Immunoaalysis of Keratan Sulfate Proteoglycan From Corneal Scars

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Corneal keratan sulfate proteoglycan (KSPG) from scar tissue of experimental penetrating corneal wounds in rabbits was analyzed 2–8 weeks after injury using three previously characterized antibodies. Keratan sulfate (KS) was identified in 2 week scars and normal corneal tissue by indirect immunofluorescence using a monoclonal antibody against sulfated KS epitopes. KSPG was measured in unfraccionated extracts of scar and of normal corneal tissue using a “sandwich” enzyme-linked immunosorbent assay (ELISA). In extracts of 2 week scars, KSPG molecules reacting with two different anti-KS monoclonal antibodies were 55% and 82% as abundant as in normal tissue extracts. Ion exchange high performance liquid chromatography (HPLC) of tissue extracts found qualitatively similar elution profiles of KSPG antigens from both scar and normal tissues. Direct ELISA of the HPLC-purified KSPG showed identical quantitative binding of antibodies against core protein and KS from normal and scar tissue. KS in the HPLC-purified extracts was sensitive to digestion with endo-β-galactosidase, whereas core protein antigens were not affected by this enzyme, as expected. Alteration of the antigenic characteristics of the KSPG of scars was detected with a competitive immunoassay using immobilized monoclonal antibodies against KS. KS in extracts from 2, 6, and 8 week scars competed only 5–11% as effectively as KS from normal cornea, although core protein antigens in the scar extracts competed 61–80% as well as those of normal cornea. These results suggest that KSPG is present in scar tissue at a concentration moderately reduced compared to that of normal corneal tissue, but that KSPG molecules are structurally altered in scar tissue, perhaps in the pattern or degree of KS sulfation. Invest Ophthalmol Vis Sci 29:1116–1124, 1988

Proteoglycans and collagen constitute the major organic components of the corneal stroma. Corneal proteoglycans are highly charged molecules, responsible for the hydrophilic properties of the cornea.1 Their association with collagen fibrils in a highly organized manner2 appears to play an important role in the ultrastructural architecture of the stromal extracellular matrix. Two major proteoglycan types are present in the stroma of most mammals and birds. Keratan sulfate proteoglycan (KSPG), which constitutes about 60% of stromal proteoglycan, is a 100 kD compound with 1–2 KS chains N-linked to core protein through asparagine.3 This glycoprotein-like structure distinguishes corneal KSPG from KS-containing proteoglycans of other tissues.3,4 Dermatan sulfate proteoglycan, constituting 40% of corneal proteoglycan, is similar to a class of proteoglycans widely distributed in skin and other connective tissues.5,6

The importance of proteoglycans to corneal transparency is evidenced by metabolic diseases in which normal corneal proteoglycan metabolism has been disrupted. In macular corneal dystrophy biosynthesis of sulfated KSPG is blocked and opaque deposits develop in the stroma.7,8 In Hurler's and Scheie's syndromes, impaired degradation of dermatan sulfate results in dense stromal opacity.9 Changes in the structure or abundance of corneal proteoglycan, therefore, can have profound effects on corneal transparency. Corneal scar tissue, which can be opaque, also displays long-term disruption of normal proteoglycan metabolism. Early investigations of glycosaminoglycan in experimental corneal wounds reported a reduction in the amount of KS and its sulfation in corneal scars, as well as an accumulation of a highly sulfated dermatan sulfate, changes which persisted for months in the scar region.11–14 In a more recent study of the proteoglycans of corneal scar tissue synthesis of KSPG was not detected until 4 months after wounding.15 This long-term alteration of proteoglycan metabolism in corneal scar tissue may contribute to changes in the extracellular matrix of the scar which render it opaque.

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Several antibodies have been characterized which react with KS and with the core protein of KSPG.\textsuperscript{18–22} These have been useful in immunofluorescent localization of KS during embryonic development of the cornea,\textsuperscript{12–14} in quantitative analysis of KS in serum\textsuperscript{26} and developing cornea,\textsuperscript{23} and in characterization of altered KSPG in macular corneal dystrophy.\textsuperscript{7,8} Availability of specific antibodies to KSPG provides a sensitive means of assessing the amount and characteristics of this proteoglycan in corneal wounds. In this study we used three previously characterized antibodies reacting with components of corneal KSPG to identify this molecule in scar tissue of experimental wounds in rabbit cornea. KSPG antigens were detected in scars in amounts similar to normal tissues, but changes in the antigenic properties of KSPG suggest an altered KSPG structure in scar tissue.

Materials and Methods

Materials

Monoclonal antibody 122 against rabbit corneal KS,\textsuperscript{18} antibody 5-D-4 against bovine nasal cartilage KS\textsuperscript{19} and a polyclonal rabbit antiserum against purified bovine KSPG\textsuperscript{20} were described previously. The polyclonal serum was further affinity-purified on immobilized bovine corneal KSPG.\textsuperscript{23} Endo-\beta-galactosidase from \textit{E. freundii} was purified as described previously.\textsuperscript{27}

Tissue Extracts

All procedures using animals conformed to the ARVO Resolution on the Use of Animals in Research. Corneas of anesthetized albino rabbits were wounded by removal of a 2 mm full-thickness button with a trephine as previously described.\textsuperscript{11} After a healing period, rabbits were killed by an overdose of sodium pentabarbital, and corneas excised. The corneal tissue was frozen for cryostat sectioning, or scar and normal tissues were excised and stored at −70°C until extraction of proteoglycans. Twelve tissue buttons of scar or normal corneal tissue were pooled and extracted 2 × 24 hr at 4°C in 1 ml (equivalent to a buffer:tissue ratio of 100:1 (w/w)) 4 M guanidine-HCl containing protease inhibitors (0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 1 mM phenylmethysulfonyl fluoride, 10 mM EDTA, 10 mM N-ethylmaleimide) and 0.05 M acetate buffer, pH 6.0. The combined extracts were dialyzed at 4°C against 6 M deionized urea in 20 mM Tris-HCl, pH 6.8, with the same protease inhibitors as above, then filtered and stored at 4°C. Protein content of the extract was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).\textsuperscript{28} Approximately equal amounts of protein per 2 mm button were extracted from scar and normal cornea with this procedure.

ELISA

Immunoassays were carried out at 25°C in NUNC polystyrene 96-well ELISA plates (Cat. #439454, Vanguard International, Neptune, NJ), with two to three rinses after each incubation in PBS (0.15 M NaCl, 10 mM sodium phosphate buffer, 0.01% (v/v) thimerosal, pH 7.4) unless otherwise noted. Samples and antibodies were diluted in PBS containing 1% (w/v) bovine serum albumin and 0.1% Tween 20 (v/v) (blocking buffer). \textit{Competitive ELISA} using a biotin-labeled purified corneal KSPG standard was described previously.\textsuperscript{23} The KSPG standard in assays with polyclonal antibody was purified further by affinity to antibody 122-agarose as described previously.\textsuperscript{18} \textit{Direct ELISA} of HPLC samples was carried out by coating an ELISA plate for 30 min with 50 μl 0.7% glutaraldehyde freshly made in 0.1 M phosphate buffer, pH 5.0. HPLC column fractions or aliquots of pooled fractions diluted to 50 μl in 6 M urea were bound to the plate overnight, and additional binding was blocked by incubation with blocking buffer for 1 hr. Anti-KSPG antibody in 50 μl was added for 2 hr, followed by incubation with peroxidase-labeled goat anti-mouse or anti-rabbit IgG F(ab\textsubscript{2}) (TAGO, Burlingame, CA) for 1 hr. Color was developed with 3,3',5,5'-tetramethylbenzidine\textsuperscript{29} for 10 min. \textit{Double-antibody ELISA}\textsuperscript{30} was carried out by adsorption of monoclonal 122 or 5-D-4 to ELISA plates overnight at 4°C in 0.1 M NaHCO\textsubscript{3} buffer, pH 9. After blocking, primary incubation was carried out for 2 hr with tissue extracts diluted in blocking buffer. Binding of the affinity-purified polyclonal antibody to the antigen monoclonal antibody complex then was assessed by a 2 hr incubation with polyclonal antibody, followed by reaction with peroxidase-labeled goat anti-rabbit IgG F(ab\textsubscript{2}) and color development as described above.

Ion Exchange HPLC

One milliliter aliquots of the guanidine-HCl tissue extract were chromatographed on a 5 ml column of Sephadex G-25 in 6 M urea, 20 mM Tris-SO\textsubscript{4}, pH 6.8. The fractions containing protein were pooled and filtered, and 0.5 ml of each was loaded on a 1 ml Mono Q anion exchange HPLC column (Pharmacia Inc., Piscataway, NJ). Elution was carried out with 6 M urea, 20 mM Tris-SO\textsubscript{4}, pH 6.8, using a gradient of increasing slope from 0–1.5 M Na\textsubscript{2}SO\textsubscript{4} at a flow rate of 1 ml per min at room temperature. Fractions of 0.3 ml were collected.
Fig. 1. Immunofluorescence of corneal scar and normal corneal tissue to detect binding of anti-KS monoclonal antibody 122. (A) normal stroma. (B) 2 week scar tissue. (C) stromal-scar junction. (D) normal stroma with nonimmune mouse IgG replacing antibody 122. (Bar, 60 μm).

Endo-β-galactosidase Treatment

Pooled peak fractions from HPLC columns were digested with *E. freundii* endo-β-galactosidase in 0.2 M sodium acetate, pH 5.8, containing four protease inhibitors.18 Dilutions of digestion mixture were bound onto a glutaraldehyde-treated ELISA plate and assayed with 5-D-4, 122 or polyclonal antibodies in direct ELISA as described above.

Immunofluorescence

Normal and 2 week rabbit scar tissues were frozen in O.C.T. Compound (Miles Inc., Naperville, IL), and 6 μm cryostat sections were mounted on formalin-fixed, gelatin-coated slides, air-dried, and stored frozen. Before staining, sections were washed three times for 5 min each in PBS containing 1.5% bovine serum albumin (BSA-PBS) and dried at 37°C overnight. Monoclonal antibody 122 or nonimmune mouse IgG controls, both at 20 μg/ml in BSA-PBS, were incubated on the sections overnight at room temperature followed by washing, as above. Fluorescein-labeled goat anti-mouse IgG (Cappel Laboratories, Malvern, PA), 20 μg/ml in BSA-PBS, was incubated at room temperature for 30 min, followed by washing as above and mounting in 90% glycerol:10% PBS containing 10 μg/ml p-phenylenediamine.
Results

Immunohistology of normal rabbit cornea using anti-KS monoclonal antibody 122 stained the stromal lamellae, giving a smooth ribbon-like fluorescence (Fig. 1A). In corneas with 2 week wounds, abrupt transition was apparent from the normal lamellar staining to staining characteristic of scar tissue (Fig. 1C), lacking the lamellar organization of the normal tissue. Central portions of the scar (Fig. 1B) also reacted with the anti-KS antibody, showing even greater disruption of matrix regularity. Replacement of the anti-KS monoclonal antibody with nonimmune mouse IgG gave no staining (Fig. 1D).

The reactivity of scar tissue with anti-KS antibody demonstrates the presence of KS in the scar tissue, but immunofluorescence does not necessarily provide accurate information on the amount of KS. For quantitation, proteoglycans were extracted from scar and normal corneal tissues and assayed in a double-antibody immunoassay. This “sandwich” assay (Fig. 2A) requires molecules capable of reacting simultaneously with both anti-KS monoclonal antibody and anti-core protein polyclonal antibody. The assay proved to be highly specific for intact proteoglycan molecules: neither purified KS glycosaminoglycan nor chemically deglycosylated KSPG core protein showed reactivity (data not shown). With unpurified corneal extracts this assay detected less KSPG in extracts of corneal scar tissue than in extracts of normal corneal tissue (Fig. 3). This difference was found with two different anti-KS monoclonal antibodies: 122, prepared against rabbit corneal KS, and 5-D-4, prepared against cartilage KS. These antibodies recognize similar but nonidentical epitopes in KS chains of corneal KSPG and in KS from proteoglycans of other tissues.30 KSPG molecules containing epitopes which bind antibody 122 were 55% as abundant in scar as in normal tissue extract, whereas KSPG molecules binding antibody 5-D-4 were 82% as abundant in scar as in normal tissue extracts.

KSPG from corneal scars was characterized by anion exchange HPLC of the tissue extracts (Fig. 4). KSPG antigens in the column effluents were detected by a direct ELISA procedure (Fig. 2B). More than 90% of the KS antigens recognized by monoclonal antibody 5-D-4 eluted in a broad peak of activity with 0.35–1.5 M Na₂SO₄. This region of the gradient (fractions 60–90) corresponds to the elution position of purified bovine KSPG (horizontal bar in Fig. 4). When assayed with the polyclonal antibody (Fig. 4C,D), the scar and corneal extracts also had similar chromatographic profiles. Each extract contained antigens co-eluting with the KS-antigen and also heterogeneous components eluting at lower ionic strength.

Quantitative distribution of the core protein antigens differed somewhat between scar and normal tissues, with 50% of the core protein antigen of scar extracts co-eluting with KS (fractions 60–90) but with only 36% of the core protein antigens from normal cornea eluting in this region of the gradient.
Protein (ng)

Fig. 3. Double-antibody ELISA of KSPG in unfractionated extracts of normal cornea and corneal scars. Error bars showing standard deviation of quadruplicate analyses were omitted if deviation was less than the diameter of the symbol. Lines show second order regression analysis of the plotted points. Normal cornea, solid circles; 2 week corneal scar, open circles. (A) Monoclonal antibody 122. (B) Monoclonal antibody 5-D-4.

Figure 5 shows a quantitative comparison of antigens in the pooled HPLC fractions (fractions 60–90) containing the proteoglycan antigens. When normalized to the amount of protein chromatographed, the purified proteoglycan from normal and scar tissue bound identical amounts of antibody. The similarity of the binding ability of the pooled fractions was consistent for each of the three anti-KSPG antibodies. Pretreatment of these purified KSPG fractions with endo-β-galactosidase before ELISA virtually eliminated binding of the monoclonal antibodies (Figs. 5A,B), confirming that the antigens recognized in the tissue extracts were KS. Endo-β-galactosidase, as expected, had no effect on binding of the polyclonal antibody (Fig. 5C), which recognizes core protein and not KS antigens.

Quantitative comparisons of KSPG using direct ELISA (Figs. 4, 5) and double-antibody ELISA (Fig. 3) demonstrated relatively small differences between scar and normal corneal tissue, but a third immunoassay procedure, a competitive assay, detected a much larger difference. In this procedure (Fig. 2C), KSPG in unfractionated extracts competed with purified, biotin-labeled corneal KSPG for binding of immobilized antibody. Extracts from normal cornea and corneal scar both competed for the binding of all three antibodies against KSPG (Fig. 6), but scar extracts were much less effective competitors for the anti-KS monoclonal antibodies than were normal corneal extracts. Quantitative evaluation of the data from Figure 6 and from older scar tissue (Table 1) shows that extracts of scar tissue were only 5–11% as effective as normal tissue extracts in a competition for the monoclonal antibodies. This altered antigenic character persisted in scar tissue at least 8 weeks after wounding. In contrast, core protein antigens in corneal scar tissue, as recognized by the polyclonal antibody, were 61–80% as effective competitors as those in normal tissue. As expected for this type of assay, mixing of normal and scar extracts resulted in a competition curve intermediate between those generated by the individual extracts (data not shown).

Discussion

KSPG components were detected in normal corneas and in 2-week-old corneal scar tissue by immunofluorescence histology of the tissues and by solid-phase immunoassays of tissue extracts with three different antibodies. Elution profiles from ion exchange chromatography of scar tissue KSPG antigens were qualitatively similar to those of normal corneal tissue. The HPLC-purified antigens from corneal scar displayed sensitivity to endo-β-galactosidase similar to KSPG antigens from normal corneal tissue. Two types of ELISA which quantitate binding of anti-KSPG antibodies detected a moderate reduction of KSPG antigens in scar tissue extracts compared to extracts of normal cornea. Similarly, a third ELISA measuring competition between KSPG from extracts and biotin-labeled KSPG measured a moderate reduction in scar tissue core protein antigens compared to normal corneal antigens. Use of anti-KS monoclonal antibodies in the competitive ELISA, however, showed KS antigens from scar tissue to be very poor competitors compared to KS antigens from normal corneas.

The antigens detected in corneal scar tissue appeared to be genuine KSPG as judged by several crite-
Fig. 4. Ion exchange HPLC of KSPG antigens from normal cornea and from corneal scars. Unfractionated extract with (A, C) 70 μg protein from normal cornea, and (B, D) 92 μg protein from 2 week scar were chromatographed on Pharmacia Mono Q ion exchange columns. Solid lines: KSPG antigens detected by direct ELISA with two different antibodies. (A, B) Anti-KS monoclonal 5-D-4. (C, D) Affinity-purified polyclonal antibody. The dotted lines show concentration of sulfate eluant. Solid bars in A and C show fractions corresponding to elution of bovine KSPG. These fractions were pooled for further analysis.

ria: (1) Use of antibodies against separate parts of the KSPG molecule (core protein and KS) showed that both antigens were present (Fig. 5), and that these two antigenic components co-chromatographed on ion exchange HPLC (Fig. 4). (2) Physical linkage between the KS and core protein antigens was demonstrated by the double-antibody “sandwich” assay (Fig. 3). (3) The KSPG antigens from scars eluted from ion exchange HPLC with a profile similar to KSPG antigens from normal cornea and to purified KSPG (Fig. 4). (4) KS antigens in the HPLC-purified proteoglycan were eliminated by digestion with endo-β-galactosidase (Fig. 5), an enzyme specific for the internal galactosidic bonds of KS.27 These data leave little doubt that legitimate corneal KSPG is present in corneal scar tissue as soon as 2 weeks after wounding.

Previous studies have not been unanimous in concluding that KSPG is present in corneal scar tissue. Glucosamine-containing material with solubility properties of KS was found in corneal scars,11-13 but a more recent investigation of the intact proteoglycans of the scar15 was unable to detect KSPG until 4 months after wounding. The data presented here confirm inferences from the earlier studies that corneal KSPG (both KS and core protein) is indeed present in the early stages of corneal wound healing. In addition, the current work is the first to demonstrate KS in early scar tissue using structurally specific reagents (antibodies and endo-β-galactosidase) and the first to show that scar KS is associated with a core protein antigenically similar to that of normal corneal KSPG.

In the direct ELISA, purified proteoglycan was immobilized by nonspecific adsorption to plastic, and the amount of antibody binding the immobilized proteoglycan was used as a measure of the amount of antigen present in the original solution (Fig. 2B). When binding was normalized to the protein content of the original extract, scar proteoglycan bound as much of each of the three antibodies as normal tissue proteoglycan. Protein content of the extracts from normal and scarred tissue were similar; consequently we infer that approximately equal amounts of KSPG can be purified from both scar and normal corneal tissue. In the double antibody ELISA, KSPG was immobilized from crude extracts by binding to one of two anti-KS monoclonal antibodies (Fig. 2A). Reactivity in this assay is limited, therefore, to KSPG molecules which contain epitopes recognized by these antibodies. The KS epitopes recognized by these anti-
bodies are not identical, and are not equally distributed in all KS molecules.\textsuperscript{30} Core proteins bearing these epitopes were 55\% (I22) and 82\% (5-D-4) as abundant in extracts of scar tissue as in normal corneal extracts.

In a third type of ELISA immunoassay, KSPG in the unfractionated extracts competed with a biotin-labeled purified KSPG standard for binding of immobilized antibody. In the case of the core protein antibodies, KSPG antigens in extracts of corneal scars competed 61–80\% as effectively as antigens from normal corneal extracts (Table 1). These results suggest a moderate reduction of total KSPG in the scar tissue. In contrast to the core protein antigens, KS antigens in the corneal scar extracts were very poor competitors, 10–20-fold less effective than KS antigens in normal corneal extracts in binding anti-
KS monoclonal antibodies. The KS glycosaminoglycan of scar appears to be present in tissue extracts at nearly normal levels, as evidenced by the amount of anti-KS antibodies which can be bound by scar tissue extracts (Fig. 5). The results of the competitive assay show, therefore, that in a mixture of KSPG from scar and KSPG from normal cornea, the anti-KS monoclonal antibodies bind preferentially to normal KS rather than to KS from scars.

The preferential binding of anti-KS monoclonal antibodies to normal tissue KS probably results from the ability of these antibodies to recognize multiple epitopic structures on the KS molecule and to bind these epitopes with varying affinity. Feizi and co-workers, using chemically characterized KS fragments with anti-KS monoclonal antibodies (including 5-D-4), found that competitive ability of an epitope was related to its length and sulfation. KS fragments containing from three to five fully-sulfated disaccharides varied in competitive abilities according to their chain length, with the decasaccharide greater than 100-fold more effective than the hexasaccharide. Fragments smaller than hexasaccharides and unsulfated fragments did not compete at all for antibody binding. Longer regions of KS sulfation appear, therefore, to form complexes with the anti-KS monoclonals which, at equilibrium, are more thermodynamically stable, and result in preferential binding of antibody to these epitopes. As a result, different arrangements of sulfate groups on apparently similar KS molecules could result in large differences in the ability of these molecules to compete for anti-KS antibodies. In support of this hypothesis is the recent demonstration that competitive abilities of purified KS from cornea and cartilage of several different species vary over a 200-fold range using the antibodies and assay protocol used here. A reasonable interpretation of the behavior of KSPG from corneal scars in these assays is that the reduced competitive ability of the scar KSPG results not from a reduced abundance of KS glycosaminoglycan chains in scar tissue per se, but rather from a structural alteration of the scar KSPG. Structural changes that might alter the competitive ability of KSPG include a decreased or altered pattern of sulfation, decreased KS-chain length, and a reduced number of KS-chains per core protein molecule. Of these changes, reduced sulfation seems the most likely. Reducion sulfation of scar KS has been suggested in earlier biosynthetic studies of corneal wound healing. Reduced sulfation might sufficiently alter the physical properties of scar KSPG so that it would not have been detected in a previous study. In this earlier study biosynthetically labeled proteoglycans were fractioned extensively before analysis of glycosaminoglycan compo-

Table 1. Relative competitive ability of KSPG antigens in extracts of cornea and corneal scars*

<table>
<thead>
<tr>
<th>Tissue extract</th>
<th>Antibody</th>
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<tbody>
<tr>
<td>Cornea</td>
<td>100</td>
</tr>
<tr>
<td>2 week scar</td>
<td>11</td>
</tr>
<tr>
<td>6 week scar</td>
<td>8</td>
</tr>
<tr>
<td>8 week scar</td>
<td>10</td>
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* The relative amount of KSPG antigen was calculated from data similar to those in Figure 6 by comparing amounts of extract protein required to reduce binding of the biotin-KSPG standard to 50% of the uninhibited value.

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