Ultrastructural Changes in a Murine Model of Graded Bruch Membrane Lipoidal Degeneration and Corresponding VEGF$_{164}$ Detection

Ursula Schmidt-Erfurth,1 Martin Rudolf,2,3 Marion Funk,1 Carmen Hofmann-Rummelt,4 Nikola-Simone Franz-Haas,4 Zoubair Aberrahrou,5 and Ursula Schlötzer-Schrehardt4

PURPOSE. To evaluate ultrastructural changes in low-density lipoprotein (LDL) receptor knockout (R$^{-/-}$) mice consuming different diets as a potential model of Bruch membrane (BM) lipoidal degeneration and to determine the distribution and concentration of VEGF$_{164}$ in this mouse model.

METHODS. Eight-month-old LDL-R$^{-/-}$ mice and wild-type controls were fed a standard or a high-fat diet. Animals were killed, and plasma cholesterol levels were determined. Using transmission electron microscopy, BM thickness, lipid vacuole size, and retinal pigment epithelial height were measured. Degenerative alterations of choriocapillaris, RPE, and photoreceptors were described and graded. Using light microscopy, VEGF$_{164}$ immunohistoreactivity was graded. Neutral lipids were detected with oil red O.

RESULTS. Choriocapillaris, BM, RPE, and photoreceptors of standard diet control animals showed a regular architecture. LDL-R$^{-/-}$ mice fed a standard diet showed more diffuse focal alterations than control mice fed a high-fat diet. Within the choriocapillaris, the basement membrane was thickened, endothelial fenestration numbers were reduced, and lumina narrowed. BM thickness increased with a loss of regular structure. With pronounced BM degeneration, lipid inclusions increased in number and size. A decrease in retinal pigment epithelial cell height was accompanied by signs of intracellular degeneration. Photoreceptor outer segments showed focal degeneration and the formation of vacuoles. All these changes were most pronounced in LDL-R$^{-/-}$ mice after a high-fat diet. VEGF$_{164}$ was found exclusively in the choriocapillaris, positively correlating with the amount of lipid accumulation in BM.

CONCLUSIONS. Feeding a standard or a high-fat diet to LDL-R$^{-/-}$ mice and wild-type controls resulted in a reproducible model of graded BM lipoidal degeneration that resembled alterations in aged human eyes. This model provides a valuable tool for investigating biological responses to lipoidal degeneration. (Invest Ophthalmol Vis Sci. 2008;49: 390–398) DOI:10.1167/iovs.07-0227

Age-related macular degeneration (AMD) is the leading cause of permanent vision loss in elderly persons living in developed countries. It is considered a multifactorial and complex disorder that results from environmental and genetic effects and from aging. Despite its high prevalence, its etiology remains largely unknown. The most prominent clinical and histopathologic features involve the retinal pigment epithelium (RPE), Bruch membrane (BM), and the choriocapillaris.1–2 BM is a thin, five-layer connective tissue between the metabolically active RPE and the choriocapillaris that provides the RPE and the outer retina with nutrients. Its central position shows how essential this structure is for the maintenance and health in adjacent tissues. With age, the human BM undergoes distinct, well-established changes thought to compromise metabolic transport through it.3 Human BM thickness increases significantly with age, and structural degeneration of the elastic and collagenous layers also increases.3–5 Simultaneous with these changes, neutral lipids accumulate in BM.1,6 The source of the accumulating lipids is still uncertain.7–8 Because of its unique position, BM is subject to possible changes from two sides, blood-derived lipids on the choriocapillaris side and metabolically derived lipids secreted on the retinal pigment epithelial side. Which portion each side contributes to the observed age-related lipid accumulation in BM is still under investigation. Studies on BM lipid composition provide information that BM lipids do not derive exclusively from the blood, as they do in atherosclerotic lesions.7,9,10

Although the connection between AMD and atherosclerosis and its risk factors remains inconclusive despite numerous studies, it is still thought that these multifactorial, complex diseases may share common pathways.8,11 In general, both diseases are marked by accumulations of extracellular lipids, oxidative stress, and inflammation. In atherosclerosis, the accumulation of low-density lipoprotein (LDL) in vascular walls and its subsequent oxidation and ingestion by macrophages is thought to be the principal cause of atherosclerotic plaque formation. Damage to the endothelial vascular lining appears to be the earliest detectable injury to the vascular wall in atherosclerosis. Free radicals, as produced by oxidized LDL or activated leukocytes, may subsequently disrupt the continuity of the intraluminal surface.11

In the early stages of AMD, cholesterol also accumulates in the BM and may lead to the subsequent oxidation of cholesterol and to concomitant inflammation and damage to the BM, RPE, and choriocapillar layer, either directly or through immune-mediated processes.1,11 Several experimental and clinical findings indicate that cumulative oxidative damage may play an important role in the pathogenesis of AMD.12

The use of atherosclerotic mouse models in ophthalmic research may help to elucidate aspects in the pathogenesis of AMD.13,14 Therefore, we investigated ocular alterations in LDL-R$^{-/-}$ mice, a well-established model of hypercholesterolemia.

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in atherosclerosis research.15,16 LDL receptors are found on all body cells.17 In general, LDL receptors largely regulate the uptake of plasma LDL into the body cells for cholesterol supply.17 Deleting this receptor inhibits the efficient clearance of plasma LDL, resulting in substantially increased plasma total cholesterol levels even under standard diet.15 Additionally, plasma cholesterol levels can be modified through a high-fat diet. Wild-type C57BL/6J mice were used as controls.

The RPE is a very active cell layer that secretes a variety of extracellular matrix proteins, complement factors, protease inhibitors, and other regulatory proteins. It reacts to environmental changes by adapting its secretion rates.18 We were particularly interested in proangiogenic agents, which play an important role in late-stage neovascular AMD and lead to sudden and irreversible loss of vision. The best-studied proangiogenic agent is vascular endothelial growth factor A (VEGF-A), a major stimulus of pathologic ocular angiogenesis.19 On the other hand, VEGF-A is known to be constitutively produced by retinal pigment epithelial cells and secreted to their basal side, serving as a paracrine signal pathway between the RPE and the choriocapillaris.19,20 It is a known survival factor for endothelial cells and induces endothelial fenestration.19-21 In a pilot study, we already found elevated VEGF-A levels (specifically isoforms 164,189 and 206a) in the basal RPE, outer plexiform layer, and photoreceptor inner segments, correlating with increased lipid accumulations in BM.15 Nevertheless this pattern, especially intraretinal expression of VEGF-A, addressed in part only the potential pathogenesis of neovascular AMD. For a more detailed view, we chose to investigate separately the diffusible VEGF isoform 164, which is the rodent equivalent to the human isoform 165. Higher VEGF isoforms are thought to be cell associated and unlikely to diffuse. In humans, VEGF165 is the most prevalent isoform and is abundantly found in surgically excised choroidal neovascularization (CNV) and AMD eyes at autopsy.22-25 It is also a predominant target of pegaptanib, an effective inhibitor of VEGF-induced CNV growth.19 In 2004, pegaptanib was the first FDA-approved VEGF inhibitor for the treatment of subfoveal CNV secondary to AMD.

In this study, we sought to determine whether graded BM lipoidal degeneration in LDL-R−/− mice and wild-type controls was reproducible and valuable for investigating biological responses. To characterize the BM alterations and the effect on adjacent structures, we evaluated transmission electron microscopy (TEM) images and measured retinal pigment epithelial height, BM thickness, lipid vacuole size, and endothelial fenestration grade. With oil red O, we could show that murine BM stained positive for neutral lipids, the suspected content of the vacuoles in TEM images. Finally, we performed immunohistochemical staining for VEGF165 to identify the distribution and pattern of this angiogenic agent according to the degree of BM lipoidal degeneration.

Materials and Methods

Animals

Female wild-type C57BL/6J and LDL receptor-deficient C57BL/6J-LDL-R−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ethics committee approval was obtained. Animals were kept in plastic cages with regular light-dark cycles and had continuous free access to water and food. Until month 6, all animals were on a regular rodent chow diet (Altromin 1324-pellet; Altromin, Lage, Germany) containing 19% protein, 4% fat, 6% fiber, 7% ash, 15.5% moisture, 0.9% calcium, 0.7% phosphorus, 15,000 IE vitamin A, 600 IE vitamin D3, 75 mg vitamin E, and 5 mg copper. At 6 months, animals were subdivided into two groups, each consisting of 10 animals. One group contained five mice of each genotype continued on the regular diet. The other group of 10 animals also included five C57BL/6J and five C57BL/6J-LDL-R−/− mice, but the diet was changed for 2 months to a high-cholesterol, Western-style diet containing 75% Purina chow plus 15% fat (primarily cocoa butter), 1.25% cholesterol, and 0.5% sodium cholate (TD 90221; Teklad Research Diets, Madison, WI). All mice were humanely killed at 8 months of age.

In summary, our study design resulted in four groups of different genotypes and diets. Each group consisted of five animals. Ten eyes per group were available for histologic examination (ntotal = 40); seven eyes per group (ntotal = 28) were used for both TEM and immunohistochemistry, and three eyes per group (ntotal = 12) were used for oil red O staining. Only five eyes per group, obtained from different animals, were subjected to morphometric analysis of ultrastructural parameters.

Plasma Cholesterol Levels

All animals were subject to plasma lipid analysis. Plasma from the overnight fasted mice was collected by retro-orbital venous puncture with heparinized capillary tubes (Becton Dickinson, Franklin Lakes, NJ) under isoflurane anesthesia before the animals were killed. Plasma total cholesterol and triglyceride levels were measured according to the instructions of the manufacturer using cholesterol and triglyceride kits from Sigma (Munich, Germany; ID 352-4) and Wako Pure Chemicals (Richmond, VA; ID 933-37994), respectively. The principle of these enzymatic tests is, as the first step, to convert the sample to a substrate. In the second step, an enzyme uses the substrate to converting a colorless reagent to a color whose intensity can be measured quantitatively with a photometer. For cholesterol, 20 μL plasma samples, standards (Sigma, Munich, Germany), and negative controls (water) were pipetted into a 1.5-mL tube and mixed with 500 μL cholesterol reagents (Sigma). After 5-minute incubation at 37°C, the absorbance at 500 nm was determined. High-density lipoprotein (HDL) levels were determined after precipitation of very low-density lipoprotein (VLDL) and LDL with an HDL precipitation reagent (Sigma; ID 352-4). For triglyceride, 1 mL reagent (reagent-1) was added to 12 μL plasma samples, standards, and negative controls and was incubated for 5 minutes at 37°C. A second reagent (reagent-2) was then added, mixed, and incubated at 37°C for 5 minutes, and the absorbance was determined at 600 nm.

Transmission Electron Microscopy

Immediately after the animals were killed, their eyes were enucleated. Without delay, seven eyes from each of the four groups (ntotal = 28) were briefly fixed in 4% buffered paraformaldehyde and bisected for further processing for TEM and immunohistochemical analysis. The halves used for TEM were fixed in 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 2% buffered osmium tetroxide, dehydrated in a graded ethanol series, and embedded in epoxy resin according to standard protocols. Semithin sections (1-μm thick) were stained with toluidine blue; ultrathin sections were stained with uranyl acetate and lead citrate and were examined with an electron microscope (LEO 906E; Carl Zeiss; Oberkochen, Germany).

For quantitative analysis of ultrastructural parameters, an automated image-processing system (Digivision; Carl Zeiss) with an integrated software package (Analysis; Soft Imaging Systems, Münster, Germany) was used. From each specimen, three sections, including the central retina, were investigated. Measurements were performed according to a random sampling procedure using the bars of the supporting grid as points of reference, by which 10 consecutive areas adjacent to the right side of a grid bar were analyzed (total, 30 measurements per eye). The investigator (NF) performing electron microscopic evaluations and measurements was masked regarding the origin of the specimens.
Immunohistochemical Analysis

The second halves of the bisected 28 eyes were fixed in buffered 4% paraformaldehyde solution for 24 hours and embedded in paraffin according to standard protocols. Immunohistochemistry was performed on 5-μm-thick paraffin sections by the peroxidase-labeled streptavidin-biotin method, with the use of a kit in accordance with the manufacturer’s instructions (LSAB Plus; DAKO, Glostrup, Denmark), and a purified polyclonal rabbit antibody against VEGF165 at a concentration of 10 μg/ml (Abcam, Cambridge, UK). Briefly, sections were incubated with primary antibody, biotinylated link antibody, and peroxidase-conjugated streptavidin for 30 minutes each. 3-Amino 9-ethyl carbazole (AEC) was used as a chromogenic substrate, and Mayer hemalum was used as a counterstain. In negative control experiments, the primary antibody was omitted or was replaced by equimolar concentrations of preimmune rabbit immunoglobulin G. In positive control experiments, formalin-fixed paraffin-embedded angiosarcoma sections known to express VEGF were used. From each eye, three sections, including the central retina, taken at 300-μm steps were stained and evaluated in a masked fashion (NF).

Oil Red O Staining

For localization of neutral lipids, the oil red O staining method was used. Three eyes from each of the four groups (n_total = 12) were embedded in OCT compound and frozen in liquid nitrogen. Cryosections (5-μm thick) were air dried, rinsed in PBS, and stained with oil red O saturated in 70% ethanol for 10 minutes, followed by repeated washing with PBS. Counterstaining of nuclei was performed with Mayer hemalum. Finally, the sections were mounted with glycerine jelly (Aquatex; Merck, Darmstadt, Germany) and analyzed by light microscopy for bright red-stained neutral lipids. We used orbital fat as an internal positive control.

Statistical Analysis

Mean (±SD) plasma cholesterol levels were calculated. The Shapiro-Wilk test was used to test whether plasma cholesterol levels were distributed normally. Two-way ANOVA was performed to determine statistically significant differences in cholesterol levels according to diet and LDL receptor deficiency. For statistical analysis of the morphologic parameters from TEM imaging, the mean (±SD) of the individual 30 measurements was calculated for each eye. Mean values were compared between groups using the nonparametric Mann-Whitney U test; differences were considered significant at P < 0.05. We used a software program (SPSS for Windows, version 6.0.1; SPSS Inc., Chicago, IL) for statistical analysis.

RESULTS

In this model, a graded increase of plasma total cholesterol could be realized. Lipid levels of the controls on a standard diet were our reference (Table 1). High-fat diets for 2 months already increased the plasma total cholesterol level significantly in controls. In comparison, LDL-R−/− mice exhibited elevated plasma total cholesterol levels even with a standard diet, which further increased under a high-fat diet (Table 1).

All control animals exhibited a uniform overall BM thickness of approximately 0.5 μm (Table 2). The regular pentilaminar architecture of BM, consisting of basal lamina of RPE, inner collagenous layer, elastic layer, outer collagenous layer, and basal lamina of the choriorapillaris, could be distinguished in TEM (Fig. 1A). In three eyes of control mice following the high-fat diet, focal vacuoles approximately 0.2 μm in size began to appear within the BM (Fig. 1B; Table 2). Discrete condensation of collagenous and elastic fibers was also observed in these specimens. In contrast, the entire BM of all LDL-R−/− mice was substantially thickened and reached a maximum of 0.8 μm after a high-fat diet (Table 2). The condensation of the collagenous and elastic fibers was enhanced. The orderly lamination of BM was no longer distinguishable (Figs. 1C, 1D). Round to horizontally oval vacuoles (approximately 0.5 μm in diameter) appearing electron lucent in TEM were diffusely distributed in all BM of LDLR−/− mice, increasing in number and size (up to 0.8 μm) in knockout animals after a high-fat diet (Figs. 1C, 1D; Table 2). Oil red O staining revealed, by bright-field microscopy examination neutral lipids, the presumed content of the electron-lucent vacuoles in TEM images. Consistent with TEM findings, none of the controls had any detectable oil red O staining (Fig. 2A). All LDL-R−/− mice showed variable amounts of oil red O-positive inclusions in BM, which displayed a nodular or laminar arrangement (Fig. 2B) and were diffusely distributed throughout the peripheral and central areas of the posterior pole.

Table 1. Plasma Levels of Control Mice and LDL Receptor Knockout Mice

<table>
<thead>
<tr>
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<th>Controls</th>
<th>LDL-R−/−</th>
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<tbody>
<tr>
<td></td>
<td>Chow (n = 10)</td>
<td>HF (n = 10)</td>
<td>Chow (n = 10)</td>
<td>HF (n = 10)</td>
<td></td>
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<tr>
<td>Plasma total cholesterol</td>
<td>90 ± 35</td>
<td>151 ± 48*</td>
<td>196 ± 91*</td>
<td>489 ± 145*</td>
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<tr>
<td>Triglycerides</td>
<td>27 ± 7</td>
<td>42 ± 7</td>
<td>75 ± 26*</td>
<td>144 ± 39*</td>
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<tr>
<td>High-density lipoprotein cholesterol</td>
<td>63 ± 4</td>
<td>71 ± 8</td>
<td>73 ± 6</td>
<td>68 ± 1</td>
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All values are given in mg/dL. HF, high-fat diet.

*P < 0.05 (referring to data in preceding column).

Table 2. Degenerative Changes of Bruch Membrane in Control Mice and LDL-R−/− Mice by TEM

<table>
<thead>
<tr>
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<th>Controls</th>
<th>LDL-R−/−</th>
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<tbody>
<tr>
<td></td>
<td>Chow (n = 5)</td>
<td>HF (n = 5)</td>
<td>Chow (n = 5)</td>
<td>HF (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Thickness of Bruch membrane (μm)</td>
<td>0.54 ± 0.12</td>
<td>0.56 ± 0.09</td>
<td>0.63 ± 0.23*</td>
<td>0.72 ± 0.19*</td>
<td></td>
</tr>
<tr>
<td>Presence of vacuoles</td>
<td>−</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td></td>
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<tr>
<td>Diameter of vacuoles (μm)</td>
<td>−</td>
<td>0.17 ± 0.07</td>
<td>0.39 ± 0.16*</td>
<td>0.58 ± 0.21*</td>
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HF, high-fat diet; +, rare; ++, moderate; ++++, frequent.

*P < 0.05 (referring to chow controls).
The choriocapillaris of control animals on the standard diet exhibited a normal ultrastructure with a regular basal lamina, a thin and heavily fenestrated endothelial lining, and widely open lumina (Table 2; Fig. 3A). Alterations of the choriocapillary layer could be documented to a minor degree in 3 of 5 control animals after a high-fat diet and to a much greater extent in all LDL-R<sup>−/−</sup> mice (Table 3). These alterations included a significant increase in the thickness of the endothelial basal lamina, a significant reduction in the number of fenestrations per 100 μm of endothelial cell membrane, and a marked narrowing of capillary lumina (Table 3; Figs. 3B, 3C).

Retinal pigment epithelial cells of control mice showed a normal ultrastructure with numerous melanosomes, phagosomes, mitochondria, cisterns of endoplasmic reticulum, and apical microvilli surrounding the photoreceptor outer segments (Fig. 3D). The basal cell membrane of the RPE was characterized by regular,

**FIGURE 1.** Transmission electron micrographs showing alterations of BM in LDL-R<sup>−/−</sup> mice (C, D) compared with control mice (A, B). (A) Normal structure of BM in control mice with chow diet. (B) After a high-fat diet, control mice showed focal minute translucent inclusions (asterisk) and a condensation of BM. (C) Thickening of BM by accumulation of vacuolar inclusions (asterisks) in LDL-R<sup>−/−</sup> mice on a chow diet. (D) In LDL-R<sup>−/−</sup> animals on a high-fat diet, the vacuolar inclusions (asterisks) within BM increased in size and density. CC, choriocapillaris. Scale bar, 1 μm.

**FIGURE 2.** Oil red O-positive inclusions within BM and RPE of control mice (A) and LDL-R<sup>−/−</sup> mice (B–D). (A) No detectable oil red O staining in control mice. (B) Focal occurrence of oil red O-positive particles (arrow) in BM of LDL-R<sup>−/−</sup> mice with chow diet; inset: positive inclusions in higher magnification. (C) Almost continuous laminar accumulation of oil red O-positive structures (arrows) in BM of LDL-R<sup>−/−</sup> animals on a high-fat diet. (D) Oil red O-positive droplets (arrows) within RPE of LDL-R<sup>−/−</sup> mice after a high-fat diet. Original magnifications: (A–D) ×100; (inset) ×250.
tightly packed infoldings (Fig. 3G). In comparison, the RPE of LDL-R<sup>−/−</sup> mice exhibited distinct ultrastructural changes. Retinal pigment epithelial cell height decreased significantly in concert with an increase in intracellular alterations (Table 3). The structure and regularity of the basal membrane infoldings were significantly reduced, as were infolding height and packing density (Table 3; Figs. 3H, 3I). Furthermore, the retinal pigment epithelial cytoplasm contained numerous round vacuoles up to 1.8 μm in diameter in LDL-R<sup>−/−</sup> mice (Figs. 3F, 3G). An incipient vacuolar degeneration was already seen in 3 of 5 control mice after a high-fat diet with a few electron-lucent vacuoles 0.2 to 0.6 μm in diameter. In control animals, regular structured microvilli were found on the apical side of retinal pigment epithelial cells (not shown). These cytoplasmic extensions, which facilitate photoreceptor segment phagocytosis, were reduced in number and size in all LDL-R<sup>−/−</sup> mice (Fig. 3F). Additionally, these mice had mild degenerative changes of the choriocapillaris and RPE in control mice and LDL-R<sup>−/−</sup> mice by TEM

### Table 3. Degenerative Changes of the Choriocapillaris and RPE in Control Mice and LDL-R−/− Mice by TEM

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>LDL-R−/−</th>
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<tbody>
<tr>
<td></td>
<td>Chow (n = 5)</td>
<td>HF (n = 5)</td>
</tr>
<tr>
<td>Thickness of endothelial basal lamina (μm)</td>
<td>0.06 ± 0.03</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>Number of fenestrations/100 μm</td>
<td>305 ± 79.8</td>
<td>255 ± 73.6</td>
</tr>
<tr>
<td>Diameter of vessel lumina (μm)</td>
<td>3.85 ± 0.86</td>
<td>2.23 ± 0.55</td>
</tr>
<tr>
<td>Height of RPE (μm)</td>
<td>7.74 ± 1.31</td>
<td>8.28 ± 1.45</td>
</tr>
<tr>
<td>Height of basal membrane infoldings (μm)</td>
<td>1.28 ± 0.24</td>
<td>1.13 ± 0.13</td>
</tr>
<tr>
<td>Presence of vacuoles in RPE</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

HF, high-fat diet; +, rare; ++, moderate; ++++, frequent.

*P < 0.05 (referring to chow controls).
photoreceptor outer segment degeneration and irregular disk arrangement that became advanced in mice following the high-fat diet (Figs. 3K, 3L).

With the use of immunohistological staining, VEGF164 was found in the choriocapillaris of control animals on a chow diet. (C, D) After high-fat diet, control mice disclosed increased but still focal VEGF164 staining of the choriocapillaris. (E, F) In LDL-R−/− mice on a chow diet, VEGF164 detection in choriocapillaris and deeper choroidal vessels was markedly increased. (G, H) LDL-R−/− mice on a high-fat diet showed continuous VEGF164 detection in the choriocapillaris. (I) Angiosarcoma-positive control showing VEGF immunopositivity of tumor cells. (J) Negative control using preimmune rabbit serum instead of VEGF antibody. Original magnifications: (A, C, E, G) ×100; (I, J) ×150; (B, D, F, H) ×250.

**DISCUSSION**

The pathogenesis of AMD is clearly driven by the interaction of environmental factors and genetic background. With regard to
lipids, hypercholesterolemia, increased dietary intake of saturated fat, monounsaturated and polyunsaturated fat, and cholesterol have been associated with early and late AMD in various studies. Furthermore, recent studies indicate that cholesterol-lowering medication such as statins may reduce the risk of early and late AMD. A genetic condition predisposing for AMD includes different polymorphisms of apolipoprotein E. Other apolipoproteins, such as apoB and apoA-I, may also have an impact on the development of AMD.

Lipid deposits in BM. Lipid accumulation and inflammatory tissue response are known to interact in atherosclerosis and in AMD.

In the present study, we characterized ultrastructural changes of BM and adjacent tissues in an established mouse model for human atherosclerosis. Eyes of C57BL/6J mice with a complete deletion of LDLR-/- and C57BL/6J wild-type mice were used. The modifiable parameter in this model was elevated plasma total cholesterol, which was further modified by a Western-style, high-fat diet. The LDL receptor is responsible for taking up plasma lipids into body cells; its deficiency, as seen in human familial hypercholesterolemia, causes an impaired clearance of lipids from plasma. Although this alone already raises plasma lipid levels, a diet high in fat and cholesterol exacerbates this effect.

In atherosclerosis research, plasma lipid levels are dependent on the chosen knockout model and the composition of the high-fat diet. LDLR-/- mice exhibited cholesterol levels elevated, on average, two to three times above those of wild-type animals on a chow diet. These levels can be significantly modified and increased with a 1.25% high-cholesterol diet. This is also a common diet of other vision scientists who use hypercholesterolemia as an additional modification feature in their models of aging and AMD. The average fat content ranged from 7.5% to 15%, higher than that in the chow diet (approximately 4%). Another classic mouse model of atherosclerosis used in vision research is ApoE-deficient mice. These animals are able to exhibit higher plasma lipid levels after chow or high-fat diets than LDLR-/- mice under the same conditions. We preferred to use LDLR-/- mice because we previously observed BM changes in them, and the plasma cholesterol levels obtained were reproducible and consistent with previous findings. Overall, mouse models with significantly elevated plasma lipids exhibit some kind of BM alterations, which could include BM thickening and deposition of additional debris such as basal laminar deposits. Even though the source of lipids in human Bruch membrane is still controversial, it seems that degenerative Bruch membrane alterations in mice are directly modifiable by hypercholesterolemia.

In our experimental setting, we obtained four distinct levels of plasma total cholesterol. C57BL/6J wild-type mice on a standard rodent diet were our controls; they had normal plasma total cholesterol levels. Plasma total cholesterol was significantly increased by LDL receptor deficiency itself and after the high-fat diet. LDLR-/- mice after high-fat diet showed the highest values for plasma total cholesterol, which were 5.5 times higher than those measured in wild-type mice fed a standard diet. It is thought that endothelial cells, RPE, and retina—like skeletal muscles and brain—take up LDL in part independently of the LDL receptor and that they meet cellular needs for cholesterol by cellular cholesterol de novo synthesis. It is a great advantage of this model that the LDL receptor does not seem to be essential for the normal function of the examined tissues and that the observed degenerative changes can be attributed to the degree of BM lipoidal degeneration.

Characteristic features of early AMD in human eyes include thickening of BM with accumulation of lipids, degenerative alterations in the retinal pigment epithelial layer and choriocapillaris. Using this mouse model we found distinct degenerative alterations in choriocapillaris, BM, RPE, and photoreceptor outer segments as well, which showed a strong positive relationship with plasma total cholesterol levels (Figs. 1–3). These degenerations were accelerated in LDLR-/- mice and most pronounced as a result of the high-fat diet. BM was significantly thickened with a condensation of collagenous and elastic fibers and loss of regular layer arrangement. These findings are comparable to alterations in aging human eyes and eyes with early AMD. With age, the thickness of human BM increases up to 135% in 10 decades, thus elongating the diffusion path length between RPE and choriocapillaris. At the same time, the integrity and thickness of the elastic layer is decreased.

The solubility of collagen in human BM declines by 60% over 9 decades and contributes to debris accumulation in BM. Simultaneously, these processes are accompanied by an increasing accumulation of neutral lipids. Our TEM data indicated an accumulation of translucent vacuoles, consistent with lipid depositions in BM. These vacuoles were directly correlated in amount and size with elevated plasma total cholesterol. In humans, it has been shown that the observed translucent vacuoles derive from extraction of solid lipid-rich particles that develop by conventional tissue processing for TEM. In our murine model, we could also demonstrate in BM the accumulation of oil red O binding material, which is characteristic for neutral lipids.

In addition to this distinct BM lipoidal degeneration, our mice exhibited specific alterations in the adjacent tissues that are also observed in humans. With aging the choriocapillaris becomes atrophic, and both capillary density and lumen diameter significantly decrease. In AMD eyes, the density and lumen diameter of the choriocapillaris were even lower. We also found in our mice an increasingly atrophic choriocapillaris. Although in general control animals did not exhibit any visible changes, LDLR-/- mice showed a thickened basement membrane, reduced endothelial fenestration, and a narrowed lumen. These changes were even more pronounced after the high-fat diet.

On the inner side of BM, the RPE and photoreceptors were also affected. Although retinal pigment epithelial cells in control mice were in general normal, the RPE became increasingly atrophic in LDLR-/- mice. Cell height was significantly decreased, and important intercellular transport structures, such as the basal infolding labyrinth or the apical fringe of microvilli, were significantly reduced. To the same degree, we found the photoreceptor outer segments altered, with sectoral loss of regular disc arrangements. Histologic studies in humans with early AMD revealed that the RPE becomes atrophic and undergoes pigmentedary changes, loss of regular cell shape, normal layer arrangement, and density affecting the photoreceptor structure.

The choriocapillaris supplies the metabolically active RPE, which maintains photoreceptor homeostasis. Structural alterations of BM, especially the accumulation of neutral lipids, affect its diffusion characteristics and result in a decline of hydraulic conductivity. Compromised metabolic exchange between choriocapillaris and RPE through the BM may cause metabolic stress and local hypoxia in the RPE. Reactive oxygen intermediates causing oxidative stress, hypoxia, and advanced glycation end products are all well-known stimuli of VEGF gene expression in retinal pigment epithelial cells. VEGF is secreted by the RPE physiologically. Retinal pigment epithelial secretion of VEGF is polarized toward BM and is essential for the trophic maintenance of choriocapillaris vasculature.
Vascular endothelial growth factor (VEGF) is a key mediator in the pathogenesis of age-related macular degeneration (AMD), a leading cause of visual impairment and blindness worldwide. Studies have shown that VEGF plays a crucial role in the development of AMD, particularly in the formation of choroidal neovascularization (CNV) lesions.

VEGF-A, one of the isoforms of VEGF, is highly expressed in the retina and choroid, and its overexpression is linked to AMD progression. VEGF-A interacts with its receptor, VEGFR-1, which is expressed in the choriocapillaris, a capillary plexus that supplies nutrients to the photoreceptors and ganglion cells.

The role of VEGF in AMD is multifaceted. It promotes angiogenesis and permeability, which are hallmark features of AMD. VEGF-A is a potent angiogenic factor and can stimulate the proliferation and migration of endothelial cells, leading to the formation of new blood vessels in the choroid. This process, known as neovascular AMD, is a major cause of vision loss in patients with AMD.

VEGF-A also plays a role in the pathogenesis of dry AMD (geographic atrophy), where it may contribute to the breakdown of the outer blood-retinal barrier and the formation of atrophic lesions.

VEGF-A is regulated by various factors, including oxidative stress, inflammation, and altered lipid metabolism. These factors can lead to an upregulation of VEGF-A expression, further exacerbating the disease process.

In conclusion, VEGF-A is a critical mediator in the pathogenesis of AMD, and understanding its role is essential for developing targeted therapeutic strategies to prevent and treat this devastating disease.


