Identification of RALDH2 as a Visually Regulated Retinoic Acid Synthesizing Enzyme in the Chick Choroid

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PURPOSE. All-*trans*-retinoic acid (atRA) has been implicated in the local regulation of scleral proteoglycan synthesis in vivo. The purpose of the present study was to identify the enzymes involved in the synthesis of atRA during visually guided ocular growth, the cells involved in modulation of atRA biosynthesis in the choroid, and the effect of choroid-derived atRA on scleral proteoglycan synthesis.

METHODS. Myopia was induced in White leghorn chicks by form deprivation for 10 days, followed by up to 15 days of unrestricted vision (recovery). Expression of atRA synthesizing enzymes was evaluated by semiquantitative qRT-PCR, in situ hybridization, and immunohistochemistry. atRA synthesis was measured in organ cultures of isolated choroids using LC-tandem MS quantification. Scleral proteoglycan synthesis was measured in vitro by the incorporation of ³⁵SO₄ in CPC-pre-cipitable glycosaminoglycans.

RESULTS. *RALDH2* was the predominant *RALDH* transcript in the choroid (>100-fold that of *RALDH3*). *RALDH2* mRNA was elevated after 12 and 24 hours of recovery (60% and 188%, respectively; *P* < 0.01). The atRA concentration was significantly higher in cultures of choroids from 24-hour to 15-day recovering eyes than in paired controls (~195%; *P* < 0.01). Choroid conditioned medium from recovering choroids inhibited proteoglycan synthesis to 43% of controls (*P* < 0.02, paired *t*-test; *n* = 16) and produced a relative inhibition corresponding to a RA concentration of 7.20×10^{-8} M.

CONCLUSIONS. The results of this study suggest that *RALDH2* is the major retinal dehydrogenase in the chick choroid and is responsible for increased atRA synthesis in response to myopic defocus. (*Invest Ophthalmol Vis Sci.* 2012;53:1649–1662) DOI:10.1167/iov.11-8444

Strong evidence from clinical and experimental studies indicates the presence of a vision-dependent emmetropization mechanism that acts to minimize refractive error through the

coordinated regulation of the growth of the ocular tissues.^{1,2} In humans, the emmetropization mechanism often breaks down, leading to the development of nearsightedness or myopia usually because of excessive growth of the posterior portion of the eye. Direct evidence of emmetropization has been provided by numerous animal studies in which modulation of the refractive target with plus and minus lenses results in changes in vitreous chamber depth to align the retinal photoreceptors with the focal plane of the eye. Interruption of the emmetropization process as a result of the distortion of visual image quality, either through ocular pathology in humans^{1–4} or application of translucent occluders in animal models (form deprivation⁵), results in axial elongation and the development of myopia. Form deprivation-induced myopia is reversible; restoration of unrestricted vision (and the resultant myopic defocus) results in a temporary cessation of axial growth, eventually leading to the reestablishment of emmetropia (recovery) in the formerly deprived eye.⁶ The response to deprivation and defocus is rapid, leading to detectable changes in vitreous chamber depth within hours.⁷

It has been well established that in chicks these visually induced changes in ocular growth are directly associated with changes in proteoglycan synthesis and proteoglycan accumulation in the sclera at the posterior pole of the eye.^{8–12} Unlike the sclera of placental mammals, the chick sclera is dominated by a cartilaginous layer that is responsible for approximately 90% of total proteoglycan synthesis, primarily as a result of increased aggrecan (the cartilage proteoglycan) core protein synthesis.⁸ The visually guided scleral response is rapid, with significant changes in scleral proteoglycan synthesis occurring within 6 hours of recovery from a previous period of form deprivation.¹³

However, despite the identification of several molecular events occurring in the retina, RPE, choroid, and sclera in association with visually guided ocular growth, the chemical signals directly responsible for regulating changes in scleral extracellular matrix synthesis during myopia development and emmetropization have not been identified. Studies in animal models suggest that all-*trans*-retinoic acid (atRA) may be an important molecular signal in the postnatal control of eye size.^{14–17} In an elegant study, Mertz and Wallman¹⁴ demonstrated that the choroidal synthesis of atRA was increased in chick eyes during recovery from induced myopia and during compensation for imposed myopic defocus. Moreover, atRA was shown to be decreased in eyes undergoing form deprivation myopia and compensation for hyperopic defocus compared with the fellow control eye, suggesting that choroidal atRA synthesis could represent a molecular basis for emmetropization. atRA is an attractive candidate for a visually regulated ocular growth regulator because it is readily diffusible, has pronounced effects on scleral extracellular matrix metabolism, and exerts its effects through highly regulated,

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locally controlled synthesis and degradation. Tissue concentrations of atRA are tightly controlled by the activities of synthesizing enzymes, retinol dehydrogenase (RDH), the retinaldehyde dehydrogenases (RALDH1, RALDH2, and RALDH3; also known as Aldh1A1, Aldh1A2, and Aldh1A3, respectively), and the atRA-metabolizing enzyme CYP26, a member of the cytochrome P450 family.¹⁸ Cyp1B1 may also contribute to atRA synthesis in the chick embryo.¹⁹ Furthermore, the rate of these dehydrogenation reactions is regulated by substrate availability, enzyme accessibility, and enzyme catalytic activity.²⁰ Through autocrine or paracrine (or both) interactions, atRA binds with nuclear retinoic acid receptor complexes to directly control the transcriptional activity of more than 100 target genes.²¹

The molecular events involved in regulating atRA synthesis during recovery from induced myopia have yet to be characterized. Therefore, this study was undertaken to identify the enzymes involved in the synthesis of atRA during visually guided ocular growth, to identify the cells involved in the modulation of atRA biosynthesis in the choroid, and to demonstrate the effect of choroid-derived atRA on scleral proteoglycan synthesis.

MATERIALS AND METHODS

Animals

White Leghorn male chicks (*Gallus gallus*) were obtained as 2-day-old hatchlings from Ideal Breeding Poultry Farms (Cameron, TX). Chicks were housed in temperature-controlled brooders with a 12-hour light/12-hour dark cycle and were given food and water ad libitum. Form-deprivation myopia was induced in 2-day-old chicks by applying translucent plastic goggles, as previously described.⁸ Goggles remained in place for 10 days, after which time either the chicks were euthanized for isolation of ocular tissues from control (contralateral untreated eyes) and form-deprived eyes or the goggles were removed and chicks were allowed to experience unrestricted vision (recover) for up to 15 days. Generally, chicks were euthanized between 9 am and 12 pm, with the exception of the 6-hour and 12-hour time points, during which the chicks were euthanized at 3 pm and 9 pm, respectively. The left eyes of all the chicks were never goggled and were used as controls. Chicks were checked daily for the condition of the occluders.

Animals were maintained and used in accordance with the Animal Welfare Act, National Institutes of Health Guidelines, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center.

Tissue Preparation

Chicks were euthanized by an overdose of isoflurane inhalant anesthetic (Iso-Thesia; Vetus Animal Health, Rockville Center, NY), and eyes were enucleated. Each eye was divided into anterior and posterior hemispheres by a circumferential cut around the equator, and the posterior hemisphere was gently cleaned of all vitreous, pecten, and muscle. One 5-mm (for RNA extraction) or 8-mm (for retinoic acid measurement and coculture experiments) tissue punch was excised from the posterior pole using a dermal biopsy punch (Miltex Instrument Company, Inc.; Bethpage, NY). Punches (containing the sclera, choroid, RPE and neural retina) were transferred to Petri plates containing one drop of PBS for choroid and retina/RPE isolation. To isolate retina/RPE and choroids, the retina and retinal pigment epithelium (RPE) were removed as a sheet with a small spatula and snap frozen in microfuge tubes. Choroids were cleaned of adherent RPE by gentle brushing with a sable brush and rinsing with PBS. After the removal of all visible pigment, choroids were separated from the sclera with a fine spatula and snap frozen in microfuge tubes in liquid nitrogen. For retinoic acid measurements and preparation of choroid conditioned medium (CCM), choroids (8-mm punches) were isolated from control

and recovering eyes (24 hours) under dim red light and were incubated in 80 μ L N2 medium (DMEM/Ham's F-12 1:1 containing N2 supplement; Invitrogen, Carlsbad, CA) for 3 hours at 37°C. For in situ hybridization and immunohistochemistry, tissues were fixed by transcardial vascular perfusion of deeply anesthetized chicks with 4% paraformaldehyde in PBS. Eyes were enucleated and hemisected, and 5-mm punches were excised from the posterior pole of control and treated eyes. Tissue punches were fixed overnight at 4°C, followed by immersion in 18% sucrose overnight at 4°C and subsequent immersion in sucrose/embedding medium 1:1 (OCT; Tissue Tek; Miles, Elkhart, IN) overnight at 4°C. Tissue punches were then embedded in OCT and rapidly frozen using liquid nitrogen. Sagittal sections (7–10- μ m thick) were cut on a cryostat microtome and collected on glass slides (Superfrost; Fisher Scientific, Fair Lawn, NJ).

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Choroids were isolated from pairs of control and treated eyes after 0 hour, 6 hours, 12 hours, 1 day, 4 days, 8 days, and 15 days of recovery and were snap frozen in liquid nitrogen. Additionally, retinas and RPE were isolated from eyes after 0 hour, 6 hours, 12 hours, and 24 hours of recovery. Total RNA was isolated from choroids and retinas/RPE using reagent (TRIzol; Invitrogen, Carlsbad, CA), followed by DNase treatment (DNA-free; Ambion, Foster City, CA). RNA concentration and purity were determined by the optical density ratio of 260/280 using a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE) and were stored at –80°C until use. cDNA was generated from 1 μ L total RNA (containing 34 to 94 ng total RNA per reaction) by reverse transcription using MuLV reverse transcriptase and random hexamers according to the manufacturer's protocol (GeneAmp RNA PCR Kit; Applied Biosystems). Semiquantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in triplicate using 1 μ L cDNA reaction product and gene-specific chicken primers together with SYBR Green (Molecular Probes, Eugene, OR) in a 96-well plate format, using a multicolor, real-time PCR detection system (iCycler iQTM; Bio-Rad, Hercules, CA). PCR was carried out in a total reaction volume of 25 μ L/well. The PCR protocol consisted of an initial denaturation step at 95°C for 90 seconds, followed by 40 cycles of amplification at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 60 seconds. Samples were again denatured at 95°C for 2 minutes. Melt curve analysis was carried out after PCR amplification by slowly heating samples from 60° to 95°C (70 cycles, increasing temperature 0.5° every 2 minutes). Samples were then maintained on a hold cycle at 15°C until they were collected. During heating, emission of SYBR green was continuously monitored at 490 nm.

Primers were selected from chick sequences of retinaldehyde dehydrogenases 1, 2, and 3 (*RALDH1*, *RALDH2*, *RALDH3*), retinol dehydrogenase 10 (*RDH10*), cytochrome P450 1B1 (*CYP1B1*), and cytochrome P450 (*CYP26*), α -smooth muscle actin (*ACTA2*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and 18S rRNA (*18S*) using BLAST, Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and σ -Genosys (St. Louis, MO) (Table 1). The chicken *GAPDH* gene was used as a reference gene to normalize for variation in starting cDNA between samples. We have previously quantified *GAPDH* mRNA copy number in choroid RNA samples from contralateral control and treated eyes²² and found no significant differences in *GAPDH* expression between contralateral control and treated eyes throughout a 0- to 15-day recovery period (paired *t*-test). Relative levels of gene expression were determined using the mean normalized expression values, as previously described.²³ Correct product size, and lack of primer-dimer formation were confirmed by DNA agarose-gel electrophoresis and melt curve analysis, respectively. As an additional control for genomic DNA contamination, reverse transcriptase was omitted from some choroid RNA samples before reverse transcription. Primer efficiencies were determined for *RALDH1*, *RALDH2*, *RALDH3*, *RDH10*, *CYP1B1*, *CYP26*, and *GAPDH* using a dilution series of retina or choroid cDNA (Table 1). PCR products were confirmed by DNA sequencing using a

TABLE 1. Gene Primers

Gene Name Accession No.	Forward Primer	Reverse Primer	Product Size (bp)	Primer Efficiency (%)
Retinaldehyde dehydrogenase 1 (<i>RALDH1</i>) NM_204577.4	5'-AATATTGTGTCACAGGCTTTGG-3'	5'-GCCCTTGGTGGTAAAAACAGA-3'	250	109.7
Retinaldehyde dehydrogenase 2 (<i>RALDH2</i>) NM_204995.1	5'-GGCTCAAGGATTTACGTGA-3' (qRT-PCR) 5'-CCCCGAGACTATGACTTCCA-3' (ISH)	5'-TCCTTCAGTAATGCGGCTCT-3' (qRT-PCR) 5'-CCAAACAATTGGACCAAAATCC-3' (ISH)	180 750	99.0
Retinaldehyde dehydrogenase 3 (<i>RALDH3</i>) AF246710	5'-CCTACTGGCAGCATAGCACA-3'	5'-ACCAAGTGAACACCCAAAGTGC-3'	226	95.0
Cytochrome P450 - 1B1 (<i>CYP1B1</i>) XM_419515.2	5'-TGGCTTTCCTGTACGAAATCC-3'	5'-TCTGGGTGGACCATTTAGC-3'	169	95.0
Cytochrome P450 26 (<i>CYP 26</i>) AF199462	5'-GAGAGAGCTCAACATGCAG-3'	5'-CTTGGTTGGGACCAAGACAGT-3'	180	97.1
Retinol dehydrogenase 10 (<i>RDH10</i>) NM_001199459.1	5'-TTCTGGCCCTTTGAAGCAGTT-3'	5'-ACACAGGTTGAGGACCCAG-3'	255	95.9
α -Smooth muscle actin (<i>ACTA2</i>) NM_001031229.1	5'-GTCCCTGTATGCTTCTGGGG-3'	5'-TGATCTTCATCTTCATGGTG-3'	569	ND
Glyceraldehyde 3-phosphate dehydrogenase (<i>GAPDH</i>) NM_204305.1	5'-TGCTAAGGCTGTGGGAAAGTC-3'	5'-CAAGGTGGAGGAATGGTGTGTC-3'	248	108.6

qRT-PCR, primer pairs used for mRNA quantification; ISH, primer pairs for in situ hybridization; ND, not determined.

capillary DNA sequencer (ABI 3730; Applied Biosystems). All PCRs were run in triplicate and repeated on three to five separate semiquantitative qRT-PCR procedures.

Riboprobe Synthesis

Sense and antisense riboprobes were generated from choroid mRNA using PCR products designed with the T7 promoter sequence (GGTA-ATACGACTCACTATAGG) at the 5' end of either forward or reverse PCR primers, respectively. PCR products were purified using a nucleotide removal kit (QIAquick; Qiagen) and transcribed with T7 RNA polymerase using digoxigenin-labeled nucleotides (Roche Applied Science, Indianapolis, IN). Templates were subsequently digested with RNase-free DNase (Ambion, Austin, TX) and purified using a column (RNeasy; Qiagen). Riboprobe concentration and purity were determined at an optical density ratio of 260/280 using a spectrophotometer (ND-1000; NanoDrop Technologies), and riboprobe integrity was evaluated after electrophoresis on 1% formaldehyde-agarose gels and ethidium bromide staining. The *RALDH2* 750 and *ACTA2* PCR products used for riboprobe synthesis were additionally cloned into a vector (PBluescript SK+; Stratagene, La Jolla, CA) according to manufacturer's protocol, and their sequences were confirmed by DNA sequencing using a capillary DNA sequencer (ABI 3730; Applied BiosystemsA). Dig-labeled *RALDH2* probe efficiency was evaluated by Southern blot analysis using the 750 *RALDH2* PCR product.

Northern Blot Analysis

For Northern blot analysis, total RNA isolated from choroid (1 μ g) was separated on 1% formaldehyde-agarose gels, blotted overnight onto a positively charged nylon membrane (Roche Applied Science, Mannheim, Germany), and UV-cross-linked. Blots were prehybridized for at least 1 hour at 68°C with prewarmed hybridization solution (DIGeasy; Roche Applied Science) in accordance with the manufacturer's instructions. Blots were hybridized for 16 $\pm$ 20 hours with 100 ng/mL DIG-labeled probe in prewarmed hybridization solution (DIGeasy; Roche Applied Science) at 68°C using a rotisserie-style hybridization oven. Northern blots were washed in a series of prewarmed washing solutions of increasing stringency (from 2 $\times$ standard sodium citrate [SSC]/0.1% SDS at room temperature (RT) – 0.1 $\times$ SSC/0.1% SDS at 65°C). Blots were then incubated with anti-digoxigenin-AP, Fab fragments (Roche Applied Science), diluted 1:20,000 in blocking buffer (1% blocking reagent [Roche Applied Science] in MABT [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]) for 1 hour at RT, followed by washes (2 $\times$ 15 minutes each) with MABT + containing 0.3% Tween-20, pH 7.5 at RT.

Chemiluminescence detection was performed using chemiluminescent substrate (CDP-star; Tropix, Bedford, MA) and was imaged using a bioimaging system (ChemiGenius²; SynGene, Frederick, MD).

In Situ Hybridization

Nonradioactive in situ hybridization was carried out on frozen sections (7–10- μ m thick) of paraformaldehyde-fixed tissues using digoxigenin-labeled RNA probes, as described. Slides were postfixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes at RT. Sections were then washed 2 $\times$ with PBS and permeabilized with proteinase K (30 μ g/mL) in PK buffer (0.05 M Tris-HCl, pH 7.5, containing 0.005 M EDTA) for 8 minutes at RT. Slides were washed in PBS (5 minutes at RT) and fixed again with 4% paraformaldehyde in PBS (15 minutes at RT). After a brief rinse in PBS, sections were acetylated with 0.1 M triethanolamine-HCl/0.25% acetic anhydride for 10 minutes at RT with stirring in a Coplin jar. Slides were prehybridized for 3 hours at 60°C in hybridization buffer (50% formamide, 5 $\times$ SSC buffer, yeast tRNA [100 μ g/mL], heparin [100 μ g/mL], 0.2% Tween-20, 0.5% CHAPS, and 0.005 M EDTA).

Hybridization was carried out for 48 hours using digoxigenin-labeled cRNA probe (200 ng/mL) in hybridization buffer, in plastic slide mailers containing sufficient probe solution to immerse the part of the slides containing the sections. Slides were then washed exten-

sively with SSC (1× SSC, 55°C, 10 minutes; 1.5× SSC, 55°C, 10 minutes; 2× SSC, 37°C, 20 minutes; 1× PBS + 0.1% Tween-20, 55°C, 10 minutes; 1× PBS + 0.1% Tween-20 at RT, for 10 minutes; 1× PBS + 2 mg/mL BSA + 0.1% Triton X-100 at RT for 15 minutes). Slides were blocked with blocking buffer (2% sheep serum in PBS containing 2 mg/mL BSA and 0.1% Triton X-100 for 1 to 5 hours at RT, followed by incubation overnight with anti-digoxigenin-AP, Fab fragments (Roche Applied Science) diluted 1:1500 in blocking buffer at 4°C. After washes in blocking buffer (2 × 30 minutes each at RT) and alkaline phosphatase buffer (0.1 M Tris [0.05 M MgCl₂, 0.1 M NaCl, 0.1% Tween 20]; 2 × 20 minutes at RT), the alkaline phosphatase reaction product was developed using NBT/BCIP (Roche Applied Science) according to manufacturer's instructions. Slides were coverslipped using mounting medium with Tris buffer (Fluoro-Gel; Electron Microscopy Sciences, Hatfield, PA) and imaged on a Nikon microscope (80i; Nikon, Melville, NY) using Nikon software (NIS Elements; Nikon).

Immunohistochemistry

For immunohistochemical detection of RALDH2 and αSMA, cryostat sections (taken as described in Tissue Preparation) were rinsed in PBS and then incubated in blocking buffer (1% bovine serum albumin, 0.2% Triton X-100, and 0.004% sodium azide in PBS) for 30 minutes at RT. Sections were incubated with rabbit anti-aldehyde dehydrogenase 2 (anti-ALDH1A2) antibody (Sigma), diluted 1:200 in blocking buffer or mouse anti-αSMA (clone 1A4; Sigma) diluted 1:50 in blocking buffer for 3 days at 4°C. For negative controls, the tissue sections were incubated in 2 μg/mL nonimmune mouse immunoglobulin (Sigma) instead of the primary antibody. After incubation with the primary antibody, the sections were rinsed in PBS and incubated in 5 μg/mL Alexa Fluor 488 (green) conjugated to goat anti-rabbit antibody (Molecular Probes, Eugene, OR) for 30 minutes at RT. For dual immunolabeling, tissue was incubated with both anti-rabbit Alexa Fluor 488 (5 μg/mL, green) and anti-mouse Alexa Fluor 568 (5 μg/mL, red) for 30 minutes at RT. Sections were rinsed in PBS, then incubated with 0.0005% DAPI nuclear stain for 10 seconds at RT, followed by a final rinse in PBS. Coverslips were mounted onto the slides with reagent containing DAPI (Prolong Gold Antifade; Invitrogen, Eugene, OR), and the immunolabeled sections were viewed by confocal microscopy, using a laser scanning confocal microscope (FluoView 1000; Olympus, Center Valley, PA).

For dual in situ hybridization/immunohistochemistry experiments, in situ hybridization was carried out as described with the omission of the proteinase K digestion step. After detection of Dig-labeled probes with NBT/BCIP, sections were processed for immunohistochemistry as described and were coverslipped using antifade reagent with DAPI (Prolong Gold; Invitrogen). NBT/BCIP reaction product, Alex Fluor 488, and DAPI labeling were visualized simultaneously using a confocal microscope (FluoView 1000; Olympus) equipped with a DIC/Nomarski filter. NBT/BCIP-labeled images were subsequently pseudocolored in (Photoshop CS5; Adobe, Mountain View, CA) and merged with identical immunolabeled and DAPI-stained images.

Western Blot Analysis

Choroids were isolated from 5-mm punches obtained from the posterior poles of recovering eyes (24 hours) and contralateral control eyes. Total protein was extracted separately from each tissue by vigorous mixing in 2% SDS (100 μL/extract). After centrifugation at 14,000 rpm for 10 minutes, aliquots of protein extracts were directly applied to SDS-PAGE gels (10% Bis-Tris Gel NuPAGE; Invitrogen, Carlsbad, CA). Gel samples were electrophoresed under reducing conditions and electroblotted onto a nitrocellulose membrane using an electrotransfer unit (XCELL Sureback; Electrophoresis Cell, Invitrogen) or were stained (SimplyBlue SafeStain; Invitrogen) according to manufacturer's instructions. Blots were probed with rabbit anti-human RALDH2 polyclonal antibody at a 1:500 dilution in blocking buffer (PBS containing 0.1% Tween-20 and 0.2% I-Block [Sigma Chemical Co., St. Louis, MO]) for 4 hours at RT, followed by incubation with goat anti-rabbit IgG

(whole-molecule) conjugated to alkaline phosphatase (Sigma Chemical Co.) at a dilution of 1:1000 for 1 hour at RT. Between incubations the blot was washed three times for 10 minutes per wash with 1× PBS containing 0.05% Tween 20. Chemiluminescence reagent (CDP-Star Ready-to-Use; Tropix, Bedford, MA) with substrate (Nitro-Block II; Tropix) was added to the blot for 5 minutes and then exposed on film and imaged using a bioimaging system (ChemiGenius²; SynGene, Frederick, MD).

Measurement of Tissue Retinoids by Mass Spectrometry

Choroids were isolated from control and treated eyes as described under dim red light and were incubated in 80 μL N2 medium for 3 hours at 37°C. A 3-hour incubation period was selected for atRA quantification based on the results of Mertz and Wallman¹⁴ in which they demonstrated that the rate of synthesis of atRA by isolated choroids in vitro plateaus after approximately 3 hours of incubation. Choroids and medium were collected in amber microfuge tubes and stored at −80°C before retinoid analyses. Retinol, retinal esters, and atRA levels were determined in organ-cultured choroids and CCM, as described previously.^{24,25} Briefly, choroids together with N2 medium were homogenized in 0.5 mL of 0.9% NaCl. A volume of 1 mL of 0.025 M KOH in ethanol was added to tissue homogenates, followed by extraction with 10 mL hexane. The top (hexane) layer containing retinol and retinol ester (RE) was removed and evaporated under nitrogen and then resolved by reverse-phase high-performance liquid chromatography (HPLC) and quantified by ultraviolet absorbance at 325 nm. The bottom layer was then extracted with 4 N HCl and followed by 10 mL hexane. The atRA in the resultant top layer was quantified by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) with atmospheric pressure chemical ionization. Retinol and RE were quantified by HPLC.²⁶ Retinoids were handled under yellow lights using only glass or stainless steel containers, pipettes, and syringes. Results are averages of triplicates and are reported as picomole (or nanomole)/(punch + medium).

Scleral Sulfated Glycosaminoglycan Synthesis

Eyes of untreated chicks, aged 10 to 14 days, were isolated, and the cornea, scleral ossicles, and vitreous body were removed. Five or six punches (5-mm diameter each) were obtained from the equatorial and posterior poles using a dermal punch (Miltex Instrument Co., Bethpage, NY), and each punch was cleaned of retina, RPE, choroid, and extraocular muscle. Scleral punches were initially placed into wells of a 96-well culture plate with 50 μL N2 medium (Ham's F-12/DMEM containing 1× N2 supplement; Stem Cell Technologies, Vancouver, BC, Canada) until all sclera were obtained for the experiment. Sclera were randomized among wells of the plate to ensure that sclera from multiple chicks were included in each treatment group. Under dim red light, scleral punches were then transferred to 96-well culture plates containing atRA (10^{−10}–10^{−5} M) in N2 medium or CCM and incubated overnight at 37°C. Scleral punches were then transferred to N2 medium containing ³⁵SO₄ (100 μCi/mL; New England Nuclear, Beverly, MA) and were incubated for 3 hours at 37°C. Radiolabeled scleral punches were digested with proteinase K (protease type XXVIII; Sigma), 0.05% wt/vol in 10 mM EDTA, 0.1 M sodium phosphate, pH 6.5, overnight at 60°C. ³⁵SO₄-labeled glycosaminoglycans were precipitated by the addition of 0.5% cetylpyridinium chloride in 0.002 M Na₂SO₄ in the presence of unlabeled carrier chondroitin sulfate (1 mg/mL in dH₂O). The samples were incubated for 30 minutes at 37°C, and precipitated glycosaminoglycans were collected on Whatman filters (GF/F) using a Millipore 12-port sampling manifold, as previously described.²⁷ Radioactivity was measured directly on the filters by liquid scintillation counting.

Statistical Analysis

Comparisons of gene expression and retinoic acid concentrations between treated and contralateral control eyes were made using Stu-

dent's *t*-test for matched pairs or the Wilcoxon matched pairs signed rank test (Prism; GraphPad, San Diego, CA). Comparisons between groups were made with one-way ANOVA using Scheffé comparisons and Bonferroni adjustments for multiple comparisons (GB Stat; Dynamic Microsystems, Inc., Silver Spring, MD).

RESULTS

Retinal and Choroidal Expression of Retinoic Acid Metabolizing Enzymes

RALDH1, *RALDH2*, and *RALDH3* gene expression were compared in the retina/RPE and choroids of control and treated eyes after 0 hour (10 days of form deprivation), 6 hours, 12 hours, 24 hours, 2 days, 4 days, and 15 days of recovery by semiquantitative qRT-PCR using *GAPDH* as a reference gene (Fig. 1). Semiquantitative qRT-PCR results indicated that *RALDH1*, *RALDH2*, and *RALDH3* were expressed in the retina/RPE of control and treated eyes after 12 hours of recovery (Fig. 1A). *RALDH1* and *RALDH3* expression was similar in retina/RPE of control and treated eyes, whereas *RALDH2* expression in the retina/RPE was significantly decreased in treated eyes after 12 hours of recovery; Fig. 1A) ($P < 0.01$). *RALDH2* expression in the retina/RPE was significantly increased in treated eyes after 10 days of form deprivation (0 hours of recovery; Fig. 1B) ($P < 0.05$). *RALDH2* mRNA pools were reduced significantly below contralateral control levels after 12 hours of recovery (Figs. 1A, 1B) ($P < 0.01$, paired *t*-test).

Choroidal expression of *RALDH1* could not be detected by semiquantitative qRT-PCR. *RALDH3* expression was detected within the choroid, at similar levels between control and treated eyes throughout 10 days of recovery (Fig. 1C). *RALDH2* was expressed at relatively high levels in the choroid compared with *RALDH3* (Fig. 1D) and was significantly increased in treated eyes after 12 hours of recovery ($P < 0.01$, paired *t*-test), reaching a maximum increase at 24 hours ($P < 0.001$, paired *t*-test). No significant changes between control and recovering eyes were detected after 15 days.

No significant differences were detected in the expression of *RDH10*, an enzyme that converts retinol to all-*trans*-retinaldehyde, between the choroids of control and treated eyes (0–4 days of recovery) (Fig. 2A). However, *RDH10* mRNA was significantly elevated in the choroids of both control and treated eyes at the 12-hour time point compared with *RHD10* mRNA in control choroids at all time points ($P < 0.01$, Scheffé comparison and Bonferroni correction ANOVA). No significant differences were detected in the expression of *CYP11B1*, an enzyme demonstrated to catalyze both the oxidation of retinol to all-*trans*-retinaldehyde and the oxidation of all-*trans*-retinaldehyde to atRA¹⁹ (Fig. 2B). Additionally, the expression of the atRA degrading enzyme, *CYP26*, was compared in control and recovering eyes. No significant differences were detected in mRNA levels of *CYP26* between control and treated eyes from any time points examined (0 hour to 15 days of recovery) (Fig. 2C). CT values for *GAPDH* were not significantly different between control and treated eyes ($P > 0.05$; paired *t*-test) or

FIGURE 1. Real-time PCR quantification of RALDH gene expression in retina/RPEs and choroids of control and treated chick eyes. (A) *RALDH1*, *RALDH2*, and *RALDH3* mRNA abundance in retina/RPEs of control and treated chick eyes after 12 hours of unrestricted vision preceded by 10 days of form deprivation (recovery). (B) *RALDH2* abundance in retina/RPEs of control and treated eyes after 0 to 24 hours of recovery. (C) *RALDH3* mRNA levels in choroids of control and treated eyes after 0 to 10 days of recovery. (D) *RALDH2* gene expression in choroids after 0 hour to 15 days of recovery. Graphs represent the mean normalized expression (MNE) calculated for *RALDH1*, *RALDH2*, and *RALDH3* and normalized to the reference gene, *GAPDH*. All reactions were run in triplicate and repeated on three to five separate semiquantitative qRT-PCR procedures with 5 to 20 chicks in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for treated eyes compared with paired contralateral controls (Student's paired *t*-test); ns, not significant. Numbers above each set of data points indicate the number of chicks used for each group.

FIGURE 2. Real-time PCR quantification of *RDH-10*, *CYP11B1*, and *CYP26* gene expression in choroids of control and treated chick eyes. (A) Abundance of *RDH-10* mRNA in choroids of control and treated chick eyes after 12 hours to 15 days of unrestricted vision, preceded by 10 days of form deprivation (recovery). (B) *CYP11B1* mRNA levels in choroids of control and treated eyes after 0 hour to 15 days of recovery. (C) *CYP26* mRNA abundance in choroids of control and treated eyes after 0 hour to 15 days of recovery. Relative gene expression was normalized to *GAPDH* mRNA (B, C). All reactions were run in triplicate and repeated on three to four separate semiquantitative qRT-PCR procedures. No significant differences were detected in *RDH-10*, *CYP11B1*, or *CYP26* gene expression between treated and contralateral control eyes at any time point. Numbers above each set of data points indicate the number of chicks used for each group.

between treatment groups ($P > 0.05$, Scheffé comparison and Bonferroni correction ANOVA) (data not shown).

In Situ Hybridization

Because *RALDH2* was the most abundant *RALDH* transcript in the choroid and because *RALDH2* mRNA pools within the choroid changed significantly during recovery, in situ hybridization was carried out to determine the distribution of *RALDH2*-expressing cells within the chick choroid (Fig. 3). For this purpose, cryosections of chick choroid were incubated

with digoxigenin-labeled sense or antisense probes for a 750-bp region of the *RALDH2* gene. Hybridized probes were then detected by successive treatment with anti-DIG-AP antibodies and NBT/BCIP substrate. *RALDH2*-positive cells were detected in treated eyes after 10 days of form deprivation (0 hour of recovery; Fig. 3A), 11 hours of recovery (Fig. 3D), and 24 hours of recovery (Figs. 3E, 3F, arrows). The antisense probe detected *RALDH2* transcripts in cells located between the large choroidal blood vessels (BVs) and the choroidal lacunae (L). *RALDH*-positive cells were also detected immediately adjacent to the sclera (S) (Figs. 3A, 3F, arrows). Labeling of the choroids with the *RALDH2* sense probe produced minimal signal (Fig. 3B). *RALDH2* mRNA was not detected in the retinas of treated eyes after 11 hours of recovery (Fig. 3G) and was not detected in the neural retinas of control eyes or of eyes treated for 0 hour and 24 hours (data not shown). It is possible that *RALDH2* is expressed in cells of the RPE, but visualization of the NBT/BCIP reaction product is obscured by pigment within the RPE. The *RALDH2* cDNA probe used for in situ hybridization experiments detected a single ~4.5-kb band by Northern blot analysis (Fig. 3C) that was larger than the expected message size for *RALDH2* at 3.55 kb. The presence of only one band on the Northern blot analysis suggested that the probe was specific. Southern blot analyses indicated that our probe bound with high affinity to the *RALDH2* 750 PCR product (data not shown). Although alternative splicing information is not available for chick *RALDH2*, we suspect that tissue-specific pre-mRNA splicing differences or alternative splicing events associated with *RALDH2* transcription in embryonic versus post-hatch chicks may account for this discrepancy, as has been shown for the human *RALDH2* gene (Aldehyde Dehydrogenase Gene Superfamily Database; www.aldh.org).

In a parallel set of experiments, tissue sections were treated with polyclonal antibodies raised against recombinant human *RALDH2* peptide (122 amino acids). *RALDH2*-expressing cells were then detected with Alexa Fluor 488-conjugated secondary antibodies. Minimal *RALDH2* labeling was detected on sections of contralateral control eyes (Figs. 4A, 4E). *RALDH2* protein was readily detected in round to spindle-shaped cells between choroidal blood vessels and choroidal lacunae in the choroids of treated eyes after 24 hours of recovery (Figs. 4C, 4D). This antibody recognized a band with an apparent molecular weight of approximately 57 kDa in whole choroid tissue extracts (Fig. 4B). Minimal signal was detected in sections in which nonimmune rabbit IgG was substituted for the anti-*RALDH2* antibody as the primary antibody (Fig. 4F).

Based on the location of *RALDH2*-expressing cells, we hypothesized that *RALDH2* was expressed by extravascular smooth muscle cells, located within the choroidal stroma. Therefore, in situ hybridization was carried out using sense and antisense probes to label α -smooth muscle actin (*ACTA2*) to identify smooth muscle cells in 11-hour recovering and contralateral control eyes (Fig. 5). *ACTA2*-expressing cells were clearly visible in the choroidal stroma of recovering eyes (Fig. 5A, arrows) and around larger blood vessels. *ACTA2*-expressing cells were also visible in the choroidal stroma of contralateral control chick eyes (Fig. 5B, arrows). Labeling of recovering and control choroids with *ACTA2* sense probe produced minimal signal (Figs. 5C, 5D, respectively).

To determine whether choroid stromal cells coexpress *RALDH2* and *ACTA2*, in situ hybridization was carried out using sense and antisense probes to *ACTA2* followed by immunolocalization of *RALDH2* protein. Hybridized probes were then detected by successive treatment with alkaline phosphatase-conjugated anti-DIG antibodies and NBT/BCIP substrate, followed by immunolabeling with anti-*RALDH2* and detection with Alexa Fluor 488-conjugated secondary antibodies. Because strong NBT/BCIP labeling can absorb the exciting light

and emitting fluorescence, RALDH2 labeling was weak in intensely labeled *ACTA2*-containing cells, and RALDH2/*ACTA2* dual labeling was detected only within cells more weakly stained with *ACTA2*. Nevertheless, *ACTA2* mRNA expression was detected in cells within the choroid stroma of control (C) and 24 hour-recovering (R) eyes and in the walls of larger blood vessels (Fig. 6, red-labeled cells). Dual in situ hybridization/immunolabeling experiments indicated that the majority of RALDH2-expressing cells (green-labeled cells) also expressed *ACTA2* (Figs. 6A-F, arrows). A subpopulation of RALDH2-positive stromal cells was also detected that did not express *ACTA2* (Figs. 6A-F, arrowheads). *ACTA2*-positive cells located in BV walls were negative for RALDH2 (Figs. 6E, 6F, asterisks). No immunolabeling or probe hybridization was detected when sections were incubated with nonimmune rabbit IgG as the primary antibody and the sense *ACTA2* RNA probe (Figs. 6G-I). These results were confirmed by double immunolabeling of chick choroid with anti-RALDH2 antibodies, together with mouse monoclonal antibodies that recognized alpha smooth muscle actin (*ACTA2*) (Fig. 7). Most RALDH2-positive cells also expressed *ACTA2* (arrows), but some RALDH2-positive cells were negative for *ACTA2* (arrowheads). Some *ACTA2*-containing cells in the choroid stroma and all those associated with BVs were negative for RALDH2 (asterisks). Figures 7D to 7F show higher magnification of an additional confocal slice (0.4 μ m), demonstrating choroid stromal cells coexpressing RALDH2 and *ACTA2* (arrows) and cells that expressed only *ACTA2* (asterisks).

Quantification of Choroidal Retinoids

To determine whether increased expression of RALDH2 in recovering choroids resulted in increased retinoic acid synthesis, atRA was measured in organ cultures of choroids during the period in which RALDH2 expression was increasing, using an ultrasensitive procedure making use of LC/MS/MS quantification (Fig. 8A). Choroids (8-mm punches) isolated from control and treated eyes after recovery periods ranging from 3 hours to 15 days were incubated in N2 culture medium (80 μ L) for 3 hours, and total atRA was measured in medium together with

the choroid punch. Control samples contained on average 0.33 to 0.60 pmol atRA, corresponding to concentrations of approximately 4×10^{-9} to 7×10^{-9} M in organ cultures. After 6 hours of recovery, atRA concentrations began to increase in treated eyes. The large SE is due to one choroid from a recovering eye that synthesized nearly fourfold more RA than the other samples. RA concentration was significantly higher in cultures of choroids from 24-hour recovering eyes than in paired controls ($\sim 195\%$; $P < 0.01$, paired *t*-test) and remained significantly elevated throughout the 15-day recovery period (averaging 0.97–1.16 pmol/punch + medium). To determine the amount of atRA synthesized in the 3-hour culture period and how much represented endogenous retinoic acid within the choroids at the time of tissue isolation, control and treated choroids, isolated after 24 hours and 10 days of recovery, were incubated in the presence of disulfiram (100 μ M), a compound that blocks the conversion of retinaldehyde to atRA by inhibiting aldehyde dehydrogenase.²⁸ No significant differences in atRA were detected between control and recovering eyes after incubation with disulfiram at the 24-hour or 10-day time points. Furthermore, no significant differences were detected in disulfiram-treated cultures and any of the six control groups (3-hour–15-day) ($P > 0.05$, Scheffé comparison and Bonferroni correction). These results suggest that endogenous levels of atRA are similar in the choroids of control and treated eyes (mean, 0.52 ± 0.06 and 0.69 ± 0.05 pmol, respectively), that control choroids synthesize little to no atRA in vitro, and that the increased retinoic acid measured in recovering samples was largely due to increased retinoic acid synthesis.

We also measured retinyl ester (RE) and retinol in all but the 15-day samples (Figs. 8B, 8C). The levels of RE, a storage form of retinoids, did not vary significantly between choroids of control and treated eyes in a 7-day period (Fig. 8B). In contrast, retinol concentration in organ cultures varied significantly between control and treated eyes. Retinol was significantly lower in cultures of treated eyes after 3 and 6 hours of recovery compared with paired contralateral controls and then subsequently increased to levels significantly higher than those of

FIGURE 4. Confocal images of RALDH2 protein (green) in choroids of control and recovering chick eyes after immunolabeling with anti-RALDH2 antibodies. (A) Montage of five images of choroids from control eyes showing minimal immunolabeling. (B) Western blot of choroid extracts from control and recovering eyes (24 hours) immunoblotted with anti-RALDH2. (C) Montage of six images of choroids from a recovering eye (24-hour recovery) demonstrating robust labeling for RALDH2 in cells within choroidal stroma. (D, E) Higher magnification of ocular tissue sections labeled with anti-RALDH2. (F) Negative control section of a recovering eye (24 hours) in which non-immune rabbit IgG was used in the first incubation, followed by incubation in Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin. Scale bars: (A, C) 40 μm; (D-F) 30 μm. Nuclei were counterstained with DAPI (blue).

control eyes after 24 hours and 4 days of recovery. These relative changes may be a reflection of changes in choroidal vascular permeability and extravasation of serum (and serum retinol) into the choroidal stroma and culture medium.^{13,29} Based on our previous estimation of the amount of serum extravasation by isolated choroids into culture medium (~12.5% serum in cultures of 5-day recovering choroids) and the concentration of serum retinol previously reported (1.97 ± 0.15 μg/mL in 1–13-day-old posthatch chicks),³⁰ we would predict retinol concentrations in the range reported in the present study (0.04–0.08 nmol/culture).

Effect of Retinoic Acid on Scleral Proteoglycan Synthesis

To determine whether choroidal synthesis of atRA could mediate changes in scleral proteoglycan synthesis, we examined the effect of atRA on ³⁵SO₄-glycosaminoglycan synthesis in vitro (Fig. 9). After incubation with retinoic acid (10^{-10} – 10^{-5} M), scleral proteoglycan synthesis was significantly inhibited in a dose-dependent manner. Based on the dose-response curve, the IC₅₀ for retinoic acid was 8×10^{-9} M. To determine whether choroid-derived atRA could similarly inhibit scleral proteoglycan synthesis, scleral punches (5 mm) were incubated with CCM, obtained under the exact conditions used for choroid samples for retinoid quantification (Fig. 10A). Scleral proteoglycan synthesis was significantly inhibited by CCM from control and recovering choroids compared with scleral samples incubated with N2 medium alone

($P < 0.001$, ANOVA with Bonferroni correction). Moreover, CCM from recovering choroids was significantly more inhibitory than CCM from control choroids ($P < 0.02$, paired *t*-test $n = 16$). Based on comparisons with our dose-response curve calculated for retinoic acid on scleral proteoglycan inhibition, the amount of inhibition by CCM corresponded to those for predicted atRA concentrations of 2.57×10^{-7} M to 2.34×10^{-5} M in control (C) and recovering (R) cultures, respectively, compared with sclera treated with N2 medium alone (N2) (Fig. 10B). When scleral proteoglycan inhibition was compared in cultures treated with CCM from recovering choroids relative to that of control CCM (R%C), scleral synthesis was inhibited to 43% of controls, corresponding to an atRA concentration of 7.20×10^{-8} M, a value within the range of the measured atRA synthesized by recovering choroids in organ cultures (4×10^{-7} M to 3×10^{-8} M).

DISCUSSION

Visual Regulation of Intraocular Retinoic Acid Synthesis

Previous work has demonstrated that in both chicks and primates, the choroid synthesizes atRA, and the rate of atRA synthesis is modulated by the refractive state.^{14,15} It has therefore been suggested that atRA could act as a locally produced (within the eye) scleral growth modulator during visually guided ocular growth. However, neither the source of the atRA

FIGURE 5. *ACTA2* expression in chick choroids. (A) Recovering eye (11-hour recovery). *ACTA2* mRNA was detected in cells located in the stroma between BVs (arrows) and in lining BVs. (B) Contralateral control eye demonstrating *ACTA2*-expressing cells in the choroid stroma (arrows). (C) Negative control using sense *ACTA2* cRNA probe to label choroid from an 11-hour recovering eye. (D) Negative control using sense *ACTA2* cRNA probe to label choroid from the contralateral control eye. Scale bar: (A–D) 100 μ m.

in the choroid nor the molecular events that control the tissue concentrations of atRA in the choroid have been established. Our semiquantitative qRT-PCR results indicate that of the three *RALDH* genes, only *RALDH2* mRNA pools are modulated during recovery from induced myopia. *RALDH2* mRNA pools are

significantly increased after 12 hours of unrestricted vision (recovery) compared with contralateral control eyes, are maximally increased by 24 hours of recovery, and return to levels close to those of controls within 4 days of recovery. These results contradict the PCR results of Bitzer et al.,³¹; in their

FIGURE 6. Dual labeling for *RALDH2* and *ACTA2* using anti-*RALDH2* antibodies and antisense RNA probes for *ACTA2*. (A, D) *RALDH2* protein (green) was detected in extravascular stromal cells in control (C) and recovering (R) choroids. (B, E) *ACTA2* mRNA (red) was detected in choroidal stromal cells that also expressed *RALDH2* (arrows) and in cells lining BVs that did not express *RALDH2* (asterisk). (C, F) Merged images indicating colocalization of *RALDH2* and *ACTA2* (arrows), *RALDH2*-expressing cells that did not express *ACTA2* (arrowheads), and *ACTA2*-expressing cells that did not express *RALDH2* protein (asterisk). (G, I) Minimal immunolabeling and probe hybridization were detected when sections were labeled with nonimmune IgG and sense *ACTA2* RNA probes. Nuclei were counterstained with DAPI (blue).

FIGURE 7. Confocal image of RALDH2 and ACTA2 expressing cells in chick choroid after immunolabeling with anti-RALDH2 and anti-ACTA2 antibodies. (A–C) One confocal slice (0.4 μm) of a recovering choroid fixed and labeled for RALDH2 (green), ACTA2 (red), and DAPI (blue) and after merging of the three channels (merge). Arrows: RALDH2-positive cells that coexpressed ACTA2. Solid arrowheads: RALDH2-expressing cell without ACTA2 expression. (D–F) Higher magnification of an additional confocal slice (0.4 μm) demonstrating choroid stromal cells coexpressing RALDH2 and ACTA2 (arrows) and cells that only expressed ACTA2 (asterisks). No immunolabeling was detected in negative controls in which nonimmune rabbit IgG was substituted for primary antibodies (data not shown). N, nerve.

study, *RALDH2* was shown to be expressed at very low levels in the choroid but to be relatively strongly expressed in the retina. Furthermore, they detected expression of *RALDH1* in the retina and choroid, with significant upregulation of retinal *RALDH1* mRNA in response to minus lens treatment. The underlying reasons for these discrepancies in these studies are unclear. No significant changes were observed in mRNA pools for the other retinoic acid-synthesizing enzymes (*RALDH3*, *CYP 1B1*, *RDH10*) or the retinoic acid-degrading enzyme (*CYP26*) in control and treated choroids.

The results of the present study indicate that endogenous concentrations of atRA are similar in the choroids of control and treated eyes ($\sim 6 \times 10^{-9}$ M), and the increased atRA concentration observed in the conditioned medium of recovering choroids is the result of increased atRA synthesis in vitro. These findings extend those of Mertz and Wallman¹⁴ and

indicate that the increased ^3H -atRA synthesis by recovering choroids they originally reported resulted in increased concentrations of secreted atRA. The increase in *RALDH2* mRNA pools in recovering choroids corresponded temporally to the increased synthesis of atRA by isolated choroids of recovering eyes. Moreover, in vitro assays indicated that the recovery-induced increase in atRA synthesis was inhibited by the aldehyde dehydrogenase inhibitor disulfiram, consistent with the singular role of RALDH2 in mediating choroidal synthesis of atRA during recovery. Although *RALDH2* mRNA returned to control levels by 14 days, RA synthesis in vitro remained elevated. This suggests that RALDH2 protein concentration or enzyme activity remains elevated after the return of *RALDH2* mRNA pools to control levels. It is also possible that an aldehyde dehydrogenase other than RALDH2 is responsible for atRA synthesis in the

FIGURE 8. Quantification of atRA, RE, and retinol in chick choroids. Chick choroids (8-mm punches) from control and recovering eyes were cultured for 3 hours in N2 medium at 37°C. atRA (A), RE (B), and retinol (C) were quantified in tissue punches, together with culture medium by LC/MS/MS. Disulfiram (DS, 100 μ M) was added to cultures of control and recovering choroids before incubation and subsequent atRA quantification. Ordinates on the x-axis represent hours (hrs) and days of unrestricted vision before choroid isolation. Values represent mean $\pm$ SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001 for treated eyes compared with paired contralateral controls (Student's paired t -test). Numbers above each set of data points indicate the number of chicks used for each group.

choroid; however, no changes were detected in mRNA pools for any of the known atRA-synthesizing enzymes over an extended period of recovery.

Within the retina/RPE, *RALDH1*, *RALDH2*, and *RALDH3* mRNA pools appeared to be modulated in the retina/RPE of form-deprived and recovering eyes; *RALDH2* was significantly higher in the retina/RPE of form-deprived eyes than in contralateral controls, and expression declined to levels significantly below contralateral control eyes after 12 hours of recovery. The modulation of *RALDH2* within the retina/RPE corresponds to previously reported changes in atRA concentration in chick retinas from form-deprived chick eyes,¹⁷ suggesting that in addition to the choroid, *RALDH2* may modulate atRA synthesis in the chick retina/RPE during visually guided ocular growth. We suspect that *RALDH2* mRNA and protein might have been expressed by the chick RPE but was undetectable in the present study because of the interference of RPE-associated pigment with our fluorescent and chromogenic detection methods. Additional studies are in progress to determine the role of the RPE in *RALDH2*-mediated atRA synthesis.

RALDH2 Expression by Choroid Extravascular Smooth Muscle Cells

The results of the present study indicate that most *RALDH2*-positive cells in the choroid coexpress ACTA2. Within the choroid, ACTA2-positive cells include "typical" smooth muscle cells that are organized as bundles of interconnected cells running along the longitudinal axes of BVs and myofibroblasts that appear as isolated spindle- to star-shaped cells located in the suprachoroid.³²⁻³⁵ *RALDH2*/ACTA2 cells appeared as isolated ovoid to spindle-shaped cells located in the stroma between larger blood and lymphatic vessels and as cells immediately adjacent to the sclera. Based on the appearance and distribution of *RALDH2*-containing cells, we suspect that retinoic acid is synthesized by a subpopulation of choroidal myofibroblasts located in the choroid stroma and in the lamina (membrana) fusca, a thin layer of pigmented connective tissue on the inner surface of the sclera, connecting it with the choroid. Given that ACTA2 mRNA and protein were not detected in some *RALDH2*-positive cells, it is possible that another population of unidentified choroidal cells also expressed *RALDH2*. Our in situ hybridization and immunolocalization results indicate that atRA is synthesized by ACTA2-containing

FIGURE 9. Effect of atRA on scleral proteoglycan synthesis in vitro. Scleral punches were isolated from untreated eyes and incubated in atRA (10^{-10} M to 10^{-5} M) for 24 hours. After incubation with retinoic acid, proteoglycan synthesis was estimated by the amount of $^{35}\text{SO}_4$ incorporation into glycosaminoglycans. Each data point represents the mean percentage of incorporation ($\pm$ SEM) relative to that of untreated sclera, incubated in the absence of retinoic acid. Numbers above each set of data points indicate the number of scleral samples used for each group.

FIGURE 10. Effect of CCM on scleral proteoglycan synthesis. (A) Choroids were isolated from control and recovering (24-hour) eyes and were cultured for 3 hours in N2 medium. Scleral punches were isolated from untreated eyes and incubated for 24 hours in CCM from control choroids, recovering choroids, and N2 medium alone (untreated) for 24 hours. After incubation with conditioned medium, proteoglycan synthesis was estimated by the amount of $^{35}\text{SO}_4$ incorporation into glycosaminoglycans. $***P < 0.001$, ANOVA; $*P < 0.05$, Student's paired *t*-test. (B) The percentage of proteoglycan inhibition by CCM relative to that N2 medium, calculated from data in Figure 10A, was superimposed on the dose-response curve established for retinoic acid on scleral proteoglycan synthesis (Fig. 9). N2, N2 medium only; C, percentage of inhibition ($\pm$ SEM) by CCM from control choroids relative to that of untreated sclera (N2); R, percentage of inhibition ($\pm$ SEM) by CCM from recovering eyes relative to that of untreated sclera (N2); R%C, percentage of inhibition ($\pm$ SEM) of CCM from recovering eyes relative to that of conditioned medium from control eyes. Numbers in parentheses indicate the number of chicks used for each group.

cells located in the choroid stroma, some of which are closely associated with the sclera. This arrangement may provide extravascular channels that facilitate the delivery of atRA directly to the sclera, bypassing the vascular and lymphatic circulation.

Although there are few reports of retinoic acid synthesis by myofibroblasts in other systems, retinoid autofluorescence and retinoid metabolism-related gene expression have been described in the myofibroblast-like hepatic stellate (Ito) cells of the liver.^{36,37} Our results on the localization of RALDH2 in the chick choroid differ from those of Fischer et al.,³⁸ in which RALDH-positive cells were identified in the choroid stroma and walls of choroidal blood vessels and in the inner nuclear layer, ganglion layer, inner plexiform layer, and photoreceptor ellipsoids of the retina. The anti-RALDH2 antibody used in the present study did not label cells associated with choroidal blood vessel walls, nor did it label any cells in the neural retina. This distribution of RALDH2-expressing cells in the chick choroid was confirmed in the present study by *in situ* hybridization using a specific antisense *RALDH2* cRNA probe, providing

additional confidence for the specificity of the anti-RALDH2 antibody used in this study. Therefore, we speculate that the differences between our results and those of Fischer et al.³⁸ may be attributed to differences in antibody specificity to the three closely related RALDH isoforms.

Retinoic Acid on Scleral Proteoglycan Synthesis

Of the three possible tissue targets for atRA within the eye (retina, choroid, sclera), the sclera is a leading candidate. We have previously shown that the rate of scleral proteoglycan synthesis in the cartilaginous layer of the chick eye is largely responsible for visually guided changes in eye size^{9,39}; proteoglycan synthesis is rapidly upregulated in the sclera during the development of myopia and is rapidly downregulated in the posterior sclera to levels significantly below those of fellow controls within 12 hours on recovery from induced myopia.^{13,40} Interestingly, *RALDH2* mRNA pools in the chick choroid increase and decrease in a manner that inversely correlates with changes in scleral proteoglycan synthesis in recovering eyes. It is well known that atRA is a potent inhibitor of proteoglycan biosynthesis by chondrocytes^{41,42} and that it facilitates cartilage catabolism through the increased synthesis of matrix-degrading enzymes.^{43–45} The results of the present study show that atRA inhibits scleral proteoglycan synthesis in a dose-dependent manner with an IC_{50} of 8×10^{-9} M. Although this IC_{50} is a log unit smaller than that previously reported by Mertz and Wallman¹⁴ ($\text{IC}_{50} = 8 \times 10^{-8}$ M), our calculated IC_{50} was similar to the IC_{50} reported for atRA on proteoglycan biosynthesis by articular cartilage explants (6×10^{-9} M)⁴¹ and is very close to the measured endogenous levels of atRA in choroid organ cultures in this study (4×10^{-9} to 7×10^{-9} M). At this concentration, atRA would be able to regulate scleral growth matrix remodeling through the stimulation or repression of transcription factors, extracellular matrix constituents, and MMPs or TIMPs.

We previously reported that recovery from induced myopia is characterized by a rapid decline in proteoglycan synthesis, which occurs within the first 12 hours of unrestricted vision and is followed by a slower, more gradual decline over the next 4 days¹³. *In vitro*, choroid explants or suprachoroidal fluid isolated from recovering eyes have been shown to inhibit sclera proteoglycan synthesis to a greater extent than do choroids or suprachoroidal fluid isolated from control eyes,^{13,29,46} suggesting that the scleral inhibition associated with recovery is regulated by choroidally derived factors. The scleral proteoglycan inhibition previously observed *in vitro* occurred with choroids isolated after 3 or more days of recovery, during the second, later phase of proteoglycan synthesis decline and is most likely related to the increased choroidal permeability and the subsequent increased release of inhibitory serum proteins into the culture medium. However, lower levels of these serum proteins are present in the culture supernatants of all choroids, regardless of the treatment,¹³ and we suspect that these serum proteins in CCM are responsible for the greater inhibition in proteoglycan synthesis by CCM than predicted by measurements of atRA concentrations alone. Results of the present study show choroidal synthesis of atRA *in vitro* is increased within 6 hours of recovery to levels that would be expected to inhibit scleral proteoglycan synthesis during the rapid phase of decline in proteoglycan synthesis. It should be noted that significant scleral proteoglycan inhibition was observed only when sclera were incubated overnight with CCM; no significant inhibition was detected using direct coculture of sclera with choroids isolated during the rapid phase of recovery.¹³ We speculate that longer periods of choroid organ culture (>3 hours) result in the accumulation of retinoid binding proteins or atRA-degrading enzymes in the medium that reduce the inhibitory action of atRA on scleral proteoglycan synthesis.

Role of Retinoic Acid in Postnatal Ocular Growth

Interpretation of studies on chicks and mammals examining the role of atRA on postnatal ocular growth are complicated by species differences in the mechanisms by which scleral remodeling and ocular length are modulated by visual stimuli and by the multiple targets and widespread effects of retinoic acid within the eye. In mammals with a fibrous sclera, ocular elongation is the consequence of decreased sulfated glycosaminoglycan synthesis, scleral thinning, and increased scleral distensibility.^{15,47,48} In chicks, ocular elongation is the result of growth of the cartilaginous sclera, with increases in sulfated glycosaminoglycan synthesis, protein synthesis, and total scleral mass.^{9–12} In chicks, increased synthesis of atRA by the choroid during recovery from induced myopia may act to inhibit proteoglycan synthesis within the cartilaginous sclera, slowing the rate of ocular elongation. In guinea pigs¹⁶ and primates,¹⁵ choroidal atRA synthesis is increased during the development of myopia, a condition that is also associated with decreased scleral proteoglycan synthesis but that, in contrast to chicks, results in increased axial elongation. Given the known inhibitory effect of atRA on scleral proteoglycan synthesis in both chicks and primates,^{14,15} choroid changes in atRA are consistent with changes scleral proteoglycan synthesis in both species and suggest that the inhibition of scleral proteoglycan synthesis by choroidally derived atRA represents a mechanism to regulate ocular length and refraction common to multiple species.

Retina-derived atRA is not likely to act directly on the sclera, at least in chicks, because retinal synthesis of atRA is increased during the development of myopia, yet scleral proteoglycan synthesis is increased, rather than decreased as would be expected if atRA acted to inhibit scleral proteoglycan synthesis. Retina-derived atRA may act upstream of choroid-derived atRA in the signaling cascade controlling eye size to regulate gene expression in the retina, RPE, or choroid or it may represent an event independent of scleral growth regulation, as has been demonstrated for atRA synthesis by RPE in response to light.⁴⁹

Because atRA is likely to affect the cellular functions of other ocular tissues in addition to the sclera, interpretation of experiments in which atRA agonists and atRA synthesis inhibitors are delivered either systemically or intraocularly is also complicated. Ocular elongation is increased after dietary administration of atRA to chicks and is decreased after administration of citral, an inhibitor of atRA synthesis.⁵⁰ Similarly, intraocular administration of the atRA synthesis inhibitor disulfiram has been shown to inhibit the development of form-deprivation myopia,³¹ a result generally opposite what would be predicated if atRA acted to inhibit ocular elongation in chicks. It is likely that untargeted administration of atRA or atRA synthesis inhibitors leads to multicellular effects that may differ from those mediated by endogenous atRA. We suspect that within the choroid, atRA concentrations are tightly regulated both spatially and temporally to generate localized effects on scleral proteoglycan synthesis and ocular size.

The results of the present study indicate that the regulation of choroid concentrations of atRA is likely to involve changes in *RALDH2* transcription and enzyme activity in extravascular α SMA-containing cells. Accordingly, these results identify potential cellular and molecular targets for the regulation of choroidal retinoic acid synthesis and ocular growth control. Furthermore, the identification of the trans-acting elements involved in the regulation of *RALDH2* gene transcription by this subpopulation of choroid stromal cells is likely to elucidate another step upstream in the signaling cascade controlling visually guided eye growth.

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