Growth of the Two Layers of the Chick Sclera Is Modulated Reciprocally by Visual Conditions

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Purpose. Although visual deprivation causes increased ocular elongation and myopia in both birds and mammals, changes in sclera appear to be in opposite directions. Because avian sclera has a cartilaginous layer as well as the fibrous layer found in mammals, we examined whether the scleral responses to various visual manipulations differ between the two layers.

Methods. To produce increases in ocular elongation and myopia, monocular diffusers or negative lenses were fitted to eyes. Conversely, to produce decreases in ocular elongation, diffusers were removed (restoring normal vision) or monocular positive lenses were fitted. Scleral layers were then dissected apart, and incorporation of labeled precursors into glycosaminoglycans (GAGs), DNA, and protein was assessed. Tissue coculture experiments were used to assess humoral interactions between scleral layers and with the choroid.

Results. In the cartilaginous layers, the incorporation of label into proteoglycans and DNA was significantly higher in eyes elongating faster than normal because of wearing diffusers or negative lenses and significantly lower than normal in eyes elongating slower than normal because of removal of the diffuser or wearing positive lenses. In the fibrous layers, the reverse was the case. Coculturing cartilaginous sclera from normal eyes with fibrous sclera from myopic or recovering eyes produced the same increase or decrease in sulfate incorporation into GAGs in the cartilaginous layer as though the tissue measured was from the animal providing the conditioning tissue. Coculturing with choroid, especially from recovering eyes, also inhibited cartilaginous sclera.

Conclusions. The fibrous layer of the avian sclera shows changes in sulfate incorporation into GAGs during deprivation and recovery from deprivation in the same direction as does the mammalian sclera, whereas the cartilaginous layer changes in the opposite direction. The responses of the cartilaginous layer may be controlled by the fibrous layer, although they are influenced by the choroid as well. Invest Ophthalmol Vis Sci. 1997;38:1726-1739.

Powerful evidence that visual stimulation affects eye growth comes from the fact that in many species of bird and mammal, visual form deprivation by means of diffusers or closed lids causes increased ocular elongation and myopia.1-7 Although the phenomenon of form-deprivation myopia seems similar in birds and mammals, the scleral changes seem to be in opposite directions.

In birds, the increased ocular elongation caused by form deprivation is associated with increased growth of the sclera, as shown by increased dry weight and increased incorporation of precursors into DNA, protein, and glycosaminoglycans (GAGs).8,9 Conversely, if a visually deprived eye has form vision restored, the eye slows its rate of elongation below normal until the length appropriate for its age and focal length is attained.10,11 Correspondingly, during this recovery period, incorporation of precursors into GAGs, protein, and DNA is reduced below normal levels.8,12,13

In mammals, in contrast, visual deprivation is associated with decreased scleral dry weight, decreased proteoglycan content, and decreased rate of incorporation of precursors into GAGs.14,15 Conversely, eyes recovering from visual deprivation show increased incorporation of precursors into GAGs, but no change in DNA or GAG levels, relative to nondeprived eyes.16
We suggest that the mechanism of scleral remodeling is similar in birds and mammals in these experimental situations, and that the apparent difference arises from the fact that the chick sclera is composed of two layers: an outer fibrous layer (like that in mammals), which contains collagen type I and small proteoglycans such as decorin, and an inner cartilaginous layer, which contains collagen types II and IV and aggrecan as the predominant proteoglycan. In the experiments presented here, we separated the two layers and found that the fibrous layer resembles the mammalian sclera in that it decreases incorporation of precursors into GAGs and DNA as the eye elongates and becomes myopic during visual deprivation, whereas the cartilaginous layer increases incorporation into GAGs and DNA under these conditions. Conversely, when the rate of ocular elongation is reduced below normal by giving deprived eyes normal visual conditions, incorporation into GAGs decreases in the cartilaginous layer and increases in the fibrous layer.

The rate of elongation of avian and mammalian eyes can also be increased or decreased by imposing hyperopic or myopic defocus by negative or positive spectacle lenses, respectively. In chicks, wearing lenses causes opposite changes in sulfate incorporation into scleral GAGs. We show that the two scleral layers show opposite changes with both types of lenses.

Finally, we attempt to address the question of which layers in the posterior wall of the globe are the controllers of growth and which are the ones controlled. We cocultured normal scleral layers with either the other scleral layer or with choroid from eyes in which the visual experience had been varied. We found that the fibrous sclera appears to control the rate of sulfate incorporation into cartilage GAGs, but that the choroid also can influence both scleral layers.

MATERIALS AND METHODS

Animals and Visual Manipulations

White Leghorn chickens (Gallus gallus domesticus), obtained as day-old chicks from Truslow Farms (Chesertown, MD), were reared in our animal facility on a 14:10-hour light:dark cycle. Care and use of the animals conformed to the ARVO Resolution on the Use of Animals in Research.

Induction of Refractive Errors. To deprive eyes of form vision, white translucent plastic diffusers were glued to the feathers around one eye. To impose refractive errors to provoke compensatory changes, spectacle lenses of polymethylmethacrylate with a base curve radius of 7 mm and an optic zone diameter of 10 mm in powers of ±15 D (Conforma Contact Lenses, Norfolk, VA) were glued to rigid plastic washers attached to Velcro rings, and complementary Velcro rings were glued to feathers around the eyes, giving a field of view of approximately 70° to 90°. Feed was sifted to remove dust particles, and lenses were cleaned approximately every 3 to 4 hours during the light phase each day. Unless otherwise indicated, each chick was fitted with a diffuser or lens over one eye; the fellow eye was left uncovered.

Refractive Error Measurements

Refractive error was measured in chicks wearing spectacle lenses (chicks fitted with diffusers were assumed to be highly myopic on the basis of many previous studies). Chicks were anesthetized with a mixture of...
The punch was located so that it nearly contacted the overdose of intraperitoneal sodium pentobarbital. Mydriasis (and presumably cycloplegia) was obtained by administering 1 drop/min for 5 to 10 minutes of 10 mg/ml vecuronium bromide (Norcuron; Organon, West Orange, NJ) and benzalkonium chloride (0.26 mg/ml) in saline. Refractive error was measured using a modified Hartinger refractometer (ausjena, Jena, Germany); data were not corrected for the artifact of retinoscopy.

**Tissue Dissection and Explant Culture**

The culture medium used in these experiments was the chemically defined medium (N2) of Bottenstein. This medium consists of Dulbecco's modified Eagle's medium and Ham's F-12 with the addition of sodium bicarbonate, sodium selenite, progesterone, putrescine, pyruvate, glutamine, and insulin. Antibiotics used were penicillin, streptomycin, and amphotericin B (Gibco, Grand Island, NY). Evidence that our culture methods maintained healthy tissue over the 48-hour period used here is indicated by the finding that isolated pieces of sclera cultured for 72 hours in continuously flowing N2 show consistently high levels of sulfate incorporation into secreted GAGs throughout the culture period.

In addition, pieces of sclera cultured for 48 hours have similar incorporation, regardless of whether the medium is refreshed at 24 hours (compare results of experiments 3 and 4).

A punch 7 mm in diameter was taken from the posterior sclera of each eye of chicks killed with an overdose of intraperitoneal sodium pentobarbital. The punch was located so that it nearly contacted the central extremity of the pecten, such that the long axis of the pecten formed a tangent to the circular punch, which lay on the nasal side of the pecten. The fibrous and cartilaginous scleral layers were separated by peeling away the fibrous from cartilaginous sclera in cold Ca++/Mg++-free Hank's balanced salt solution (Sigma, St. Louis, MO) under a dissecting microscope. In another set of birds, the choroid was isolated in cold Ca++/Mg++-free Hank's balanced salt solution by first peeling away the neural retina, then gently brushing away the retinal pigment epithelium, and finally peeling the choroid away from the sclera. Tissues were in the dissecting medium for approximately 5 to 30 minutes and then were transferred to tissue culture medium (without insulin) until all dissections were completed.

**Scleral Growth Assays**

Because we find greater variability in all label incorporation measures between individual animals than between the eyes of an individual, we organized the experiments so that the data collected are measures on a treated eye compared with the untreated fellow eye of the same individual. There may be a disadvantage to this procedure, however. In treatment with spectacle lenses, the refractive error of the fellow eye changes slightly in the same direction as the lens-treated eye. If the same were true for incorporation of precursors into GAGs, our procedure might underestimate the magnitude of the treatment effects.

We assessed the incorporation of Na$_2^{35}$SO$_4$ into GAGs as a reflection of the rate of proteoglycan synthesis. Scleral layers were incubated with Na$_2^{35}$SO$_4$ (10 μCi/ml) for either 3 or 20 hours, digested overnight at 57°C in 0.5 ml of proteinase-K (protease type XXVII, Sigma, in 10 mM EDTA, 0.1 M sodium phosphate [pH = 6.5]), centrifuged for 5 minutes at 7500g, and divided into two aliquots. In one aliquot (200 μl), GAGs were precipitated for 1 hour at 37°C with cetylpyridinium chloride in 2 mM Na$_2$SO$_4$ in the presence of unlabeled chondroitin sulfate (1 mg/ml), captured by filtration (Whatman GF/F), and scintillation counted using CytoScint (Fisher Biotech, Pittsburgh, PA). In the other aliquot (50 μl), DNA content was measured by fluorimetry using Hoechst 33258 dye (Polysciences, Warrington, PA).

Precursor incorporation into protein and DNA was measured by labeling scleral layers with $^3$H-proline (5 μCi/ml) or $^3$H-thymidine (5 μCi/ml), respectively, for 20 hours. Subsequently, the tissues were agitated in four changes of cold 5% trichloroacetic acid, each lasting 24 hours, to remove unincorporated radiochemical. In each experiment, we confirmed that the last wash did not contain radioactivity above background levels. Tissues were then digested in 0.5 ml of proteinase, centrifuged for 5 minutes at 7500g, and divided into two aliquots. One aliquot (100 μl) was scintillation counted to assess precursor incorporation, and the other aliquot (50 μl) was used to determine the DNA content by fluorimetry. Uptake of precursors was expressed as normalized to DNA content to reflect the synthetic activity per cell. This was desirable because visual deprivation decreases the cell density in the posterior scleral region from which the punches were taken. The range of counts for all samples (measurements of sulfate, proline, and thymidine incorporation) was fibrous sclera, 550 to 5,250 cpm, cartilaginous sclera, 7,150 to 27,020 cpm, and background 30 to 50 cpm.

We measured only the newly synthesized GAGs that were incorporated into the scleral tissue; by separately assaying the culture medium in a few cases, we estimate that the amount lost to the medium, which we did not process, was small (roughly 10%) in cartilaginous sclera but larger in the fibrous sclera. Perhaps because the proteoglycans from the fibrous sclera are smaller, they diffuse out of the tissue more readily.

**Data Analysis**

As mentioned, uptake of $^{35}$S-sulfate, $^3$H-proline, and $^3$H-thymidine into tissues was normalized to DNA con-
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tent and values were expressed as femtomoles of radioc- 
chemically incorporated/ ng DNA. We calculated femto- 
moles incorporated by comparing the sample of pro- 
cessed tissue with a standard well without tissue 
containing a known quantity of radiocchemical. For 
comparisons between scleras of treated and untreated 
eyes of the same bird, data are expressed as ratios of 
the paired treated/control layers, after each datum 
was divided by the amount of DNA in the sample. A 
paired Student's t-test, comparing the ratios to a null 
hypothesis of mean = 1, was performed for all data 
expressed as ratios.

PROTOCOLS

Experiment 1: Form Deprivation and Recovery 
From Form Deprivation

To assess the effect of form deprivation, day-old chicks 
had one eye covered by a diffuser for 14 days (other 
chicks had sulfate incorporation measured after 7 days 
of form deprivation). To assess the effect of recovery 
from form deprivation, chicks had one eye covered by 
a diffuser for 11 days, followed by diffuser removal 
and 3 days of normal vision. In all groups, the fellow 
eyes were left untreated. In a similar study, the mean 
refractive error after form deprivation exceeded —15 
D; after 3 days of recovery, it was about —6 D.25 After 
enucleation, we separated the fibrous and cartilagi- 
 nous scleral layers of a posterior scleral punch. Precur- 
sor incorporation into GAGs, DNA, and protein of 
the fibrous and cartilaginous layers was measured as 
described above, after incubation with Na235SO4, 3H-
thymidine, or 3H-proline for 20 hours. The number 
of subjects in each group is indicated in Figure 4.

Experiment 2: Effect of Defocusing Spectacle 
Lenses

Day-old chicks wore either —15 D lenses (n = 11), 
which imposed hyperopic defocus, or +15 D lenses 
(n = 13), which imposed myopic defocus (except at 
very close distances); fellow eyes were left untreated. 
On day 9, lenses were removed and refractive error 
was measured. This was followed by enucleation and 
separation of fibrous and cartilaginous scleral layers 
of a posterior scleral punch. Paired fibrous and cartilagi- 
nous layers were then separately cultured in N2 
for 20 hours with Na235SO4 and processed to quantify 
incorporation into GAGs and DNA content.

Experiment 3: Tissue Interactions in Coculture

To assess whether the effects of different visual manip- 
ulations on the scleral layers were the result of the 
independent actions on each layer of substances dif- 
fusing from elsewhere or whether one layer could in- 
fluence the synthetic activities of other layers, we co-
cultured normal fibrous or cartilaginous tissue from 
untreated eyes (measured tissues) with either the cho- 
roid or the complementary scleral layer (conditioning 
tissues) from normal eyes, eyes wearing diffusers 
termed form-deprived eyes), or eyes that had pre- 
viously worn diffusers (termed recovering eyes). The 
visual manipulations for these animals were as de- 
scribed for experiment 1; the number of subjects in 
each group is indicated in Figures 7 and 8.

As shown in Figure 2, the measured tissues con- 
isted of fibrous and cartilaginous layers from normal eyes, 
separated as described in methods. This yielded two 
pieces of cartilaginous and two pieces of fibrous scleral tissue for 
each animal. These pieces were cocultured for 20 hours with 
different conditioning layers from the eyes of an animal that 
had one experimental (form-deprived or recovering from 
form deprivation) and one untreated fellow eye. Tissue com- 
binations during coculture were conditioning fibrous with 
measured cartilaginous sclera or vice versa, or either mea- 
sured fibrous or cartilaginous sclera with conditioning cho- 
roid. After the 20-hour coculture, the conditioning tissues 
were discarded and labeled sulfate was added to the existing 
conditioned media for 20 hours (fibrous sclera) or for 3 
hours (cartilaginous sclera).
discarded and Na$_2^{35}$SO$_4$ was added to the existing conditioned media for 20 hours (fibrous sclera) or 3 hours (cartilaginous sclera). To reduce the effect of individual variability, we constructed these experiments so that the two eyes of one animal provided the conditioning tissues compared (form-deprived versus normal or recovering versus normal) and the two eyes of another animal provided the normal tissues measured.

For one experiment, we tested conditioning tissues from recovering and untreated eyes on measured tissues from recovering, rather than normal, eyes. To retain the advantage just described of having the conditioning and measured tissues each come from a single animal, we obtained the conditioning tissue as follows. Diffusers with a small nasal opening that restricted vision to a small portion of the temporal retina were placed over both eyes of 4-day-old chicks for 11 days ($n=7$), resulting in myopic refractions on the optic axis (mean refractive error: right eyes $-13.8$ D, left eyes $-15.8$ D). Diffusers were then removed and the eyes were permitted 3 days of normal vision. We know that restoring vision to locally deprived parts of the retina causes the refractions and the eye shape to return to normal.36

For another experiment, we again departed from our paradigm by testing conditioning tissue from a normal eye on measured tissue from a normal eye of another animal; the measured tissue from the fellow normal eye was incubated without any conditioning tissue.

**Experiment 4: Effect of Separation of Fibrous From Cartilaginous Sclera**

To assess the effect of separation of the scleral layers per se, we used the protocol shown in Figure 3. Paired normal eyes from chicks ages 1 ($n=17$ or 18), 7 ($n=11$), 8 ($n=18$), 14 ($n=12$), and 23 days old ($n=11$) were enucleated and scleral punches taken. In the punch from one eye of each animal (designated initially separated), scleral layers were separated and incubated separately in N2 for 20 hours. In the punch from the other eye (designated initially intact), the intact scleral punches were incubated in N2 for 20 hours, after which the scleral layers were separated. Then, scleral layers were incubated separately in fresh N2 with Na$_2^{35}$SO$_4$ for 3 or 20 hours (all ages), $^3$H-proline ($n=11$; 7 days old), or $^3$H-thymidine ($n=10$; 7 days old) for an additional 20 hours.

**RESULTS**

**Experiment 1: Form Deprivation and Recovery From Form Deprivation**

Our principal discoveries are that sulfate incorporation into GAGs is affected in opposite directions in two respects. First, for a given visual manipulation (form deprivation or recovery), the two scleral layers change in opposite directions. Second, for a given scleral layer (fibrous or cartilaginous sclera), the changes are in opposite directions for form deprivation and recovery from form deprivation. Specifically, in the cartilaginous layer from form-deprived eyes, the sulfate incorporation is nearly three times that of the untreated fellow eye (291% at day 7 and 297% at day 14; Fig. 4A), whereas in the cartilaginous layer from recovering eyes, it is significantly decreased (to 81% of untreated eyes at 14 days of age; Fig. 4E). The opposite is true of sulfate incorporation in the fibrous layer. In form-deprived eyes it is decreased to 74% of untreated eyes, and in recovering eyes it is increased to 177% of untreated eyes.

This general bidirectional pattern is partly evident in $^3$H-thymidine incorporation into DNA as well. In scleral tissues from form-deprived eyes, $^3$H-thymidine incorporation changes in the same direction and to similar extents as does sulfate incorporation into GAGs (to 262% of untreated in cartilage, down to 77% in fibrous; Fig. 4B). In scleral tissues from recovering eyes, the $^3$H-thymidine incorporation in cartilage changes in the opposite direction as during form deprivation (down to 77%; Fig. 4F), but the recovering fibrous sclera does not show a significant change (104% of normal).

The pattern of $^3$H-proline incorporation into protein in cartilaginous sclera also resembles that of sul-
fate incorporation into GAGs. Incorporation increased to 145% in tissue from form-deprived eyes and decreased to 81% in tissue from recovering eyes relative to tissue from fellow untreated eyes (Figs. 4C, 4G). In contrast, the fibrous sclera from form-deprived and recovering eyes incorporated significantly more ³H-proline (179% and 215%, respectively). This suggests that both increased and decreased ocular elongation may involve increased protein synthesis in the chick fibrous sclera.

An independent confirmation of the general pattern of opposite responses in the two scleral layers is that the total amount of DNA per scleral disk shows small nonsignificant trends that are opposite in cartilaginous and fibrous layers: form-deprived eyes, lower in cartilage and higher in fibrous (Fig. 4D), and recovering eyes, higher in cartilage and lower in fibrous (Fig. 4H). Only the decrease in cartilage from myopic eyes is statistically significant. Thus, across groups, the changes in DNA content tend to be opposite to the changes in the rate of DNA synthesis, as evaluated by thymidine incorporation. This pattern is probably a consequence of the cartilage's enlarging by a greater increase in extracellular matrix synthesis than in cell division, so that the density of cells is reduced, whereas in the fibrous layer the cell division increases more than does the extracellular matrix synthesis.

Experiment 2: Effect of Defocusing Spectacle Lenses

Eyes can be made to grow in the myopic direction not only by form deprivation but also by allowing them to compensate for hyperopic refractive error imposed by negative spectacle lenses. In these experiments, we find the same pattern of sulfate incorporation into GAGs as in form-deprived eyes—that is, increased (to 150%) in the cartilaginous layer and decreased (to 68%) in the fibrous layer, relative to untreated fellow eyes (Fig. 5A). We know that the lenses were being compensated for because negative lens wear resulted in a myopic mean refractive error of −5.1 D, but un-
Negative lens wear

Positive lens wear

FIGURE 5. Sulfate incorporation into GAGs and DNA content in fibrous and cartilaginous scleral layers in response to negative (A,B) and positive (C,D) lens wear. Nine-day-old chicks wore a lens over one eye since postnatal day 1. Data shown are mean (±SEM). Vertical axis is logarithmic. An asterisk indicates statistical significance at the level of P < 0.05.

treated fellow eyes had a mean refractive error of 1.2 D (Fig. 6).

Conversely, eyes can be made to grow in the hyperopic direction not only by being given normal vision after form deprivation (recovery) but also by allowing them to compensate for myopic refractive error imposed by positive spectacle lenses. Here, too, we find the same pattern as in eyes recovering from visual deprivation. Eyes wearing positive lenses had sulfate incorporation in the cartilaginous layer decreased to 65% and sulfate incorporation in the fibrous layer increased to 133% relative to untreated fellow eyes (Fig. 5C). Positive lens wear resulted in a hyperopic mean refractive error of +11.2 D; untreated fellow eyes had a mean refractive error of +1.0 D (Fig. 6). As in the previous experiment, there were nonsignificant trends in DNA content opposite to the changes in sulfate incorporation (Figs. 5B and 5D). These results suggest that the same mechanisms are used for growth in myopic and hyperopic directions, whether caused by visual deprivation, by recovery from its effects, or by lenses.

Experiment 3: Tissue Interactions in Coculture

To ascertain whether the two scleral layers respond oppositely because they have independent responses to signals from another tissue or because one layer determines the response of the other layer, we cocultured fibrous or cartilaginous sclera with the complementary scleral layer from form-deprived or recovering eyes (methods shown in Fig. 2). We found a remarkable asymmetry between the effects of the scleral layers on each other (Figs. 7A and 7B).

The fibrous sclera effectively imposed the visual conditions of its eye of origin on the cartilaginous sclera with which it was cultured. This is shown by three results. First, scleral cartilage from normal eyes cocultured with fibrous sclera from form-deprived eyes had 143% more sulfate incorporation into GAGs than did cartilage cocultured with fibrous sclera from untreated eyes (Fig. 7A, row B, right panel). Thus, coculture with fibrous sclera (conditioning tissue) from form-deprived eyes caused normal cartilage to increase sulfate incorporation, making it similar to cartilage from form-deprived eyes (compare Fig. 7A, row B, right panel and Fig. 4A). Second, coculture with fibrous sclera from eyes recovering from form-deprivation myopia caused sulfate incorporation to decrease to 76% in the cartilaginous sclera from normal eyes, making it similar to cartilage from recovering eyes (compare Fig. 7A, row C, right panel and Fig. 4E). These effects are bidirectional, as can be seen by comparing the experimental tissues to those cultured alone (Fig. 7A, left panel). If we express the effect of fibrous sclera from deprived eyes on scleral cartilage from normal eyes relative to being incubated

FIGURE 6. Refractive errors of chicks wearing negative or positive lens over one eye (filled bars), and the untreated fellow eyes (open bars). Nine-day-old chicks wore a lens over one eye since postnatal day 1. Data shown are mean (±SEM). An asterisk indicates statistical significance at the level of P < 0.05.
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EFFECT OF FIBROUS SCLERA ON CARTILAGINOUS SCLERA

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<th>Conditioning Tissues</th>
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A

Sulfate incorporation into GAG (femtomoles sulfate / ng DNA)

Ratio of sulfate incorporation into GAG

B

Sulfate incorporation into GAG (femtomoles sulfate / ng DNA)

Ratio of sulfate incorporation into GAG

FIGURE 7. Sulfate incorporation into GAGs in (A) cartilaginous and (B) fibrous scleral layers from paired normal eyes of the same animal cocultured with complementary scleral layers (fibrous in the case of cartilaginous, cartilaginous in the case of fibrous) from eyes in different visual conditions. Each bar represents the mean (±SEM) sulfate incorporation into GAGs/ng DNA (left panels) and the mean ratio of the pairs (±SEM) (right panels) of the measured tissues cocultured with the conditioning tissues shown at left. The bars in row A are unfilled to emphasize that they represent a different ratio (cocultured with normal conditioning tissue relative to no conditioning tissue). An asterisk indicates statistical significance at the level of \( P < 0.05 \). In both scleral layers, sulfate incorporation into GAGs (left panels) in normal tissues cocultured with untreated tissues (rows B and C) is not significantly different (two-sample \( t \)-test) from normal tissues cocultured with normal tissues (row A).

with no other tissue (that is, multiplying the “Form-Deprived/Untreated” ratio by the “Normal/None” ratio in Fig. 7A), the ratio is 1.60 (Form-Deprived/Normal × Normal/None = 1.43 × 1.12 = 1.60). If we form the equivalent ratio for the effect of the fibrous sclera from eyes recovering from deprivation, the ratio is 0.85 (Recovering/Normal × Normal/None = 0.76 × 1.12 = 0.85). Third, a similar decrease was seen if the fibrous sclera from recovering and untreated eyes was cocultured with scleral cartilage from recovering eyes. This decrease occurs because the cartilage from recovering eyes cocultured with untreated fibrous increased its incorporation to normal levels (Fig. 7A, row D, left panel).

In contrast, the cartilaginous sclera did not impose the visual conditions of its eye of origin on the fibrous sclera. Instead, cartilage from normal eyes inhibited fibrous sclera sulfate incorporation into GAGs relative to being cultured alone, and cartilage from either form-deprived or recovering eyes reduced it 42% more (Fig. 7B, right panel). Taken together, these results are consistent with the fibrous sclera’s being the determinant of the pattern of response of both layers of the sclera.

However, the effect of coculture with choroid of different origins suggests that the choroid also affects the cartilage. As was the case with coculture with fibrous sclera, sulfate incorporation into GAGs of cartilaginous sclera from normal eyes was increased (to 117%) by coculture with choroid from form-deprived...
FIGURE 8. Sulfate incorporation into GAGs in (A) cartilaginous and (B) fibrous scleral layers cocultured with choroid from eyes in different visual conditions. Data shown are mean (±SEM) sulfate incorporation into GAGs/ng DNA (left panels) and mean ratio of the pairs (±SEM) (right panels), as in Figure 7. The bars in row A are unfilled to emphasize that they represent a different ratio (cocultured with normal choroid relative to no conditioning tissue). An asterisk indicates statistical significance at the level of $P < 0.05$. In both scleral layers, sulfate incorporation into GAGs (left panels) in normal tissues cocultured with untreated tissues (rows B and C) is not significantly different (two-sample $t$-test) from normal tissues cocultured with normal tissues (row A).

eyes (Fig. 8A, row B, right panel) and decreased (to 59%) by coculture with choroid from recovering eyes (Fig. 8A, row C, right panel). However, because choroid from normal eyes inhibited cartilage from normal eyes to 78% of the sulfate incorporation level of cartilage incubated alone (Fig. 8A, row A, right panel), we view the choroid results as being composed only of inhibition, as can be seen by comparing the experimental tissues to those cultured alone (Fig. 8A, right panel). Thus, the choroid from form-deprived eyes inhibits the cartilage to 91% relative to being incubated alone (Form-Deprived/Normal $\times$ Normal/None = 1.17 $\times$ 0.78 = 0.91; Fig. 8A, rows A and B, right panel), and the choroid from recovering eyes inhibits it to 46% (Recovering/Normal $\times$ Normal/None = 0.59 $\times$ 0.78 = 0.46; Fig. 8A, rows A and C, right panel). In contrast, the response of cartilage to coculture with fibrous sclera is truly bidirectional.

The effect of choroid on fibrous sclera is also unidirectional: the choroid from normal eyes stimulates sulfate incorporation in fibrous sclera from normal eyes to 133% of fibrous incubated alone (Fig. 8B, row A, right panel), and the myopic choroid has the same effect (Fig. 8B, row B, right panel), whereas the choroid from recovering eyes stimulates it to 264% (Fig. 8B, row C, right panel). The fact that the choroid inhibits the cartilage and stimulates the fibrous is further evidence for the opposite responses of the two scleral layers and for the independence of their responses to extrinsic chemical signals.

Experiment 4: Effect of Separation of Fibrous From Cartilaginous Sclera

The sulfate incorporation into GAGs in the fibrous and cartilaginous scleral layers from normal eyes changed in opposite directions with age. Sulfate incorporation in the fibrous sclera decreased 10-fold after postnatal day 1 and showed smaller, if any, changes thereafter (Fig. 9A). In contrast, sulfate incorporation in the cartilaginous sclera increased monotonically from day 7 to day 23 (Fig. 9B). These age-dependent changes in sulfate incorporation into GAGs are statistically significant by
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FIGURE 9. Precursor incorporation into GAGs, DNA, and protein in (A) fibrous and (B) cartilaginous scleral tissues either initially separated or initially left intact during first 20 hours in culture. Scleral tissues were obtained from paired untreated eyes of chicks ages 1 (n = 17 or 18), 7 (n = 11), 8 (n = 18), 14 (n = 12), and 23 (n = 11) days. Data shown are mean (±SEM). A two-sample t-test was performed for unpaired comparisons. An asterisk indicates statistical significance (two-sample t-test) at the level of \( P < 0.05 \). The "x" indicates a single outlier not included in statistical analysis.

Mechanical separation of the sclera from normal eyes into its two component layers by itself caused an increase in sulfate incorporation into GAGs, as assessed by comparing tissue separated initially with that separated after 20 hours in culture together (initially intact).

Linear regression for fibrous (slope < 0, \( P < 0.0001 \)) and cartilaginous tissues (slope > 0, \( P < 0.0001 \)), whether they were separated initially or separated after 20 hours in culture together (initially intact).

Does Precursor Incorporation Into GAGs in Separated Scleral Layers Reflect Normal Growth Processes?

We have shown consistent and opposite changes in incorporation of sulfate into GAGs in the two scleral layers. We view these as probably indicating corresponding changes in proteoglycan synthesis. We recognize that in some proteoglycans, the level of sulfation may change independently of core protein synthesis.

linear regression for fibrous (slope < 0, \( P < 0.0001 \)) and cartilaginous tissues (slope > 0, \( P < 0.0001 \)), whether they were separated initially or separated after 20 hours in culture together (initially intact).

Second, the effects of visual conditions on growth of the scleral cartilage appear to be controlled by the fibrous sclera. The stimulatory effect of form deprivation and the inhibitory effect of recovery from form deprivation can be mimicked by coculturing normal cartilaginous sclera with fibrous sclera from form-deprived or recovering eyes. Furthermore, the inhibitory effect on the scleral cartilage of recovery from form deprivation can also be mimicked by coculture with choroid from recovering eyes. In contrast, the fibrous sclera is stimulated by coculture with choroid, especially from recovering eyes, but is inhibited by donor cartilaginous sclera regardless of the visual conditions of the eye of origin.

DISCUSSION

These results show that the two layers of the chick sclera respond differently in all situations examined and that both layers must be studied to understand how vision controls the growth of the chick eye. Specifically, we have shown two main effects.

First, when the rate of elongation of the eye is altered by visual manipulations, the cartilaginous and fibrous layers of the eye show opposite modulations of growth, as indicated by sulfate incorporation into GAGs. In the cartilaginous layer of the sclera, the precursor incorporation into GAGs increases with more rapid ocular elongation (visual deprivation or wearing negative lenses) and decreases with less rapid ocular elongation (recovery from visual deprivation or wearing positive lenses). In the fibrous layer of the sclera, the opposite changes occur. Thus, the changes in the fibrous sclera are in the same direction as in form-deprived and recovering tree shrews.14-16 Our results are also consistent with those of Gottlieb et al17 showing that visual deprivation causes the cartilaginous sclera to thicken and the fibrous sclera to thin and with evidence of decreased scleral thickness in form-deprived tree shrews18,19 and monkeys,20 and in myopic humans.21-23

Second, the effects of visual conditions on growth of the scleral cartilage appear to be controlled by the fibrous sclera. The stimulatory effect of form deprivation and the inhibitory effect of recovery from form deprivation can be mimicked by coculturing normal cartilaginous sclera with fibrous sclera from form-deprived or recovering eyes. Furthermore, the inhibitory effect on the scleral cartilage of recovery from form deprivation can also be mimicked by coculture with choroid from recovering eyes. In contrast, the fibrous sclera is stimulated by coculture with choroid, especially from recovering eyes, but is inhibited by donor cartilaginous sclera regardless of the visual conditions of the eye of origin.

Does Precursor Incorporation Into GAGs in Separated Scleral Layers Reflect Normal Growth Processes?

We have shown consistent and opposite changes in incorporation of sulfate into GAGs in the two scleral layers. We view these as probably indicating corresponding changes in proteoglycan synthesis. We recognize that in some proteoglycans, the level of sulfation may change independently of core protein synthesis. In cartilage, however, the predominant proteoglycan, aggrecan, is fully sulfated.24 Further-
more, because the GAGs in sclera (predominantly the cartilaginous sclera) have a half-life of 10 days, whereas our measurements of sulfate incorporation into GAGs took no longer than 20 hours, the rate of incorporation reflects mostly new net synthesis, at least in the case of cartilaginous sclera.

One of the difficulties in interpreting this work is that the experiments necessitated physical separation of the scleral layers to assess their separate synthetic activities. We must consider, therefore, how precise the separation was and how great an effect this separation is likely to have had on the sclera. To answer the first question, the separation is unlikely to have left significant amounts of cartilage on the fibrous layer, because the variability of precursor incorporation into GAGs in cartilage and fibrous sclera is comparable, although the cartilage has 100-fold greater radioactivity (Fig. 9). If the separation left fibrous sclera on the cartilaginous sclera, this would have had a negligible effect on our measurements of the cartilage for the same reason. Indeed, faulty separation cannot be responsible for the changes in precursor incorporation into GAGs in our experimental eyes, because the amount of DNA hardly changes and the changes that occur are in the opposite direction.

Furthermore, it appears that the act of separation does not drastically affect the response of the tissues, because the precursor incorporation into DNA, protein, and GAGs is similar in tissues separated at the start of incubation compared to ones incubated together first and separated later (Fig. 9). This similarity is maintained over a 4-week range of ages, even though the absolute levels change 10-fold in the fibrous sclera over that period and the degree of adhesion between the scleral layers changes enormously (layers come apart easily at hatching but are difficult to separate at 3 weeks of age).

Nonetheless, there is an effect of separation of the layers. Those separated initially consistently incorporate somewhat more sulfate than those separated later (Fig. 9). We cannot distinguish whether this is caused by synthetic activities related to repair of tissue damage or by the fact that both layers are released from a mutual inhibition. If we deliberately increased the tissue damage by making a second 4-mm-diameter punch within the 7-mm punch used in the experiments reported here, the rate of sulfate incorporation into the unseparated sclera nearly doubles.

However, we are encouraged to think that our opposite results from fibrous and cartilaginous sclera are real phenomena because they are so precisely mimicked by coculturing the separate layers from normal eyes with their complementary layers from eyes with different visual experiences and are consistent with results from coculture with choroids on scleral punches that had not had their layers separated (see Opposite Responses in Cartilaginous and Fibrous Sclera).

Relation to Compensation for Spectacle Lenses

We find that negative and positive lenses modulate precursor incorporation into scleral GAGs in opposite directions, both in the cartilaginous and fibrous sclera. This confirms a recent study on unseparated sclera. Because the +15 D lenses would cause profound myopic defocus for all but the closest objects and the −15 D lenses would cause hyperopic defocus that would not be fully compensated by accommodation, we take our findings as evidence for bidirectional growth modulation of the sclera rather than as a variant of deprivation myopia, as some have attempted to do. We discuss this issue more fully elsewhere.

Furthermore, the similarity of responses to lenses and to form deprivation and recovery suggests that these manipulations, despite the differences they produce in visual processing, share a final path in the control of ocular elongation.

Opposite Responses in Cartilaginous and Fibrous Sclera

Three simple humoral explanations could account for the opposite responses of the two scleral layers to deprivation or defocus. First, two different sets of extrinsic signals from the retina or elsewhere could separately guide the growth of the two layers. Second, a single set of signals could have opposite effects on the two layers. Third, one layer could receive the extrinsic signals and control the other layer. Our results suggest that this last alternative may suffice. We find that coculture of normal scleral cartilage with fibrous sclera from form-deprived or recovering eyes causes growth effects similar to those seen in the cartilage of eyes that were themselves form-deprived or recovering, respectively. On this basis, one could argue that cartilage need only respond to the fibrous sclera and that the fibrous sclera could be the only scleral layer that responds to extrinsic signals.

However, we find that the choroid from form-deprived and recovering eyes influences cartilaginous sclera in the same direction as does the fibrous sclera. We argue, however, that they have different roles, the fibrous sclera’s having both stimulatory and inhibitory roles and the choroid’s having an inhibitory role. This latter result is consistent with a study of the effect of choroid-conditioned medium on sulfate incorporation into GAGs of pieces of sclera, which showed a dose-dependent inhibition by choroids from recovering eyes and no stimulation from choroids from visually deprived eyes. Finally, the fact that the co-

* In this experiment, because the scleral layers were not separated, the precursor incorporation into GAGs measured would largely reflect the cartilaginous sclera, although the fibrous sclera could have been a modulatory influence.
culture with normal fibrous sclera causes an increase in the sulfate incorporation into GAGs of the scleral cartilage, but coculture with normal choroid causes a decrease, argues that these two tissues play distinct roles. These findings do not identify either the choroid or the fibrous sclera as the source of the signaling molecules. Either tissue might be acting as a sponge, accumulating the secretions of yet another tissue.

In summary, these coculture studies make plausible the notion that the retina or retinal pigment epithelium produces diffusible signals that act on the choroid and fibrous sclera, and that these tissues in turn control the growth of the cartilaginous sclera.

Beyond this humoral explanation, there might be a mechanical explanation for the opposite responses of the cartilaginous and fibrous scleral layers. It is well known that rhythmic mechanical forces result in an increase in proteoglycan synthesis of cartilage [review by Urban58]. Such forces cause hyperpolarization in chondrocytes but depolarization in fibroblasts. This may change in intraocular pressure, either diurnal or caused by fluctuations in ocular accommodation, provoke opposite changes in proteoglycan synthesis in chondrocytes and fibroblasts.

Finally, there is evidence that the cartilaginous and fibrous layers of the sclera should be viewed as a single tissue, with cells at the boundary between the layers switching identity between chondrocytes or fibroblasts as circumstances demand—that is, increased chondrogenesis during increased ocular elongation and increased fibrogenesis during decreased ocular elongation. At the boundary with the fibrous sclera, scleral cartilage from form-deprived eyes incorporates unusually large amounts of sulfate and the boundary between the layers becomes indistinct, suggesting active chondrogenesis. In bovine fibrocartilaginous tendon, compressive loading increased the synthesis of large proteoglycans such as aggrecan, the cartilage proteoglycan, and decreased the synthesis of small proteoglycans, such as decorin, found in the fibrous sclera; unloaded tendon showed the opposite changes.

Why does the fibrous sclera decrease its rate of precursor incorporation into both DNA and GAGs when the eye is elongating especially rapidly? In other connective tissues, decreased GAG synthesis is associated with decreased mechanical stiffness. This may be the case in the fibrous sclera as well, at least in the tree shrew, as shown by the association of decreased GAG synthesis with increased extensibility during applied constant force in form-deprived eyes. Furthermore, if the sclera is artificially weakened by treatment with lathyritic agents, tree shrew ocular elongation is enhanced. Thus, it seems plausible that the stiffness of the fibrous sclera must be reduced for the eye to elongate rapidly and that this is done by decreased GAG synthesis, presumably accompanied by increased proteolysis. Cartilage, in contrast, increases its GAG synthesis during rapid ocular elongation (the turnover also increases somewhat compared to normal). In the chick, the loss of stiffness of the fibrous sclera of form-deprived eyes might be compensated for by increased stiffness of the cartilage, associated with the increased GAG synthesis. This might help explain why lathyritic agents do not enhance ocular elongation resulting from form deprivation in chicks. These remodeling activities in both layers may be mediated by changes in the activation and inhibition of gelatinases, as has been shown for both chicks and tree shrews during form deprivation.

Differences Between Birds and Mammals

The pattern of results presented here resolves the apparent difference between birds and mammals shown by other studies. The fibrous sclera of the chick shows decreased sulfate incorporation into GAGs during the increased ocular elongation brought about by form deprivation, as has been shown for the fibrous sclera of the tree shrew. The difference in the chick is that it also has a layer of cartilage, which shows opposite responses. When the sulfate incorporation into GAGs is measured without separating the scleral layers, the cartilage greatly dominates because its proteoglycans are much more highly sulfated, giving the impression that the chick sclera shows opposite responses to those of the mammalian sclera.

In fact, the anthropomorphic tendency to regard the avian sclera as aberrant and distinct from the mammalian sclera ought to be resisted. Walls argues that a sclera with cartilage, being stiffer than a fibrous sclera and thus permitting larger and less spherical eyes, is the general vertebrate form and that scleral cartilage has been lost only in three groups (snakes, salamanders, and nonmonotreme mammals). He suggests that in mammals with exceptionally large eyes (e.g., whales and elephants), the lack of cartilage requires the sclera to be so thick that it occupies most of the volume of the eye. Finally, even the presence of cartilage in the avian sclera is not so great a difference from the mammalian sclera as it appears, in that mammalian sclera also expresses two of the three principal phenotypic markers for cartilage.

If we combine Walls’ speculations with the ones we made earlier in this discussion, we can view the fibrous sclera as needing to soften to grow. As a consequence, in mammals rapid elongation leaves the sclera vulnerable to losing the rigidity necessary to withstand expansion driven by the normal intraocular pressure; if this happens, a posterior staphyloma may result. In contrast, the general vertebrate pattern of a sclera reinforced with cartilage may be able to grow without this risk. This might explain why chicks with 20 to 30
D of myopia for long periods have stable eye shapes and why chicks in which the sclera is weakened by lathyrinic agents do not develop longer eyes after visual deprivation. Furthermore, one could view the Russian therapy of injecting foreign substances behind the eye to stimulate inflammatory connective tissue growth as a step toward giving humans the structural benefits of a cartilaginous sclera.

In conclusion, how the retina adjusts the growth of the sclera, and thereby the size and shape of the eye, remains a mystery, but all evidence points to a single solution for both birds and mammals, one that involves bidirectional modulation of scleral remodeling to enhance or reduce the rate of ocular elongation during growth, thereby continuously adjusting the eye toward functional emmetropia.

Key Words
cartilage, choroid, hyperopia, myopia, proteoglycans

Acknowledgments

The authors thank Debora L. Nickla and Sek-Jin Chew for a critical reading of an earlier version of this manuscript and Tadayo Kusakari for the use of Figure 1.

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