Murine High-Fat Diet and Laser Photochemical Model of Basal Deposits in Bruch Membrane

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Objective: To examine the histologic, histochemical, and ultrastructural changes in Bruch membrane in mice on a high-fat diet with and without laser photochemical injury.

Methods: Five groups of C57BL/6 mice were studied. Group 1 included 2-month-old mice on a normal diet; group 2 included 8-month-old mice on a normal diet; group 3 included 8-month-old mice on a high-fat diet; groups 4 and 5 included 8-month-old mice on a normal diet or high-fat diet, respectively, that underwent laser application of one eye with argon blue laser (488 nm). The mice were killed and plasma lipid levels were measured. The eyes were examined by standard electron microscopy, filipin histochemistry for unesterified cholesterol (UC) and esterified cholesterol (EC), and the osmium–tannic acid–phenylenediamine method for preserving extracellular lipid particles.

Results: The plasma cholesterol level was significantly higher in the mice on the high-fat diet than the controls (P< .001). Bruch membrane was thicker in group 2 than group 1 (P = .04) and group 3 had a thicker Bruch membrane than group 2 (P = .003). All eyes in group 3 exhibited accumulation of electron-lucent debris. There was no histochemical and ultrastructural evidence that this material represented accumulated UC or EC. Seven of 9 laser-injured eyes in group 5 accumulated basal laminar deposit (BlamD)–like material in Bruch membrane (P = .02).

Conclusions: Electron-lucent debris accumulates in murine Bruch membrane, and the amount correlates with age and high-fat diet. This debris has some similarities with basal linear deposits, although the debris does not form a discrete layer external to the basement membrane of the retinal pigment epithelium as occurs in basal linear deposits. These deposits do not appear to be UC or EC. Laser photochemical injury of the retinal pigment epithelium may result in the appearance of BlamD-like deposits in eyes with electron-lucent debris. The BlamD-like deposits in this model are similar to the basal laminar deposits that occur in age-related macular degeneration.

Clinical Relevance: This is an animal model of ultrastructural BlamD-like material in Bruch membrane that is very similar to the deposits that occur in age-related macular degeneration.


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MATERIALS AND METHODS

MICE

Two-month-old, female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Me). All experiments were conducted according to the Declaration of Helsinki and Guiding Principles in the Care and Use of Animals.15 Five groups of mice were studied: 2-month-old mice on a normal diet (n=10, group 1); 8-month-old mice on a normal diet (n=10, group 2); 8-month-old mice on a high-fat diet for 6 months (n=10, group 3); 8-month-old mice on a normal diet for 6 months that underwent laser photochemical injury in one eye (n=4, group 4); and 8-month-old mice on a high-fat diet for 6 months that underwent argon laser photochemical injury in one eye (n=9, group 5).

DIET

Groups of mice were fed either a standard rodent chow (laboratory rodent diet 5001; PMI Nutrition Int Inc, Brentwood, Mo) or a high-fat atherogenic diet. The synthetic high-fat diet was delivered by ICN Biochemicals Inc (Aurora, Ohio) and contained the following constituents: 50% sucrose, 15% cocoa butter, 1.25% cholesterol, 0.5% sodium cholate, 1% corn oil, 20% casein, 4.82% alphacel non-nutritive bulk, 5% American Institute of Nutrition mineral mix, 1% American Institute of Nutrition vitamin mix, 1% cholin chloride, 0.3% dl-methionine, and 0.13% vitamin E. This diet is recommended for raising lipid levels without causing liver damage and gallstone formation.16 The mice had free access to food and water, were housed in plastic cages, and were kept on a 12-hour light-dark cycle. All mice were housed approximately 1 m from the floor where standard day lighting was 50 foot-candles.

ARGON LASER PHOTOCHEMICAL INJURY

Eight-month-old C57BL6 mice fed a normal (group 4) or high-fat (group 5) diet for 6 months were exposed to argon laser photochemical injury in the right eye similar to a previously described protocol.19-21 The mice were exposed to blue light argon laser (488 nm) attached to an indirect ophthalmoscope. The eye was dilated with 2.5% phenylephrine, and the light was focused with a 20-diopter lens onto the center of the retina in the posterior pole. The exposure time was 7 seconds, the power was 20 mJ, and the spot size was 50 µm. This laser exposure was given every other day for 14 days. The wavelength and power settings were chosen to compromise RPE function without causing thermal or phototoxic injury.22

PLASMA LIPID CHEMISTRY

At the end of the experiment, the mice were killed and blood samples for lipid analyses were taken. An enzymatic method was used to determine total triglyceride and cholesterol levels. Plasma samples from animals from the same groups were pooled together and then fractionated over a fast protein liquid chromatography column.23

TISSUE PREPARATION

At the time of death, the 12-o’clock position of the eyes was marked. The eyes were then enucleated, and the right eyes were fixed in 2.5% phosphate-buffered glutaraldehyde. The left eyes and livers of mice in groups 2 and 3 were fixed with 4% paraformaldehyde in phosphate-buffered saline and evaluated for extracellular lipids using filipin histochemistry and osmium–tannic acid–phenylenediamine (OTAP) methods. After 24 hours, the central area of the posterior pole was identified according to the 12-o’clock mark, and a rectangular piece of tissue measuring 1.5×1 mm, including the optic nerve head, was cut off. Central areas of the right eyes from mice in groups 1 through 4 were again placed in 2.5% glutaraldehyde and processed for electron microscopy.

CONVENTIONAL ELECTRON MICROSCOPY

For electron microscopy, tissue was postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer. Standard dehydration was performed, the specimen was embedded in epoxy resin (LX-112; Ladd Research Industries Inc, Burlington, Vt), and semithin sections (1.0 µm) were cut and stained with 2% toluidine blue in 2% sodium borate. Ultrathin (silver) sections were cut, stained with uranyl acetate and lead citrate, and examined using an electron microscope (JEOL 100CXII; JEOL, Peabody, Mass). A section containing the optic nerve head and central area was scanned at 1900 magnification with ×10 binoculars. Representative

membrane of hypercholesterolemic mice, either induced by apolipoprotein E (apo E) deficiency14 or a high-fat diet.15 To investigate if the electron-lucent debris in Bruch membrane of hypercholesterolemic mice is composed of UC and/or EC, we examined the histochemical and ultrastructural changes in Bruch membrane of different aged mice on normal vs high-fat diets. In addition, because we have observed BlaD-like material in the eye of a patient who had a phototoxic injury,16 we sought to determine whether argon blue laser photochemical injury of the RPE enhanced the accumulation of BlaD-like material in mice on either normal or high-fat diets.

RESULTS

The results for groups 1 through 3 are summarized in Figures 1, 2, and 3. Total plasma cholesterol level was significantly greater in 8-month-old mice on a high-fat diet than 8-month-old mice on a normal diet (P<.001) (Figure 1). All lipoprotein classes examined were elevated in the mice on the high-fat diet, with the greatest increase for the very low- and low-density lipoproteins. The thickness of Bruch membrane of 8-month-old mice on a normal diet was significantly greater than 2-month-old mice (P=.04). The difference in membrane thickness of 8-month-old mice on a normal diet vs high-fat diet was also significant, with mice on the high-fat having thicker membranes (P=.003) (Figure 2).

There were ultrastructural changes in Bruch membrane associated with age and diet. Non–membrane-bound electron-lucent particles were round, occasionally confluent, and scattered throughout both collagenous layers of Bruch membrane (Figure 4). This material was present in Bruch membrane in 2 of 10 two-month-old
photographs were taken at approximately 1 mm from the optic nerve head at ×29000 and printed at ×72500 magnification. The thickness of Bruch membrane was measured in 2 representative photomicrographs per case. The smallest and largest part of Bruch membrane was measured and the average thickness was determined.

Ultrastructural changes within and internal to Bruch membrane were compared to those previously described for human eyes and for the eyes of apo E-deficient mice. The Bruch membrane of apo E-deficient mice contains electron-lucent debris of 2 forms: non-membrane-bound profiles that are small, round, and numerous and membrane-bound profiles that are larger, more irregular, and sparse. For simplicity of presentation in this report, the term electron-lucent debris includes both membrane-bound and non-membrane-bound material.

STERELOGIC METHODS

Stereologic methods were used to quantify the amount of electron-lucent debris present in Bruch membrane for groups 1 through 3. Conventional electron micrographs at ×72500 magnification were used. The Bruch membrane included the basement membranes of the RPE and choriocapillaris, inner and outer collagenous zones, and middle elastic layer. A transparent grid, with grids measuring approximately double the average diameter of the vacuoles, was placed over the micrograph. The number of grid points overlying Bruch membrane and the number overlying any type of debris were determined. The volume fraction of Bruch membrane occupied by electron-lucent debris was the ratio of grid points overlying debris to total grid points. At least 46 grid points were counted on each micrograph to allow for 3% error.

FILIPIN HISTOCHEMISTRY FOR DETECTING UC AND EC

Cholesterol was revealed in tissue sections with filipin, a fluorescent compound that is specific for the 3β-hydroxy group of cholesterol. Filipin histochemical analysis was performed on the left eyes of mice in groups 2 (n=6) and 3 (n=9). Livers from mice in group 3 (n=6) served as a positive control for EC. Eye and liver tissues were cryosectioned at 10 μm. Sections for UC were hydrated and incubated for 30 minutes in a filipin solution (5 mg of filipin [Sigma-Aldrich, St Louis, Mo], dissolved in 1 mL of dimethylformamide, and diluted in 100 mL of phosphate-buffered saline). Sections for EC were processed as follows: (1) native UC was extracted by two 5-minute rinses in 70% ethanol; (2) native EC was hydrolyzed with cholesterol esterase (EC 3.1.1.13; Boehringer-Mannheim, Mannheim, Germany) at a concentration of 1.65 U/mL in 0.1 M potassium phosphate buffer, pH 7.4, for 3 hours at 37°C; (3) UC newly released by the hydrolysis of EC was then stained with filipin as described herein. Controls consisted of incubating sections in the enzyme vehicle only. Sections were viewed by epifluorescence microscopy using a 420-nm excitation filter and 520-nm barrier filter.

LIPID-PRESERVING ELECTRON MICROSCOPY

The eyes of mice in group 3 (n=7) were processed by the OTAP method to preserve small droplets and vesicles of extracellular lipids. Tissues were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (2.5 hours), 1% tannic acid in 0.5 M sodium cacodylate buffer (30 minutes), and fresh 1% p-phenylenediamine in 70% ethanol (30 minutes). Sections 1 μm thick that were stained with toluidine blue were assessed; silver sections were cut with an ultramicrotome, placed on mesh grids, and stained with uranyl acetate and lead citrate; and transmission electron microscopy was performed.

STATISTICAL ANALYSIS

The Wilks-Shapiro test was used to test whether plasma cholesterol level, thickness of Bruch membrane, and amount of electron-lucent debris were normally distributed. From these results, the Wilcoxon rank sum test was used to determine if mean cholesterol level was higher in mice on the high-fat diet than in control mice. Wilcoxon rank sum test was also used to compare the mean average Bruch membrane thickness in group 2 vs group 1 and group 3 vs group 2. With the assumption that the amount of electron-lucent debris was distributed normally in each mouse group, 2 tests were performed to compare the amount of debris in group 2 vs group 1 and group 3 vs group 2. A Fisher exact test was used to compare groups 4 and 5. P<.05 was considered significant.
4 and 7 of 9 eyes in group 5 (Figure 9) showed evidence of this material. Membranous debris was present in all of the eyes with BlamD-like material (Figure 9).

This debris was present throughout the BlamD-like material. The RPE in group 5 mice exhibited intercellular vacuolations and intracytoplasmic condensations.

The major finding of our study is that mice exposed to laser photochemical injury following 6 months on a high-fat diet exhibited a sub-RPE material similar to the BlamD present in human eyes (Figure 9). The accumulation of BlamD-like material in our hypercholesterolemic mice on a high-fat diet after laser photochemical injury of the RPE is similar to that shown in other, preliminary studies.19-21 Those previous studies used 488-nm argon blue laser exposure of the RPE in mice with apolipoprotein B overexpression19,21 or mice on a high-fat diet20 and found that age and high-fat diet predispose mice to BlamD-like material accumulation. Plasma lipidemia and deposit severity were weakly correlated in one of those studies.21 The mechanisms by which laser photochemical injury and high-fat diet interact to produce a BlamD-like lesion are unknown, but a plausible process is com-
promise of RPE metabolism of material that accumulates in Bruch membrane.

Our study showed that membrane-bound and non-membrane-bound electron-lucent debris accumulates with age in murine Bruch membrane and that this accumulation is enhanced by a high-fat diet. This material was distributed throughout Bruch membrane and did not form a discrete layer external to the basement membrane of the RPE, as does BlinD. Interestingly, the ultrastructural changes seen in Bruch membrane of the C57/B16 mice consuming high-fat diets are similar to those we previously reported for apo E–deficient mice. Apolipoprotein E–deficient mice also have elevated plasma cholesterol levels, but because apo E is expressed by the RPE, it was also possible that the effect on Bruch membrane was due to local changes at the level of the RPE. The present study using mice subjected to dietary manipulation of plasma cholesterol suggests that the ultrastructural changes in Bruch membrane of apo E–deficient mice are due to the altered plasma lipid profile rather than RPE dysfunction. Furthermore, because Bruch membrane changes are similar in these 2 animal models, we suspect that laser photochemical injury to the RPE will elicit deposition of a BlinD-like material in any hypercholesterolemic mouse, regardless of whether the hypercholesterolemia is genetically induced or induced by diet.

A thickening of Bruch membrane and accumulation of debris with age and high-fat diet in C57/BL6 mice have also been reported by Miceli and coworkers. However, the ultrastructure of the accumulated material in that study looked less organized and more vesicular than the material seen in our study. Miceli and coworkers found more autofluorescent and autophagocytic vacuoles in the cytoplasm of RPE in mice on high-fat diets than in mice on normal diets. We did not examine autofluorescence in our study. Those authors also observed RPE vacuolations in mice on high-fat diets vs control mice, which we did not observe. These differences may be explained by different high-fat diets and/or artifactual changes.

The present study is the first, to our knowledge, to examine Bruch membrane of hypercholesterolemic mice with histochemical methods specific for cholesterol. The results of filipin staining indicated that the electron-lucent debris in hypercholesterolemic mice does not con-

Figure 6. Eight-month-old mice fed a high-fat diet (group 3) have a thicker Bruch membrane (between arrowheads) than 2-month-old mice. The Bruch membrane contains electron-lucent particles (asterisks) and membranous debris (curved arrow). The retinal pigment epithelium (RPE) and choriocapillaris endothelium (END) appear normal (original magnification ×49300).

Figure 7. A, Intense filipin fluorescence of esterified cholesterol in the liver of an 8-month-old mouse fed a high-fat diet (group 3). Lack of fluorescence in the liver (B) and Bruch membrane (C, arrowheads) in an 8-month-old mouse fed a normal diet (group 2). Filipin fluorescence for unesterified cholesterol in a group 2 (D) and group 3 (E) mouse. Cellular membranes of the retinal pigment epithelium and choriocapillaris endothelium are labeled. The Bruch membrane is a dim line between the fluorescent basal retinal pigment epithelium and choriocapillaris endothelium (arrowheads, D and E).
tain significant amounts of EC or UC. Furthermore, the small number of lipid particles labeled by the OTAP method was insufficient to account for the large number of electron-lucent profiles seen in Bruch membrane by conventional electron microscopy. It is possible that triglycerides, membrane phospholipids, and/or by-products of cholesterol metabolism correlate with the electron-lucent debris in Bruch membrane. Triglycerides and phospholipids increase with age in human Bruch membrane. Human Bruch membrane in normal-aged donors contains abundant 100-nm-diameter droplets that are enriched in EC relative to UC. Although Bruch membrane cholesterol is biochemically and morphologically identical to that in other connective tissues where plasma low-density lipoprotein is the known source, the present study indicates that factors other than elevated plasma low-density lipoprotein levels are required to produce cholesterol accumulation in Bruch membrane of mice. One should note that plasma lipid profiles in normal mice and humans are qualitatively similar, except that cholesterol is primarily transported into cells as high-density lipoprotein in mice and low-density lipoprotein in humans.

Previous epidemiological studies have yielded conflicting data regarding the association of cardiovascular risk factors and age-related maculopathy. High-fat diet and elevated plasma cholesterol levels have been associated with AMD in some studies but not others. There is evidence that changes in or damage to large arteries is associated with late age-related maculopathy. C57BL6 mice develop atherosclerotic lesions in large arteries when fed a high-fat diet to produce total plasma cholesterol levels of 200 to 300 mg/dL (5.17-7.76 mmol/L), similar to those in our study. In our study, the high-fat diet consistently resulted in a significant thickening of Bruch membrane and enhanced accumulation of electron-lucent debris. Furthermore, an atheromatous-like plaque was present on the external aspect of Bruch membrane in 1 mouse in group 3 (Figure 6). There may be some degree of homology between the pathogenesis of changes in mice and in humans with age, although the data regarding an association between AMD and hypercholesterolemia are inconsistent. Our model shows that ultrastructural changes that are similar to components of AMD can be generated in mice. This provides valuable information, regardless of the pathogenesis of AMD. It is possible that constituents of murine Bruch membrane and BlamD originate from systemic lipids in the choriocapillaris in a similar, possibly parallel, mechanism as the pathogenesis of atherosclerosis. Alternatively, these lipids may originate from local cellular sources. Further studies are needed to address these questions.

In summary, our experiments showed that an age-related accumulation of electron-lucent debris in Bruch membrane is enhanced by high-fat diet in C57BL6 mice. Although this debris remains to be characterized biochemically, the high-fat diet was a necessary condition for the production of a BlamD-like material subsequent to laser photochemical injury of the RPE. Because thick BlamD is also associated with AMD, it is significant that...
a highly similar lesion can now be reproduced reliably in an animal model. Although BlinD is associated with AMD, BlinD appears to be specific for AMD. The lack of the specific development of BlinD is a limitation of our model.

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REFERENCES