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Topographic and age-related changes of the retinal epithelium and Bruch's membrane of rhesus monkeys

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Abstract

Purpose—To examine structural differences in the retinal pigmented epithelium (RPE) and Bruch's membrane of rhesus monkeys (*Macaca mulatta*) as a function of topography and age.

Methods—The retinas of two old (24 and 26 years old) and two young (1 and 6 years old) female monkeys were examined by light, fluorescence and electron microscopy at the macula, equator and ora serrata.

Results—All monkeys lacked fluorescence and lipofuscin granules in the RPE at the ora serrata where photoreceptors are absent. The equator and macula showed intense fluorescence and many lipofuscin granules in the RPE of the old but not the young monkeys. At the ora, the RPE contained many dense round melanin granules throughout the cell. At the equator and macula, melanin granules were more apical, less frequent and often elongated. Mitochondria were clustered at the basal side of the RPE cell near infolds of the plasma membrane. Both mitochondria and infolds tended to increase toward the macula. In all regions, the basal lamina of the RPE did not penetrate the extracellular space adjacent to infolds. The elastin layer of Bruch's membrane was wide at the ora and equator and thin at the macula. In the old monkeys, drusen were found at all retinal regions between the basal lamina and the internal collagen layer of Bruch's membrane. They were often membrane bound with a basal lamina and contained material resembling structures in the RPE. Severe drusenoid-like degeneration was found at the ora serrata of the oldest monkey.

Conclusions—Lack of fluorescence and lipofuscin in the RPE at the ora serrata, where photoreceptors are absent, confirms that RPE fluorescence depends on outer segment phagocytosis. Mitochondrial clustering indicates that the basal side of the RPE cell uses most energy and this becomes maximal at the macula. The presence of age-related degenerative changes and drusen at all retinal locations in the older monkeys, even at the ora where RPE lipofuscin was absent, indicates that these processes are not dependent on local lipofuscin accumulation. Therefore lipofuscin toxicity may not be the sole cause of age-related RPE degeneration.

Introduction

Many degenerative changes of the retina are due to abnormalities of the retinal pigmented epithelium (RPE). Several of these changes tend to be more severe in the macula than at more peripheral areas of the retina, but the reasons for the macula's selective vulnerability are unknown. These degenerative changes often develop slowly during adult life and are related to a general senescence of the retinal epithelium, which in some cases leads to age-related macular

degeneration (6,13,28). Rhesus monkeys also develop an age-related drusenoid maculopathy, which closely resembles the human disease (10,11). In order to determine whether structural differences between the macula and other areas of the retina could explain the macula's greater vulnerability, we examined the autofluorescence and ultrastructure of the RPE and Bruch's membrane at the ora serrata, equator, and macula of young and old rhesus monkeys.

Methods

The retinas of four female rhesus monkeys (*Macaca mulatta*) were examined by light and electron microscopy. The monkeys were categorized as either young (1 and 6 years old) or old (24 and 26 years old). Monkeys were euthanized for experimental or clinical reasons and the eyes were enucleated within a few minutes after death. One eye was fixed by immersion in 3% glutaraldehyde and the other in 4% paraformaldehyde in phosphate buffered saline, after the globes were pierced to facilitate diffusion of the fixative into the vitreal cavity. After storage for several weeks in fixative, the eyes were washed in buffer and dissected with the aid of a surgical microscope. The posterior segment was dissected into pieces about 1 cm², which included segments from the macula, the temporal equator, and temporal ora serrata. All segments were post-fixed in 1% osmium tetroxide for 1 hour, dehydrated, and embedded in epon. Unstained sections were examined by light and fluorescence microscopy. The excitation light for fluorescence came from a mercury arc lamp using 480±20 and 545±15 nanometers excitation with barrier filters of 535±25 and 620±30 nanometers. After fluorescence imaging, the sections were stained by toluidine blue and re-examined by light microscopy. At selected sites, ultra-thin sections were cut, post-stained with uranyl acetate and lead citrate, and examined by electron microscopy (Zeiss EM 10C/CR or JEOL 1200 EX2). Negatives obtained by photography from the electron microscope were digitized by a Microtek 5 scanner and transferred to a computer where they were examined at different magnifications.

All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Primate Research Center at Oregon Health & Science University and the Gerontology Research Center, NIA, and conformed to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended.

Results

Auto-fluorescence

Figure 1 shows unstained bright field (A&C) and fluorescence (B&D) images of the retina of the one-year-old monkey at the ora serrata (A&B) and equator (C&D) with arrows marking identical sites in the two images of each section. There was no fluorescence of the RPE at either the ora (Figure 1B) or the equator (Figure 1D). However, at the equator fluorescence was seen in the inner segments of cones and more weakly in rods. The fluorescence in the photoreceptors was stronger to blue (480 nm) than to green (545 nm) excitation whereas the converse was so for the RPE, indicating a different source of fluorescence. The retina of the 6-year old was similar to the one-year old monkey. The retina of the 26-year old monkey (Figure 2A–D) also lacked fluorescence at the ora (Figure 2B) but showed strong fluorescence at the equator (Figure 2D) and macula; the 24-year old monkey was similar. There was no obvious difference in the strength of the inner segment fluorescence between young and old monkeys.

Ora Serrata

Electron microscopy of the ora serrata of the one-year old monkey showed many round melanin granules (M) throughout the cytoplasm of the RPE from the apical to basal side (Figure 3A&B).

There was an absence of lipofuscin bodies that concurred with the lack of autofluorescence (7). At this extreme peripheral location there were no photoreceptors (O). A magnified view of the image within the white box (Figure 3B) at the basal side of the cell showed incisures of the basal lamina coupled with the basal plasma membrane (white arrow). The basal lamina did not enter the extracellular space within the multiple infolds of the basal plasma membrane. There were no mitochondria in the cytoplasm. In all four monkeys, a relatively wide band of elastin (e) was seen in Bruch's membrane at both the ora and equator but not the macula. The RPE at the ora of the 6-year old was similar to that of the one-year old monkey.

Electron microscopy of the RPE layer at the ora serrata of the 26-year old monkey is shown in Figure 4A&B. This region had many degenerative changes mainly at the basal side of the RPE layer including both basal laminar and basal linear debris (13, 19). There were many melanin granules but no lipofuscin bodies in the RPE, which concurred with the absence of auto-fluorescence in the same animal (Figure 2B). A wide elastin layer (e) was present in Bruch's membrane (Figure 4A). A magnified view of the boxed area (Figure 4B) showed mitochondria and basal infolds (small white arrow) of the plasma membrane. The basal lamina (large white arrow) was interrupted by debris (larger two asterisks) that extended through the plasma membrane to Bruch's membrane through the basal lamina, making this debris both a basal laminar as well as a basal linear deposit (11). There is considerable debris (small asterisk) in a drusenoid-like pattern in Bruch's membrane. This degeneration extended over several RPE cells at the ora serrata where autofluorescence and photoreceptors were absent (Figure 2B). The RPE of the 24-year-old monkey at the ora was not as degenerated as the 26-year-old monkey but had drusen (see below).

The Equator

The RPE at the equator of the one-year-old monkey (Figure 5A&B) had no lipofuscin bodies, corresponding to a lack of auto-fluorescence (Figure 1B) but had abundant melanin granules (M). A magnified view (Figure 5B) shows numerous mitochondria (m) adjacent to basal infolds (white arrow). A thin basal lamina touched the tips of the cell's plasma membrane but did not enter the extracellular space adjacent to infolds. A wide elastin layer (e) was found, again characteristic of non-macular retina.

The RPE of the 6-year-old monkey (Figure 6A&B) had virtually no lipofuscin bodies but contained melanin granules and mitochondria, the latter again at the basal side of the cell. A magnified view (Figure 6B) shows infolds (if) of the plasma membrane (short arrow), adjacent to the basal lamina (long arrow). Collagen fibers (arrowhead) and a wide elastin layer (e) are seen in Bruch's membrane.

At the equator, the RPE of the 26-year-old monkey showed less degeneration than at the ora (Figure 7A&B). The many lipofuscin bodies (L) in the cytoplasm corresponded with the strong auto-fluorescence at the equator (Figure 2D). A large cytoplasmic vacuole (V), seen often in RPE, may represent a site of retinyl esters. A magnified view (Figure 7B) revealed mitochondria (m) close to basal infolds (white arrow) and a prominent elastin layer (e) in Bruch's membrane, again typical of extra-macular retina. It is noteworthy that considerably more debris is seen in Bruch's membrane than in the younger monkeys at the equatorial region.

The Macula

Figure 8A&B show RPE from the macula of the 6-year-old monkey. Again the virtual absence of lipofuscin bodies in the cytoplasm concurred with its absent autofluorescence. There were fewer melanin granules than at more peripheral locations. A magnified view (Figure 8B) shows many mitochondria (m) adjacent to basal infolds (if). The basal lamina (long arrow) was thin and straight, just touching the tips of the invaginated basal plasma membrane (short arrow).

The conspicuously wide elastin layer seen in Bruch's membrane of more peripheral retina was absent. There were many collagen fibers (arrowhead) some contacting the basal lamina.

Figure 9A&B shows RPE in the macula of the 26-year-old monkey with numerous lipofuscin bodies (L), a paucity of dense melanin granules (M), but less degeneration than at the ora serrata. A magnified view (Figure 9B) showed many mitochondria (m) next to basal infolds (long arrow) with a slightly thickened basal lamina (short arrow). A peroxisome (P) was present next to the mitochondria. There was a break (asterisk) in the basal lamina, not found in the younger monkeys. Bruch's membrane contained much debris not seen in the younger monkeys. There were no parallel arrays of collagen fibers as seen in the younger monkeys and the prominent elastin layer was absent.

Drusen

Drusen were found in all retinal regions of the old monkeys but none were found in the young monkeys. Figure 10A&B show drusen, one at the ora serrata (A) and the other at the equator (B), of the 24-year-old monkey. These drusen were relatively small, the size of an RPE cell, but not likely to be detectable by ophthalmoscopy. The druse at the ora (Figure 10A) was associated with a break in the basal lamina (arrow) and had formed adjacent to an RPE cell containing no lipofuscin. Few mitochondria were seen in the vicinity. The material in this druse was not membrane-bound and not identifiable. The druse at the equator (Figure 10B) was formed adjacent to an RPE cell loaded with lipofuscin (L). This druse formed where mitochondria (m) were present, again next to basal infolds. The material in the druse was not membrane-bound and not identifiable as cytoplasmic structures seen in the RPE.

Figure 11 shows drusen (D) at the equator of the 26-year-old monkey where RPE cells also had much lipofuscin. The druse of Figure 11A is composed of two distinct membrane-bound structures, one of which has an element (lower white arrowhead) that seems identical to another in the cytoplasm of the adjacent RPE cell (upper white arrowhead). This druse formed adjacent to a region containing clusters of mitochondria (m) and many basal infolds. It was bound above by the basal lamina of the RPE and below by numerous collagen fibers (one example marked by black arrowhead); having separated these two inner components of Bruch's membrane, which normally are in contact. A wide elastin layer (e) again is present at the equator. Just beneath it are macrophage-like cells (ma), which are rarely seen in Bruch's membrane. The druse of Figure 11B is not only membrane-bound but also has a basal lamina (thin black arrow) which is slightly thinner than the basal lamina (thicker black arrow) of the RPE. The druse has formed adjacent to basal infolds (if) and clusters of mitochondria (m). It is interesting that the basal infolds have large areas of extracellular space separating them. Again, the druse has formed between the basal lamina of the RPE above and the inner layer of collagen fibers (arrowhead) below.

Figure 12 shows two more drusen found at the equator of the 26-year-old monkey demonstrating how they were consistently formed between the basal lamina of the RPE above and the inner collagen layer (12A arrowheads) below and adjacent to basal infolds with clusters of mitochondria (m). The wide elastic layer (e) of the peripheral retina was seen in Bruch's membrane; just below was a macrophage-like cell (ma), suggesting inflammation. The druse of Figure 12B is membrane-bound and also has a basal lamina producing a double basal lamina (two white arrows) at its upper edge.

Discussion

Results from this study confirmed that lipofuscin, the main source of autofluorescence in the RPE, resulted from the phagocytosis of outer segments (1,7). RPE autofluorescence and lipofuscin were strikingly absent at the ora serrata, where there were no photoreceptors, even

in monkeys of advanced age that showed abundant autofluorescence and lipofuscin at the equator and in the macula. This finding agrees with earlier in vivo studies that showed an absence of autofluorescence at the periphery of the human retina (27).

It is notable that despite the lack of lipofuscin, there was degeneration of the RPE at the ora serrata in both old monkeys. Pathological changes were especially severe in the 26-year-old monkey, which showed a relatively large area of RPE deterioration, an abnormality also seen in human subjects and referred to as tapetoretinal degeneration (3,23). Furthermore, drusen were present in all regions, including the ora serrata of the old but not the young monkeys. These findings imply that these degenerative changes were age-related but occurred independent of lipofuscin accumulation.

Examination of the ultra structure of the retina provided insight into the formation of drusen. These characteristically dome shaped structures are found invariably between the basal lamina of the RPE and the internal collagen layer of Bruch's membrane (18,19). Hogan et al (12) theorized that drusen originated from segments of the RPE cell and suggested that drusen "occupied" the internal collagen layer. Our results also suggest an origination in the RPE; however, the inner collagen layer remained intact and was always external to drusen. It is possible that a druse is being pushed into the inner collagen layer perhaps by oncotic pressure, displacing the collagen further into Bruch's membrane. Alternatively, an isolated segment from the basal side of the parent RPE cell may form without displacing the inner collagen layer. Our findings are suggestive of this latter theory, which resembles the "budding" hypothesis proposed by Burns and Feeney (2) for humans and seen by Ishibashi et al. (14) in monkeys. "Budding" suggests that a segment of the RPE cell loses contact with the cell's nucleus and degenerates to form the material comprising a druse, which is also membrane-bound. By this mechanism, the druse remains between the basal lamina and the internal collagen layer. Observations in the current study suggest the same mechanism because some cell organelles found in the cytoplasm of the RPE are apparent in the degenerating druse (Figure 11A) and some basal laminar material characteristic of the host RPE cell is present around the druse (Figure 11B and 12B). Such biogenesis of drusen, however, would require breaks in the basal lamina so that a druse could separate from its parent cell by crossing the basal lamina. We present evidence that such breaks occurred in monkeys (Figures 4B and 11A). Hogan et al. (12) also showed some examples of basal lamina breaks in human tissue. This scenario resembles an autophagic process in which the RPE cell is eaten away from its basal side. The presence of two types of druse, some that are membrane-bound with a basal lamina and others considered more degenerated and without such structures, suggests early and late stages in the genesis of drusen. It is interesting that macrophage-like cells tend to be present at the hypothetical early phase where they may contribute to the dissolution of these membranous structures.

The question remains as to why this degenerative process occurs. One hypothesis is that A2E, a pyridinium bisretinoid, contained in phagocytized outer segments accumulates in the RPE cell as a major component of lipofuscin. The toxic actions of A2E could then cause degeneration of the RPE cell by several proposed mechanisms (21). However, this hypothesis does not explain why drusen form. At the ora serrata, no lipofuscin was present and yet an age-related drusenoid-like degeneration of the RPE layer occurred. Thus, there are clearly factors other than lipofuscin toxicity that lead to an age-related degeneration of the RPE.

Both oxidative stress and gene mutations have been linked to the development of drusen. We found that virtually all the mitochondria in the RPE were located at the basal side of the cell, close to basal membrane infolds. This suggested that the greatest energy demand for the RPE cell occurred near these infolds. The metabolically active basal side of the RPE cell is also the primary site where drusen form. Oxidative damage has been shown to accumulate with age

(4,24) and an increase in severity has been linked to somatic mitochondrial mutations, which are more frequently detected in subjects with age-related macular degeneration (25). Similarly polymorphisms in the complement factor H (*CFH*) and several other immune system-related genes also modify the risk for developing age-related macular degeneration in humans. An immune response at the site of degenerating cellular segments which form a druse may interact with the oxidative damage at the basal side of the RPE cell. Thus far, the *CFH* gene has not been implicated in maculopathy in non-human primates. There are two other genes, however, which are linked to both human and monkey maculopathy (9,20,26), the age-related maculopathy susceptibility gene (*ARMS 2*) and the HtrA serine peptidase 1 (*HTRA1*) gene. Both genes are located on chromosome 10 yet the role of the proteins these genes code for is unclear (26). *ARMS2* is particularly interesting because it exists only in primates with maculas (9).

A serendipitous finding in our study was the strong autofluorescence detected in the inner segments of the photoreceptors, especially cones. This fluorescence can be attributed to flavoproteins and NAD(P)H in mitochondria (15–17). Stress of mitochondria is thought to increase this fluorescence (8). We found that the fluorescence of the inner segments of the photoreceptors was similar in both young and old monkeys implying that age did not increase the stress on these photoreceptor organelles. We did not detect RPE fluorescence at the ora in any monkey and not at any location in the one-year-old monkey even though all of these RPE cells contained mitochondria. Thus there seems to be more fluorescence from the mitochondria of photoreceptor inner segments than from mitochondria in RPE. This may be due to a larger number of mitochondria or a denser collection of these organelles in the inner segments of photoreceptors than at the basal side of RPE cells. It is interesting that even with these high concentrations of mitochondria in the inner segments of photoreceptors, age-related degeneration of these structures does not seem to be as common as that of the RPE. Fluorescent lipofuscin precursors have also been reported in photoreceptors (22) but mainly in the outer rather than the inner segments. The fluorescence in the inner segments in our study did not increase with age and was therefore presumably not age-related.

The present study was done to determine if examination of the ultra structure of the RPE and Bruch's membrane of old and young monkeys at various retinal sites could provide a better understanding of the genesis of drusenoid degeneration. The most striking observation was that age related degeneration of the RPE layer can occur in the absence of lipofuscin, indicating that this substance is not the only cause of age related RPE degeneration.

At all locations examined, virtually all of the mitochondria in the RPE were located at the basal side adjacent to numerous infolds of the basal membrane. Thus, the metabolic load must be greatest at these infolds, which was where drusen appeared to originate. Drusen and drusenoid-like degenerations were common in all areas of the retina of old monkeys but were undetectable in the young monkeys. In the old monkeys there was a greater density and size of drusen in the macula than at more peripheral locations. However, there is evidence that the total number of drusen may be greater in peripheral retina of human subjects (18) probably due to its greater size. Ultrastructure appeared qualitatively different in the macula than at the ora serrata or the equator. For example, a greater concentration of mitochondria and basal membrane infolds was found at the macula than at more peripheral locations, and may increase oxidative stress at the macula. Additionally, there was a much thinner elastin layer in Bruch's membrane at the macula than at more peripheral locations. Chong et al (5) previously detected this difference in the elastin layer and suggested that it might influence the vulnerability of the macula to degeneration. Although the mechanism is unknown, perhaps a thinner elastin layer permits greater oxygen flow into the retina and thus increases oxidative stress.

It would seem that a better understanding of age-related macular degeneration in monkeys, as well as in humans, will depend upon more knowledge of the molecular biology of the energy demanding basal side of the RPE cell and its relationship to the large concentrations of mitochondria in this same location.

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Figure 1.

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