Reduced Phagosomal Content of the Retinal Pigment Epithelium in Response to Retinoid Deprivation

Martin L. Katz, Michael Norberg, and Holly J. Stientjes

Previous investigations have shown that lipofuscin accumulation in the retinal pigment epithelium (RPE) is reduced greatly as a consequence of vitamin A deprivation. The mechanism by which vitamin A regulates RPE lipofuscin deposition remains to be determined. It is possible that retinoids are direct precursors of this substance. Alternatively, vitamin A deficiency may reduce the uptake and processing of other potential precursors. In retinas lacking photoreceptor cells, RPE lipofuscin accumulation is decreased substantially. This finding suggested that components of phagocytosed photoreceptor outer segments may be precursors for RPE lipofuscin. The effect of vitamin A deprivation on RPE lipofuscin content therefore could be the result of reduced outer segment phagocytosis by the RPE of vitamin A-deprived animals. To evaluate this possibility, experiments were conducted to determine whether vitamin A deprivation altered the phagosomal content of the RPE. Rats were fed diets containing or lacking retinoid precursors of 11-cis retinal. Retinoic acid was included in the diets of the vitamin A-deprived animals. After both 10 and 26 weeks, the RPE phagosomal contents were determined in animals from each dietary group. Photoreceptor cell densities also were measured in these rats. At both time points, the RPE phagosomal content was lower significantly in the retinoid-deprived animals than in those fed a vitamin A precursor of the visual pigment chromophore. This reduction was not the result of photoreceptor cell death; the density of these cells was not affected significantly by dietary vitamin A. Thus, it appears that retinoid deprivation reduces the rate of photoreceptor outer segment turnover and, consequently, outer segment phagocytosis by the RPE. This finding is consistent with other data showing that components of phagocytosed photoreceptor outer segments are precursors for RPE lipofuscin.


During senescence, there is a progressive accumulation of autofluorescent lysosomal storage bodies in the retinal pigment epithelia (RPE) of mammalian retinas.1-3 Similar storage bodies, commonly called lipofuscin, are deposited in numerous other postmitotic cell types during the aging process.4 In the RPE, it appears that the major source of lipofuscin precursors is phagocytosed photoreceptor outer segments. In animals without a source of outer segment material as a result of photoreceptor cell degeneration, RPE lipofuscin accumulation is reduced greatly.5,6 A similar dependence of RPE lipofuscin accumulation on the presence of photoreceptor cells was observed in human eyes.7,8 In addition, if the degradation of outer segment material by the RPE is blocked, either before or after it is phagocytosed, this material develops a lipofuscin-like fluorescence.5,9 Thus, it appears that the precursors for lipofuscin fluorophores, in particular, are components of the outer segments.

What molecular components of the outer segments are likely candidates for these fluorophore precursors? Previous investigations suggest that the retinoids involved in the visual cycle play a role in RPE lipofuscin fluorophore formation. Animals that consume diets lacking these retinoids accumulate substantially less RPE lipofuscin than do those fed nutritionally complete diets.10,11 In addition, when degradation of outer segment material by the RPE is blocked, the fluorescence that develops in this undegraded material is highly dependent on the availability of retinoids in the retina.12,13 This latter finding suggests that retinoids are involved directly in RPE lipofuscin formation. However, retinoid deficiency also may indirectly affect the availability of other molecular components of the outer segments that are potential precursors for lipofuscin formation. For example, retinoid deficiency may slow the rate of synthesis of the entire outer segment, thus reducing uptake by the RPE of all outer segment components. We conducted experi-
ments to determine whether retinoid deficiency alters outer segment phagocytosis by the RPE.

Materials and Methods

Animals and Treatments

Male Fischer 344 albino rats were obtained at 21 days of age from Harlan Sprague-Dawley (Indianapolis, IN). On arrival, the animals were divided into two treatment groups. One group of rats was fed a synthetic diet that contained adequate levels of all nutrients known to be required by the rat. This diet (+A) contained vitamin A in the form of retinyl palmitate, which can be metabolized to all the retinoids involved in the visual process and to those used by other tissues. The remaining rats were fed an identical diet, except that retinoic acid was substituted for retinyl palmitate (−A).14 Retinoic acid can satisfy the metabolic requirements of most tissues for vitamin A, but it cannot be converted metabolically into the retinoids involved in vision.

The animals were housed in clear plastic cages in the same room under 12-hr dark–12-hr light cycles. Illumination was provided by 75-W incandescent bulbs. Light levels in the cages were determined with a Lutron model LX-101 light meter (Markson Science, Phoenix, AZ) with the probe placed face-up on the cage bottoms. During the light phase of the lighting cycle, the average illumination measured at the cage bottoms was 10–20 lux. This level was below that which produces detectable light damage to the retina.15 Total darkness was maintained during each 12-hr dark cycle. The room in which the animals were housed was maintained at a constant temperature of 20°C. All investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Morphologic Analyses

Analyses were done to determine the effects of retinoid deprivation on RPE phagosomal content and photoreceptor cell densities. After both 10 and 26 weeks, the animals in each treatment group were killed with carbon dioxide gas 1–2 hr after the onset of the daily lighting cycle. Their eyes were enucleated and immediately placed in a cacodylate-buffered aldehyde fixative. The corneas, irises, and lenses were removed; the remaining portions of the eyes were agitated gently in the fixative at room temperature for a minimum of 24 hr. For ultrastructural analyses, one eye from each animal was dissected to obtain a region with one edge extending approximately 2.5 mm from the optic nerve head toward the superior ora serrata. These tissues were washed in 0.17 mol/l sodium cacodylate (pH 7.4), postfixed in 1% OsO₄, and embedded in Epon-Araldite resin (EMS, Fort Washington, PA).11 Ultrathin sections of the embedded tissues containing regions of the retinas 1–2 mm superior to the optic nerve head were cut in planes parallel to the long axes of the photoreceptors. The sections were stained with uranyl acetate and lead citrate; they were examined with a JEM 1200 EX electron microscope (JEOL Ltd., Tokyo, Japan).

We quantified the RPE phagosomal content using an Image-1 image analysis system (Universal Imaging, West Chester, PA). Electron micrographs of the RPE from each animal were made at a magnification of 4000×. A series of micrographs of adjacent non-overlapping regions of the RPE from each sample were made. These micrographs were obtained from a minimum of 225 μm of RPE length (measured along the RPE basal lamina) from each sample. The number of phagosomes in each measured area was determined, and the area of each phagosome was measured with the assistance of the image-analysis system.

Photoreceptor cell densities were determined in the regions of the contralateral eye from each animal that corresponded with the regions from which RPE phagosomal contents were determined. The eyes were bisected along the superior–inferior meridian, and one-half of each eye was embedded in glycol methacrylate. Sections along this meridian passing through the optic nerve were cut at a thickness of 2 μm with an LKB Historange microtome (Bromma, Sweden) and stained with toluidine blue. With the assistance of an Analytical Imaging Concepts image-analysis system (Irvine, CA) interfaced to a light microscope, the number of photoreceptor cell nuclei was counted in a 153-μm length of retina corresponding to the region in which the RPE phagosomal content was determined. Three sections from each retina were analyzed, and the average photoreceptor densities obtained from these analyses were determined.

Statistical Analyses

Determination of whether the retinoid intake had a significant influence on the measured parameters at each time point was accomplished using analysis of variance. Comparisons between individual treatment groups were done using the Newman-Keuls procedure.

Results

Dietary deprivation of the retinoids involved in the visual process resulted in a substantial reduction in RPE phagosomal content (Figs. 1, 2). In rats fed the −A diet for only 10 weeks, the mean total phagosomal cross-sectional area was less than one half that of rats fed the +A diet for the same length of time (P < 0.01, Fig. 1). In the +A group, there was a decrease in RPE
phagosomal content between 10 and 26 weeks. No such age-related decrease in RPE phagosomal content was observed in the −A rats. However, after 26 weeks of treatment, the RPEs of the −A animals still contained only approximately 55% as much phagosomal material as did the +A rat RPEs (P < 0.005, Fig. 1). This vitamin A-related reduction in RPE phagosomal content resulted from a combination of decreases in both number of phagosomes per unit length of RPE and average phagosomal size. After 10 weeks, the −A rats had approximately 40% fewer phagosomes in the region of RPE examined than did the +A animals (P < 0.01, Fig. 3). As observed with the total phagosomal area measurements, the mean number of phagosomes per unit length of RPE declined between 10 and 26 weeks in the +A group. No such age-related decrease occurred in the −A animals (Fig. 3). Even after 26 weeks of treatment, however, there were still fewer phagosomes in the area of the RPE from the −A rats that was examined than in the corresponding area of the RPE from the +A animals (P < 0.05, Fig. 3). The mean phagosomal size was approximately 22% greater in the +A group than in the −A animals after 10 weeks (P < 0.05, Fig. 4). Although mean phagosomal size increased somewhat between 10 and 26 weeks in the +A group, the increase was not statistically significant. No age-related change was observed in phagosomal size in the −A rats. Thus, after 26 weeks, the mean phagosomal size in the −A group was only approximately 70% of that in the +A animals (P < 0.01, Fig. 4).

**Discussion**

An age-related accumulation of the autofluorescent pigment lipofuscin occurs in the RPEs of humans and all other vertebrate species studied. The mechanisms involved in lipofuscin formation are not understood for the RPE or for any other tissue in which this pigment accumulates during aging. However, significant progress has been made in identifying the precursors of RPE lipofuscin. It appears that these precursors are predominantly molecular components of the photoreceptor outer segments. Among the evidence for this conclusion is the finding that, in animals with reduced RPE uptake of outer segment material, RPE lipofuscin accumulation is reduced greatly. In the Royal College of Surgeons (RCS) strain of rat, the photoreceptor cells develop normally until they reach maturity; after this, they specifically degenerate as a result of a defect in phagocytosis by the RPE. After the animals are a few months of age, their retinas have few remaining photoreceptor cells. Thus, for most of their lives, the RPE does not take up outer segment material as a result of both the phagocytic defect and photoreceptor cell loss in RCS rats. The RPEs of these animals contain much less lipofuscin than is found in the RPEs of congenic age-matched rats with normal retinas. The photoreceptor cells also can be removed specifically from the retinas of normal rats at an early age by exposing the animals to relatively high-intensity light under the appropriate conditions. In the latter instance, removal of the photoreceptor cells also is accompanied by a substantial decrease in RPE lipofuscin accumulation.

These observations by themselves merely indicated that photoreceptor cells are involved in RPE lipofuscin formation, but they did not show the mechanism by which these cells promote accumulation of lipofuscin in the RPE. Evidence suggested that components of the outer segments are converted directly into the contents of RPE lipofuscin granules. During the period of photoreceptor degeneration in RCS rats, material derived from the outer segments is trapped in the retina adjacent to the RPE. This material was found to develop a lipofuscin-like fluorescence, indicating that lipofuscin fluorophores could be generated directly from components of the outer segments with-
Fig. 2. Electron micrographs illustrating the effect of retinoid deprivation on retinal pigment epithelium (RPE) phagosome content. Representative phagosomes are indicated by arrows. Phagosomes in the RPE of the -A rats were smaller and less numerous than those in the RPE of the +A animals. Both micrographs are of tissues obtained from rats that had been fed the defined diets for 10 wk.
out RPE participation. Additional evidence that RPE lipofuscin fluorophores are derived from the photoreceptor outer segments was provided by experiments in which RPE degradation of phagocytosed outer segments was blocked by the protease inhibitor leupeptin. Like the outer segment material that accumulated in the retinas of RCS rats, the undegraded phagosomes quickly developed a lipofuscinc-like fluorescence. It therefore appears that at least the fluorophores of RPE lipofuscin were derived from the molecular components of the photoreceptor outer segments.

The outer segments contain several molecular constituents that are potential precursors for lipofuscin fluorophores. The phospholipids of the outer segment membranes are rich in highly unsaturated fatty acids. These fatty acids are susceptible to oxidative degradation. Some of the products of oxidative degradation of fatty acids are themselves fluorescent, and other products can generate fluorophores after reactions with amino groups of proteins or other cellular constituents. On this basis, it was proposed that lipofuscin fluorophores were primarily products of lipid autoxidation. However, it has been shown that lipofuscin fluorophores and the fluorescent products of lipid autoxidation have fluorescence and chromatographic properties that are distinctly different. Thus, it is unlikely that the outer segment phospholipids are major precursors for RPE lipofuscin fluorophores. Proteins can develop fluorescence through covalent modification (eg, nonenzymatic glycation). Covalent modifications that generate protein-linked fluorophores also can block lysosomal degradation of the modified proteins and allow accumulation of fluorescent compounds in lipofuscin. A third component of
the outer segments that was considered to be a candidate for a RPE lipofuscin fluorophore precursor was vitamin A. As indicated earlier, animals deficient in the retinoids involved in the visual process showed a dramatic reduction in the age-related accumulation of lipofuscin in the RPE.\textsuperscript{11} In addition, the fluorescence that developed in the outer segment material of degenerating photoreceptors in RCS rats was reduced greatly if the animals were fed retinoid-deficient diets.\textsuperscript{12} The fluorescence of the degraded phagosomes that accumulated in the RPE in response to leupeptin treatment also was reduced as a result of retinoid deprivation.\textsuperscript{13}

Based on these latter two observations, it appears likely that vitamin A is involved directly in RPE lipofuscin fluorophore formation. However, it also is possible that vitamin A deficiency indirectly reduced the generation of these fluorophores by reducing the outer segment content of some other component that was involved in fluorophore formation. For example, retinoid deprivation might reduce the protein content of the outer segments in a manner analogous to that which occurs in the photoreceptors of retinoid-deprived insects.\textsuperscript{29,30} Experiments to test this possibility indicated that, in rats, retinoid deficiency had no effect on the opsin density in the outer segment membranes.\textsuperscript{31} Thus, the protein composition of the outer segments probably was relatively normal in retinoid-deprived animals, so the reduced fluorophore formation was unlikely to result from a specific decrease in the availability of a protein precursor.

Although vitamin A appears to be involved directly in RPE lipofuscin fluorophore formation, retinoid deprivation also could reduce RPE lipofuscin accumulation by decreasing the uptake of all outer segment components by the RPE. In a previous study, it was found that outer segment diameter decreased as a result of retinoid deprivation.\textsuperscript{31} It also was reported that the linear rate of outer segment growth was slowed in vitamin A-deprived animals.\textsuperscript{32} Thus, the rate of production of all outer segment components appears to be reduced as a consequence of vitamin A deprivation. Our findings are consistent with this conclusion. For the outer segments to remain at a steady-state size, the rates of disc membrane synthesis and phagocytosis by the RPE must be balanced. Therefore, the finding that RPE phagosomal content was reduced as a consequence of vitamin A deficiency at the time of day when phagocytosis was occurring at its peak rate indicated that the rate of outer segment turnover was reduced. As indicated by our data, the reduced phagosomal content of the RPE was the result of less outer segment production per photoreceptor cell, not of a reduction in the number of photoreceptor cells.

Aside from the implications of these findings for our understanding of the mechanisms involved in RPE lipofuscin formation, they also suggest a role for retinoids other than retinoic acid in regulating aspects of photoreceptor and RPE cell metabolism in addition to transduction. At least one of the retinoids involved in the visual cycle apparently controls the rate of outer segment turnover. There are various points in the turnover process where vitamin A might exert its regulatory effect. For example, a retinoid might directly regulate the rate of transcription of one or more genes coding for protein components of the outer segments. The rate of disc membrane assembly, in turn, might depend on the availability of these proteins. Preliminary evidence suggests that, in Drosophila, retinoids directly regulate the rate of expression of the opsin gene.\textsuperscript{33} Substantial research will be required to define the precise mechanism by which retinoids regulate photoreceptor outer segment turnover in vertebrates.

**Key words:** vitamin A, retinal pigment epithelium, retina, photoreceptors, lipofuscin

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