Vitamin A and Interstitial Retinol-Binding Protein in an Eye with Recessive Retinitis Pigmentosa

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The composition and amount of vitamin A stored in the retinal pigment epithelium and choroid (RPE-Ch) was evaluated in postmortem donor eyes from a patient with retinitis pigmentosa that was probably inherited by an autosomal recessive mode. Additionally, the soluble proteins in the neural retina and RPE-Ch cytosols and interphotoreceptor matrix were examined collectively for the presence of interstitial retinol-binding protein (IRBP). Although there was depletion of the amount of vitamin A stored in the RPE, this was commensurate with the histopathologic findings on the RPE extent and thickness. No evidence was found for an accumulation of free retinol. Nearly all of the vitamin A stored in the RPE was esterified. As in normal eyes, the retinyl esters consisted mainly of palmitate mixed with a small proportion of stearate. Eleven-cis retinyl esters were present, although their proportion was lower than that reported for normals. IRBP could not be detected in stained gels of the soluble proteins, or by autoradiography of these gels after treatment with 125I-concanavalin A. These findings suggest that depletion of stored vitamin A, accumulation of free retinol, or deficiency of 11-cis isomer are unlikely to be causative factors in the retinal degeneration examined here. Although the depletion of IRBP seen at this advanced stage might be secondary to the advanced loss of photoreceptors, the authors cannot rule out the possibility that a relative deficiency or abnormality in this protein at earlier disease stages may contribute to the pathogenesis of retinitis pigmentosa.

We present here some recent studies on vitamin A and its putative interphotoreceptor matrix transport protein (IRBP) in the eye from a donor with retinitis pigmentosa. Preliminary reports of some of this work have been published.

Materials and Methods

The morphologic and biochemical analyses described in this report were conducted on eyes from a 69-year-old white man with retinitis pigmentosa. His condition was probably inherited by an autosomal recessive mode because the family history revealed that the donor's maternal and paternal grandmothers were sisters. There was no history of retinitis pigmentosa in other members of the pedigree.

The patient was first examined in the Electroretinography Service of the Massachusetts Eye and Ear Infirmary at age 58. He reported that symptoms of night-blindness had developed at about 25 years of age, and at that time a diagnosis of retinitis pigmentosa was first established. Between the ages of 37 and 54, his visual acuity declined from 20/20 OD and 20/25 OS to 20/40 OU, and at age 47, classic ring scotomas were first noted. The patient was aware of loss of peripheral vision but denied any difficulty with hearing or history of polydactyly.

When first examined at the Massachusetts Eye and Ear Infirmary, his best corrected visual acuity was 20/50 OU. His refractive error was OD +6.00 -1.50 × 165° and OS +6.00 -1.50 × 176°. Kinetic visual field testing with a Goldman perimeter revealed a concentric constriction to the 10° isopter with a 1V-4e white test light OU, and a constriction to the 5° isopter with a 1-4e white test light. Large midperipheral ring scotomas were observed. The patient retained an inferior crescent of field, approximately 10° in diameter, both inferonasally and inferotemporally in each eye with a 1V-4e white test light. Slit-lamp examination revealed a slight nuclear sclerosis and small central posterior subcapsular cataracts OU. Intraocular tensions by applanation were normal. Fundus examination revealed slight waxy pallor of each disc, granularity of each macula, attenuation of retinal vessels, and moderately heavy intraretinal bone-spicule pigment distributed 360° around the midperiphery. Dark-adaptation testing with an 11° white test light, centrally fixated, revealed a threshold elevated 2.5 log units above normal after 45 min of dark-adaptation. Conventionally recorded, full-field electroretinograms to single flashes of white light under dark-adapted conditions and to 30 Hz white flicker were not detectable (ie, less than 5 μV).

Over the next 10 years, the patient’s visual acuity decreased to 20/70 in the right eye and remained at 20/40 to 20/50 in the left eye. His dark-adaptation threshold showed further elevation to 3.5 log units above normal in the right eye and 4.0 log units above normal in the left eye. Over this period his central visual field narrowed to the 8° isopter and the peripheral crescents of field, though still present, were diminished in extent as monitored with confrontation testing. Because he experienced difficulty with reading, his right cataract was removed without complication 3 months prior to his death. Postoperatively, his vision was reported to be 20/40 in that eye.

The donor died of liver failure due to metastatic carcinoma. During the 2 years preceding his death he was maintained on a chemotherapeutic regime that consisted of six series, lasting 5–8 weeks each, of weekly intravenous injections of 5-fluorouracil. During his final hospitalization his medications included indomethacin, oxazepam, cimetidine, prochlorperazine, furosemide, and oxycodon hydrochloride. Both eyes were enucleated approximately 45 min after death. The right eye, designated as Berman-Gund Laboratory Specimen H-91, was immediately fixed in 1% formaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4 and was subsequently postfixed in 2% osmium tetroxide in the same buffer, dehydrated in graded ethanols, and embedded in Epon (Ladd Research Industries, Inc; Burlington, VT). All quadrants of the eye were systematically surveyed in semithin (1 μm-thick) sections stained with toluidine blue. Particular attention was paid to those areas that corresponded to the tissues of the companion eye that were used for the biochemical studies of this report. Thin sections from the fovea and from the midperipheral retina just superior to the temporal horizontal meridian were prepared and...
examined with a JEOL 100C electron microscope (Jeol; Tokyo, Japan).

The left eye was opened and dissected according to the plan presented in Figure 1. The tissues used for the present study included the retina, RPE, choroid and sclera of sector 1, and the RPE, choroid and sclera of sectors 2 and 3. These were frozen at $-80^\circ$C within an hour of enucleation and shipped on dry ice to the Cullen Eye Institute, where they were designated RP 179; the biochemical analyses described here were initiated within 12 hr of the donor's death.

Sector 1 consisted of retina, RPE-Ch, and sclera (scleral area, 0.86 cm$^2$). The sclera was removed and its area measured on a Zeiss Videoplan (Carl Zeiss, Inc; New York, NY). The remaining combined tissues were homogenized in 0.15 M NaCl, 0.01 M Na$_2$Si$_6$O$_5$ and Spherisorb CN in series 17; mobile phase, 0.4% diethyl phosphate (pH 7.4; PBS) and centrifuged for 1 hr at 100,000 $\times$ g. The supernatant, which contained retinal cytosol, interphotoreceptor matrix, and RPE-Ch cytosol, was treated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 5–20% gradient gels according to Liou et al. The binding of $^{125}$I-concanavalin A to these gels was carried out as described by Bridges and Fong.

For comparison, sectors of similar area and location were cut from a normal donor eye and treated identically. Sectors 2 and 3 consisted only of RPE-Ch and sclera (total sclera area, 3.0 cm$^2$). The RPE-Ch was peeled away from the sclera and the vitamin A extracted with acetone and quantitated by the Carr-Price reaction as described by Bridges et al. High-performance liquid chromatography (HPLC) of this extract was carried out using the equipment and procedures described by Bridges and Alvarez. Data from this analysis were compared with our published data for normal donor eyes. The quantities of 11-cis and all-trans esters were obtained by measuring the areas under the HPLC peaks (Fig. 2) and comparing them with those obtained from standard mixtures of authentic 11-cis and all-trans retinyl stearates and palmitates. Sectors 4 and 5 and the neural retina from sectors 2 and 3 are still being studied at the Berman-Gund Laboratory, with specific reference to a comparison of the in vitro cyclic nucleotide phosphodiesterase activities and the protein synthetic capacities of postmortem retinal tissues of normal donors and donors with retinitis pigmentosa.

**Results**

**Histopathology of the Right Eye**

The results of histologic examination of the right eye were consistent with the clinical diagnosis of advanced retinitis pigmentosa. The only substantive population of surviving photoreceptor cells was the foveal cones (Fig. 3A). No photoreceptor cells could be positively identified by morphologic criteria in the near and midperiphery (Fig. 3B, C), and in the far
In the periphery of the eye, the atrophic photoreceptor cells present were separated from each other by large gaps in the outer nuclear layer (Fig. 3D). At the center of the foveal pit, the outer nuclear layer was approximately two nuclei deep. The inner segments of these surviving photoreceptors were separated from each other by large gaps in the outer nuclear layer. Anterior to this region, widely scattered photoreceptor cells were completely absent from an area extending from 2.2 mm eccentric to the fovea to within 4.5 mm of the ora serrata throughout the superior hemisphere (Fig. 3B, C). Within this zone the outer margin of the neural retina was formed by what appeared to be Muller cell processes and cells of the inner nuclear layer. Anterior to this region, widely scattered photoreceptor cells in advanced stages of atrophy were present. Only a few of these cells had inner segments that barely protruded beyond the outer limiting membrane. No outer segment material could be discerned at the light microscopic level. Recognizable photoreceptor cells were completely absent from an area extending from 2.2 mm eccentric to the fovea to within 4.5 mm of the ora serrata throughout the superior hemisphere (Fig. 3B, C). Within this zone the outer margin of the neural retina was formed by what appeared to be Muller cell processes and cells of the inner nuclear layer. Anterior to this region, widely scattered photoreceptor cells in advanced stages of atrophy were present. Only a few of these cells had inner segments that barely protruded beyond the outer limiting membrane, and there was no evidence for outer segment material in the subretinal space (Fig. 3D).

The appearance of the retinal pigment epithelium (RPE) paralleled the condition of the overlying photoreceptors. The foveal RPE was relatively normal in appearance. The cells measured approximately 15 \( \mu m \) in height and contained large numbers of lipofuscin, melanin, and melanolysosome granules, and a few phagosomes. In the near periphery and midperiphery the RPE was hypopigmented and thinned (averaging approximately 7 \( \mu m \) in height; Fig. 3B, C). In some sections through the superior hemisphere there were small gaps in the pigment epithelium, where the outer limiting membrane of the neural retina directly abutted Bruch's membrane (Fig. 3C). However, such areas constituted no more than 10% of the length of RPE present in any one section. In the far periphery the RPE was continuous but thin, and a confluent layer of drusen-like material was present between the basal surface of the RPE and Bruch's membrane (Fig. 3D). The choriocapillaris appeared to be normal throughout the eye.

### Vitamin A Content and Composition

As shown by Table 1, vitamin A was present in the RPE-Ch from sectors 2 and 3 of the left eye. The quantity per unit area was approximately half that of normal donor eyes, and the estimated amount for the whole eye was more than one standard deviation lower than normal. Virtually all of the vitamin A was esterified, and the esters consisted mainly of retinyl stearate and palmitate in proportions similar to those found in normal eyes. No accumulation of free retinol was apparent.

Unlike the eye examined by Bridges and Alvarez, there was evidence for only mild depletion of the 11-cis isomer. The presence of 11-cis retinyl stearate and palmitate in the RPE-Ch is shown in Fig. 2, using an HPLC system that was optimized for isomer separation. Two major peaks are evident. Peak 1 corresponds to 11-cis retinyl palmitate: the shoulder on the leading edge is 11-cis retinyl stearate. Peak 2 corresponds to all-trans retinyl palmitate: the presence of the all-trans retinyl stearate is apparent from the shoulder on the rising phase of peak 2.

### Interstitial Retinol-Binding Protein (IRBP)

Figure 4 compares the SDS-gel pattern for the soluble proteins from the combined retina and RPE-Ch (including any interphotoreceptor matrix) from RP 179 with that from a comparable tissue sample from a normal eye. While several proteins appear to be depleted or absent in the sample from the dystrophic eye, the most noticeable is the 135K IRBP that has been purified and characterized from normal human interphotoreceptor matrix by Fong et al. At the time these studies were carried out, antiretino IRBP antiserum was not available. However, human IRBP is a glycoprotein with a strong affinity for concanavalin A, and we utilized this property to develop a sensitive technique for detecting its presence. The same gel was incubated with \( ^{125}I \)-labeled concanavalin A. The autoradiogram showed that there was prominent binding of the lectin at the 135K position in the track from the normal eye: none was evident in the track from the dystrophic tissue (data

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**Table 1. Amount and composition of vitamin A stored in the RPE-Ch (sectors 2, 3) of the left eye (RP 179) compared with normal controls**

<table>
<thead>
<tr>
<th>Vitamin A</th>
<th>Normals*</th>
<th>RP 179</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/cm²</td>
<td>0.55</td>
<td>0.28</td>
</tr>
<tr>
<td>nmol/eye</td>
<td>7.9 ± 4.5</td>
<td>(2.5)⁴</td>
</tr>
<tr>
<td>Fraction esterified</td>
<td>0.98 ± 0.26</td>
<td>1.0</td>
</tr>
<tr>
<td>All-trans stearate 18:0/all-trans palmitate 16:0</td>
<td>0.25</td>
<td>0.3</td>
</tr>
<tr>
<td>11-cis/all-trans</td>
<td>1.52 ± 0.48</td>
<td>0.33</td>
</tr>
</tbody>
</table>

* See Bridges et al. and Bridges and Alvarez.⁴ Based on an estimated total area.
not shown) confirming its absence in the stained pattern.

Discussion

Given the clinical evidence for symmetric disease progression in this donor (see Materials and Methods), it is likely that the histologic findings in the right eye represent an accurate measure of the structural alterations present in the left eye. Assuming that this is the case, then the retina and RPE-Ch sample of sector 1 from the left eye (Fig. 1), used for biochemical analyses, contained in its most peripheral segments a small population of photoreceptor cells that either lacked or had severely shortened inner segments, but had no outer segment membranes. Sectors 2 and 3 were largely composed of thinned and hypopigmented RPE from the near and midperipheral regions, together with a smaller amount of better preserved RPE from the far periphery. Because the RPE was thinned in areas of the companion eye homologous to sectors 2 and 3 of the left eye, the total RPE cell volume per unit of retinal surface area in these sectors was probably substantially reduced in comparison with that found in normal eyes.

Considered in the light of the histopathologic findings, the reduction of total vitamin A stores in this tissue per unit retinal surface area (Table 1) would approximate what one would expect were there normal vitamin A concentrations in the remaining volume of RPE. It further appears that the end stages of retinal disease in this individual were not associated with abnormalities of the forms of vitamin A stored within the RPE-Ch. Previous observations on the Royal College of Surgeons (RCS) rat model of hereditary retinal dystrophy\(^\text{31}\) prompted the suggestion that in retinitis pigmentosa there might be a defect in the ability of the RPE to esterify retinol with consequent damage to photoreceptor cells by release of lysosomal enzymes\(^\text{32}\) (see Berman et al\(^\text{33}\) for further discussion of this point). However, the present results, since they demonstrate that the vitamin A stored in the RPE of this patient consisted almost entirely of retinyl esters rather than retinol, do not support this hypothesis. A possible failure to isomerize all-trans to 11-cis retinoid was first explored in a clinical trial by Chatzinoff et al.\(^\text{14}\) Eleven-cis vitamin A (the retinoid used was not reported) was administered intramuscularly to retinitis pigmentosa patients over a 3-year period. This group was compared with a parallel group of patients that had received the all-trans isomer. No beneficial effect of 11-cis retinoid was found. However, the ease with which the 11-cis isomer is converted back to all-trans when dispersed in tissue preparations (see Bridges\(^\text{27}\) for review) casts doubt on the likelihood that in this study 11-cis retinol would have survived to be delivered to the RPE. More recently, this question was raised again. Bridges and Alvarez\(^\text{17}\) found that while the vitamin A in the RPE of a patient with a retinal degeneration resembling sector retinitis pigmentosa\(^\text{34,35}\)
was essentially normal in amount, it was selectively depleted in the 11-cis isomer. It could not be determined whether this depletion was a primary cause of degeneration or if it was secondary to loss of photoreceptor cells, given the suggested involvement of photoreceptors in the formation of 11-cis isomer.36 The present findings, however, show in at least this one case of retinitis pigmentosa that, even when photoreceptor cell degeneration has advanced to the point where only a small population of cones survive, there may be substantial amounts of 11-cis retinyl ester in the RPE.

There is strong evidence that IRBP is involved in the transport of retinol between the RPE and retina.18-20,25 Deficiency or molecular alteration of IRBP could therefore result in deficiencies of vitamin A within the neural retina, despite the presence of normal vitamin A stores in the RPE, with consequent photoreceptor cell death.1,2 Alternatively, the deficiency of IRBP could result in the toxic accumulation of excessive free retinol in the photoreceptor layer (rather than in the RPE), another mechanism that might lead to photoreceptor cell death. It has been recently shown that IRBP can also bind α-tocopherol.25 An impairment of vitamin E delivery to the neural retina secondary to a deficiency of IRBP could therefore deprive photoreceptors of adequate supplies of this important antioxidant.37

The involvement of IRBP in the pathogenesis of retinal degenerations in animal models of hereditary retinal degeneration is as yet unclear. During the first three postnatal weeks of life the amount of IRBP in RCS rats increases in parallel with the elongation of rod outer segments, but subsequently (as demonstrated by immunocytochemical and biochemical methods) it declines rapidly in amount.38-40 The appearance of pyknotic photoreceptor cell nuclei in the outer nuclear layer precedes detectable decrements in IRBP levels, and there is thus no evidence that abnormalities of IRBP are responsible for the initial stages of photoreceptor cell degeneration in these dystrophic rats. However, in RCS rats the retina ceases to synthesize and secrete IRBP when the photoreceptors have degenerated, suggesting both that these cells are its source (see also Hollyfield et al41) and that its loss in the diseased human eye of the present study and the absence of 75 "receptor" for exogenous retinol (possibly identical to IRBP) seen in an earlier study42 was secondary to the degeneration of photoreceptor cells. Future measurement of IRBP in earlier stages (with more photoreceptor cells present) with more sensitive immunological techniques might resolve whether a deficiency or modification of this protein contributes to the pathogenesis of some forms of retinitis pigmentosa.

Key words: retinitis pigmentosa, vitamin A, interstitial retinol-binding protein, 11-cis isomer, esterification

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References