CONCLUSIONS. The results of this study suggest that increased choroidal permeability coincides with a decrease in the rate of scleral glycosaminoglycan synthesis during recovery from myopia. The authors speculate that increased choroidal permeability may represent a mechanism for controlling the rate of delivery of bioactive factors to the sclera to regulate the rate of glycosaminoglycan synthesis in the posterior sclera. (Invest Ophthalmol Vis Sci. 2007;48:2957–2966) DOI:10.1167/iovs.06-1051

Strong evidence from clinical and experimental studies indicates the presence of a vision-dependent emmetropization mechanism that acts to minimize refractive error through the coordinated regulation of the growth of ocular tissues.1–2 Deprivation of form vision, either through ocular abnormality in humans3–6 or as a result of the application of translucent occluders in chicks, tree shrews, or nonhuman primates,7 results in an interruption of this emmetropization mechanism, leading to increased axial elongation and the development of myopia.

In chicks, the development of myopia is associated with increased scleral growth at the posterior pole of the eye, as evidenced by increases in total protein accumulation, proteoglycan synthesis, and proteoglycan accumulation.8–11 Inhibition of proteoglycan synthesis by systemic administration of β-xyloside inhibits approximately 75% of form deprivation–induced ocular growth and slows vitreous chamber growth in control eyes, suggesting that proteoglycan synthesis is a major factor controlling the postnatal growth of the chick eye during the development of myopia.12 These scleral extracellular matrix changes are reversible; restoration of unrestricted vision (and the resultant myopia) results in a temporary cessation of axial growth, eventually leading to the reestablishment of emmetropia (recovery) in the formerly deprived eye.13 We have previously shown10 that the restoration of unrestricted vision from previously form-deprived eyes (recovery) results in a rapid downregulation (within 1 day) in the rate of proteoglycan synthesis in the posterior sclera. This downregulation of scleral proteoglycan synthesis just precedes the deceleration in ocular elongation rate in the formerly deprived (myopic) eye and is presumed to be largely responsible for the deceleration in the rate of scleral vitreous chamber elongation and recovery from myopia. Of much interest is the mechanism by which visual image quality can regulate scleral extracellular matrix remodeling in a rapid and reversible manner.

Because of its proximity to the sclera, the choroid, a highly vascularized layer located between the retina and the sclera, has been implicated in the regulation of scleral metabolism.14,15 As does the mammalian choroid, the chick choroid consists of a layer of choriocapillaries that are adjacent to Bruch membrane and larger blood vessels nearer the sclera.16,17 Additionally, the chick choroid contains numerous thin-walled, endothelial-lined vessels that exhibit structural features of lymphatic vessels (lymphatic lacunae).16,17 The lacunae are largest and most prominent toward the sclera in a region termed the suprachoroida,16 and they contain a clear liquid, termed suprachoroidal fluid, that is easily extracted.20 Similar sparse lymphatic-like structures have occasionally been reported in the primate choroid eye.21,22

Studies by Wallman et al.14 and Liang et al.23 have shown that the chick choroid undergoes a rapid and dramatic increase in thickness in response to myopic defocus. As visualized histologically by light and electron microscopy, the visually driven thickening of the chick choroid results, at least in part, from swelling of the choroidal lacunae14,18 and from extravascular tissue edema.19 Similar changes in choroid thickness have been observed in tree shrews and primates, but to a lesser degree.24–26

It is hypothesized that this increase in choroidal thickness is a rapid mechanism for reducing refractive error by pushing the retina to the focal point.13 This thickness change may be the
result of changes in choroidal blood flow\textsuperscript{27} or vascular permeability\textsuperscript{19,20} in the production of osmotically active molecules, such as glycosaminoglycans, that draw water into lymphatic lacunae present in the choroidal stroma\textsuperscript{14,28} or in the contraction and relaxation of nonvascular smooth muscle cells in the choroidal stroma.\textsuperscript{16,29} Concomitant with choroidal thickening, the rate of vitreous chamber elongation slows dramatically\textsuperscript{30} as does proteoglycan synthesis in the chick sclera.\textsuperscript{10}

We have previously shown that organ-cultured choroids isolated from recovering eyes synthesize, accumulate, and release large amounts of ovotransferrin into the culture medium.\textsuperscript{30} This increase in ovotransferrin accumulation and release is not simply a reflection of choroidal thickening because ovotransferrin levels in culture medium return to control levels, whereas choroidal thickness remains significantly elevated in recovering eyes. Based on a previous report that demonstrated decreased choroidal vascular permeability during the development of form deprivation myopia and marked increases in choroidal vascular permeability during recovery from myopia,\textsuperscript{20} we speculate that the increased ovotransferrin accumulation and release during the recovery process coincides temporally with the deceleration in axial elongation,\textsuperscript{30} we hypothesize that myopia stimulates an increase in choroidal permeability that facilitates the transport of potential ocular growth regulators (such as ovotransferrin) to the sclera, which inhibit scleral glycosaminoglycan synthesis and temporarily slow the rate of axial elongation, facilitating recovery from induced myopia. The present study was designed to test this hypothesis by measuring choroidal permeability and scleral sulfated glycosaminoglycan synthesis at several time points during recovery from myopia. Results of these studies indicate that reestablishment of unrestricted vision from prior form deprivation (recovery) is associated with significant increases in choroidal permeability that coincide with a significant downregulation of glycosaminoglycan synthesis to levels significantly below those of control eyes. Furthermore, suprachoroidal fluid removed from recovering choroids significantly inhibits scleral glycosaminoglycan synthesis in vitro compared with that of fluid isolated from control choroids. Together, these data suggest that changes in choroidal permeability may represent a mechanism for the regulation of ocular growth by controlling the rate of delivery of bioactive factors to the sclera to regulate the rate of scleral glycosaminoglycan synthesis at the posterior pole. The results of the present study have been reported previously in abstract form (Rada JA, et al. IOVS 2006;47:ARVO E-Abstract 1805).

**Materials and Methods**

**Animals**

White Leghorn cockerels (\textit{Gallus gallus}) were obtained as 2-day-old hatchlings from Ideal Breeding Poultry Farms (Cameron, TX). Form deprivation myopia was induced in 2-day-old chicks by applying transparent plastic goggles, as previously described.\textsuperscript{8,10} Briefly, chicks were lightly anesthetized with isoflurane (Vedco Inc., St. Joseph, MO), and hemispheric goggles, cut from the bottom of 15-ml round-bottom test tubes, were affixed with cyanoacrylate adhesive to the feathers around the right eyes of 2-day-old chicks. Goggles remained in place for 10 days, after which chicks were humanely killed either for permeability or for glycosaminoglycan synthesis experiments (0 days of recovery; \( n = 6 \)), or goggles were removed and chicks were allowed to experience unrestricted vision (recovery) for 1, 4, 7, and 15 days (\( n = 4 - 6 \) birds at each time point). The left eyes of all the chicks were never goggled and were used as controls. Previous studies have demonstrated that the rate of glycosaminoglycan synthesis in the posterior sclera and the rate of ocular elongation are significantly increased after 2 days of form deprivation.\textsuperscript{10,15} Upon the restoration of unrestricted vision (from prior 10–14 days of form deprivation), glycosaminoglycan synthesis in the posterior sclera and the rate of ocular elongation decreased to their lowest levels after 4 and 7 days of recovery, respectively.\textsuperscript{10,30} Based on these previous studies, the visual conditions selected for the present study were designed to induce maximal changes in scleral glycosaminoglycan synthesis. Birds were housed in temperature-controlled brooders with a 12-hour light/12-hour dark cycle and were fed food and water ad libitum. Chicks were checked twice daily and were maintained and used in accordance with the Animal Welfare Act, the 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.

**Measurement of Choroidal Permeability**

Choroidal permeability was quantified by measuring albumin leakage from blood vessels into the choroid using Evans blue, as previously described\textsuperscript{31} with some modifications. Evans blue dye (Sigma, St. Louis, MO) was dissolved in normal saline (30 mg/mL), sonicated for 5 minutes, and filtered through a 0.45-\textmu m filter. Chicks (\( n = 4 - 6 \) chicks at each of five time points) were anesthetized with isoflurane (Vedco Inc.) inhalation anesthesia (0.8% in oxygen), and Evans blue (125 mg/kg) was injected through the wing vein using a glass capillary under microscopic inspection. Evans blue noncovalently binds to plasma albumin in the bloodstream and elsewhere and has been previously used to measure vascular permeability.\textsuperscript{32} One hour after intraocular injection, the chicks were anesthetized with isoflurane and killed while still under anesthesia. Immediately afterward, trunk blood was obtained and the eyes were enucleated. Serum was obtained from trunk blood by collection of the supernatant after centrifugation at 1100 rpm for 15 minutes. Vitreous humor and suprachoroidal fluid were withdrawn separately from enucleated eyes using a 50-gauge needle on a Hamilton syringe. Suprachoroidal fluid was collected from the central posterior pole of enucleated chick eyes, as previously described,\textsuperscript{20} by inserting the needle, bevel side up, just beneath the sclera, in the suprachoroidal space. Fluid (10–40 \( \mu \)L) was withdrawn from each eye. Vitreous fluid was withdrawn from a second intraocular site through an injection near the equator of the eye. The concentration of Evans blue in serum (diluted 1:10 with physiological saline), vitreous, and suprachoroidal fluid was determined with a spectrophotometer (ND-1000, Nanodrop, Wilmington, DE) with reference to a standard curve of Evans blue in saline at 620 nm (1.2 \( \mu \)g/mL-1.2 mg/mL). At 620 nm, the absorbance of vitreous, suprachoroidal fluid, and serum (diluted 1:10 in saline) is negligible and similar to that of physiological saline (absorbances of \(-0.009\), \(-0.001\), and \(-0.007\), respectively, when calibrated against saline), and therefore a calibration curve of Evans blue with saline was used to quantify the concentration of Evans blue in vitreous and suprachoroidal fluid.

**Scleral Sulfated Glycosaminoglycan Synthesis**

The posterior hemispheres of eyes of form-deprived chicks (0 days of recovery), eyes from chicks recovering from myopia for 1 to 15 days, and contralateral controls (also used in permeability experiments) were obtained and gently cleaned of all retina, RPE, choroid, vitreous, pectin, and muscle. One 5-mm tissue punch was excised from the posterior sclera with the use of a dermal punch (Miltex Instrument Co., Bethpage, NY). Scleral punches were placed into wells of a 96-well culture plate with 50 \( \mu \)L Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), antibiotic/antimycotic (100 IU penicillin, 100 \( \mu \)g/mL streptomycin, 25 \( \mu \)g/mL amphotericin B), and \( ^{35} \)SO\textsubscript{4} (100 \( \mu \)Ci/mL; New England Nuclear, Beverly, MA) and were incubated for 4 hours at 37°C.

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In some experiments, ocular fluids (vitreous and suprachoroidal fluid) and serum were tested for their ability to alter the rate of scleral glycosaminoglycan synthesis. For these experiments, scleral punches from 10-day-old normal chick eyes (n = 4–7 scleral punches for each condition) were placed in wells of a 96-well culture plate containing 50 μL ocular fluid (or DMEM alone) diluted 1:1 with DMEM containing antibiotic/antimycotic and 35SO4 (200 μCi/mL; New England Nuclear) and were incubated for 24 hours at 37°C.

Radiolabeled scleral punches were digested with proteinase K (protease type XXVIII; Sigma), 0.05% wt/vol in 10 mM EDTA, 0.1 M sodium phosphate, pH 6.5, overnight at 60°C. Glycosaminoglycans ([35S]SO4 labeled) were precipitated by the addition of 0.5% cetylpyridinium chloride (CPC) in 0.002 M Na2SO4 in the presence of unlabeled carrageenan and monosodium sulfate (1 mg/mL in dH2O). Samples were incubated for 30 minutes at 37°C, and precipitated glycosaminoglycans were collected on Whatman filters (GF/F) using a 12-port sampling manifold (Millipore, Bedford, MA) as previously described.10 Radioactivity was measured directly on the filters by liquid scintillation counting.

**Cell Culture**

Human sclerae obtained, through the National Disease Research Interchange (NDRI), from human donor eyes within 48 hours of death were isolated from both eyes of a 26-year-old donor, cleaned of adhering muscle, fat, retina, conjunctiva, and vitreous, and minced into pieces smaller than 2 mm2. Scleral pieces were then placed in 60-mm culture dishes and were covered with sterile coverslips to hold the sclerae in place. Fibroblasts were grown from the explanted donor sclerae in DMEM with 15% fetal bovine serum. After 2 to 4 weeks of culture, the coverslips and scleral explants were removed, and cultures were allowed to become confluent. For measurements of sulfated glycosaminoglycan synthesis rates, cultures were incubated in the presence of [35SO4 (100 μCi/mL; PerkinElmer Inc., Wellesley, MA) for 24 hours at 37°C in suprachoroidal fluid from control and recovering eyes diluted 1:2 with DMEM containing 1× antibiotic/antimycotic (n = 4 fibroblast cultures per condition). Sulfated glycosaminoglycan synthesis was assessed by measuring the amount of [35SO4 incorporated into CPC-precipitable glycosaminoglycans from aliquots of the culture medium, as described. This research was conducted in accordance with the tenets of the Declaration of Helsinki.

**Suprachoroidal Fluid Characterization**

After 10 days of monocular form deprivation and 3 days of unrestricted vision, control and experimental eyes were enucleated, and 10 to 30 μL suprachoroidal fluid was removed from the posterior pole of control and experimental eyes using a 30-gauge needle on a Hamilton syringe, with the aid of a dissection microscope. In some cases, vitreous fluid was withdrawn separately from the vitreous chamber with the Hamilton syringe. Suprachoroidal fluid and vitreous fluid were pooled separately for three batches of control and recovering eyes using 50 chicks per batch. Suprachoroidal fluid and vitreous fluid were centrifuged at 4°C for 15 minutes to pellet cellular debris. In some cases, suprachoroidal fluid from control and recovering eyes was fractionated using a 10-kDa molecular weight cutoff filter (Millipore) by centrifugation at 4°C for 30 minutes to 2 hours, until no fluid was visible in the retentate chamber. The retentate (more than 10-kDa molecular weight fraction) was reconstituted to the original volume with 0.85% NaCl and was used in glycosaminoglycan synthesis assays. The filtrate (less than 10-kDa molecular weight fraction) was then separated using a 3-kDa molecular weight cutoff filter (Millipore) by centrifugation at 4°C for 30 minutes. The filtrate (less than 3-kDa molecular weight fraction) and the retentate (3- to 10-kDa molecular weight fraction) were used in scleral glycosaminoglycan synthesis assays.

**Proteinase Digestion**

In one series of experiments, aliquots of suprachoroidal fluid from control and recovering eyes were separated into 3- to 10-kDa fractions (digested with proteinase K [0.5 mg/mL; Trichirachium album; Amresco, Solon, OH] at 60°C overnight) and fractions that were smaller than 3 kDa. The proteinase was removed from the 3- to 10-kDa fraction after digestion by microcentrifugation using a 10-kDa molecular weight cut-off filter, and the resultant filtrate was collected for subsequent scleral glycosaminoglycan synthesis experiments.

**Statistical Analysis**

Comparisons of sulfated glycosaminoglycan synthesis rates and choroidal permeability between control and recovering eyes were made using Student’s two-tailed t-tests for matched pairs. Comparisons of effects of suprachoroidal fluid or serum on scleral glycosaminoglycan synthesis rates were made using the one-factor Scheffe’ F test for multiple comparisons with Bonferroni correction.

**RESULTS**

Through a technique described by Pendrak et al.,20 suprachoroidal fluid was readily extracted from the posterior poles of enucleated chick eyes. The suprachoroidal fluid was clear, and there were no visible signs of contamination with blood. Occasional pigment was aspirated but was removed by centrifugation before biochemical analyses. In contrast to the results reported by Pendrak et al.,20 a greater volume of suprachoroidal fluid could be removed from recovering eyes (approximately 15–20 μL) than from contralateral control eyes (approximately 10 μL), and substantially lower volumes of suprachoroidal fluid could be removed from form-deprived eyes (≤5 μL).

**Changes in Choroidal Permeability during Recovery**

Evans blue concentration was measured in aliquots of suprachoroidal fluid extracted from enucleated eyes of chicks after 0, 1, 4, 7, and 15 days of recovery from induced myopia (Fig. 1A). Evans blue concentration in suprachoroidal fluid of control eyes after 10 days of form deprivation (0 days of recovery) was significantly lower in treated eyes (18.9 ± 4.4 μg/mL) than in contralateral control eyes (79.1 ± 19.9 μg/mL; P = 0.05; paired t-test). Evans blue concentration was not significantly elevated in eyes after 1 day of unrestricted vision (recovering eyes, 58.9 ± 22.5 μg/mL) compared with paired contralateral control eyes (20.9 ± 5.9 μg/mL; P = 0.135; paired t-test). After 4 days of unrestricted vision, the suprachoroidal fluid levels of Evans blue were significantly elevated in recovering eyes compared with levels in contralateral controls (224.5 ± 20.0 μg/mL vs. 20.2 ± 5.6 μg/mL; P < 0.01; paired t-test). Evans blue levels remained elevated in the suprachoroidal fluid of recovering eyes after 7 days of recovery (180.4 ± 38.7 μg/mL) compared with those of contralateral controls (20.1 ± 3.7 μg/mL; P < 0.05; paired t-test) but returned to levels similar to those of controls after 15 days of recovery (130.0 ± 47.8 μg/mL; P > 0.05; paired t-test). Similar results were obtained when the Evans blue concentration in suprachoroidal fluid was normalized to the plasma concentration of Evans blue (Fig. 1B). Comparison of normalized Evans blue levels in the suprachoroidal fluid of control eyes of all treatment groups (0–15 days of recovery) suggested that little change occurred in choroidal permeability in untreated control eyes. Comparison of normalized Evans blue levels in the suprachoroidal fluid of treated eyes after 1 day of unrestricted vision, compared with normalized Evans blue levels in suprachoroidal fluid from form-deprived eyes (0 days of recovery), indicated that significant increases in choroidal permeability occurred in the treated eye after 1 day of unrestricted vision (P < 0.05; ANOVA). Results of these experiments confirm and extend the studies of Pendrak et al.,20 who demonstrated increased vas-
cular permeability in the choroids of chick eyes recovering from form deprivation myopia for 7 days.

**Changes in Scleral Sulfated Glycosaminoglycan Synthesis during Recovery**

After extraction of suprachoroidal fluid, sulfated glycosaminoglycan synthesis was measured as the rate of $^{35}$SO$_4$ incorporated into CPC-precipitable glycosaminoglycans in organ-cultured, 5-mm scleral punches excised from the posterior poles of enucleated chick eyes (Fig. 2). After 10 days of form deprivation, the rate of sulfated glycosaminoglycan synthesis in the posterior sclera of deprived eyes was significantly higher than that of control eyes (P < 0.05; paired t-test). After 1 day of unrestricted vision, no significant differences were detected in the rate of sulfated glycosaminoglycan synthesis between recovering and control eyes (P = 0.772; paired t-test). In contrast, after 4 days of unrestricted vision, the rate of scleral glycosaminoglycan synthesis was significantly lower in recovering eyes than in control eyes (P < 0.05; paired t-test). After 7 days of recovery, scleral glycosaminoglycan synthesis rates in recovering eyes returned to levels similar to those of controls (P = 0.588). No significant differences were detected in glycosaminoglycan synthesis rates between recovering and control eyes after 15 days of unrestricted vision, though a trend toward an increased rate of sulfated glycosaminoglycan synthesis was observed in the posterior sclera of recovering eyes compared with control eyes (P = 0.071; paired t-test). Comparison of the relative increase in choroidal permeability in recovering eyes with that of relative decrease in scleral glycosaminoglycan synthesis in recovering eyes suggested that the increase in choroidal permeability coincided with a significant reduction in the rate of scleral glycosaminoglycan synthesis in the posterior sclera (Fig. 3).

**Biological Activity of Suprachoroidal Fluid**

Suprachoroidal fluid and vitreous fluid were isolated from experimental chick eyes (after 10 days of form deprivation with or without 3 additional days of unrestricted vision) and control eyes, pooled separately for experimental and control eyes, and applied to scleral punches, isolated from untreated chicks, in organ culture. Sulfated glycosaminoglycan synthesis was significantly increased in form-deprived eyes compared with control eyes (P < 0.05; paired t-test). In contrast, incubation of scleral punches with suprachoroidal fluid from control eyes resulted in a significant decrease in the rate of sulfated glycosaminoglycan synthesis after 24 hours of radiolabeling with $^{35}$SO$_4$ compared with suprachoroidal fluid from control eyes (P < 0.01; ANOVA with Bonferroni correction; Fig. 4). No significant differences in scleral glycosaminoglycan synthesis were observed when scleral punches were incubated with vitreous fluid from control eyes compared with vitreous fluid from recovering eyes or DMEM alone. In contrast, incubation of scleral punches with suprachoroidal fluid isolated from myopic eyes (after 10 days of form deprivation) resulted in a significant increase in scleral proteoglycan synthesis compared with incubation of scleral punches with suprachoroidal fluid isolated from control eyes or DMEM alone (+31% and +48%; P < 0.05 and
Unlike the sclera of placental mammals, the chick sclera contains an outer cartilaginous layer in addition to an inner fibrous scleral layer structurally similar to that of mammals and humans. Therefore, to gain insight into the effect of suprachoroidal fluid on cells of the mammalian fibrous sclera, cultures of human scleral fibroblasts were incubated with suprachoroidal fluid extracted from recovering and control chick eyes. Incubation of cultures of human scleral fibroblasts with suprachoroidal fluid from recovering chick eyes resulted in a significant increase in sulfated glycosaminoglycan synthesis compared with fibroblast cultures incubated with suprachoroidal fluid from control chick eyes (Fig. 6; *P ≤ 0.01; t-test), indicating that the inhibition of sulfated glycosaminoglycan synthesis by recovering suprachoroidal fluid is not a general effect; rather, it is specific to chick sclera.

Centrifugal Fractionation of Suprachoroidal Fluid

Suprachoroidal fluid was fractionated using 10-kDa molecular weight cutoff filters, and the filtrate (≤ 10 kDa) and retentate (≥ 10 kDa) were assessed for glycosaminoglycan synthesis inhibition (Fig. 7A). The ≥ 10-kDa fractions were generally inhibitory to scleral glycosaminoglycan synthesis compared with DMEM alone (*P ≤ 0.01); however, no significant differences in sulfated glycosaminoglycan synthesis were detected when sclera were incubated with ≤ 10-kDa suprachoroidal fluid fractions from recovering eyes compared with those from control eyes (*P = 0.237). In contrast, the ≤ 10-kDa suprachoroidal fluid
Fraction from recovering eyes demonstrated significant inhibition of scleral glycosaminoglycan synthesis compared with the <10 kDa suprachoroidal fluid fraction from control eyes (-56%; *P < 0.001). Chick sclera incubated with the <10 kDa suprachoroidal fluid fraction from control eyes demonstrated rates of glycosaminoglycan synthesis similar to those of sclera incubated in DMEM alone. These results suggest that the inhibitory activity observed in suprachoroidal fluid from recovering eyes is likely the result of bioactive factors with a mass of <10 kDa. Therefore, the <10 kDa suprachoroidal fluid fractions were further separated into a <3 kDa fraction and a 3- to 10 kDa fraction. No significant differences were detected in sulfated glycosaminoglycan synthesis rates when chick sclera were incubated with the <3 kDa fractions from control eyes compared with those of recovering eyes or the 3- to 10 kDa suprachoroidal fluid fractions from control eyes compared with those from recovering eyes (Fig. 7B). Moreover, scleral glycosaminoglycan synthesis activity in the 3- to 10 kDa suprachoroidal fluid fractions from control and recovering eyes was not significantly different from that of DMEM alone (*P = 0.566; ANOVA). Interestingly, the <3 kDa fractions from control and recovering eyes demonstrated significant inhibitory activity on the rate of scleral glycosaminoglycan synthesis compared with the 3- to 10 kDa fractions or DMEM alone (*P < 0.01 for control samples; *P < 0.001 for recovering samples). These results suggest that the strong inhibitory activity of the <3 kDa fractions from control and recovering eyes is differentially regulated by compounds in the 3- to 10 kDa suprachoroidal fluid fractions.

To determine whether an interaction between compounds in the <3 kDa suprachoroidal fluid fraction and proteins in the 3- to 10 kDa fraction of suprachoroidal fluid were involved in the inhibition of scleral glycosaminoglycan synthesis, the 3- to 10 kDa suprachoroidal fluid fractions from control and recovering eyes were digested with proteinase K or were incubated in enzyme buffer alone before recombination with their respective <3 kDa fractions and were tested for effects on sulfated glycosaminoglycan synthesis inhibition (Fig. 8). Unseparated fractions represented suprachoroidal fluid isolated from control and recovering eyes and applied directly scleral punches; undigested fractions represented suprachoroidal fluid incubated with enzyme buffer at 60°C.

Digestion of the 3- to 10 kDa fraction of suprachoroidal fluid from control eyes followed by recombination with the <3 kDa fraction from control eyes and subsequent organ culture with chick sclera in the presence of 35SO4 resulted in significant inhibition in the rate of sulfated glycosaminoglycan synthesis compared with suprachoroidal fluid from control eyes in which the 3- to 10 kDa fraction was incubated in enzyme buffer only and recombined with the <3 kDa fraction from control eyes (undigested; -52%; *P < 0.01).

Similarly, digestion of the 3- to 10 kDa fractions of suprachoroidal fluid from recovering eyes before recombination with the <3 kDa fraction resulted in a significant reduction in glycosaminoglycan synthesis compared with undigested recombinant fractions from control and recovering eyes. **P ≤ 0.001; ***P ≤ 0.01. DMEM, rate of sulfated glycosaminoglycan synthesis in scleral organ cultures incubated in DMEM alone (mean ± SEM for n = 6 scleral punches in each group: ANOVA).

![Figure 7](image-url)  
**Figure 7.** Centrifugal fractionation of suprachoroidal fluid. (A) Suprachoroidal fluid from control and recovering eyes was passed through a 10-kDa centrifugal filter, and the filtrate (<10 kDa) and retentate (>10 kDa) were tested on cultures of chick sclera for their effects on scleral sulfated glycosaminoglycan (GAG) synthesis. n.s., not significant; ***P < 0.01; ANOVA. (B) Further separation of suprachoroidal fluid using 3-kDa centrifugal filters indicated that significant sulfated glycosaminoglycan synthesis inhibitory activity was present in <3-kDa fractions of suprachoroidal fluid from control and recovering eyes. **P ≤ 0.001; ***P ≤ 0.01. DMEM, rate of sulfated glycosaminoglycan synthesis in scleral organ cultures incubated in DMEM alone (mean ± SEM for n = 6 scleral punches in each group: ANOVA).

![Figure 8](image-url)  
**Figure 8.** Effect of proteinase digestion on bioactivity of suprachoroidal fluid. The >3-kDa/10-kDa fractions of suprachoroidal fluid from control and recovering eyes were subjected to proteinase K digestion before reconstitution with the <3-kDa fractions and were assessed for scleral sulfated glycosaminoglycan (GAG) synthesis inhibition in organ cultures with chick sclera in the presence of 35SO4. Digestion of the >3-kDa/10-kDa fractions of suprachoroidal fluid from control and recovering eyes resulted in significant inhibition in the rate of sulfated glycosaminoglycan synthesis compared with undigested fractions (**P < 0.01; *P < 0.05). Unseparated (suprachoroidal fluid tested directly) and undigested (fractionated and incubated in proteinase buffer) suprachoroidal fluid from recovering eyes was significantly more inhibitory for scleral sulfated glycosaminoglycan synthesis compared with suprachoroidal fluid from control eyes (**P < 0.01; mean ± SEM for n = 6 scleral punches for each condition).
sclera to levels 33% to 128% above those of contralateral control eyes.10

Because scleral glycosaminoglycan synthesis rates were similar after incubation with suprachoroidal fluid containing protease-digested 3- to 10-kDa fractions from control and recovering eyes, we speculate that proteins in the 3- to 10-kDa fractions of suprachoroidal fluid from control eyes act to disassociate scleral glycosaminoglycan synthesis, possibly through interactions with inhibitory molecules in the <3-kDa fractions. For comparison, normal chick sclera was also incubated with unseparated, undigested suprachoroidal fluid from control and recovering eyes (unseparated). As expected, undigested suprachoroidal fluid from recovering eyes was significantly more inhibitory for scleral glycosaminoglycan synthesis than was suprachoroidal fluid from control eyes (\( \*P < 0.01; \text{Student's } t \)-test). Similarly, undigested suprachoroidal fluid from recovering eyes (separated on 10-kDa and 3-kDa centrifugal filters and incubated at 60°C in proteinase buffer) was significantly more inhibitory for scleral glycosaminoglycan synthesis than was suprachoroidal fluid from control eyes (\( \*\*P < 0.01; \text{Student's } t \)-test). The increase in scleral proteoglycan synthesis observed after incubation with undigested fractions of suprachoroidal fluid from control and recovering eyes most likely reflects the dilution (1:10) of the undigested suprachoroidal fluid with the enzyme buffer and the possible effects of incubation temperature (60°C) on the activity of the 3- to 10-kDa factors.

Because scleral glycosaminoglycan synthesis rates were similar after incubation with suprachoroidal fluid containing protease-digested 3- to 10-kDa fractions from control and recovering eyes, we speculate that proteins in the 3- to 10-kDa fractions are largely responsible for the differences in bioactivity of suprachoroidal fluid from recovering and control eyes.

**Effect of Serum on Scleral Sulfated Glycosaminoglycan Synthesis**

We hypothesize that changes in choroidal vascular permeability alter the rate of delivery of bioactive factors to the sclera to regulate the rate of proteoglycan synthesis in the posterior sclera. These bioactive factors may be synthesized by the choroid, or they may be present in the general circulation. To determine whether serum can similarly regulate scleral glycosaminoglycan synthesis, sulfated glycosaminoglycan synthesis was measured in punches of normal chick sclera after incubation with chicken serum at concentrations ranging from 0% to 100% (Fig. 9A). Significant inhibition of sulfated glycosaminoglycan synthesis was observed after incubation of chick scleral punches with 50% to 100% chicken serum (\( P < 0.05 \) and \( P < 0.01 \), respectively; ANOVA). Scleral glycosaminoglycan synthesis was assessed on scleral punches after incubation with chick serum and suprachoroidal fluid after separation into 3- to 10-kDa and <3-kDa fractions (Fig. 9B). Similar to results shown in Figure 7B, the <3-kDa fractions of suprachoroidal fluid from control and recovering eyes and from chick serum demonstrated significant inhibitory activity on the rate of scleral glycosaminoglycan synthesis compared with the 3- to 10-kDa fractions of each fluid or DMEM alone (\( P < 0.01 \); ANOVA). No significant differences were detected between DMEM alone and any of the 3- to 10-kDa fractions (\( P > 0.05 \); Scheffé comparisons). Thus, both in serum and in suprachoroidal fluid, the <3-kDa fraction is highly inhibitory compared with whole serum or with the >3-kDa fraction.

**DISCUSSION**

Of much interest is the mechanism by which visual image quality can regulate scleral extracellular matrix remodeling in a rapid and reversible manner to effect dramatic changes in the rate of vitreous chamber elongation and the refractive state of the eye. The observations that the choroid undergoes dramatic increases in thickness14,23 and permeability20 during recovery from induced myopia and that it demonstrates thinning14,23 and lowered permeability20 during the development of myopia suggest this ocular tissue may play a role in controlling ocular growth under these visual conditions. Moreover, isolated choroids cultured in the presence of chick sclera have been shown to upregulate and downregulate scleral proteoglycan synthesis, depending on the visual condition of the eye from which the choroid was isolated.15 Here, we report that the increased choroidal permeability associated with recovery from induced myopia coincides temporally with significant inhibition in scleral glycosaminoglycan synthesis compared with contralateral control eyes.

We have previously shown that form deprivation for 1 to 10 days stimulates the synthesis of proteoglycans in the posterior sclera to levels 33% to 128% above those of contralateral control eyes10 and that restoration of unrestricted vision (myopic defocus) for 1 day results in rapid downregulation of
scleral proteoglycan synthesis to control levels. After 2 days of recovery, scleral proteoglycan synthesis levels continue to fall to levels significantly below (~32%) those of contralateral controls. The upregulation and downregulation of scleral proteoglycan synthesis rates in response to form deprivation and myopic defocus immediately precede the acceleration and deceleration of vitreous chamber elongation rates associated with the development of and the recovery from myopia, respectively, suggesting that the rate of proteoglycan synthesis in the posterior chick sclera is directly related to the rate of vitreous chamber elongation.

Results from the present study show that significant increases in choroidal permeability occurred within 1 day of unrestricted vision after previous form deprivation. Choroidal permeability was further increased in treated eyes after recovery periods up to 7 days and then decreased to levels similar to permeability levels in control eyes after 15 days of recovery. The increased choroidal permeability observed in treated eyes after 1 to 7 days of recovery coincided with a rapid and significant downregulation of scleral proteoglycan synthesis rates in recovering eyes. Based on these results, we speculate that when choroidal permeability is lower in treated eyes than in control eyes (as in form-deprived eyes), proteoglycan synthesis and the rate of ocular elongation will be higher in experimental eyes than in contralateral control eyes. When choroidal permeability in treated eyes is similar to that of control eyes, scleral proteoglycan synthesis and ocular elongation rates will be similar in the two eyes, as was observed after 1 day of unrestricted vision. When choroidal permeability in treated eyes is higher than in control eyes, scleral proteoglycan synthesis will be lower than in contralateral control eyes (and in 4-day recovering eyes), and ocular elongation rates will be significantly slower than in contralateral control eyes.

Previous studies have demonstrated that choroidal thickening in response to myopic defocus is associated with dilatation of choroidal lymphatic vessels in addition to swelling of the choroidal stroma. Results of Pendrak et al. and from the present study suggest that much of the increased volume of suprachoroidal fluid is derived from plasma. We speculate that increased vascular permeability of choroidal blood vessels leads to accumulation of plasma proteins and water within the choroidal stroma and within choroidal lymphatic vessels, either through channeling fluid to lymphatic vessels from the choroidal extravascular stroma or through direct lacunae-venous connections, as have been previously described.

It is possible that these lymphatic vessels act to regulate the delivery of choroidally derived growth factors to the sclera and that the dilution of these vessels during recovery from induced myopia represents a mechanism to channel scleral growth regulators away from the sclera. Although we cannot exclude this possibility, our data demonstrate that suprachoroidal fluid contains scleral growth regulators that can upregulate and downregulate scleral proteoglycan synthesis in a manner similar to that observed in vivo during visually guided ocular growth, and we suggest that this fluid is delivered to the sclera in vivo to regulate the rate of vitreous chamber elongation.

We hypothesize that choroidal permeability is a mechanism for the regulation of ocular growth by controlling the rate of delivery of bioactive factors to the sclera to regulate the rate of proteoglycan synthesis in the posterior sclera. Changes in choroidal permeability may represent a direct mechanism for the control of the rate of ocular elongation, or they may be the secondary effect of other visually induced choroidal changes. Our hypothesis is most strongly supported by our observations that suprachoroidal fluid isolated from eyes recovering from induced myopia significantly inhibited sulfated glycosaminoglycan synthesis by chick sclera compared with suprachoroidal fluid and vitreous fluid from control eyes and that suprachoroidal fluid isolated from eyes with induced myopia significantly stimulated the rate of scleral glycosaminoglycan synthesis compared with contralateral control eyes.

Suprachoroidal fluid was subsequently fractionated on centrifugal filters to identify fractions with bioactivity when assessed for altering scleral glycosaminoglycan synthesis rates. Although >10 kDa fractions from control and recovering eyes were generally inhibitory to scleral glycosaminoglycan synthesis, no differences were observed in glycosaminoglycan synthesis rates when sclerae were incubated with >10 kDa fractions of suprachoroidal fluid from control eyes compared with recovering eyes. We previously identified ovotransferrin as an abundant protein in the suprachoroidal fluid with proteoglycan synthesis-inhibiting activity. Based on the relatively large mass of ovotransferrin (78 kDa), this protein would be present in the >10 kDa fractions of suprachoroidal fluid of control, recovering, and myopic eyes and would not be modulated by vision-dependent mechanisms. In contrast, the <10 kDa fraction from recovering eyes demonstrated significant inhibitory activity compared with that from control eyes, nearly identical with inhibitory activity observed in unfractionated suprachoroidal fluid from recovering eyes (56% inhibition with fractionated fluid, 54% inhibition with unfractonated fluid). These results suggest that the vision-dependent inhibitory activity observed in suprachoroidal fluid from recovering eyes was probably caused by bioactive factors with a mass of <10 kDa. However, further separation of the suprachoroidal fluid samples into <3 kDa and 3–10 kDa fractions resulted in a loss of recovery-specific inhibitory activity. Interestingly, the <3 kDa fractions from control and recovering eyes demonstrated significant glycosaminoglycan synthesis inhibition compared with the 3–10 kDa suprachoroidal fluid fractions or DMEM alone. These results suggest an interaction between factors present in the 3–10 kDa fraction and the <3 kDa fraction. Moreover, the strong inhibition of sulfated glycosaminoglycan synthesis by the <3 kDa fractions of suprachoroidal fluid from control and recovering eyes suggests that compounds in the 3–10 kDa fractions act to disinhibit the activity of the <3 kDa fractions, and this interaction is responsible for the differences in glycosaminoglycan synthesis regulation by recovering and control suprachoroidal fluid. This interaction was supported by experiments in which proteins in the 3–10 kDa fractions from control and recovering eyes were digested with proteinase K before recombination with the <3 kDa fraction. Digestion of proteins in the 3–10 kDa fractions of suprachoroidal fluid resulted in approximately 52% and 36% inhibition of scleral glycosaminoglycan synthesis rates in control and recovering fractions (digested), respectively, resulting in similar activity levels between control and recovering suprachoroidal fluid fractions. Taken together, these results suggest that differences in bioactivity of suprachoroidal fluid from recovering and control eyes are caused by alterations in the interactions of inhibitory factors in the <3 kDa fraction with suprachoroidal fluid proteins in the 3–10 kDa fraction, which normally act to inhibit the action of inhibitory small molecules (<3 kDa) present in suprachoroidal fluid from control and recovering eyes.

Interestingly, chicken serum demonstrated significant inhibitory activity on scleral glycosaminoglycan synthesis when present in concentrations of 50% or greater, and fractionation of serum into <3 kDa and 3–10 kDa fractions indicated that the <3 kDa fraction was highly inhibitory compared with the 3–10 kDa fraction and similar in inhibitory activity to the <3 kDa fractions of suprachoroidal fluid from control and recovering eyes. These results suggest that variations in serum protein concentration in the suprachoroidal fluid, as a result of vascular permeability changes, may be responsible for the
differential effects of control, myopic, and recovering suprachoroidal fluid on scleral glycosaminoglycan synthesis rates in vitro.

Retinoic acid has been suggested as a chemical signal between the choroid and sclera involved in ocular growth regulation because choroidal retinoic acid synthesis is increased in choroids of chick eyes recovering from myopia, and all-trans retinoic acid inhibits scleral proteoglycan synthesis in vitro. In plasma and within the cell, retinoids are bound to distinct transport proteins, respectively, the plasma retinol-binding protein (RBP) and the cellular retinol-binding proteins (CRBPs). Therefore, although free retinoic acid is of sufficiently small molar mass (300.42 g/mol) to be in the <3-kDa fractions of suprachoroidal fluid, retinoic acid complexed with its binding protein(s) would most likely be absent from the inhibitory <10-kDa fractions of control and recovering suprachoroidal fluid. Interestingly, suprachoroidal fluid from recovering eyes of chicks was stimulatory for sulfated glycosaminoglycan synthesis by human scleral fibroblasts compared with suprachoroidal fluid from controls, indicating that the inhibition observed on chick sclera was not a reflection of a general inhibitory activity of suprachoroidal fluid but rather was specific for chick sclera. It is important to note that the chick sclera is composed of an inner layer of cartilage absent in most mammals and an outer fibrous layer similar in its composition to the fibrous type I collagen–dominated extracellular matrix of the human sclera. In chicks, the increased ocular enlargement associated with the development of myopia results from growth of the inner cartilaginous region, whereas the fibrous sclera in chick and in mammals shows little sign of induced growth; instead, the fibrous sclera exhibits decreased synthesis of proteoglycans and collagens and overall thinning during myopia development.

In chicks and mammals, recovery is characterized by a reversal of the processes occurring during the development of myopia; recovery is associated with decreased growth and decreased sulfated glycosaminoglycan synthesis in the posterior cartilaginous sclera of chicks and increased growth and sulfated glycosaminoglycan synthesis in the fibrous sclera. Our finding that suprachoroidal fluid from recovering chick eyes stimulates, rather than inhibits, sulfated glycosaminoglycan synthesis by human scleral fibroblasts suggests that the choroidal response associated with recovery from myopia may represent a general signaling mechanism to slow the rate of ocular elongation during recovery from induced myopia, regardless of scleral structure. Changes in choroidal vascular permeability coincident with changes in scleral glycosaminoglycan synthesis suggest that bioactive factors in suprachoroidal fluid are derived from serum and de novo choroidal synthesis. We cannot exclude the possibility, however, that bioactive factors present in suprachoroidal fluid are derived partially or in total by diffusion from the retina, RPE, or sclera because each of these ocular tissues has been shown to undergo protein or gene expression changes in association with visually guided ocular growth. Limitations in the volume of suprachoroidal fluid available for analyses has curtailed identification of bioactive compounds in the suprachoroidal fluid. Continued studies using molecular and proteomic approaches are under way to elucidate the choroidal regulation of scleral extracellular matrix remodeling.

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References

2. van Alphen GW. Emmetropization in the primate eye. CIBA Found Symp 1990;155:115–120.


**ERRATUM**


The fifth author’s name should be listed as Mahiul Muhammed Khan Muqit.