Biochemical Analyses of Proteoglycans in Rabbit Corneal Scars

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Macromolecules from normal rabbit cornea and cornea containing a 2-mm diameter button of scar tissue were biosynthetically labeled with 35S-sulfate and 3H-glucosamine in vivo and in organ culture. Labeled macromolecules, including proteoglycans (PGs) extracted from the normal cornea, scar tissue, and corneal tissue adjacent to the scar with guanidine hydrochloride were chromatographed on DEAE-Sepharose CL-6B columns and eluted with increasing concentrations of NaCl. The elution pattern of corneal macromolecules synthesized in vitro was remarkably similar to that in vivo. In another experiment, corneas having 2-, 4-, and 8-week-old scars were labeled in organ culture and also extracted. Scars synthesized PGs with lower sulfation than those of adjacent corneal tissue. Although PG synthesis in scar decreased with wound age, the synthesis in adjacent cornea remained the same. In a third experiment, PGs extracted from pools of unlabeled 2- and 4-week-old scars, adjacent corneal tissue, and normal corneas were chromatographed on ion-exchange columns and analyzed chemically. The quantity of PGs in scar and adjacent cornea increased with healing time. The ratios of keratan sulfate PG to dermatan sulfate PG in normal cornea, scar, and adjacent cornea was 2.3, 0.6, and 1.5, respectively. The PGs from adjacent corneal tissue had a higher charge density than those from scar. The predominant adjacent-cornea dermatan sulfate PG had a higher charge density than that in normal cornea. The authors conclude that cornea adjacent to the healing wound synthesizes PGs measurably different from those in scar and normal cornea. Invest Ophthalmol Vis Sci 31:1975-1981, 1990

The major proteoglycans (PGs) in cornea contain keratan sulfate (KSPG) and dermatan sulfate (DSPG).1–6 Interest in these macromolecules stems from their important role in maintaining the structure and functional properties of the cornea.7–11

The major glycosaminoglycan (GAG) in the cornea, KS is a heteropolysaccharide that contains a repeating unit of N-acetyllactosamine, with variable degrees of glucosamine sulfation;12–14 DS contains repeating disaccharide units of sulfated N-acetylgalactosamine and hexuronic acid. The name “dermatan sulfate proteoglycan” is currently used because iduronate and glucuronate are present in the polymer.1,4,5 Although the chemistry of normal corneal PGs has been studied extensively,1,4,5,6,12,15–19 considerably less is known about PGs in healing tissues.

Incorporation of 35SO4-label along the edge of perforation wounds in the rabbit cornea during the healing process indicates rapid synthesis of corneal PGs.20,21 Normal high-sulfated KS and low-sulfated DS are markedly reduced in healing corneal wounds, but high-sulfated DS and an immunochemically different KS accumulate.22–27

Analyses of radiolabeled PGs from young rabbit corneal scars indicate synthesis of an unusually large DSPG with GAG side chains of normal size.28 Normal high charge-density KSPGs appear late in scar formation, and the normal pattern of PGs is eventually regenerated along with the return of transparency.

We studied the chemical properties of PGs synthesized in healing rabbit cornea and adjacent corneal tissue. Our results confirm the existence of an unusually large DSPG in scar tissue and show, for the first time to our knowledge, that the proportion of KSPG to DSPG, their sizes, and degree of sulfation in scar differ from those in adjacent and normal cornea.

Materials and Methods

Rabbits used in this study were treated in compliance with the Guiding Principles in the Care and Use of Animals, DHEW Publication, NIH 85-23, and in accordance with the ARVO Resolution on the Use of Animals in Research.

We compared the synthesis of macromolecules in...
corneas in vitro and in vivo. The corneas of six rabbits with a central 2-mm diameter, 2-week-old scar in each right eye and the contralateral normal cornea were kept in organ culture to incorporate carrier-free 35S-sulfate (140 μCi/ml) and 3H-glucosamine (28 μCi/ml, 19 mCi/mmol) (New England Nuclear, Boston, MA) into glycoproteins and PGs. To incorporate label in vivo, the right eyes of six adult albino rabbits were wounded allowed to heal for 2 weeks to produce corneal scar tissue. Rabbits were anesthetized with topical proparacaine before injection of 100 μCi of 3H-glucosamine and 125 μCi of 35S-sulfate in 20 μl of phosphate-buffered saline, pH 7.0, into the anterior chamber of each eye on 4 consecutive days. Animals were then killed with an intravenous overdose of pentobarbitonal, and the corneas were collected. The cultured corneas and the corneas labeled in vivo were briefly washed in phosphate-buffered saline, and the 2-mm diameter scar button and adjacent corneal tissue were separated in preparation for PG extraction.

In another experiment, 11, nine, and nine rabbit eyes having 2-, 4-, and 8-week-old scars, respectively, were cultured in the presence of radiolabel. After incubation, scar tissues were separated from cornea adjacent to the healing wounds. Pooled scar tissue and adjacent cornea were prepared for PG extraction.

The PGs in all tissues in both series of experiments were extracted with guanidine hydrochloride and fractionated on a DEAE-Sepharose CL-6B (Sigma Chemical Co., St. Louis, MO) column with increasing concentrations of NaCl. The PG-associated hexosamines from scar and adjacent cornea increased during healing. Although the ratio of total GlcN/GalN in normal corneal PGs was 2.3, the ratio in scar and adjacent cornea was about 0.6. This ratio was 1.9, 1.3, and 1.2 in corneas adjacent to 2-, 4-, and 8-week scars, respectively. Most PG-associated hexosamines from scar eluted at 0.25 and 0.50 M NaCl, whereas hexosamines from adjacent cornea eluted at 1.0 M NaCl. Moreover, the elution pattern in these two healing tissues differed from that in normal cornea. In particular, the predominant adjacent-cornea DSPG had a higher charge density than the major DSPG from normal cornea. In contrast to the scar, in adjacent tissue the incorporation rate of 3H and 35S showed no decreasing trend. Nevertheless, the 35S/3H ratio decreased with time. A more uniform distribution of 3H-glucosamine into the three eluted fractions was evident in adjacent cornea in comparison to scar, indicating a greater percentage of PGs with lower charge densities in the scar.

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**Results**

**Synthesis and Quantitative Analyses of PGs**

Radiolabeled macromolecules from organ-cultured normal corneas and corneas with 2-week-old scars (scar tissue and adjacent corneal tissue) were eluted from ion-exchange columns in patterns similar to macromolecules from comparable tissues labeled in vivo (Figs. 1, 2). Since samples were from pooled tissues, no statistical analyses were available. Previous studies indicate glycoproteins elute at 0.0 M NaCl, and PGs of increasing charge density elute at 0.25-1.0 M NaCl.3

In a separate series of experiments, we compared the incorporation of radiolabeled precursors into PGs synthesized in scar and adjacent cornea during organ culture. Whereas the adjacent-cornea data were expressed as dpm/mg wet tissue/24 hr, we chose not to express the data on scar tissue in the same manner because of the inherent high probability of errors in wet-weight determinations of very small amounts of tissue. Thus, comparisons were made of trends in each tissue with time of healing. Incorporation rates of 3H-glucosamine and 35S-sulfate into PGs of organ-cultured 2-, 4-, and 8-week-old scars and their 35S/3H ratios decreased with wound age.

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Molecular Size of PGs

Normal cornea and 2- and 4-week-old scar PGs eluted from ion-exchange columns with 0.5 and 1.0 M NaCl, were subjected to Sepharose CL-4B gel chromatography. Most of the GlcN- and GalN-containing macromolecules from normal cornea, representing KSPG and DSPG, respectively, were identified by their sensitivity to GAG-specific enzymes and were eluted as single peaks in the retarded zone.
Fig. 3. PGs of 0.5 M NaCl fractions from DEAE-Sepharose CL-6B columns were chromatographed on Sepharose CL-4B (A) PGs from normal corneas. (B) PGs from 2-week-old scars. Glucosamine (GlcN) and galactosamine (GalN) represent KSPG and DSPG, respectively. Fractions were separated into three zones, a, b, and c.

Fig. 4. PGs of 1.0 M NaCl fractions from DEAE-Sepharose CL-6B columns were chromatographed on Sepharose CL-4B. (A) PGs from normal corneas. (B) PGs from 2-week-old scars. Glucosamine (GlcN) and galactosamine (GalN) represent KSPG and DSPG, respectively.

Discussion

Previous studies show that rabbit corneas maintained in organ culture synthesize labeled PGs similar to those labeled in vivo. Using similar organ culture procedures with some modifications, we studied the synthesis of PGs in healing corneas. Similarities in the pattern of macromolecular synthesis in organ-

Normal KSPG derived from 0.5 M (Fig. 3A) and 1.0 M NaCl (Fig. 4A) fractions were smaller (Kav, 0.54 and 0.41) than DSPGs from the same fractions (Kav, 0.34 and 0.28). The PGs from normal cornea, however, were intermediate in size compared with their corresponding PGs in 2-week-old scar tissue (Figs. 3B, 4B). The KSPG in the 0.5 M NaCl fraction from scar appeared as two peaks with a major component eluting at Kav 0.78 and a minor peak at 0.11 (Fig. 3B). The DSPG from the same NaCl fraction eluted as two large peaks with Kav 0.56 and 0.17. The unusual GalN-containing peak near the void volume was identified as DSPG by its sensitivity to chondroitinase ABC.

Each PG type of the 1.0 M NaCl fraction from scar tissue eluted as two peaks, similar to those in the 0.5 M NaCl fraction. The KSPGs from the 1.0 M fraction eluted as minor and major peaks (Kav 0.06 and 0.56), whereas DSPGs eluted as a major peak (Kav 0.06) and a broad peak (Kav about 0.33) (Fig. 4B). The large DSPG peak (zone a) and retarded fractions (zone b) obtained from scar (Fig. 3B) were subjected to reduction and alkylation and chromatographed on Sepharose CL-4B under dissociative conditions (Fig. 5). The GalN-containing PG eluted at the V₀ (Fig. 5, zone a), indicating that this large-molecular-weight DSPG did not result from aggregation of the molecule or from polymerization with itself or other proteins through disulfide linkages. The DSPGs of zone b remained in its original position after reduction and alkylation (Fig. 5, zone b). Similar gel-chromatographic analyses of 4-week-old scar PGs also indicated the presence of this unusually large DSPG (Fig. 6).
cultured and in vivo corneal tissues attest to the relevance of our findings to corneal scar formation in the eye. Variability in the results from one series of experiments to the other, however, allowed us to make reasonable comparisons only in groups of tissues radiolabeled and fractionated simultaneously.

The $^{35}$S/$^3$H ratios and the distribution of label and hexosamines in NaCl fractions from ion-exchange columns indicate that scars synthesize predominantly low-sulfated PGs in comparison with adjacent corneal tissues. Unlike adjacent cornea, the rate of PG synthesis in scar decreases with time of healing. Recalculation of the data (not shown) to include changes in cell number with healing indicates that the rate of PG synthesis per cell also decreases with time, suggesting cellular down-regulation of PG synthesis during wound healing.

Stromal cells within an edematous stroma, in invading a graft, or in the stroma adjacent to the edge of a wound, as shown in the current study, synthesize GAGs without scar formation. Corneal tissue adjacent to the wound loses substantial amounts of PGs soon after wounding (unpublished observations). Regeneration of these macromolecules results in the synthesis of PGs that are proportionately and chemically different from those in the scar or normal adult cornea. Our studies suggest that the proportion of KSPG to DSPG in adjacent corneal tissue is between those in normal cornea and scar tissue. Moreover, the charge densities, as determined by ion-exchange chromatography, of some PGs in these tissues differ. Thus, a greater proportion of PGs in scar tissue are low sulfated in comparison to PGs in adjacent corneal tissue. Although the corneal tissue adjacent to a 2-mm trephination wound is initially edematous and translucent, it is transparent by week 8 of healing (unpublished observations). Therefore, the alterations in PGs in this tissue do not alter corneal transparency. The lack of transparency in scar must be due to changes in PGs unique to this tissue or to other factors.

Stromal cells in the adjacent stroma and corneal wound are confronted with different matrices. In the former tissue, the cells are surrounded by a collagenous matrix, albeit partially depleted of PGs. In the perforated wound, collagen fibrils are replaced by a fibrin matrix. The pattern of PG synthesis by stromal cells in these two closely associated tissues may be determined partially by the physical and chemical properties of the extracellular matrices. Although the macromolecular contribution of endothelium-derived cells to the posterior scar is largely unknown, an alternative or additional explanation for the differences in PGs in scar and the adjacent corneal tissue may involve the synthesis of distinctively different PGs by these cells during scar formation.

Quantitative analysis of the PG hexosamines is consistent with previous studies showing a prepon-
derance of DS and minor amounts of KS in scar.\textsuperscript{23-25} Our study shows that KSPG in scar tissue has a low charge density, probably due to the degree of sulfation, and generally lower than KSPG from normal cornea. This may have resulted in low values for KS in earlier studies. These molecular characteristics may also be related to the immunochemically aberrant KSPG in scar tissue.\textsuperscript{26}

The pattern of PG synthesis in scar is similar to that in fetal cornea.\textsuperscript{31,39} Indeed, despite the differences in environment, the early developing cornea and the scar are alike in the synthesis of several extracellular matrix molecules.\textsuperscript{37,40-43} However, DSPGs from scar tissue are composed of two distinct populations, one smaller and one larger than DSPG from developing cornea. Our observation that scar tissue synthesizes\textsuperscript{28} and accumulates unusually large PGs, their absence from normal developing corneas,\textsuperscript{44} and current concepts of the mechanism of corneal transparency\textsuperscript{44} suggest that these macromolecules, by virtue of their hydrodynamic volume, play a role in the opacity of scar tissue.\textsuperscript{28}

The dissimilarity in size of scar DSPGs is not due to differences in GAG chain length.\textsuperscript{28} Instead, the core-protein length or number of GAG chains per molecule may account for the difference. The latter situation accounts for the difference in size of bone PGs, biglycan, and decorin.\textsuperscript{45} Although decorin, containing one GAG chain, is immunochemically similar to DSPG in normal bovine cornea, biglycan, containing two GAG chains, is not present in this tissue. It is tempting to speculate that the large biglycan may be identical to the large DSPG in corneal scar tissue.

**Key words:** proteoglycans, wound healing, scar, organ culture, cornea

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