Published in final edited form as:

Exp Eye Res. 2011 May ; 92(5): 394–400. doi:10.1016/j.exer.2011.02.011.

Regulation of the Biphasic Decline in Scleral Proteoglycan Synthesis During the Recovery From Induced Myopia

Jody A. Summers Rada^{a,b} and Lindsey R. Hollaway^a

Jody A. Summers Rada: Jody-summersrada@ouhsc.edu; Lindsey R. Hollaway: Lindsey-Hollaway@ouhsc.edu

^a Department of Cell Biology, University of Oklahoma Health Sciences Center, 975 NE 10th Street, Room 266, Oklahoma City, Oklahoma, 73104

^b Oklahoma Center of Neuroscience, University of Oklahoma Health Sciences Center, 975 NE 10th Street, Room 266, Oklahoma City, Oklahoma, 73104

Abstract

During the recovery from form deprivation myopia (myopic defocus), the rate of proteoglycan synthesis in the posterior sclera decreases co-incident with a deceleration of axial elongation. The choroid has been implicated in the regulation of scleral proteoglycan synthesis, possibly through the synthesis and secretion of scleral growth inhibitors. Therefore these studies were carried out to attempt to establish a causal relationship between choroidal secretion and the inhibition of scleral proteoglycan synthesis during the recovery from induced myopia. Chicks were form vision deprived for 10 days followed by a recovery period (3 hours – 20 days) of unrestricted vision. Scleras and choroids (5 mm punches) were isolated from control and treated eyes. The rate of proteoglycan synthesis was estimated by the incorporation of ³⁵SO₄ in cetyl pyridinium chloride-precipitable glycosaminoglycans by isolated sclera of control and treated eyes. Additionally, choroids from control and treated eyes were placed in co-culture with untreated age-matched normal chick sclera for 20 – 24 hours, after which time scleras were removed and scleral proteoglycan synthesis rates determined. Following removal of occluders, a biphasic decline was observed in scleral proteoglycan synthesis: A rapid decline in proteoglycan synthesis (–7.6 % per hour; $r^2 = 0.923$) was observed over the first 12 hours of recovery, followed by a slow decline extending from 12 – 96 hours (–0.3% per hour; $r^2 = 0.735$). Proteoglycan synthesis rates gradually increased to control levels over the next 96 hours at a rate of + 0.3 % per hour. No relative proteoglycan inhibition was observed when untreated sclera were co-cultured with choroids from eyes recovering for 0 – 4 days, whereas co-culture of untreated sclera with choroids from eyes recovering for 5 and 8 days resulted in significant inhibition of sclera proteoglycan synthesis, relative to that of sclera co-cultured with choroids from control eyes ($\approx -24\%$, $p < 0.05$, paired t-test). In conclusion, recovery from induced myopia is characterized by a rapid decline in proteoglycan synthesis which occurs within the first 12 hours of unrestricted vision as well as a slower more gradual decline that occurs over the next four days. Choroidal inhibition of scleral proteoglycan synthesis *in vitro* occurs during the second phase of decline and is most likely related to increased choroidal permeability; whereas the rapid decline in proteoglycan synthesis that

Corresponding Author: Jody Summers Rada, Ph.D., Department of Cell Biology, University of Oklahoma Health Sciences Center, 975 NE 10th Street, Room 266, Oklahoma City, Oklahoma, 73104, jody-summersrada@ouhsc.edu, Phone: (405) 271-8001 ext. 46478, Fax: (405) 271-3548.

No commercial relationships

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

occurs during the first 12 hours of recovery is regulated by an independent, yet to be identified mechanism.

Keywords

sclera; choroid; myopia; proteoglycans; glycosaminoglycans; form deprivation

1. INTRODUCTION

In a variety of vertebrate species, postnatal ocular growth is regulated, in large part, by the quality of the visual environment. Form vision deprivation (FD) or hyperopic defocus is associated with increased ocular growth and myopia development (Goss and Criswell, 1981; Rada et al., 2006; Schaeffel et al., 1988) whereas imposed myopic defocus or recovery from FD myopia results in a slowing of ocular elongation (Schaeffel et al., 1988; Wallman and Adams, 1987). It has been convincingly shown that visually induced changes in ocular growth are associated with changes in sclera proteoglycan synthesis and accumulation at the posterior pole of the eye. In placental mammals and primates, the rate of vitreous chamber elongation is inversely related to the rate of proteoglycan synthesis in the posterior sclera (McBrien et al., 2000; Moring et al., 2007; Norton and Rada, 1995; Rada et al., 2000; Troilo et al., 2006) while in the chick, increased axial elongation and development of myopia are associated with significant increases in proteoglycan synthesis in the cartilaginous layer of the sclera (Christensen and Wallman, 1991; Gentle et al., 2001; Marzani and Wallman, 1997; McBrien et al., 1991; Rada et al., 1991, 1994). In both chicks and primates, visually induced changes in ocular elongation and sclera proteoglycan synthesis are reversible; restoration of unrestricted vision (and the resultant myopia) results in a temporary cessation of axial growth, eventually leading to re-establishment of emmetropia ("recovery") in the formerly deprived eye (McBrien et al., 2000; Siegart and Norton, 1998; Qiao-Grider, et al., 2004; Rada et al., 1992; Wallman and Adams, 1987). Moreover, the ocular response to FD or defocus is rapid, leading to detectable changes in vitreous chamber depth within hours (Winawer and Wallman, 2005). Significant changes in scleral proteoglycan synthesis have also been detected following one or two days of FD or recovery, respectively, although earlier time points have not yet been assessed (Rada et al., 1992). Therefore, of much interest is the mechanism by which myopic defocus can rapidly down-regulate scleral proteoglycan synthesis and lead to recovery from induced myopia.

The choroid has been implicated as a tissue involved in mediating changes in scleral proteoglycan synthesis during recovery from induced myopia. Recovery is associated with choroidal thickening (Liang et al., 1996; Wallman et al., 1995), increased choroidal synthesis of retinoic acid (Mertz and Wallman, 2000), hyaluronan accumulation in the choroidal stroma (Summers Rada et al., 2010), and a marked increase in choroidal vascular permeability (Pendrak et al., 2000; Rada et al., 2001; Rada and Palmer, 2007). *In vitro*, choroid explants or suprachoroidal fluid isolated from recovering eyes have been shown to inhibit scleral proteoglycan synthesis to a greater extent than that of choroids or suprachoroidal fluid isolated from control eyes (Marzani and Wallman, 1997; Rada and Palmer, 2007). The scleral proteoglycan inhibition observed *in vitro* has been shown to be related to an increased concentration of serum-derived factors in the suprachoroidal fluid of recovering choroids (Rada and Palmer, 2007). Based on these studies, it has been speculated that in addition to facilitating choroidal thickening, changes in choroidal permeability may regulate the delivery of secreted growth factors to the sclera to regulate scleral proteoglycan synthesis (Rada and Palmer, 2007).

In the present study, we extend our previous work and hypothesize that choroidally-derived and secreted factors are directly responsible for the inhibition of scleral proteoglycan synthesis observed during the recovery from induced myopia. To test this hypothesis, choroids were isolated from control and recovering eyes at several time points between 0 and 8 days of recovery and co-cultured with punches of normal untreated sclera. Proteoglycan synthesis rates were compared in sclera co-cultured with choroids from control and recovering eyes as well as in sclera isolated directly from control and recovering eyes. If secreted factors from the choroid are directly responsible for the inhibition of scleral proteoglycan synthesis during recovery, we predict that the relative inhibition of scleral proteoglycan synthesis by isolated choroids in vitro will be temporally similar to that observed in sclera directly isolated from recovering and control eyes. By using this approach, we may identify vision-dependent tissue interactions between the choroid and sclera that regulate scleral proteoglycan synthesis during periods of accelerated or decelerated ocular growth, as well as during the regulation of normal growth associated with emmetropization.

The results of the present study demonstrate that the inhibition of proteoglycan synthesis during recovery from induced myopia occurs in two phases; a rapid decline in proteoglycan synthesis which occurs within the first 12 hours of unrestricted vision as well as a slower, more gradual decline that occurs over the next four days. We conclude that the choroidal inhibition of scleral proteoglycan synthesis previously observed (Marzani and Wallman, 1997; Rada and Palmer, 2007) corresponds to the second phase of decline and is most likely related to increased choroidal permeability; whereas the rapid phase of decline is regulated by an independent, yet to be identified mechanism.

2. MATERIALS AND METHODS

2.1. Animals

White Leghorn cockerels (*Gallus gallus*) were obtained as 2 - day old hatchlings from Ideal Breeding Poultry Farms (Cameron, Texas, U.S.A.). Form deprivation (FD) myopia was induced in two day old chicks by applying translucent plastic goggles as previously described (Rada et al., 1991). Briefly, chicks were lightly anesthetized with isoflurane (Vedco Inc., St. Joseph, MO) and hemispheric goggles, cut from the bottoms of 15 ml round bottom test tubes were affixed to the feathers around the right eye of two day old chicks with cyanoacrylate adhesive. Goggles remained in place for 10 days after which time, goggles were removed and chicks were allowed to experience unrestricted vision (recover) for 0 – 20 days. The left eyes of all the chicks were never goggled and were used as controls. Birds were housed in temperature-controlled brooders with a light/dark cycle of 12 hr L/12 hr D, and fed food and water *ad libitum*. Chicks were checked twice a day. Sclera and/or choroids were isolated from control and treated eyes of birds following 10 days of form deprivation (= 0 hr recovery), as well as from birds recovering for 3 hrs, 6 hrs, 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs (5 days), 360 hrs (15 days) and 480 hrs (20 days). Additionally, sclera were isolated from untreated 12 – 20 day old chicks for co-culture and organ culture experiments. The chicks were maintained and used in accordance with the Animal Welfare Act and National Institutes of Health Guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures adhered to the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center.

2.2. Scleral Sulfated Glycosaminoglycan Synthesis

The posterior hemispheres of eyes of FD chicks (= 0 days of recovery), or from eyes from chicks recovering from FD myopia for 1 – 20 days and contralateral controls were obtained and one 5 mm tissue punch was excised from the posterior sclera of control and treated eyes

using a dermal punch (Miltex Instrument Co., Bethpage, NY). All retina, RPE, choroid, vitreous, pecten, and muscle were gently cleaned from each sclera punch. Scleral punches were initially placed into wells of a 96 well culture plate with 50 μ l of N2 medium [Ham's F-12/DMEM containing 1x N2 supplement (Stem Cell Technologies, Vancouver, BC) and glucose (8 mg/ml)] until all sclera were obtained. Scleral punches were then transferred to N2 medium containing $^{35}\text{SO}_4$ (100 μ Ci/ml; New England Nuclear, MA) and incubated for 3 hr. at 37°C. Radiolabelled scleral punches were digested with proteinase K (protease type XXVIII, Sigma, St. Louis, MO), (0.05% w/v in 10 mM EDTA, 0.1 M sodium phosphate, pH 6.5) overnight at 60°C. $^{35}\text{SO}_4$ – labeled glycosaminoglycans (GAGs) were precipitated by the addition of 0.5% cetylpyridinium chloride (CPC) in 0.002 M Na_2SO_4 in the presence of unlabeled carrier chondroitin sulfate (1mg/ml in dH_2O). The samples were incubated for 30 min at 37°C and precipitated GAGs were collected on Whatman filters (GF/F) using a Millipore 12-port sampling manifold as previously described (Rada et al., 1992). Radioactivity was measured directly on the filters by liquid scintillation counting.

2.3. Sclera/Choroid Co-Culture

Sclera/Choroid co-cultures were carried out in 96 well plates containing permeable inserts (3.0 μ m pore size; polycarbonate, (HTS Transwell®-96 Permeable Support System, Corning Incorporated, Corning NY). Scleral punches (5 mm) were isolated from right and left eyes of normal untreated 12 – 20 day old chicks and placed in wells of a 96 well transwell receiver plate containing 235 μ l of N2 and kept on ice. The 96 well- permeable support was placed over the receiver plate and 75 μ l N2 was added to each well of the insert. To isolate choroids, 5 mm punches were taken from the posterior poles of control and treated eyes and placed in a plastic petri dish containing a small volume of N2. With the aid of a dissecting microscope, the retina and retinal pigment epithelium (RPE) were removed as a sheet with a small spatula and remaining small bits of RPE were removed by gentle brushing with a sable brush and rinsing with N2. Following removal of all visible pigment, choroids were separated from the sclera with a fine spatula and placed in the permeable insert containing N2 medium. The transwell system was covered with a lid and incubated at 37°C for 20 – 24 hours. Immediately following co-culture, sclera were removed from the transwell system and radiolabelled in fresh N2 containing $^{35}\text{SO}_4$ for proteoglycan synthesis as described above. Aliquots of culture medium from the insert and receiver plate were analyzed by SDS-PAGE using 10% bis-tris gels (Invitrogen, Corp., Carlsbad, CA). Following staining of gels with SimplyBlue SafeStain (Invitrogen Corp), gels were imaged with a Chemigenius imager (Syngene USA, Frederick, MD), and densitometry was performed using GeneTools version 3.08.01 (Synoptics Ltd/Syngene USA, Frederick MD).

2.4. Statistical Analyses

Comparisons between treated and contralateral control eyes were made using Student's *t*-test for matched pairs (GB Stat, Dynamic Microsystems, Inc, Silver Spring, MD). Linear regression analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). Comparisons between groups were made with one way ANOVA using Scheffe's comparisons and Bonferroni adjustments for multiple comparisons (GB Stat, Dynamic Microsystems, Inc, Silver Spring, MD).

3. RESULTS

3.1. Recovery In Vivo

In order to estimate scleral proteoglycan synthesis rates in control and recovering eyes *in vivo*, $^{35}\text{SO}_4$ incorporation into CPC-precipitable glycosaminoglycans (GAGs) was measured in posterior scleral punches isolated from control eyes and treated eyes after 10 days of monocular form deprivation (FD) followed by 0 – 20 days of unrestricted vision (recovery)

over a three hour period of radiolabeling in organ culture (Figure 1). Initially, $^{35}\text{SO}_4$ -GAG synthesis was significantly higher in treated eyes as compared with paired controls ($+63.59 \pm 16.22\%$, $p < 0.0001$, paired t-test) and remained significantly elevated following three hours of recovery ($+63.63 \pm 16.28\%$, $p < 0.001$) and after 6 hours of recovery ($+38.60 \pm 10.82\%$, $p < 0.001$). However, following 12 hours of recovery, $^{35}\text{SO}_4$ -GAG synthesis was significantly lower in treated eyes as compared with controls ($-22.62 \pm 7.28\%$, $p < 0.001$). $^{35}\text{SO}_4$ -GAG synthesis was also significantly lower in treated eyes following 96 hrs (4 days) and 192 hrs (8 days) as compared with contralateral controls ($-44.96 \pm 4.92\%$ and $-18.33 \pm 4.61\%$; $p < 0.0001$ and $p < 0.001$, respectively). $^{35}\text{SO}_4$ -GAG synthesis was not significantly different between control and treated eyes following 24 hrs, 360 hrs (15 days) or 480 hrs of recovery (20 days) ($p = 0.16$, 0.24 and 0.49 , respectively). Based on analyses of the slope(s) of temporal changes in the relative proteoglycan synthesis shown in Figure 1, three phases of recovery were identified. The first phase was characterized by a rapid decline in $^{35}\text{SO}_4$ -GAG synthesis over the first 12 hours of recovery ($-7.63 \pm 1.56\%$ per hr; $r^2 = 0.923$; $p = 0.04$). The second phase was characterized by a continued gradual decline in $^{35}\text{SO}_4$ -GAG synthesis that reached a minimum by four days of recovery ($-0.35 \pm 0.21\%$ per hr; $r^2 = 0.735$; n.s.). The third phase was characterized by a gradual increase in $^{35}\text{SO}_4$ -GAG synthesis that reached control levels by 15 days of recovery ($0.15 \pm 0.02\%$ per/hr; $r^2 = 0.948$; $p = 0.03$).

3.2. Recovery in vitro

In order to determine whether the rapid decline in $^{35}\text{SO}_4$ -GAG synthesis observed during the first 12 hours of recovery (Figure 1) was due to an *in vivo* mechanism that results in deceleration in eye growth or due to a passive mechanism as a result of removal of the FD stimulus, $^{35}\text{SO}_4$ -GAG synthesis was measured on sclera punches isolated from control and FD eyes following 0 – 48 hours of organ culture (Figure 2). Following 6 hrs of organ culture, $^{35}\text{SO}_4$ -GAG synthesis increased by approximately 48% in sclera isolated from control eyes and remained significantly elevated throughout the 48 hr culture period (dashed line; $p < 0.05$, ANOVA with Bonferroni adjustment). $^{35}\text{SO}_4$ -GAG synthesis by FD sclera also significantly increased when placed in culture, as compared to FD sclera that were not organ cultured ($p < 0.01$, ANOVA with Bonferroni adjustment). Interestingly, no rapid decline in $^{35}\text{SO}_4$ -GAG synthesis was observed in sclera isolated from FD eyes when placed in organ culture. $^{35}\text{SO}_4$ -GAG synthesis remained significantly elevated in sclera isolated from FD eyes following at least 24 hrs of organ culture and returned to levels not significantly different from that of control sclera after 48 hrs of organ culture. Increased variability in $^{35}\text{SO}_4$ -GAG synthesis measurements was observed in sclera from FD eyes with increasing time in organ culture. These results suggest that the rapid decline in $^{35}\text{SO}_4$ -GAG synthesis observed *in vivo* is the result of an active vision-dependent mechanism involving additional ocular tissues or organ systems, not present when sclera are isolated and placed in organ culture.

3.3. Choroid co-culture

To test the hypothesis that choroid-derived and secreted factors are responsible for the rapid down-regulation of $^{35}\text{SO}_4$ -GAG synthesis observed in the sclera of recovering eyes, sclera from untreated chick eyes were co-cultured with choroids isolated from eyes recovering from FD myopia for 0 – 8 days using transwell culture systems (Figure 3). Co-cultures were incubated overnight at 37°C and scleral $^{35}\text{SO}_4$ -GAG synthesis was measured following co-culture as described above. Scleral $^{35}\text{SO}_4$ -GAG synthesis was similar following co-culture with choroids isolated from birds experiencing 0 – 24 hrs of recovery, although a slight non-significant increase in scleral $^{35}\text{SO}_4$ -GAG synthesis was noted when sclera were co-cultured with choroids from 6 hr recovering eyes. Scleral $^{35}\text{SO}_4$ -GAG synthesis was significantly lower in sclera co-cultured with choroids from eyes recovering for 5 days and 8 days as

compared with contralateral controls (−23.72% and −24.08%, respectively; $p < 0.05$, paired t-test).

3.4. Choroid permeability and $^{35}\text{SO}_4$ -GAG synthesis

Profiles of secreted proteins were compared between choroids of control and recovering eyes using SDS-PAGE following 24 hrs of organ culture in transwell plates. Recovery was associated with a marked increase in serum protein concentration in the culture medium in the insert (upper chamber) of the transwell system (Figure 4). Similar increases in serum protein concentration were also observed in the culture medium within the receiver plate (lower chamber) of the transwell system (data not shown). The elevated levels of serum proteins released into the culture medium are most likely the result of increased choroidal vascular permeability observed during the recovery from induced myopia (Pendrak et al., 2000; Rada et al., 2001; Rada and Palmer, 2007). Comparison of choroid culture supernatants with a dilution series of normal chicken serum indicated that serum concentration in supernatants of 5 day recovering chick choroids was approximately 12.5% (Figure 4). In the present study, choroid permeability was estimated by quantification of the 66 kDa serum albumin band on SDS-PAGE gels (arrow, Figure 5A,B). Image analyses of SDS-PAGE gels indicated that relative serum albumin concentration was significantly lower in culture medium of choroid/sclera co-cultures following 6 hr of recovery as compared to that of paired controls ($p < 0.05$; paired t-test for $n =$ five pairs of medium obtained from control and treated co-cultures). Serum albumin was significantly elevated in the culture medium of treated choroids following 4, 5 and 8 days of recovery ($p < 0.05$, paired t-test). We have previously shown that scleral $^{35}\text{SO}_4$ -GAG synthesis *in vitro* is inhibited by the presence of chicken serum (Rada et al., 2001; Rada and Palmer, 2007). In order to determine whether the inhibition in scleral $^{35}\text{SO}_4$ -GAG synthesis observed following co-culture with 5 day choroids could be a result of the presence of chicken serum, scleral $^{35}\text{SO}_4$ -GAG synthesis was measured in the presence and absence of equivalent amounts of chicken serum to that present in choroid-sclera co-cultures ($\approx 12.5\%$ serum; Figure 6). Incubation of sclera from untreated eyes with 12.5% chicken serum for 24 hrs caused a significant inhibition of $^{35}\text{SO}_4$ -GAG synthesis as compared with sclera cultured in N2 medium alone (−46.7% $p < 0.05$, paired t-test). These results suggest that most, if not all, inhibition in $^{35}\text{SO}_4$ -GAG synthesis observed in normal sclera co-cultured with 5-day recovering choroids could be accounted for by the inhibitory effect of chicken serum.

4. DISCUSSION

Previous studies have suggested that choroid-derived factors can regulate the rate of sclera proteoglycan synthesis, based largely on results obtained from experiments using sclera/choroid co-cultures under normal laboratory conditions (Marzani and Wallman, 1997; Rada and Palmer, 2007). In an elegant study, Marzani and Wallman (1997) demonstrated that co-culture of normal cartilaginous sclera with choroids isolated from eyes following 3 days of recovery (with prior FD for 11 days) resulted in an approximately 50% inhibition of scleral proteoglycan synthesis as compared to cartilaginous sclera co-cultured with choroids isolated from control eyes. The opposite effect was observed when choroids were co-cultured with fibrous sclera (approximately 50% stimulation of scleral proteoglycan synthesis when co-cultured with recovering choroids), although the absolute levels of proteoglycan synthesis were nearly 100 fold lower in fibrous sclera as compared with cartilaginous sclera. Consistent with these results were our previous results that demonstrated suprachoroidal fluid, isolated from eyes following 3 days of recovery (with prior FD for 10 days), also caused significant inhibition in scleral proteoglycan synthesis (Rada and Palmer, 2007). If secreted factors from the choroid are directly responsible for the inhibition of scleral proteoglycan synthesis during recovery, we hypothesized that co-culture

of normal sclera with choroids isolated from recovering eyes would result in a relative inhibition of scleral proteoglycan synthesis similar to that observed in sclera directly isolated from recovering eyes.

Based on the results of the present study, recovery from induced myopia could be described in three phases: An initial, rapid down-regulation that occurred over the first 12 hours of recovery, an intervening slow deceleration phase from 12 hrs – 4 days, and a final gradual return to control levels by 20 days of recovery. The initial phase of decline was not simply due to removal of the FD stimulus as a result of enucleation of the eye and placement of the treated sclera in organ culture; sclera from control and FD eyes demonstrated *increased* proteoglycan synthesis when placed in organ culture, as compared with control and FD sclera that were not organ-cultured. These results suggest that placement of sclera in organ culture results in disinhibition of proteoglycan synthesis, whereas *in vivo*, scleral proteoglycan synthesis is generally inhibited during periods of normal ocular growth and exposure to myopic defocus results in an even greater, relatively rapid, vision dependent inhibition.

Based on the rapid down-regulation of sclera proteoglycan synthesis observed during the first 12 hrs of recovery, we predicted that choroids isolated from recovering eyes during the first 12 hrs of recovery would be most effective at inhibiting proteoglycan synthesis by normal untreated sclera *in vitro*. Surprisingly, choroids isolated from eyes recovering for 3 – 12 hrs were not effective at inhibiting sclera proteoglycan synthesis in co-cultures, and in fact, resulted in a slight increase in sclera PG synthesis following 6 hrs of recovery. Only following 120 hrs (5 days) of recovery was statistically significant inhibition observed in sclera co-cultured with recovering choroids as compared to sclera co-cultured with paired control choroids. Co-culture of recovering choroids (6 hr of recovery) with sclera isolated from 10 day FD eyes produced very similar results to those obtained using untreated sclera (Summers Rada and Folger, 2009), indicating that the lack of inhibitory effect by choroids isolated from eyes recovering for 3 – 12 hours was not the result of using sclera from untreated chicks in organ cultures. Our results from choroid/sclera co-cultures are consistent with the co-culture experiments reported by Marzani and Wallman (1997) as well as those by Rada and Palmer (2007) in which significant inhibition of proteoglycan synthesis was observed when cartilaginous or intact sclera was co-cultured with choroids or suprachoroidal fluid following three days of recovery.

Comparison of culture supernatants from choroid co-cultures indicated that serum protein concentration was significantly increased in the medium of choroids following 4 – 8 days of recovery. The increase in serum proteins released into the culture medium following 4 days of recovery is most likely a reflection of increased choroidal vascular permeability associated with 4 – 15 days of recovery from FD myopia (Pendrak et al., 2000, Rada and Palmer, 2007). We suspect that increased vascular permeability leads to increased extravascular accumulation of serum proteins that are passively released into the culture medium during organ culture. Additionally, increased vascular permeability may facilitate the release of serum proteins from the choroidal vasculature of the choroid explant during organ culture. The presence of serum protein in choroid/sclera co-cultures is responsible for most, if not all of the observed PG synthesis inhibition observed following co-culture with choroids from 4 – 8 days of recovery, as incubation with a concentration of chicken serum comparable to that observed from culture with 5-day recovering choroids resulted in as much, if not more inhibition than that observed in 5 –day sclera/choroid co-cultures (16 – 62% inhibition for 12.5% serum as compared with 21 – 49% for 5 day co-cultures respectively). Additional support for the inhibitory role of serum protein on sclera PG synthesis is the finding that a small, but significant decrease in serum albumin was observed in culture supernatants of 6 hr recovering eyes, which corresponded to a slight increase in

sclera proteoglycan synthesis in co-cultures of sclera with 6 hr treated choroids. The inhibitory effect of serum on scleral proteoglycan synthesis may be due to the presence of ovotransferrin as well as small molecules of less than 3 kDa, both of which have been shown to inhibit scleral proteoglycan synthesis *in vitro* (Rada et al., 2001; Rada and Palmer, 2007).

The rapid deceleration in sclera PG synthesis during the first 12 hrs of recovery from FD myopia followed by a slower deceleration phase over the next four days is suggestive of separate and distinguishable mechanisms that regulate sclera PG synthesis during these two phases. The results of the present study suggest that increased choroidal permeability may assist in the down-regulation of sclera PG synthesis that occurs during the slow deceleration phase following the next 4 days of recovery, however our results indicate that choroid permeability does not play a role in the rapid deceleration phase of scleral proteoglycan synthesis associated with the early phase of recovery from myopia

Previous studies have shown that significant increases in choroid thickness can be detected within 2 hrs of a short period of myopic (positive lens-induced) defocus (Zhu et al., 2005), suggesting that changes in choroid gene expression and tissue architecture occur immediately as a direct result of myopic defocus. Furthermore, we have identified significant changes in choroid expression of HAS2 following 6 hrs of recovery, which mediates changes in hyaluronan synthesis and accumulation within the choroid stroma (Summers Rada, et al., 2010). It remains to be determined, however, as to the role of the choroid in the rapid deceleration of scleral proteoglycan synthesis observed during the recovery from induced myopia. If the choroid is directly involved the rapid down-regulation of scleral proteoglycan synthesis, we suspect that this choroid/sclera tissue interaction occurs locally (e.g. at the choroid-sclera interface) and is tightly regulated, as has been demonstrated in several developmental systems such as diencephalon differentiation (Guinazu et al., 2007), somite formation (Vilhais-Neto et al., 2010), and limb patterning (Mackem, 2006). We therefore suggest that the identification of a direct choroid/sclera interaction will require the development of experimental systems more similar to the *in vivo* condition.

Acknowledgments

This grant was supported by a grant from the National Eye Institute (EYO9391; JSR). The authors thank Drs. Allan Wiechmann, Leo Tsiokas, Marie Hanigan and Eric Howard (Department of Cell Biology, University of Oklahoma Health Sciences Center) for their helpful scientific discussions and Wengtse Lam for her technical assistance.

ABBREVIATIONS

FD	form vision deprivation
GAGs	glycosaminoglycans

References

- Christensen AM, Wallman J. Evidence that increased scleral growth underlies visual deprivation myopia in chicks. *Invest Ophthalmol Vis Sci.* 1991; 32:2143–2150. [PubMed: 2055705]
- Gentle A, Truong HT, McBrien NA. Glycosaminoglycan synthesis in the separate layers of the chick sclera during myopic eye growth: comparison with mammals. *Curr Eye Res.* 2001; 23:179–184. [PubMed: 11803479]
- Goss DA, Criswell MH. Myopia development in experimental animals-a literature review. *Am J Optom Physiol Opt.* 1981; 58:859–869. [PubMed: 7304715]

- Guinazu MF, Chambers D, Lumsden A, Kiecker C. Tissue interactions in the developing chick diencephalon. *Neural Development*. 2007; 2:25. [PubMed: 17999760]
- Liang H, Crewther SG, Crewther DP, Pirie B. Morphology of the recovery from form deprivation myopia in the chick. *Aust N Z J Ophthalmol*. 1996; 24:41–44. (Suppl). [PubMed: 8811241]
- Machem, S. Limb development. In: Unsicker, K.; Kriegstein, K., editors. *Cell Signaling and Growth Factors in Development*. Wiley-VCH Verlag GmbH & Co; 2006. p. 523–618.
- Marzani D, Wallman J. Growth of the two layers of the chick sclera is modulated reciprocally by visual conditions. *Invest Ophthalmol Vis Sci*. 1997; 38:1726–1739. [PubMed: 9286261]
- McBrien NA, Lawlor P, Gentle A. Scleral remodeling during the development of and recovery from axial myopia in the tree shrew. *Invest Ophthalmol Vis Sci*. 2000; 41:3713–3719. [PubMed: 11053267]
- McBrien NA, Moghaddam HO, Reeder AP, Moules S. Structural and biochemical changes in the sclera of experimentally myopic eyes. *Biochem Soc Trans*. 1991; 19:861–865. [PubMed: 1794573]
- Mertz JR, Wallman J. Choroidal retinoic acid synthesis: a possible mediator between refractive error and compensatory eye growth. *Exp Eye Res*. 2000; 70:519–527. [PubMed: 10866000]
- Moring AG, Baker JR, Norton TT. Modulation of glycosaminoglycan levels in tree shrew sclera during lens-induced myopia development and recovery. *Invest Ophthalmol Vis Sci*. 2007; 48:2947–2956.
- Norton TT, Rada JA. Reduced extracellular matrix in mammalian sclera with induced myopia. *Vision Res*. 1995; 35:1271–1281. [PubMed: 7610587]
- Pendrak K, Papastergiou GI, Lin T, Laties AM, Stone RA. Choroidal vascular permeability in visually regulated eye growth. *Exp Eye Res*. 2000; 70:629–637. Erratum in: *Exp Eye Res*. 71:329, 2000. [PubMed: 10870521]
- Qiao-Grider Y, Hung LF, Kee CS, Ramamirtham R, Smith EL 3rd. Recovery from form- deprivation myopia in rhesus monkeys. *Invest Ophthalmol Vis Sci*. 2004; 45:3361–3372. [PubMed: 15452037]
- Rada JA, Huang Y, Rada KG. Identification of choroidal ovotransferrin as a potential ocular growth regulator. *Curr Eye Res*. 2001; 22:121–132. [PubMed: 11402389]
- Rada JA, Johnson JM, Achen VR, Rada KG. Inhibition of scleral proteoglycan synthesis blocks deprivation-induced axial elongation in chicks. *Exp Eye Res*. 2002; 74:205–215. [PubMed: 11950231]
- Rada JA, Matthews AL, Brenza H. Regional proteoglycan synthesis in the sclera of experimentally myopic chicks. *Exp Eye Res*. 1994; 59:747–760. [PubMed: 7698268]
- Rada JA, McFarland AL, Cornuet PK, Hassell JR. Proteoglycan synthesis by scleral chondrocytes is modulated by a vision dependent mechanism. *Curr Eye Res*. 1992; 11:767–782. [PubMed: 1424721]
- Rada JA, Nickla DL, Troilo D. Decreased proteoglycan synthesis associated with form deprivation myopia in mature primate eyes. *Invest Ophthalmol Vis Sci*. 2000; 41:2050–2058.
- Rada JA, Palmer L. Choroidal regulation of scleral glycosaminoglycan synthesis during recovery from induced myopia. *Invest Ophthalmol Vis Sci*. 2007; 48:2957–2966. [PubMed: 17591860]
- Rada JA, Shelton S, Norton TT. The sclera and myopia. *Exp Eye Res*. 2006; 82:185–200. [PubMed: 16202407]
- Rada JA, Thoft RA, Hassell JR. Increased aggrecan (cartilage proteoglycan) production in the sclera of myopic chicks. *Dev Biol*. 1991; 147:303–312. [PubMed: 1916012]
- Schaeffel F, Glasser A, Howland HC. Accommodation, refractive error and eye growth in chickens. *Vision Res*. 1988; 28:639–657. [PubMed: 3195068]
- Sieglwart JT Jr, Norton TT. The susceptible period for deprivation-induced myopia in tree shrew. *Vision Res*. 1998; 38:3505–3515. [PubMed: 9893785]
- Summers Rada JA, Folger RH. Biphasic scleral response during the recovery from induced myopia: is the choroid involved? *ARVO Meeting Abstracts*. 2009; 50:3930.
- Summers Rada JA, Wiechmann AF, Hollaway LR, Baggenstoss BA, Weigel PH. Increased hyaluronan synthase-2 mRNA transcripts and hyaluronan accumulation accompany choroidal

- thickening during the recovery from induced myopia. *Invest Ophthalmol Vis Sci J.* 2010; 51:6172–6179.
- Troilo D, Nickla DL, Mertz JR, Summers Rada JA. Change in the synthesis rates of ocular retinoic acid and scleral glycosaminoglycan during experimentally altered eye growth in marmosets. *Invest Ophthalmol Vis Sci.* 2006; 47:1768–1777. [PubMed: 16638980]
- Vilhais-Neto GC, Maruhashi M, Smith KT, Vasseur-Cognet M, Peterson AS, Workman JL, Pourquié O. Rere controls retinoic acid signalling and somite bilateral symmetry. *Nature.* 2010; 463:953–957. [PubMed: 20164929]
- Wallman J, Adams JI. Developmental aspects of experimental myopia in chicks: Susceptibility, recovery and relation to emmetropization. *Vision Res.* 1987; 27:1139–1163. [PubMed: 3660666]
- Wallman J, Wildsoet C, Xu A, Gottlieb MD, Nickla DL, Marran L, Krebs W, Christensen AM. Moving the retina: choroidal modulation of refractive state. *Vision Res.* 1995; 35:37–50. [PubMed: 7839608]
- Zhu X, Park TW, Winawer J, Wallman J. In a matter of minutes, the eye can know which way to grow. *Invest Ophthalmol Vis Sci.* 2005; 46:2238–2241. [PubMed: 15980206]

Figure 1.

Linear regression analyses of relative scleral proteoglycan synthesis rates. Proteoglycan synthesis rates (**inset**, Figure 1) were divided into three phases; a rapid deceleration over the first 12 hours of recovery (Phase 1; -7.63 ± 1.56 % per hr; $r^2 = 0.9226$; $p = 0.0395$), an intermediate slow deceleration phase that occurred from 12 – 96 hrs (Phase 2; -0.35 ± 0.21 % per hr, $r^2 = 0.7350$, ns.) and a final slow acceleration phase to control levels from 96 – 480 hrs (Phase 3; 0.15 ± 0.02 % per hr, $r^2 = 0.9479$; $p = 0.0264$). Regression was performed on the mean Y values at each time point and P values test the null hypothesis that the overall slope of each line is zero. **Inset:** $^{35}\text{SO}_4$ -GAG synthesis in posterior scleral punches isolated from control and recovering eyes used for regression analyses. Each data point represents the mean percentage difference ($\pm$ S.E.M.) of $^{35}\text{SO}_4$ -incorporation of treated samples relative to that of controls ($\text{exp-control/control} \times 100$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, Students paired t-test for treated vs. control eyes for $n = 5 - 20$ birds in each group.

Figure 2.

Effect of organ culture on $^{35}\text{SO}_4$ -GAG synthesis by sclera from FD eyes. $^{35}\text{SO}_4$ -GAG synthesis was measured on sclera punches isolated from control (dashed line) and FD eyes (10 days of deprivation, solid line) following 0 – 48 hours of organ culture. **Inset:** Relative change in $^{35}\text{SO}_4$ -GAG synthesis in treated eyes as compared with paired controls. Each data point (■) represents the mean percentage difference ($\pm$ S.E.M.) of $^{35}\text{SO}_4$ -incorporation of treated samples relative to that of controls ($\text{exp-control/control} \times 100$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, students paired t-test for comparisons between treated vs. control eyes; $^\dagger p < 0.05$ for comparisons of control sclera from 6 – 48 hr time points as compared with those from 0 hr time point (ANOVA with Bonferroni correction). * $p < 0.01$ for comparisons of FD sclera from 6 – 24 hr time points as compared with those from 0 hr time point (ANOVA with Bonferroni correction). $n = 5$ birds in each group

Figure 3.

Effect of choroid co-culture on $^{35}\text{SO}_4$ -GAG synthesis by untreated sclera. $^{35}\text{SO}_4$ -GAG synthesis was measured on untreated sclera punches following 20 – 24 hours of co-culture with choroids isolated from control and treated eyes. Each data point (■) represents the mean percentage difference ($\pm$ S.E.M.) of $^{35}\text{SO}_4$ -incorporation of sclera co-cultured with treated choroids relative to that of sclera co-cultured with contralateral controls (exp-control/control $\times 100$). * $p < 0.05$, students paired t-test for comparisons between sclera co-cultured with treated vs. control choroids, for $n = 5 - 14$ samples at each time point.

Figure 4.

Serum protein in choroid conditioned medium. Aliquots (10 μ l) of culture medium from the transwell inserts of choroid/sclera co-cultures were analyzed by SDS-PAGE and compared to serial dilutions of chicken serum (3 – 12%). Choroids were isolated from control (C) and 5 day recovering (5 dr) eyes and co-cultured with untreated sclera for 24 hrs.

Figure 5.

Quantification of 66 kD protein in culture medium. **A)** Culture medium from the transwell inserts of choroid/sclera co-cultures (0 hr – 8 days) was analyzed by SDS-PAGE. Gels were digitized and the densities of the ≈ 66 kD band (serum albumin, arrow) were compared between control (C) and recovering (R) eyes. **B)** Each data point ($\bullet$) represents the mean percentage difference ($\pm$ S.E.M.) of relative albumin concentration as determined by densitometry ($\text{exp-control/control} \times 100$). To normalize for overall staining differences, values were normalized to the average of the control band density for each gel. * $p < 0.05$, students paired t-test for comparisons of albumin concentration in co-cultures using treated vs. control choroids, for $n = 5$ at each time point.

Figure 6.

Effect of serum on scleral $^{35}\text{SO}_4\text{-GAG}$ synthesis. $^{35}\text{SO}_4\text{-GAG}$ synthesis was measured in digests of untreated sclera following overnight incubation with normal chicken serum at a final concentration of 12.5% or with N2 medium alone. * $p < 0.05$. (mean $\pm$ SEM for $n = 6$ sclera in each group, ANOVA).