Cell Surface-Associated Keratan Sulfate on Normal and Migrating Corneal Endothelium

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Purpose. To investigate cell surface-associated keratan sulfate on the corneal endothelium.

Methods. Immunolabeling techniques were used at the light, scanning, and transmission electron microscopic level to localize keratan sulfate on the corneal endothelium. The investigation included human, bovine, and rabbit corneal endothelia. A quantitative study of the relationship between cell size and keratan sulfate levels was conducted on normal bovine corneal endothelium. Changes in the distribution of keratan sulfate and chondroitin sulfate on endothelial cell surfaces were investigated on organ cultured bovine corneas during endothelial wound healing. Changes in the levels of keratan sulfate during endothelial wound healing were investigated in organ cultured human corneas and in vivo in rabbit corneas. Inhibition-enzyme-linked immunosorbent assay also was used to detect keratan sulfate in the aqueous humor.

Results. A variegated distribution of keratan sulfate was revealed on normal human, bovine, and rabbit corneal endothelia. Some cells had high levels of keratan sulfate on their surfaces whereas others, sometimes immediately adjacent, had little or none. Wound healing experiments resulted in changes of keratan sulfate levels on the migrating endothelial cells in bovine, human, and rabbit. In wounded organ cultured bovine corneas, there was a decrease in keratan sulfate levels and an increase in chondroitin sulfate levels on migrating endothelial cells. Keratan sulfate was detected in bovine aqueous humor.

Conclusions. The pattern of occurrence of keratan sulfate and chondroitin sulfate on the corneal endothelial cells in normal and wounded cornea suggests that these glycosaminoglycans have differing roles in endothelial adhesion and migration. Invest Ophthalmol Vis Sci. 1996;37:1256–1270.

The importance of keratan sulfate proteoglycan (PG) in relation to the fibril organization in the corneal stroma has long been recognized. The transparency of the corneal stroma is dependent on the regular diameter and spacing of the collagen fibrils.¹ Loss of transparency in the scarred corneal stroma is the result of disorganization of the fibril arrangement and is associated with the loss of keratan sulfate. The return of transparency during wound healing is dependent on the restoration of fibril order and is associated with the return of keratan sulfate.²⁻³ Keratan sulfate PG also has been shown to be important in the acquisition of transparency during development.⁴⁻⁵ Molecular biology studies have shown that three types of keratan sulfate PG are present in the corneal stroma; two of these have a core protein size of 37 kDa, and the other has a core protein size of 25 kDa.⁶

Although the role of keratan sulfate in fibril organization has long been known, little work has been conducted on cell surface-associated keratan sulfate. Only fairly recently have workers realized the importance of cell surface PGs and their involvement in the control of cell adhesion, migration, and proliferation.⁷ Cell surface PGs can be involved in both the promotion and the inhibition of cell adhesion to the extracel-
The apical surface of the endothelial cells is readily wound repair, and because of this the effect of cell faces. By contrast, the presence of large interstitial lular matrix. For example, cell surface heparan sulfate PGs on the cell surface may block the interaction of cell surface ligands to extracellular matrix components by steric exclusion. Cell surface-associated PGs also may interact with anti-adhesive molecules such as thrombospondin to destabilize cell contact points.

Proteoglycans have been implicated in cell migration, which is associated with the constant breakage and reformation of cell adhesion sites. The importance of chondroitin sulfate PG is well known. Removal of chondroitin sulfate PG from the surface of mouse melanoma cells prevents their migration into type I collagen gels. In other tissues, chondroitin sulfate PG prevents cell migration—for example, in neural crest migration in developing chick embryos.

Proteoglycans indirectly can influence cell migration, proliferation, or both, by interacting with and modulating the activity of the various growth factors that control these events. Proteoglycans may sequester growth factors in a form protected from proteolysis and thereby regulate their availability. This interaction can be through the glycosaminoglycan chain or through the protein core.

Currently, little is known about the role keratan sulfate plays on cell surfaces. It has been shown that keratan sulfate distribution changes during the development of different cell groups in the epithelium of chick nasal passages. Keratan sulfate is known to occur on the surface of a human embryonic carcinoma cell line, and it has been suggested that this strongly implies that it is involved in growth factor binding or modulation. Recently, the core protein of the keratan sulfate PG, fibromodulin, has been shown to bind to transforming growth factor β (TGFβ).

The adult corneal endothelium is an excellent system in which to study the role of cell surface molecules. The endothelial cells form a monolayer on Descemet’s membrane, and, because the tissue is avascular, it is relatively easy to maintain in organ culture. The apical surface of the endothelial cells is readily accessible for antibody labeling and enzyme digestion methods. For wound healing studies, the corneal endothelium can be reproducibly damaged using a freeze injury technique. Migration of endothelial cells to the wound area is the primary mechanism for wound repair, and because of this the effect of cell migration on cell surface molecules can be investigated.

We report the results of an investigation into cell surface-associated keratan sulfate on endothelial cells in normal and wounded cornea and present evidence indicating that levels of cell surface keratan sulfate are linked to wound healing.

MATERIALS AND METHODS

Specimens

Fresh bovine corneas, obtained from the local abattoir within 2 hours of death, were immediately dissected out and immersed in fixative. Normal and wound healing studies on rabbit were performed at the Anheuser-Busch Eye Institute (St. Louis, MO). The normal human corneas for the wound healing study were obtained from the Manchester Eye Bank (Manchester, UK).

Antibodies

Keratan Sulfate. The antibody (5-D-4) to the keratan sulfate glycosaminoglycan chain was obtained from ICN Biochemicals Ltd (Thame, UK). The monoclonal anti-keratan sulfate antibodies BCD-7 and KPC-190 were kindly donated by Dr. Tibor Giant of St. Luke’s Medical Center (Chicago, IL). The monoclonal anti-keratan sulfate antibody MZ-15 was a gift from Dr. Tim Hardingham of The Kennedy Institute of Rheumatology (London, UK). A secondary goat anti-mouse IgG 5-nm gold-conjugated antibody (British Biocell International, Cardiff, UK) was used to visualize the primary antibodies.

Chondroitin Sulfate. The antibody (CS-56) to the chondroitin sulfate glycosaminoglycan chain was obtained from Sigma Chemical (Poole, UK). A secondary goat anti-mouse immunoglobulin M (IgM) 5-nm gold-conjugated antibody (British Biocell International) was used to visualize the primary antibody.

Immunolabeling

Fixation for all specimens was carried out in 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline (PBS) for a minimum of 30 minutes.

Labeling Procedure. The labeling was carried out on intact corneas. After fixation, the samples were washed thoroughly in PBS buffer placed in 0.1 M glycine for 15 minutes and then washed again in PBS. For samples undergoing overnight enzyme digestion, this procedure was performed at this point, with equivalent samples incubated in buffer alone for the same period. The labeling procedure started with incubating the corneal samples at room temperature with neat, normal goat serum for 20 minutes. The goat serum was then removed, and the corneal endothelium was incubated for 2 hours at room temperature with the primary antibody at 1:100 dilution (5-D-4), 1:50 dilution (MZ-15, BCD-7, and KPC-190), and 1:40 dilution (CS-56) in PBS buffer at pH 7.4 containing 1% bovine serum albumin (BSA) and 1% Tween 20. This was followed by a 30-minute wash under agitation in two changes of the buffer. The samples were then incubated in the secondary antibody for 2 hours at a dilution of 1:100 in PBS at pH 8.2 containing 0.5 %.
BSA, 0.5% normal goat serum, 1% sodium chloride, 1% fish gelatin, and 1% Tween 20. This was followed by at least three 30-minute washes in buffer alone, which was followed immediately by five 5-minute washes in distilled water.

**Light Microscopy.** The 5-nm colloidal gold particles were enhanced for light microscopy by the use of a silver enhancement kit (British Biocell International). The enhancement procedure was performed for 10 to 15 minutes at room temperature. Corneas were then washed in distilled water and photographed. Under the light microscope, labeled regions of the specimen appeared dark and unlabeled regions appeared light.

**Scanning Electron Microscopy.** Immunolabeled samples were silver enhanced for 5 to 10 minutes at room temperature by the use of a silver enhancement kit (British Biocell International), after which the samples were washed in distilled water and dehydrated through an ethanol series. Then the samples were air dried immediately or were substituted with liquid Pel-dri II (Agar Scientific, Stansted, UK), which was allowed to solidify before it was sublimated off the sample under vacuum. The use of Peldri II in this way has been shown to produce equivalent results to critical point drying. The samples were mounted on stubs and were sputter coated with gold before being examined under a JEOL (Tokyo, Japan) 840 scanning electron microscope. Under the scanning electron microscope, labeled regions of the specimen appeared bright and unlabeled regions of the specimen appeared dark.

**Transmission Electron Microscopy.** Post-embedding labeling. After fixation, specimens were dehydrated through an ethanol series and embedded in Unicryl resin (British Biocell International). Polymerization was conducted at 52°C. Ultrathin sections were cut and collected on gold-coated grids. The labeling was carried out by floating the grids, section side down, on droplets in a moist chamber. The sections were first incubated at room temperature on droplets of normal goat serum for 20 minutes, after which the serum was flicked off and the grids were transferred to a droplet containing the primary antibody at 1:50 dilution in PBS buffer, pH 7.4, containing 1% BSA and 1% Tween 20, for 1 to 2 hours. This was followed by floating the grids sequentially for 2 minutes, each time on 5 droplets of buffer solution. The grids were then floated on droplets containing the secondary antibody at a dilution of 1:50 in PBS, pH 8.2, containing 1% BSA, 1% normal goat serum, and 1% Tween 20, for 2 hours. The grids were floated for 2 minutes, each time in 5 droplets of the buffer alone, followed by three 2-minute washes in droplets of distilled water. After the final wash, the sections were examined under a JEOL 100cx transmission electron microscope; some grids were counterstained with aqueous uranyl acetate and phosphotungstic acid.

**Pre-embedding labeling.** This labeling procedure was as described for light microscopy until the specimens were washed in buffer after labeling with the secondary antibody. After this stage, the specimens were fixed again in 2% glutaraldehyde, which was followed by a wash in buffer and post-fixation in 1% osmium tetroxide. This was followed by a conventional embedding procedure in Agar 100 resin (Agar Scientific). Ultrathin sections were cut, collected on naked copper grids, and counterstained with uranyl acetate and Reynolds lead citrate before examination under the transmission electron microscope.

**Controls**

Three different controls were used for the immunolabeling procedures:

1. The primary antibody was omitted during the labeling procedure.
2. The primary antibody was replaced by control mouse ascites fluid at an equivalent dilution.
3. Pre-labeling enzyme digestion was carried out as detailed immediately below.

**Keratanase Digestion.** The endothelial surface of the corneas was digested at room temperature for 18 hours by keratanase (Sigma) at a concentration of 1 U/ml in 0.1 M sodium acetate buffer, pH 7.4, containing 5 mM benzamidine–HCl and 0.1 M 6-amino-caproic acid. Labeling on these keratanase control samples was compared with samples that had been incubated for an equivalent time in the buffer alone.

**Chondroitinase ABC Lyase Digestion.** The endothelial surface of the corneas was digested at room temperature for 18 hours by chondroitinase ABC lyase (Sigma) at a concentration of 1 U/ml in 0.5% BSA-enriched Tris buffer, pH 8, containing 5 mM benzamidine–HCl and 0.1 M 6-amino-caproic acid. Labeling on these chondroitinase ABC lyase control samples was compared with samples that had been incubated for the same period in the buffer alone.

**Cell Size Measurements**

Measurement of cell sizes was carried out on light microscopic prints at ×400 magnification using a caliper with 0.1-mm graduations. The maximum axis of each cell was measured. Levels of keratan sulfate labeling were graded from grade 1 (intense staining) to grade 5 (little or no staining). Statistical analysis was carried out using one-way analysis of variance on the SPSS (Chicago, IL) for Windows package.
**Wound Healing Experiments**

**Cultured Bovine Corneas.** Fresh bovine eyes were obtained from the abattoir, and muscle and conjunctival remnants were dissected away. For corneal culturing, a method modified from the procedure used by the Manchester Eye Bank was used. The eyes were immersed in 0.5% polyvinylpyrrolidone for 2 minutes, followed by a 1-minute immersion in 0.1% sodium thiosulfate. After this, the eyes were washed in sterile saline (0.9% sodium chloride) containing penicillin (200 U/ml), streptomycin (0.2 mg/ml), and neomycin (0.4 mg/ml). The cornea was then removed under sterile conditions in a laminar flow hood and placed in a culture dish.

Wounding was carried out by pressing a liquid nitrogen-cooled 4-mm diameter brass rod against the endothelial surface for several seconds.

After wounding, the corneas were placed in a culture dish containing approximately 70 ml of organ culture medium and incubated at 34°C. The medium consisted of Eagles minimum essential medium with Earle’s salts and Hapes buffer, with 4% heat-inactivated fetal calf serum, 2 mM sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.2 U/ml neomycin. The media were changed every 2 to 3 days.

**Cultured Human Corneas.** Wound healing experiments were conducted on six normal human corneas that had been in organ culture for 1 to 2 weeks. The human corneas had no history of corneal edema and came from individuals ranging from 67 to 89 years of age (average, 81 years). The organ culture system method was the standard procedure used by the Manchester Eye Bank; media did not contain chondroitin sulfate. Corneas were damaged by pressing a liquid nitrogen-cooled 3-mm diameter rod against the endothelial cell surface. Corneas then were returned to fresh organ culture media for 4 days before they were removed, fixed, and labeled.

**Rabbit Corneas.** The wounding of the rabbit corneas was carried out at the Anheuser–Busch Eye Institute. Two mature New Zealand white rabbits were anesthetized with an intramuscular injection of ketamine (40 mg/kg) and xylazine (7 mg/kg). A small incision was made in the limbus of one eye of each animal, and a 1-mm Suarez spreader (Storz Instruments, St. Louis, MO) was inserted into the anterior chamber. The corneal endothelium and some of Descemet’s membrane were debrided with a single pass of the Suarez spreader running along the center of the cornea. The wound was closed using two 10-0 nylon sutures. Postoperative treatment consisted of a subconjunctival injection of gentamicin sulfate (12 mg). Clinical examinations at postoperative days 1 and 3 revealed endothelial wounding the eyes were free from infection. At postoperative day 7, the animals were sedated with acepromazine maleate (2 mg/kg) and killed with Sleepaway (Fort Dodge Labs, Fort Dodge, IA). Corneal–scleral discs from the wounded and unwounded eyes were dissected out carefully and placed in fixative before they were immunolabeled for keratan sulfate. At all times, the animals were treated in accordance with the guidelines established by the St. Louis University Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Inhibition–Enzyme-Linked Immunosorbent Assay for Keratan Sulfate Detection**

Inhibition enzyme-linked immunosorbent assays (ELISA) were carried out on bovine aqueous humor samples to determine whether keratan sulfate was present. Before that, however, 0.01 U/ml of keratanase II (Seikagaku, Tokyo, Japan) was added to one sample of aqueous humor while an equivalent volume of 10 mM sodium acetate buffer was added to an identical sample. Both samples were incubated for 24 hours at a temperature of 35°C. Keratanase II is known to digest the antigenic region on keratan sulfate, specific for the 5-D-4 antibody. A 96-well ELISA plate was coated with the antigen bovine articular cartilage (AID1 fraction) in a carbonate buffer containing 0.1 M sodium bicarbonate and 0.025 M sodium carbonate, pH 9.2, and was left to incubate at room temperature overnight.

The inhibition mixture, consisting of triplicates of a serial dilution of each of the two aqueous humor samples, plus the primary antibody to keratan sulfate (5-D-4), was incubated at room temperature for 1 hour and then overnight at 4°C. 5-D-4 was used at a concentration of 1:6000 in a diluent PBS buffer containing 1% BSA, 0.05% Tween 20, and 0.05 M ethylenediaminetetraacetic acid, pH 5.3. A standard also was prepared consisting of serial dilutions of a keratan sulfate fraction extracted from bovine articular cartilage.

The next day the plate was washed automatically with a PBS buffer containing 0.05% Tween 20, pH 6, and coated with the diluent buffer for 1 hour at room temperature to block nonspecific binding. The inhibition mixtures were allowed to reach room temperature and were added to the washed plate after the blocking step. The plate was incubated at room temperature for 10 minutes before washing. The secondary antibody, goat anti-mouse peroxidase IgG (Sigma), in the diluent buffer, was incubated in the plate for 1 hour at room temperature. The plate was again washed and the enzyme substrate, tetramethylbenzidine dihydrochloride (Sigma), with hydrogen peroxide in a citrate buffer containing 0.05 M citric acid and 0.1 M disodium hydrogen orthophosphate (Sor-
FIGURE 1. Light micrograph of bovine cornea immunolabeled for keratan sulfate. Using the light microscope, cells appeared darker with more intense labeling. Magnification, ×160. (a) The distribution of keratan sulfate labeling on the apical surface of normal bovine endothelial cells. (b) Control bovine cornea incubated overnight with keratanase before labeling. The surface of the endothelial cells did not label.

**RESULTS**

Light microscopic results clearly show labeling for keratan sulfate on the surface of bovine endothelial cells (Fig. 1a) using the antibody 5-D-4. Samples preincubated overnight with keratanase show no labeling (Fig. 1b), whereas equivalent samples incubated overnight in the buffer alone show normal labeling. The most striking finding is that the level of labeling varies from cell to cell. In extreme cases, adjacent cells vary between heavy labeling to almost no labeling. The pattern does not appear to be random. Rather, cells with approximately the same level of staining tend to occur.
in groups or chains. This pattern of staining was also observed in normal human and rabbit corneas.

This variegated pattern of staining was observed on normal bovine cornea using the anti-keratan sulfate antibodies BCD-7, KPC-190, and MZ-15. Control, keratanase-digested samples showed no labeling with these antibodies.

Scanning electron micrographs (Fig. 2a) show the distribution of keratan sulfate to be even over the surface of individual normal bovine endothelial cells, with some evidence of increased labeling at the cell borders. Corneas incubated overnight in keratanase before labeling had only a few silver-enhanced gold particles on their surfaces, as shown in Figure 2b.

Transmission electron microscopic results from the post-embedding labeling show some labeling of the apical endothelial membrane, together with labeling inside the endothelial cells. The quality of ultrastructural preservation is poor using this technique because of suboptimal fixation, and little detail can be seen inside the cells. The region of Descemet's membrane adjacent to the base of the endothelial cells labels for keratan sulfate, as demonstrated in Figure 3.

The results from the pre-embedding technique showed positive labeling of the apical endothelial cell surface. Control samples were incubated overnight in keratanase before they were labeled and processed for electron microscopy. There were virtually no gold particles in these samples (Fig. 4). Small invaginations
often were observed on the apical surface of the endothelial cells, sometimes associated with vesicles inside the cell. Fibrillar material was seen on the surface of some endothelial cells, including those that had undergone keratanase digestion. In contrast to the control samples, the corneal samples incubated overnight in buffer, without keratanase before labeling for keratan sulfate, had numerous immunogold particles along the apical membrane of the endothelium (Figs. 5 to 8).

The immunogold particles appear some approximately 10 to 50 nm above the cell surface. Figure 5 shows dense labeling along the apical border region between two endothelial cells. In heavily counterstained sections, there was evidence that the labeling was associated with filaments projecting from the cell surface (Fig. 6). Frequently, labeling was associated with vesicles either fusing with the apical cell membrane or just below the apical surface of the cells (Figs. 7, 8).

The variegated pattern of cell labeling was observed in normal and in organ-cultured human corneas. In human organ-cultured specimens, many of the cells with high levels of keratan sulfate appeared
to overlap their adjacent cells more often than cells that did not label heavily. Similarly, organ cultured bovine corneas appeared to label more heavily at their cell borders.

**Bovine Cornea Wound Healing**

In the wound healing experiments on organ-cultured bovine cornea, the corneas were labeled for chondroitin sulfate as well as for keratan sulfate.

**Keratan Sulfate.** During wound healing, endothelial cells migrate inward, usually taking 7 to 10 days to cover the 4-mm diameter wounded area. Figure 9a shows this area immediately after wounding. Normal cells surrounding the wounded area labeled heavily for keratan sulfate. Figure 9b shows that after 1.5 days, some cells had migrated to the wound area and had much lower levels of keratan sulfate than those cells outside the wound area. Sometimes the edge of the wound area was marked by a narrow band of cells that labeled heavily for keratan sulfate (Fig. 9b). The endothelial cells away from the wounded area had normal levels of keratan sulfate. The central wounded area, consisting of freeze-damaged necrotic cells, also labeled heavily. Figure 9c shows that by day 4, the cells migrated approximately halfway into the original wound area and that the majority of endothelial cells that migrated into the wounded area did not label for keratan sulfate. This is in contrast to the normal level of labeling of those endothelial cells outside the original wound area.

**Chondroitin Sulfate.** Chondroitin sulfate labeling has a pattern almost opposite that for keratan sulfate labeling. Figure 10a shows that just after wounding, there was no labeling of the normal endothelial cells surrounding the wound area. The wound area shows some background labeling of the freeze-damaged necrotic cells. Figure 10b shows that 1.5 days after wounding, the endothelial cells have started migrating inward to the wound area. Most of these cells label strongly for chondroitin sulfate, whereas those outside the original wound area do not. After 4 days (Fig. 10c), the cells have migrated approximately halfway to the original wound area; the majority of these cells label heavily for chon-
Wound Healing Controls. Enzyme digest experiments were conducted on 4-day-old wounded bovine cornea. Corneas predigested with keratanase showed no labeling for keratan sulfate (Fig. 11b), and corneas predigested with chondroitinase ABC lyase did not label for chondroitin sulfate (Fig. 12b). The only labeling that was still present was the labeling of the freeze-damaged necrotic cells in the center of the wound area. There was also a small amount of background labeling just outside the central wound area. Examination of this region under the scanning electron microscope revealed that nearly all of this background resulted from labeling of ruptured necrotic cells that had been in the original wound area and that had been migrated under by inwardly moving endothelial cells. Colloidal gold particles are well known to produce background labeling on damaged or necrotic cells. Equivalent corneas incubated overnight in the buffer alone showed the normal pattern of labeling of a 4-day-old wounded cornea for keratan sulfate (Fig. 11a) and chondroitin sulfate (Fig. 12a). However, in general, wounded corneas incubated overnight in buffer before processing did have slightly more background labeling than equivalent corneas processed immediately after removal from organ culture. It seems probable that overnight incubation may result in damage or rupturing of some cells, thus causing a small increase in background labeling by colloidal gold particles. Results from other enzyme digestion experiments showed that predigestion with keratanase did not change the pattern of labeling for chondroitin sulfate.

Scanning Electron Microscopy. Scanning electron microscopy showed clearly that the majority of the inwardly migrating cells did not label for keratan sulfate (Fig. 13a) and did label intensely for chondroitin sulfate (Fig. 13b). In contrast, Descemet's membrane labeled more heavily for keratan sulfate (Fig. 13a) than for chondroitin sulfate (Fig. 13b). Migrating cells were enlarged, flattened, and irregular. Posteriorly they often had filipodia, whereas anteriorly they had a lamellipodium (Fig. 14). The pattern of chondroitin sulfate labeling on the surface of these migrating cells was not always even. Some cells had areas of increased chondroitin sulfate labeling on
FIGURE 11. Light micrographs showing 4-day-old wounded bovine corneas immunolabeled for keratan sulfate. Using the light microscope, cells appeared darker with more intense labeling. Magnification, X15 (a) This specimen shows the normal pattern of labeling for keratan sulfate on a 4-day-old wound. There were normal levels of labeling on the endothelial cells outside the original wound area (B) and much reduced labeling of those endothelial cells that migrated to the wound area (A). A thin band of heavily labeled endothelial cells is visible at the edge of the original wound area (arrow). (b) This control specimen was incubated overnight in keratanase before labeling (the specimen in a was incubated in buffer alone). The only labeling was background labeling of the freeze-damaged necrotic cells in the central wound area (W) not covered by inwardly migrating endothelial cells.

that migrated to the wounded area had low levels of keratan sulfate on their surfaces. However, at the edge of the wound area was a band of cells that labeled heavily (Fig. 16). The change in labeling for keratan sulfate between cells at the edge of the wound and those that migrated to it was seen more clearly under the scanning electron microscope (Fig. 17) because the atomic number contrast produced by the silver-enhanced gold particles was much higher than the contrast produced with the light microscope. With the scanning electron microscope, the change in keratan sulfate levels from the edge of the wound to the wounded area was very clear.

Analysis of Cell Size With Intensity of Labeling

Results of this investigation show that there is no direct correlation between cell size and intensity of labeling for keratan sulfate, although one-way analysis of variance did reveal that the cell populations with different labeling levels did have significantly different mean cell sizes.

Inhibition-Enzyme-Linked Immunosorbent Assay

Figure 18 shows that bovine aqueous humor inhibits the binding of 5-D-4 to ELISA plate wells coated with

FIGURE 12. Light micrograph showing 4-day-old wounded bovine corneas immunolabeled for chondroitin sulfate. Using the light microscope, cells appeared darker with more intense labeling. Magnification, X15. (a) This specimen shows the normal pattern of labeling for chondroitin sulfate on a 4-day-old wound. The endothelial cells outside the wound area (B) do not label for chondroitin sulfate. In contrast, the majority of cells that had migrated to the wound area (A) had high levels of labeling (A). (b) This control specimen was incubated overnight in chondroitinase ABC lyase (the specimen in a was incubated in buffer alone). This specimen had no labeling except for background labeling of the freeze-damaged necrotic cells in the central wound area (W) not covered by inwardly migrating endothelial cells.

their surfaces, usually toward the posterior of the cell (Fig. 14). The leading edge of the lamellipodium also labeled heavily for chondroitin sulfate. As the cells migrate inward to the wounded areas, they move underneath any wound-damaged cells or debris in their path.

Human Wound Healing

Results from the wound healing experiments of organ-cultured human cornea showed less consistent changes than those observed by bovine cornea. Of six human corneas, two showed a clear reduction in keratan sulfate levels on the migrating cells, whereas the remaining corneas showed no clear change in labeling. In the corneas that showed distinct changes, the pattern of labeling was similar to that observed in bovine corneas; the majority of the cells that migrated to the wound area showed reduced labeling for keratan sulfate (Fig. 15). As with bovine cornea, the edge of the wound area was associated with a narrow band of cells labeling more heavily than normal.

Rabbit Wound Healing

Results from the in vivo study on rabbit cornea wounding showed that the enlarged endothelial cells
FIGURE 13. Scanning electron micrographs of bovine cornea 3 days after wounding show the leading edge of the inwardly migrating endothelial cells. These micrographs show the direction of migration to be from bottom to top (arrowheads). Using the scanning electron microscope, the silver-intensified immunogold label appeared bright. Magnification, ×300. (a) Wounded bovine cornea immunolabeled for keratan sulfate. There was almost no labeling for keratan sulfate on these cell surfaces, and so they appear dark using the scanning electron microscope. A typical cell is indicated by the large arrow. The exposed Descemet's membrane (D) in the wound area did label for keratan sulfate and so appears bright. (b) Wounded bovine cornea immunolabeled for chondroitin sulfate. The micrograph shows the leading edge of the migrating endothelial cells that had high levels of labeling for chondroitin sulfate and so appear bright. A typical cell is indicated by the large arrow. Descemet's membrane (D) had less labeling than endothelial cell surface.

FIGURE 14. Scanning electron micrograph of an endothelial cell at the edge of a 3-day-old wound. The cell labeled for chondroitin sulfate. The leading edge of the cell was round, forming a lamellipodium, and the rear of the cell exhibited filopodia (arrowhead). This micrograph shows the direction of migration of the cell to be from left to right (large arrow). Labeling on the surface was uneven; the front of the cell (f) had less labeling than the rear (r). Magnification, ×1500.

FIGURE 15. Light micrograph of organ-cultured human cornea 4 days after wounding. At this stage, the inwardly migrating endothelial cells migrated halfway to the wound (W) area. The majority of the endothelial cells that migrated into the wound area (A) labeled less heavily for keratan sulfate than those outside the wound area (B). A narrow band of cells is visible at the edge of the original wound area, which labeled more heavily than normal (arrow). Magnification, ×19.

keratan sulfate. This inhibition is destroyed by keratanase II digestion, thus confirming that keratan sulfate in the aqueous humor produces this inhibition.

DISCUSSION

Results from this study clearly indicate that the keratan sulfate glycosaminoglycan is present on the surface of corneal endothelial cells. Labeling on the surface of the cells often is associated with loose fibrillar material. It seems likely that this material may form part of the glycoprotein layer recently reported to be present on the apical surface of corneal endothelial cells. The fact that the labeling is often some distance above the cell surface, up to 50 nm (Fig. 5), suggests the possibility that the keratan sulfate glycosaminoglycan...
FIGURE 16. Light micrograph of the surface of rabbit corneal endothelium, 7 days after wounding, labeled for keratan sulfate. Cells that migrated to the wound area had reduced labeling for keratan sulfate (A). Cells outside the wound area had normal levels of labeling for keratan sulfate (B). A band of endothelial cells at the edge of the wound area labeled heavily for keratan sulfate (joined arrowheads) and so appeared darker under the light microscope. The central area of the wound (W), consisting of exposed Descemet's membrane, also labeled heavily. This micrograph shows the direction of migration of the cells to be from right to left. Magnification, ×200.

The ELISA results show that keratan sulfate is present in the aqueous humor, to which the apical surface of the corneal endothelium is exposed. With transmission electron microscopy, vesicles frequently were observed just below the apical surface of the cell or fusing with the apical membrane. Many of these vesicles labeled for keratan sulfate (Figs. 7, 8). These pictures strongly suggest that keratan sulfate is released or taken up by the endothelium. Previous work on corneal endothelial cells in culture has indicated that they synthesize and release both keratan sulfate and chondroitin sulfate. Furthermore, the fact that the apical surface of normal corneal endothelial cells still labels for keratan sulfate after several days in corneal organ culture certainly suggests that these cells actively produce keratan sulfate.

The cause of the differential distribution of keratan sulfate on the surface of normal endothelial cells is not directly apparent. Differential distribution of keratan sulfate has been reported in chondrocytes, but, in this case, it is correlated with differences in depth in cartilage. However, in corneal endothelial cells, there can be radically different levels of keratan sulfate present in adjacent cells (Figs. 1a, 2a). A similar differential distribution of neural cell adhesion molecule and the intracellular adhesion molecule have been observed on normal human endothelial cells. Furthermore, a differential distribution of the chondroitin sulfate PG, CD-44, was reported on cultured human endothelial cells. Recent work on endothelial cells in rabbit carotid arteries has shown that these cells have a differential distribution of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 and that expression of these adhesion molecules changes in response to shear stress. It is possible that the differential distribution of keratan sulfate on the corneal endothelium is the result of the small changes in the adhesion, movement, or both, of individual cells needed to maintain the regularity of cell size and shape in the endothelial monolayer.

Results from the wound healing experiments on cultured bovine corneas show that the inwardly migrating endothelial cells have reduced keratan sulfate and increased chondroitin sulfate on their apical surfaces. Although it is beyond the scope of this article to determine the exact function of these changes, there are some obvious possibilities. The simplest explanation is that the migration of endothelial cells to the wound area is facilitated by these changes in cell surface glycosaminoglycans. As mentioned in the introduction, there are many ways in which PGs are linked to the migration and adhesion of cells. They can promote cell adhesion, as occurs with heparan sulfate, or they may block cell adhesion by steric inhibition.

FIGURE 17. Scanning electron micrograph of rabbit endothelium, 7 days after wounding, labeled for keratan sulfate. Cells that migrated to the wound area had much reduced labeling for keratan sulfate (A). Cells outside the wound area had normal levels of labeling for keratan sulfate (B). A band of endothelial cells at the edge of the wound area labeled heavily (joined arrowheads) and so appeared very bright under the scanning electron microscope. The change in the level of labeling between cells at the edge of the wound and cells in the wound area was very clear under the scanning electron microscope. Magnification, ×420.
FIGURE 18. Inhibition curves obtained in enzyme-linked immunosorbent inhibition assays using 5-D-4. The assays were performed in triplicate on bovine aqueous humor that had been preincubated at 35°C with 0.01 U/ml of keratanase II in sodium acetate buffer (●) and bovine aqueous humor that was preincubated in sodium acetate buffer alone (■). A standard consisting of serial dilutions of a keratan sulfate-rich fraction purified from bovine articular cartilage also was performed on the same plate.

Although this study is the first to investigate cell surface glycosaminoglycans, previous work examined changes in lectin binding during endothelial wound healing in rat. These investigators found changes in the binding pattern of wheat germ agglutinin, concanavalin A, Ricinus communis, and soybean agglutinin. It was proposed that the changes in lectin binding were related to the migration of the endothelial cells because this is the primary method of wound repair. However, it also was suggested that some of the changes might reflect the cell cycle phase of the endothelial cells because injury can stimulate some endothelial cells, which normally reside in the G0 phase, to undergo cell cycling and mitosis. This raises the possibility that the changes in glycosaminoglycan levels, observed during endothelial wound healing in this study, could be linked to endothelial cell cycle changes.

Although the changes in glycosaminoglycan levels observed in this investigation have been on the apical surface of the cells, this may well reflect similar changes on the basal surface of the cells. For example, some integrins involved in forming adhesion points on the basal surface of migrating fibroblasts then detach and move to the apical surface of the fibroblasts as they break these adhesion points in moving forward. Another possibility is that the chondroitin sulfate present on the apical surface of the migrating corneal endothelial cells helps these cells to migrate underneath and detach the freeze-damaged necrotic cells in the wound area.

Because this project has used antibody labeling techniques, the changes observed are changes in specific epitopes of keratan sulfate and chondroitin sulfate and not necessarily changes of overall levels of keratan sulfate and chondroitin sulfate glycosaminoglycans. For example, it is known that the keratan sulfate antibody used in this study (5-D-4) binds to the sulfated regions of the glycosaminoglycan chains and does not bind to undersulfated keratan sulfate. Thus, it is possible that the changes in labeling that occur during wound healing reflect changes in the sulfation levels of keratan sulfate rather than changes in overall levels of keratan sulfate.

Little is known about the role of cell surface-associated keratan sulfate. It has been shown that an undersulfated form of keratan sulfate is involved in macrophage adhesion. Other workers have shown that oligosaccharides derived from keratan sulfate bind to the leukocyte adhesion molecule, L-selectin. It has been proposed that an interaction between L-selectin on the surface of lymphocytes and glycosaminoglycans on the surface of endothelial cells could be involved in the formation of inflammatory lesions on the cornea. The significance of these studies is not related directly to the findings in this article, but they do...
serve to indicate that keratan sulfate is involved in cell adhesion.

Results from the organ-cultured human cornea show that changes in levels of keratan sulfate also occurred during cell migration in human cornea. Changes in keratan sulfate labeling were similar to those observed in bovine cornea. Although the wounding experiments with bovine cornea consistently showed a clear reduction in labeling for keratan sulfate, such a clear change in keratan sulfate levels was not always observed in the human samples. There are a number of possible reasons to explain why the results from the human wound healing were not as consistent as the results from bovine cornea. Human corneas came from elderly persons, and it was only possible to carry out the wounding after the corneas had been in culture for at least 1 week. The bovine corneas, on the other hand, were wounded before they were placed in culture.

The rabbit study has shown that changes in keratan sulfate levels during wound healing also occur in vivo. The general pattern is similar to that observed in cultured bovine corneas: The innermost migrating cells had abnormally low levels of keratan sulfate and were surrounded by a ring of migrating cells at the edge of the wound area, which showed higher than normal levels of keratan sulfate.

In conclusion, this investigation has revealed that keratan sulfate is unevenly distributed over the corneal endothelium and is present in the aqueous humor. In wound healing experiments, cell surface keratan sulfate levels appear to be reduced, whereas (in bovine cornea) chondroitin sulfate levels increase. A similar pattern of keratan sulfate changes was observed in some human corneas and in vivo in rabbit corneas. It seems possible that (in bovine cornea) cell surface keratan sulfate and chondroitin sulfate have different roles in endothelial adhesion and migration, perhaps through interactions with cell adhesion molecules or growth factors.

Key Words
adhesion, cornea, endothelium, migration, proteoglycans

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References


