Altered Antigenicity of Keratan Sulfate Proteoglycan in Selected Corneal Diseases

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Monoclonal antibody against keratan sulfate (KS) was used for immunofluorescent staining of sections of human corneas from 8 normal eyes, 19 with keratoconus, 4 with pellucid marginal degeneration, 5 with primary macular corneal dystrophy, and 1 with recurrent macular corneal dystrophy. The anti-KS monoclonal antibody did not stain the corneas with primary macular corneal dystrophy, but stained all other corneas to varying degrees. Staining intensity was weaker than normal in most keratoconus and pellucid marginal degeneration corneas, and was very weak in a case of macular corneal dystrophy that had recurred in a transplanted normal cornea. In several corneas with keratoconus, normal staining was seen at the periphery, and staining intensity decreased in the thinned central portion of the stroma. The decreased KS staining was not localized in stromal scar tissue found in the keratoconus and pellucid marginal degeneration corneas. Quantitation of relative staining intensity found keratoconus and pellucid marginal degeneration corneas to be 49% and 40% as intensely stained, respectively, as normal corneas, a statistically significant decrease (P < 0.01). Distribution of staining intensities of the keratoconus corneas demonstrated a single modality. These results are in agreement with findings of previous biochemical studies, which show reduction of highly sulfated keratan sulfate epitopes in corneas from keratoconus and pellucid marginal degeneration, and absence of sulfated keratan sulfate epitopes in macular corneal dystrophy. Invest Ophthalmol Vis Sci 31:419–428, 1990

Keratoconus and pellucid marginal degeneration are disorders of the cornea involving progressive thinning of the stroma.1,2 In keratoconus, stromal thinning is central and accompanied by corneal ectasia, whereas in the less frequent condition of pellucid marginal degeneration, thinning occurs in the inferior peripheral stroma.1,2 In both cases, disruption of Bowman’s layer and stromal scarring occur. Stromal thinning may involve modification of stromal extracellular matrix metabolism, and even though no biochemical characteristic of the stroma has been linked to the etiology of these diseases, abnormalities in keratoconus have been identified in both protein and carbohydrate components of the stromal extracellular matrix. Work on this subject has been reviewed.3 In some keratoconus corneas, protein and collagen in the stroma are reduced.4-6 Keratoconus corneas also contain increased levels of hexosamine and uronic acid,5,7 and stain abnormally with a cationic dye, Safranin O, suggesting an increase of glycosaminoglycans in the stroma.4

Glycosaminoglycans are the carbohydrate moieties of proteoglycans, components of the stroma which are second only to collagen in abundance in the cornea. Proteoglycans appear to be important in the maintenance of the ultrastructural integrity of the stroma, and alterations of corneal proteoglycans have been identified in several corneal abnormalities. In macular corneal dystrophy, a defect in synthesis of keratan sulfate (KS) results in production of an unsulfated KS proteoglycan (KSPG)-related glycoprotein in place of fully sulfated KSPG.8 Corneas with lattice dystrophy exhibit an unusual, highly sulfated dermatan sulfate,9 and stromal scar tissue formed after penetrating wounds contains alterations in both KSPG and dermatan sulfate proteoglycans.10-12 Despite the apparent importance of proteoglycans, neither the role of the altered proteoglycans in the pathology of these conditions nor the metabolic changes involved in producing altered proteoglycans has been elucidated. Development of antibodies to proteoglycans has provided a sensitive and accurate means of identify-
ing these major components of connective tissues in extracts and in histologic sections. Monoclonal antibodies against KS have been useful in identifying KS during normal corneal embryonic development\(^1\)\(^3\) and in healing corneal wounds.\(^1\)\(^9\) A recent study found that pooled extracts of keratoconus stromas showed a reduced ability to bind anti-KS monoclonal antibodies.\(^1\)\(^4\) In the current study, individual sections of normal corneas and corneas from patients with keratoconus, pellucid marginal degeneration, and macular corneal dystrophy were examined with an anti-KS monoclonal antibody using immunofluorescence. A significant reduction in the binding of anti-KS monoclonal antibody to the diseased corneas was found. A preliminary report of this work has been presented.\(^1\)\(^5\)

### Materials and Methods

#### Tissue

Corneal buttons from patients with keratoconus, pellucid marginal degeneration, and macular corneal dystrophy were removed during keratoplasty and were fixed immediately in 10% formalin (3.7% w/v formaldehyde), 0.04 M sodium phosphate, pH 7.2, at room temperature for 48–72 hr, before dehydration and embedding in paraffin. Normal corneas of eyes obtained from the University of Iowa eye bank were processed in a manner similar to that for the diseased tissue.

#### Staining

Staining was carried out in batches, with sections (8-\(\mu\)) of two to five experimental corneas and one control cornea, each on separate albuminized slides. The control was one of a series of sections from the central region of a single normal cornea, and was included with each staining. After deparaffinization, the sections were immediately blocked, and then were incubated with monoclonal antibody 122 at 20 \(\mu\)g/ml for 2 hr, as described previously.\(^1\)\(^3\) After incubation with primary antibody, sections were incubated with phycoerythrin-labeled goat anti-mouse IgG (Accurate Chemical and Scientific, Westbury, NY) at 100 \(\mu\)g/ml, in 1% (w/v) bovine serum albumin, 0.02 M Tris-HCl, pH 7.2, 0.15 M NaCl, 0.01% (w/v) sodium azide for 60 min at room temperature in the dark. These staining conditions maximized antibody-specific to nonspecific staining. Slides were rinsed and counterstained with bisbenzimide to stain nuclei,\(^1\)\(^2\) then and were mounted in 20% (w/v) polyvinyl alcohol, 10% (v/v) glycerol in 0.05 M Tris-HCl, pH 8, containing 100 \(\mu\)g/ml p-phenylenediamine and 10 \(\mu\)g/ml ascorbic acid. The slides were stored 4°C for 14–18 hr in the dark, and then photographed under a standardized protocol with a Zeiss (Oberkochen, West Germany) epifluorescence microscope. Phycoerythrin fluorescence was detected with an excitation filter of 530–560 nm and an emission filter of 565–580 nm. Bisbenzimide fluorescence was detected with excitation < 360 nm and emission at >485 nm. Negatives were produced on T-max film (Eastman Kodak, Rochester, NY) and developed at ASA 400 with Kodak D-76 developer, 22–24°C, for lengths of time recommended by the manufacturer. Negatives were printed on Agfa BEH 310 PE contrast paper, using exposure times matched to produce equal brightness for the corneal control section in each group.

#### Quantitation of Staining Intensity

The optical density of negatives taken through a 25X objective was determined at 450 nm using 96-well microplate spectrophotometer (model EL 307; Bio-Tek Instruments, Winookki, VT), illuminating a portion of the negative with a diameter equivalent to 40 \(\mu\)m of the corneal section. The average optical density for a negative was found to be a linear function of the exposure time for optical densities in the range 0–0.8. Intensity of stromal fluorescence was estimated by averaging three optical density measurements randomly chosen from the central stromal region of each negative. Variation in the optical density within a single stroma typically gave standard deviations of less than 10% of the mean. The relative staining intensity of each experimental cornea was normalized by comparison with the appropriate control cornea. Interbatch variation, assessed by optical densities of the negatives from sections of control cornea stained over a period of several months, gave a standard deviation of 14% of the mean.

### Results

Monoclonal antibody 122 has been found to be a highly specific reagent for immunohistochemical staining of corneal KS in formalin-fixed, paraffin-embedded sections of chicken tissues.\(^1\)\(^3\) Figure 1 demonstrates the effectiveness of this antibody in staining KS in paraffin sections of formalin-fixed human corneas. The corneal stroma, including Bowman's layer and Descemet's membrane, was brightly stained. Occasional light staining of the epithelial cells, as well as moderate variation in staining intensity within the stroma (Figure 1F) was observed. Staining was eliminated by substitution of nonspecific mouse IgG (Figure 1E) or by preincubation of the antibody with an excess of KS (Figure 1G). Preincubation with heparin, a similar sulfated glycosaminoglycan (Figure 1H), had no effect on staining.
Fig. 1. Specificity of KS staining in human corneas with monoclonal antibody 122. Four serial sections of a normal human cornea were stained with anti-KS monoclonal antibody 122 (E–H) and counterstained with bisbenzimide to visualize cell nuclei (A–D), as described in Materials and Methods. (A, E) Nonspecific mouse IgG was substituted for antibody 122. (B, F) Stained with antibody 122. (C, G) Antibody 122 was preincubated with 1 mg/ml bovine corneal KS, 25°C, 30 min before staining. (D, H) Antibody 122 was preincubated with 1 mg/ml heparin as in (C) and (G). Bar, 100 μm.

Staining was eliminated also by incubation of the tissue sections with endo-β-galactosidase, an enzyme that digests KS (data not shown).

Antibody 122 was used to stain sections of 37 human corneas, both normal and those from patients with keratoconus, pellucid marginal degeneration, and macular corneal dystrophy, and revealed a variety of staining patterns. Normal corneas (Figures 2A, E, I–L) exhibited bright uniform stromal staining, occasionally more intense in separations between stromal lamellae. Keratoconus corneas (Figure 2B–D, F–H) typically were much less intense in their staining, which often was limited to anomalously bright interlamellar staining, giving these corneas a striped or spotted look (Figure 2F, G). The intensity of staining in these corneas was more heterogeneous than that of normal corneas, ranging from normal intensity to virtually no staining (2C, D). In several keratoconus sections, a gradient of staining intensity was present: intensity was nearly normal at the periphery and decreased in the thinned central region of the stroma. Figure 3 presents montage photographs illustrating sections of two corneas in which this gradient of staining was readily apparent.

In Figure 4, staining of pellucid marginal degeneration corneas is shown. Like keratoconus, these corneas exhibited a noticeable decrease in staining intensity compared to normal corneas. Unlike keratoconus, however, regions of stromal thinning were not observed to stain in a manner different from the remainder of the stroma; rather, the staining was consistently weak throughout the stroma (not shown).
Fig. 2. Comparison of KS staining in normal and keratoconus corneas. Four batches of slides containing three experimental sections and one control cornea section were stained with monoclonal antibody 122, as described in Materials and Methods. Experimental corneas are the second, third, and fourth figures in each horizontal row. On the left (A, E, I) are sections of the same normal cornea, included in each batch as an internal control. The top two rows show staining of keratoconus corneas (B-D, F-H) and the bottom row (J-L) shows three normal corneas. Bar, 100 μm.
Figure 3. Association of reduced KS staining with the thinned region of keratoconus corneas. Montage photographs of two keratoconus corneas (A, B) show staining with anti-KS antibody 122 (upper sections) and nuclear staining (lower sections) of the same sections. Bar, 500 μm.

Figure 5 illustrates the staining of corneas from macular corneal dystrophy. Primary macular corneal dystrophy (Figure 5C, F) had no detectable staining in sections of the five corneas tested. A corneal graft removed after recurrence of the stromal deposits characteristic of macular corneal dystrophy (Figure 5B, E) showed weak positive staining.

Keratoconus corneas develop ruptures in Bowman’s layer, leading to stromal disruption and scarring. As shown in Figure 6, for keratoconus, the stroma under the breaks in Bowman’s layer was characterized by reduced cellularity (Fig. 6B, E), disruption of the lamellar regularity (Fig. 6A, D), and an uneven staining with anti-KS antibody (Fig. 6C, F). It was not unusual to find material staining with the anti-KS antibody present in the epithelium at these locations (Fig. 6C, F). Staining in the scarred regions of the stroma was not significantly reduced compared to surrounding nonscarred stroma.

The absence of staining in macular corneal dystrophy corneas by anti-KS antibody was unambiguous. On the other hand, the major observation regarding staining of keratoconus and pellucid marginal degeneration was that the stroma in these corneas generally had a reduced intensity of staining. This conclusion was documented quantitatively by measurement of the optical density of photographic negatives taken of the corneas under carefully standardized conditions. These data, summarized in Figure 7, support the initial assessment that corneal sec-
Fig. 4. Staining of KS in corneas from pellucid marginal degeneration. Two corneas with pellucid marginal degeneration (B, E; and C, F) were compared to the normal control (A, D) for KS staining. (A–C) Bisbenzimide counterstain for nuclei. (D–F) Anti-KS antibody 122 staining for KS, as described in Materials and Methods. Bar, 100 μm.

The data presented here show that corneas from keratoconus and pellucid marginal degeneration had reduced binding of anti-KS monoclonal antibody compared to a group of normal corneas, as assayed by immunohistochemical staining. Corneas from macular corneal dystrophy showed no binding of this antibody. The reduced staining in the keratoconus corneas appeared to be associated with the central thinned region in sections where both normal thickness and thinned regions were present. Irregular staining was found in scar tissue near disruptions in Bowman's layer, and KS-antigenic material was sometimes found in the epithelium near these points.

Biochemical studies have demonstrated that corneas from macular corneal dystrophy patients do not produce normally sulfated KSPG. These patients appear to lack sulfated KS in their serum as well. In a previous study, immunohistology of a large number of corneas from macular corneal dystrophy patients showed that corneas from 58% of cases reacted with at least one of five different anti-KS monoclonal anti-
In the current study, 11 total primary cases of macular corneal dystrophy were examined (5 as reported in Results and 6 more corneal sections provided by Drs. J. Baum and N. Panjwani, Tufts University) with I22, a well-characterized monoclonal antibody against sulfated KS, different from the antibodies used in the earlier study. None of the cases in the current study showed reactivity with I22, suggesting that the epitope recognized by this antibody may be more consistently absent in macular corneal dystrophy than are those of antibodies used in the earlier study. The extremely weak staining of the cornea in which macular corneal dystrophy had recurred in a normal graft suggests that the sulfated KS present in the grafted tissue was degraded, and was not replaced by host keratocytes which presumably repopulated the stroma. Our results support the conclusion of the original biosynthetic studies that sulfated KS (defined here by the sulfated epitope recognized by antibody I22) is not present in corneas with macular corneal dystrophy. In addition, the results with a recurrent case support the idea that this disease results from an error in synthesis, and not from degradation of sulfated KS.
Keratoconus and pellucid marginal degeneration corneas present a pattern distinctly different from that of macular corneal dystrophy, in that staining with monoclonal antibody I22 was not absent but instead was present in reduced amounts compared with normals. This conclusion arises from simple observation of the photomicrographs in Figures 2, 3, and 4, as well as from the quantitative (diseased vs normal) comparisons of the photographic negatives produced under standardized conditions (Figs. 7, 8). The quantitative technique measures fluorescence indirectly and was used to determine the relative degree to which the fluorescence of the diseased corneas differed from that of normals. In a previous study, KS antigen in pooled extracts of keratoconus corneas was found to be reduced by 52% compared to KS in normal corneas by using a quantitative immunoassay. The 51% decrease in staining intensity of keratoconus corneas found in the current study is in close agreement with the quantitative immunoassay, suggesting that changes in staining reflect actual changes in abundance of KS epitope in the stroma.

Monoclonal antibody I22 has been shown to bind sulfated epitopes in the KS chain and to be almost inactive with unsulfated KS. The reduction in I22 binding may result from KSPG that has reduced sulfation or that contains a pattern of sulfation not recognized by the antibody. Reduced antigenicity may result also from KSPG molecules with fewer sulfated KS chains per protein core, or with KS chain lengths shorter than normal. Anseth found no change in the abundance of keratan sulfate sugar residues in kera-
Normal Keratoconus Pellucid

Staining Intensity (%)

0 50 100 150

Normal Keratoconus Pellucid

Staining Intensity (%)

Fig. 7. Intensity of KS staining of normal and diseased corneas. Relative staining intensity with anti-KS monoclonal antibody 122 was calculated as described in Materials and Methods for normal (squares), keratoconus (circles), and pellucid marginal degeneration (triangles) corneas. Data are presented as a scatter diagram showing each value (open symbols) and as the mean and standard deviation of the group (solid symbols). The student t-test showed a significant decrease from normal of both keratoconus ($P < 0.001$) and pellucid marginal degeneration ($P < 0.01$).

toconus corneas; therefore, it would appear that a change in KS sulfation is the most likely cause for our current findings.

Corneal scar tissue resulting from penetrating wounds contains a reduction in sulfated KS antigen similar to that of keratoconus. This similarity raises the possibility that stromal scar tissue present in many keratoconus corneas is responsible for the altered KS content in this disease. In scarred regions of the cornea, as seen in Figure 6, KS immunofluorescence was irregular, but binding of antibody 122 was not reduced compared to the surrounding nonscarred stromal tissue, suggesting that the loss of sulfated KS antigen in keratoconus was not limited to the scarred regions of these corneas. Pellucid marginal degeneration has similarities to keratoconus, but stromal scarring is infrequent and stromal thinning is restricted to a portion of the inferior peripheral stroma in pellucid marginal degeneration. All four pellucid marginal degeneration corneas showed significantly decreased KS staining, but the intensity of staining had no apparent relationship to the region of stromal thinning. The loss of KS antigen in keratoconus and pellucid degeneration therefore does not appear to be a phenomenon associated with localized stromal scarring, but instead appears to occur throughout cornea. The only pattern of staining localization which could be identified was in 3 of the 19 keratoconus corneas, in which the loss of KS was more pronounced in the thinned region (Figure 3).

Accumulation of an antigenically altered KSPG in keratoconus and pellucid marginal degeneration would not seem to be a likely primary event in the pathologic process of these diseases. Rather, this alteration may reflect a response of the keratocytes to an abnormal extracellular environment. Secretion of abundant, highly sulfated KS appears to be a tightly regulated function carried out by keratocytes only under conditions present in the normal stroma. In healing wounds, viral keratitis, and lattice dystrophy, and in corneal grafts undergoing rejection, the stroma contains KS with reduced sulfation and dermatan sulfate with increased sulfation. A similar pattern of altered glycosaminoglycan synthesis occurs in
cultured stromal fibroblasts and in corneal organ cultures in which the epithelium has been damaged.\textsuperscript{20,21} The alteration of KSPG documented in the current study may be a similar response of keratocytes to an abnormal stromal extracellular environment. The recent demonstration by Matsuda et al\textsuperscript{22} that irregularities in endothelial cell shape precede all other symptoms of keratoconus, suggests a source of the altered stromal environment. The idea that keratoconus (and possibly pellucid marginal degeneration) are metabolic consequences of endothelial abnormalities is compelling but as yet unsupported by specific experimental data. To address the implications of the data presented in the current study, better understanding is needed of the factors regulating proteoglycan biosynthesis, accumulation, and turnover in both normal and diseased corneas.

**Key words:** cornea, keratoconus, keratan sulfate, macular corneal dystrophy, pellucid marginal degeneration

**References**