Proteoglycan Molecules in Keratoconus Corneas

Shoichi Sawaguchi, Beatrice Y. J. T. Yue, Isabel Chang, Joel Sugar, and Jeffrey Robin

Proteoglycan molecules in keratoconus corneas were studied by immunohistochemical and electron microscopic histochemical methods. Compared with normal human control subjects, the staining intensity with monoclonal antibody 9-A-2 was enhanced in the stroma of scarred keratoconus corneas, whereas the intensity with antibody J-19 was reduced. The 9-A-2 experiment showed an increased immunoreactivity of dermatan sulfate proteoglycan epitopes, and the J-19 experiment indicated a decreased immunoreactivity of sulfated keratan sulfate epitopes. Uronic acid analyses were consistent with the 9-A-2 data. Electron microscopy performed after cuprolinic blue staining showed apparent accumulation of abnormally thick, chondroitinase ABC-sensitive, dermatan sulfate proteoglycan filaments in keratoconus corneas. Such filaments were especially prominent in scarred areas. In addition, keratan sulfate proteoglycan filaments appeared to be less abundant than those found in normal control corneas. Similar alterations of both types of proteoglycan molecules were also seen and reported in scarred corneas. The similarity suggests that the proteoglycan abnormalities found in keratoconus corneas may be secondary, at least in part, to scarring. Invest Ophthalmol Vis Sci 32:1846-1853, 1991

Keratoconus is a potentially blinding disease that thins and scars the central portion of the cornea. The cause of this disease is unknown. Yue et al, Yue and others reported a decrease of total protein and an increase of nonproteinaceous component in corneas affected by keratoconus. Subsequent histochemical studies using Safranin O, a dye specific for polyanions such as glycosaminoglycans, have shown an enhanced staining, indicating an accumulation of proteoglycan molecules in keratoconus corneas. In normal corneas, keratan sulfate proteoglycan is the major proteoglycan of the cornea, and dermatan sulfate proteoglycan is the remainder. Funderburgh et al found decreased antigenicity of keratan sulfate in keratoconus corneas. By a solid-phase immunoassay, they also showed that keratan sulfate in keratoconus corneas has a modified structure. The keratan sulfate proteoglycan contains fewer or shorter sulfated keratan sulfate chains, but the amount of keratan sulfate core protein remains unaltered.

This study assessed further the possible proteoglycan alterations in keratoconus corneas. Immunohistochemical techniques with the use of monoclonal antibodies against chondroitin/dermatan sulfate and against keratan sulfate proteoglycans were used. In addition, sulfated proteoglycan molecules were visualized under an electron microscope after cuprolinic blue-dye binding.

Materials and Methods

Immunohistochemical Analysis

Nineteen corneas were obtained after penetrating keratoplasty from 19 patients who had keratoconus. Fifteen normal human corneas were obtained from the Illinois Eye Bank (Chicago). These corneas were received within 24 hr of death from individuals who had no known ocular diseases, and all of the corneas were clear and had no remarkable features. The number of corneas used for each experiment and the age and clinical records of patients, including duration of their disease, are shown in Table 1. Corneal manifestations, such as iron ring and mild to moderate scarring, were reported for every case of keratoconus. The diagnosis was confirmed by pathologic examination. Disruption of Bowman's condensation and Fleischer's ring were seen in all keratoconus specimens.

For immunohistochemical studies, keratoconus buttons and normal human corneas were fixed in 10% buffered formaldehyde solution, processed, and embedded in paraffin. As another set of control specimens, paraffin sections of corneas were obtained from donors with histopathologically diagnosed cases of corneal scar (donor ages, 21 and 63 yr old, trauma...
Table 1. Clinical characteristics of donors of keratoconus and normal human corneas*

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Keratoconus</th>
<th>Normal human controls</th>
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<tbody>
<tr>
<td></td>
<td>Number of patients</td>
<td>Age at surgery, yr</td>
</tr>
<tr>
<td>Immunostaining for 9-A-2</td>
<td>8†</td>
<td>35 ± 14 (range: 14-57)</td>
</tr>
<tr>
<td>Immunostaining for J-19</td>
<td>8†</td>
<td>35 ± 14 (range: 14-57)</td>
</tr>
<tr>
<td>Uronic acid analysis</td>
<td>9</td>
<td>39 ± 17 (range: 16-67)</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>2</td>
<td>17, 49</td>
</tr>
</tbody>
</table>

* All patients with keratoconus had typical manifestations of iron ring and mild to moderate scarring.
† Corneas from the same eight patients were used for 9-A-2 and J-19 immunostaining experiments.

Cases of keratoconus (age 16 and 64 yr old) and Fuchs' corneal dystrophy (donor ages, 66 and 74 yr old) were studied in parallel.

Immunostaining was performed with monoclonal antibody 9-A-2 (ICN, Lisle, IL), which recognizes 4-sulfated disaccharide of chondroitin and dermatan sulfates. This antibody was used in the form of ascitic fluid and was raised against chondroitinase ABC-digested rat chondrosarcoma proteoglycans. It reacts with the resulting unsaturated disaccharide near the linkage region between the oligosaccharide and the core protein.12,13 The specificity of the antibody recognition relies on the previous digestion of antigens on tissue sections with either chondroitinase AC or ABC.13 Staining was also done using monoclonal antibody J-19 in the form of ascites fluid. This antibody is specific for sulfated epitopes in keratan sulfate proteoglycan.14,15

All specimens from keratoconus and from normal human corneas were processed in the same manner and stained simultaneously with either monoclonal antibody 9-A-2 or J-19. Before the staining, 6-μm-thick sections were deparaffinized and rehydrated. In preparation for the staining with 9-A-2, sections were treated for 2 hr at 37°C with either 0.2 units/ml of chondroitinase ABC (Sigma Chemical Co., St. Louis, MO) in enriched Tris buffer (0.25M Tris hydrochloride, 0.18 M NaCl, 0.05% bovine serum albumin, 5 mM benzamidine hydrochloride and 0.1 M 6-aminocaproic acid, pH 8.0)13 or with buffer alone (negative control). All sections were then incubated in 5% blocking normal goat serum for 20 min, rinsed three times with phosphate buffered saline (PBS) for 5 min each, and allowed to react for 90 min with 9-A-2 (1:10). After they were rinsed with PBS, the sections were incubated further for 30 min with 1:500 biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) and washed again with PBS. For blocking of the endogenous peroxidase, corneal sections were soaked in 0.3% hydrogen peroxide-methanol at room temperature for 30 min. They were subsequently incubated with avidin-biotin-horseradish-peroxidase complex (Vector) for 30 min, and the color reaction was developed with 0.01% hydrogen peroxide, 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma) in 50 mM Tris buffer (pH 7.6) as previously described.16 The sections were dehydrated, mounted with Permount (Fisher Scientific, Itasca, IL), and photographed.

The staining with J-19 was performed similarly, except that the enzyme digestion step was omitted. The antibody was used at a 1:100 dilution,16 and the secondary antibody used was biotinylated goat anti-mouse IgG. For negative control specimens, normal mouse serum, mouse ascites, or J-19 preincubated13 for 30 min with 1 mg/ml of bovine corneal keratan sulfate (Seikagaku Kogyo Co., Tokyo, Japan) was used in place of the primary antibodies. These specimens were uniformly stained negative. Some sections were also treated with 1 unit/ml of keratanase (Sigma) at 37°C for 1 hr17 before staining. Immunostaining experiments were repeated twice.

A video camera (Dage series 68, Dage-MTI, Michigan City, IN) was used to visualize the immunostaining, and the image was analyzed by an image processing system (Zeiss SEM-IPS, Carl Zeiss, Thornwood, NY), which was established in Dr. Paul Knepper's laboratory18 (Children's Memorial Hospital, Chicago). Measurements were made as the percent transmission with a 40×/1.0 oil-immersed objective using a scanning spectrophotometer (Zeiss) with the wavelength set at 500 ± 10 nm. A zero transmission value was measured as a camera blank with a black background. A 100% transmission value was measured as the average value of an empty field. For the corneal specimens, at least five measurements were made on stromal cells and stromal lamellae of different fields. The absorbance (A) values were calculated from the percent transmission (T) values by A = −log (1/T) and were calibrated against the nonspecific background values obtained with nonimmune serum. Data ob-
tained from the central portion of keratoconus specimens and normal human corneas were analyzed by grouped student’s t-tests.

Uronic Acid Analysis

Before analyses, dry weights of keratoconus and normal human corneas were obtained by drying at 60°C in vacuo for 8 hr. The tissues were cut into small pieces, and the proteoglycan contained therein was extracted with 5 ml of 4 M guanidine hydrochloride and 0.05 M sodium acetate (pH 5.8), to which protease inhibitors 0.1 M 6-aminohexanoic acid, 10 mM EDTA, and 5 mM benzamidine hydrochloride were added. The extraction was carried out at 4°C for 18 hr and repeated twice. After centrifugation, the extracts were dialyzed, lyophilized, and resolubilized. Aliquots of the samples were then taken for uronic acid analysis by the carbazole assay as previously described. Chondroitin 4-sulfate (ICN) from whale cartilage (molecular weight, 25,000–50,000) was used as the standard.

Electron Microscopic Histochemistry

Two keratoconus corneas and two normal human corneas were fixed overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Sorensen’s buffer (pH 7.4). The corneas were further dissected into 2–3 mm blocks and again fixed in paraformaldehyde-glutaraldehyde for 1–4 days.

Portions of the corneas from each group were subjected to enzyme treatments. They were kept in PBS overnight, then divided into three groups: chondroitinase ABC, keratanase, and control specimens. The three groups of specimens were processed in this manner: (1) chondroitinase ABC: after being stored in cold enriched Tris buffer for 24 hr, the tissues were incubated in enriched Tris buffer with 0.1 units/ml of chondroitinase ABC for 24 hr at 37°C; (2) keratanase (endo-beta-galactosidase, ICN): pieces of corneal sections were first stored in cold, 50 mM sodium acetate buffer (pH 5.8), 5 mM benzamidine hydrochloride, and 0.1M 6-aminocaproic acid for 24 hr, then digested in sodium acetate buffer with 0.0001 units/ml of keratanase for 24 hr at 37°C; (3) control specimens: the tissues were treated as described above, but without enzymes.

After the enzyme treatments, all tissues were rinsed with cold PBS for 2 hr with several exchanges of PBS. They were then stained with cuprolinic blue (Gallard-Schlesinger Industries Inc, Carle Place, NY) at a critical electrolyte concentration, as described by Scott and Tawara et al. To summarize briefly, the fixed corneal tissues were equilibrated for 1 hr in several changes of 25 mM sodium acetate, 0.1 M MgCl₂ and 2.5% (W:V) glutaraldehyde (pH 5.7), placed in 0.05% cuprolinic blue in the sodium acetate fixative, and stained overnight at room temperature. Tissues were rinsed three times in the sodium acetate fixative without the dye, washed three times in 0.5% sodium tungstate in water, and washed three times in 0.5% sodium tungstate in 50% (V:V) ethanol. Tissues were then dehydrated through graded concentrations of ethanol and embedded in epoxy resin. Ultrathin sections were examined under electron microscopy using uranyl acetate staining.

Results

Figure 1 shows the immunohistochemical staining of normal human and keratoconus corneas using monoclonal antibody 9-A-2. The antibody is specific for the 4-sulfated disaccharide of chondroitin sulfate and dermatan sulfate proteoglycans. Since it reacts only with the resulting unsaturated disaccharide near the linkage region between the oligosaccharide and the core protein, the 9-A-2 staining in the stroma of all five normal human corneas (Fig. 1A) was, as would be expected, weakly positive. Compared with the control tissues (Fig. 1A), the staining in the stroma of eight scarred keratoconus corneas was markedly enhanced (Fig. 1B). Such an elevation in 9-A-2 staining was also seen in scarred corneas (photographs not shown) of two patients who had undergone trauma.

The epithelium of most of the corneal specimens reacted with 9-A-2 either weakly or negatively. One normal human cornea and two keratoconus corneas showed a positive reaction in the epithelium. As a group, however, no abnormality in terms of epithelial staining was evident in keratoconus corneas.

The immunohistochemical staining was further analyzed and the intensities of reaction products were measured with the use of an image processing system. Statistical analyses (Table 2) showed that in keratoconus, the stromal lamellae had a significantly higher level of 9-A-2 staining than that found in the central portion of normal control corneas. The increase was seen in six of the eight specimens examined. The average 9-A-2 intensity in the corneal stromal cells in keratoconus, however, was similar to that in control tissues. The two scarred corneas of trauma cases yielded similar results to those of the keratoconus corneas.

Figure 2 shows the immunostaining of corneas with monoclonal antibody J-19. Normal human corneal stroma (Fig. 2A) stained positively with the sulfated keratan sulfate antibody. The staining was eliminated by preincubation of the antibody with keratan sulfate. In addition, the staining intensity was markedly reduced when corneal sections were pretreated with keratanase.
Fig. 1. Immunostaining of corneas with monoclonal antibody 9-A-2. Corneas were obtained from (A) a 25-yr-old normal subject and (B) a 39-yr-old patient with keratoconus. Positive reaction products appeared as brown deposits. Staining in the stroma of the eight keratoconus corneas examined was stronger than that in normal human corneas. Two keratoconus corneas also showed positive staining in the epithelial layer. As a group, however, the epithelial staining in keratoconus corneas was similar to that in normal controls (magnification ×950).

Fig. 2. Immunostaining of corneas with monoclonal antibody J-19. Corneas were obtained from (A) a 44-yr-old normal subject and (B) a 27-yr-old patient with keratoconus. Positive reaction products appeared as brown deposits. Compared with normal human control subjects, staining in the stromal lamellae was reduced in the eight keratoconus corneas examined. The average staining intensity in the keratoconus stromal cells was similar to that in control cells. Although some focal cells appeared to react with J-19, the epithelium of most of the corneal specimens stained faintly or negatively (magnification ×780).
Table 2. Staining intensity in corneas from patients with keratoconus*

<table>
<thead>
<tr>
<th></th>
<th>Normal human corneas (n = 5)</th>
<th>Keratoconus (n = 8)</th>
<th>Scarred corneas (n = 2)</th>
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</thead>
<tbody>
<tr>
<td>9-A-2 staining</td>
<td></td>
<td></td>
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<tr>
<td>Stromal lamellae</td>
<td>0.026 ± 0.008</td>
<td>0.090 ± 0.015† (6)</td>
<td>0.146 ± 0.044‡ (2)</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>0.112 ± 0.030</td>
<td>0.202 ± 0.034§ (4)</td>
<td>0.315 ± 0.016§ (2)</td>
</tr>
<tr>
<td>J-19 staining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal lamellae</td>
<td>0.240 ± 0.030</td>
<td>0.107 ± 0.014‡ (8)</td>
<td>0.131 ± 0.009† (2)</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>0.521 ± 0.055</td>
<td>0.437 ± 0.037§ (2)</td>
<td>0.377 ± 0.022§ (1)</td>
</tr>
</tbody>
</table>

* Data (mean ± SEM) are optical densities obtained by an image processing system (see Materials and Methods). Values in parentheses indicate the number of specimens that showed a value either significantly higher (for 9-A-2 staining) or significantly lower (for J-19 staining) than that of normal human controls.
† P < 0.012 compared with normal human control subjects.
‡ P < 0.0008 compared with normal human control subjects.
§ Not significantly different from normal human control subjects.

The J-19 staining in the epithelium, unlike that in the stroma, was positive in two of the five normal human corneas examined. One of these two specimens was found to have immunoreactivity due to nonspecific reactions. Thus, it was concluded that the staining in the epithelium of one specimen may not genuinely indicate the presence of sulfated keratan sulfate epitopes in this corneal layer.

The epithelium of seven of the eight keratoconus corneas examined did not stain with J-19, whereas the stroma (Fig. 2B) of all of these corneas reacted with J-19 positively. The intensity in the stroma was, however, much weaker than that seen in normal human control subjects. A similar pattern was likewise found in two scarred corneas (photographs not shown). By contrast, surgical specimens obtained from patients with Fuchs' corneal dystrophy had normal stromal staining. Image analyses (Table 2) confirmed these conclusions.

The uronic acid content (Table 3) in keratoconus corneas was found to be significantly higher than that in normal control corneas. Keratan sulfate does not contain uronic acid. These results therefore indicated that keratoconus corneas had an increased dermatan sulfate proteoglycan content.

By electron microscopy, a thin (CB-1) and a medium (CB-2) type of cuprolinic blue-positive filaments were visualized in normal human corneas (Fig. 3A). They ran transverse to the direction of collagen fibers. Filaments CB-2 were completely eliminated by chondroitinase ABC treatment (Fig. 3B) and were identified as dermatan sulfate proteoglycan. The thin CB-1 filaments were removed by keratanase (Fig. 3C), representing keratan sulfate proteoglycan. In the two keratoconus corneas, an apparent accumulation of abnormally thick filaments (CB-3) was seen surrounding the keratocytes and in stromal lamellae (Fig. 3D). These filaments were especially prominent in scarred areas. Since these assemblies were chondroitinase ABC-sensitive (Fig. 3E), but not keratanase-sensitive (Fig. 3F), they might be aggregates of an unusual, perhaps highly sulfated form of dermatan sulfate proteoglycan. In keratoconus specimens, the CB-1 filaments appeared to be less abundant than those seen in normal corneas (Figs. 3B and 3E).

Discussion

This study shows that proteoglycan molecules in the stroma of scarred keratoconus corneas are altered. Results of staining with monoclonal antibody 9-A-2 showed that the immunoreactive dermatan sulfate proteoglycan epitopes seen in keratoconus corneas were increased, whereas the experiments with monoclonal antibody J-19 showed a decrease in the immunoreactive sulfated keratan sulfate epitopes. The guanidine hydrochloride extract from keratoconus corneas contained a higher level of uronic acid, which was consistent with the 9-A-2 data. These studies suggest that the enhanced Safranin O staining found previously in keratoconus corneas probably reflects the greater representation of dermatan sulfate proteoglycan molecules.

Our J-19 immunostaining data agree with findings by Funderburgh et al\textsuperscript{10,11} of decreased or altered keratan sulfate molecules in corneas of patients with keratoconus. Like the antibody I22 used by Funderburgh et al, J-19 binds to the sulfated epitope of keratan sulfate proteoglycan. The reduced antigenicity may
Fig. 3. Electron micrographs. (A) A cornea, obtained from a 26-yr-old normal subject, after staining for proteoglycans with cuprolinic blue. Thin-type (arrowheads, CB-1) and medium-type (short arrows, CB-2) cuprolinic blue-positive filaments are seen. (B) The same specimen, treated with chondroitinase ABC before the cuprolinic blue staining. CB-2 filaments disappeared, and CB-1 filaments (arrowheads) remained. (C) The same specimen, pretreated with keratanase. CB-2 filaments (short arrows) are seen. (D) A cornea, obtained from a 19-yr-old patient with keratoconus, after staining for proteoglycans with cuprolinic blue. In addition to the thin and the medium types of cuprolinic blue-positive filaments, an apparent accumulation of a thick-type filament (large arrows, CB-3) is seen. (E) The same keratoconus specimen as in Figure 3D, pretreated with chondroitinase ABC. CB-2 and CB-3 filaments disappeared, and CB-1 filaments (arrowheads) remained. (F) The same keratoconus specimen as in Figure 3D and Figure 3E, pretreated with keratanase. CB-3 filaments were not digested. Bar = 0.1 μm.
be a result of a decreased degree of sulfation, a shortened keratan sulfate chain length, or a reduced number of keratan sulfate chains per proteoglycan molecule. However, Funderburgh et al.\textsuperscript{10} concluded that a decreased sulfation level was the most likely possibility because the content of glucosamine in keratan sulfate molecules was found to be normal in keratoconus corneas in an earlier study.\textsuperscript{23}

A cationic dye, cuprolinic blue, was used to study the proteoglycan molecules ultrastructurally. When applied to tissues at a critical electrolyte concentration, this dye binds selectively to the sulfated glycosaminoglycan residues of proteoglycans.\textsuperscript{20-22} An apparent accumulation of abnormally thick, chondroitinase-sensitive dermatan sulfate proteoglycan filaments (CB-3) was shown in the stroma of keratoconus corneas. Their presence was particularly evident in scarred areas. By contrast, the thin CB-1 keratan sulfate proteoglycan filaments appeared to be in reduced amounts in keratoconus corneas. This finding indicated an increased dermatan sulfate proteoglycan content and a reduced sulfated keratan sulfate content in keratoconus corneas. The thick dermatan sulfate filaments resemble those identified in corneas of patients with macular corneal dys trophy.\textsuperscript{24}

A marked decrease in keratan sulfate proteoglycan and accumulation of a high-sulfate, high-iduronic acid dermatan sulfate were reported in scarred corneas.\textsuperscript{23,25-29} The scarring can be a result of traumatic injury,\textsuperscript{3,25-27} penetrating wounds,\textsuperscript{28} nonperforating wounds,\textsuperscript{29} or diseases such as keratitis and lattice corneal dystrophy.\textsuperscript{23} Increased 9-A-2 staining was seen in the two scarred corneas from our trauma cases, but J-19 staining diminished. The strikingly similar change in the pattern of proteoglycan molecules led us to conclude that the alteration seen in our keratoconus specimens may be attributed, at least in part, to scarring. Previously, Anseth\textsuperscript{23} showed no discernible glycosaminoglycan abnormality in keratoconus corneas that had no scarring, Andreasen et al.\textsuperscript{30} also found normal uronic acid content in six keratoconus corneas that had no scars.

Dermatan sulfate binds water more tightly than keratan sulfate proteoglycan.\textsuperscript{31} The increased dermatan sulfate proteoglycan concentration in local areas has been suggested to be the basis for the long-term excess hydration of corneal scar tissue.\textsuperscript{32} In line with these observations, keratoconus corneas were more hydrated (having a reduced percentage of dry weight) than normal control subjects.\textsuperscript{2} It remains, however, to be determined whether this and other proteoglycan-related alterations affect the pathologic development in keratoconus, including thinning of the cornea.

**Key words:** cornea, cuprolinic blue staining, immunostaining, keratoconus, proteoglycan

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