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Improvement in Rod and Cone Function in Mouse Model of *Fundus albipunctatus* after Pharmacologic Treatment with 9-*cis*-Retinal

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Abstract

Purpose—To assess changes in rod and cone visual functions in a mouse model of *Fundus albipunctatus* with disrupted 11-*cis*-retinol dehydrogenase (*RDH*) genes after pharmacologic treatment with an artificial retinal chromophore.

Methods—Retinoid levels and photoreceptor functions of *Rdh5*^{−/−}*Rdh11*^{−/−} mice at a variety of light intensities were analyzed with normal-phase HPLC and ERG techniques. Production of 11-*cis*-retinal, the visual pigment chromophore, was suppressed with a potent inhibitor of the retinoid cycle, all-*trans*-retinylamine (Ret-NH₂). The chromophore was replaced by a functional geometric isomer, 9-*cis*-retinal, delivered by oral gavage.

Results—Aberrant cone responses were detected in 12-month-old *Rdh5*^{−/−}*Rdh11*^{−/−} mice raised in a 12-hour light/12-hour dark cycle. This cone defect was exacerbated in conditions of low levels of 11-*cis*-retinal. Administration of 9-*cis*-retinal increased the rate of dark adaptation and improved cone function in *Rdh5*^{−/−}*Rdh11*^{−/−} mice.

Conclusions—Disruption of 11-*cis*-RDHs causes a slowly developing cone dystrophy caused by inefficient cone pigment regeneration. Rod and cone visual function improved significantly in the mouse model of *F. albipunctatus* after treatment with 9-*cis*-retinal, suggesting a potential approach to slow the progression of cone dystrophy in affected humans.

The final oxidation step in a metabolic pathway, called the retinoid (visual) cycle, is catalyzed by 11-*cis*-retinol dehydrogenases (RDHs).^{1,2} This cycle of reactions is responsible for the production of 11-*cis*-retinal, a chromophore of visual pigments.^{3,4} Disruption of the main RDH enzyme in humans, RDH5, causes *Fundus albipunctatus*.⁵ Initially, it was thought that *F. albipunctatus* was an autosomal recessive form of congenital stationary night blindness characterized by the appearance of numerous small white dots in the retinal pigment epithelium (RPE) and by delays in dark adaptation.⁶ However, more recently it was demonstrated that progressive cone dystrophy developed in some patients with *F. albipunctatus*.^{7–9} In mice, RDH5 is responsible for most 11-*cis*-RDH activity, but a second RDH—RDH11—plays a complementary role.¹⁰ The phenotype of RDH5 knockout in mice is milder than it is in humans; thus, human conditions are mimicked more closely in *Rdh5*^{−/−}*Rdh11*^{−/−} mice, and these mice were chosen for our study.¹¹ These mouse models, with disrupted genes encoding 11-*cis*-RDH, showed delayed dark adaptation with slow 11-*cis*-retinal regeneration but lacked the retinal structural abnormalities that are typically observed in human diseases with mutation

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in the *RDH5* gene.^{10,12,13} Interestingly, an accumulation of *cis*-retinyl esters, mainly 13-*cis*-retinyl ester, was detected in mice with disrupted 11-*cis*-RDH genes,^{12,13} and this accumulation can be related to the pathology of *F. albipunctatus*.¹¹

Mutations in RPE65 and lecithin/retinol acyltransferase (LRAT) cause severe blindness from birth or early childhood^{14–16} because RPE65 and LRAT are essential for the production of chromophore in the RPE.^{17,18} Retinal degeneration was observed when visual pigments were not formed,^{19,20} but cone cells were more significantly affected, as demonstrated in an RPE65-deficient mouse model.^{21,22} The main causes of the retinal abnormalities were associated with a lack of 11-*cis*-retinal production because of the absence of RPE65 or LRAT enzymes. Treatment with 9-*cis*-retinoids successfully rescued visual function and structure in RPE65- and LRAT-deficient mice.^{23–26}

In this study, we examined the pharmacologic approach with 9-*cis*-retinal to rescue cone function in mice with the combined deletions of RDH5 and RDH11 (*Rdh5*^{−/−}*Rdh11*^{−/−} [11-*cis*-RDHs]). Electrophysiologic and biochemical measurements demonstrate that the deletion of 11-*cis*-RDH results in delayed dark adaptation at an early age and cone dystrophy after 12 months. Delayed dark adaptation and cone functional abnormalities were improved substantially with oral 9-*cis*-retinal treatment. This study suggests a potential treatment of *F. albipunctatus* to prevent cone dystrophy.

Materials and Methods

Animals

All animal experiments were conducted in accordance with procedures approved by the Case Western Reserve University Animal Care Committee and conformed to the recommendations of the American Veterinary Medical Association Panel on Euthanasia and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were maintained in complete darkness or in a 12-hour light/ 12-hour dark cycle. All manipulations were performed under filtered dim red light (no. 1 safelight filter; Eastman Kodak, Rochester, NY). Transmittance was greater than 560 nm.

Rdh11^{−/−} mice were generated as described previously.¹⁰ Genotyping of these mice was carried out by PCR using primers O9 (5'-CTATGGCGTGCATGTGGAAGT-3') and O10 (5'-TCTCCTTCCCAATGCCTGTG-3') for wild type (WT; 389 bp) and O9 and O11 (5'-GCTAAAGCGCATGCTCCAGA-3') for targeted deletion (300 bp). PCR product with primers O9 and O11 was cloned and sequenced to verify the proper targeting of *Rdh11*.

Rdh5^{−/−} mice were previously generated and characterized by Carola A. Driessen and Jacques J. Janssen.^{12,13} Genotypes of *Rdh5*^{−/−} mice were determined by PCR using primers KORDH-s1 (5'-GGGCAGCTGCAGTCTGCACCATC-3') and KORDH-a1 (5'-GGGCAAGACCTGACCTGGGGGC-3'), which produce a 2.9-kb fragment for the WT allele and a 1.5-kb fragment for the disrupted allele. *Rdh5*^{−/−}*Rdh11*^{−/−} mice were generated by cross-breeding *Rdh5*^{−/−} and *Rdh11*^{−/−} mice.

Analyses of Retinoids

All experimental procedures related to the extraction, derivatization, and separation of retinoids from dissected mouse eyes were carried out as described previously.^{25–28} Briefly, the tissues were treated with NH₂OH, and retinoids were extracted with hexane and analyzed on a normal-phase HPLC column. All reactions involving retinoids were carried out under dim red light. Retinoids were separated by normal-phase HPLC (4.6 μm × 250 mm; Ultrasphere-Si; Beckman, Fullerton, CA) with a mobile phase consisting of 10% ethyl acetate and 90% hexane at a flow rate of 1.4 mL/min and detection at 325 nm (HP1100 HPLC; Hewlett-Packard, Palo

Alto, CA) with a diode array detector and appropriate software (HP Chemstation A.03.03; Hewlett-Packard).

Electroretinograms

Before recordings, mice were dark adapted for more than 24 hours. Under safety light, mice were anesthetized by intraperitoneal injection using 20 μ L/g body weight of 6 mg/mL ketamine and 0.44 mg/mL xylazine in 10 mM sodium phosphate, pH 7.2, containing 100 mM NaCl. Pupils were dilated with 1% tropicamide. A contact lens electrode was placed on the eye, and a reference electrode and a ground electrode were placed in the ear and on the tail, respectively. ERGs were recorded with a universal testing and electrophysiologic system (UTAS E-3000; LKC Technologies, Inc., Gaithersburg, MD). Light intensity was calibrated by the manufacturer of the ERG and was computer controlled. Mice were placed in a Ganzfeld chamber, and scotopic and photopic responses to flash stimuli were obtained from both eyes simultaneously. Single-flash recording, flicker ERG, and recording after intense constant illumination were performed using methods described previously.¹⁰

Retinoid, 9-*cis*-Retinal, and Ret-NH₂ Gavage

9-*cis*-Retinal was purchased (Sigma, St. Louis, MO), and retinylamine^{29,30} Oral (Ret-NH₂) was synthesized by the method recently reported. Gavage was carried out as described previously.²⁵ Briefly, retinoids were dissolved in vegetable oil, and less than 180 μ L solution was delivered per each animal through a gavage needle.

Results

Retinoid Analysis

Patients with *F. albipunctatus* show delayed dark adaptation as a consequence of slower 11-*cis*-retinal regeneration.⁶ To investigate the retinoid levels in 11-*cis*-RDH (RDH5 and RDH11) double-knockout mice, HPLC analysis of the eyes from *Rdh5*^{-/-}*Rdh11*^{-/-} and WT mice was performed 48 hours after dark adaptation and 15 minutes after probe flash. In dark-adapted conditions, compared with WT mice, *Rdh5*^{-/-}*Rdh11*^{-/-} mice had elevated levels of 11- and 13-*cis*-retinyl esters (mainly 13-*cis*-retinyl ester) that further increased 15 minutes after a single light flash that bleached approximately 35% of rhodopsin, in agreement with previously published data^{10,12,13} (and data not shown). After the first 15 minutes of dark adaptation, the 11-*cis*-retinal chromophore regeneration in double-knockout mice was similar to the level found in WT mice. To examine retinoid composition under stronger light stress, approximately 98% of rhodopsin was bleached by 500 cd/m² for 48 minutes, and the amount of recovered 11-*cis*-retinal was determined. Regeneration kinetics of WT mice showed a typical logarithmic curve and reached 50% of the total regeneration at approximately 3 hours, whereas double-knockout mice displayed a sigmoidal regeneration pattern with 50% of the total regeneration occurring 6 hours after bleaching. Twenty-four hours of dark adaptation was sufficient for the complete 11-*cis*-retinal regeneration in mice of both genetic backgrounds (Fig. 1). Thus, under intense bleaching, delayed recovery of 11-*cis*-retinal was more profound. This observation is reminiscent of the mild delayed dark adaptation kinetics observed on ERG in patients with *F. albipunctatus*.⁶

Clearance of the accumulated 13-*cis*-retinyl ester and 11-*cis*-retinal regeneration was also examined when approximately 98% of rhodopsin was bleached by intense light. Immediately after light exposure, similar amounts of all-*trans*-retinal and all-*trans*-retinyl esters were detected in WT and *Rdh5*^{-/-}*Rdh11*^{-/-} mice (data not shown). One day after exposure to light, the amounts of 11-*cis*-retinal and all-*trans*-retinyl esters returned to normal levels and were the same as in dark-adapted *Rdh5*^{-/-}*Rdh11*^{-/-} mice not exposed to light. However, 13-*cis*-retinyl ester was elevated in *Rdh5*^{-/-}*Rdh11*^{-/-} mice and did not show any significant reduction

compared with those measured immediately after the intense bleach (Fig. 2A). No decrease in 13-*cis*-retinyl esters was observed even 7 days after illumination. To determine whether 13-*cis*-retinyl ester elevation occurred, *Rdh5*^{-/-}*Rdh11*^{-/-} mice dark adapted for 24 hours after exposure to the first 48 minutes of illumination were subjected to a second exposure of 500 cd/m⁻² for 48 minutes. Unexpectedly, no further accumulation of 13-*cis*-retinyl ester was detected when compared with that of the singly bleached *Rdh5*^{-/-}*Rdh11*^{-/-} mice. 13-*cis*-Retinyl ester levels maintained the same level as before the second exposure to the intense light, suggesting that there can be a limit to the storage of 13-*cis*-retinyl ester in the RPE (Fig. 2B).

ERG Analysis of Photoreceptor Function

To evaluate rod and cone photoreceptor function in *Rdh5*^{-/-}*Rdh11*^{-/-} mice, single-flash ERG recordings were performed under scotopic and photopic conditions. *Rdh5*^{-/-}*Rdh11*^{-/-} mice raised in a 12-hour light/12-hour dark cycle or raised in complete darkness showed similar a- and b- wave amplitudes compared with WT mice kept in a 12-hour light/12-hour dark cycle under scotopic conditions (Figs. 3A, 3B). Rod photoreceptor function did not appear to be affected by genetic background or light conditions. When photopic ERG representing cone function was recorded, *Rdh5*^{-/-}*Rdh11*^{-/-} mice raised in the dark did not display significant differences compared with WT mice kept in a 12-hour light/12-hour dark cycle, whereas *Rdh5*^{-/-}*Rdh11*^{-/-} mice from a 12-hour light/12-hour dark cycle showed slightly lower a- and b- wave amplitudes under high-intensity light (Figs. 3C, 3D). To assess cone function by other tests, flicker ERG examination was performed with these mice. As shown in Figure 4, a lower amplitude of flicker ERG was observed in *Rdh5*^{-/-}*Rdh11*^{-/-} mice raised in a 12-hour light/12-hour dark cycle. Significant aberrations were detected in photopic ERGs; the 30-Hz flicker ERG with high-intensity illumination (2.5 log cd · s · m⁻²; Fig. 4) suggested that cone abnormalities in *Rdh5*^{-/-}*Rdh11*^{-/-} mice were caused by light and developed slowly over the course of 12 months because no changes to single-flash or flicker ERG were detected in 6-month-old mice (data not shown).

Low Levels of Visual Chromophore Production and Photoreceptor Function

To investigate whether low levels of visual pigment were sufficient to cause cone dystrophy, mice underwent gavage with Ret-NH₂ every week for 1 month to induce low levels of 11-*cis*-retinal, the visual chromophore. ERG recordings from treated WT mice did not show attenuated a- and b-wave amplitudes in scotopic or photopic ERG (data not shown). In double-knockout mice, scotopic ERG was not affected, in contrast to what occurred in nontreated mice (Figs. 5A, 5B); however, photopic ERG recordings were reduced significantly in a- and b- wave amplitudes (Figs. 5C, 5D). When the chromophore in the form of 9-*cis*-retinal was supplied, Ret-NH₂ and 9-*cis*-retinal treated mice did not show cone dystrophy (Figs. 5C, 5D). These data suggest that the cone aberrations of *Rdh5*^{-/-}*Rdh11*^{-/-} mice were induced by the low level of the visual chromophore.

9-*cis*-Retinal Supplementation and Dark Adaptation in *Rdh5*^{-/-}*Rdh11*^{-/-} Mice

To improve the delayed dark adaptation, treatment with 9-*cis*-retinal by oral gavage was used. Three days after gavage, ERGs were recorded with an intense bleaching condition (500 cd/m⁻² for 3 minutes) before recovery of the a-wave amplitude was monitored. A-wave amplitudes in the recovery phase after the bleach for WT, *Rdh5*^{-/-}*Rdh11*^{-/-}, and 9-*cis*-retinal gavage *Rdh5*^{-/-}*Rdh11*^{-/-} mice are shown in Figure 6A. Recovery in a-wave amplitude was clearly improved in the 9-*cis*-retinal gavage *Rdh5*^{-/-}*Rdh11*^{-/-} mice ($P < 0.001$), though it was still suppressed compared with WT mice. To assess isorhodopsin generation in mice after gavage, retinoid analysis was performed. After 5 hours of dark adaptation following 20 minutes of intense light exposure, 9-*cis*-retinal was detected by HPLC separation in the organic solvent

extracts of the eye (Fig. 6B). These results suggest that 9-*cis*-retinal was incorporated into visual pigments during the course of the experiment.

Discussion

In the past, *F. albipunctatus* was thought to be a stationary disease. Recent reports, however, indicate that cone dystrophy gradually develops in some patients.^{7,8,31,32} A study of photoreceptor function in patients with *F. albipunctatus* conducted by Cideciyan et al.⁹ demonstrates that cone photoresponse is reduced to approximately 60% of the normal amplitudes, and Niwa et al.³¹ report that 38% of patients with *F. albipunctatus* have extensive cone dystrophy. In humans, *F. albipunctatus* is caused by a mutation in the *RDH5* gene, but symptoms in *Rdh5* knockout mice are not fully similar to those observed in humans. In the knockout mice, delayed dark adaptation kinetics are also milder than those observed in humans.^{10,12} With the use of *Rdh5*^{-/-}*Rdh11*^{-/-} mice and their further impairment of 11-*cis*-RDH activity, mild cone dystrophy was observed in 12-month-old mice.

The gradual development of cone dystrophy in patients with *F. albipunctatus* may result from their slower rate of regeneration of cone pigments because low levels of 11-*cis*-retinal induced by a potent inhibitor of the visual cycle, Ret-NH₂,^{29,30} in the 11-*cis*-RDH double-knockout mice produced NH₂, severe cone abnormalities, whereas simultaneous administration of the artificial chromophore, 9-*cis*-retinal, with Ret-NH₂ spared the cones. Rod photoreceptor function was not affected after the same treatment with Ret-NH₂. This observation also suggested that cone photoreceptors are more sensitive to a shortage of the visual chromophore than are rod photoreceptor cells. Cideciyan et al.⁹ report that, as a consequence of the *RDH5* null mutation, the rate of cone function recovery is slower than the rate of rod function recovery. Hence, less efficient regeneration of cone pigments can be one of the mechanisms for late-onset cone dystrophy in patients with *F. albipunctatus*.

In this study, significant differences between *Rdh5*^{-/-}*Rdh11*^{-/-} raised in the dark (low level of 13-*cis*-retinyl ester) and during the 12-hour light/12-hour dark cycle (high level of 13-*cis*-retinyl ester) point to the possibility of a potentially pathogenic role of accumulated 13-*cis*-retinyl esters in the RPE. 11-*cis*-RDH double-knockout mice showed accumulated 13-*cis*-retinyl esters after light bleach that were not detected in WT mice. Retinosomes are among the storage sites of all-*trans*-retinyl esters in the RPE,^{24,33} and 13-*cis*-retinyl esters are also found in these structures.¹¹ Even mice kept in a 12-hour light/12-hour dark cycle, where the light intensity was at normal room light level (less than 50 lux), exhibited high levels of 13-*cis*-retinyl ester accumulation. Surprisingly, the accumulated 13-*cis*-retinyl ester after light bleach remained in the RPE for more than 1 week under dark conditions. Based on these facts, the typical appearance of numerous small white dots located in the RPE of patients with *F. albipunctatus* might correspond to the accumulation of 13-*cis*-retinyl ester in the RPE of 11-*cis*-RDH double-knockout mice. Human retinoid sample analysis indicated that the amount of ester stored in human eyes might be greater than in the *Rdh5*^{-/-} mice and appear as the white dots in a patient's fundus (data not shown). Although the pathogenesis of cone degeneration by the accumulated lipid in the retinosome is unclear, it is suggested that lipid oxidation products in the RPE contribute to slow-onset retinal degeneration, especially cone photoreceptor degeneration.^{34,35} Therefore, reduction of 13-*cis*-retinyl ester in the RPE can help to prevent cone dystrophy.

In summary, 9-*cis*-retinal treatment has the potential to become a therapeutic approach to preserve cone function in patients with *F. albipunctatus*. Ret-NH₂ successfully inhibits the accumulation of 13-*cis*-retinyl ester,¹¹ and 9-*cis*-retinal could improve delayed dark adaptation and cone dystrophy resulting from the shortage of visual pigments.

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Figure 1.

Recovery of 11-*cis*-retinal from intense bleach in WT and *Rdh5*^{-/-}*Rdh11*^{-/-} mice. Mice were dark adapted for 48 hours, and rhodopsin was bleached greater than 98% with intense white light at 500 cd/m⁻² for 48 minutes. Bleach level was determined by retinoid analysis. Regeneration of 11-*cis*-retinal, corresponding to the recovery of rhodopsin, was measured during dark adaptation. Error bars, SEM ($n = 3$). The recovery of the chromophore was delayed in *Rdh5*^{-/-} *Rdh11*^{-/-} mice.

Figure 2.

Clearance of 13-*cis*-retinyl esters after intense bleach in WT and *Rdh5*^{-/-}*Rdh11*^{-/-} mice. Mice were dark adapted for 48 hours, and rhodopsin was bleached greater than 98% with intense white light at 500 cd/m⁻² for 48 minutes. Retinoid levels were monitored for 7 days after bleaching in *Rdh5*^{-/-}*Rdh11*^{-/-} mice. 13-*cis*-Retinyl esters persisted during this period in the knockout mice (A), whereas they were absent at any time point in WT mice (data not shown). Rhodopsin was bleached greater than 98% with intense white light at 500 cd/m⁻² for 48 minutes after 48 hours of dark adaptation, and retinoid levels were examined with HPLC methods (48 min L). Mice that underwent 48 minutes of illumination were dark adapted for 24 hours (24 h DA) and retinoid analysis or a second 48-minute illumination. Then retinoid levels of 2 times-48 minutes illuminated mice were analyzed directly after the exposure to light (48 min L × 2) (B). Error bars, SEM (*n* = 3). These results suggest that 13-*cis*-retinyl esters are elevated to a stable level and that multiple bleaching did not cause further accumulation.

Figure 3.

Rod and cone photoreceptor analysis by ERG. Twelve-month-old mice raised in the dark (D) or in 12-hour light/12-hour dark cycle (L/D) were dark adapted for 48 hours, and scotopic and photopic ERGs were recorded. Scotopic conditions are shown in (A) and (B), and photopic ERGs are indicated in (C) and (D). Error bars, SEM ($n = 3$). Significant changes were only observed in photopic conditions at high intensities.

Figure 4.

Cone functional analysis by flicker ERG. Twelve-month-old mice raised in complete darkness (D) or in a 12-hour light/12-hour dark cycle (L/D) were dark adapted for 48 hours, and flicker ERGs were recorded. Photopic 30-Hz flicker ERG with $2.5 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$ stimuli was performed. Error bars, SEM ($n = 3$). Significant changes in cone responses were observed in 12-hour light/12-hour dark (L/D) conditions.

Figure 5.

Effects of lower visual pigment levels on photoreceptor functions in *Rdh5^{-/-}Rdh11^{-/-}* mice. Mice were gavaged with Ret-NH₂ (1 mg) and/or 9-*cis*-retinal (2.5 mg) once a week for 1 month (gavage, four times) and were kept in 12-hour light/12-hour dark light conditions. Treated mice were dark adapted for 48 hours, and scotopic and photopic ERGs were recorded. Scotopic conditions are shown in (A) and (B), and photopic ERGs are shown in (C) and (D). Error bars, SEM ($n = 3$).

Figure 6.

Pharmacologic treatment of *Rdh5*^{-/-}*Rdh11*^{-/-} mice with 9-*cis*-retinal. A single dose (2.5 mg) of 9-*cis*-retinal was administered to *Rdh5*^{-/-}*Rdh11*^{-/-} mice by oral gavage 3 days before ERG recording. These mice were dark adapted for 3 days after gavage and then were bleached with intense constant illumination (500 cd/m²) for 3 minutes. Recovery of a-wave amplitudes was monitored with single-flash ERG (-0.2 cd · s · m⁻²) for 60 minutes, and recovery ratios were plotted. These conditions led to bleaching approximately 70% of rhodopsin, as determined by retinoid analysis. The ratio was significantly improved in 9-*cis*-retinal *Rdh5*^{-/-}*Rdh11*^{-/-} after gavage ($P < 0.001$) compared with nontreated mice. Error bars, SEM ($n = 3$) (A). A single dose (2.5 mg) of 9-*cis*-retinal was administered to *Rdh5*^{-/-}*Rdh11*^{-/-} mice by oral gavage, and intense light (500 cd/m²) was illuminated for 20 minutes at 2 hours after gavage. Retinoid analysis was performed following 5-hour dark adaptation after light exposure. Representative HPLC data from measurements of three mice are shown (B).