Current Eye Research

ISSN: 0271-3683 (Print) 1460-2202 (Online) Journal homepage: http://www.tandfonline.com/loi/icey20

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To cite this article: David A. DiLoreto Jr., Caesar Luo, David J. Calkins & Manuel del Cerro (2006) An Ultrastructural Study of the Pathology of the Retinal Pigment Epithelium, Bruch's Membrane, and the Choriocapillaris in the Aged Fischer 344 Rat, Current Eye Research, 31:9, 749-763

To link to this article: http://dx.doi.org/10.1080/02713680600864782

Published online: 02 Jul 2009.

Current Eye Research, 31:749–763, 2006

ISSN: 0271-3683 print / 1460-2202 online
DOI: 10.1080/02713680600864782

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An Ultrastructural Study of the Pathology of the Retinal Pigment Epithelium, Bruch's Membrane, and the Choriocapillaris in the Aged Fischer 344 Rat

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ABSTRACT *Purpose*: The neural retinal degeneration in the aging Fischer 344 (F344) rat has been previously characterized. Here we describe the ultrastructural changes that occur in the retinal pigment epithelium (RPE), Bruch's membrane, and choriocapillaris in the periphery of the aged Fischer 344 rat. *Methods*: F344 eyes from 24-month-old animals (n = 4 animals, 8 eyes) were fixed and embedded for ultrastructural study. Serial mid-sagittal sections were taken from the superior peripheral retinas within 300 μm of the ora serrata. Pathology within the RPE, Bruch's membrane, and choriocapillaris was described. *Results*: Progressive changes were seen in the RPE/Bruch's/choriocapillaris complex, increasing anteriorly as the ora serrata was approached. Early pathology of the RPE included increased number of basal infoldings, increased number of phagolysosomes and lipofuscin deposits, attenuation, inclusion of vasculature, vesicle formation, and whirling extensions of the basement membrane into the cytoplasm. Bruch's membrane showed spots of considerable thinning, but most prominent was the nodular thickening. The choriocapillaris was found to have severe endothelial degeneration and transformation to fibrous tissue in the most severely affected regions. Lipofuscin was also found in areas of degenerated choriocapillaris. *Conclusions*: Prior work focused on the neural retina, documented photoreceptor cell loss, and showed that Müller cell changes preceded that loss in the periphery of the F344 rat. It is now evident that the pathology in the RPE/Bruch's membrane/choriocapillaris complex may also be a critical component of the overall degenerative process. A possible mechanism for the extensive peripheral retinal degeneration in the F344 is presented.

KEYWORDS aging; Bruch's membrane; choriocapillaris; choroid; degeneration; electron microscopy; Müller cell; photoreceptor; pigment epithelium; rat; retina

Received 9 August 2005
Accepted 10 June 2006

Supporting Grant: KO8 EY016742-01 Award (DD), Research to Prevent Blindness (UREI)

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INTRODUCTION

Previously we characterized the age-related retinal changes in the Fischer 344 (F344) rat. Initially we used histomorphometry to quantify cell loss which

demonstrated a precipitous, selective photoreceptor cell loss between 12 to18 months of age, most dramatic in the superior peripheral retina.[1] While photoreceptor cell loss occurs with age and is more pronounced in the peripheral retina of most species, the loss we documented in the F344 rat retina was accelerated and more severe compared to age-matched Sprague-Dawley controls.[1] Subsequent functional testing correlated visual function to retinal histology, suggesting a possible interaction with the Müller cell.[2] Further characterization using immunocytochemistry and one- and two-dimensional gel electrophoresis showed that Müller cell hypertrophy and activation, specifically glial fibrillary acidic protein (GFAP) up-regulation, preceded chronic photoreceptor cell degeneration in the F344 rat.[3]

Our ultrastructural descriptions were limited to the degenerating photoreceptor cell nuclei, inner and outer segments, the reactive Müller cells, the breakdown of the outer limiting membrane, and lipofuscin accumulation in the retinal pigment epithelial cell.[3] Others have also briefly described ultrastructural changes in the RPE with age in this model.[4] Using electron microscopy, our observations from previous studies have been extended to areas outside of the neural retina. Presently we recorded the ultrastructural age-related pathology in the peripheral retina at the level of the retinal pigment epithelium (RPE), Bruch's membrane, and choriocapillaris. These findings may help elucidate the mechanism of extensive photoreceptor cell loss in the peripheral retina of the aging Fischer 344 rat.

MATERIALS AND METHODS

Fischer 344 rats were purchased at specific ages from the National Institute on Aging (Harlan Sprague Dawley, Indianapolis, IN, USA). Housing conditions were as follows: 22°C to 24°C, 12 h/12 h light/dark cycle, lighting intensity of less than 40 ftcd. All animals were handled in strict accordance with the ARVO statement on the use of laboratory animals. The animals were sacrificed one week after housing at the University of Rochester vivarium. Eyes from four female Fischer 344 rats (n = 8) were processed for electron microscopy as previously published.[1] Briefly, for enucleation, rats were anesthetized using ketalar, IM (Ketamine, Parke-Davis, Morris Plains, NJ, USA) at a dose of 90 mg/kg and a concentration of 100 mg/ml and pentobarbital, IP (Nembutal, Abbot Laboratories, Chicago, IL, USA) at a dose of 20 mg/kg and a concentration of 50 mg/ml.

With surgical cautery the eyes were marked on the limbus at the twelve o'clock position. They were then enucleated and placed in 6% glutaraldehyde with 0.1 M sodium cacodylate at 40°C. A sagittal slit was made through the cautery mark, in line with the optic nerve to allow penetration of the fixative. The eyes were left in the fixative for 48 h, then hemisected in 70% ethanol through the cautery mark and the optic nerve along the twelve to six o'clock plane. The two hemispheres were then embedded in Durcupan (Fluka Chemie, Switzeland).

The eyes were hemisected in a midsagittal plane (in line with the cautery mark on the limbus at 12:00 and through the optic nerve) producing a nasal and temporal hemisphere. Each hemisphere was evaluated in the following way: A 1-micrometer-thick section was taken for light microscopical screening from the superior half of each hemisphere.[5] Once the light microscopic section was deemed satisfactory, three ultrathin sections were taken consecutively and mounted on a slot grid to include the ora serrata. One hundred microns were then cut and a new set of 3 consecutive sections were taken. This was done 4 times for each hemisphere, thus producing 5 representative samples for analysis spanning a distance of at least 800 microns through each eye.

For transmission electron microscopic analysis, two eyes from each animal were used (n = 4 animals, 8 eyes). The distance from the ora serrata to the area of measurement was 300 microns. Ultrathin sections were taken of this area and the thickness of Bruch's membrane was observed. Changes near or within the RPE, Bruch's membrane, and choriocapillaris were described. Specifically, every section was sampled for the following: attenuation of the RPE, basement membrane proliferation of the RPE, vascularization of the RPE, variations in the thickness of Bruch's membrane, presence of focal thickening within Bruch's membrane, degeneration of the choriocapillaris, and presence of lipofuscin in the choroid.

RESULTS

The eyes of all 24-month-old F344 rats showed progressive changes in the RPE/Bruch's/choriocapillaris complex increasing in severity, anteriorly as the ora serrata was approached. Posterior from the ora serrata, areas of degenerating photoreceptor cells were seen with prominent Müller cell processes within the inner nuclear layer (Fig. 1A). In these areas the outer

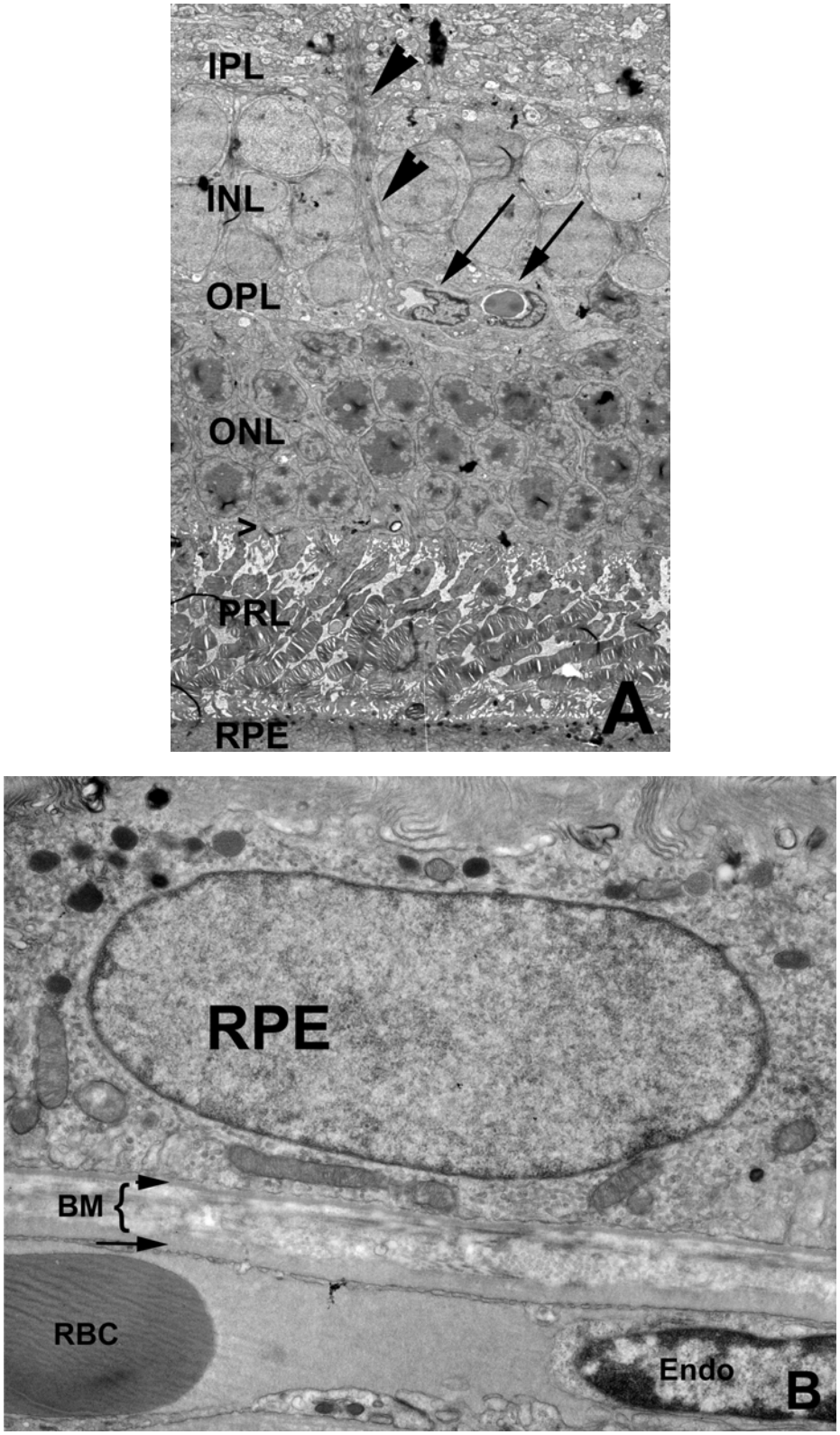


FIGURE 1 **Ultrastructure of the peripheral retina of the Fischer 344 rat. (A) An area of degenerating retina is shown from the inner plexiform layer (IPL) to retinal pigment epithelium (RPE). A Müller cell process (arrowheads) can be seen running from the inner nuclear layer (INL) to the IPL. Blood vessels (arrows) are seen within the outer plexiform layer (OPL). Photoreceptors (PRL) are disorganized and fragmented. (ONL = outer nuclear layer; >= outer limiting membrane; original magnification 1000×). (B) Normal appearing retinal pigment epithelial cell (RPE), Bruch's membrane (BM), and choriocapillaris complex. RPE cell nucleus (RPE), uniform Bruch's membrane (BM), and a red blood cell (RBC), within a fenestrated choriocapillaris blood vessel (Endo = endothelial cell nucleus) can be seen. In Bruch's membrane, the basement membrane of the RPE cell (arrowhead), middle collagenous/elastic zone ({), and basement membrane of the endothelial cell (arrow) are identified (original magnification 9000×).**

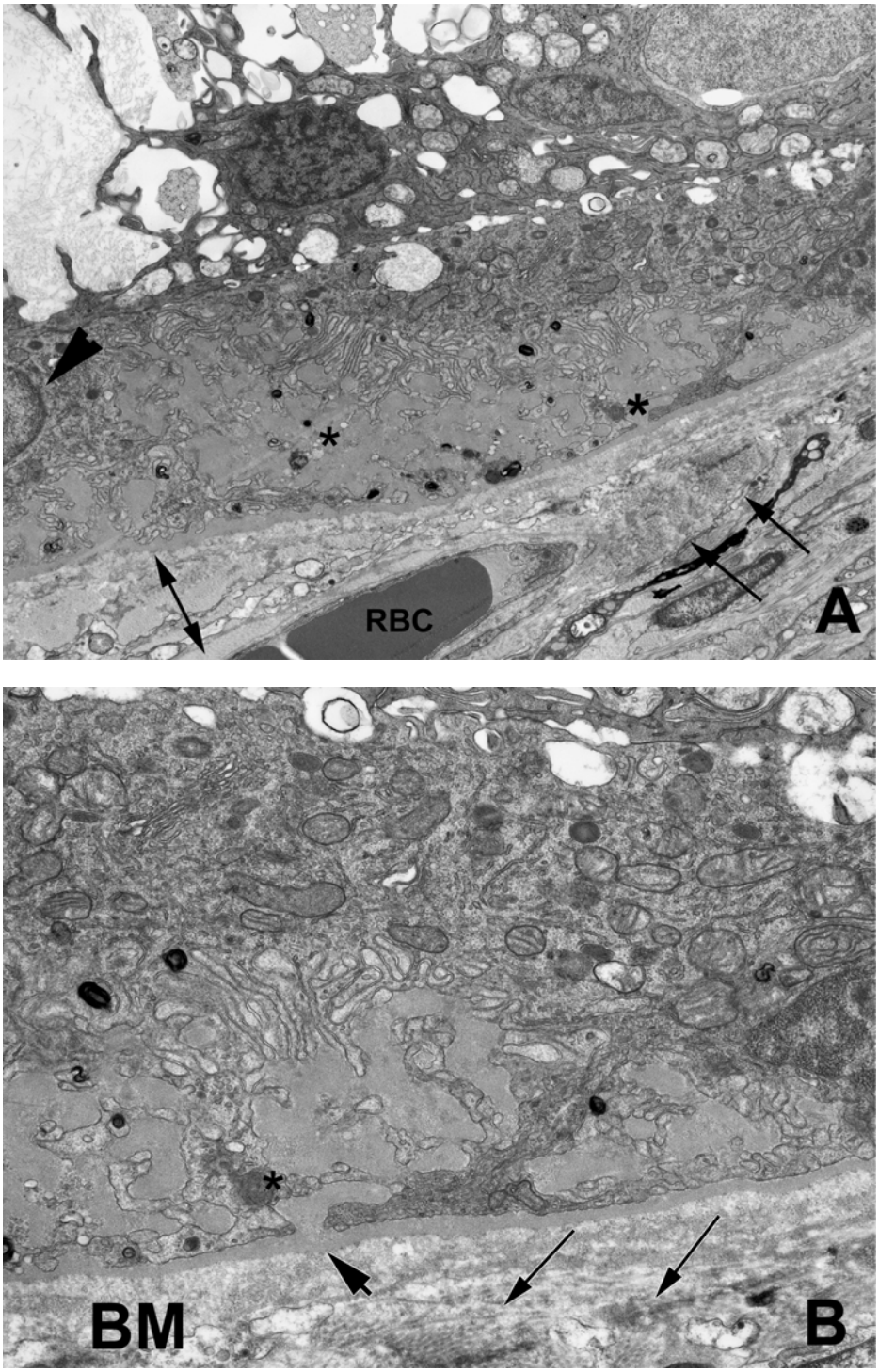


FIGURE 2 **Basement membrane proliferation of the retinal pigment epithelium (RPE) in areas of age-related retinal peripheral degeneration of the Fischer 344 rat. (A) Lower power view showing the RPE cell nucleus (arrowhead), overlying degeneration and disorganization of the neuroretina, and thickening of Bruch's membrane (double-headed arrow) and fibrosis of the choriocapillaris as evidenced by collagen deposition (arrows) at the border of an endothelial cell. The RPE basement membrane can be seen as whirling extensions (*) into the RPE cytoplasm (RBC = red blood cell; original magnification 4000×). (B) Higher power view of the RPE basement membrane proliferation as seen in Fig. 2A. Asterisk in Fig. 2A corresponds to asterisk seen here. Arrowhead highlights the continuity of the amorphous material with the basement membrane intruding into abnormal basal infoldings of the RPE cell. (BM = Bruch's membrane; original magnification 9000×). (C) Another example of basement membrane proliferation of an RPE. Electron dense remnants mark the breakdown of the outer limiting membrane (arrowheads). Below this is the RPE cell with extensive basement membrane proliferation (asterisk). (BM = Bruch's membrane; RBC = red blood cells; Endo = endothelial cell of the choriocapillaris; original magnification 3000×). (D) Higher power view of a portion of Fig. 2C. Asterisk here corresponds to the position marked by the asterisk in Fig. 2C. The arrowhead marks a spot where the whirling intracytoplasmic amorphous material is continuous with the basement membrane of the RPE cell. (BM = Bruch's membrane; original magnification 12000×).** ***(Continued)***

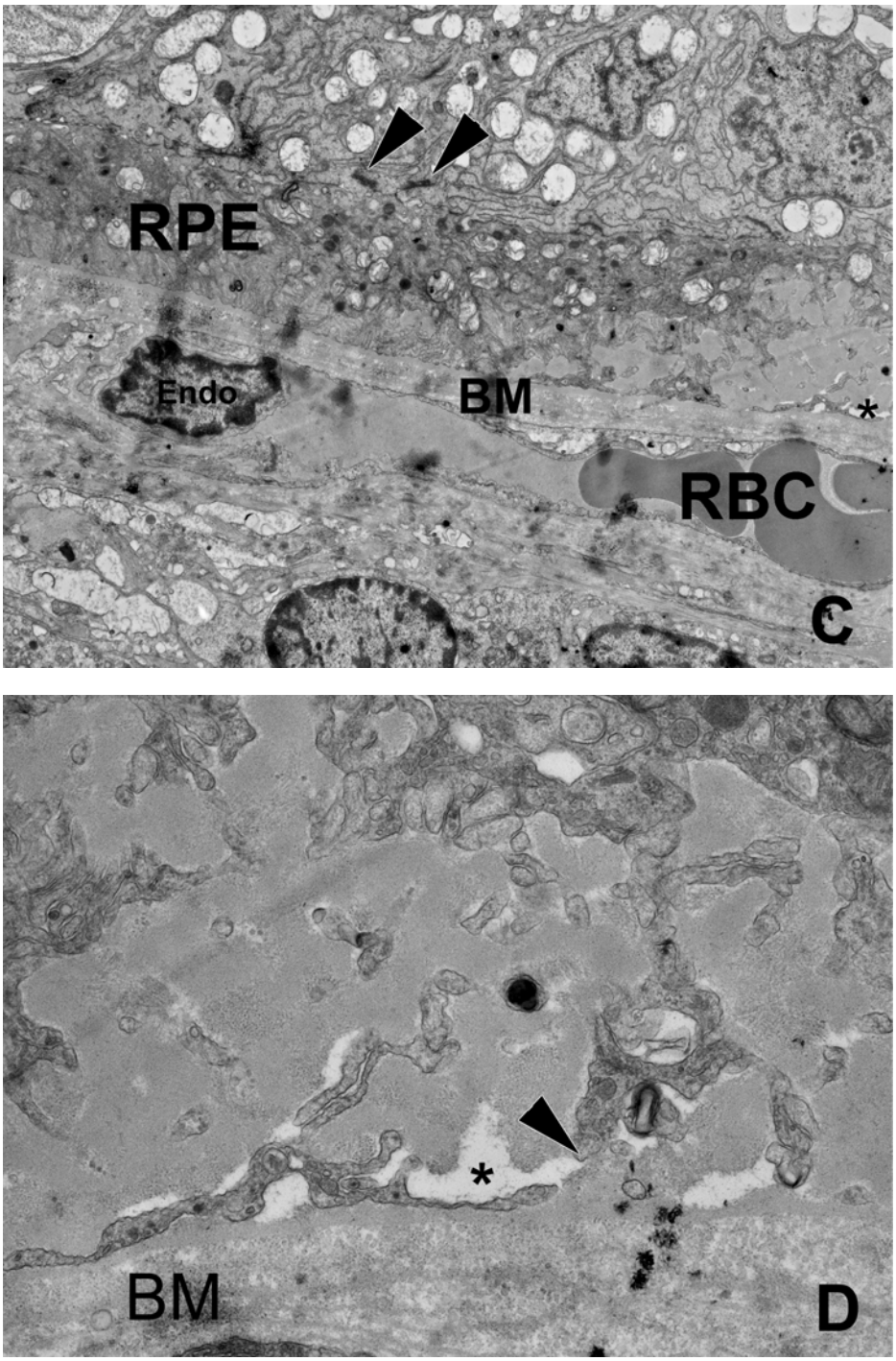


FIGURE 2 **(Continued)**

limiting membrane was still intact. In the subretinal space, macrophages were noted to be engaged in active phagocytosis of degenerating outer segments (not shown). Cell bodies from photoreceptor cells were also seen in the subretinal space. In some instances, the photoreceptor cell body could be seen "squeezing" through the outer limiting membrane into the subretinal space. In these distal areas of degeneration where the outer nuclear layer was still formed and the photoreceptors showed inner segments, the underlying Bruch's membrane appeared normal (Fig. 1B).

Early peripheral changes of the RPE included increased number of basal infoldings, increased number of phagolysosomes and lipofuscin deposits, atrophy, and whirling extensions of the basement membrane into the cytoplasm (Fig. 2). This process was most prominent near the ora serrata. Underlying these areas was pronounced thickening of Bruch's membrane

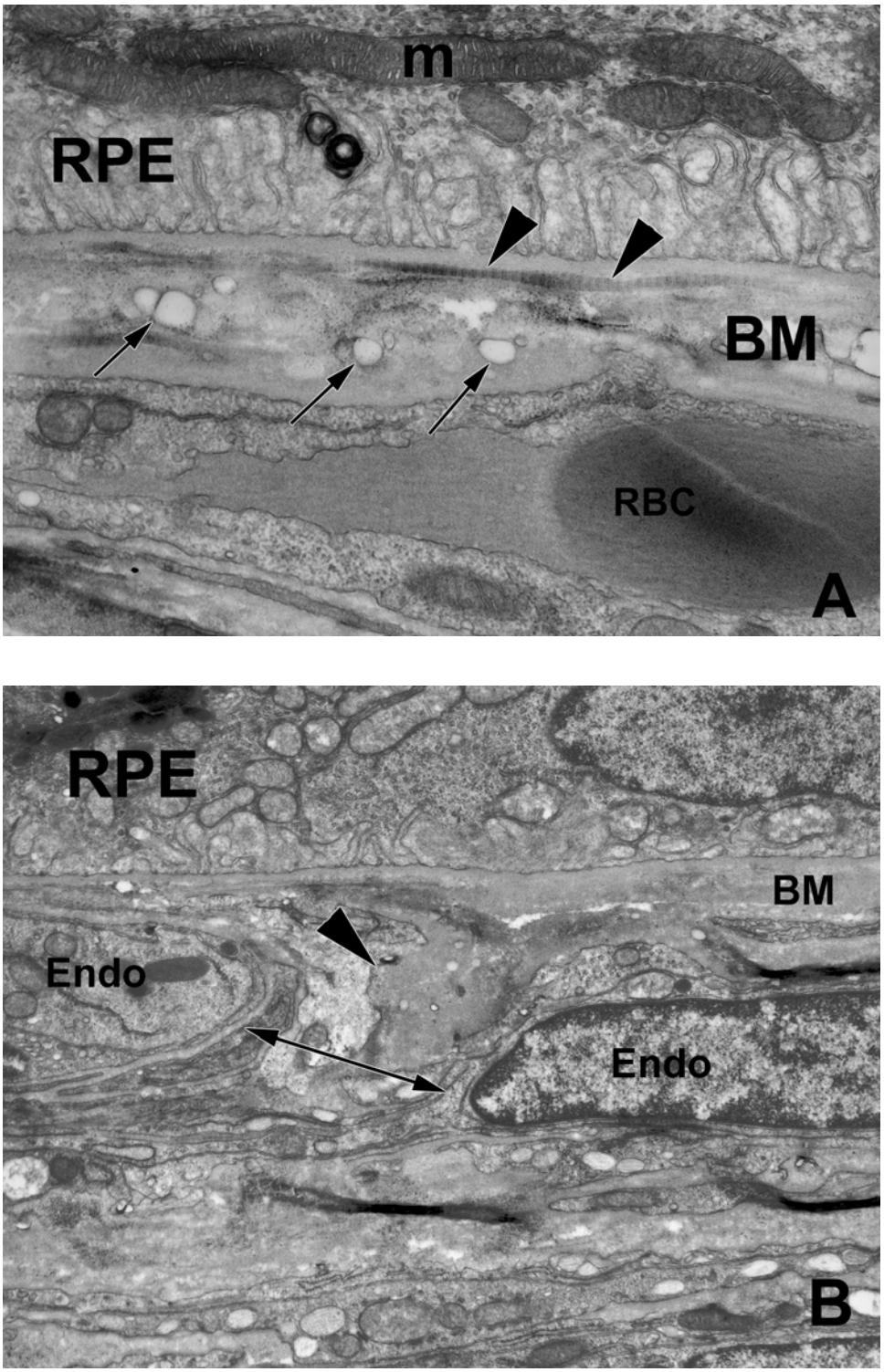


FIGURE 3 **Variations in thickening of Bruch's membrane in the areas of age-related peripheral retinal degeneration of the Fischer 344 rat. (A) Areas of diffuse thickening in Bruch's membrane were seen in all eyes. Electron lucent inclusions (arrows) were prominent in the thickened areas. Also, prominent collagen strands (arrowheads) that appeared widely spaced were found (original magnification 17000×). (B) Irregularities of Bruch's membrane seemed pronounced at interendothelial areas of the choriocapillaris. These "pegs" were seen to thicken (arrowhead) between (double-headed arrow) degenerating endothelial cells (Endo) (original magnification 9000×). (C) Focal areas of thickening in Bruch's membrane were seen in many eyes. These focal areas (arrow) were in the outer portion of Bruch's membrane (BM) protruding into the lumen of blood vessels (BV) of the choriocapillaris. Endothelial cell cytoplasm can be seen (arrowhead). (RPE = retinal pigment epithelium; original magnification 9000×).** ***(Continued)***

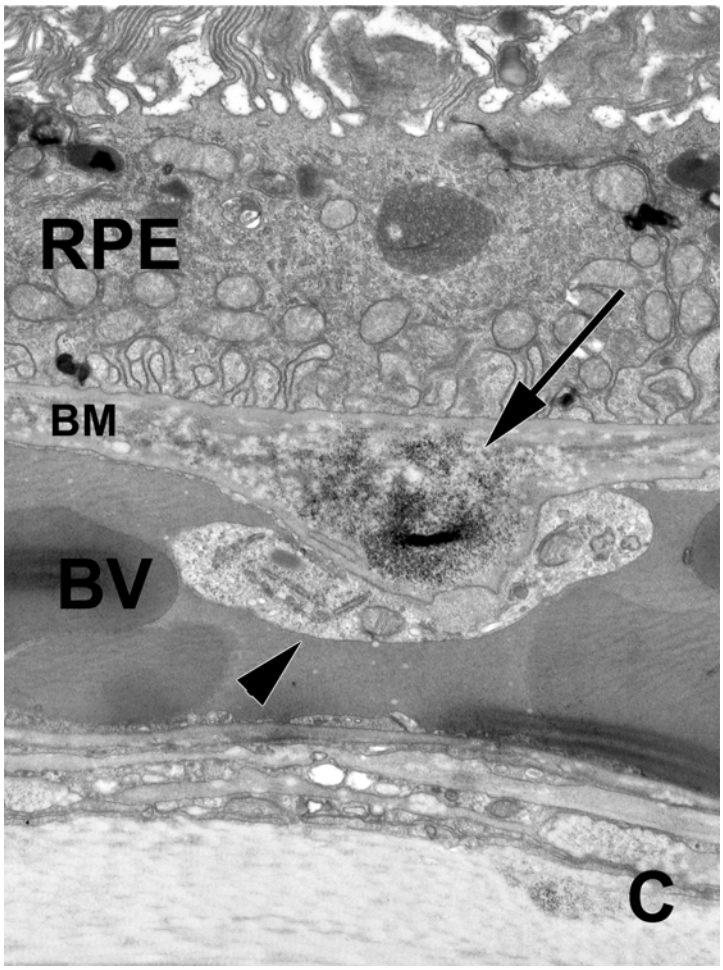
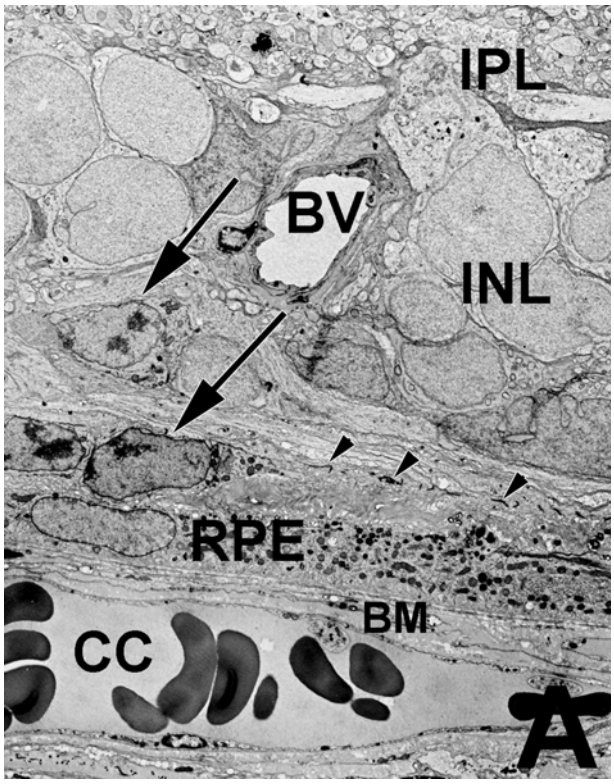


FIGURE 3 **(Continued)**

with excess collagen deposition. Many endothelial cells within the choriocapillaris showed severe degenerative changes in these areas. Overall, Bruch's membrane showed sporadic stretches of considerable thinning, but most prominent was the thickening (Fig. 3). Both diffuse thickening as well as focal thickening were seen. Within the areas of diffuse thickening were accumulations of electron lucent particles and widely spaced collagen (Fig. 3A). The areas between the endothelial cells within the choriocapillaris were extremely affected. These degenerating areas often showed prominent collagen deposition that was continuous with Bruch's membrane and surrounded the endothelial cells (Fig. 3B). The areas of focal thickening were confined to collagen deposition in the outer layers of Bruch's membrane, often protruding into the endothelial cells within the choriocapillaris (Fig. 3C).

As the photoreceptor cells degenerated and the outer nuclear layer and photoreceptor cell layers disappeared, the RPE often appeared vascularized. Choroidal neovascularization was a likely source.[6] However, careful inspection of the continuum of degeneration revealed another possible origin. As the outer retina degenerated, vessels from the inner retina migrated toward the RPE (Fig. 4A). Once the photoreceptor cells degenerated and disappeared, this allowed the vasculature that originated from the inner nuclear layer or outer plexiform layer to move outward, becoming engulfed by the RPE (Fig. 4B). Once within the RPE, the basement membrane of the vessels was seen to proliferate within the cytoplasm of the RPE. No blood vessels were ever seen crossing Bruch's membrane.

Atypical vesicle formation within the RPE was noted in areas of degeneration. While no obvious drusen were detected, degenerating mitochondria were seen to accumulate near the basement membrane of the RPE (Fig. 5), and the resulting material had the appearance of some types of drusen including electron lucent and electron dense inclusions and curvilinear profiles.



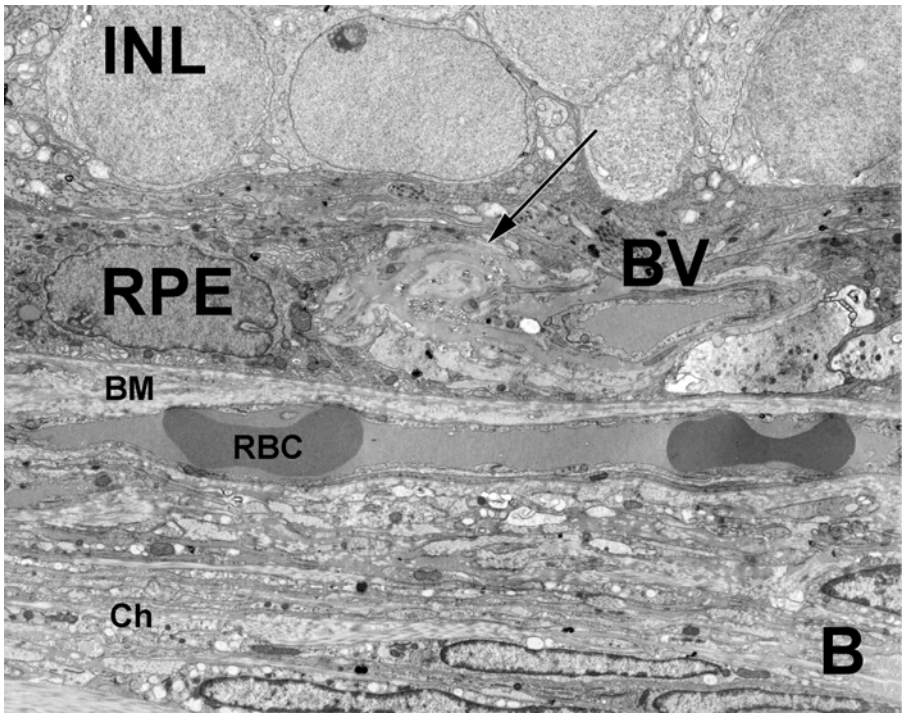


FIGURE 4 Photoreceptor cell loss and vascularization of the RPE. Blood vessels seen within the RPE most likely originated from in the inner retina. No blood vessels were seen to cross Bruch's membrane. (A) Area of near complete loss of photoreceptor cells. The outer nuclear layer (INL) has come into contact with the retinal pigment epithelium (RPE). Photoreceptor cell nuclei can be seen (arrows) and are identified by the characteristic chromatin pattern. The outer limiting membrane (arrowheads) is discontinuous and can be seen contacting the microvilli of the RPE. With loss of the photoreceptors, a blood vessel (BV) originating in the INL can be seen approaching the RPE (IPL = inner plexiform layer; Bruch's membrane; CC = blood vessel of the choriocapillaris; original magnification 2000×). (B) An area of complete loss of photoreceptor cells. The inner nuclear layer (INL) is seen apposing the retinal pigment epithelium (RPE). A non-fenestrated blood vessel (BV) that most likely originated in the INL or outer plexiform layer seems to be engulfed by an attenuated retinal pigment epithelial cell (RPE). The endothelial cell of the blood vessel is degenerating and its basement membrane in extending into the cytoplasm of the RPE cell (arrow). In contrast to the non-fenestrated blood vessels found in the neural retinal, the blood vessels within the choriocapillaris are fenestrated (original magnification 3000×).

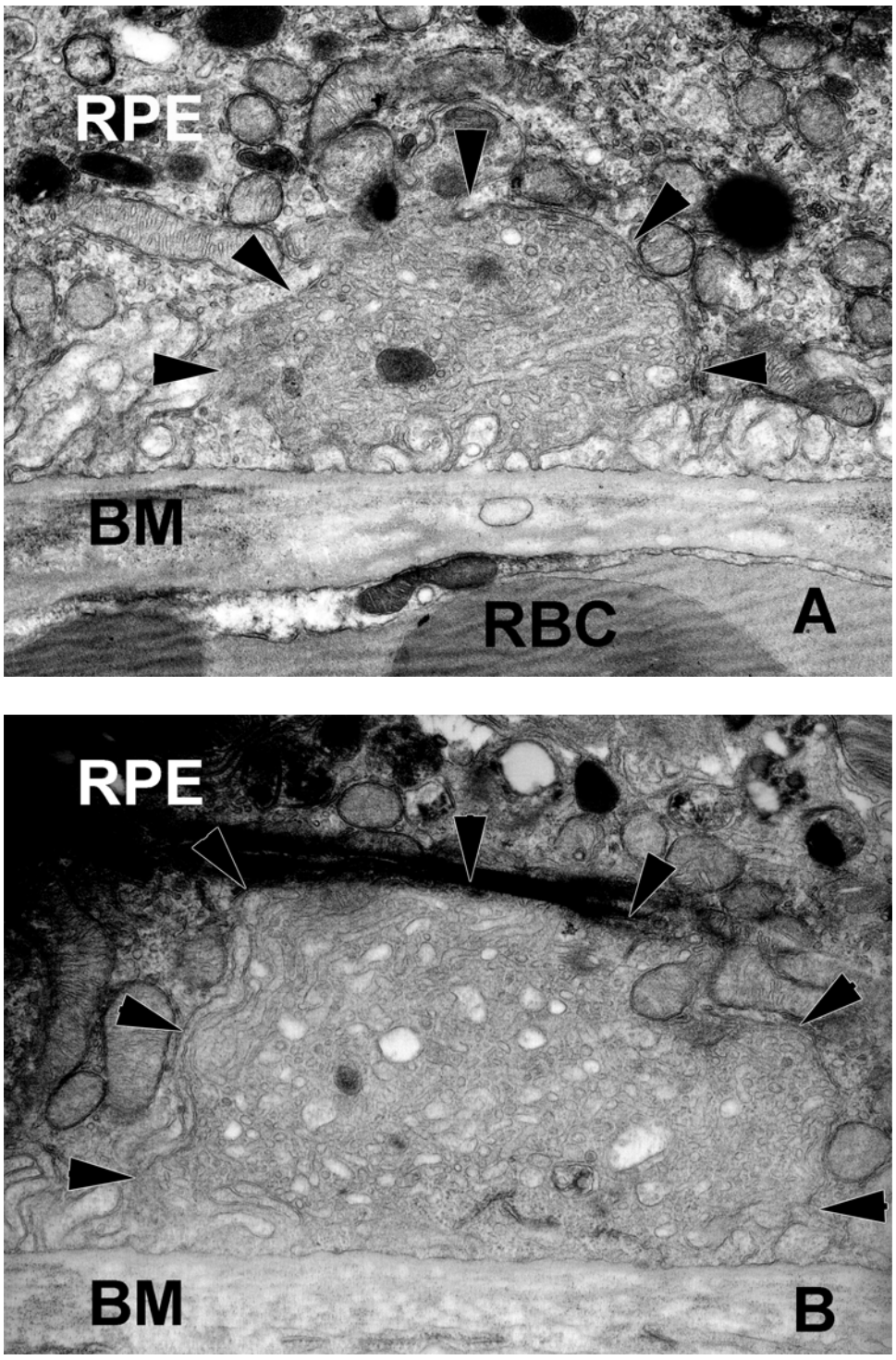


FIGURE 5 **Intracellular inclusion in the retinal pigment epithelium. (A) Lipid droplets, lipofuscin, and degenerating organelles were seen throughout the RPE of the aging rats. In addition, two particular types of inclusions were seen in the basal portion of the RPE cells resting against its basement membrane. One type of inclusion is seen in Figs. 5A and 5B (arrowheads). These inclusions seem to be organization of degenerating mitochondria. A double layered membrane characteristic of mitochondria is seen in both A and B outlining the debris. Also, multiple degenerating mitochondria are seen surrounding both inclusions (BM = Bruch's membrane; RPE = retinal pigment epithelium; RBC = red blood cell; original magnification for both A and B is 15000×). (B) F344 druse, low power, with overlying photoreceptor outer segment degeneration. (C) A different type of inclusion is seen here. Although this inclusion (arrowhead) is not within Bruch's membrane as many drusen are, there are drusenlike components to the debris including electron lucent and electron dense inclusions and curvilinear profiles. Also, there is associated basement membrane proliferation of the RPE surrounding the inclusion (arrow) (PR = outer segment of the photoreceptors; * = Bruch's membrane; RBC = red blood cell within the choriocapillaris; Ch = choroids; original magnification 3500×).** ***(Continued)***

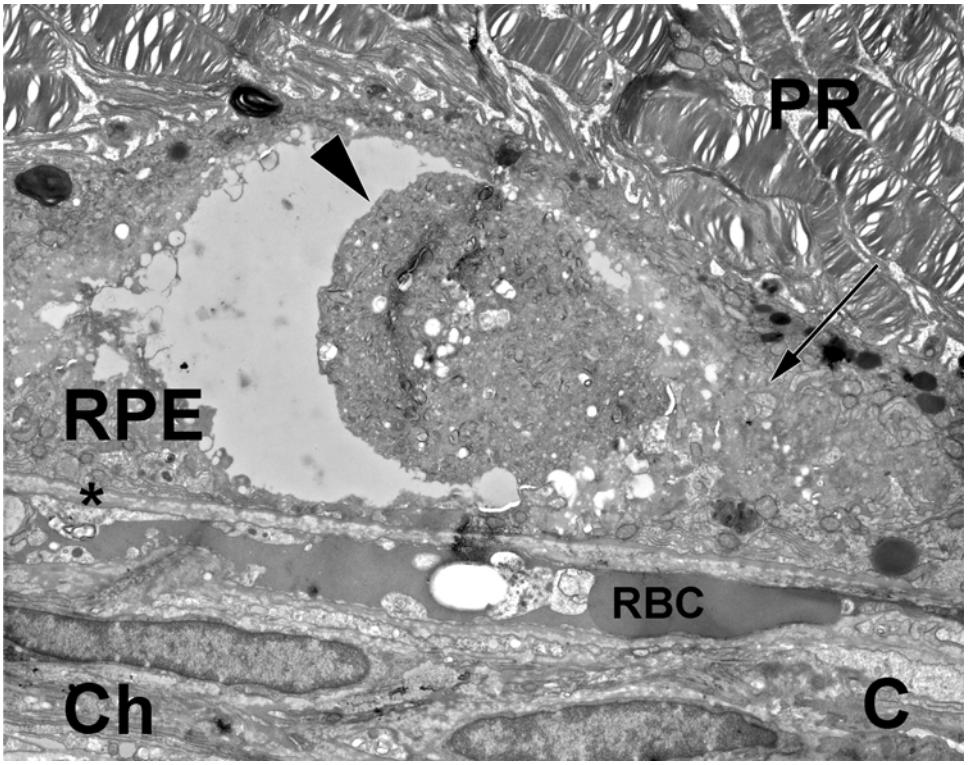


FIGURE 5 **(Continued)**

The choriocapillaris was found to undergo severe degeneration with subsequent transformation to fibrous tissue in the most severely affected regions. Endothelial cells were seen with scant cytoplasm; excess collagen deposition occurred around them that became continuous with Bruch's membrane (Fig. 6A). Although no basal laminar deposits were seen within the RPE side of Bruch's membrane, we did see typical basal laminar deposits within areas of degenerating choriocapillaris (Figs. 6B and 6C). Another interesting finding of the choriocapillaris that has usually been associated with the RPE was the presence of lipofuscin in areas of degenerated choriocapillaris. It appeared that the degenerating endothelial cells within the choriocapillaris contained phagolysosomes and lipofuscin (Figs. 7A and 7B). Around the lipofuscin deposits there was extensive collagen deposition (Fig. 7C).

DISCUSSION

We have identified and described numerous age-related changes at the ultrastructural level within the RPE/Bruch's membrane/choriocapillaris complex in the Fischer 344 eye. Prior work on the retinal degeneration in the F344 has focused on the photoreceptor cell[3,7,8] and only sporadic ultrastructural data have been shown.[3,4] Here, a more extensive analysis was performed to identify changes outside the neural retina that may have implications for the mechanism of photoreceptor cell loss. Based on past studies it was assumed that the accelerated loss of photoreceptor cells in the periphery of the Fischer 344 rat was caused by cellular signaling within the neural retina between photoreceptor cells and Müller cells.[3] It is now clear the pathology outside the neural retina, in the RPE/Bruch's membrane/choriocapillaris complex, may also play a role. This role most likely has to do with oxygen delivery.[9] Because the outer retina is avascular, the photoreceptors are supplied with oxygen and nutrients that diffuse from the choriocapillaris across Bruch's membrane and RPE. Changes in any one of these three structures could have profound effects on the oxygen levels that the photoreceptor cells are exposed to.

Some notable changes reported in this paper within this RPE/Bruch's membrane/choriocapillaris complex that could influence oxygen diffusion included both diffuse and nodular thickening of Bruch's membrane as well as vacuole accumulation and collagen deposition. This is in line with previous reports of peripheral retinal changes with age in other species such as

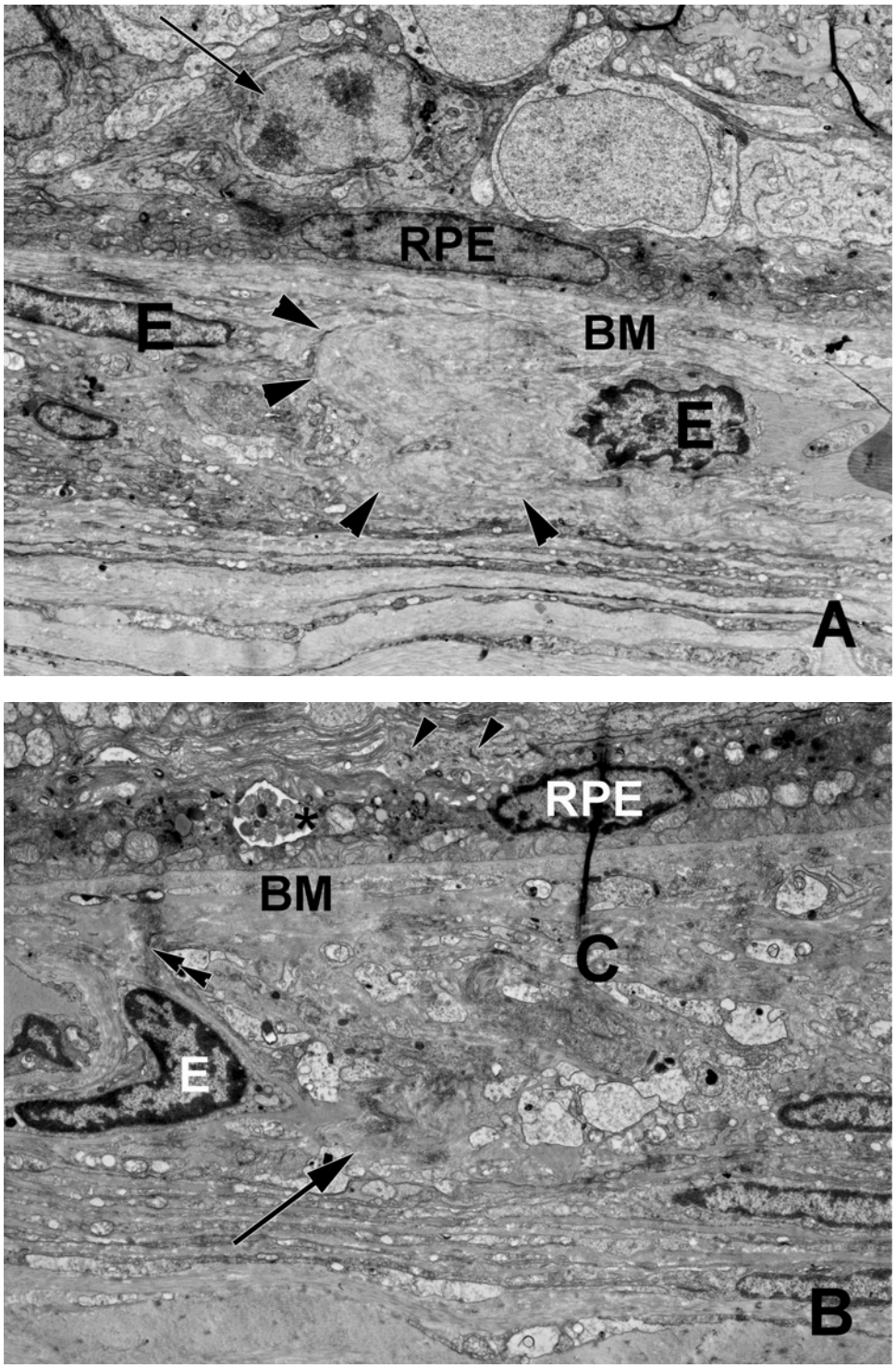


FIGURE 6 **Degeneration of the choriocapillaris with collagen deposition. (A) One photoreceptor cell nucleus is seen (arrow) above a severely attenuated retinal pigment epithelial (RPE) cell. Bruch's membrane (BM) has lost its delineation and a large collagen deposition (arrowheads) is seen between two degenerating endothelial cells (E) of the choriocapillaris (original magnification 3000×). (B) Here no photoreceptor cell nuclei are seen. The outer limiting membrane (arrowheads) is discontinuous above an attenuated RPE cell. An intracytoplasmic inclusion is seen within the RPE cell (*). Bruch's membrane is severely thickened with collagen deposition (double arrowhead) around degenerating endothelial cells (E) of the choriocapillaris. Basement membrane proliferation of the endothelial cells are seen, contributing to thickening of Bruch's membrane. Basal laminar like deposition is seen in the choriocapillaris (arrow) sandwiched between basement membrane proliferation of an endothelial cell (E) and collagen deposition (C) (original magnification 3000×). (C) Higher power view centered on the basal laminar like deposits in the choriocapillaris. The distinction parallel arrangement of striped electron dense material alternating with electron lucent material and surrounded by amorphous material is characteristic of basal laminar deposits of age-related macular degeneration which are typically seen within in the outer RPE, not in the choriocapillaris (original magnification 12000×).** ***(Continued)***

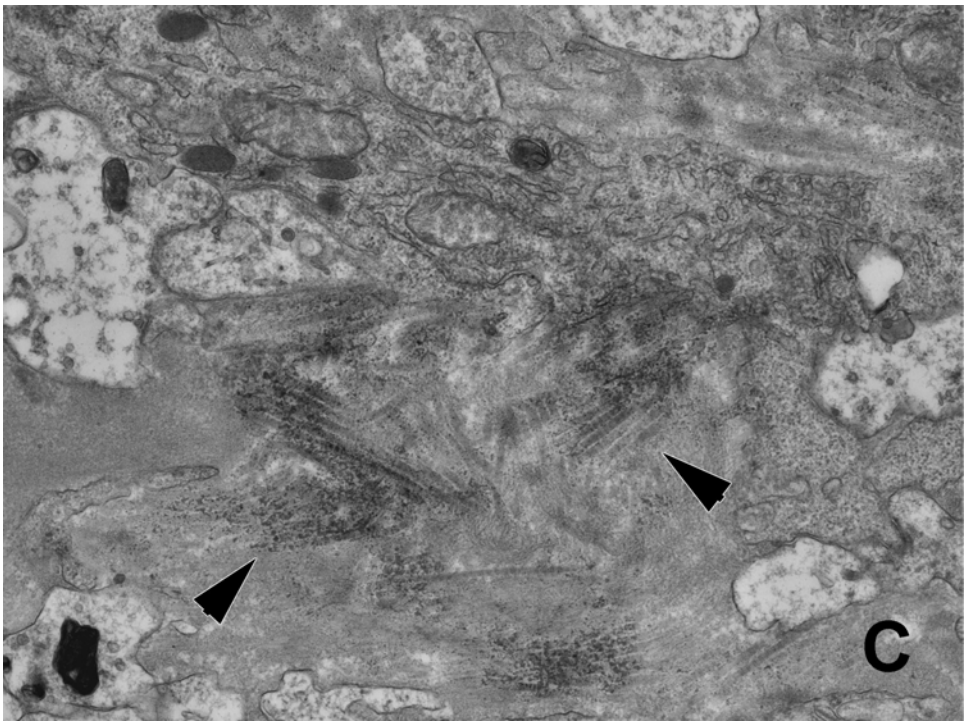


FIGURE 6 **(Continued)**

mouse[10,11] and human.[6,12–19] RPE changes included proliferation of basement membrane and accumulations of broken down organelles, mostly mitochondria, which had ultrastructural similarities to the components of drusen. While the composition of drusen is under active investigation,[20,21] it is believed that drusen are formed from extracellular matrix proteins. This report shows that organelles released from degenerating RPE cells may also be involved in the formation of drusen. Severe endothelial cell degeneration was seen in the choriocapillaris along with excess collagen deposition, both of which have been documented in human disease. A novel finding in this work is the presence of basal laminar deposits and lipofuscin (both previously reported in the RPE) within the choriocapillaris, showing that degenerating endothelial cells of the choriocapillaris may undergo similar degenerative changes as the RPE cell with age. Another interesting finding was that the source of RPE vascularization was most likely from engulfed inner retinal vasculature that was displaced as the outer retina degenerated and not from choroidal neovascularization, since no breaks in Bruch's membrane were identified.

It is known that photoreceptor cells in the retina are extremely sensitive to changes in oxygen levels from development through adulthood.[9,22] For example, most of the developing rat retina undergoes photoreceptor cell loss due to hypoxia, but the edge or most peripheral retina near the ora serrata reacts differently.[9,22] The edge is subjected to chronic stress from hyperoxia from post-natal day 20 through adulthood.[22] It is this chronic low-grade hyperoxia that is believed to be responsible for upregulation of trophic factors such as fibroblast growth factor 2 (FGF-2) and ciliary neurotrophic factor (CNTF) that make the far peripheral retina more resistant to further degenerative insults.[23–25] The hyperoxia is believed to be due to increased oxygen flow from the choriocapillaris anterior to the edge of the retina. Also contributing to the increased oxygen levels in this area are the anastomoses near the ora serrata of the anterior ciliary arteries from the iris and ciliary body with the posterior ciliary arteries from the choriocapillaris. Why then does the Fischer 344 rat undergo an accelerated loss of photoreceptor cells in the far periphery with age compared to age-matched control animals such as the Sprague-Dawley rat (1)? It is our belief that the changes described within this paper regarding the degeneration of the RPE/Bruch's/choriocapillaris complex significantly affects the oxygen levels contributing to photoreceptor cell loss.

Several features of our observations suggest that pathology of the RPE/Bruch's/choriocapillaris complex contributes to the process of photoreceptor cell degeneration. These findings were not only seen at the

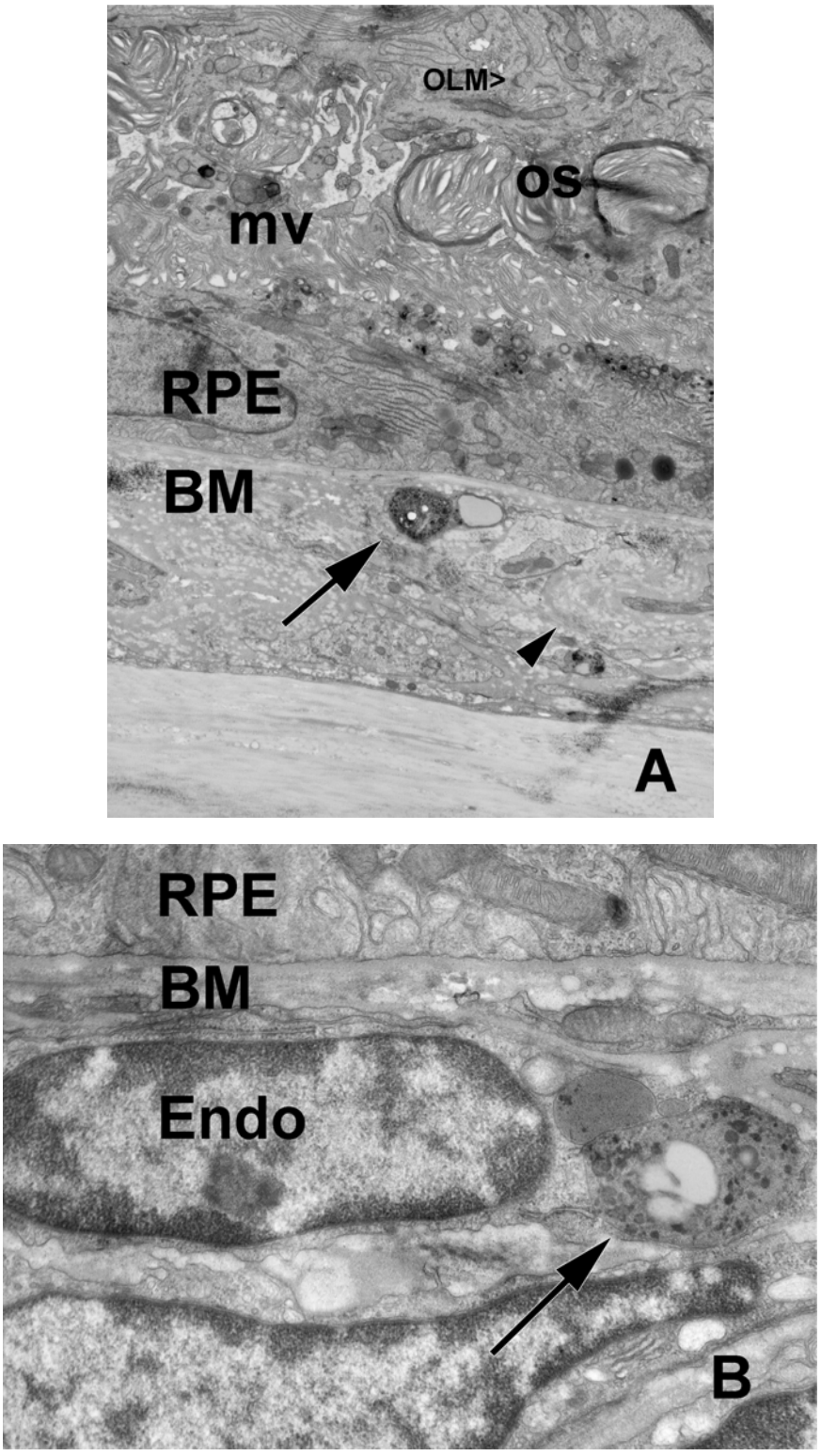


FIGURE 7 **Lipofuscin deposition in the choriocapillaris. Lipofuscin was seen external to Bruch's membrane in all of the animals, especially in areas of choriocapillaris fibrosis and endothelial cell degeneration. The lipofuscin may be within endothelial cell processes. (A) Area of degenerated photoreceptors revealing only a discontinuous outer limiting membrane (OLM) and outer segments (OS) being engulfed by the microvilli (mv) or the retinal pigment epithelium (RPE). Bruch's membrane (BM) in this area is extremely thickened. A phagolysosome (arrow) is seen here with early lipofuscin granules in an area of degenerated and fibrosed choriocapillaris (original magnification = 5000×). (B) Higher power view of a phagolysosome in the choriocapillaris adjacent to an endothelial cell nucleus (Endo). Again, early lipofuscin granules are seen within the lysosome (RPE = retinal pigment epithelium; BM = Bruch's membrane; original magnification = 15000×). (C) Lipofuscin (arrow) deposit seen within fibrosing choriocapillaris. Bruch's membrane's (BM) boundaries are unclear as excess collagen (arrowheads) is deposited around the lipofuscin. There were often prominent collagen bands surrounding the lipofuscin (Endo = endothelial cell of choriocapillaris; RBC = red blood cell; original magnification = 9000×).** ***(Continued)***

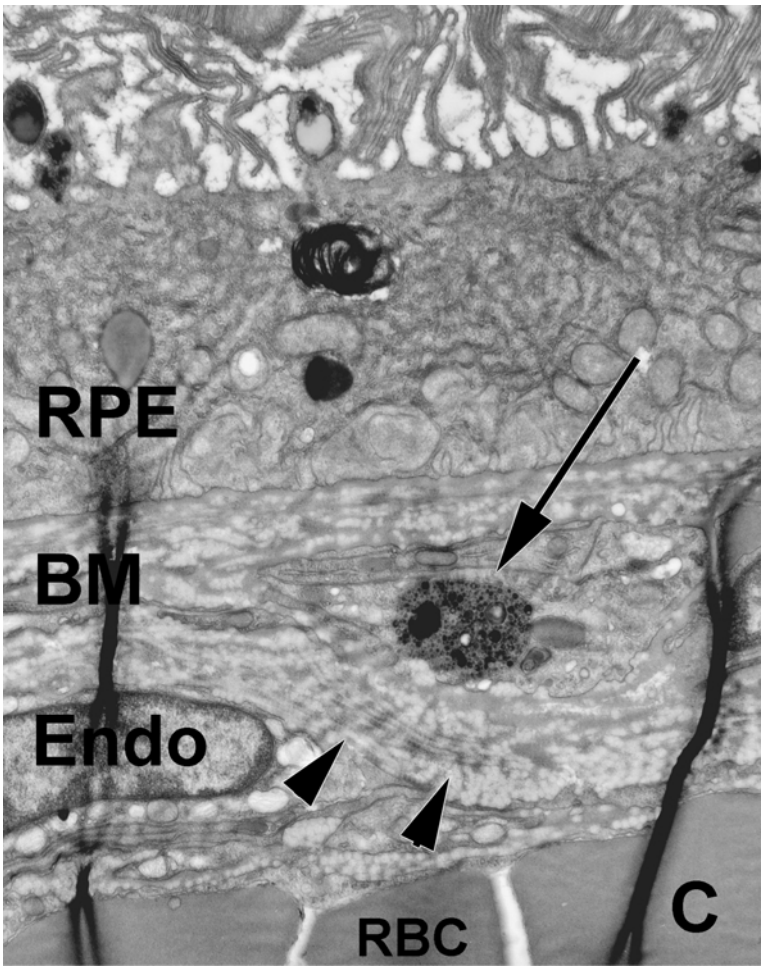


FIGURE 7 **(Continued)**

edge or most peripheral portion of the retina but also in areas toward the equator, prior to extensive loss of photoreceptors. Together, these would alter the oxygen distribution to the peripheral retina from the beneficial one of chronic hyperoxia to a detrimental one of hypoxia. These changes included:

(1) thickening of Bruch's membrane with
    (a) basement membrane proliferation of the RPE, and
    (b) diffuse and nodular thickening of Bruch's membrane;
(2) increased intracellular accumulations of waste materials in the RPE;
(3) degeneration of the choriocapillaris with
    (a) endothelial cell loss,
    (b) collagen deposition, and
    (c) lipofuscin accumulation.

Two other factors that contribute to this hypoxic state are decreased perfusion of the choroidal circulation with age[26] and the fact that the peripheral retina is surrounded by a vascular water-shed zone between anterior ciliary and posterior ciliary arteries, making it particularly prone to ischemic insult.[6] Taken together, these factors would change the level of oxygenation of the peripheral retina with age. This age-related change from hyperoxia to hypoxia would eliminate the protective advantage that the peripheral retina maintains through most of adulthood. Because of this new oxygen state, the peripheral retina would be particularly vulnerable to hypoxia, driving an accelerated loss of photoreceptor cells much like what is seen during development.

Mechanisms for the loss of peripheral retinal photoreceptors are beginning to be discovered. One of the leading theories on the cause of photoreceptor cell loss is the regulation of photoreceptor cell populations based on oxygen supply. Developmentally, photoreceptor cells are lost due to hypoxia, while peripheral photoreceptors near the ora serrata are afforded

"protection" from insult by a chronic level of hyperoxia. Based on careful ultrastructural observations, we have shown how changes outside the neural retina may affect oxygen diffusion and how this may contribute to the accelerated photoreceptor cell loss in the peripheral retina of the aging F344 rat. Whether the mechanism involves FGF-2, CNTF, and GFAP as in other animal models has yet to be determined. Having a readily available animal model to study and manipulate will help to shed light on the complex interaction between age-related photoreceptor cell loss, Müller cell reactivity, RPE degeneration, and choriocapillaris involution.

ACKNOWLEDGMENTS

The authors would like to thank Thurma McDaniel and Karen Bentley for their technical assistance.

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