Developmental Changes in Proteoglycans of Rabbit Corneal Stroma

John D. Gregory,* Shridhar P. Damle,* Henry I. Covington,† and Charles Cintron†

Proteoglycans have been extracted from rabbit corneal stromas at developmental stages from fetal to adult. Ion exchange fractionation and gel chromatography show that proteoglycans decrease in sulfation and relative amount and proteoglycans increase in sulfation and relative amount during development. There are small increases in size of all of the proteoglycans up to 2 weeks after birth, and the final adult composition is achieved by 8 weeks. Invest Ophthalmol Vis Sci 29:1413–1417, 1988

Since the achievement of corneal transparency is the outcome of the developmental changes that occur in the stroma, it is of interest to observe changes in macromolecular components that may contribute to this end. The proteoglycans (PG) of the stroma consist of proteoglycans sulfates (PKS) and proteoglycans sulfates, and they appear to play an important part in the organization of the connective tissue matrix.1 The term proteoglycan sulfate (PDS) will be used here because the glycosaminoglycans (GAG) of the PDS fractions have been shown2 to contain about 40% of their uronic acid as iduronic; the possibility that some PG molecules may contain chondroitin sulfate has not been excluded. The early, careful histochemical work of Smelser and Ozanics3,4 on rabbit corneal development, in which they used metachromatic staining, incorporation of labeled sulfate, and hyaluronidase susceptibility, set the stage for more detailed chemical analyses. Later studies have been mainly performed on rabbit, bovine and chick corneas, from which glycosaminoglycans (GAGs), which are the polysaccharide components of PGs, have been isolated.5–7 More recently, the intact PGs rather than the GAGs have been fractionated in a variety of ways.8,9 Monkey and human corneas have also been examined,9,10 and loss of transparency in the human disease, macular corneal dystrophy has been attrib-ututed to a metabolic defect in the synthesis of proteoglycan sulfates.10 A very intimate and specific interaction of the PGs with collagen fibrils is indicated by the electron microscopic studies of Scott and Haigh,11 and this is consistent with the long-standing idea that the uniform spacing of collagen fibrils, which is essential to transparency, is regulated by the PGs.

Changes in collagen type and content have been reported during development of bovine12 and rabbit13 corneas. In chick corneas, changes in the GAGs have been observed5 and also changes at the PG level in the chick14 and in the rabbit15 by immunohistochemistry, but little has been done by chemical analysis of PGs in mammalian corneal development. In this report, some data are given on changes in the PGs of rabbit corneal stroma as observed by use of a fractionation scheme previously described.2

Materials and Methods

Materials

New Zealand White rabbits, about 3 kg, were bred at the Eye Research Institute (Boston, MA). At fetal age of 24 days, at birth, and at 2 and 8 weeks and 6 and 12 months, they were killed by intraperitoneal pentobarbital injection, and corneas were dissected and kept at — 20°C. All animal care and treatment were in compliance with the ARVO Resolution on the Use of Animals in Research.

Chondroitinase ABC (Proteus vulgaris) was obtained from Miles Laboratories (Elkhart, IN), and endo-β-galactosidase (keratanase)16 was a gift from Dr. Y.-T. Li, Tulane University Medical Center, New Orleans, Louisiana. Papain, twice crystallized, was from Worthington (Freehold, NJ). Sepharose CL-4B, DEAE-Sepharose CL-6B, and Sephadex G-50 were from Pharmacia (Piscataway, NJ), and Bio-Gel P-2 from Bio-Rad (Rockville Center, NY). Guanidine-
Concentration of NaCl (M) in eluent

Fig. 1. Chromatography of extracts of rabbit corneal stromas of various ages on DEAE-Sepharose CL-6B eluted with increasing concentrations of NaCl in buffered 7 M urea. Several points (not fetal) were repeated with similar results. Fractions were analyzed for glucosamine (open bars) and galactosamine (cross-hatched bars).

HC1 (Grade I), benzamidine-HCl, and 6-aminohexanoic acid were from Sigma (St. Louis, MO). Urea (A.C.S., Fisher, Fair Lawn, NJ), as an 8 M solution, was passed through a Barnstead high-capacity deionizing cartridge just before use.17 All other chemicals were reagent grade.

Extraction of Proteoglycans

For extraction, the corneas (except the fetal) were scraped to remove epithelium and endothelium, cut into small pieces and weighed. Eighteen fetal corneas were used, eight at each intermediate age, and four at 1 year. The total wet weights varied from 90-330 mg at various ages. They were gently stirred overnight at 4°C with ten volumes of 4 M guanidine-HCl, 50 mM sodium acetate, pH 5.8, containing 100 mM 6-aminohexanoic acid, 10 mM EDTA and 5 mM benzamidine-HCl as protease inhibitors.18 The extraction was repeated twice, and the combined extracts were dialyzed three times overnight against ten volumes of 7 M urea, 50 mM Tris-HCl, 50 mM EDTA, pH 7.0. For estimation of extraction efficiency, each residue was digested with papain (100:1 w/w) in 0.5 M sodium acetate, 0.1 M potassium phosphate, 10 mM EDTA, 10 mM dithiothreitol, pH 6.9, at 64°C overnight, and dialyzed for hexosamine analysis as a measure of unextracted PG.

Chromatography

Each extract was passed through a column (0.6 ml bed volume) of DEAE-Sepharose CL-6B equilibrated in the buffered urea described above. Elution was done stepwise with five-column volumes each of 0 M, 0.25 M, 0.5 M, 1.0 M and 2.0 M NaCl in buffered urea.2 Effluent fractions were diazylized against water for hexosamine analysis, or in some cases for concentration and gel chromatography.

A column (0.7 X 10 cm) of Sepharose CL-4B was equilibrated in 4 M guanidine-HCl, 50 mM sodium acetate, pH 5.8, and used for analysis of some of the above PGs. Fractions of 1 ml were collected, dialyzed against water and used for hexosamine analysis.

For analysis of enzyme digests, a column (0.6 X 39 cm) of Sephadex G-50, fine, was equilibrated in 0.2 M acetic acid-pyridine, pH 5.0. Pooled fractions corresponding to the void volume (V0, defined by chromatography of Blue Dextran), the intermediate zone, and the total volume (Vt, defined by glucurone) were collected. The pools were evaporated, evacuated in a dessicator over NaOH and H2SO4 to remove pyridine, and analyzed for hexosamines.

Analyses and Enzymatic Methods

Hexosamines were determined on a modified Technicon TSM amino acid analyzer (Technicon Instrument Corp., Tarrytown, NY) after hydrolysis of the sample in 4 M HCl at 100°C for 8 hr,19 and uronic acid was determined by a variation of the carbazole method.20 Digestion with chondroitinase ABC (0.05 unit/mg of substrate) was done in 0.1 M sodium acetate, 0.1 M Tris-HCl, pH 7.3, at 37°C overnight.21 Endo-β-galactosidase was used at 1 unit/mg of substrate in 0.05 M sodium acetate, pH 6.0, at 37°C overnight.

Appropriate PG fractions from ion exchange chromatography were dialyzed against water, concentrated, and digested either with chondroitinase ABC or with endo-β-galactosidase. The digest was analyzed by passage through the column of Sephadex G-50, as above. The percent of galactosamine of or of glucosamine, respectively, that moved from the V0 zone to a retarded position in the column is reported as the percent digested.

Results

Extraction and Chromatography

All extractions of corneal stromas yielded more than 80% of the glucosamine and more than 87% of the galactosamine. When the extracts were fractionated by ion exchange chromatography on DEAE-Sepharose CL-6B, the patterns shown in Figure 1 were obtained. In the PG fractions (0.5 M and 1.0 M
NaCl), galactosamine is a measure of PDS, as shown by the fact that the galactosamine-containing material can be completely digested to small fragments by treatment with chondroitinase ABC. The glucosamine is largely contained in PKS, which is digestible by endo-β-galactosidase (keratanase), but a small amount of the glucosamine represents oligosaccharide groups that are part of both PG structures. In the case of mature corneas, almost all of the PGs emerge at NaCl concentrations of 0.5 M and 1.0 M; the glucosamine that emerges earlier probably represents unrelated glycoproteins and hyaluronic acid.

If the contents of hexosamines in the 0.50 M and 1.0 M NaCl eluates (Fig. 1) are summed and expressed as μmol per cornea instead of per wet weight, the curves shown in Figure 2 appear. This clearly illustrates the reversal in the ratio of PKS to PDS that occurs at 2 weeks. It also emphasizes the overall growth of the corneas that is taking place during the experimental period and beyond, a change that is not apparent when the data are expressed relative to wet weight (Fig. 1).

Of the total PKS from fetal or newborn corneas, about 30% was eluted by 0.25 M NaCl compared to 3% at 1 year. In contrast, the amount eluted by 1.0 M NaCl increased from less than 10% at the early stages to 70% at 1 year. In the case of PDS, there was a small reduction in the proportion that was eluted by 1.0 M NaCl over the time span, but a large increase in the amount eluted at 0.50 M relative to 1.0 M NaCl. There was also a large increase in the total amount of glucosamine relative to galactosamine during development. Thus the overall ratio of PKS to PDS increased from 0.34 at the fetal stage to 1.7 at 2 weeks and thereafter.

The PG fractions from ion exchange chromatography were further analyzed by gel chromatography on Sepharose CL-4B in a dissociating solvent, as shown in Figure 3. Analysis for uronic acid, which is present only in PDS, gives curves (not shown) exactly corresponding to the galactosamine. It can be seen that at all ages the PKSs are smaller than the PDSs, and that the sizes of the PGs change after birth, but are constant after about 2 weeks.
by which time the eyes have opened and the corneas
the adult pattern appears by the 8th week. In addi-
charge density. After birth a shift is seen toward the
(Fig. 3) indicates that most of the PKS and PDS are of
the large proportion eluted by 1.0 M NaCl at the early
sulfate with a low content of sulfate.16 The proportion
degraded, as given in Table 1, shows that 80% or
more of the material eluted at 0.25 M NaCl is digest-
ible and hence is a low-sulfated PKS.
In the case of the galactosamine-containing PG,
the large proportion eluted by 1.0 M NaCl at the early
stages was characterized by treatment with chondroi-
tinase ABC. After digestion and chromatography on
Sephadex G-50, all of the galactosamine was found in
small fragments, indicating that this fraction was
composed entirely of PDS.

### Enzymatic Analyses

Most of the glucosamine-containing components
at early stages (fetal and newborn) are eluted at lower
NaCl concentrations than would be expected of PGs
with a normal, adult degree of sulfation. In order to
confirm that these are a form of PKS and not HA,
material from these fractions was digested with an
endo-β-galactosidase that is active toward keratan
sulfate with a low content of sulfate.16 The proportion
degraded, as given in Table 1, shows that 80% or
more of the material eluted at 0.25 M NaCl is digest-
able and hence is a low-sulfated PKS.

In the case of the galactosamine-containing PG,
the large proportion eluted by 1.0 M NaCl at the early
stages was characterized by treatment with chondroi-
tinase ABC. After digestion and chromatography on
Sephadex G-50, all of the galactosamine was found in
small fragments, indicating that this fraction was
composed entirely of PDS.

### Discussion

It is clear from the elution patterns of stromal PGs
(Fig. 1) that there are prominent changes during de-
velopment. Our results suggest that PKS in fetal cor-
nea is undersulfated and present in low quantities
relative to PDS. Postnatally, the charge density of
PKS and its relative quantity increase, reaching the
adult level between the 2nd and 8th week after birth,
by which time the eyes have opened and the corneas
have become transparent.

Almost all of the PDS in fetal cornea has a high
charge density. After birth a shift is seen toward the
deposition of PDS with a lower charge density, and
the adult pattern appears by the 8th week. In addi-
tion, gel chromatography of newborn corneal PGs
(Fig. 3) indicates that most of the PKS and PDS are of
smaller size than they are at 2 weeks and beyond.

The increase in PKS and PDS content of the tissue
that occurs during development results from growth
and not from a progressive loss of water, and is ac-
companied by a marked change in the ratio of PKS to
PDS during the first 8 weeks of life (Fig. 2). The ratio
of PGs then remains constant during further growth
to the adult state.

Differences in the tissue distribution and water ab-
sorption properties of the two GAGs of bovine cor-
nea have been seen,22 and it has been hypothesized
that these properties play a role in the maintenance of
normal corneal hydration. The question arises
whether the water-absorptive and water-retentive
properties of the PGs in neonate corneas are similar
to those in the adult, even though the proportions
and charge densities of PGs differ at these two stages.
Immunohistochemical studies in our laboratory have
indicated the presence of both chondroitin (or der-
matan) 6-sulfate and 4-sulfate in developing rabbit
corneas (unpublished). Moreover, chondroitin-6-sul-
fate has been shown to have absorptive and retentive
properties similar to those of adult keratan sulfate.23
Possibly the neonatal PG mixture, containing both
chondroitin (or dermatan) 6-sulfate and 4-sulfate,
can compensate for the lower quantities of PKS and
can maintain normal adult hydration.

Although developmental events in chick cornea
may not be completely analogous to those in rabbit
cornea, it may be mentioned that Conrad6 and Hart7
also found that a keratan sulfate is synthesized at a
very early stage and that its degree of sulfation in-
creases in the course of maturation in chick. Hart24
suggested that a lack of sulfate donor might account
for the low sulfation of PKS during early develop-
ment. In rabbit, however, this is not likely to be the
case because the early formation of highly sulfated
PDS indicates that the sulfate donor must have been
present in sufficient amount.

To be in accord with the various chemical and his-
tochemical observations of rabbit corneal develop-
ment made over 30 years, a sequence may be sug-
gested as follows. Very early during development,
PDS with a high charge density is the major PG in the
stroma, with considerably smaller amounts of a low-
sulfated PKS.6,7,15 PGs of higher sulfation then in-
crease from the posterior to the anterior portion of the
cornea.3,4 At birth a mixture of small and adult-
size PGs is present. The highly charged PDS observed
by us and by Anseth is accompanied by increasing
amounts of more highly sulfated PKS, as we and
Smelser3,4 have seen. PGs of normal adult size are
present by 2 weeks after birth, but the ratio of PKS to
PDS has not yet reached its adult value (Fig. 2). At
this time PDS of lower charge is also being deposited.
By about the 3rd week after birth, metachromasia
and sulfate incorporation become uniform through-
out the stroma, and the mature balance of PG quan-
tity and charge is present by 8 weeks (this report).

Although changes in the relative amounts of the

### Table 1. Susceptibility of PG fractions to digestion
by endo-β-galactosidase (see Methods)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn, 0.25 M-NaCl eluate</td>
<td>80</td>
</tr>
<tr>
<td>Newborn, 0.50 M-NaCl eluate</td>
<td>88</td>
</tr>
<tr>
<td>Newborn, 1.00 M-NaCl eluate</td>
<td>100</td>
</tr>
<tr>
<td>2-week-old, 0.25 M-NaCl eluate</td>
<td>86</td>
</tr>
<tr>
<td>2-week-old, 0.50 M-NaCl eluate</td>
<td>86</td>
</tr>
<tr>
<td>1-year-old, 0.50 M-NaCl eluate</td>
<td>86</td>
</tr>
<tr>
<td>1-year-old, 1.00 M-NaCl eluate</td>
<td>88</td>
</tr>
</tbody>
</table>
PGs and in their degree of sulfation are major events during corneal development, we do not yet know whether other properties may change. Altered control of posttranslational steps might change the nature and quantity of the oligosaccharide groups or change the iduronic acid content of the dermatan sulfate, for example. Continuing study of the rabbit cornea will include radiolabeling of the PGs at early stages, by established methods,\(^\text{25}\) in organ culture or in vivo, for added analytical sensitivity. Molecular cloning of cDNA is also being undertaken by us to furnish probes for in situ hybridization of the protein cores of the PGs for a more detailed analysis of their sequence of appearance and for identification of the cell types that synthesize them during development. Such analyses will also be useful in studying corneal wound healing, since similar progressive changes in PG composition have been seen.\(^\text{26}\)

**Key words:** corneal stroma, development, proteoglycan, rabbit

**References**