Title

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Journal

Ultrastructural pathology, 44(2)

ISSN

0191-3123

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Publication Date

2020-03-01

DOI

10.1080/01913123.2020.1731039

Peer reviewed

HHS Public Access

Author manuscript

Ultrastruct Pathol. Author manuscript; available in PMC 2023 February 15.

Published in final edited form as:

Ultrastruct Pathol. 2020 March 03; 44(2): 174–181. doi:10.1080/01913123.2020.1731039.

Retinal and choroidal capillaries contribution to age-related macular degeneration (AMD) phenotypes in murine models of the disease

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Abstract

Mouse models of age-related macular degeneration (AMD) such as $Ccl2^{-/-}$ and $Ccl2^{-/-}/Cx3cr1^{-/-}$ have not yet been fully characterized ultrastructurally. Although we have previously shown extranuclear DNA (enDNA) leakage into the cytoplasm and damaged mitochondria in the retinal pigment epithelium (RPE) of these AMD mouse models, little is known about the state of their vascular capillaries of the retina and choroid. Our ultrastructural survey shows that the aberrations were not restricted to the RPE cells, but also extended to the vasculature of the retina and choroid. Their endothelial aberrations included cytoplasmic degeneration, pyknotic DNA, hypertrophic nuclei, and loss of fenestration in addition to duplication of basement membrane and loss of density in Bruch's membrane. Moreover, the state of the vasculature in the mutant mice models suggests that the capillaries could also be active contributors to the pathological findings seen in AMD. The goal of this study is to gain insights into the early events of AMD that may lead to a better understanding of AMD's pathogenesis, improve our preventative measures, and formulate designed therapeutic regimens that are tailored to target the initial pathological events.

Keywords

Transgenic mice; blood vessel; capillaries; choriocapillaris; endothelial cell; endothelial fenestration; Bruch's membrane; retinal pigment epithelium (RPE); retina

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Declaration of Interest

The authors declare they have no conflicting financial interests.

Introduction

Age-related macular degeneration (AMD) is a common degenerative eye condition, primarily affecting people age 50 and older, and is more prevalent among Caucasians than in black or Latino populations.[1] AMD has a genetic correlation which places those with a family history at higher risk of developing the disease.[2] There are many risk factors associated with AMD susceptibility which include aging, genetics, environmental factors, diet, smoking, and oxidative damage.[3] Currently, the initial events of AMD are still speculative; furthermore, there is a dearth of data on the early histopathological changes that occur in AMD and particularly in regard to choroidal integrity.[4]

Defining early detectable signs of AMD can be useful in clinical settings for diagnosis and management of the disease. Current translational and clinical research is primarily focused on the retinal pigment epithelium (RPE) as a course of therapeutic application in AMD. Previous studies have sought to slow or halt the progression of the disease using various therapeutic modalities such as antioxidants, lasers, and VEGF inhibitors.[5] However, without a fundamental understanding of the early events of AMD pathogenesis, these interventions may be ineffective.

Healthy retinal metabolism is reliant on a steady supply of oxygen, and has two vascular conduits, the choroidal and the retinal capillaries. The areas that are affected by the progression of AMD are the RPE and Bruch's membrane, which are both supported by the eye's vascular tunic, the choriocapillaris. The choriocapillaris provides the eye with its blood supply, allowing the retinal tissue to obtain necessary oxygen and nutrients. The retina is metabolically active and requires vast amounts of oxygen and nutrients to function.[6] The choroid supplies an estimated 80% of the required nutrients to the retina, while the other 20% is covered by the retinal vasculature.[7] The choriocapillaris is further segmented into discrete capillary beds called lobules.[8] The fenestrations found throughout the endothelium of this capillary network lend the vascular tunic a uniquely efficient means of delivering oxygen and vital nutrients to the underlying retinal epithelium. The vasculature also provides a means for waste disposal and the delivery of immune scavenger cells such as macrophages to the macular area.[9] Choroidal capillary dilapidation may precipitate the events in AMD, and set the ground for drusen accumulation in patients with macular degeneration.[4] Thus far, little histopathological findings at the ultrastructural level have been reported on the vasculature status of AMD in the murine models. For this reason, our comparative study seeks to define the vascular structural changes in AMD mouse models in order to gain insights into the pathological events that occur at the ultrastructural level.

Currently, there are two primary competing hypotheses that speculate on the early events that initiate AMD. The first hypothesis is fundamentally focused on the RPE integrity, and its subsequent degradation, which is hypothesized to precipitate the macular dysfunction and its ultimate breakdown.[10] The second hypothesis, called Freidman's hemodynamic theory, posits that impaired blood flow is the pathogenic precursor that is responsible for the early derangement seen in AMD. This theory attributes this impairment of blood flow to a systemic atherosclerosis process that causes choroidal dropout, and loss of endothelial

fenestration of the choriocapillaris.[10–12] Reduced blood flow and obstructed perfusion have been observed in human AMD cases.[13]

Our research investigates vasculature integrity at the ultrastructural level in the choriocapillaris and the neural retina to assess its contribution to the AMD phenotype in our mouse models.[14] Although mice eyes lack macula, their AMD models contribute to our understanding of the physiology and pathology of AMD. Variants of Ccl2 and Cx3cr1 have been shown to play a role in age-related complex diseases.[15,16] Therefore, AMD has been modeled in Ccl2/Cx3cr1 knockout mice because polymorphisms in these genes have been associated with increased risk of developing AMD in humans.[14,16,17] Additionally, these mouse models have been demonstrated to illustrate AMD signs and drusen deposits in their retina.[18,19]

Materials & methods

Donor mice

The following mouse strains were used in the study:[1] C57BL/6J wild type negative control (n = 6);[2] C57BL/6N (*Crb1*^{rd8} mutation) positive control (n = 6);[3] *CCl2*$^{-/-}$single knockout (n = 6); and[4] *CCl2*$^{-/-}$/*Cx3Cr1*$^{-/-}$ double knockout (n = 6). The last two strains were on C57BL/6N background.[14]

Mice were 1.5–6 months old. The 1.5 months mice represented the double knockout mice because these started showing their retinal lesions around this time while the single knockouts took longer time, up to 6 months. The presence of retinal lesions was confirmed by fundoscopy prior to enucleation of the eyes.[20]

The C57BL/6N (*Crb1*^{rd8} mutation) is not a model of AMD but rather a model of Crb1-associated focal retinal degeneration with earlier onset of AMD characteristics such as focal photoreceptor atrophy, RPE degeneration, elevated ocular A2E levels, complement deposition, as well as retinal dystrophy.[21] Chemokine ligand-2 (*CCl2*) plays an important role in recruitment of monocytes from peripheral blood, and its dysfunction is implicated in inflammatory processes that result in retinal lesions.[15] Single nucleotide polymorphisms (SNPs) in the chemokine receptor *Cx3Cr1* are associated with AMD and other degenerative diseases because they affects microglia's functions, thus altering homeostasis.[16,22]

Single knockout mice of *CCl2*$^{-/-}$ or *Cx3Cr1*$^{-/-}$develop key characteristics of human AMD, while the double knockout *CCl2*$^{-/-}$/*Cx3Cr1*$^{-/-}$ strain shows earlier onset of these characteristics, higher penetrance and more lesions; thus, this strain serves as an acceptable model for studying AMD and screening therapies.[16,20,21]

Tissue preparation

The eyes were enucleated, fixed in 2.5% buffered glutaraldehyde, then processed for transmission electron microscopy (TEM) according to Ogilvy et al.[20] Briefly, specimens were washed in phosphate buffered saline (PBS), post-fixed in 0.5% osmium tetroxide (OsO_4), rinsed, dehydrated, then embedded in epoxy resin. Blocks were sectioned at ~90 nm thickness on a Leica EM UC6 ultramicrotome (Leica, Austria), double-stained with uranyl

acetate and lead citrate, and imaged with JEOL JEM-1010 electron microscope (JEOL, Japan).

Results

C57BL/6J wild type negative control

The choriocapillaris of the wild type C57BL/6J strain showed no ultrastructural aberrations (Figure 1a,b). The lumina appeared well expanded and contained healthy red blood cells (RBCs) without unusual inclusions or cellular debris. Capillaries' lumina were fully lined with endothelial cells lacking any abnormalities. The apical side of endothelial cells showed normal fenestrations. The endothelial nuclei were located posteriorly close to the basement membrane or laterally at the junctions between the endothelial cells and were normal in size. Basement membranes were unremarkable and did not show any splitting or duplication as was seen in the knockout or the positive control mice (Figure 1c–h). Pericytes were also normal in shape and location. Similarly, the Bruch's membrane was regular in thickness and intact.

The vessels of the neural retina lacked ultrastructural abnormalities (Figure 2a,b). The cross-sectional images showed vessels with patent lumen containing normal RBCs; the endothelial cells completely lined the vessels' lumina and their nuclei were lying flat against the basement membrane. Thin basement membranes were continuous and surrounded the vessels and mural cells. Mural cells and retinal tissues around the vessels appeared healthy.

C57BL/6N (Crb1^{rd8} mutation) positive control

Unlike in the "negative control" mouse model, choriocapillaris of the positive control mice had several abnormalities including degenerate endothelial cytoplasm and hypertrophic nuclei, absent fenestrations, and split basement membranes (Figure 1c,d). The hypertrophic endothelial nuclei (HEN in 1C & D) were anteriorly relocated, an abnormal position that blocks a stretch of the exchange area with the RPE, thus reducing perfusion efficacy. Capillaries' lumina appeared constricted with fewer RBCs in comparison to those in the wild type. Pericytes appeared degenerate (DPC in 1C)). Bruch's membrane was disintegrated and RPE cells were necrotic (Figure 1c,d).

Vessels of the neural retinal in the outer plexiform layer (OPL) and inner plexiform layer (IPL) had several obvious aberrations (Figure 2c–e). The vessels had degenerate endothelial cells; thickened and split basement membranes, as well as collapsed or occluded lumina. The cytoplasm of mural cells appeared degenerate (Figure 2c,d). In general, most of the vessels appeared dysfunctional and retinal cells adjacent to the vessels were necrotic and edematous.

Ccl2$^{-/-}$ single knockout

In the single knockout (*Ccl2*$^{-/-}$) mouse eyes, the choriocapillaris showed several ultrastructural aberrations (Figure 1e,f). The endothelial cells' cytoplasm was degenerate or lacking and their anterior fenestrations were lost. The nuclei were hypertrophic (HEN) with pyknotic chromatin and abnormally relocated anteriorly. The capillaries' lumina

were constricted or totally occluded; basement membrane was thin, split, and irregular in appearance. Pericytes were degenerate (DPC). Bruch's membrane (BM) appeared intact, while the RPE cells were necrotic.

In the neural retina, vessels of the OPL (Figure 2f,g) lacked normal-looking endothelial cells; the cytoplasm of mural cells was necrotic with shrunken nuclei and pyknotic chromatin. Basement membranes were degenerate and split. The capillary lumina were either constricted or totally occluded. Retinal cells adjacent to the vessels were necrotic.

Ccl2$^{-/-}$/Cx3cr1$^{-/-}$ double knockout

The ultrastructural findings in the eyes of the double knockout (*Ccl2* $^{-/-}$*/Cx3cr1* $^{-/-}$) mice showed similar aberrations as those seen in the single knockout ones, however, these mice showed these aberrations as early as 6 weeks. The choriocapillaris showed several ultrastructural aberrations (Figure 1g,h). The endothelial cells had degenerate cytoplasm, absent anterior fenestrations, and hypertrophic nuclei that migrated to abnormal anterior location. The lumina of capillaries were constricted and contained inclusions of cellular debris. The basement membranes were split into pockets that occasionally contained pericytic nuclei that were hypertrophic (Figure 1g); pericytes were necrotic. Bruch's membrane (BM) appeared intact, while RPE cells seemed necrotic and had degenerate basal infoldings.

The retinal vessels (Figure 2h,i) showed degenerate cytoplasm in endothelial and mural cells. The lumina of the retinal vessels of the IPL, OPL, ganglion cell layer (GCL) and nerve fiber layer (NFL) were constricted or occluded, and no RBCs were seen in their lumina. The basement membrane was split into pockets containing cellular debris. The surrounding retinal cells were necrotic and edematous (Figure 1i).

Discussion

Our ultrastructural survey shows that the aberrations in the studied AMD mouse models extend beyond degenerate mitochondria and extranuclear DNA (enDNA) of RPE to the vasculature of both retinal and choroidal tissues of the eye. The aberrations in the capillaries of the mutant mice suggest that these vascular abnormalities are the major causes for the pathogenesis and the development of AMD phenotype. The negative control mouse model used in our study depicted normal choriocapillary characteristics, including thin and fenestrated anterior endothelial wall abutting Bruch's membrane, posteriorly or laterally located healthy endothelial nuclei, morphologically normal RBCs in the lumina, sparse pericytes on the scleral side, and numerous melanocytes in choroidal tissue. However, the single and double knockout mouse models showed a breakdown of the choroidal structure, many abnormalities in the choriocapillaris, and degenerate retinal capillaries.

Studies have established that the integrity of neural retinal vasculature is crucial for a healthy fovea, whereas pathologic disruptions of its integrity results in derangement of metabolic processes in the macular region.[23] Aberrant blood flow in retinal capillaries has long been speculated to play a major role in the pathogenesis of AMD.[24–26]

In our two mouse models of AMD, the retinal vessels appeared atrophic with collapsed lumina that severely restricted blood flow. Mural cells were atrophic with pyknotic nuclei. The basement membranes were degenerate with debris in their pockets. These abnormal ultrastructural changes caused atherosclerotic narrowing of the blood vessels that led to impaired perfusion and consequent ischemic necrosis of the retinal tissue.

Many studies have implicated impaired choroidal perfusion of the RPE with its impact on the metabolic processes in the macular area as a major cause of the deterioration and the drusen formation that are seen in AMD.[4,10,12] Interestingly, choriocapillaris density correlates with functionality, thus disease progression indicates a mechanistic phenomenon occurring at the choriocapillaris that possibly drives AMD pathogenesis.[27] There is also increasing support for the hypothesis that there may be a potential link between AMD pathology and atherosclerosis progression.[28] Our ultrastructural survey has correlated the single and double knockout mutations (*Ccl2*$^{-/-}$ and *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$) with damaged choriocapillaris and the development of an AMD phenotype. Therefore, our observations lend support to the hemodynamic theory of AMD since the development of an AMD phenotype was associated with damaged choriocapillaris and restricted blood flow.

In these two AMD mouse models, the damage was not restricted to the vasculatures of the choriocapillaris and neural retina. We have previously shown that these models have damaged DNA and mitochondria in their RPE cells.[20] The DNA damage was characterized by chromatin leakage into the cytoplasm and the formation of chromatin vesicles, a phenomenon that we have usually observed in human specimens of AMD.[1,20] Furthermore, in a situation analogous to these mouse models, we have shown mitochondrial susceptibility to damage in AMD patients' eyes, and demonstrated the same findings in RPE cell cultures as an inflammatory model of AMD.[1,20] The ultrastructural aberrations of these AMD models mirror the same pathological changes that we have observed in human AMD eyes; both share aberrant choroidal and retinal vasculature, enDNA, and mitochondrial degeneration. Without a doubt, the *Ccl2*$^{-/-}$ and *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$knockout mice represent an analogous disease model of AMD.

Conclusion

Overall, our ultrastructural analysis of mouse models of AMD has shown that the integrity of the choroidal and retinal vasculatures does in fact degrade in lockstep with progression of an AMD phenotype. While this conclusion does not determine the mechanisms for AMD pathology, it does, however, provide supportive evidence for the hemodynamic theory of AMD. Further research in macular degeneration, with a focus on the choriocapillary aberrations is needed to provide insights into the early events and mechanistic pathways that ignite the relentless pathogenic progression that is generally seen in AMD.

Acknowledgments

The mouse model, *CCl2*$^{-/-}$*/Cx3Cr1*$^{-/-}$ double knockout, was generated at Dr. Chi Chao Chan's laboratory at the National Eye Institute, NIH, Bethesda, MD.(14) We are thankful to Ms. Heba Mohammed for her editorial assistance.

Funding

The research was supported by the intramural program of the National Eye Institute.

Abbreviations:

AMD	age-related macular degeneration
BM	Bruch's membrane
DPC	degenerate pericyte
EN	endothelial nucleus
enDNA	extranuclear DNA
GCL	ganglion cell layer
HEN	hypertrophic endothelial nucleus
IPL	inner plexiform layer
NFL	nerve fiber layer
OPL	outer plexiform layer
RBC	red blood cell
RPE	retinal pigment epithelium
SNPs	Single nucleotide polymorphisms

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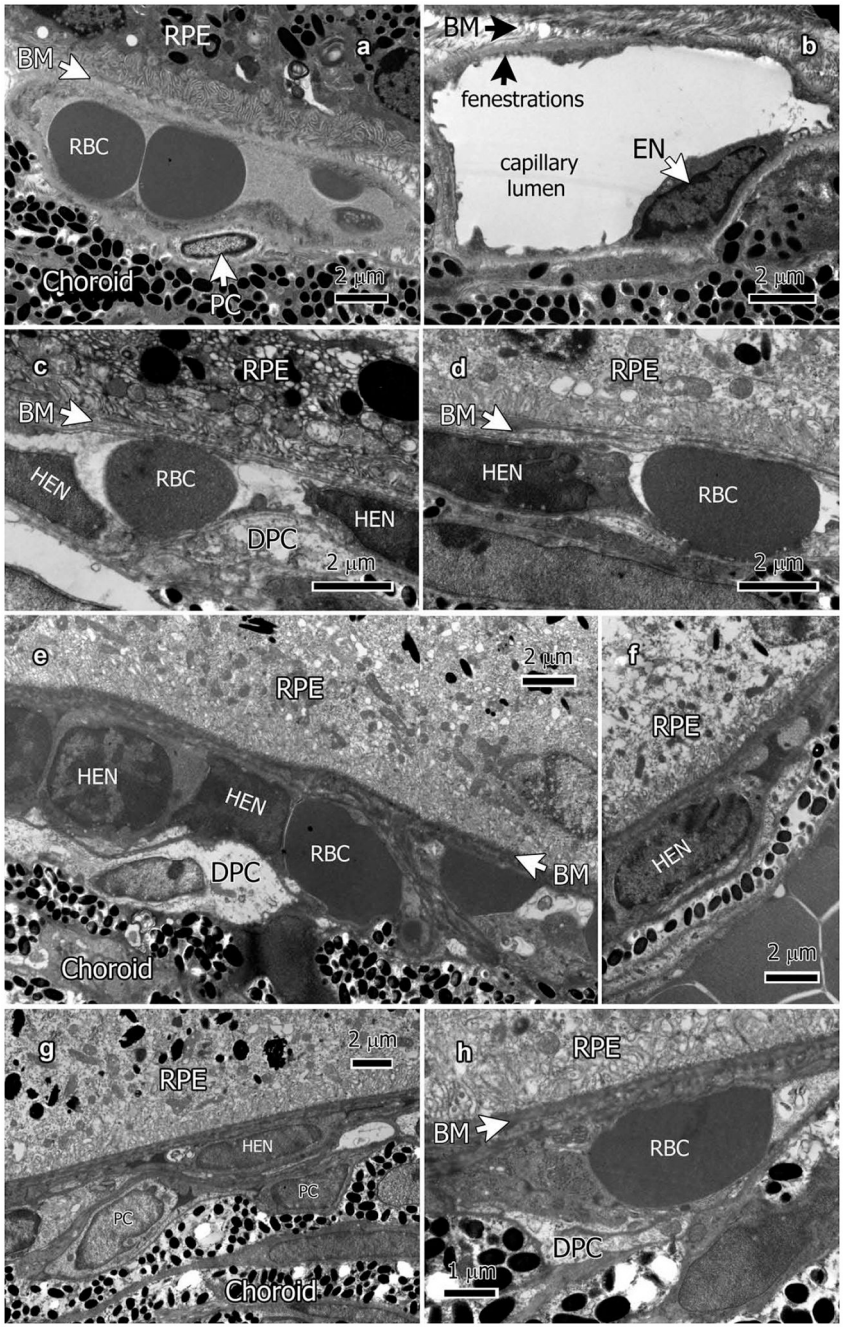

Figure 1.

Representative TEM micrographs of choroidal capillaries of the 4 mouse strains. (a, b) Wild type C57BL/6J, choroidal capillaries lack abnormalities. Endothelial cytoplasm is regular in appearance. Fenestrations are present in their regular anterior location (black arrow in B upper left corner). Nuclei of regular size and located posteriorly on the scleral side. Basement membrane regulr and no spliting or thickening were seen. (c, d) In the C57BL/6N, endothelial cells had degenerate cytoplasm, fenestrations were absent, and hypertrophic nuclei were abnormally anteriorly located. Splits in the basement membrane were visibles (d, lower right). Bruch's membrane had disintegrated and lost density (white arrows in

c, d). Some RPE cells were necrotic, and others had reduced number of melanosomes. (e, f) In the single knockout Ccl2−/− endothelial cytoplasm was scant, degenerate, or absent; the endothelial nuclei were pyknotic, hypertrophic and anteriorly relocated, and fenestrations were absent. The basement membrane was split, vessels' lumina were reduced, and pericytes were degenerate (DPC in e). (g, h) In the double knockout Ccl2 −/−/Cx3cr1−/−, the capillaries contained inclusions of cellular debris, and the basement membranes were split into pockets that occasionally contained pericytic nuclei (g). The lumina were constricted with hypertrophic endothelial nuclei (g) or cellular debris (h). Capillaries lacked fenestrations at the interface with Bruch's membrane. The hypertrophic cytoplasm of endothelial cells was degenerate and contained mitochondria with abnormal degenerate appearance. The RPE was necrotic.

Abbreviations: BM = Bruch's membrane; DPC = degenerate pericyte; EN = endothelial nucleus; HEN = hypertrophic endothelial nucleus; RBC = red blood cell; RPE = retinal pigment epithelium.

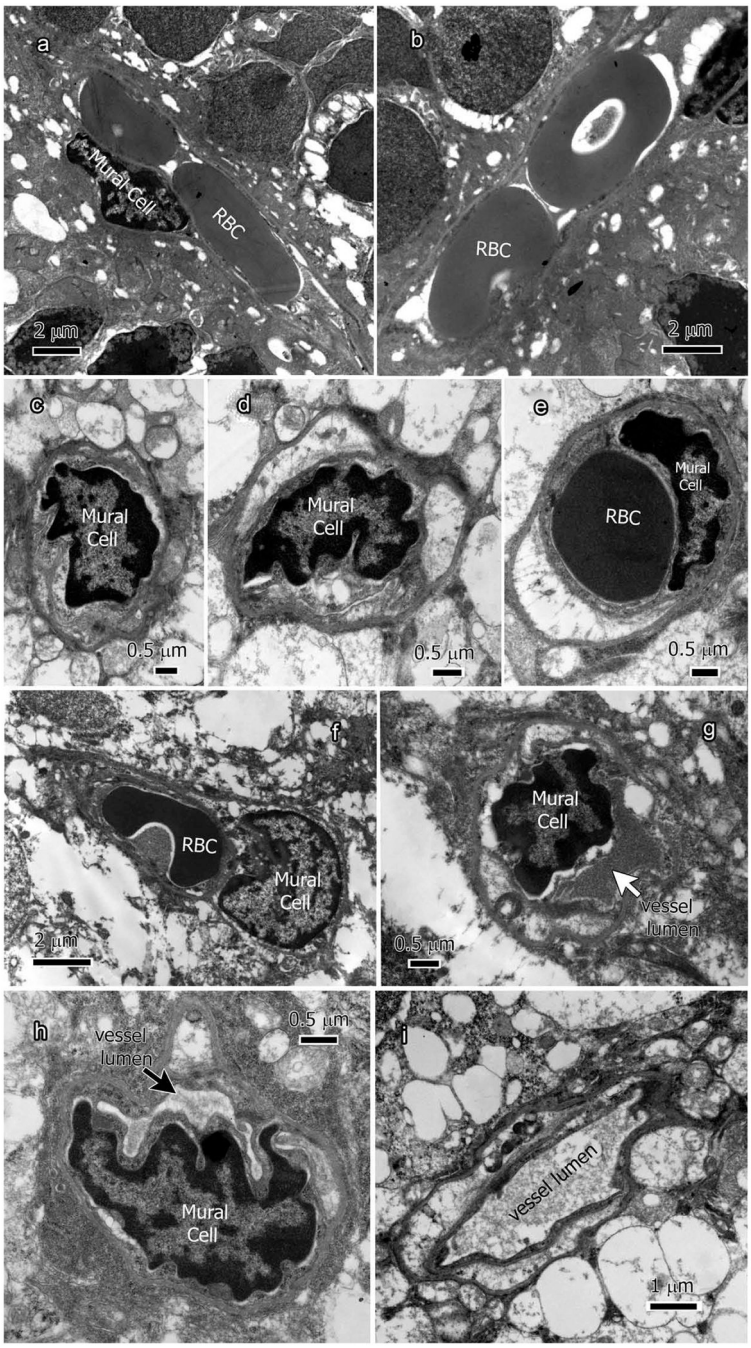

Figure 2.

Representative TEM micrographs of retinal vessels of the 4 mouse strains. (a, b) Wild type C57BL/6J vessels in the outer plexiform layer (OPL) were unremarkable and lacked ultrastructural abnormalities. Vessels' lumina were wide, fully lined with endothelial cells, filled with RBCs, and clasped with mural cells. (c–e) Retinal vessels of the C57BL/6N mouse strain in OPL and inner plexiform layer (IPL) showed degenerate endothelial cells, mural cells with disintegrated cytoplasm, constricted or collapsed lumina (c, d), as well as thickened and split basement membranes. The surrounding retinal cells were highly

degenerate and lacked internal structures. (f, g) In the Ccl2−/− knockout mice, OPL vessels had split and, in some places, disintegrated basement membranes, and both mural cells and endothelial cells had scant and degenerate cytoplasm. Surrounding retinal cells were necrotic. (h, i) The retinal vessels of the double knockout Ccl2 −/−/Cx3cr1 −/− mouse strain showed degenerate endothelial cells, mural cells had disintegrated cytoplasm, and the lumina were reduced and constricted with thick proteinaceous accumulation (i). The basement membrane was split into pockets containing cellular debris. Surrounding retinal cells were necrotic and edematous.