Visual Deprivation Upregulates Extracellular Matrix Synthesis by Chick Scleral Chondrocytes

Jody A. Rada and Amy L. Matthews

Purpose. To characterize the cellular events responsible for the exaggerated ocular growth associated with experimental myopia in chicks, the accumulation and synthesis of proteoglycans and collagen were measured in the posterior sclera of control and form vision-deprived chick eyes.

Methods. Buttons (10 mm) from the posterior sclera of control and deprived eyes were used for biochemical measurements of glycosaminoglycans and hydroxyproline to estimate proteoglycan and collagen accumulation, respectively. The synthesis of proteoglycan, collagen, total protein, and RNA were measured in cultures of scleral chondrocytes isolated from posterior scleral buttons of control and deprived eyes by measuring the specific incorporation of $^{35}$SO$_4$, $[^3H]$proline, $[^3S]$methionine, and [5-$^3H$]uridine, respectively. The relative rate of aggrecan precursor protein synthesis was measured in cultures of control and deprived chondrocytes using immunoprecipitation assays.

Results. Form deprivation resulted in increased accumulation of proteoglycans but not collagen within the posterior sclera. In contrast, chondrocytes isolated from the posterior sclera of form-deprived eyes maintained elevated rates compared with controls of proteoglycan synthesis (+143%) and collagen synthesis (155%), as well as total protein synthesis (115%) and total RNA synthesis (44%). Because total protein synthesis was higher in cultures of deprived chondrocytes, the rate of aggrecan precursor protein synthesis, relative to total protein synthesis, was similar for both populations of cells. Pretreatment of scleral chondrocytes with actinomycin D, an inhibitor of RNA synthesis, resulted in a 112% increase in the rate of proteoglycan synthesis by control chondrocytes, but had no significant effect on the rate of proteoglycan synthesis by chondrocytes isolated from form-deprived eyes.

Conclusions. Because proteoglycans accumulate within the posterior sclera of deprived eyes to a greater extent than collagen, yet form deprivation stimulates the synthesis of collagen and total protein as well as proteoglycans, these data suggest that collagen, and perhaps other scleral components, are selectively remodeled within the posterior sclera during the process of ocular elongation. Furthermore, experiments with actinomycin D suggest that the general upregulation observed in form-deprived chondrocytes may be due to the absence of an inhibitor normally present under conditions of form vision. Invest Ophthalmol Vis Sci. 1994; 35:2436-2447.

Form vision deprivation, either experimentally induced in animals or associated with ocular injury or pathology in humans, has been shown to result in axial elongation of the ocular globe and subsequent myopia. Studies on form-deprivation myopia in chicks indicate that ocular elongation involves scleral growth, which is largely due to the synthesis and accumulation of extracellular matrix within the posterior region of the ocular globe.

Unlike the mammalian sclera, normal chick sclera consists of a thick inner cartilaginous layer as well as an outer fibrous layer. Scleral cartilage, like that of normal cartilage, consists of chondrocytes embedded in a matrix consisting of the proteoglycans aggrecan, decorin, biglycan, and fibromodulin and collagens type II, IX, and X. Aggrecan is the major proteoglycan of
cartilage where it functions to supply the osmotic swelling pressure of cartilage, whereas the collagenous components provide cartilage with its tensile strength. Biochemical analyses of chick sclera indicate that form deprivation stimulates the synthesis and accumulation of aggrecan within the cartilaginous sclera of the posterior pole of the eye. The synthesis of decorin, a small chondroitin–dermatan sulfate proteoglycan is also increased; however, no net accumulation of decorin was detected from extracts of the posterior sclera. Significant increases in the rate of aggrecan synthesis have been reported to occur as early as 1 day after form deprivation, and synthesis levels remain elevated during the deprivation period. This effect is rapidly reversible. Removal of the occluder causes proteoglycan synthesis to fall below control levels within 24 hours, resulting in a temporary cessation of ocular growth. These results indicate that changes in proteoglycan synthesis in the posterior cartilaginous sclera are modulated, albeit indirectly, by the quality of the visual environment.

In contrast to the changes observed in proteoglycan accumulation, no significant differences were detected in collagen accumulation when measured on the anterior or posterior scleral hemispheres of control and deprived eyes. However, because the orbit is rapidly enlarging in response to form deprivation, we would expect the existing collagenous components of the deprived sclera to be actively degraded and new collagens synthesized to accommodate proteoglycan accumulation and expansion of the sclera. To determine if form deprivation specifically alters proteoglycan synthesis or similarly effects the synthesis of other scleral components, the present study examines the rates of collagen and proteoglycan synthesis in vitro, under normal conditions as well as under conditions of form deprivation.

We have previously shown that primary cultures of scleral chondrocytes, isolated from form-deprived eyes, maintain an elevated rate of proteoglycan synthesis for up to 24 hours in culture, at levels similar to those observed for scleral buttons isolated from form-deprived and control eyes, and radiolabeled in organ culture. Isolated scleral chondrocytes have a typical chondrocytic morphology, accumulate alcian blue-positive material, and produce aggrecan in culture, confirming the chondrocytic phenotype. In the present study, we have taken advantage of this cell culture system to investigate the cellular and molecular mechanisms involved in regulating the scleral growth processes associated with form-deprivation myopia. Although the mammalian sclera is structurally different from that of the chick, and studies on tree shrews have shown that ocular elongation is not accompanied by increases in scleral extracellular matrix, form-deprivation myopia in chicks and mammals has been shown to be regulated by similar mechanisms.

Experimental myopia can be induced in chicks and tree shrews by the administration of the dopamine against apomorphine, suggesting that similar neurochemical events are occurring in the deprived retinas of these species. Because form deprivation induces the most dramatic biochemical changes within the cartilaginous portion of the chick sclera, an understanding of the cellular mechanisms regulating extracellular matrix synthesis by scleral chondrocytes will help to elucidate the relationship between retinal image quality and scleral growth. A portion of this work has appeared in abbreviated form.

**METHODS**

**Induction of Myopia**

Form-deprivation myopia was induced in 2-day-old white leghorn chicks (Carey Farms; Marion, OH) by applying translucent plastic goggles, as previously described. We have previously shown that this method of form deprivation induces significant axial elongation after 5 days of occlusion similar to that reported by others. The goggles remained in place for 10 days, and chicks were maintained on a 12 hours light:12 hours dark cycle. Birds whose goggles fell off were not included in the study. The chicks were maintained and used in accordance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Accumulation**

The accumulation of DNA, collagen, and proteoglycans was measured from posterior scleral buttons, excised from control and form vision-deprived eyes. Eyes from 10 chicks that had been form vision-deprived for 10 days were divided into anterior and posterior hemispheres by making a circumferential cut around the equator of the eye globe. The anterior and posterior hemispheres were gently cleaned of all vitreous, retina, pigmented epithelium, pecten, choroid, and muscle. A 10 mm surgical trephine (Weck Ophthalmic, Research Triangle Park, NC) was used to excise scleral buttons of exactly the same size from the posterior poles of normal and form-deprived eyes. The optic nerve head, usually adherent to an edge of the button, was trimmed at its exit from the sclera. These scleral buttons represented 32% of the total scleral wet weight in deprived eyes and 36% of the scleral wet weight in control eyes. Posterior scleral buttons were
digested with 0.05% (wt/vol) proteinase K (protease type XXVIII, Sigma, St. Louis, MO) in 10 mM EDTA, 0.1 M sodium phosphate, pH 6.5 overnight at 60°C.

The DNA content of digested scleral samples was determined using bisbenzimidazole (Hoechst No. 33258, St. Louis, MO). Fluorescence was measured immediately with a DNA fluorometer (Hoefer Scientific, San Francisco, CA) and compared with a standard curve prepared with 2.5 to 20 ng of calf thymus DNA. Hydroxyproline was quantitated by the method of Woessner using chloramine T after hydrolysis of proteinase K digested samples in 6 N HCl at 100°C overnight. Glycosaminoglycan (GAG) concentrations were measured on aliquots of the proteinase K digests using the metachromatic dimethylmethylene blue assay. Absorbance values were compared with a standard curve prepared with 2.5 to 25 μg of porcine rib cartilage chondroitin sulfate.

**Measurement of Synthesis**

The rate of synthesis of proteoglycans, aggrecan precursor protein, collagen, total protein, and total RNA were measured from cultures of scleral chondrocytes.

**Isolation of Scleral Chondrocytes.** In this report, we modified the cell isolation procedures previously described by isolating chondrocytes only from a 10 mm punch taken from the posterior pole of the eye, where the majority of biosynthetic changes are occurring in response to form deprivation (manuscript in preparation). After visual deprivation for 10 days, 20 to 30 chicks were sacrificed, and control and form-deprived eyes were enucleated. Corneas and bony ossicles were removed from each eye and the cartilaginous sclera was cleaned of all non-scleral tissues. Buttons (10 mm) were excised from the posterior sclera of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact. Scleral chondrocytes were then isolated from the scleral cartilage of each eye as described above. The posterior scleral buttons as well as the remainder of the cartilaginous sclera were pooled separately for deprived and control eyes and predigested with 0.4% collagenase (type II; Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution containing 1.3 mM Ca++ and 0.9 mM Mg++ at 37°C for 15 minutes (without stirring) to remove most of the fibrous sclera while leaving the scleral cartilage intact.
Extracellular Matrix Synthesis by Scleral Chondrocytes

Immunoprecipitation. Antibodies specific for the core protein of aggrecan, the cartilage proteoglycans, were used to measure the synthesis of the aggrecan precursor protein by isolated scleral chondrocytes using immunoprecipitation assays. The DNA content of chondrocyte cultures was measured from proteinase K-digests of each culture by fluorometry using Hoechst reagent, as described above.

Aggrecan was isolated from a total of 40 g (=100 eyes) of adult chicken sclera (obtained from a local butcher) by 4 M guanidine extraction and CsCl density gradient centrifugation, as described by Hascall and Sajdera. The bottom third of each CsCl gradient was pooled, dialyzed exhaustively against water, and lyophilized. The pooled fraction was reconstituted in 6 M urea containing 0.05 M Tris, pH 6.8, 0.1% CHAPS, and 0.15 M NaCl and applied to DEAE-Sepharose equilibrated with the same solvent. The unbound or glycoprotein fraction was washed through, and the bound or proteoglycan material was eluted with 6 M urea containing 1.15 M NaCl. The elution position of proteoglycans was determined by measuring the GAG concentration in each fraction. Tubes containing proteoglycans were pooled, dialyzed, lyophilized, and reconstituted in 4 M guanidine-HCl containing 0.05 M Tris, pH 6.8, and 0.1% CHAPS. Aggrecan was separated from smaller proteoglycans by chromatography on Sepharose CL-2B (100 x 1.6 cm) equilibrated in 4 M guanidine-HCl containing 0.05 M Tris, pH 6.8, and 0.1% CHAPS. After dialysis of each fraction, aliquots were digested with chondroitinase and keratanase (Seikagaku America Inc., Rockville, MD) as previously described. Digested samples were electrophoresed on 5% polyacrylamide gels, and fractions containing the 350 kDa core protein were pooled and lyophilized. Polyclonal antibodies against the intact proteoglycan were raised in rabbits (Hazelton Research Products, Denver, PA), and the titer of the antisera was determined by enzyme-linked immunosorbent assays. Specificity of the antisera was shown by immunoprecipitation of the aggrecan precursor protein synthesized by chick sternal and scleral chondrocytes.

Immunoprecipitation and identification of the aggrecan precursor protein was done as described previously. In brief, scleral chondrocytes isolated from control and form-deprived eyes were plated in 24 well culture plates with standard chondrocyte medium at a density of 500,000 cells/well and allowed to attach for 3 hours at 37°C. Plating medium was removed and replaced with serum-free, methionine-free Dulbecco's modified eagle's medium (DMEM) for 20 minutes at 37°C to deplete intracellular pools of methionine. This medium was removed and replaced with serum-free, methionine-free DMEM containing 35S-methionine (500 μCi/500 μl), and chondrocytes were pulsed for 1 hour at 37°C. After radiolabeling, culture medium was removed and the cell layer was extracted with lysing buffer (0.1 M Tris, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.01% Aprotinin, 0.02% Na azide). Cell lysates from 35S-methionine pulsed cells were sonicated, and cell debris was removed by centrifugation. An aliquot of the lysate was precipitated with trichloroacetic acid (TCA), washed, and solubilized in NaOH to measure total incorporated radioactivity. Aliquots of cell lysates from control and form-deprived cultures containing equal amounts of incorporated radioactivity were incubated with protein A-Sepharose beads previously charged with specific antisera or preimmune sera, for negative controls. The beads then were washed, and the bound immunoglobulin G and radiolabeled proteins were released by boiling in Laemmi sample buffer containing 2% SDS and 97 mM dithiothreitol. Immunoprecipitated material was separated by SDS-PAGE and visualized by fluorography after treatment of the acrylamide gel with Fluoro-hance (RPI, Mount Prospect, IL), drying, and exposure to X-Omat film (Eastman Kodak Co., Rochester, NY) at -70°C for 2 days. The amount of immunoprecipitated core protein in control and form-deprived cultures was determined by scanning the 350 kDa bands on the autoradiogram and expressing the results as the integrated area of the peaks.

DNA measurements could not be made directly on cell lysates used for immunoprecipitation because the extraction buffer was not compatible with the fluorometric assay. To determine the amount of 35S-methionine labeled proteins synthesized per cell during the 1 hour pulse, scleral chondrocytes from control and deprived eyes were plated in 96 well plates, and six wells of each population were radiolabeled with 35S-methionine and total incorporated radioactivity determined by TCA precipitation, as described above. In the same experiment, six wells of each cell population were plated and processed identically to those cultures radiolabeled with 35S-methionine, with the exception that scleral chondrocytes were harvested in 1 X proteinase K and the DNA content determined using Hoechst reagent. The ratio of total 35S-methionine labeled protein/DNA was determined from measurements from adjacent wells, assigned before the experiment was carried out. Total protein synthesis/DNA values obtained by this method were determined to be unbiased and representative, as determined by permutation analyses of all possible combinations.

Collagen Biosynthesis by Chondrocytes. Collagen synthesis was determined on cultures of scleral chondrocytes isolated from control and form-deprived eyes using a modified version of the method described by Diegelmann et al. Scleral chondrocytes were plated at a density of 80,000 cells/well in 96-well plates for 3 hours. After the attachment period, the plating medium was removed and replaced with Ham's F-12 me-
medium containing 30 μCi [2,3-3H]proline/well (Amersham, Arlington Heights, IL) ascorbic acid (50 μg/ml), β-aminopropionitrile (25 μg/ml), penicillin (50,000 μ/l), streptomycin (50,000 μ/l), and 0.1% gentamycin. After a 6-hour radiolabeling period, the culture supernatants were collected in microfuge tubes and frozen for analysis at a later time. The cell layers were digested with proteinase K, and the DNA content of each culture was determined as described above.

To proceed with the analysis of collagen synthesis, the culture supernatants were digested with chondroitinase ABC (0.375 U/ml) and keratanase (0.19 U/ml) for 15.5 hours at 37°C. Chick embryo carrier protein (1.5 mg) was added to each tube. Total protein was precipitated with cold TCA, added to a final concentration of 10%. After centrifugation, TCA was extracted with 5X cold 10% TCA, resuspended, and divided in two equal portions—one for a blank and one to be collagenase digested. After centrifugation, TCA was extracted with ethanol:ethyl ether (3:1). Sample blanks were incubated in 440 μl of incubation cocktail containing 10 μmol of [5-3H]uridine (5 μCi/ml) with or without cycloheximide (20 μg/ml) to inhibit protein synthesis, or actinomycin D (20 μg/ml) to inhibit RNA synthesis for 6 hours. After the radiolabeling period, the pulse medium was removed, the cell layer was rinsed with cold acid precipitation solution (1 M HCl, 0.1 M sodium pyrophosphate), and cells were harvested in acid precipitation solution by scraping. Cell suspensions were then sonicated in microfuge tubes, an equal volume of carrier salmon sperm was added to the tubes (5 mg/ml), and precipitated RNA was recovered on filters with the aid of a 12-port filtration manifold (Millipore, Bedford, MA). The filters were then washed three times with 5 ml of acid precipitation solution, once with 3 ml of ethanol, and counted for radioactivity by liquid scintillation.

**Statistical Analyses.** Comparisons between control and form-deprived eyes were made using Student’s two-tailed t-tests for matched pairs. Comparisons between groups were made using the Scheffe F-test for multiple comparisons.

**RESULTS**

**Accumulation**

We have previously shown that proteoglycans accumulate within the posterior scleral hemisphere in response to form deprivation, whereas no significant changes occur in the anterior sclera. Although no significant differences were detected in the DNA content of posterior scleral hemispheres between control and deprived eyes, the DNA content of 10 mm buttons, excised from the posterior poles of eyes form deprived for 10 days, has been shown to be reduced by ≈20%, as compared with the same region of paired controls eyes. A linear relationship exists between chondrocyte number and DNA content, yielding a value of 6 to 7 pg DNA per cell, for control and deprived chondrocytes (data not shown). Therefore, to standardize our data and to assess biosynthesis on a cellular level, we expressed our data in terms of DNA content. After 10 days of form deprivation, GAG levels were 47.78% higher than those of controls (P < 0.001, Student’s paired t-test) (Fig. 1A). When GAGs were expressed per DNA, the GAG/DNA ratio was also significantly higher in scleral buttons from form-deprived eyes (29.41 μg/μg DNA ± 1.56 SEM), as compared with controls (17.89 μg/μg DNA ± 1.16 SEM, P < 0.001) (data not shown). To estimate collagen accumulation, the hydroxyproline content was determined on the same 10 mm scleral buttons (Fig. 1B). The hydroxypro-
Extracellular Matrix Synthesis by Scleral Chondrocytes

Proteoglycan Synthesis by Scleral Chondrocytes

Proteoglycan synthesis was measured in chondrocytes isolated from posterior scleral buttons, as well as the remaining anterior and equatorial regions ("rest of sclera") from control and form-deprived eyes and expressed per \( \mu g \) DNA (Fig. 2). After a 3-hour attachment period and a 6-hour radiolabeling period with \( ^{35}S \)-methionine, the control chondrocytes from both scleral regions synthesized proteoglycans at similar rates. However, rate of proteoglycan synthesis in chondrocytes isolated from the posterior sclera of form-deprived eyes (59,035 cpm/\( \mu g \) DNA) was 143% higher than that of control chondrocytes isolated from the same scleral region (24,312 cpm/\( \mu g \) DNA) \((P \leq 0.001, \text{ANOVA})\)

(Fig. 2A). In contrast, the rates of proteoglycan synthesis in cells isolated from the anterior and equatorial regions of sclera ("rest of sclera") from deprived eyes were similar to that of controls (Fig. 2B).

Aggrecan Precursor Protein Synthesis

Antibodies were used to immunoprecipitate the aggrecan precursor protein from cell lysates of 35S-methionine-pulsed scleral chondrocytes to measure the rate of aggrecan precursor protein synthesis, relative to the rate of total protein synthesis. The amount of incorporated radiolabel in lysates of control and form-deprived chondrocytes was determined after precipitating proteins in an aliquot of the lysate of medium with 5% TCA and counting the radioactivity in the precipitate. When expressed per cell, chondrocyte lysates isolated from form-deprived eyes incorporated significantly more radiolabel into total TCA precipitated protein, as compared to controls ($P < 0.001$, ANOVA) (Fig. 3). Aggrecan was immunoprecipitated from equal amounts of incorporated radioactivity from control and form-deprived chondrocyte lysates, and the immunoprecipitated material was examined by SDS-PAGE followed by autoradiography (Fig. 4A). Antibodies to aggrecan immunoprecipitated the 350 kDa precursor protein, as well as a 180 kDa band in control and form-deprived chondrocyte cultures. The 180 kDa band represents a protein that co-precipitates with aggrecan and was not included in quantitative analyses. Because aggrecan was immunoprecipitated from equal amounts of incorporated radioactivity in control and form-deprived chondrocyte lysates, the bands on the autoradiogram represent the levels of aggrecan synthesis relative to total protein synthesis. No significant differences were observed in the rates of aggrecan precursor protein synthesis when measured as density on the autoradiogram (Fig. 4B) or amount of radioactivity in the gel band (data not shown), indicating that relative to total protein synthesis, the rate of aggrecan synthesis is similar in control and form-deprived chondrocytes. Because the rate of total protein synthesis per cell in deprived cultures is increased 115% over that of controls (Fig. 5), it can be estimated that the absolute level of aggrecan core protein synthesis per cell in deprived cultures is also increased by this amount.

Collagen Synthesis by Scleral Chondrocytes

Collagen synthesis was also measured in chondrocytes isolated from posterior scleral buttons of control and form-deprived eyes (Fig. 5). After a 3-hour attachment period and a 6-hour radiolabeling period with [3H]-proline, the absolute rate of collagen synthesis per DNA was 154.95% higher in cultures of chondrocytes from form-deprived eyes, as compared with control.
Extracellular Matrix Synthesis by Scleral Chondrocytes

FIGURE 5. Absolute and relative collagen synthesis by scleral chondrocytes. Collagen synthesis was measured in control and form-deprived chondrocyte cultures as the amount of $^3$H-proline-labeled protein sensitive to collagenase. (A) Collagen synthesis/μg DNA (x ± SEM). **P ≤ 0.01, for n = 6 cell wells, ANOVA). (B) % Collagen synthesis/total protein synthesis. N.S., not significant.

chondrocytes ($P ≤ 0.01$, ANOVA) (Fig. 5A). Because the rate of total protein synthesis was also significantly higher in cultures of form-deprived chondrocytes (95,267 dpm/μg DNA ± 7969.2 SEM) as compared with controls over this labeling period (40,115 dpm/μg DNA ± 2933.0 SEM, data not shown), the rate of collagen synthesis relative to total protein synthesis was not significantly different between control and form-deprived chondrocyte cultures ($P > 0.9$), representing 6% to 7% of total protein synthesis by scleral chondrocytes (Fig. 5B).

RNA Synthesis by Scleral Chondrocytes

The rate of total RNA synthesis was determined over a 6-hour radiolabeling period after the 3-hour attachment period in scleral chondrocytes from control and form-deprived eyes (Fig. 6). Total RNA synthesis was 43.6% higher in form-deprived chondrocyte cultures, when expressed per DNA, as compared with controls ($P ≤ 0.04$, ANOVA). Treatment of the cultures with cycloheximide reduced the rate of RNA synthesis in both control and form-deprived chondrocyte cultures by 68% and 69%, respectively. Furthermore, the rates

FIGURE 6. Total RNA synthesis in scleral chondrocyte cultures. RNA synthesis was determined as the amount of $^3$H-uridine incorporated into RNA, relative to total DNA (μg) (x ± SEM, for n = 6 cell wells in each group). Some cultures were pretreated with cycloheximide (20 μg/ml) for 30 minutes to block protein synthesis, followed by labeling with $^3$H-uridine in the presence of cycloheximide (20 μg/ml) for 6 hrs. *P ≤ 0.04, compared to untreated controls; N.S., not significant, between control and deprived treated groups (ANOVA).

FIGURE 7. Effect of actinomycin D on proteoglycan synthesis. Chondrocytes isolated from control and form-deprived eyes were pretreated with actinomycin D (20 μg/ml) for 30 minutes to block RNA synthesis, followed by labeling with $^{35}$SO$_4$ in the presence of actinomycin D (20 μg/ml) for 6 hours (x ± SEM). **P ≤ 0.01, compared to controls treated with vehicle alone. N.S., not significant. Significance was determined by ANOVA for n = 5 cell wells of 500,000 cells each, per group.
of RNA synthesis/μg DNA in control and form-deprived chondrocyte cultures after treatment of the cultures with cycloheximide were not significantly different.

The Effect of Actinomycin D on Proteoglycan Synthesis

To determine if the increases in proteoglycan synthesis observed in form-deprived chondrocyte cultures were due to increases in the rate of transcription, chondrocyte cultures were treated with actinomycin D or vehicle alone, and the rate of proteoglycan synthesis was determined by measuring the amount of 35SO₄ incorporated into GAGs (Fig. 7). Measurement of RNA synthesis with and without treatment with actinomycin D indicated that treatment with actinomycin D resulted in a 98% drop in RNA synthesis rates (data not shown). Surprisingly, treatment of control chondrocyte cultures with actinomycin D resulted in a 112% increase in proteoglycan synthesis/μg DNA (P ≤ 0.01, ANOVA). In contrast, the rate of proteoglycan synthesis in form-deprived chondrocyte cultures treated with actinomycin D (38,033.0 cpm/μg DNA ± 1126.4 SEM) was not significantly different from that of untreated cultures of form-deprived chondrocytes (48,713.3 cpm/μg DNA ± 7240.0 SEM).

DISCUSSION

In this study, we have examined the effect of form deprivation on the accumulation of proteoglycans and collagen in vivo and on the synthesis of proteoglycan and collagen by scleral chondrocytes in vitro. Furthermore, the scleral chondrocyte cell culture system was used to initiate investigations on the mechanisms that regulate extracellular matrix synthesis under conditions of visual deprivation.

Accumulation

We have previously shown that application of a translucent occluder to one eye of a newly hatched chick results in significant axial elongation after 5 days of visual occlusion and is accompanied by significant increases in scleral dry weight from 5 to 15 days of form deprivation as compared to age-matched controls. Measurements of DNA content within the posterior sclera indicated that normal ocular growth is associated with a decrease in cell density from 7 to 17 days after hatching. Furthermore, the DNA content of the posterior buttons from deprived eyes is ≈20% lower than that of paired controls of the same age after 10 and 15 days of form deprivation, suggesting that visual deprivation results in an exaggeration of normal ocular growth. In this report, we limited our analyses to a 10 mm scleral button from the posterior pole of the orbit because this region has been shown to undergo the greatest biosynthetic changes in response to form deprivation (manuscript in preparation). Results from biochemical measurements in the present study indicate that proteoglycans accumulate in the posterior scleral pole of form-deprived eyes to a greater extent than they do in the same region of control eyes, when expressed either in absolute amounts or per DNA. Because these measurements were made on the same size scleral buttons and the thickness of the chick sclera is similar between control and form-deprived eyes, this suggests that the increased accumulation of proteoglycans within the cartilaginous and fibrous posterior sclera is displacing the cellular components of the posterior sclera. This increased accumulation may result from an increased synthesis of proteoglycan, decreased rate of degradation, or a combination of both processes.

In contrast, measurement of total hydroxyproline from the posterior scleral buttons indicated that the amount of collagen accumulation is similar within the posterior scleral pole of control and form-deprived eyes. Because collagen exists in the scleral extracellular matrix as a highly cross-linked network of fibrils, we would expect that scleral collagen is being actively remodeled (degraded and resynthesized) to accommodate the newly synthesized proteoglycans.

Synthesis

We used a cell culture system to investigate the biosynthesis of extracellular matrix components by scleral chondrocytes under normal conditions, as well as under conditions of form deprivation. Chondrocytes isolated from the posterior pole of form-deprived eyes synthesized proteoglycans at a rate 143% higher than that of controls. In contrast, cells isolated from the rest of the sclera (equatorial and anterior regions) of form-deprived eyes synthesized proteoglycans at a rate similar to that of control chondrocytes from the same region. Similar results have been obtained from ex vivo studies on the rates of proteoglycan synthesis in different regions of the sclera from control and form-deprived eyes measured in organ culture (manuscript in preparation), further confirming the validity of this cell culture system as representative of that which occurs in vivo. We have previously demonstrated by Sepharose CL-4B chromatography and fluorography that aggrecan is the primary proteoglycan synthesized by scleral chondrocytes in culture. In the present study, we used polyclonal antibodies specific for the core protein of aggrecan to measure the rate of synthesis of the core protein of aggrecan in control and form-deprived chondrocytes, relative to the rate of total protein synthesis. The precursor protein of aggrecan was detected in cultures of both control and form-
deprived chondrocytes as a band migrating at \( \approx 350 \) kDa. Similar amounts of the aggrecan precursor protein were synthesized by control and deprived chondrocytes, relative to total protein, indicating that the increases in proteoglycan synthesis observed by measuring \( {^{35}}\text{SO}_4 \) incorporation into GAGs are probably a reflection of the increased rate of total protein synthesis in deprived chondrocytes.

The rate of collagen synthesis was examined in cultures of scleral chondrocytes by measuring the incorporation of \( {^3}\text{H-proline} \) into collagenase-sensitive material. When expressed in absolute amounts, the rate of collagen synthesis/DNA was significantly higher in form-deprived chondrocytes as compared with controls. However, because total protein synthesis was also significantly higher in chondrocytes isolated from form-deprived eyes, the rate of collagen synthesis relative to total protein synthesis was similar for scleral chondrocytes isolated from control and form-deprived eyes. In contrast to the increases in proteoglycan accumulation observed in the posterior sclera of deprived eyes, no increases in collagen accumulation were observed in the sclera of form-deprived eyes. These results suggest that although total protein synthesis is increased in the posterior sclera of form-deprived eyes, a specific remodeling process is occurring that allows certain components (e.g., proteoglycans) to accumulate in the posterior sclera of deprived eyes, whereas the collagenous components are turned over and do not accumulate over control levels. Our measurements of extracellular matrix synthesis were made on chondrocyte cultures from which the fibrous sclera had been removed. It is possible that collagen accumulation is higher within the cartilaginous sclera of deprived eyes but is counterbalanced by a decrease in collagen accumulation within the fibrous sclera. The observation that the outer fibrous layer is thinner in deprived eyes supports this idea.

Regulation

In an effort to determine the level at which form deprivation mediates the observed changes in protein synthesis, the synthesis of total RNA was measured by control and form-deprived chondrocytes. Measurement of \( {^3}\text{H-juridine} \) into total RNA indicates that the rate of total RNA synthesis/DNA was 44% higher in chondrocyte cultures from form-deprived eyes, as compared with controls \( (P \leq 0.04) \). Pretreatment of the cultures with cycloheximide resulted in a significant drop in the rate of RNA synthesis in both populations of cells, indicating that protein synthesis is required for RNA synthesis by both control and form-deprived chondrocytes. This secondary response may be due to the presence of a growth factor that acts in an autocrine fashion to stimulate RNA synthesis in the same cell. Furthermore, the levels of RNA synthesis are similar in control and form-deprived chondrocyte cultures after treatment with cycloheximide, indicating that the increased rate of RNA synthesis in the form-deprived chondrocyte cultures is dependent upon the synthesis of a growth factor. Of the many factors known to influence cartilage metabolism, several reports have implicated the local production of the insulin-like growth factors in articular cartilage as well as in the developing chick sclera. Furthermore, studies on isolated chondrocytes in culture show that insulin-like growth factors act in vitro to stimulate RNA, total protein, and proteoglycan synthesis in a manner similar to that observed in the present study.

Because form deprivation stimulates proteoglycan synthesis to the same extent as protein synthesis, we monitored proteoglycan synthesis as one index of general biosynthesis levels under conditions where RNA synthesis was blocked with actinomycin D. Because actinomycin D binds to DNA and prevents transcription, this agent was used to determine if form deprivation stimulates proteoglycan synthesis at the transcriptional level (requiring RNA synthesis) or at a level of post-transcriptional regulation. If the stimulation of proteoglycan synthesis observed in form-deprived eyes is dependent upon transcription of new mRNA, proteoglycan synthesis would be dramatically lower in cultures of scleral chondrocytes from form-deprived eyes after transcription was blocked with actinomycin D. Actinomycin D had no significant effect on the rate of proteoglycan synthesis in form-deprived eyes, suggesting that the increased rate of proteoglycan synthesis observed in form-deprived chondrocyte cultures is due to a post-transcriptional mechanism. Surprisingly, the addition of actinomycin D to cultures of control chondrocytes resulted in a 112% increase in proteoglycan synthesis to levels similar to those observed in untreated deprived chondrocyte cultures. This effect suggests stabilization or existing mRNA in control chondrocytes in a manner similar to that observed with decorin mRNA expression by bovine chondrocytes after treatment with actinomycin D or retinoic acid. Taken together, these data suggest that control chondrocytes synthesize an inhibitor with a relatively short half life \(< 6\) hours) that acts via a post-transcriptional mechanism to lower the rate of proteoglycan synthesis (and perhaps other chondrocyte-specific messages). This inhibitor may act to destabilize specific mRNAs to result in reduced levels of translation of chondrocyte proteoglycan core proteins, or it may inhibit the synthesis of enzymes required for the assembly of GAGs. If the synthesis of this protein is blocked with actinomycin D, the rate of proteoglycan synthesis increases due to stabilization of existing mRNA.
Form-deprived chondrocytes may fail to express this post-transcriptional inhibitor and, therefore, demonstrate an increased rate of proteoglycan synthesis relative to controls. Alternatively, chondrocytes from form-deprived eyes may express this factor, but they may already be maximally upregulated before treatment with actinomycin D and are unable to synthesize proteoglycans at higher rates.

In conclusion, form deprivation stimulates the synthesis of proteoglycans, collagens as well as total protein, by scleral chondrocytes. Although total RNA synthesis is increased in chondrocytes from form-deprived eyes, the increased rate of proteoglycan synthesis observed in form-deprivation myopia appears not to be a direct consequence of increased transcription; instead, it is due to a post-transcriptional event. Experiments with actinomycin D indicate that this post-transcriptional event may be the production of a scleral growth inhibitor present in scleral cells when formed images are presented on the retina. The production of this growth inhibitor by scleral cells may be one mechanism of regulating scleral growth and maintaining emmetropia.

Key Words
myopia, chondrocytes, proteoglycans, collagen, sclera

Acknowledgments
The authors thank Dr. John R. Hassell (The Eye and Ear Institute, Pittsburgh, Pennsylvania) for his helpful discussions and suggestions, and Dr. Robert F. Diegelmann, (Wound Healing Center, Richmond, Virginia) for his advice with the collagen synthesis assays. Thanks are extended to Dr. Richard Day and Doug Landsittel for assistance with statistical analyses. The authors also thank Judy Smith and Geri Gutkowski for assistance in manuscript preparation.

References
26. Kang RN, Norton TT. Alteration of scleral morphol-
Extracellular Matrix Synthesis by Scleral Chondrocytes


