibodies at appropriate dilutions using an indirect fluorescein isothiocyanate (FITC)-conjugated technique described earlier. The secondary antibody was FITC-conjugated goat anti-rabbit immunoglobulin G (1:40 dilution; Cappel, Malvern, PA). Immunofluorescence-immunoglobulin G from normal rabbit serum at a concentration of 10 µg/ml was used in place of specific antibodies as a control to monitor nonspecific background staining.

Results

Fractionation of 35SO4-radiolabeled proteoglycans from whole intact corneas on a column of Superose 6 produced a single major peak eluting in the middle of the fractionation range of the resin (Fig. 1A, peak C-1). Analysis of the glycosaminoglycan types contained in this peak indicated that it consisted of 56%
chondroitin and dermatan sulfate and 22% keratan sulfate, representing glycosaminoglycans on decorin and KSPG core proteins, respectively (Fig. 1B). Less than 7% of the incorporated sulfate was in heparan sulfate. The material resistant to digestion may be regions of keratan sulfate that are sulfated poorly. These regions would not be degraded by keratanase because it requires the presence of sulfate esters for cleavage. These partially degraded glycosaminoglycans would be larger than di- or tetrasaccharides and, as a result, would elute in high molecular weight fractions by Sephadex G-50 chromatography and be considered undegraded. Fractionation of the proteoglycans synthesized by corneal fibroblasts in culture produced a
Fig. 2. Immunostaining of human corneas. Cryostat sections of human corneas were immunostained with affinity-purified antibodies against keratan sulfate proteoglycan protein (A) or anti-decorin antibodies (B) using an indirect immunofluorescence staining technique. Note that both these antibodies immunoreacted with the stromal matrix but not with the epithelium (arrowhead) or Bowman’s membrane (arrow).

The antibodies next were used to immunoprecipitate the precursor proteins to these proteoglycans from lysates of 35S-methionine-pulsed corneal fibroblasts. With this short radiolabeling procedure, most of the incorporated radiolabel is in proteins that are

stoma in tissue sections (Fig. 2A) and reacted with a 50,000 relative molecular weight (Mr) core protein generated by keratanase digestion of the intact proteoglycan (Fig. 3, lane 2) in western blots. Because KSPG migrates on SDS-PAGE as an extended smear (from 200,000 to 70,000 Mr), the intact proteoglycan (Fig. 3, lane 1) does not appear on western blot analysis as readily as the core. Antibodies to decorin also intensely stained the corneal stroma (Fig. 2B) and reacted with the intact decorin proteoglycan (Fig. 3, lane 3) and the 43,000 Mr core protein generated by chondroitinase ABC digestion of decorin (Fig. 3, lane 4). The antibodies next were used to immunoprecipitate the precursor proteins to these proteoglycans from lysates of 35S-methionine-pulsed corneal fibroblasts. With this short radiolabeling procedure, most of the incorporated radiolabel is in proteins that are
still in the rough endoplasmic reticulum and have not
toved to the Golgi apparatus where the glycosaminoglycans are added to the precursor proteins. The
immunoprecipitated material was examined by SDS-PAGE followed by autoradiography. The antibodies to
decorin immunoprecipitated a doublet around Mr = 43,000 (Fig. 4, lane 2). The immunoprecipitation of
this doublet was blocked by unlabeled decorin core protein (Fig. 4, lanes 3 and 4), thereby confirming it as the
precursor protein to decorin. When antibodies to KSPG were used to immunoprecipitate the core protein
from an equivalent amount of incorporated radioactivity, a faint band at Mr = 46,000 could be detected on
autoradiograms exposed for a length of time comparable to the decorin immunoprecipitations (lane 6, arrow). If this material is the precursor protein to
KSPG, it is produced at substantially lower levels than the precursor protein to decorin and migrates to a lower position on SDS-PAGE, most likely as a result of the presence of the immunoglobulin G used in the
immunoprecipitation.

Because a large proportion of heparan sulfate proteoglycan was found to be produced by corneal fibroblasts in culture (Fig. 1), we also used antibodies to the
core protein of perlecan, a heparan sulfate proteoglycan found in basement membranes, in these immunoprecipitation reactions. The antibodies to perlecan
immunoprecipitated a major band of 400,000 Mr and two lower Mr bands of approximately 220,000 and 300,000 Mr (Fig. 5, lane 3) from lysates of 35S-methionine-pulsed corneal fibroblasts. These lower molecular weight bands were two of the three major proteins precipitated with antibodies to laminin (Fig. 5, lane 2), and their immunoprecipitation was blocked with increasing amounts of unlabeled laminin (Fig. 6, lanes 3 and 4). This coprecipitation of laminin with proteoglycan has been observed previously
and could be caused by an interaction between laminin and perlecan or the presence of related epitopes because regions of these proteins are known to be homologous. The precursor protein to perlecan (Fig. 5, lane 6) and laminin (Fig. 5, lane 5) also were immunoprecipitated from lysates of 35S-methionine-pulsed corneal epithelial cells. However, the bands were not as intense as those obtained from lysates of corneal fibroblasts even though they were precipitated from equal amounts of incorporated radiolabel. This suggests that the corneal fibroblasts in culture make relatively more basement membrane components than corneal epithelial cells in culture. Furthermore, the autoradiogram demonstrating laminin and perlecan synthesis (Fig. 5) was made from a 3-day exposure; autoradiograms showing decorin and keratan sulfate
Fig. 6. Immunoprecipitation of laminin synthesized by corneal fibroblasts. Corneal fibroblasts were pulse-labeled with \(^{35}\)S-methionine, and laminin in the cell lysate was immunoprecipitated using anti-laminin antibodies together with protein A-Sepharose beads. Lane 1, preimmune serum. Lane 2, immunoprecipitation of laminin chains with anti-laminin antibodies. Lanes 3 and 4, immunoprecipitation with anti-laminin antibodies that were blocked with 5 and 50 \(\mu\)g of laminin, respectively. Note that the immunoprecipitation of laminin is prevented by blocking the antibodies with excess amounts of laminin (lane 4).

synthesis were made from 10-day (Fig. 4, lanes 1 and 2) and 34-day (Fig. 4, lanes 3-6) exposures, even though all immunoprecipitations were done with equal amounts of incorporated radiolabel. This suggests that the synthesis of basement membrane components by corneal fibroblasts is at least equivalent to and likely greater than the synthesis of decorin and KSPG.

The identity of the precursor protein to perlecan was investigated further by pulse-chase radiolabeling of corneal fibroblasts (Fig. 7). Two dishes of cells were pulsed with \(^{35}\)S-methionine for 30 min. The cell layer from one dish was harvested at that point; the media in the other dish was replaced with media lacking radiolabel. After a 2-hr chase period, the cell layer and media in the second dish were harvested separately. Antibodies to perlecan immunoprecipitated the 400,000 M\(_s\), precursor protein from lysates of pulsed cells as before (Fig. 7, lane 2). This protein was absent from the cell layer and the media after the 2-hr chase (Fig. 7, lanes 3 and 4). Heparitinase digestion of the immunoprecipitates from the media caused some of the radiolabel to enter the top of the gel (arrowhead), but it did not produce a 400,000 M\(_s\), core protein (Fig. 7, lane 7). Digestion with chondroitinase ABC, however, produced a band at 400,000 M\(_s\), (Fig. 7, lane 8). This suggests that at least a portion of the precursor protein identified with antibodies to perlecan was actually converted to a chondroitin sulfate proteoglycan and not a heparan sulfate proteoglycan.

The antibodies to perlecan and laminin also used to immunostain cryostat-cut sections of cornea (Fig. 8). Both antibodies immunostained the epithelial basement membrane (Figs. 8A, 8D) and Descemet's membrane (Figs. 8B, 8E). Additionally, a punctate pattern of immunostaining could be detected in the corneal stroma (Figs. 8C, 8F). These antibodies also reacted with the cell surface and the extracellular areas of corneal fibroblasts in culture (Figs. 9B-C).

Discussion

Our results showed that corneal fibroblasts in culture produce proteoglycans in a different proportion than do corneas in organ culture. Based on glycosaminoglycan content, the intact corneas synthesized mostly decorin and KSPG; corneal fibroblasts in culture synthesized mostly chondroitin and heparan sulfate with only trace amounts of keratan sulfate. This was consistent with the observations of others using bovine and rabbit corneal fibroblasts.\(^{32,33}\) We confirmed and extended the interpretations of glycosaminoglycan analyses by using antibodies to the core proteins of decorin, KSPG, and perlecan to immunoprecipitate the precursor proteins produced by fibro-
blasts. The precursor protein to decorin was detected in corneal fibroblasts although it was produced in lesser amounts than that by skin fibroblasts (data not shown). Only trace levels of the precursor protein to KSPG could be detected in corneal fibroblasts, suggesting that these cells are phenotypically altered when placed in tissue culture.

Antibodies to laminin, nidogen (entactin), and perlecan have been shown previously to localize in mouse corneas to the epithelial basement membrane, to Descemet's membrane, and to basement membrane-like plaques in the stroma. We found a similar distribution for laminin and perlecan in human corneas. Furthermore, we confirmed the identity and source of this immunoreactive material by demonstrating the synthesis of laminin and perlecan precursor proteins by corneal fibroblasts in culture. Furthermore, the fibroblasts make at least as much and possi-
Fig. 9. Immunostaining of corneal fibroblasts in culture. Corneal fibroblasts, cultured in chamber slides, were immunostained with either preimmune rabbit serum (A), anti-HSPG antibodies (B), or anti-laminin antibodies (C). Corresponding phase contrast micrographs are shown in the right panel (A', B', C'). Note that both antibodies reacted with the cell surface and extracellular matrix of the cultured cells (×200).

bly more laminin and perlecan in culture than they make decorin and KSPG, the major proteoglycans of the adult corneal stroma. These corneal fibroblasts also make more laminin and perlecan than do corneal epithelial cells in culture.

Perlecan originally was isolated from a murine basement membrane-producing tumor. It was shown to contain heparan sulfate side chains and be synthesized from a 400,000 M, precursor protein. Antibodies against the core protein of perlecan have been
shown to react with all basement membranes and with heparan sulfate proteoglycans produced by various basement membrane-producing cells, including glomerular cells.26,35 We showed antibodies to perlecan immunoprecipitated a 400,000 M₄, precursor protein produced by corneal epithelial cells and corneal fibroblasts. At least some of this protein, however, was converted by fibroblasts to a chondroitin sulfate proteoglycan rather than a heparan sulfate proteoglycan. Furthermore, heparitinase digestion of the proteoglycans immunoprecipitated with antibodies to perlecan resulted in the appearance of a band at the top of the gel. Because only one 400,000 M₄, perlecan precursor protein was detected, the perlecan population may possess both heparan sulfate and another proteoglycan with more than one type of glycosaminoglycan side chain. There are two possible explanations for these results. The first is that this is the same gene product as that made by “authentic” basement membrane producing cells and that it receives chondroitin sulfate side chains instead of, or in addition to, heparan sulfate side chains. The other possibility is that the precursor protein made by corneal fibroblasts is a gene product that is similar to the core protein of perlecan in size and contains some epitopes in common with perlecan. As a result, it is immunoprecipitated by the antibodies to perlecan. Although it is not possible to confirm either of these possibilities now, it should be noted that southern blot analysis of perlecan gene using complementary DNA clones has indicated that in mice there is only one gene for perlecan.31

Most of the high molecular weight proteoglycans produced by corneal fibroblasts in culture (Figs. 1C, 1e; C-1 and M-1 fractions) contained heparan sulfate and/or chondroitin sulfate side chains. These eluted in the void volume and would be in the size range for perlecan regardless of the type or number of glycosaminoglycan side chains. Decorin (M₄, ≈ 116,000) would be included in the C-2 and M-2 fractions, and most of the incorporated sulfate in those fractions was chondroitin sulfate. There was also substantial amounts of heparan sulfate in the C-2, C-3, and M-2 fractions; however, this material was too small to be perlecan even if it did have heparan sulfate side chains. This material is probably another heparan sulfate proteoglycan (ie, a different core protein with heparan sulfate side chains).

The loss of phenotype during cell culture has been documented for a number of cells, but most notably in cartilage, where chondrocytes in culture lose the ability to make aggrecan (cartilage-specific proteoglycan), collagen type II, and link protein. The loss of phenotype during cell culture has been documented for a number of cells, but most notably in cartilage, where chondrocytes in culture lose the ability to make aggrecan (cartilage-specific proteoglycan), collagen type II, and link protein.44 Our results showed that corneal fibroblasts in culture have lost the ability to produce KSPG, a unique marker for corneal stroma, and have increased their production of laminin and perlecan, components of basement membranes. Analysis of the collagens produced by corneal fibroblasts in culture show that they produce collagen type I, the major collagen of corneal stroma.27 Thus, only the glycoprotein and proteoglycan gene products of corneal fibroblasts appear to be disregulated in culture.

In a series of experiments similar to these but using chick corneas and chick corneal fibroblasts from day 15 chick embryos, we easily detected corneal KSPG precursor protein synthesis by corneal fibroblasts in culture although the precursor protein was not converted to a proteoglycan and remained as a glycoprotein.32 The disregulation of proteoglycan production in human corneal fibroblasts therefore is a consequence of cell culture because intact corneas in organ culture produce both proteoglycan types. There are at least two possible causes for this disregulation. The age of the donor may be important. The fibroblasts used in this study were grown from human corneas received from donors 46 yr of age and older and may not be as phenotypically stable as those from embryos. The method of cell isolation also may affect its phenotype. The human corneal fibroblasts were grown from stromal pieces; the chick cells were isolated after collagenase digestion of the stroma and used within 1 week of plating. The growth of cells from stromal explants, as in this study, could select for one particular type of fibroblast or require the cells to undergo extensive proliferation, even in primary cultures, which could cause a loss of their original phenotype. Using KSPG precursor protein production as a marker for phenotypically correct corneal fibroblasts will be useful for identifying (1) the elements or processes that regulate the synthesis of corneal proteoglycans and (2) the mechanisms of disregulation that lead to corneal opacity and blindness.

Key words: proteoglycans, fibroblasts, epithelium, basement membrane, stroma

Acknowledgments

The authors thank Gena Volas for technical assistance.

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