Differential Relative Sulfation of Keratan Sulfate Glycosaminoglycan in the Chick Cornea during Embryonic Development

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PURPOSE. To investigate structural remodeling of the developing corneal stroma concomitant with changing sulfation patterns of keratan sulfate (KS) glycosaminoglycan (GAG) epitopes during embryogenesis and the onset of corneal transparency.

METHODS. Developing chick corneas were obtained from embryonic day (E)12 to E18 of incubation. Extracellular matrix composition and collagen fibril spacing were evaluated by synchrotron x-ray diffraction, hydroxyproline assay, ELISA (with antibodies against lesser and more highly sulfated KS), and transmission electron microscopy with specific proteoglycan staining.

RESULTS. A significant relative increase in highly sulfated KS epitope labeling occurred with respect to hydroxyproline content in the final week of chick development, as mean collagen interfibrillar distance decreased. Small KS PG filaments increased in frequency with development and were predominantly fibril associated.

CONCLUSIONS. The accumulation of highly sulfated KS during the E12 to E18 timeframe could serve to fine tune local matrix hydration and collagen fibril spacing during corneal growth, as gross dehydration and compaction of the stroma progress through the action of the nascent endothelial pump. (Invest Ophthalmol Vis Sci. 2010;51:1365–1372) DOI:10.1167/iovs.09-4004

The cornea is a connective tissue with remarkable transparency that forms the primary refractive surface of the eye; yet, how this property arises in development remains little understood. The most widely studied model of corneal development is that of the embryonic chicken. In this tissue, orthogonal fibril bundles that later form into lamellae. At embryonic day (E)14, the chick cornea transmits only approximately 40% of white light, but then begins to increase in transparency so that, at E19, transmission is more than 95%, similar to the adult condition. The transparency increase after E14 is accompanied by significant dehydration of the cornea, flattening of keratocytes, and compaction of stromal collagen fibrils into a spatially ordered array. This restructuring of the stroma is key for the acquisition of corneal transparency.

Small leucine-rich proteoglycans (PGs) interact with collagen fibrils in the corneal stroma and are thought to help control fibril size and spatial organization. PGs are composed of a core protein covalently bound to sulfated glycosaminoglycan (GAG) side chains. The major GAG in the cornea is keratan sulfate (KS), and three types of PG bear these side chains: lumican, keratocan, and mimecan (or osteoglycin). By E18, in the chick stroma KS PGs bear two to three GAG chains of approximately 15 kDa each, and the PGs lumican and keratocan have three of five potential linkage sites variably substituted with KS GAGs. Collagen–PG interactions are thought to occur via the PG core protein, with sulfated GAG chains extending into the interfibrillar space, where they confer stromal water-binding and sorptive tendencies that help define the hydrophilicity and swelling properties, and thus the ultrastructure, of the stromal matrix.

Conical PGs, especially those carrying KS chains, fulfill an important role in the establishment of a properly formed stroma during corneal embryogenesis. The importance of these molecules is illustrated by the fact that the corruption of sulfate motifs on KS is associated with structural matrix alterations in the corneas of humans with the inherited disease macular corneal dystrophy, and in the corneas of mice with gene-targeted deletions in lumican or Chst5, a KS sulfotransferase enzyme. Some investigators have reported the accumulation of KS GAGs and KS PGs during embryonic development. Molecular studies have shown that levels of mRNA for keratocan, first expressed at E6, exhibit a steady decline from E9 to E18, whereas, over the same time period, lumican mRNA levels remain several-fold higher. Moreover, an approximate threefold increase in lumican core protein occurs between E7 and E9, with a two- to threefold increase in mRNA for beta-1,4-galactosyltransferase, an enzyme involved in sulfated KS synthesis, reported between E8 and E13. Biochemical analyses have also shown that KS is synthesized by the chick cornea between E5 and E7, but that it only becomes highly sulfated by E14, with another study indicating a switch in the production of unsulfated to sulfated KS between E12 and E15.

The current investigation was designed to test the hypothesis that sulfation of KS provides an environment of hydration conducive to the deposition of collagen fibrils and the establishment of a highly ordered stromal matrix in the developing chick. To this end, experiments were conducted to quantify
collagen biosynthesis, KS sulfation status, collagen fibril spacing, and collagen-KS PG associations during the latter stages of chick corneal morphogenesis.

Methods

Specimens

For x-ray diffraction and biochemical studies, a series of 69 embryonic chick corneas was obtained that comprised daily incremental stages from E12 to E18. Corneas were excised by an incision around the limbus in eyes of embryonic chicks obtained from Hamburger-Hamilton (HH)-staged, fertilized eggs collected from a commercial hatchery (Hy-Line UK, Warwickshire, UK) approximately 2 hours earlier, where E12 = HH38, E13 = HH39, E14 = HH40, E15 = HH41, E16 = HH42, E17 = HH43 and E18 = HH44. Immediately on excision, the corneas were sandwiched between layers of clingfilm to minimize dehydration and were placed on dry ice. Frozen corneas were transferred to −80°C storage until the synchrotron x-ray diffraction experiments could be conducted. For electron microscopy, a smaller group of fertilized eggs (Henry Stewart & Co. Ltd., Louth, Lincolnshire, UK) was incubated at 37°C until staging and excision. All work was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as with local ethics guidelines.

Synchrotron X-Ray Diffraction

Individual corneas (n = 69), still secured in cling film, were positioned in a sealed specimen holder between sheets of x-ray-transparent mylar where they were allowed to thaw. In turn, each cornea (n = 8-11 daily from E12 to E18) was then placed into the path of a focused (1 × 1 mm) monochromatic (λ = 0.154 nm) x-ray beam on beamline 2.1 of the Synchrotron Radiation Source, Daresbury Laboratory (Cheshire, UK). All corneas were exposed for 2 minutes each, and the resulting fiber diffraction patterns were recorded on a multiwire, gas proportional area detector plate positioned 8.25 m behind the cornea. Immediately after x-ray exposure, the corneas were refrozen and transported to the home laboratories for biochemical and immunological analyses (described in the following sections). Diffraction patterns (512 × 512 pixels) were analyzed with purpose-written software (Unix-based; Sun Microsystems, Mountain View, CA) and graphics and statistics packages (Unix; Optimas, Bothell, WA; Statistica; Statsoft, Tulsa, OK, Excel; Microsoft, Redmond, WA), as described previously. The first-order equatorial (i.e., interfibrillar) reflection, calibrated to the 67-nm axial D-periodic repeat of collagen in wet rat tail tendon, was used to provide a measure of the mean center-to-center collagen fibril Bragg spacing as an average throughout the whole thickness of the central cornea. Statistical significance was ascertained by using statistical tests for large data groups: one-way ANOVA with post-hoc Tukey HSD.

After x-ray analysis, the corneas were refrozen for transportation to the home laboratory and digested for 17 hours at 60°C with 1 mg/mL papain (Sigma-Aldrich, Poole, UK) in 0.05 M sodium acetate buffer (pH 5.6), containing 0.025 M EDTA and 5 mM cysteine HCl. The enzyme was inactivated at 100°C, and the digests stored at −20°C until further analysis.

Hydroxyproline Assay

Papain digests from individual corneas were hydrolyzed by using equal volumes of 11.7 N concentration HCl to supernatant at 110°C overnight. Specimens were then lyophilized. Dried hydrolysates were assayed in triplicate as previously described, against known standards and read at 540 nm after 10 to 20 minutes incubation at 70°C. Statistical significance was ascertained by using statistical tests for large data groups: one-way ANOVA with post-hoc Tukey HSD.

KS Quantification by Competitive ELISA

Optimized, competitive ELISAs were developed to quantify pentasulfated hexaxacharides and tetrasulfated hexaxacharides as the smallest linear structures in KS chains, as recognized by the monoclonal antibodies 5D4 and 1B4, respectively. Ninety-six-well EIA microtiter plates (MP Biomedicals, Cambridge, UK) were coated by passive adsorption with a 250 ng/mL chondroitinase ABC-digested bovine articular cartilage aggrecan (BAC ABC core) antigen in a 20-mM sodium carbonate coating buffer (pH 9.6), for 14 hours at 37°C. Native aggrecan core protein is substituted with both chondroitin sulfate (CS) and KS chains, and CS was selectively removed by chondroitinase digestion to leave a KS-linked coating protein. The plates were washed with Tris saline azide (TSA) and the unreacted sites blocked with the addition of 1% (wt/vol) bovine serum albumin (BSA) in TSA. All incubations were performed for 1 hour at 37°C.

Papain digests from single corneas were serially diluted and allowed to bind with an equal volume of 5D4 (1:8000 dilution in 1% BSA/TSA) and incubated to compete against the BAC ABC core. A standard curve was generated from serial dilutions of BAC ABC core/5D4. The plates were then washed with TSA before incubation with alkaline phosphatase–conjugated goat anti-mouse antibody (1:5000 dilution; Promega, Madison, WI). The plates were again washed before alkaline phosphatase substrate (p-nitrophenyl phosphate, 1 mg/mL) was applied in DEA buffer (0.126 mM MgCl₂, 1 mM diethanolamine, pH corrected to 9.8). Color development was quantified on a plate reader (Multiskan MS; Labsystems, Helsinki, Finland) at 405 nm, to determine the inhibition of binding.

The same stack corneal extracts were then assayed for lesser sulfated KS by using the 1B-4 monoclonal antibody. Optimization of conditions established that microtiter plates were coated with 125 ng/mL BAC ABC core and ELISAs performed as described above, with a 1:4000 1B-4 dilution. Statistical significance was ascertained with statistical tests for large data groups: one-way ANOVA with post-hoc Tukey HSD.

Electron Microscopy

On excision, corneas from E12 to E18 chicks (n = 21) were prepared for electron microscopy, as described previously. A series of E12 to E18 corneas was fixed in 2.5% glutaraldehyde in 25 mM sodium acetate buffer with 0.05% cuprophanic blue (0.1 M MgCl₂; pH 5.7), to stain the sulfated PGs. Another series was fixed for 10 minutes in 4% paraformaldehyde (pH 7.2). These corneas were then washed twice in chondroitinase buffer (50 mM Tris, [pH 8], 60 mM sodium acetate, and 0.02% BSA) and quartered. Corneal pieces were incubated for 4 hours at 37°C in chondroitinase buffer containing 2.5 U/mL chondroitinase ABC (from Proteus vulgaris; Sigma-Aldrich) to remove the chondroitin-dermatan-sulfate GAG chains from the tissue. Commercially prepared protease inhibitor cocktail for general use (Sigma-Aldrich) was also added to 1% total volume to inhibit endogenous PG-degradative activity. The control corneas were similarly processed in buffer minus enzyme. The samples were washed in chondroitinase buffer (minus enzyme) before fixation and staining overnight, as before. All corneas were then contrast enhanced with sodium tungstate and dehydrated and embedded in Araldite, according to standard protocols. Ultrathin sections (~90-nm thick) were cut on glass knives, collected on copper grids, and stained with 1% aqueous phosphotungstic acid and saturated aqueous uranyl acetate before examination by transmission electron microscopy (JEM-1010; JEOL, Tokyo, Japan) equipped with an 11-megapixel CCD camera (Orius SC1000 CCD camera; Gatan Ltd., UK, Cambridge, UK).

Results

Mesenchymally derived presumptive keratocytes that populate the developing chick cornea are responsible for establishing the secondary stroma by synthesizing and depositing hybrid collagen type I/IV fibrils. Quantification of collagen as hy-
**TABLE 1.** Average Amount of Hydroxyproline and Average Collagen Interfibrillar Spacing in Embryonic Chick Corneas

<table>
<thead>
<tr>
<th></th>
<th>E12 (n = 8)</th>
<th>E13 (n = 10)</th>
<th>E14 (n = 11)</th>
<th>E15 (n = 10)</th>
<th>E16 (n = 11)</th>
<th>E17 (n = 10)</th>
<th>E18 (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline, μg/mL</td>
<td>4.38 ± 0.18</td>
<td>8.06 ± 1.07</td>
<td>7.43 ± 0.48</td>
<td>17.48 ± 1.46</td>
<td>17.23 ± 0.74</td>
<td>34.52 ± 4.11</td>
<td>34.69 ± 1.64</td>
</tr>
<tr>
<td>Collagen fibril Bragg spacing, nm</td>
<td>60.8 ± 0.60</td>
<td>63.1 ± 1.01</td>
<td>63.9 ± 0.99</td>
<td>61.9 ± 1.22</td>
<td>59.8 ± 1.53</td>
<td>57.0 ± 0.87</td>
<td>53.7 ± 0.94</td>
</tr>
</tbody>
</table>

Embryonic days E12 to E18 are equivalent to Hamburger-Hamilton stages 38 to 44 of development. Hydroxyproline measurements are also representative of total collagen content per cornea. Data are expressed as the mean ± SE.

**DISCUSSION**

The changing sulfation patterns of KS in the developing chick cornea raise several questions regarding the likely functional role of this molecule with respect to its sulfation status. Sulfa-

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**TABLE 2.** The Average Amounts of 1B4- and 5D4-Labeled KS Epitope Relative to BAC-ABC Core after Normalization to Hydroxyproline Content in Embryonic Chick Corneas

<table>
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<th>E17 (n = 10)</th>
<th>E18 (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesser sulfated KS (1B4-labeled epitope/hydroxyproline)</td>
<td>0.125 ± 0.064</td>
<td>0.128 ± 0.032</td>
<td>0.153 ± 0.046</td>
<td>0.129 ± 0.024</td>
<td>0.109 ± 0.016</td>
<td>0.114 ± 0.022</td>
<td>0.118 ± 0.024</td>
</tr>
<tr>
<td>Highly sulfated KS (5D4-labeled epitope/hydroxyproline)</td>
<td>0.775 ± 0.261</td>
<td>0.958 ± 0.152</td>
<td>0.865 ± 0.160</td>
<td>0.840 ± 0.111</td>
<td>1.879 ± 0.167</td>
<td>1.449 ± 0.143</td>
<td>1.874 ± 0.206</td>
</tr>
</tbody>
</table>

Data are the mean ± SE. Embryonic days E12 to E18 of development are equivalent to Hamburger-Hamilton stages 38 to 44.
Figure 1. Transmission electron micrographs of developing chick corneal stroma. Tissue compaction progressed from loose bundles of collagen fibrils at E12 to a mature lamellar form at E18. Scale bar, 1 μm.
FIGURE 2. Transmission electron micrographs of chondroitinase digested developing chick corneal stroma. Increased deposition of cuprolinic blue-stained sulfated KS PG filaments occurs in the E12–E18 timeframe. Scale bar, 1 μm.
tion of individual corneal KS chains is not homogeneous, and unsulfated disaccharides, which are predominant nearer the linkage region, become increasingly sulfated toward the non-reducing terminal. Moreover, KS chains undergo sequential elongation and sulfation during synthesis. The absence of sulfotransferase enzyme in human corneas with macular corneal dystrophy, as in mutant corneas of Chst5-knockout mice, results in an immature, truncated GAG and an atypical corneal phenotype. Thus, KS chain length and sulfation are dictated by tissue-specific factors such as the presence of processing enzymes. During chick development, galactose-transferase activity increases with KS biosynthesis and is maintained at an unusually high level in adult cells. The sequential mechanism of GAG subunit sulfation appears consistent with the absence of the sulfotransferase enzyme, when neither GlcNAc nor Gal residues are sulfated. The negative charge provided by sulfation could serve to prevent KS GAG chain collapse during elongation and perhaps offer an explanation of the differential sulfation between shorter and longer KS chains. The presence of increasing amounts of highly sulfated KS during embryonic development is possibly linked to the appearance of the sulfate donor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS). The measured elevation of 5D4 labeling at the time of increased fibrillogenesis and matrix compaction could be a result of PAPS availability facilitating increases in KS chain length and greater sulfation. The combination of these events could enhance organized stroma formation through the binding of core proteins and the hydrophilic influence of sulfate groups.

A major purpose of the present study was to investigate the structural remodeling of the stroma in relation to changing sulfation patterns of constitutive KS epitope during chick corneal morphogenesis. The results confirm previous synchrotron x-ray diffraction studies that documented the progressive compaction of corneal collagen fibrils with embryonic growth in the chick. In the dataset examined herein, compaction occurred after E14 (Table 1), with electron microscopy indicating that the compaction happened within bundles of collagen fibrils in which fibrils are already fairly well organized spatially (Fig. 1). The x-ray diffraction and electron microscopy findings, when considered together, imply that collagen bundles are coalescing into lamellae, whereas, at the same time, fibrils within bundles are becoming more closely spaced. Between E15 and E16, the stroma experiences a significant increase in highly sulfated KS epitope that is disproportionate to the measured increase in hydroxyproline (Table 2). This observation is illustrative of an accelerated deposition of highly sulfated KS post-E15 which exceeds the anticipated in ovo corneal growth rate, as implied by hydroxyproline deposition. Thus, as the chick cornea develops and becomes transparent and compacted, the nature of KS antigenicity changes, with the highly sulfated KS fraction accumulating preferentially over that of the lesser-sulfated isoform and from a higher starting point. It seems counterintuitive to reason that negatively charged, highly sulfated KS GAG would accumulate as the stroma condenses and act as a biological driving force for tissue dehydration. Quite the opposite: The water-retentive properties of this hydrophilic macromolecule would be expected to swell the corneal matrix, rather than to aid in its compaction. Indeed, the current data show that the stroma was already undergoing compaction when the cornea, at E15, experienced the first sizable increase in highly sulfated KS epitope.

This result suggests that the emergence of more highly sulfated KS GAG in the chick cornea as it matures in ovo is not a driving force correlating with the homogeneous compaction of collagen fibrils in the coalescing collagen bundles and developing stromal lamellae and perhaps points to the influence of other modulating factors that help drive the structural remodeling of the developing corneal stroma in the week before hatching. Activity of the bicarbonate pump in the corneal endothelium is critical for controlling corneal hydration and thickness and thus for maintaining transparency in the adult eye. It is also likely to be important in stromal water regulation and compaction in the developing cornea. However, the precise stage at which the endothelial pump mechanism becomes active is unknown. Histologic investigations have shown that an intact endothelial monolayer is established by E5 to E6.

Stromal fluid at this time is mostly immobilized by hyaluronic acid.
acid; later, small leucine-rich PGs, particularly KS PGS most likely influence the water-binding characteristics of the stroma. Conrad et al. 18,19 studying gene expression associated with chick corneal development, including those genes involved in endothelial transport, found 5- to 10-fold increases in sodium-potassium ATPase transporter, sodium bicarbonate transporter, and carbonic anhydrase II (CA2), alongside increases in KS gene expression from E9 to E16. CA2 in particular, through increased transport of Na⁺/HCO₃⁻ and Cl⁻ would be expected to drive thinning of the cornea. Perhaps the accumulation of oversulfated KS during this time serves to maintain local collagen hydration, as gross dehydration and compaction progress through the action of the nascent endothelial pump.

References

35. Akama TO, Nakayama J, Nishida K, et al. Human corneal GlcNac 6-O-sulfotransferase and mouse intestinal GlcNac 6-O-sulfotrans-


