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# Synthesis of Stromal Glycosaminoglycans in Response to Injury

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**Abstract** Our goal is to examine the synthesis and deposition of corneal glycosaminoglycans (GAGs) in response to a wound created by the insertion of porous discs into stromal interlamellar pockets. The disc and the surrounding stromal tissue were assayed and compared to contralateral control stroma and to sham operated corneas at 14, 42, and 84 days. The tissue and/or discs were removed and labeled with $^{35}$S-sulfate for 18 h; GAGs were extracted with 4 M guanidine–HCl. Extracts were chromatographed on Q-Sepharose columns, bound proteoglycans were eluted with a linear salt gradient, and radioactive fractions were analyzed. Total GAG content was determined colorimetrically, using dimethylmethylene blue. Specific GAGs were determined using enzymatic digestion with selective polysaccharide lyases and protein cores were examined using SDS–PAGE. The nonbound fractions from the chromatography were assayed for TGF-β using Western blot analysis and for hyaluronic acid using an $^{125}$I-radiometric assay. Specific GAGs were localized 42 days after the disc had been implanted in the stroma. The placement of the discs into the stroma resulted in a decrease in the total amount of GAG. However, the ratio of dermatan–chondroitin sulfate and heparan sulfate to keratan sulfate increased in the surrounding tissue and disc. Hyaluronic acid was elevated at day 14 in the surrounding tissue, and not until day 84 in the disc. Western blot analysis of surrounding tissue extracts revealed forms of TGF-β that migrated with an apparent molecular mass of 63 and 43 kDa. The results indicate that the insertion of discs into interlamellar pockets causes changes in the sulfation and proportion of the glycosaminoglycans in the surrounding tissue and the disc. These changes are coincident with the appearance of TGF-β. After 84 days, the population of glycosaminoglycans in the disc begins to resemble the surrounding stroma. This model will allow us to examine further the synthesis and deposition of proteins following an extensive wound in which cells must migrate to the wound site and then undergo extensive remodeling. 

**Key words:** GAGs, TGF-β, stroma, remodeling

The extracellular matrix (ECM) of the corneal stroma is composed of a variety of macromolecules that include collagens (I, V, and VI), fibronectin, laminin, and proteoglycans. Interactions between collagens and proteoglycans are thought to provide for the structural and optical properties of the stroma. TGF-β has been shown to be a potent mediator of numerous cellular and physiological processes, including the control of the ECM. Immunohistochemical studies have shown that keratocytes stain positively for TGF-β during corneal wound healing in vitamin A-deficient rats [Hyashi et al., 1989]. TGF-β mRNA is expressed in cultured stromal fibroblasts [Wilson et al., 1992]. The presence of TGF-β and its receptors suggests that it plays a role in regulating the synthesis of stromal ECM components. While TGF-β has been detected in vivo and in vitro, the relationship between the expression of TGF-β and the expression of collagen and proteoglycans in the wounded stroma has not been fully elucidated.

The corneal stroma contains two main groups of proteoglycans, one with chondroitin–dermatan sulfate side chains and the other with keratan sulfate side chains [Axelsson and Heinegard, 1978; Hassell et al., 1979; Gregory et al., 1982; Funderburgh and Conrad, 1990]. The corneal chondroitin/dermatan sulfate proteoglycan protein core has been recently cloned from chick cornea and identified as decorin [Li et al., 1992]. Corneal keratan sulfate proteoglycan protein core (lumican) also has been recently cloned from chick cornea, and its primary structure has been found to resemble decorin, fibromodulin and biglycan [Blochberger et al., 1992].

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The ECM in wounded stromal tissue has been shown to have distinct structural and biochemical properties. Opaque human corneal scars contain collagen fibrils with abnormally large diameters [Schwarz and Keyserling, 1969]. Irregular spaced collagen fibrils also have been observed in human and rabbit scars [Cintron et al., 1978]. The disruption of fibrillar organization in corneal scars is accompanied by changes in the distribution and chemical properties of the proteoglycans and by a reduction in the synthesis of proteoglycans. Wounded corneas synthesize unusually large chondroitin–dermatan sulfate proteoglycans [Hassell et al., 1983] possessing glycosaminoglycan side chains with higher sulfation and increased amounts of iduronic acid [Funderburgh and Chandler, 1989]. Keratan sulfate molecules in corneal scars have been shown to have increased size and a lower sulfation and to be antigenically distinct [Funderburgh et al., 1988]. The ratio of keratan sulfate to chondroitin-dermatan sulfate has been shown to decrease after wounding and heparan sulfate has been detected in corneal scars [Cintron et al., 1990].

To investigate the remodeling that occurs in an extensive wound, we have developed a model using a porous disc in which cells must migrate into the interstices of the material and establish a matrix. The model allows one to follow wound repair in delineated regions, as the cells that have migrated into the disc can be evaluated separately from those adjacent to the wound site and also from those a distance from the wound site.

The web that we have designed allows for the eventual anchoring of a keratoprosthetic device to the cornea via extensive fibroplasia [Trinkaus-Randall et al., 1990, 1991, 1994]. Specifically, our goal was to compare the deposition of GAGs, their associated core proteins, and TGF-β in the disc to the surrounding tissue and unwounded corneas. The results indicate that after 42 days the population of GAGs is characteristic of corneal wounds. However, after 84 days the profiles of GAGs in the disc resemble that of a normal cornea.

## MATERIALS AND METHODS

### Materials

Guanidine–HCl was obtained from Gibco BRL (Gaithersburg, MD). Dimethylmethylene blue (DMB) and benzamidine hydrochloride hydrate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Q-Sepharose Fast-Flow, Ponceau-S, sodium acetate, heparinase III, chondroitinases AC Flavo and ABC, and heparan sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Chondroitin sulfates A and B (super special grade) standards and keratanase II were purchased from Seikagaku America (Rockville, MD). Endo-β-galactosidase was purchased from Boehringer Mannheim (Indianapolis, IN). Monoclonal antibody (mAb) specific to TGF-β (1, 2, and 3) was obtained from Genzyme (Cambridge, MA). Both the ECL chemiluminescence detection kit and horseradish peroxidase (HRP)-conjugated sheep antimouse IgG were purchased from Amersham (Arlington Heights, IL). The hyaluronic acid kit was purchased from Pharmacia Diagnostics (Uppsala, Sweden).

### Placement of the Discs

New Zealand rabbits (3–4 lb) were anesthetized with intramuscularly administered ketamine hydrochloride (35 mg/kg) (Aveco Co., Fort Dodge, IA) and Xylazene hydrochloride (5 mg/kg) (Mobay Corp., Shawnee, KS). Discs of polybutylene/polypropylene were inserted into central interlamellar stromal pockets, using a surgical procedure described previously [Trinkaus-Randall et al., 1991]. A minimum of five discs were inserted for each time point and condition. To control for the insertion of the disc, sham operations were performed in which lamellar pockets were created, but no disc inserted. To examine the response of the cornea to the material, neither antibiotic nor corticosteroid was administered.

### Tissue Preparation

Central corneas were removed with a 10-mm trephine. The discs and surrounding tissue were teased apart, and separate analyses were carried out on each. Analysis was performed on similar regions of the contralateral and sham operated corneas. After excision the epithelium and endothelium were removed. The tissue and disc were radiolabeled for 18 h at 37°C in serum free, sulfate free media containing $^{35}$S-sodium sulfate. After incubation, the samples were washed, and lyophilized and the dry weight measured.

### Dimethylmethylene Blue Colorimetric Assay

Total sulfated GAGs were quantitated colorimetrically using the dimethylmethylene blue (DMB) assay [Farndale et al., 1986]. Samples were mixed with the DMB reagent (1:25), and

absorbance at 525 nm was immediately read. Concentrations were determined from standard curves of highly purified specific GAGs.

### Extraction and Purification of Proteoglycans

The samples were homogenized using a Polytron (Brinkman Inst., Westbury, NY) extracted for 18 h at 4°C (in excess of 100 v/w) in a solution of 4 M guanidine HCl containing 0.05 M sodium acetate pH 6.0 and the following protease inhibitors: 0.1 M aminocaproic acid, 1 mM benzamidine HCl, 1 mM phenylmethylsulfonylfluoride, 10 mM ethylenediametetraacetic acid (EDTA), and 10 mM N-ethylmaleimide [Gregory et al., 1982]. The extracts were centrifuged for 30 min at $4 \times 10^4$ *g* and the pellet re-extracted until negligible GAG was detected in the supernatant by DMB analysis. The supernatants were dialyzed exhaustively against deionized water, lyophilized and resuspended in deionized 7.0 M urea, 0.05 M Tris HCl, and 0.05 M EDTA, at pH 7.2. Extracts were loaded on a 2-ml Q-Sepharose Fast-Flow anion-exchange column equilibrated with urea buffer [Midura et al., 1989]. Conductivity and absorbance at 280 nm were continuously monitored. The proteoglycans were eluted using a linear 0–1.5-M NaCl gradient, and 1.3-ml fractions were collected. The elution profile of the proteoglycans was assessed using DMB analysis and liquid scintillation. Fractions containing GAG were pooled, concentrated, and desalted with dialysis, followed by lyophilization. The nonbound fractions were collected dialyzed against deionized water, lyophilized, and resuspended in phosphate-buffered saline (PBS).

### Enzymatic Digestion

Purified GAGs were identified and quantified by treatment with selective polysaccharide lyases. Digestion conditions were optimized with time, temperature, concentration, and specificity studies. Aliquots of each sample were subjected to digestion for 3 h at 37°C in 70 mM Tris–HCl. The concentration of GAG in the digestion mixture was adjusted to approximately 80 μg/ml (determined by DMB analysis), and the pH and concentration were adjusted to the optimum for each enzyme: chondroitinase ABC (1 U/ml), pH 8.0:chondroitinase AC (0.5 U/ml), pH 7.3; keratanase II and endo-β-galactosidase (0.1 U/ml), pH 5.9, and heparinase III (10 U/ml), pH 7.0. Specific GAG content was determined as the concentration difference between each digest and control lacking enzyme. Each data point represented pooled samples for each time point and region.

### Gel Electrophoresis and Western Immunoblotting

Sodium dodecyl sulfate–polyaerylamide gel electrophoresis (SDS–PAGE) was performed [Laemmli, 1970]. Proteoglycans were digested with either chondroitinase ABC or keratanase II and endo-β galactosidase. Sample volumes were normalized to tissue dry weight. Digests were electrophoresed on 4–15% linear polyacrylamide gradient minigels under nonreducing conditions. After staining for 1 h in 0.25% Coomassie Blue R250 in 9% acetic acid in 45% methanol, the gels were destained.

Aliquots from the nonbound fractions (volumes normalized to tissue dry weight) were electrophoresed on 4–15% gradient minigels under nonreducing conditions. The protein was transferred to an immobilon PVDF membrane in 25 mM Tris, 192 mM glycine, using a Minitrans transfer apparatus [Towbin et al., 1979]. Transfer was verified by reversible staining of the membrane with Ponceau S [Aebersold et al., 1987]. The membrane was blocked with 5% nonfat dry milk and 0.1% Tween in PBS. Blots were incubated overnight with a mAb specific to TGF-$\beta_{1-3}$ diluted 1:1,000 in PBS containing 1% milk and 0.1% Tween, at 4°C. After washing, the membranes were incubated for 1 h in peroxidase-conjugated sheep antimouse IgG diluted 1:3,000 in the above diluent. The presence of antibody was visualized using ECL, a chemiluminescence detection kit.

### Immunohistochemistry

After excision, the corneas were frozen in OCT, and 10-μm sections were cut. The sections were allowed to dry and adhered to Vectabond-coated slides. Slides were rehydrated in PBS. Epitopes for dermatan–chondroitin sulfate proteoglycans were unmasked by digestion with chondroitinase ABC or chondroitinase AC in the presence of protease inhibitors as described above and incubated in PBS containing 3% nonfat milk. The following primary antibodies were applied to the slides at room temperature for 1 h: antikeratan sulfate (Sundar-Raj) mAb J19 (1:500) and antichondroitin-4-sulfate and dermatan sulfate mAb LQ ΔDi 4S clone 2-B-6 IgG (1:50). The dilutions were made up in PBS containing 1% nonfat milk. Slides were washed in

PBS containing 1% milk between all steps. Appropriate secondary antibodies were applied for 30 min at room temperature, followed by 15-min incubation with avidin–FITC complex. The slides were washed with PBS and coverslipped using a fluorescent mounting medium. Fluorescence and phase-contrast photographs were taken on a Nikon-inverted Diaphot microscope at 10× magnification, using Kodak Tri X Pan film. Film exposures were kept constant for each antibody series.

### Radiometric Assay

Aliquots from the non bound fractions were analyzed for hyaluronic acid using a commercially available $^{125}$I-radiometric assay (Pharmacia, Sweden).

## RESULTS

### Experimental Design

Porous discs were implanted into corneal stromal interlamellar pockets. The synthesis and deposition of sulfated GAGs in the disc, the tissue surrounding the disc, sham-operated corneas, and contralateral control corneas were compared after 14, 42, and 84 days of wound healing.

### Elution Profile of GAGs

To evaluate the relative sulfation states of the GAG preparations, the guanidine extracts were loaded onto Q-Sepharose anion-exchange columns. A linear salt gradient was applied to the column, and bound proteoglycans were eluted on the basis of the charge density of the GAGs. The elution profiles of sulfated GAGs were determined using DMB analysis (Fig. 1) and populations of the newly synthesized proteoglycans were assessed by incorporation of $^{35}$S-S04 (Fig. 2). The unbound fractions were assayed for TGF-β and hyaluronic acid.

The chromatographic profiles from the wounded and adjacent regions were compared to control tissue to examine changes in the distribution of charge density of GAGs. GAG in the fractions from the disc extracts at days 14 and 42 was detected at low concentrations that could not be quantitated accurately using either the DMB assay or liquid scintillation counting (not shown). At day 14, both the GAG deposited in the specific regions and the newly synthesized GAG eluted between 0.3 and 0.9 M NaCl (Figs. 1A, 2A). Minor differences were detected in the

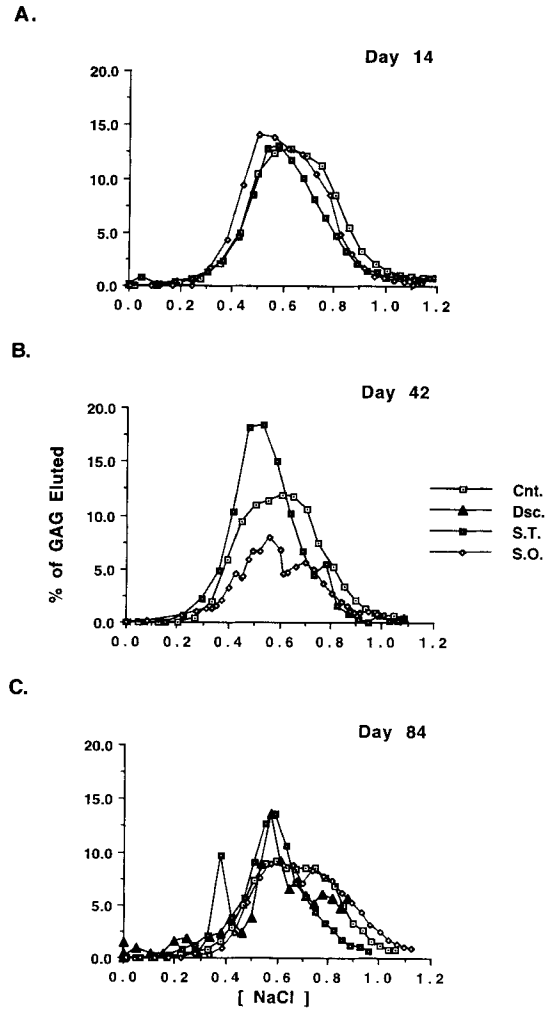


**Fig. 1.** Anion-exchange chromatographs of sulfated GAG present in the tissue extracts (DMB assay). Guanidine extracts were fractionated on Q-Sepharose anion-exchange columns and eluted with increasing molarity of NaCl. At each salt concentration, fractions were assayed for sulfated GAG present in the different regions at the three time points. Each data point represents the percentage of the eluted GAG for each sample. **A:** Day 14. **B:** Day 42. **C:** Day 84. Cnt, control; Dsc, disc; ST, surrounding tissue; SO, sham operated.

elution patterns of the sulfated GAG that had been deposited in the three regions over a period of 14 days; 68% of the control eluted between 0.5 and 0.8 M, while 65% and 72% of the surrounding tissue and sham extracts, respectively, were eluted (average control values for all three time points 69.2 ± 7.3) (Fig. 1A). After 42 days, the differences in the elution profiles were greater. By this time, most of the GAGs from the surrounding tissue eluted at salt concentrations lower than control (62% of the surrounding tissue extract eluted between 0.4 and 0.55 M),

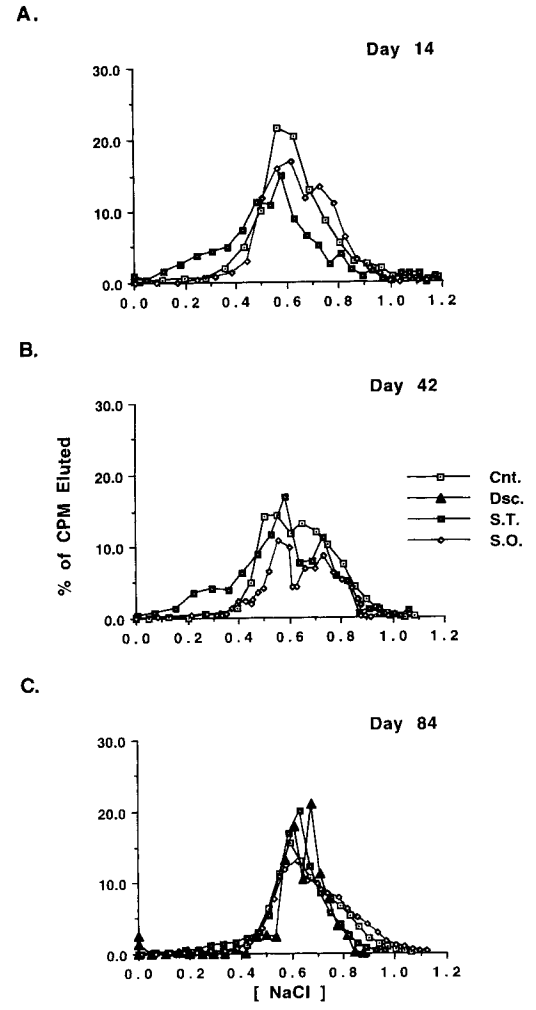


**Fig. 2.** Anion-exchange chromatographs of newly synthesized sulfated GAG. Guanidine extracts were fractionated on Q-Sepharose anion-exchange columns and eluted with NaCl. Fractions were assayed for $^{35}S$-$SO_4$ incorporation into GAG using liquid scintillation. **A:** Day 14. **B:** Day 42. **C:** Day 84. Cnt, control; Dsc, disc; ST, surrounding tissue; SO, sham operated.

while only 38% of the control eluted at the lower molarity), indicating an increase in the proportion of lower sulfated species (Fig. 1B). However, after 84 days, the elution profiles of sulfated GAG that had been deposited in the disc and surrounding tissue were similar again (disc: 72%; surrounding tissue: 66.4%; sham-operated: 62%; with the control having an average and standard deviation of 69.2 ± 7.3) (Fig. 1C). No significant differences were detected (Student's *t*-test).

When the newly synthesized GAG was analyzed, differences were detected in the elution profiles at days 14 and 42. The average control value was 72.9 ± 4.2%. From day 13 to 14, 79% of the newly synthesized sulfated GAG extracted from the control tissue eluted between 0.5 and 0.8 M. By contrast, 87% of the newly synthesized GAG from the sham tissue eluted between 0.5 and 0.8 M. The populations of newly synthesized GAGs were significantly differently in the different regions (Student's *t*-test $p < 0.5$). Similar differences were noted in newly synthesized GAG at days 41–42. By 84 days, little difference in the distribution of charge density in the populations of newly synthesized GAGs was detected (Fig. 2C).

### Total GAG Content

The average concentration of GAG in the control corneas was 23.3 ± 1.2 μg/mg dry tissue. We have defined control as uninjured tissue that has been extracted, purified, and evaluated with the DMB assay. The concentration of GAGs in the disc (μg GAG/mg dry tissue or μg GAG/mg disc; the weight of the disc before implantation was subtracted before calculations) increased from 15.3% of control at day 14 to 54.5% at day 84 (Fig. 3). We have demonstrated previously that cells migrate into the disc from the surrounding tissue and then deposit an extensive matrix [Trinkaus-Randall et al., 1991, 1994]. Therefore, the assumption is made that all GAG found in the disc is newly synthesized. The GAG in the surrounding tissue was less than control at all three time points. The level of GAG in the sham operated corneas was similar to control at day 14 (104.6%). Later time points resembled the surrounding tissue (67.8% and 60.0% at days 42 and 84, respectively), indicating a delay in the response to the injury. Since the extractions were performed on pooled tissue samples, statistical analyses were not possible.

### Enzymatic Characterization of Specific GAGs

Compositional analyses revealed changes in the proportion of specific GAGs synthesized in response to the insertion of the disc. Glycosaminoglycan content was determined as the difference between the digested and control (lacking enzyme) aliquots. Chondroitin sulfate was defined as GAGs susceptible to chondroitinase AC digestion. Dermatan sulfate was defined as GAGs susceptible to chondroitinase ABC digestion but not to chondroitinase AC digestion. Heparan sulfate and keratan sulfate were defined as GAGs susceptible to digestion with Heparanase III and keratanases, respectively.

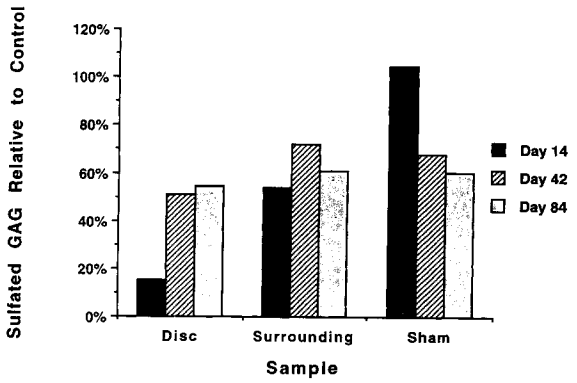


Fig. 3. Sulfated glycosaminoglycans (GAG/dry weight tissue relative to control). Samples were assayed for total sulfated GAG using DMB at days 14, 42, and 84. The percentage of sulfated GAG increased in the disc after 14 days or between 14 and 84 days. The average concentration of GAG in the control contralateral corneas was 23.3 ± 1.2 μg/mg dry tissue.

The amount of each GAG was determined and the distribution for each region at the three time points (days 14, 42, and 84) was compared. The relative proportion of specific GAGs in both the contralateral and sham operated corneas were similar at all three time points (Fig. 4). After 14 days, the proportion of GAGs in the surrounding tissue was similar to control. However, in the disc, dermatan sulfate was the only GAG detected (Fig. 4A). After 42 days, keratan sulfate was detected in the disc (Fig. 4B). The relative proportions of keratan sulfate in the disc and surrounding tissue were 44.8% and 62.7% lower than control, respectively. The proportion of dermatan sulfate in both samples was similar to the controls at day 42. Chondroitin sulfate was present at elevated levels in the surrounding tissue and absent in the disc. A significant amount of heparan sulfate was detected in the disc and surrounding tissue at day 42. By day 84, the proportion of GAGs in the surrounding tissue and the disc were similar (Fig. 4C). Levels of heparan sulfate were reduced and the ratios of keratan sulfate to chondroitin sulfate and dermatan sulfate remained lower than control after 84 days.

### Immunohistochemical Distribution of GAGs

To examine the distribution of specific GAGs within the corneas after the placement of the discs, immunohistochemistry was performed. The distribution after 42 days is shown (Fig. 5A–C). In all samples examined, the epithelium was not positive for any of the antibodies specific for GAGs.

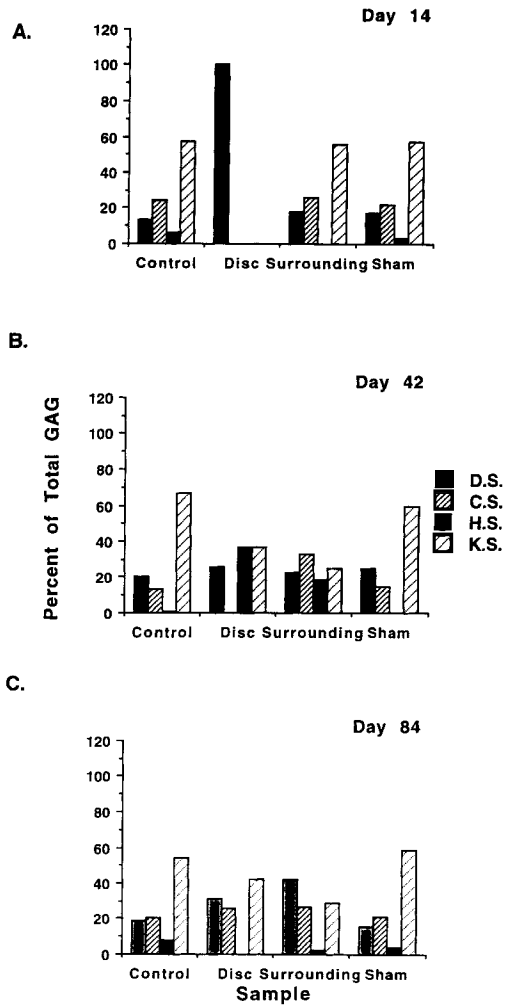


Fig. 4. Relative proportions of sulfated glycosaminoglycans. Aliquots from purified tissue extracts from four regions were assayed for dermatan sulfate (DS), chondroitin sulfate (CS), heparan sulfate (HS), and keratan sulfate (KS), using a selective polysaccharide lyase digestion assay. **A:** Day 14. **B:** Day 42. **C:** Day 84. Chondroitin sulfate was defined as GAGs susceptible to chondroitinase AC digestion. Dermatan sulfate was defined as GAGs susceptible to chondroitinase ABC digestion, and not to chondroitinase AC digestion. Heparan sulfate and keratan sulfate were defined as GAGs susceptible to digestion with Heparanase III and keratanases, respectively. Data are presented as percentage of total GAG.

Chondroitin and dermatan sulfate were localized using specific antibodies following enzymatic digestion (Fig. 5A,B). Selective digestion with chondroitinase AC or ABC removed all but the terminal disaccharide units attached to the linkage region of chondroitin sulfate or chondroitin sulfate and dermatan sulfate, respectively. After enzymatic treatment, tissue sections were incubated with antibodies specific to the terminal disaccharides (ΔDi 4S). Chondroitin and der-

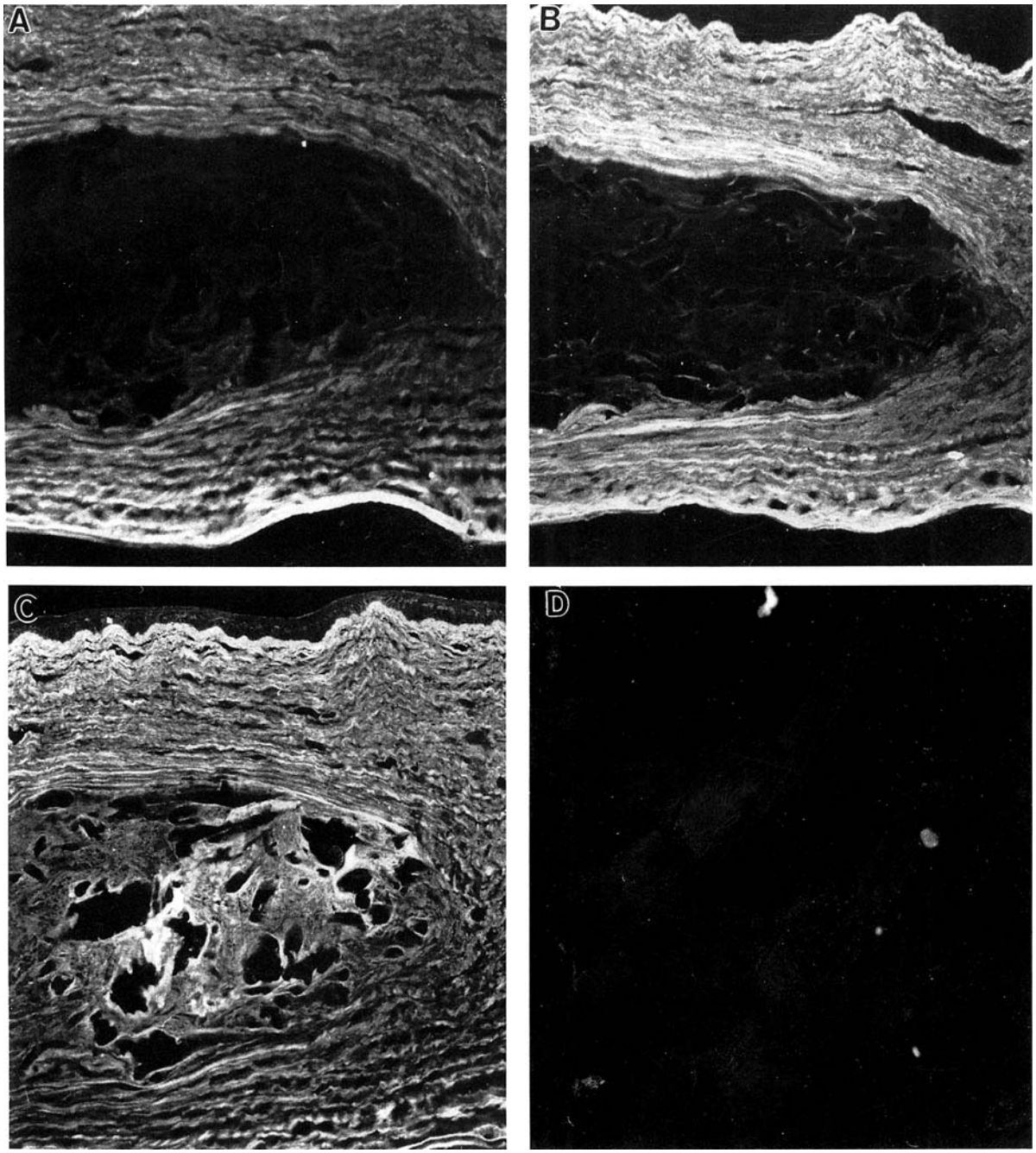


**Fig. 5.** Distribution of glycosaminoglycans. GAGs were localized using indirect immunohistochemical staining of frozen cross sections of corneas 42 days after placement of discs. Sections were stained for (**A**) dermatan sulfate, (**B**) dermatan sulfate and chondroitin sulfate, and (**C**) keratan sulfate, using specific antibodies. Staining was not detected when the primary antibody is omitted (**D**).

matan sulfate were detected throughout the stroma. Chondroitin sulfate (Fig. 5A) and dermatan sulfate (Fig. 5B) were minimal in the disc as compared to the surrounding tissue. While only a small amount of dermatan and chondroitin sulfate were detected using the immunohistochemical techniques, they were detected with the more sensitive biochemical assays. Staining was not detected in either of the undigested sections.

Keratan sulfate was detected throughout the disc and surrounding stroma (Fig. 5C). The in-

tensity of staining was similar throughout the stroma. Positive controls were conducted using cartilage and the appropriate regions stained positively (data not shown). Fluorescence was negligible when the primary antibody was omitted (Fig. 5D).

### Protein Cores

The protein cores (decorin, keratan sulfate proteoglycan) were examined using SDS–PAGE following digestion with either chondroitinase ABC or keratanase II and endo-β-galactosidase, respectively. Samples were normalized to tissue dry weight. The gels were stained with 0.2% Coomassie Blue; undigested proteoglycans were not detected, presumably due to the presence of the GAG chains [Rada et al., 1993].

One major core protein was released when proteoglycan preparations were digested with equal amounts of chondroitinase ABC prior to gel electrophoresis (Fig. 6A). This core protein has an apparent molecular mass of 45 kDa, consistent with that of decorin after chondroitinase ABC digestion [Rada et al., 1993; Fisher et al., 1989]. It was present at higher concentrations at days 42 and 84 in the surrounding tissue and only at day 84 in the sham. No additional chondroitin–dermatan sulfate protein cores were present in the wounded tissue or controls. The only other band present on the gel was chondroitinase ABC, having a molecular mass of 98 kDa that stained with equal intensity in all lanes confirming equal loading (Fig. 6A).

When the preparations were digested with a combination of keratanase II and endo-β-galactosidase, two core proteins at 42 and 46 kDa were released. These molecular masses are consistent with those reported for bovine corneal keratan sulfate proteoglycans [Funderburgh et al., 1991, 1993]. The intensity of these bands reflected the total amount of keratan sulfate present in the proteoglycan preparations (Fig. 6B). The most intensely stained band representing the keratan sulfate proteoglycan core was detected in the control uninjured tissue. No additional keratan sulfate protein cores were present in the wounded tissue. The bands at 69 and 230 kDa were present in the enzyme mixture, and the similarity in band intensity in all lanes indicated equal loading. The day 42 sample was not available for analysis.

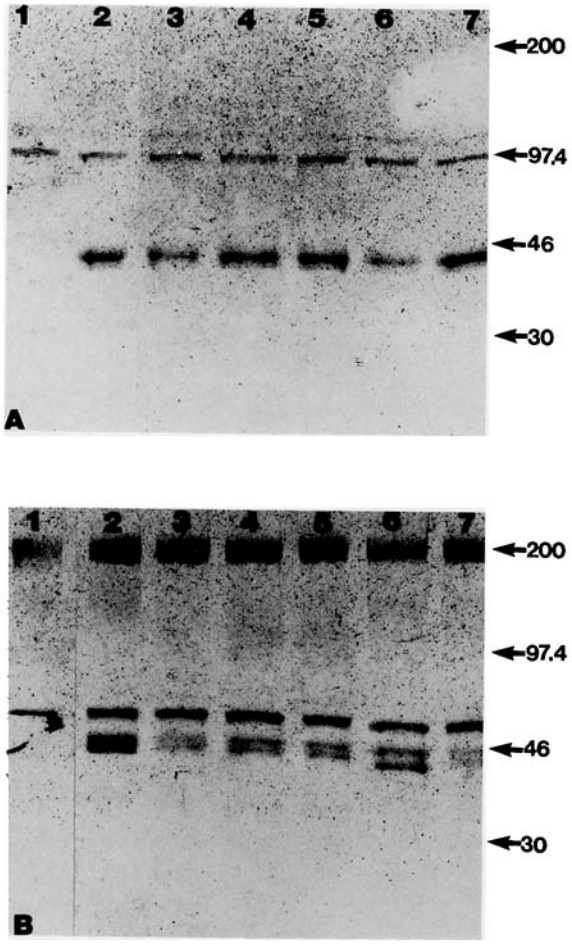


**Fig. 6.** Protein cores. The bound fractions were digested with either (**A**) chondroitinase ABC or (**B**) keratanase II and endo-β-galactosidase and electrophoresed on 5–15% gradient SDS–PAGE gels (nonreduced). Lane 1, enzyme; lane 2, control; lanes 3–5, surrounding tissue at days 14, 42, and 84, respectively; lanes 6, 7, sham tissue at days 14 and 84, respectively.

### Hyaluronic Acid

A series of preliminary experiments demonstrated the presence of hyaluronic acid in the unbound fraction. Hyaluronic acid was detected only at concentrations four orders of magnitude lower than that of the sulfated GAGs. The average value for controls was 0.40 ± 0.1 μg/mg dry tissue. After 14 days, hyaluronic acid in the surrounding tissue was elevated in response to the wound (Fig. 7). Hyaluronic acid levels in the disc were similar to control at day 14 and were not elevated until 84 days. The remaining samples were similar to control. Unfortunately, the day 42 sham sample was not available for analysis.

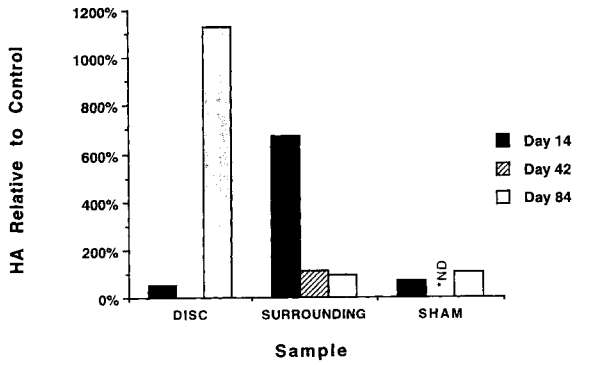


Fig. 7. Hyaluronic acid content in the different regions. The amount of hyaluronic acid relative to control tissue was determined using an $^{125}$I-radiometric assay at days 14, 42, and 84. The average concentration of HA in controls was 0.40 ± 0.1 μg/mg dry tissue. *ND, data not available.

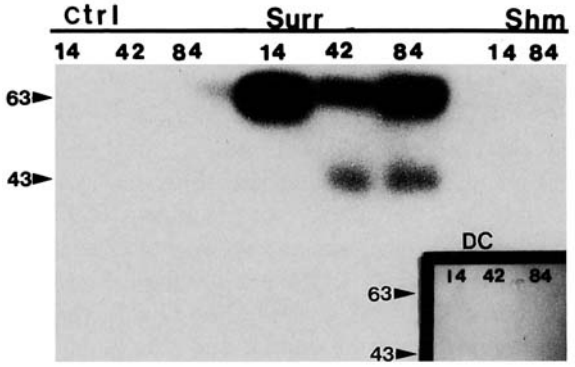


Fig. 8. Western blot analysis for TGF-β. Unbound fractions of extracts eluted from the anion-exchange chromatography were electrophoresed on 5–15% gradient SDS–PAGE gels (nonreduced), and immunoreactive TGF-β proteins were detected by Western blot analysis in the surrounding tissue only. **Insert:** TGF-β was not detected within the disc. The peptide was not detected when the primary antibody was omitted (data not shown). The numbers represent days after wounding. Ctrl, control; Surr, surrounding tissue; Shm, sham operated tissue; DC, disc.

### Presence of TGF-β

The relative amounts of TGF-β present in the tissue and disc were examined. To demonstrate that TGF-β was eluted in the unbound fraction, a stromal extract incubated with $^{125}$I-TGF-β was applied to a Q-Sepharose column, and the radioactive peak was localized to the unbound fraction. Western blot analysis was performed to determine whether endogenous TGF-β was present (Fig. 8). Sample volumes were normalized to dry weight tissue (3.0 mg) so that the relative intensities of the bands could be compared. The 63-kDa band was present in the surrounding tissue extracts at all three time points and a less intense band at 43 kDa was detected at days 42 and 84 (arrows). TGF-β was not detected in either the contralateral corneas at days 14 and 42 or the sham-operated corneas at days 14 and 84 and was minimal in the contralateral corneas at day 84. No bands were detected when extracts from the disc (normalized to 0.4 mg dry tissue) were run and probed on a separate blot (insert). The peptide was not detected when the primary antibody was omitted (not shown).

## DISCUSSION

In the past, keratoprosthetic devices have been designed that failed to support fibroplasia and the subsequent deposition of extracellular matrix proteins. The lack of integration with the host cornea may have been responsible for the complications that arose. We have previously demonstrated that cellular ingrowth into the interstices of a web does occur and is followed by collagen synthesis and deposition [Trinkaus-Randall et al., 1991, 1994]. This has now been shown by several other laboratories with other webs and other tissues and therefore seems to be a general tissue response [Legeais et al., 1992]. Our goal was to determine whether the deposition of collagen was accompanied by proteoglycan deposition, the other major matrix component synthesized by the stroma. Specifically, we wanted to determine whether the synthesis and accumulation of the GAGs resembled the well-documented wound-healing response.

Corneal wounds are associated with changes in the distribution and chemical properties of the proteoglycans and a reduction in the quantity of proteoglycans. In response to an injury, large chondroitin–dermatan sulfate proteoglycans are synthesized possessing glycosaminoglycan side chains with higher sulfation and an increased amount of iduronic acid. Keratan sulfate molecules synthesized are increased in size and have a lower sulfation [Funderburgh and Chandler, 1989; Funderburgh et al., 1988]. In addition, the ratio of keratan sulfate to chondroitin–dermatan sulfate has been shown to decrease after wounding [Cintron et al., 1990]. As the response of hyaluronic acid to injury is well documented, we examined its concentration at various times in the disc and surrounding tissue. After 14 days, hyaluronic acid was elevated in the surrounding tissue. However, it was not elevated within the disc until day 84. Other wound models analyzed histochemically

have detected hyaluronic acid in wounded rabbit stromas at 4–14 days postoperatively [Molander et al., 1993]. Regions a distance from the wound resembled that of the control. These experiments resemble those of other immunohistochemical data reporting that fibronectin is detected in the area surrounding the disc immediately after surgery and is not detected at later time points (data not shown). These results indicate that the response within the disc must allow for remodeling, and this is delayed, as cells must first enter the area. This model will provide information regarding the deposition of protein following an extensive wound in which cells must first migrate and then undergo extensive remodeling.

We examined whether the deposition of specific GAGs changed over time and whether this change mimicked a normal wound response. We found that by day 84 there was little difference in the distribution of charge density in the populations of newly synthesized GAGs. Coincident with this we demonstrated that the concentration of GAGs in the disc increased from 17% of control at 14 days to 54% of control after 84 days. At the same time points, the sulfated GAG present in the tissue surrounding the disc was 60% of control. By contrast, the sulfated GAG in the sham-operated corneas was 96% of control after 14 days and 60 ± 2% at 42 and 84 days. These results indicate that stromal keratocytes have migrated into the disc and synthesized glycosaminoglycans even though there is a delay in the wound response when the porous material is inserted into the interlamellar bed.

When the specific GAGs were examined, we found that in the disc dermatan sulfate was detected by day 14. The dermatan sulfate detected in the disc at days 14 and 42 was resistant to digestion with chondroitinase AC and may be the high iduronic acid type characteristic of corneal scars and of noncorneal connective tissue [Funderburgh and Chandler 1989; Anseth and Fransson, 1969]. By day 84, the chondroitin–dermatan sulfate susceptible to chondroitinase AC in the disc are present in proportions similar to those seen in the surrounding tissue.

Keratan sulfate is detected in the disc at day 42; by day 84, its relative abundance is similar to control corneas. It is possible that there is synthesis of nonsulfated keratan sulfate chains at day 14 in the disc that cannot be detected with the DMB assay or by $^{35}S$-$SO_4$ labeling. This form of keratan sulfate is characteristic of healing corneal wounds and has been detected in early corneal development and noncorneal keratan sulfate proteoglycans [Funderburgh et al., 1988, 1991; Hart, 1976]. The presence of both keratan sulfate and low iduronate chondroitin–dermatan sulfate in the disc at day 84 are indications that the profile of proteoglycan synthesis is beginning to resemble that of a normal cornea.

When the extracts were assayed for TGF-β using Western blot analysis, the results were intriguing. Intense bands were present in the surrounding tissue that were not present in the control or sham corneas. Nonspecific antibodies did not bind to TGF-β. Bands of similar molecular weight have been successfully competed with an excess of antigen [Gentry et al., 1987]. While the molecular masses (63 and 43 kDa) were not indicative of the active form of TGF-β these bands were similar in molecular mass to forms of TGF-β identified with Western blot analysis or immunoprecipitation [Wakefield et al., 1988; Keski-Oja et al., 1987; Lyons et al., 1988]. Our results indicate that there are two bands that stain specifically for TGF-β in the surrounding tissue.

These results are interesting as TGF-β is a potent mediator of proteoglycan synthesis and composition [Bassols and Massaque, 1988]. TGF-β influences proteoglycan production by regulating the expression of specific core proteins, by stimulating GAG synthesis and by increasing the length of GAG chains [Schonherr et al., 1993]. There is also considerable evidence that decorin is a negative-feedback regulator of TGF-β, since its core protein binds TGF-β and neutralizes its biological activity [Border et al., 1992]. The appearance of a 43-kDa form of TGF-β in the surrounding tissue at days 42 and 84 is coincident with increases in decorin and dermatan and chondroitin sulfates in this region. These data suggest that the increases may have inhibited the active form of the peptide. However, while a similar increase in decorin at 84 days is seen in the sham, the latent forms of the peptide are not detected in our assay. As we have not determined whether there are changes in the expression of decorin message at specific time points in our model, we cannot conclude from our data that decorin regulates TGF-β activity. It is possible that a significantly larger induction of proteoglycan synthesis than observed in our model is necessary for such a regulatory mechanism.

Our previous results combined with those presented here suggest that stromal keratocytes migrate from the adjacent regions into the interstices of the porous material. Collagen is deposited by day 28 [Trinkaus-Randall et al., 1991], and the synthesis and deposition of GAGs occurs at a later time. Both the sulfation and the specific GAGs resemble the control by day 84. These experiments demonstrate that it is possible to develop a wound model in which synthesis and deposition and the subsequent binding of lumican and decorin to collagen fibrils can be monitored in vivo.

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