β-D xyloside alters dermatan sulfate proteoglycan synthesis and the organization of the developing avian corneal stroma

RITA A. HAHN and DAVID E. BIRK

Robert Wood Johnson Medical School, Department of Pathology, 675 Hoes Lane, Piscataway, New Jersey 08854-5635, USA

*To whom correspondence should be addressed

Summary

Corneal transparency is dependent upon the development of an organized extracellular matrix containing small diameter collagen fibrils with regular spacing, organized as orthogonal lamellae. Proteoglycan-collagen interactions have been implicated in the regulation of collagen fibrillogenesis and matrix assembly. To determine the role of dermatan sulfate proteoglycan in the development and organization of the secondary corneal stroma, its synthesis was disrupted using β-D xyloside. The secondary corneal stroma contains two different proteoglycans, dermatan sulfate and keratan sulfate proteoglycan. β-D xyloside interferes with xylose-mediated O-linked proteoglycan synthesis, and thus disrupts dermatan sulfate proteoglycan synthesis. Corneal keratan sulfate proteoglycan, a mannose-mediated N-linked proteoglycan, should not be altered. Biochemical analysis of corneas treated both in vitro and in ovo revealed a reduced synthesis of normally glycosylated dermatan sulfate proteoglycans and an increased synthesis of free xyloside-dermatan sulfate glycosaminoglycans. Keratan sulfate proteoglycan synthesis was unaltered in both cases. Corneal stromas were studied using histochemistry and electron microscopy after in ovo treatment with β-D xyloside. The observed biochemical alterations in dermatan sulfate proteoglycans translated into disruptions in the organization of β-D xyloside-treated stromas. There was a reduction in the histochemical staining of proteoglycans, but no alteration in collagen fibril diameter. In addition, focal alterations in collagen fibril packing, and a disruption of lamellar organization were observed in β-D xyloside-treated corneas. These data suggest that dermatan sulfate proteoglycans are not involved in the regulation of corneal collagen fibril diameter, but are important in the fibril-fibril spacing as well as in lamellar organization, and cohesiveness.

Key words: cornea, stroma, proteoglycans, development, matrix organization, proteoglycan-collagen interactions, β-D xyloside.

Introduction

Corneal strength and transparency are dependent upon the development and maintenance of an organized extracellular matrix, including uniformly small diameter collagen fibrils with a consistent interfibrillar spacing. These regularly packed fibrils are organized into lamellae with adjacent layers approximately perpendicular to one another. In early avian development, an acellular primary stroma consisting of orthogonally arranged heterotypic type I and type II collagen fibrils (Trelstad and Coulombre, 1971; Linsenmayer et al., 1977, 1990; Hay, 1980) and dermatan sulfate proteoglycan (DS PG) is deposited by the corneal epithelium (Bansal et al., 1989). On day 5, this matrix is invaded by the neural crest-derived presumptive corneal fibroblasts which synthesize the secondary stroma (Hay, 1980). This secondary stroma is composed of heterotypic type I and type V collagen fibrils (Birk et al., 1988), beaded filaments of type VI collagen (Linsenmayer et al., 1986, 1990) and proteoglycans (PG). The secondary corneal stroma contains two classes of small, fibril-associated proteoglycans: one with dermatan sulfate/chondroitin sulfate side chains (DS PG) which has been shown to be decorin, and a second with keratan sulfate side chains (KS PG) which has recently been termed lumican (Hart, 1976; Funderburgh et al., 1986; Midura and Hascall, 1989; Blochberger et al., 1992). On day 14, the stroma begins to dehydrate and compact (Hay, 1980). Transparency develops, provided the stromal matrix is properly organized.

Morphological studies have shown both DS and KS PGs to be associated with specific bands of corneal collagen fibrils (Scott and Haigh, 1985, 1988). These data indicate that proteoglycan-collagen interactions may have a role in corneal fibril assembly, matrix organization and ultimately corneal transparency. Studies perturbing DS PG synthesis in other systems, suggest that DS PG plays a role in various aspects of development. These include feather pattern formation...
in chick embryo skin (Goetinck and Carlone, 1988), mesenchymal migration (Lane and Solursh, 1991), as well as branching morphogenesis in the lung (Smith et al., 1990) and kidney (Platt et al., 1987; Leloint et al., 1988). In these perturbation studies, β-D xyloside was used as a competitive site for the initiation of DS and/or CS GAG chain biosynthesis. This results in an under- or non-glycosylated core protein coupled with an increase in free xyloside-GAG chain (Okayama et al., 1973; Schwartz et al., 1974; Galligani et al., 1975).

In the secondary corneal stroma, fibrillogenesis is influenced by the collagen type I and type V composition (Birk et al., 1990; Linsenmayer et al., 1990). Corneal PGs also have been shown to influence the kinetics of fibril formation in vitro (Birk and Lande, 1981). In addition, collagen-proteoglycan interactions have been shown to affect both the rate of fibril formation as well as final fibril morphology in a variety of in vitro systems (Vogel et al., 1984; Vogel and Trotter, 1987; Rada et al., 1991).

In this report, DS PG synthesis is disrupted using β-D xyloside during secondary stromal development to determine the role of this PG in stromal matrix organization. β-D xyloside interferes with xylose-mediated O-linked PG synthesis and thus disrupts DS PG synthesis. Corneal KS PG, a mannoside-mediated N-linked PG, should remain unaltered by β-D xyloside treatment. Therefore, perturbation with β-D xyloside permits the elucidation of the role of DS PGs in corneal stromal development.

Materials and methods

β-D xyloside stock

β-D xyloside (p-nitrophenyl β-D xylopyranoside) was solubilized in 95% ethanol and mixed with 2× phosphate-buffered saline (PBS) pH 7.4, for a final concentration of 20 mg/ml. An aliquot was added, along with [35S]H2SO4 (ICN Radiochemicals), to corneal organ cultures or injected into the air sac of fertilized white leghorn chicken eggs. Control corneas and chick embryos received comparable volumes of ethanol:PBS. The methylumbreiferyl β-D xyloside analog was dissolved in DMSO alone. This analog was not used in vivo due to its insolubility at the concentrations required for these experiments.

Organ culture

Corneas were excised from 17-day chicken embryos, rinsed in PBS with antibiotics (0.1 mg/ml gentamicin, 0.005 mg/ml fungizone), and cultured in Dulbecco’s Modified Eagle Medium containing 0.5% fetal bovine serum, 0.05 mg/ml gentamicin, 2.5 μg/ml fungizone, 0.05 mg/ml ascorbate and 0.15% sodium bicarbonate. Corneas were pre-incubated with or without β-D xyloside at 37°C with 5% CO₂. After 4 hours this medium was replaced with fresh medium containing 50 μCi/ml [35S]H2SO4. Treated corneas had β-D xyloside added to their medium at final concentrations between 0.01 and 5.0 mM. Untreated corneas had comparable volumes of vehicle added to their medium. After an 18 hour incubation, the corneas were rinsed in PBS, the central corneas isolated using a 3 mm dermal punch, and the proteoglycans extracted in 4 M guanidine, 50 mM sodium acetate extraction buffer with N-ethylmaleimide, benzamidine and Na₂EDTA, pH 5.8 (Oegema et al., 1975; Birk et al., 1981).

In ovo

White leghorn chicken embryos were incubated at 37°C, in a humidified atmosphere. β-D xyloside and 35S were injected into the air sac of 10-, 12- and 14-day embryos during the development of the secondary stroma according to the following schedule: day 10, 2.8 mmoles β-D xyloside, 100 μCi 35S; day 12 and 14, 11.1 mmoles β-D xyloside, 200 μCi 35S. This resulted in 50-75% viable embryos at day 17 of development. Control chick embryos were injected with comparable quantities of the 95% ethanol:PBS and 35S without xyloside according to the same schedule. This resulted in 90-100% viable embryos at day 17. On day 17, both treated and untreated central corneas were isolated as described above.

Proteoglycan extractions

In ovo and organ-cultured corneas were minced and their proteoglycans extracted in 4 M guanidine-HCl, 50 mM sodium acetate, 10 mM Na₂EDTA, 10 mM benzamidine, 10 mM N-ethylmaleimide, 0.1 M. Me-amino-n-caproic acid, pH 5.8. The tissues were extracted in 2 ml/cornea at 4°C for 24 hours, concentrated to approximately half their volume, and extensively dialyzed against the same buffer to remove free isotope. Samples were centrifuged at 17,500 g at 4°C for 1 hour followed by an ultracentrifugation at 144,000 g at 4°C for 2 hours. In some cases, in ovo treated corneas were isolated with a 3 mm punch, cut in half, and extracted in PBS (100 μl/cornea) on ice for 2 hours with intermittent mixing. Samples were centrifuged, the supernatant reserved, and the pellet re-extracted for another 2 hours. After centrifugation, the pellet was further extracted in guanidine extraction buffer for proteoglycans and analyzed as outlined below.

Proteoglycan analysis

To undergo enzyme digestion, organ-cultured and in ovo corneal extracts were dialyzed exhaustively against 25 mM Tris-HCl, pH 7.5, at 4°C, and divided into equal sized samples for chondroitinase ABC and keratanase (Seikagaku America, Inc.) digestions (Hart, 1976). Chondroitinase ABC-treated samples were incubated at room temperature for 24 hours with inclusions of 0.1 unit/ml at 0 hours and 12 hours. Keratanase-treated samples were incubated at 37°C for 24 hours with inclusions of 0.2 units/ml at 0 hours and 12 hours. In some cases extracts were further digested to glycosaminoglycans using Pronase (Calbiochem) at 55°C for 24 hours with additions of 35 PUK/ml at 0 hours and 12 hours. After treatment, samples were lyophilized, resuspended in 4 M guanidine·HCl extraction buffer and chromatographed.

Chromatography

Extracted proteoglycans and digested samples were centrifuged in a Beckman airfuge for 20 minutes at 126,000 g prior to chromatography on a Superox 6 gel filtration column (Pharmacia LKB). The Superox 6 column possesses a separation range between 5×10⁴ and 5×10⁶ Mₘ, and globular standards were chromatographed for reference. Thyroglobulin (669×10⁶ Mₘ), catalase (232×10⁵ Mₘ), aldolase (158×10⁵ Mₘ) and bovine serum albumin (67×10⁵ Mₘ) migrated with a separation range between 5×10⁴ and 5×10⁶ Mₘ. The column had a void volume of 8.0 ml, determined from the elution of blue dextran, and a total volume of 22 ml, determined from the elution of H₂O. Chromatography was performed using a LKB HPLC system programmed for a flow rate of 0.3 ml/minute and 0.5 ml sized fractions. These fractions were
Whole cornea $^{35}$S incorporation
To determine $^{35}$S incorporation into proteoglycans, $\beta$-D xyloside-treated and untreated chick corneas were collected on day 17 and briefly rinsed in PBS. The central corneas were isolated, cut in half, and individually solubilized in Protosol according to the manufacturer’s instructions (New England Nuclear). Incorporation of $^{35}$S was determined by suspending the digests in 10 ml EcoscintA and radioactivity determined using a scintillation counter.

Electron microscopy
Whole corneas were stained histochemically for proteoglycans using cuprolinic blue (BDH Chemicals, Ltd) essentially as described by Scott (1985). An acetate buffer stock, containing 50 mM sodium acetate and 0.2 M MgCl$_2$, pH 5.7 with acetic acid, was mixed with 25% glutaraldehyde and distilled water to yield a 2.5% glutaraldehyde, 25 mM acetate, 0.05% cuprolinic blue fixative. This was filtered with a 0.45 $\mu$m filter prior to use. Initially, whole corneas from 17-day chick embryos were stained in cuprolinic blue fixative for 1 hour at room temperature. Central corneas were isolated, cut in half and placed in fresh cuprolinic blue fixative overnight. Excess stain was removed by washing the tissue in fixative without cuprolinic blue for 30 minutes followed by a 30 minute wash in 25 mM acetate buffer, pH 5.7. The tissues were enbloc stained for 1 hour with aqueous 0.5% sodium tungstate, followed by a wash in distilled water for 15 minutes. The tissues were dehydrated through a cold, graded ethanol series followed by propylene oxide. The tissues were infiltrated, embedded in a fresh mixture of Polybed 812, nadic methyl anhydride, dodecanecylic anhydride and DMP-30 (Polysciences, Inc.) and polymerized (Birk et al., 1989). Sections were cut on a Reichert Ultracut E microtome using a diamond knife, stained with 2% aqueous uranyl acetate and 0.25% phosphotungstate, followed by a wash in distilled water for 15 minutes. The tissues were observed using a JOEL 1200EX or Phillips 420 transmission electron microscope. Collagen fibril diameters were measured on photographic prints enlarged $\sim$135,000 x using a 10 x reticle. The control and experimental prints were randomized and measured blind. Measurements were from three different corneas from each of two different experiments.

Results
$\beta$-D xyloside was used to perturb the xylose-mediated glycosylation of DS core protein in an in vitro organ culture system as well as in ovo and then studied biochemically. Corneas treated in ovo were further investigated to determine if biochemical alterations translate into changes in the organization of the corneal secondary stroma.

In vitro studies were done using 17-day chick embryo corneas to evaluate the effects of $\beta$-D xyloside on corneal PG synthesis under controlled conditions. In vitro $\beta$-D xyloside treatment resulted in an alteration in PG synthesis relative to the untreated controls (Fig. 1). Chromatography of guanidine-extracted PGs from untreated corneas showed a relatively broad included peak (Fig. 1A). Enzyme digestions indicate that this peak contains both DS PG and KS PG and when these samples were sequentially digested with chondroitinase ABC and keratanase all labelled material eluted at the total volume of the Superose 6 column (data not shown). Chromatograms of the extracted PGs from corneas treated in vitro with $\beta$-D xyloside showed a broader peak shifted to a lower relative molecular mass (Fig. 1A). Subsequent enzyme digestion using keratanase or chondroitinase ABC indicated that the PG eluting in the same range as the untreated corneal PG is predominantly KS PG (Fig. 1). The lower $M_t$ region of the profile consists of DS-containing material (Fig. 1). Comparable results were obtained using both $\beta$-D xyloside analogues at a concentration of 1-5 mM. These data indicate that $\beta$-D xyloside treatment alters DS PG synthesis. Normally glycosylated DS PG is not seen while DS-containing material is present as a broad lower $M_t$ peak (Fig. 1B) in the $\beta$-D xyloside-treated corneas. This suggests $\beta$-D xyloside, acting as an alternative DS GAG attachment site from the normal core protein, may be creating free xyloside-GAG chains of varying lengths. Meanwhile KS-PG retains its profile after $\beta$-D xyloside treatment (Fig. 1C). These data indicate that $\beta$-D xyloside treatment disrupts DS PG synthesis, producing less intact DS PG and free xyloside-GAG, while KS PG synthesis is unaltered.

A second set of studies was performed to characterize the effects of $\beta$-D xyloside in ovo (Fig. 2 and Table 1). In ovo, $\beta$-D xyloside was present during day 10 to 17 of development. We considered this period to be representative of secondary corneal stroma development since it was during this period that most of the secondary stroma was synthesized and assembled. Gentle extractions with PBS were performed, on the basis that if $\beta$-D xyloside was acting as a competitive site for DS GAG attachment and creating free $\beta$-D xyloside DS GAG chains these free chains would be easily extractable. Examination of PBS-extractable material

$$
\begin{array}{lcccc}
\text{Table 1. Corneas treated in ovo with or without } & \text{Untreated control} & \text{ } & \text{ } & \text{ } \\
& \text{K$_{ave}$} & \text{Range (ml)} & \text{K$_{ave}$} & \text{Range (ml)} \\
\text{PG PEAK}^* & 0.21 & 6.5 & 0.25 & 6.5 \\
\text{DS-PG}^\dagger & 0.18 & 4.0 & 0.21 & 4.5 \\
\text{DS-Xt} & - & - & 0.37 & 8.0 \\
\text{KS-PG}^\ddagger & 0.25 & 6.0 & 0.25 & 6.0 \\
\text{DS-GAG}^\ddagger & 0.25 & 7.0 & 0.32 & 8.0 \\
\text{KS-GAG}^\ddagger & 0.50 & 7.0 & 0.50 & 7.0 \\
\end{array}
$$

Chromatography was performed on a Pharmacia Superose 6 column with a $V_o$ of 8.0 ml, and a $V_r$ of 22.0 ml. Samples were chromatographed in 4.0 M guanidine-HCl, 50 mM sodium acetate, pH 5.7, BSA (67 x 10$^3$ M), aldolase (158 x 10$^3$ M), catalase (232 x 10$^3$ M) and thyroglobulin (669 x 10$^3$ M) were run for reference, with $K_{ave}$ of 0.5, 0.35, 0.28 and 0.05 respectively.

*Guianidine-HCl extracted peak after digestion with chondroitinase  ABC.
\daggerPBS extractable DS-containing material. Determined after digestion with keratanase (see Fig. 2B).
\ddaggerDetermined after digestion with keratanase or chondroitinase ABC followed by Pronase to release GAG chains (see Fig. 2E-F).
Fig. 1. In vitro, β-D xyloside alters dermatan sulfate proteoglycan synthesis in the corneal stroma. Superose 6 gel filtration profiles of 35S-labelled proteoglycans from 17-day chick embryo corneas. Organ cultures were preincubated for 4 hours with or without 2.0 mM β-D xyloside, followed by 35S in the presence or absence of the inhibitor for 18 hours. (A) The relative molecular mass profile of guanidine-extracted PGs from organ cultured corneas illustrates an altered profile in β-D xyloside-treated corneas. Samples were digested with keratanase (B) or chondroitinase ABC (C). These enzyme digestions reveal that the low Mr material in the β-D xyloside-treated corneas contains DS and that β-D xyloside does not alter KS PG. A sample containing 10⁶ cts/minute was loaded in each case. Arrows indicate V₀ and Vₜ. Untreated (—) and β-D xyloside-treated (---) corneas.

in both β-D xyloside-treated and untreated corneas demonstrates a difference in the elution profiles (Fig. 2A). Enzyme digestions indicate that KS PG is extracted from both untreated and treated corneas (Fig. 2C) while DS-containing material is extractable only from the β-D xyloside-treated corneas (Fig. 2B). Digestion with keratanase demonstrates that untreated corneas produce very little extractable DS-PG. However, chromatograms of PBS extracts of β-D xyloside-treated corneas demonstrated a broad low Mr peak of DS-containing material (Fig. 2B). This profile mirrors the shift in Mr seen in vitro (Fig. 1B) and is limited to DS material; KS PG retains its Mr profile after in ovo β-D xyloside treatment. Again, this suggests that β-D xyloside acts as an alternative DS GAG attachment site in the cornea, both in vitro and in ovo.

Proteoglycans from in ovo control and β-D xyloside-treated corneas also were extracted with 4.0 M guanidine-HCl and characterized (Fig. 2D). PGs from β-D xyloside-treated corneas consistently chromatographed at a lower Mr when compared with untreated control corneas, Kave of 0.25 versus 0.21 respectively. The elution profile from β-D xyloside-treated corneas indicated a decrease in normally glycosylated DS PG with an increase in lower Mr DS-containing material. Keratan sulfate and dermatan sulfate glycosaminoglycans (GAG) were characterized after treatment in ovo with β-D xyloside (Fig. 2E-F). PGs were digested with keratanase or chondroitinase ABC followed by hydrolysis with Pronase. Dermatan sulfate was seen shifted to a lower Mr (Kave of 0.25 versus 0.32) in β-D xyloside-treated versus control corneas (Fig. 2E). The KS GAG profile was unaltered (ATave of 0.50) in untreated versus β-D xyloside-treated corneas (Fig. 2F).

The effect of β-D xyloside on 35S incorporation into whole corneas also was investigated. In ovo treated and untreated corneas were ethanol-acetate precipitated and collected onto GFA filters for analysis. The general trend exhibited indicates that β-D xyloside treatment reduced 35S incorporation by approximately 20% when compared to untreated control corneas: 39,790 ± 8011 [4] versus 31,557 ± 7416 [7] (mean cts/minute per cornea ± s.d. [number of corneas]) respectively. However, the differences were not statistically different between β-D xyloside-treated and untreated 35S incorporation.

Electron microscopy with histochemical staining for PGs with cuprolinic blue was done to determine if the biochemical changes in DS PG translated into morphological differences in the organization of corneal stroma. These studies demonstrated three consistent structural changes when β-D xyloside-treated 17-day corneas were compared to non-treated controls: a
β-D xyloside affects corneal stromal development

3-D xyloside affects corneal stromal development

Fig. 2. In ovo, β-D xyloside alters dermatan sulfate proteoglycan synthesis in the corneal stroma. Chromatograms of PBS-extractable material (A-C) from 17-day chick embryo corneas treated in ovo with β-D xyloside between 10 and 17 days of development. Profiles prior to enzyme digestion (A) show β-D xyloside treatment creates a broad range of low Mr. Digestion with keratanase (B) reveals that the PBS-extractable low Mr material in β-D xyloside-treated corneas contains DS, with a low Mr range similar to the organ culture corneas (Fig. 1B). Chondroitinase ABC digestion (C) indicates β-D xyloside does not alter the KS PG Mr range. Chromatograms of guanidine-extracted PGs (D) from 17-day chick embryo corneas treated in ovo with β-D xyloside beginning at 10 days of development. Proteoglycans from treated corneas exhibit a shift to a lower Mr when compared with the untreated controls. In ovo treatment with β-D xyloside reduces corneal dermatan sulfate, but not keratan sulfate glycosaminoglycan Mr. DS and KS glycosaminoglycans were characterized by digestion with keratanase (E) or chondroitinase ABC (F), both followed by Pronase. The elution profiles illustrate an effect of β-D xyloside, with DS GAGs demonstrating a lower Mr in treated versus untreated corneas (E). The KS GAG profiles were unaltered (F). Samples of $10^4$ (A), $2 \times 10^4$ (B-C) and $1.5 \times 10^4$ (D-F) cts/minute were loaded onto a Superose 6 gel filtration column; arrows indicate $V_o$ and $V_t$. Untreated (----) and β-D xyloside-treated (-----) corneas.

Reduction in proteoglycans, focal alterations in collagen fibril packing and a disruption of lamellar organization. In addition, there was no alteration in collagen fibril diameter.

The total PGs stained with cuprolinic blue in the β-D xyloside-treated corneas were reduced when compared with control corneas. The stained PGs are predominantly fibril associated in both cases (Fig. 3A,B). However, when compared with untreated corneas (Fig. 3A), there was a consistent and reproducible decrease in histochemically stained PGs associated with corneal collagen fibrils in the β-D xyloside-treated corneas (Fig. 3B). This observed decrease in PGs was consistent with the reduction in DS PG seen biochemically. Proteoglycan-collagen interactions have been suggested as important in the regulation of collagen fibril diameter. To address whether normally glycosylated DS PG was involved, fibril diameters were measured from control and β-D xyloside-treated corneas (Fig. 4). There was no correlation between fibril diameter and the presence of normally glycosylated DS PG. The mean diameters were $25.0 \pm 2.1$ nm versus $24.5 \pm 1.7$ nm (± s.d.) for
control and treated corneas respectively. The mean fibril diameters for the separate experiments were 24.8 and 25.2 nm for the controls and 23.6 and 25.3 nm for the β-D xyloside experiments.

The untreated corneal stromas were composed of fibrils with a relatively constant interfibrillar spacing (Fig. 5A). In comparison, β-D xyloside-treated corneas showed regions with normal collagen fibril packing and pockets of irregularly spaced collagen fibrils or areas devoid of fibrils (Fig. 5B). In β-D xyloside-treated stromas there were a large number of areas in which this regular fibril packing was disrupted as well as electron lucent areas separating regions of relatively normal packing. This suggested a role for DS PG in maintaining fibril spacing and packing within lamellae. In addition, the treated corneas contained numerous very large cuprolinic blue-stained structures (Fig. 5B) which were not obviously associated with collagen fibrils. These structures were rarely seen in the untreated controls.

Lamellar cohesiveness appeared altered in β-D xyloside-treated versus untreated corneas. Treated corneas exhibited a propensity to split between and within lamellae (Fig. 6B-C). This splitting within a lamella as well as the separation of adjacent lamellae was common in all corneas treated with β-D xyloside, examples were observed in virtually all low magnification fields. Control corneas very rarely demonstrated such alterations, and when present the magnitude of the disruption was considerably reduced. This disruption of lamellar integrity may be related to the alterations seen in fibril packing within lamellae. Interlamellar separations were a common observation in the β-D xyloside-treated stromas (Fig. 6B) and very infrequent in the untreated control stromas (Fig. 6A). These morphological differences also may be due to a decrease in lamellar adhesiveness which makes the tissue more susceptible to damage during processing for electron microscopy. These results suggest that DS PG plays a role in regulation of fibrillar spacing and lamellar cohesiveness.

Discussion

The role of proteoglycans, specifically DS PG, in collagen fibrillogenesis and matrix assembly during development of the secondary corneal stroma is addressed in this report. β-D xyloside was used to perturb the xylose-mediated glycosylation of the DS core protein. Dermatan sulfate PG synthesis was disrupted by treatment with β-D xyloside, producing less normally glycosylated DS PG and significant quantities of free xyloside-DS GAG while KS PG synthesis is unaltered. β-D xyloside allows for the
selective alteration of O-linked synthesis of DS PG without disturbing the synthesis of the other major stromal PG, the N-linked corneal KS PG. β-D xyloside inhibits normal DS PG synthesis by competing with core protein xylosides for GAG chain attachment, thereby creating a free xylose-DS GAG molecule (Okayama et al., 1973; Schwartz et al., 1974; Galligani et al., 1975). Biochemical analysis of corneas treated both in vitro and in ovo revealed a reduced synthesis of normally glycosylated DS PG and an increased synthesis of free xylose-DS GAG. The presence of free xylose-DS GAG was indicated since DS was easily extracted under mild physiological conditions as well as by the shift in \( M_r \) profiles of DS from β-D xyloside-treated corneas.

In addition, corneal proteoglycans were studied using histochemistry and electron microscopy after treatment with β-D xyloside. These data demonstrated that the observed biochemical alterations in DS PG translated into disruptions in the organization of β-D xyloside-treated corneal stromas. Three consistent structural changes were observed in corneas treated with β-D xyloside during 10 to 17 days of development: a reduction in proteoglycans, focal alterations in collagen fibril packing and a disruption of lamellar organization. In addition, there was no alteration in collagen fibril diameter.

The reduced synthesis of normally glycosylated DS PG correlated with the reduction in cuprolinic blue stained PGs in the treated corneal matrix. These ultrastructural analyses indicate that DS PG influences interfibrillar spacing, but has no effect on fibril diameter in the secondary stroma. In β-D xyloside-treated stromas there were a large number of areas in which regular fibril packing was disrupted as well as electron lucent areas separating regions of relatively normal packing. This implies a role for DS PG in maintaining fibril spacing and packing within lamellae. Inter- and intra-lamellar separations were common observations in the β-D xyloside-treated stromas and very infrequent in the untreated control stromas. This indicated that DS PG has a role in regulation of fibrillar spacing as well as lamellar cohesiveness. Analogously, when β-D xyloside was used to disrupt PGs during the development of the primary corneal stroma there was no alteration in fibril diameter. However, the disruption of PG synthesis resulted in abnormalities in fiber organization as well as in the gradual rotation seen in these layers of fibers from anterior to posterior (Coulombre and Coulombre, 1975).

Treatment of chick embryos in ovo with β-D xyloside does not block all subsequent DS PG synthesis in the cornea. However, our biochemical and histochemical analyses demonstrated a significant decrease when compared to controls. Our data indicates no role of DS PG in the regulation of fibril diameter, but DS PG does have a role in the development of matrix architecture, specifically the establishment of fibril packing and maintenance of lamellar integrity. However, the influence of small amounts of DS PG, which could not be inhibited completely, in fibril diameter regulation cannot be ruled out, but our data coupled with other studies (Bansal et al., 1989; Birk and Lande, 1981) indicate that DS PG is not required for normal corneal fibril assembly and diameter regulation.

Macular dystrophy, a condition distinguished by corneal opacity, is marked by improper biosynthesis of KS PG (Hassell et al., 1980; Nakazawa et al., 1984) and areas within the stromal matrix which are devoid of collagen fibrils (Newsome et al., 1982). These areas are thought to consist of accumulations of abnormal KS-containing material. The possibility that some of the structural alterations observed in β-D xyloside-treated corneas were due to accumulations of xylose-DS GAG and not to the absence of normally glycosylated DS PG cannot be ruled out entirely.
Fig. 5. β-D xyloside treatment alters collagen fibril packing. Transmission electron micrographs of untreated or β-D xyloside-treated 17-day chick embryo corneas, each from comparable areas. Untreated corneas exhibit consistently regular and tight packing of collagen fibrils (A). β-D xyloside-treated corneas (B) exhibit areas of normal packing, N, but also possess areas of abnormal packing, A, and areas devoid of collagen fibrils, *. Large CB-staining structures (curved arrow in B), not obviously associated with collagen fibrils, are seen in β-D xyloside-treated corneas. Chick embryos were treated in ovo with β-D xyloside between 10 and 17 days of development. Bar, 1 μm.

Studies of developing avian sternal cartilage treated with β-D xyloside also demonstrated an alteration in fibril organization and a compromised tensile strength of the cartilage. Fibrils in the treated sterna were morphologically similar to untreated fibrils (Hjelle and Gibson, 1979) suggesting that PGs do not participate in regulation of fibril diameter fibrillogenesis, but do participate in the spacial organization of cartilage fibrils. The disruption in collagen fibril distribution in this tissue was suggested as the cause for the reduction in tensile strength (Hjelle and Gibson, 1979). Cartilage and the corneal stroma are considerably different with respect to PG classes and amounts. Cartilage contains a large aggregating (aggrecan) PG in addition to the small fibril associated class. Although measurements of the corneal stroma's tensile strength were not done for the current report, a similar reduction in tensile strength might be extended to the β-D xyloside-treated corneal matrix. These corneas revealed a tendency to tear between and within lamella. This tearing may be the result of localized abnormal fibril packing due to alterations in the normally glycosylated DS PG. However, tearing also appears in areas of apparently normal fibril spacing. This indicates a role for DS PG in lamellar cohesiveness not related to its role in collagen fibril packing. A correlation among fibril diameter, mechanical properties, and the content and composition of glycosaminoglycans has been demonstrated (Flint et al., 1984; Merrilees et al., 1987; Parry and Craig, 1988). There was no change in corneal fibril diameter after β-D xyloside treatment, demonstrating that an alteration in proteoglycan composition alone may be sufficient to compromise a tissue’s mechanical properties.

Proteoglycan-collagen interactions have been implicated in regulation of collagen fibril diameter. Non-collagenous matrix components, including proteoglycans, influence fibril assembly and tissue organization (Birk and Lande, 1981; Vogel et al., 1984, 1987; Vogel and Trotter, 1987; Ruoslahti, 1988; Brown and Vogel, 1990). The relative content of specific proteoglycans decreases as fibril diameter increases in some tissues and these changing patterns may be partially responsible for the resulting fibril diameters (Scott, 1984). The small dermatan sulfate proteoglycans from tendon have a high affinity for type I collagen and their specificity of binding lies in the protein core (Brown and Vogel, 1990). It has been proposed that small proteoglycans
3-D xyloside affects corneal stromal development

Bound to the fibril surface are major regulators of fibril growth and that lateral fusion of fibrils might occur when such proteoglycan sheaths are removed from the fibril surface (Scott, 1984). The secondary corneal stroma contains dermamn sulfate and keratan sulfate proteoglycans, which interact with specific regions on the collagen fibril (Scott and Haigh, 1985, 1988). The effects of proteoglycans on collagen fibril formation have been studied in vitro using proteoglycans from cornea or sclera (Birk and Lande, 1981) and were shown to affect the kinetics of fibril formation differently, but no differences in the fibril diameter were observed. Other studies using the small dermatan sulfate PG from tendon (Vogel and Trotter, 1987) as well as the corneal DS and KS PG (Rada et al., 1991) also found an alteration in the kinetics of fibril formation with a modulation in fibril diameter. However, the diameters were not identical to those seen in vivo. Therefore other mechanisms may operate to regulate fibril diameter.

The interaction between molecules of different collagen types during the co-assembly into heterotypic fibrils is an important regulatory mechanism in collagen fibrillogenesis (Henkel and Glanville, 1982; Keene et al., 1987; Birk et al., 1988; Vaughan et al., 1988; van der Rest and Mayne, 1988; Mendler et al., 1989; Linsenmayer et al., 1990). The cornea contains heterotypic fibrils composed of collagens type I and V. Increasing the molar ratio of type V collagen decreases the diameters of fibrils formed in vitro. However, such fibrils are still substantially wider than the 25 nm fibrils characteristic of the corneal stroma in vivo. In vitro assembly is not as controlled as the fibril forming process is in situ. In vitro there are no cell defined domains characteristic of connective tissues actively assembling collagenous matrices (Birk and Trelstad, 1984, 1986; Birk et al., 1989). Cellular control of the mixing of different macromolecules, and postdepositional processing, probably plays an important role in the control of fibril formation. However, at present little is known about the intracellular and extracellular mechanism(s) controlling these interactions.

In summary, corneal dermamn sulfate proteoglycan synthesis was altered after treatment with β-D xyloside. The biochemical alterations in dermamn sulfate proteoglycans translated into disruptions in the organization of β-D xyloside-treated stromas. There was a reduction in the histochemical staining of proteoglycans, but no alteration in collagen fibril diameter. In addition, focal alterations in collagen fibril packing and a disruption of lamellae organization were observed in β-D xyloside-treated corneas. These data suggest that dermamn sulfate proteoglycans are not involved in the regulation of corneal collagen fibril diameter, but are important in...
fibroblast-fibril spacing as well as in lamellar organization, and cohesiveness.

We would like to thank Emanuel Zycband for expert technical assistance. This work was supported by grant EY 05129 from the National Institutes of Health.

References


(Accepted 9 March 1992)