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Modulation of Glycosaminoglycan Levels in Tree Shrew Sclera during Lens-Induced Myopia Development and Recovery

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Abstract

Purpose—In juvenile tree shrews, positioning a negative-power lens in front of an eye produces a hyperopic shift in refractive state and causes a compensatory increase in axial length over several days so that the eye is myopic when the lens is removed. During negative lens compensation, the scleral extracellular matrix is remodeled. A biomechanical property of the sclera, creep rate, increases; during recovery from induced myopia, the creep rate decreases below normal levels. Changes in glycosaminoglycan (GAG) levels, including those of hyaluronan, may participate in these changes in creep rate and, in turn, participate in controlling the axial length and refractive state. This study investigated the unsulfated and sulfated GAG composition of the sclera during compensation for a -5 diopter (D) lens and during recovery.

Methods—Capillary electrophoresis was used to assess the relative levels (ng/mg dry scleral weight) of unsulfated GAGs (hyaluronan [HA] and chondroitin [C0S]), sulfated GAGs (chondroitin-4-sulfate [C4S], chondroitin-6-sulfate [C6S], and dermatan sulfate [DS]) in the sclera of groups of tree shrews ($n = 5$ per group) that wore a monocular -5 D lens for 1, 2, 4, or 11 days or had 11 days of -5 D lens wear followed by 1, 2, or 4 days of recovery from lens wear. The fellow eye served as an untreated control. Groups of normal and plano lens-treated animals provided age-matched values.

Results—Expressed as a fraction of dry weight, levels of HA were lower after 1, 4, and 11 days of -5 D lens wear. Levels of C0S, C6S, and C4S were significantly lower after 4 and 11 days of lens wear. After 1 and 2 days of recovery, GAG levels in the treated eyes were not significantly different from those in control eyes. After 4 recovery days, HA levels were lower, but the levels of all other GAGs were not different in the recovering and control eyes. Some binocular changes also occurred.

Conclusions—The rapid differential decrease in HA levels during negative lens compensation and the absence of any difference after just 1 day of recovery suggest that HA levels may play a previously unrecognized early role in regulating the biomechanical property (creep rate) of the sclera. The reduced levels of the other GAGs, which occur when creep rate is at its peak elevation, and their rapid return to normal after 1 day of recovery suggest that they may also participate in regulating this biomechanical property of the sclera.

An important role of the sclera (Fig. 1) is to control the location of the retina by opposing intraocular pressure (IOP) that tends to expand the globe. There is strong evidence that an emmetropization mechanism exists in juvenile animals (macaque monkeys, marmosets, tree

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shrews, chicks, fish) that normally coordinates the axial length of the eye with the optical power provided by the cornea and lens by controlling the biochemical and biomechanical properties of the sclera (for recent reviews, see Wildsoet,¹ Norton,² Smith,³ Wallman and Winawer,⁴ Summers et al.,⁵ and Norton et al.⁶).

Shifting the location of the focal plane away from the cornea by positioning a negative-power lens in front of an eye produces a compensatory increase in its axial elongation rate until the retinal location matches the shifted focal plane and defocus is minimized while the animals are wearing the lens.⁷⁻¹¹ In the fibrous sclera of tree shrews (highly visual mammals closely related to primates), an increase in viscoelasticity—a biomechanical property of the sclera measured as creep rate—occurs,^{11,12} allowing normal IOP to gradually expand the globe. After 2 days of -5 diopter (D) lens wear, the scleral creep rate in tree shrews was found to be significantly higher than normal.¹¹ The creep rate peaked after 4 days of lens wear and then gradually declined toward normal as the retina approached the shifted focal plane after 11 days of treatment.¹¹

Underlying the elevation of creep rate is remodeling of the scleral extracellular matrix (ECM). There is a small overall decrease in dry weight of the sclera¹³⁻¹⁵ and a loss of type 1 collagen,^{13,16} probably at the edges of the individual lamellae (1- to 2- μ m-thick layers produced by the scleral fibroblasts; Fig. 1). Selective upregulation of mRNA occurs for some matrix metalloproteinases (MMPs) (MMP-2 and the membrane type-1 MMP), and selective downregulation of mRNA occurs for some, but not all, tissue inhibitors of MMPs (TIMP-3).^{17,18} Decreases in the levels of transforming growth factor beta (TGF β)¹⁹ and thrombospondin-1²⁰ have been found in tree shrew sclera during myopia development. Thus, evidence exists for remodeling of structural components of the sclera.

As compensation to a negative lens develops, the elongated eye is myopic if measured with the lens removed. Discontinuing lens treatment while the eye is still in the juvenile growth period^{21,22} initiates a process that slows the axial elongation rate to below normal.^{17,23,24} Because the eye's optics continue to mature, the focal plane gradually moves away from the cornea, toward the retina, producing optical recovery from the induced myopia. A similar pattern of myopia development and recovery from induced myopia in tree shrews and other species occurs if a diffuser is used to prevent images from forming on the retina (form deprivation myopia).^{8,25,26}

During recovery from induced myopia, the biomechanical and biochemical properties of the tree shrew fibrous sclera are altered in a pattern that in many ways is a mirror image of the pattern during myopia development. For instance, after 2 days of recovery from form deprivation¹¹ or recovery from negative lens-induced myopia (Sieglwart and Norton, unpublished data, 2007), the creep rate declines to below normal levels in the recovering eye. The reduction in scleral viscoelasticity is paralleled by selective changes in the biochemical composition and mRNA levels in the sclera that are generally a reversal from those seen during myopia development,^{11,13,17,27,28} with the exception that collagen levels remain slightly lower during recovery (Norton TT, et al. *IOVS* 1995;36:ARVO Abstract 3517). Thus, throughout the juvenile period, the sclera is a dynamic structure whose biochemical composition and biomechanical properties are regulated under the control of retinally derived signals.

Glycosaminoglycans (GAGs) constitute a small (approximately 0.2%) fraction of the scleral dry weight that may play a role in controlling the creep rate of the sclera. They are unbranched, linear, polysaccharide chains of repeating disaccharide units²⁹ that contain one hexosamine and either a uronic acid or a galactose residue. Human sclera contains five types of GAGs^{30,31}: hyaluronan (HA; 19%-33% of total scleral GAGs), which exists without attachment to a

protein core; chondroitin sulfate (CS; 19%-33%); dermatan sulfate (DS; 29%-49%); heparan sulfate (HS; 2%-12%); and keratan sulfate (KS), which attach to a proteoglycan core protein such as decorin, biglycan, or aggrecan.³¹ Based on keratinase sensitivity, tree shrew scleral proteoglycans contain little or no KS (J. Summers Rada, personal communication, 1997), and human and rabbit sclera contain little HS.^{31,32} Thus, the present study did not measure KS or HS levels.

Functionally, it has been suggested that GAGs, such as the DS GAG chain attached to decorin core protein, stabilize the spacing of collagen fibrils within the scleral lamellae.³³ Aggrecan, a large proteoglycan with many GAG chains, has been found in human sclera,³⁴ and mRNA for aggrecan core protein has been found in tree shrew sclera (Siegrwart JT Jr, et al. *IOVS* 2005;46:ARVO E-Abstract 3335). Aggrecan and HA levels may strongly affect the hydration levels of the sclera, particularly in the regions between the lamellae that contain the fibroblasts.^{35,36} GAG levels may be involved in modulating the mechanical properties of the ECM by controlling the slippage of the lamellae across each other, thus controlling scleral creep rate and, hence, contributing to control of the axial elongation rate.

Previous studies have reported a reduction in sulfated GAG levels after several days or weeks of negative lens wear or form deprivation in tree shrew and other species^{16,37,38} using a metachromatic dimethylmethylene blue assay.³⁹ Changes in sulfated GAG synthesis (decrease during myopia development, increase during recovery) have been measured by ³⁵S sulfate incorporation into GAGs.^{15,28,38} These assays are measures of the amount of sulfation of GAGs but do not assess levels of unsulfated GAGs. They could not detect HA because it was unsulfated.

It is now known that the onset of axial elongation and scleral creep rate elevation occur after only a few days of negative lens wear and that recovery begins on a similar time scale.^{17,26,40} Information is needed on GAG level changes that may occur soon after the onset of compensation and recovery. If changes in scleral GAG levels are causally related to the scleral creep rate changes and the axial elongation rate, they must occur rapidly after the beginning of compensation and recovery. Any GAG changes underlying the scleral creep rate change might be expected to move in opposite directions during compensation and recovery.

Because unsulfated and sulfated GAGs exist in sclera in small quantities and because tree shrew eyes are relatively small (approximately 8 mm axial length), a technique was needed that allows measurement of nanogram amounts of these substances. Capillary electrophoresis (CE) is capable of rapid, accurate, high-resolution separation of substances from low sample volumes,⁴¹ and it has been shown to yield complete separation and identification of CS and HA disaccharides.⁴² CE allows quantification of small amounts of GAGs, discrimination of the various GAG types, and measurement of the levels of unsulfated and sulfated GAGs. This study used CE to measure unsulfated and sulfated GAG levels in groups of tree shrews that compensated for a negative-power lens for various time periods or that recovered from lens-induced myopia. In addition, because the untreated control eye is not always normal,⁴⁰ GAG levels were also measured in normal, age-matched tree shrews. Results of the present study have been reported previously in abstract form (German AJ, et al. *IOVS* 1999;40: ARVO Abstract 2387; German AJ, et al. *IOVS* 2002;43:ARVO E-Abstract 215).

Methods

Experimental Groups

Juvenile tree shrews (*Tupaia glis belangeri*) were produced in our breeding colony and raised by their mothers on a 14-hour on/10 hour-off light/dark cycle. Groups of tree shrews, balanced to include males and females, were divided according to four conditions: normal animals ($n =$

3 per age group), negative lens compensation animals ($n = 5$ per group), recovery animals ($n = 5$ per group), and control animals ($n = 5$ per group). Normal animals were measured 1, 15, 24, 28, 35, 39, 45, and more than 120 days after natural eyelid opening (days of visual experience [VE]) to provide baseline values. As in previous studies,^{11,17,43,44} the negative lens compensation groups wore a monocular -5 D (spherical power) lens for 1, 2, 4, or 11 days starting at 24 ± 1 days of VE to induce axial elongation and myopia. Recovery groups also experienced -5 D lens wear for 11 days, starting at 24 ± 1 days of VE, to induce compensation. Then each lens was removed and the now myopic treated eye was allowed to recover for 1, 2, or 4 days. Normal groups at 28, 35, and 39 days of VE corresponded to 4 and 11 days of negative lens wear and to 4 days of recovery, respectively. There were two control groups. One wore a monocular plano (0-power) lens from day 24 to day 28 of VE to control for possible effects of lens wear (such as temperature or accumulated dust or body oil that could cloud the lens). Animals in the other group, the surgery control group, received (on day 21 ± 1 of VE) a dental acrylic pedestal to which a goggle frame could be attached (but was not) and were measured 3 days later on day 24 ± 1 of VE. This group controlled for any possible effects of wound healing on GAG levels⁴⁵ caused by the minor surgery to install the pedestal. All lens treatments were monocular, with the untreated fellow eye serving as a control. The procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Pedestal Attachment

At 21 ± 1 days of VE, each animal in the negative lens compensation, recovery, and control groups was anesthetized (ketamine 17.5 mg; xylazine 1.2 mg, supplemented with 0.5% to 2.0% halothane as needed) and received a dental acrylic pedestal to which a goggle frame could be attached according to procedures described by Siegwart and Norton.⁴⁶ On the morning of day 24 ± 1 of VE (3 days of postsurgery recovery), a goggle frame was clipped to the pedestal (except in the surgery control group). Each animal in the compensation and recovery groups wore a monocular -5 D lens in front of the randomly selected treated eye. Plano lens control animals wore a 0-power lens. The control eye had unrestricted vision through an open (no lens) goggle frame. The goggle was removed for approximately 3 minutes in dim illumination twice daily (approximately 9:00 AM and approximately 4:30 PM.) while the lens was cleaned. During lens cleaning and transport to and from the laboratory, the animals were kept in a darkened nest box to minimize exposure to visual stimuli. Badly scratched lenses were replaced as needed while the animal was kept in darkness (less than 30 minutes).

Axial and Refractive Measures

Ocular component dimensions were measured under anesthesia with A-scan ultrasound, as described by Norton and McBrien,²² when the pedestal was attached to the compensation, recovery, and control animals to ensure that the treated and control eyes did not differ significantly at the start of the treatment period. After the treatment/recovery period, terminal ultrasound measurements were made. Normal animals received only terminal measures.

At the end of the treatment or recovery period, cycloplegic measures of refractive state were taken with the use of an infrared autorefractor (ARK 700-A; Nidek, Fremont, CA) while the animals were awake.⁴⁷ Cycloplegia was induced with two drops of topical 1% ophthalmic atropine sulfate in each eye at least 1 hour before measurements were made, as in previous studies.^{11,16,22,43,44,48} Streak retinoscopy measures were also obtained under anesthesia and showed similar results (not reported). Measures with the autorefractor have been more reliable, perhaps because the animals were awake and maintained an intact tear film.⁴⁷

Tissue Preparation

On completion of the final refractive and ocular component measures, each animal received a lethal dose of sodium pentobarbital (approximately 333 mg/kg). While the animals were deeply anesthetized, both eyes were enucleated and placed into 4°C Dulbecco modified Eagle medium (DMEM). Extraocular muscles, conjunctiva, and orbital fat were dissected away from the globe, the cornea was removed by careful cutting along the corneoscleral junction, and the iris, lens, and vitreous were removed. The inner surface of the sclera was scraped to remove retina and choroid, and the outer surface was scraped to remove remaining extraocular tissues. Scleras were frozen in liquid nitrogen and stored at -80°C until the start of the biochemical measures.

Assay of Scleral Glycosaminoglycans

Biochemical analysis was completed without knowledge of which was the treated eye and which was the control eye. Individual scleras were thawed, blotted, cut into approximately 1-mm squares, and lyophilized overnight at 4°C. Scleras were then defatted overnight in acetone (35 vol) at 4°C, desiccated for 18 hours at room temperature (RT), and weighed, giving a dry weight. Defatted scleras (4-5 mg) were incubated with agitation in 300 µL of 0.1 M NaOH at 4°C for 16 hours to extract the GAGs. The sample was centrifuged at 15,000g for 30 minutes, and the supernatant was collected and neutralized to pH 7.4 with 2 M acetic acid. GAGs were precipitated by the addition of 4 volumes of 100% ethanol at 4°C for 16 hours. After centrifugation at 1300g for 30 minutes, pellets were dissolved in 400 µL of 50 mM Tris buffer, the sample was loaded on a diethylaminoethanol (DEAE) membrane (Multiscreen-DE plate; Millipore, Bedford, MA), and light vacuum was applied. Flow-through was discarded (earlier analysis revealed the absence of GAGs in flow-through samples). GAGs were retained on the DEAE membrane and were eluted with 200 µL of 0.2 M LiCl (fraction 1) followed by 200 µL of 1.5 M LiCl (fraction 2). Both fractions were then precipitated for 16 hours at 4°C with 4 vol of 100% ethanol. Fraction 1, containing hyaluronan, was resuspended in 40 µL hyase buffer (50 mM ammonium acetate, 10 mM CaCl₂, pH 6.5). Twenty microliters ABCase buffer (0.1 M Tris-HCl, 30 mM sodium acetate, pH 8.0) was added to fraction 2, which was then divided into two equal aliquots (2a and 2b) for subsequent ABCase and ACASE digestions. Both aliquots were ethanol precipitated for 16 hours at 4°C, and the pelleted GAGs were resuspended in 10 µL ACASE buffer (0.1 M Tris-HCl, 30 mM sodium acetate, 10 mM EDTA, pH 7.4; fraction 2a) or ABCase buffer (fraction 2b). Fraction 1 was analyzed for hyaluronan, which eluted from DEAE in 0.2 M LiCl. On digestion with group B streptococcal hyaluronidase at 37°C for 3 hours (3.75 m-units; generously provided by David Pritchard, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL), the product Δdi HA was separated by CE and quantified (Fig. 2A). Fraction 2a was analyzed for chondroitin 4- and 6-sulfates and chondroitin, which eluted from DEAE in 1.5 M LiCl. On digestion with chondroitinase AC (3.75 m-units; Sigma, St. Louis, MO) at 37°C for 3 hours (fraction 2a), the corresponding unsaturated disaccharide products Δdi 4S, Δdi 6S, and Δdi 0S were released, separated by CE, and quantified (Fig. 2B). On digestion using chondroitinase ABC (3.75 m-units, Sigma) at 37°C for 3 hours (fraction 2b), dermatan sulfate, in addition to chondroitins 4- and 6-sulfates (and chondroitin at a slow rate), was released. In this case, Δdi 4S derived from chondroitin 4-sulfate and dermatan sulfate was separated and identified and quantified by CE (Fig. 2C). Fraction 2b was analyzed for dermatan sulfate, which also eluted from DEAE in 1.5 M LiCl. Dermatan sulfate content was calculated as the difference between Δdi 4S released by chondroitinases ABC and AC.

The identification and quantification of unsaturated disaccharides in digests was performed using capillary electrophoresis (CE), as described in Carney and Osborne.³⁷ The CE system used was from Applied Biosystems (model 270A-HT; Foster City, CA). Samples were loaded hydrostatically (2 seconds) at 500 mm Hg and were separated at pH 9.8. Peaks were detected at 232 nm for unsaturated disaccharides. Benzoic acid was added as an internal standard to

compensate for any variation in injection volume, and results were calculated based on standards of a known disaccharide amount (5 µg/mL solution of Δdi HA, Δdi OS, Δdi 6S, and Δdi 4S; Fig. 2D) and were calculated as nanogram per milligram of scleral dry weight. It should be noted that chondroitin was not found as a discrete GAG; if it had been, it would have eluted exclusively in the 0.2 M LiCl fraction (Fig. 2A). Evidently, short segments of chondroitin are an integral part of hybrid chondroitin 4 and 6 chains, which results in their occurrence in the 1.5 M LiCl fraction (Fig. 2B). Each sample was run in triplicate, and the mean value was used in further analyses.

Statistical Analysis

Repeated-measures ANOVA was used to determine whether refractive, axial, dry weight, and GAG measures changed with age (in normal animals) and whether negative lens treatment and recovery affected refractive, axial, and dry weight measures (in the compensation and recovery groups). The ANOVAs had two factors; the between-subjects factor was measurement day (four compensation days, three recovery days), and the within-subject factor was eyes. Separate analyses were conducted for treated versus control and for normal left versus right eyes. If a significant main effect was found ($\alpha = 0.05$), post hoc analysis (Fishers least significant difference) was used to determine the days on which the differences occurred.

As discussed in Results, the changes in some GAG levels occurred slowly and were only evident after 4 and 11 days of treatment. Including all four negative lens compensation groups in an ANOVA would thus understate the significant changes and was not appropriate. Given that all seven groups were independent and were composed of different animals, though the treated and control eyes were not independent, it was appropriate to examine treated eye versus control eye differences with dependent (paired) *t*-tests. Because previous studies found a reduction in GAG levels during negative lens compensation, a one-tailed test was used. Independent two-tailed *t*-tests were used to examine whether there were significant differences between normal animals, with left and right eyes averaged (either treated or control groups of the same age), and between treated and control eyes at 11 days of treatment compared with 1 day of recovery.

Results

Refractive State and Ocular Component Dimensions

As was found previously,^{22,49} the refractive state in normal animals gradually became slightly less hyperopic from 15 days of VE to more than 120 days of VE, and there was no significant difference between right and left eyes in any normal group. The mean refractive value of all normal eyes in the age range 24 to 39 days of VE was (mean $\pm$ SEM) 5.6 ± 0.7 D. Based on a recent study using visual evoked potentials⁴⁷ and a study using wavefront sensing technology (Ramamirtham R, et al. *IOVS* 2003;44:ARVO E-Abstract 1986), it appears that refractive state measures made with an autorefractor (Nidek) are approximately 4 D more hyperopic than the true refractive state.⁵⁰ Thus, the eyes of the normal animals were approximately 1.6 ± 0.07 D hyperopic at this age. Eyes of the plano lens and surgery control animals did not differ significantly from age-matched normal eyes, indicating that the surgical procedure to install the pedestal and plano lens wear had no effect on refractive state or vitreous chamber depth.

As in previous studies,^{11,43,44} -5 D lens wear in this study produced myopia in the treated eyes with an overall treatment effect (ANOVA; $P < 0.05$). After 1 day of lens wear, the treated eyes were similar to the control eyes. After 2 days, the treated eyes were myopic (mean $\pm$ SEM, -0.5 ± 0.6 D, measured with the lens removed) compared with the control eyes, but the difference was not significant. After 4 days the treated eyes were significantly myopic, (-2.4 ± 0.6 D), and the vitreous chamber was significantly elongated (0.05 ± 0.03 mm; *t*-tests; $P <$

0.05). The amount of with-the-rule astigmatism was generally small (less than 1.5 D) and did not systematically change as a result of negative lens treatment. As expected,^{11,16,17} vitreous chamber enlargement accounted for nearly all the axial elongation. Eleven days of lens wear produced nearly full compensation (-4.6 ± 0.3 D) and an appropriate amount of vitreous chamber depth elongation⁴⁸ (0.08 ± 0.04 mm; *t*-test; $P < 0.05$). Refractive and ocular component measures of the control eyes were not significantly different from values in normal eyes.

As has been found in previous studies,^{17,26} discontinuing negative lens wear (recovery) produced a gradual decrease in the refractive difference and vitreous chamber difference between the treated and control eyes (to -3.1 ± 0.5 D and 0.7 ± 0.02 mm after 4 days of recovery), though recovering eyes remained significantly myopic compared with control eyes at all recovery durations (ANOVA, $P < 0.05$).

Scleral Dry Weight

Scleral dry weight provides an index of the overall amount of ECM in the sclera during normal development and during negative lens compensation and recovery. Scleral dry weight of the normal animals increased significantly as animals matured from 1 day of VE (2.3 ± 0.2 mg) to more than 120 days of VE (4.7 ± 0.4 mg; ANOVA, $P < 0.05$). Left and right eye values were similar in normal animals. Scleral dry weight of the plano lens animals and the cap control animals did not differ significantly from that of normal animals.

As shown in Figure 3, the dry weight of sclera of both eyes in the negative lens compensation and recovery groups also increased significantly over time (ANOVA, $P < 0.05$). The dry weight of the control eyes did not differ from that of normal eyes (unpaired *t*-test, $P > 0.05$). As in previous studies,^{13,15,28,51,52} the dry weight of the sclera gradually became lower in the treated eyes than in the control eyes. After 4 and 11 days of lens wear, the treated eye dry weight was lower than that of the control eye, but the difference was not statistically significant (*t*-test, $P > 0.05$). Treated eye dry weight remained lower during recovery, and differences were statistically significant in all three recovery groups (*t*-test, $P < 0.05$, indicated by the asterisks in Fig. 3). Thus, the loss of scleral material that developed in the three recovery groups during 11 days of negative lens wear remained after 1, 2, and 4 days of recovery.

It is important to note that, in this study, GAG levels are expressed as nanogram per milligram of dry weight rather than per whole sclera. This controlled for both the overall increase in dry weight with age and the differences in dry weight that developed between treated and control eyes. If the composition of the sclera had not changed, it would have been expected that all GAGs (expressed as a fraction of dry weight) would be the same in the treated and control eyes at all ages, even when the dry weight of the sclera differed across ages or between treated and control eyes.

Glycosaminoglycans Levels

Normal and Control Animals—During normal development, GAG levels were relatively stable as a fraction of scleral dry weight as the overall weight of the sclera increased because of normal growth. The dashed lines in Figures 4B to 4F show the normal values from 24 to 39 days of VE that corresponded to the period of negative lens wear and recovery. GAG values in the scleras of the plano lens animals did not differ from the values in age-matched normals (two-tailed independent *t*-test, $P > 0.05$). When the oldest group of animals (more than 120 days of VE) was included, the levels of unsulfated GAGs (HA and C6S) and one sulfated GAG (C6S) declined significantly across groups (ANOVA, $P < 0.05$).

As shown in Figure 4A, averaged across ages 24 to 39 days of VE, unsulfated GAGs constituted approximately 11% of total GAGs in the sclera (HA, 6.9%; C0S 3.7%), and sulfated GAGs constituted 89% of them (C6S, 1.5%; C4S, 21.4%; DS, 66.5%). These relative values varied little from the youngest animals (1 day of VE) to the oldest animals (more than 120 days of VE).

Compensation Animals—During negative lens compensation, there was an early change in the composition of the sclera with regard to HA after just 1 day of negative lens wear and then later changes in all GAGs except DS after 4 and 11 days of lens wear. As shown in Figure 4B, after 1 day of lens wear, the relative HA content was significantly lower ($-27.9\% \pm 6.6\%$) in treated eyes than in control eyes (t -test, $P < 0.05$). No significant changes occurred in the other GAG levels. In the group of animals that had 2 days of treatment, the difference in the treated eyes compared with the control eyes for HA was not significant. As may be seen in Figure 4B, HA levels in the control eyes of this group appeared to decrease compared with normal eyes and compared with the levels in the control eyes of the group that had 1 day and 4 days of lens wear. Similar binocular effects on control eyes have been found previously in response to negative lens wear.¹⁷ Mean levels of other GAGs (C0S, C6S, and C4S) were elevated in the treated eyes of some animals after 1 day of lens wear, but variability was very high, and the differences were not statistically significant. In the groups of animals that wore the lens for 4 days and for 11 days, the levels for HA, C0S, C6S and C4S (Figs. 4B-4E) were significantly lower in the treated eyes than in the control eyes. Levels of DS (Fig. 4F) did not change significantly during negative lens compensation. In addition, C0S levels differed from those of normal eyes in two groups; after 4 days of negative lens wear, they were significantly lower in treated eyes than in normal eyes. After 11 days, they were significantly higher in control eyes than in normal eyes.

Figure 5A shows the differences between the right eyes and left eyes of age-matched normal groups of animals for all five GAGs. In this figure, error bars indicate the 95% confidence intervals. When a difference of zero fell outside the 95% confidence interval, as with C6S in the group at 28 days of VE, the difference was generally statistically significant (one-tailed t -test, $\alpha = 0.05$). Note that the normal groups had only three animals per group, which had the effect of generally enlarging the size of the 95% confidence intervals relative to the treatment and recovery groups, which had five animals per group. This was particularly evident for DS.

Figures 5B to 5F show the treated-eye versus control-eye differences (and 95% confidence intervals) for the five GAGs measured in this study. It appears that after 1 day of negative lens treatment, biochemical changes were occurring in the sclera but were variable across animals for all GAGs except HA, which was consistently lower. After 2 days of negative lens treatment, differences in the treated eyes compared with the control eyes were near 0 for all GAGs. By 4 days of lens wear and continuing at 11 days, GAG levels were consistently lower in the treated eyes, except for DS, which showed no significant treated eye versus control eye effect.

Recovery Animals—As shown in Figures 4B to 4F and 5B to 5F, the significant decrease in GAG levels that developed after 4 and 11 days of -5 D lens wear was absent in the group that had just 1 day of recovery. Across the three recovery groups, there were no differences in treated eyes versus control eyes in the unsulfated or sulfated GAG levels except that HA levels were again significantly lower in the treated eyes after 4 days of recovery (two-tailed t -test, $P < 0.05$). As shown in Figure 4, 1 day of recovery produced binocular effects in the treated and control eye GAG levels compared with the 11-day group. In every case, GAG values after 1 day of recovery were lower in the treated and control eyes than they were in the group with 11 days of lens treatment. Decreases were significant for C0S, C6S, and DS in treated versus recovering eyes (R v T) and for C6S, CS and DS in control eyes (RC v C).

Discussion

In agreement with a previous report,¹⁵ it was found in the present study that total GAGs constitute a small percentage (0.2%) of the dry weight of the sclera in normal juvenile tree shrew eyes. Unsulfated GAGs were less abundant than sulfated GAGs, constituting a relatively stable 10.6% of the total GAGs across the range of ages examined. Nearly two thirds (65%) of the unsulfated GAGs were in the form of HA. However, HA made up only 6.9% of total GAGs, which is less than the 19% to 33% reported for human sclera.³¹ Sulfated GAGs represented most (89.4%) of the GAGs in the sclera. Of the sulfated GAGs, most (74%) existed in the form of DS. This is not surprising given the large amount of type 1 collagen in tree shrew sclera and the association of the DS proteoglycan, decorin, with type 1 collagen.⁵³⁻⁵⁵

Changes in Dry Weight

Dry weight changed over time in normal eyes, in which it gradually increased as a function of age in these growing juvenile animals. Consistent with previous reports,^{15,16,56} dry weight was lower in treated eyes after 4 and 11 days of negative lens wear, but in this study the reduction did not reach statistical significance during the period the lens was worn. It was only significantly lower in the three recovery groups. Previous studies have found larger changes at the posterior pole of the sclera than in the sclera as a whole.^{15,15,16} If our sample had been restricted to the posterior pole region, we might have found a larger, statistically significant difference between the treated and control eyes after 4 and 11 days of negative lens wear.

The absence of a rapid recovery in dry weight is interesting because it suggests that the decrease in the scleral creep rate after 2 days of recovery¹¹ can occur without a substantial increase in the amount of scleral tissue, which is largely type 1 collagen. This may suggest that other early-occurring changes, such as in levels of HA or the recovery in the other GAG levels, could participate in controlling this biomechanical property.

Changes in GAG Levels

In contrast to the relatively stable GAG levels found throughout normal development, this study found that a simple manipulation of the visual environment—wearing a monocular -5 D lens—rapidly produces a reduction, relative to the scleral dry weight (which controls for any weight differences between scleras), of approximately 25% in the HA level in the sclera of the treated eye. Within 24 hours of removal of the negative lens, HA levels were not significantly lower in the treated eyes. This was a new finding that could not have been detected in previous studies, which assessed only sulfated GAGs. Consistent with previous studies of sulfated GAG levels,^{15,16,28,37,38} we found that a reduction in the level of unsulfated (C0S) and sulfated (C6S and C4S) GAGs also occurred after 4 and 11 days of lens wear. This reduction also disappeared after just 1 day of recovery. Normal sclera has a low creep rate that is stable during development.¹¹ Similarly, GAG levels also are stable during normal development. That rapid changes can occur in GAG levels during lens compensation and recovery suggests that GAG levels are tightly controlled during normal development and, hence, are able to respond rapidly to changes in the visual environment that call for upregulation or downregulation of the axial elongation rate.

The composition of the sclera for the most abundant sulfated GAG (DS) did not show a significant differential change during treatment or recovery. This result is consistent with the suggestion that much of the DS is attached to decorin, which, in turn, is associated with type 1 collagen. To the extent there was a progressive loss of collagen in the sclera during the development of lens-induced myopia, there was a concomitant loss of decorin and its associated DS GAG chain, such that the amount of DS relative to the remaining sclera did not change.

Temporal Relationship of GAG Changes to Scleral Creep Rate Changes—How the composition of the sclera contributes to the biomechanical property (creep rate) of the sclera is unknown. However, it would logically seem more likely that scleral creep occurs by slippage at the interface between the collagen-rich lamellae rather than by displacement of the tightly packed collagen fibrils within a lamella. That the amount of collagen may not play a key role in the scleral creep rate is supported by the finding that the dry weight of the sclera did not recover after 1, 2, or 4 days of recovery in this study, yet creep rate has been found to decrease after 2 days of recovery.

If HA or other GAG levels play a role, in conjunction with other remodeling of the sclera, in producing changes in the scleral creep rate, their changes must precede, or at least be coincident with, the onset of the creep rate changes that occur during the development of induced myopia and should reverse (rebound) or at least recover to normal levels during recovery, when the creep rate changes from higher to lower than in control eyes. The reduction in HA levels in the treated eyes after 1 day of treatment occurred before the changes in refractive state, axial length, and vitreous chamber depth that were detected in the treated eyes. As shown in Figure 6, in a previous study¹¹ the scleral creep rate in treated eyes increased significantly relative to control eyes after 2 days of -5 D lens treatment and peaked at 4 days. Creep rate was lower, but still significantly elevated, after 11 days of negative lens wear. Creep rate was not measured in that study after 1 day of treatment. At 4 days of treatment, when creep rate was near its peak in the Siegwart and Norton study,¹¹ HA levels in the present study were significantly lower in the treated eyes of this study and were joined by a reduction in other sulfated and unsulfated GAGs that continued at 11 days of treatment. Thus, a consistent decrease in HA levels occurred quickly, before creep rate was elevated in the previous study, and persisted until the end of the period of increased creep rate.

No significant reduction in HA occurred after 2 days of negative lens treatment. However, as shown in Figure 4B, the treated eye values remained low at this time point. Values in the control eyes appeared to decrease compared with values in the groups after 1 and 4 days of lens treatment, so there was no treated eye versus control eye difference in the 2-day lens wear group. Such control eye changes have been noted in other studies with regard to mRNA levels¹⁷ and are of unknown origin. However, given that the refractive state and the axial elongation rate of the control eyes generally is unaffected (as in the present study), it appears that changes in isolated aspects of scleral remodeling can occur without affecting elongation and refraction. It is only when a constellation of changes occurs—including selective changes in mRNA levels for some, but not all, MMPs and TIMPs, loss of collagen, and the GAG levels changes reported here—that a change in creep rate and axial elongation rate occurs.

Changes During Recovery—During recovery from induced myopia, the difference in GAG levels between treated and control eyes quickly disappeared. The group of animals that was measured after 11 days of negative lens treatment had significantly lower levels of HA, C0S, C6S, and C4S. The group that had the same 11 days of treatment, plus 1 day of recovery, showed no significant difference. It is possible, but seems unlikely, that the 1-day recovery group differed from the 11-day treatment group by not having reduced GAG levels at 11 days (this could not be measured because the GAG determinations required enucleation). However, the consistently lower GAG levels at 4 and 11 days of treatment, taken with the absence of significant differences in the recovery groups, suggests that there were lower GAG levels in all groups after 11 days of lens wear and that the levels increased to normal within 1 day of recovery. Creep rate in sclera has not been measured before 2 days of recovery, when it was found previously to be significantly lower in eyes recovering from form deprivation than in control eyes.¹¹ Recently, we found that a similar decrease occurred after 2 days of recovery from 11 days of -5 D lens wear (Siegwart and Norton, unpublished data, 2007). It appears that the restoration of normal GAG levels within 24 hours must precede, or at least accompany, the

change in creep rate during recovery. Interestingly, if HA and other GAGs play a role in controlling creep rate, an elevation in their levels above normal must not be necessary; a return to normal levels is sufficient. Why HA levels (but no other GAG levels) were again significantly lower after 4 days of recovery is unknown.

Possible Early Role of HA in Scleral Creep Changes—Unlike other GAGs, HA is not synthesized in the Golgi; rather, it is synthesized in the plasma membrane by a membrane bound protein and is extruded into the extracellular space.⁵⁷ This mode of synthesis may be related to the rapid turnover of HA. The tissue half-life for HA ranges from 0.5 day to 2 or 3 days.^{58,59} HA is catabolized by receptor-mediated endocytosis and lysosomal degradation by CD44, a hyaluronan binding site or HA receptor.⁶⁰⁻⁶² It is possible for HA to be rapidly degraded in treated eyes such that its levels can be lower in the treated eye after only 1 day of compensation. Similarly, degradation may be quickly slowed by the release of hyaluronidases, bound to hyaluronidase inhibitors,⁶³ to allow the restoration of normal HA levels within 1 day of recovery. It would be interesting to learn in future studies whether mRNA synthesis or the activity of the various HA degrading enzymes also changes during negative lens compensation and recovery.

Because HA is secreted from the fibroblast surface, its concentration is likely to be greatest near the fibroblasts, which are located between the bundles of collagen fibrils that constitute the scleral lamellae (Fig. 1). It is between these lamellae that slippage would be most likely to occur during scleral creep. In addition, HA is a large molecule that forms aggregates with other large molecules such as aggrecan. It would be difficult for HA and aggrecan to exist between the tightly intertwined bundles of collagen and decorin within the lamellae. Recently, mRNA levels for aggrecan core protein have been found to be lower after 4 days of -5 D lens treatment (Siegwart JT Jr, et al. *IOVS* 2005;46:ARVO E-abstract 3335).⁵ HA and other GAGs may form a gel-like matrix that acts to retard the slippage of the lamellae across each other. In this restricted space, changes in HA levels, in combination with alterations in the levels of other key ECM proteins, may in turn produce the change in scleral creep rate that controls axial elongation and refractive state. The location of the other GAGs within the sclera is unknown except for much of the DS, which would be expected to be associated with decorin and, therefore, with type 1 collagen within the scleral lamellae. Thus, how the GAGs might interact with and participate in the remodeling of the sclera that results in an altered creep rate must remain for further investigations.

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FIGURE 1.

Electron micrograph of normal tree shrew sclera stained with cuproinic blue, which stains sulfated glycosaminoglycans, and showing two dark fibroblast processes (F) and several lamellae composed primarily of bundles of type 1 collagen with associated decorin and its sulfated glycosaminoglycan chain. Scale bar, 1 μ m. Photograph by Edward Clarke.

FIGURE 2.

Examples of the CE output after digestion with (A) hyaluronidase (fraction 1), (B) chondroitinase AC (fraction 2a), and (C) chondroitinase ABC (fraction 2b). (D) Disaccharide standards. BA, benzoic acid.

FIGURE 3.

(A) Scleral dry weight of left and right normal eyes (*triangles*) and of treated and control eyes (*filled and open circles*) during compensation and recovery. (B) Percentage difference in scleral dry weight between the treated and control eyes during treatment (*black bars*) and recovery (*shaded bars*). Asterisks indicate significant differences ($P < 0.05$, paired t -test).

FIGURE 4.

GAG levels. (A) Relative abundance of GAGs in normal sclera. Values are the average of normal animals at age 24, 28, 35, and 39 days of VE. Twenty-five days of VE corresponded to 1 day of negative lens treatment, and 39 days of VE corresponded to 4 days of recovery. (B, C) Unsulfated GAG levels during compensation and recovery. (D-F) Sulfated GAG levels during compensation and recovery. Treated eyes are represented as *filled circles*, and control eyes are represented as *open circles*. Normal (left and right eyes averaged) GAG levels are represented by *triangles*. Asterisks indicate a significant difference between treated and control eyes. T v N indicates a significant difference between treated and normal eyes; C v N indicates significant difference between control and normal; R v T indicates significant decrease in treated eyes between 11 days of treatment and 1 day of recovery; RC v C indicates significant decrease in control eyes between 11 days of treatment and 1 day of recovery.

FIGURE 5.

Differences in GAG levels. (A) Difference between left and right normal eyes for all five GAGs. For clarity, the plots have been shifted around the four normal ages—24, 28, 25, and 39 days of VE. Values have been normalized to DS based on relative abundance in Figure 4A. (B, C) Differences in unsulfated GAG levels during compensation and recovery. (D-F) Differences in sulfated GAG levels during compensation and recovery. Error bars indicate the 95% confidence intervals ($SEM \times 1.96$). Asterisks indicate a significant difference between treated and control eyes or right and left normal eyes.

FIGURE 6.

Comparison of the timing of changes in HA in the present study and scleral creep rate, measured previously.⁹ (A) Percentage difference (treated control eye) in HA levels during -5 D lens compensation and recovery from Figure 5. (B) Percentage difference in the average creep rate in the treated and control eyes from the previous study⁹ in which the animals underwent -5 D lens wear for similar periods).