Morphologic and Electroretinographic Phenotype of SR-BI Knockout Mice after a Long-Term Atherogenic Diet

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PURPOSE. To evaluate functional and ultrastructural changes in the retina of scavenger receptor B1 (SR-BI) knockout (KO) mice consuming a high fat cholate (HFC) diet.

METHODS. Three-month-old male KO and wild-type (WT) mice were fed an HFC diet for 30 weeks. After diet supplementation, plasma cholesterol levels and electroretinograms were analyzed. Neutral lipids were detected with oil red O, and immunohistochemistry was performed on cryostat ocular tissue sections. The retina, Bruch’s membrane (BM), retinal pigment epithelium (RPE), and choriocapillaris (CC) were analyzed by transmission electron microscopy.

RESULTS. Using the WT for reference, ultrastructural changes were recorded in HFC-fed SR-BI KO mice, including lipid inclusions, a patchy disorganization of the photoreceptor outer segment (POS) and the outer nuclear layer (ONL), and BM thickening with sparse sub-RPE deposits. Within the CC, there was abnormal disorganization of collagen fibers localized in ectopic sites with sparse and large vacuolization associated with infiltration of macrophages in the subretinal space, reflecting local inflammation. These lesions were associated with electroretinographic abnormalities, particularly increasing implicit time in a- and b-wave scotopic responses. Abnormal vascular endothelial growth factor (VEGF) staining was detected in the outer nuclear layer.

CONCLUSIONS. HFC-fed SR-BI KO mice thus presented sub-RPE lipid-rich deposits and functional and morphologic alterations similar to some features observed in dry AMD. The findings lend further support to the hypothesis that atherosclerosis causes retinal and subretinal damage that increases susceptibility to some forms of AMD. (Invest Ophthalmol Vis Sci. 2009; 50:3931–3942) DOI:10.1167/iovs.08-2527

Age-related macular degeneration (AMD) is a complex group of diseases in which the roles of many genetic and environmental factors are intertwined. Accumulation of cholesterol under the retinal pigment epithelium (RPE) and within Bruch’s membrane (BM) is a normal feature of aging, but imbalances in cholesterol homeostasis may also contribute to the development of retinal diseases, including AMD. Atherosclerotic cardiovascular diseases are characterized by extracellular lipid deposition in vessel walls, and retention of plasma lipoproteins in the intima is a key event initiating atherosclerosis. ApoB is a component of plasma lipoproteins and is associated with esterified cholesterol in sub-RPE lesions, such as drusen and basal deposits, in the human eye with age-related maculopathy.

These heterogeneous materials form particular abnormal structures, such as basal deposits, and have been detected by electron microscopy beneath the RPE in the BM of aged human eyes with AMD. Basal laminar deposits (BlinDs) are localized between the RPE basement membrane and its plasma membrane and consist of basement membrane proteins and long-spacing collagen. Membranous debris including basal linear deposits (BlamDs) are localized between the RPE basement membrane and the inner collagenous zone of BM forming focal aggregations or diffuse layers. The presence of BlinDs and early BlamDs define the cutoff at which aging becomes early AMD.

Various genetically modified animal models of atherosclerosis display AMD-like phenotypes (apoE-deficient and transgenic mice, LDLR-deficient mice, and apoB100 transgenic mice). Indeed, these animals models present lipoidal degenerations and basal deposits in the BM that resemble alterations observed in ageing human eyes. Some of these animal models also exhibit reduced electroretinographic responses.

SR-BI–deficient mice and knockout (KO) mice have been established by the laboratories of Van Berkel16 and Krieger17,18 as models for atherosclerosis. Their studies show that SR-BI KO mice fed with a high-fat, high-cholesterol diet16 or a high fat cholate (HFC) diet (supplementary data are provided in Zhang et al.19) can develop atherosclerotic lesions of the arteries and heart with high plasma cholesterol concentrations. The scavenger receptor class B, type 1 (SR-BI), is an important mediator of HDL metabolism. SR-BI is abundant in the liver and steroidalogenic tissues and mediates the selective uptake of cholesterol esters from HDL for the synthesis of bile and steroid
hormones. Studies of transgenic and knockout models have clearly implicated SR-BI in HDL metabolism and reverse cholesterol transport from peripheral tissues to the liver in vivo. SR-BI is also expressed by cells within the arterial wall, including endothelial cells, smooth muscle cells, and macrophages. Several studies have shown that SR-BI is present in ocular tissues, in particular in the retina, and RPE. We therefore studied the effect of an HFC diet in SR-BI-deficient mice. We also investigated whether the possible deleterious lesions that may be triggered by such a treatment over a long period share similarities with known features of some forms of human AMD. We determined the propensity of SR-BI KO mice to develop high-fat-diet–induced basal deposits in their eyes and assessed retinal function impairment. First, we confirmed the atherogenic phenotype by biochemical analysis of plasma lipid distribution and histochemical staining of atherosclerotic lesions by using the heart as a positive control. Our findings were entirely consistent with those described by Van Eck et al. We then analyzed the electoretiographic profiles of control and SR-BI-deficient mice. We also evaluated the structural and ultrastructural changes in the choroid, BM, RPE, and neural retina in eye tissue sections by using lipid staining, immunohistochemistry, and transmission electron microscopy.

**Materials and Methods**

**Animals and Diets**

All experimental procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. SR-BI wild-type (WT) and SR-BI KO mice (from Jackson Laboratories [Jax], Bar Harbor, ME) were B6;129S2-Scarb1tm1Kri/J (093579). This strain was first generated and described by Krieger’s laboratory (see Rigotti et al.). Heterozygous mice (SR-BI+/−) were cross-bred to generate WT (SR-BI+/+) and KO (SR-BI−/−) progeny. Targeted and WT SR-BI genotypes were verified by PCR amplification of DNA extracted from tail biopsies (Jax genotyping protocol). The animals were kept in plastic cages with a 12-hour/12-hour light-dark cycle and permanent free access to water and food.

Three-month-old SR-BI WT and KO male mice were separated into two groups for each genotype (n = 10/group). One group was fed a high-fat diet containing 15% cocoa butter, 1.25% cholesterol, and 0.5% sodium cholate (HFC group), and the second group was maintained on a normal diet (chow) with 6% fat. The diets were produced by the SAFE Company (Augy, France).

After 30 weeks’ exposure to the diet, the mice were weighed, and electoretiograms were recorded and analyzed. Blood samples were taken before starting the diet and at the time of death by cervical dislocation. The eyes and hearts were collected for histologic and molecular analyses.

**Biochemical Analyses**

Blood was collected after an overnight fast by retro-orbital puncture under isoflurane-induced anesthesia and collected on heparin or in anticoagulant-free tubes. Plasma was separated by low-speed centrifugation (3000 rpm, 15 min, 4°C) and kept at +4°C until lipid analyses (<3 days).

**Plasma Lipid Concentrations**

Plasma lipid concentrations were measured in triplicate in each sample of plasma collected from each mouse. Total cholesterol (TC) and triglyceride (TG) concentrations were measured in the individual samples (n = 8/group) with commercially available kits (BioMérieux, Marcy-l’Étoile, France). HDL-cholesterol (HDL-C) concentrations were determined after precipitation of apolipoprotein (apo) B-containing lipoproteins with phosphotungstic acid/Mg (Boehringer Mannheim, Germany). Non-HDL cholesterol (Non-HDL-C) concentrations were obtained by subtraction of HDL-C from TC values. The results reported are the mean and standard deviation for eight individual plasma samples per group, with comparison between groups.

**Cholesterol Distribution Profile**

Lipoproteins were prepared by high-speed centrifugation from pooled plasma for each animal group by gel filtration fractionation. Cholesterol and triglyceride contents were analyzed by fast protein liquid chromatography (FPLC; Superose 6HR 10/30 column; Amersham Pharmacia Biotech, Uppsala, Sweden).

**Histologic Analysis**

**Tissue Preparation.** The eyes were enucleated and the hearts excised and incubated overnight at 4°C with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The hearts were cryoprotected in PBS/50% sucrose for 48 hours. The hearts and eyes were washed, with PBS and then embedded in OCT compound (Tissue-Tek; Sakura Finetek, Villeneuve d’Ascq, France) and stored at −80°C. Serial sections of 10 aortic roots per group and five eyes per group, were cut with a cryostat (Jung CM 3000; Leica, Wetzlar, Germany). The specimens were cryosectioned at 10 μm (aortic root) or 14 μm (eyes) and stored at −80°C. We examined areas of atherosclerotic lesion stained with oil red O (ORO) and Gill’s hematoxylin on all cryostat sections starting at the tricuspid valves. Forty sections per eye (near the optic nerve) were stained with ORO and observed under light microscope to determine the distribution of neutral lipid deposits.

**Investigation of Lesions Size in the Aortic Root and in the Retina.** Lipid histochemical analysis. Lesion composition was determined in eye and heart cryosections using a monoclonal rat anti-mouse macrophage antibody (clone MOMA-2 MAB1852; Chemicon, Paris, France), a polyclonal goat anti-CD3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; and AbCys, Paris, France), and a polyclonal rabbit anti-CD5 antibody (AbD-Sec, Oxford, UK). Control slides containing sequential sections were probed with nonimmune serum buffer without primary antibody. Immunolabeled specimens were viewed by confocal laser scanning microscopy. VEGF immunolabeling was coupled with DAPI staining of the nucleus.

Twelve tissue sections per eye and per heart were used for immunohistochemistry, and hearts and eyes of five animals per group were used for each immunohistochemical experiment.

**Transmission Electron Microscopy.** Eyes from each group (three per group) were enucleated and fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4). We left the eyeballs at room temperature for 30 minutes and then dissected them at the level of the limbus and removed the lens. The posterior eyeball was fixed for a further 5 hours. Samples for electron microscopy (EM) were postfixed in 1% osmium tetroxide in cacodylate buffer (0.1 M, pH 7.4) and dehydrated in solutions of increasing ethanol concentration (50%, 70%, 95%, and 100%). Each posterior eyeball was dissected in two parts. The two half samples from each studied eye (three eyes per group) were embedded in epoxy resin. Twenty semithin sections (1 μm) from the two half samples of any given eye examined were cut on an ultramicrotome (Reichert Ultracut E; Leica) into semithin tissue sections. These semithin sections were stained with toluidine blue, and the morphology of the retina/RPE/choroid was examined under a light microscope (DMRB; Leica). We determined the ocular distribution of the sub-RPE deposits (plaques larger than 10 μm and containing neutral lipids) and the number of photoreceptor cells in the central and peripheral retina by analyzing the center and the peripheral regions of eight adjacent semithin sections per half eye (thus, 16 semissections per eye were analyzed). Ultrathin sections (80 nm) were contrasted by using uranyl acetate and lead citrate, and 10 ultrathin sections per eye were observed at 80 kV under an electron microscope.

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TABLE 1. Body Weight Gain in SR-BI WT and SR-BI KO Fed Chow and the HFC Diet

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Body Weight (g)</th>
<th>Body Weight Gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-BI WT</td>
<td>Before diet</td>
<td>29.7 ± 3.8</td>
<td>+16</td>
</tr>
<tr>
<td></td>
<td>Chow (n = 26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HFC (n = 8)</td>
<td>41.9 ± 7.6</td>
<td>+37*</td>
</tr>
<tr>
<td>SR-BI KO</td>
<td>Before diet</td>
<td>30.3 ± 2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n = 25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chow (n = 9)</td>
<td>36.9 ± 4.9</td>
<td>+22</td>
</tr>
<tr>
<td></td>
<td>HFC (n = 9)</td>
<td>35.3 ± 6.9</td>
<td>+15</td>
</tr>
</tbody>
</table>

* P < 0.01, SR-BI WT HFC versus SR-BI KO HFC.

(model 100 CX II; JEOL, Tokyo, Japan) at the Cordeliers Research Centre (Paris). Each eye was examined for changes in the retina and RPE, the presence of sub-RPE basal deposits, choroidal changes, and photoreceptor degeneration. Micrographs were taken of each ultrathin section at various locations, recorded, and analyzed (n = 20/eye) to determine BM and RPE thickness for each eye.

Electroretinography

The mice were dark-adapted overnight for each recording and prepared under dim red light. Anesthesia was induced by intramuscular injection of a mixture of ketamine (100 mg/kg Ketamine 1000; Virbac, France, Carros, France) and xylazine (10 mg/kg, Rompun 2%; Bayer Pharma, Putzbrunn, Germany) by the same investigator. The band-pass filter width was 0.3 to 300 Hz.

Light adaptation was performed with background illumination of 30 mcd/m2 to abolish rod activity. Single-flash recordings were obtained both in dark-adapted (scotopic) and light-adapted (photopic) conditions. Ten single-flash responses were emitted regularly toward the mouse eyes. We took the mean of 10 responses with an interstimulus interval (ISI) of 1.92 seconds or 0.21 Hz (for 1,000 and 3,000 mcd/m2), and of 15 responses per 1000 mcd/m2 to abolish rod activity.

To test the effect of a 30-week diet on body weight, we measured the weight of each mouse before and after the beginning of each diet (Table 1). The SR-BI KO mice (3 months old) were healthy and active when fed a chow diet, with body weights similar to those of control animals. All chow-fed 3-month-old mice had gained weight after 30 weeks of feeding (16%–22% gain). The WT mice receiving the atherogenic HFC diet gained 37% of their 3-month body weight, whereas the SR-BI KO mice gained only 15%. The HFC diet led to a greater body weight gain in the control mice than in the SR-BI KO mice (P < 0.01). All the SR-BI KO mice fed the standardized high-fat, high-cholesterol diet (HFC) exhibited an abnormal appearance (they displayed face alopecia, arched backs, thick skin, and often, genuine wounds) and died prematurely (50% before the end of the diet period). This effect of the HFC diet on SR-BI KO survival has not been described, probably because other studies did not administer this atherogenic diet for such prolonged periods.

Proatherogenic Profile in SR-BI–Deficient Mice Fed an HFC Diet

Compared with the SR-BI WT mice, the SR-BI KO mice fed a chow diet had a higher plasma concentration of total cholesterol, with higher concentrations of both the anti-atherogenic HDL-C fraction and the proatherogenic non-HDL-C fraction; there was no difference in plasma TG levels (Table 2). Exposure to a high-fat diet resulted in different lipid phenotypes in the SR-BI WT and SR-BI KO mice. After the HFC diet, the SR-BI KO mice had a higher cholesterol concentration due to an increase in both HDL-C and non-HDL-C concentrations, suggestive of a proatherogenic lipid profile (Table 2). As expected, the increased HDL-C concentration in the SR-BI KO mice was associated with increased HDL lipoprotein size, as revealed by the cholesterol distribution profile (Fig. 1).

These results are in accordance with previous studies by Krieger's team (supplementary data19) and confirm the proatherogenic lipid profile of the SR-BI KO group fed the HFC diet.

Statistical Analysis

Analysis of variance (ANOVA; Statview software; StatSoft, Inc., Tulsa, OK) was used to determine the significance of intergroup differences.

Results

Diet Supplementation and Body Weight Gain in WT and KO Mice

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All results presented in Table 2 and Figure 1 were obtained at the end of the treatments and immediately before the mice were killed.

**Effect of HFC Diet on Atherosclerosis in the Heart of SR-BI KO Mice**

We first analyzed aortic lesions using ORO staining. Consistent with previous studies, the atherogenic diet resulted in large lesions corresponding primarily, in SR-BI KO mice, to fatty streaks. These fatty streaks contained lipid-filled macrophages exhibiting heavy ORO staining (Fig. 2B). WT mice consuming HFC diets are normally resistant to atherosclerosis and did not display any lipid deposition in the arterial walls (Fig. 2A).

We examined the immunohistochemistry of cryosectioned aortic roots to study these lesions. The aortic lesions in the HFC-fed KO mice showed intense infiltration by strongly immunoreactive macrophages (labeled with the MOMA 2 antibody; Fig. 2D).

**ERG Changes in SR-BI KO Mice Exposed to the HFC Diet**

We investigated the effect of the HFC diet on ERG responses in the SR-BI KO mice and SR-BI WT mice. We present only the most significant results: the amplitude and latency of the scotopic a- and b-waves (Fig. 3). ERG data are not shown for all animals that received a normal diet.

We recorded and compared electroretinograms after 30 weeks of diet. SR-BI KO mice exposed to the HFC diet had altered scotopic ERG responses. The amplitude of the scotopic ERG a wave in the HFC-fed SR-BI KO mice appeared to be similar to that in the HFC-fed WT mice. The amplitude of the scotopic ERG b-wave in the HFC-fed SR-BI KO mice appeared to be lower than that in the HFC-fed WT mice, but the difference was not statistically significant.

Between 30 and 3000 mcd/ms, a-wave latency in the SR-BI KO mice was higher than that in the WT mice ($P < 0.05$). We also observed higher b-wave latency for all the intensities in the SR-BI KO mice (between 10 and 25,000 mcd/ms) than in the WT mice ($P < 0.05$). These results suggest the presence of an ongoing progressive retinal degenerative process.

**Effect of HFC Feeding on the Eyes of SR-BI KO Mice**

We examined the formation of sub-RPE deposits in the eye more closely by histologic analysis of semithin (1-$\mu$m plastic sections stained with toluidine blue) and cryosectioned (14-$\mu$m) slices of eyes of the SR-BI KO mice after 30 weeks on the HFC diet. The SR-BI KO mice receiving the HFC diet had sub-RPE deposits between the RPE and choriocapillaris (CC; Figs. 4B, 4C). These deposits were characterized by the formation of homogeneous material between the RPE plasma membrane and the basal lamina. ORO staining revealed sub-RPE deposits rich in neutral lipids located between the RPE and CC in tissue sections from the HFC-fed SR-BI KO mice (Figs. 4E, 4F). Eye tissue sections from both the normal diet-fed WT mice
and the HFC-fed WT mice did not show any ORO staining (Fig. 4D).

We examined the semithin sections under the light microscope and counted the sub-RPE deposits (only those larger than 10 μm were included). These deposits probably correspond to the accumulation of neutral lipids stained by ORO in cryosections. The mean number of deposits larger than 10 μm per hemiretinal, semithin tissue section was more than four (4.58 ± 0.60) in the HFC-fed SR-BI KO mice (Table 3). These lesions were sparse and mostly found (70%) in the peripheral retina. We observed, for the first time, rare deposits (0.27 ± 0.07) in the semithin sections of the HFC-fed WT mice. Immunohistochemistry of cryosectioned eyes revealed apoE staining in the RPE and in the BM of the HFC-fed SR-BI KO mice (Figs. 4H, 4I).

Ultrastructural Changes in the RPE-BM-CC Complex in SR-BI KO Mice Exposed to the HFC Diet

Histologic examination of retinal sections from the HFC-fed SR-BI KO mice under the light microscope showed deposits between the RPE and BM (Figs. 4B, 4C, 4E, 4F). We analyzed sub-RPE deposits more closely by EM. In contrast to that in the HFC-fed WT mice (Fig. 5C), basal infolding of the RPE in the HFC-fed SR-BI KO mice was disorganized and thickened, with accumulation of large amorphous electron-dense material between the folds (Fig. 5D). This material was continuous with the basal lamina of the RPE and the collagenous inner layer of the BM. Indeed, these structures displayed the same electron-dense lesions when examined by EM. BM of the HFC-fed SR-BI KO mice was much thicker (1.09 ± 0.09 μm; Figs. 5B, 5D) than that of the HFC-fed WT mice (0.49 ± 0.02 μm; Figs. 5A, 5C). This difference in thickness was due to the buildup of electron-dense and electron-lucent particles in the outer collagen layer contiguous with the CC basement membrane. These particles were also found, to a lesser extent, in the inner collagen layer. There were regions of BM thickening and accumulation of deposits between the RPE and BM of varying thickness, possibly constituting continuous lesions (Figs. 5B) or large vacuoles containing multilaminar membranes (Fig. 5E). These large vacuoles seemed to be formed by the fusion of smaller vacuoles abnormally filled with lipids. ORO staining of the sub-RPE deposits observed by light microscopy strongly suggested that the electrolucent vacuoles observed by EM probably contained neutral lipids.

Ultrastructural Changes in the CC Associated with Local Inflammation

The SR-BI KO mice fed with the HFC diet showed abnormalities affecting the whole CC layer. Comparison of electron micrographs of the HFC-fed WT (Fig. 5A) and the HFC KO mouse retinas showed major abnormalities in the CC of the HFC-fed KO mice. Choriocapillaries and their basal lamina both contain membrane-bound electrolucent vacuolization, probably corresponding to large lipid droplets (Figs. 5B, 5D, asterisks). These round, confluent particles observed in the CC exhibited the same electron density as that of sub-RPE deposits. The ultrastructural abnormalities of the CC in the HFC-fed KO mice (Fig. 5D) not seen in the HFC-fed WT mice (Fig. 5C) included reduplication (Fig. 5B, arrowhead) and thickening of the endothelial basal lamina with significant local loss of cho-
riocapillary endothelial cell fenestrations beneath either the abnormal lipid-containing lesions or large vacuoles (Fig. 5D, arrowheads).

We also observed endothelial deposits (Figs. 5B, 5E, arrows), similar to atherosclerotic lesions observed in the aortic root. These lesions considerably reduced the diameter of the lumen of the choriocapillaries.

We studied these choroidal lesions by ORO staining (Fig. 6A, arrow). Staining with specific antibodies revealed accumulation of smooth muscle cells (Fig. 6C) and infiltration of macrophages.

Table 3. Average ONL Cell Nuclei Rows per Animal and per Group and Average Sub-RPE Deposits Counted in Hemiretinal Semithin Sections per Animal and per Group on the HFC Diet

<table>
<thead>
<tr>
<th>Eye ID/Genotype</th>
<th>Number of Deposits ≥10 μm</th>
<th>Central ONL Cell Count</th>
<th>Peripheral ONL Cell Count</th>
<th>BM Thickness (μm)</th>
<th>RPE Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>52.12</td>
<td>0.43 ± 0.30</td>
<td>11.57 ± 0.48</td>
<td>7.43 ± 0.20</td>
<td>0.43 ± 0.13</td>
<td>8.24 ± 0.30</td>
</tr>
<tr>
<td>55.8</td>
<td>0.29 ± 0.18</td>
<td>9.86 ± 0.26</td>
<td>5.86 ± 0.26</td>
<td>0.53 ± 0.02</td>
<td>7.53 ± 0.17</td>
</tr>
<tr>
<td>59.17</td>
<td>0.14 ± 0.13</td>
<td>10.14 ± 0.32</td>
<td>7.14 ± 0.24</td>
<td>0.51 ± 0.02</td>
<td>7.84 ± 0.28</td>
</tr>
<tr>
<td>Average</td>
<td>0.27 ± 0.07</td>
<td>10.51 ± 0.43</td>
<td>6.80 ± 0.39</td>
<td>0.49 ± 0.02</td>
<td>7.78 ± 0.20</td>
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<tr>
<td>KO Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56.5</td>
<td>6.14 ± 0.59</td>
<td>10.43 ± 0.20</td>
<td>5.29 ± 0.18</td>
<td>0.81 ± 0.11</td>
<td>7.92 ± 0.49</td>
</tr>
<tr>
<td>59.29</td>
<td>4.00 ± 0.76</td>
<td>9.43 ± 0.20</td>
<td>4.86 ± 0.40</td>
<td>1.22 ± 0.09</td>
<td>4.78 ± 0.30</td>
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<td>57.7</td>
<td>3.78 ± 1.25</td>
<td>12.22 ± 0.91</td>
<td>8.89 ± 0.58</td>
<td>1.19 ± 0.12</td>
<td>6.40 ± 0.52</td>
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<tr>
<td>Average</td>
<td>4.58 ± 0.6*</td>
<td>10.70 ± 0.67</td>
<td>6.34 ± 1.04</td>
<td>1.09 ± 0.09*</td>
<td>6.38 ± 0.76</td>
</tr>
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RPE and BM thickness were assessed in ultrathin sections of HFC-fed SR-BI WT and KO eyes (n = 3).

* P < 0.001, SR-BI KO HFC versus SR-BI WT HFC.
(Fig. 6G, asterisk) and T-lymphocytes in the endothelium of chorioidal vessels (Fig. 6D) in the HFC-fed KO mice. These results confirm that lesions, like those in arterosclerotic roots, occurred in the CC. In sharp contrast, no mice from any of the other groups studied exhibited any ORO-positive staining or immunostaining in the BM or the choroid.

On semithin sections, we detected abnormal cell accumulation posterior to sub-RPE deposits (Fig. 6F). Some of these cells displayed features characteristic of foam cells (lipid-charged macrophages) with white intracellular vacuoles similar to lipid inclusions (Fig. 6F). EM of the CC layer near sub-RPE deposits revealed hypertrophy of the endothelial cell bodies (Fig. 6J, asterisks) and macrophage infiltration in the CC (Fig. 6I), consistent with local inflammation in this layer. We also observed large cells in the CC with numerous vacuolar inclusions and presenting characteristics similar to those of lipid-charged macrophages (Fig. 6L). Large areas containing abundant banded structures, resembling transverse and longitudinal collagen fibers, were observed both in BM and throughout the CC layer in two different eyes (Fig. 6K, asterisks). These collagen fibers appeared to fill the choriocapillary space and replace capillary vessels.

**RPE and Neuroretinal Ultrastructural Changes in SR-BI KO Mice Fed the HFC Diet**

We found major differences in RPE ultrastructure between the HFC-fed WT and HFC-fed KO mice. The greatest differences were in some areas displaying an abnormal architecture with a thickening of RPE basal infolding adjacent to BM (Figs. 5D, 6K), misaligned and disorganized RPE apical microvilli adjacent to
POS (Figs. 7A, inset; 7B, arrow) and an intracellular accumulation of vacuolar inclusions (Fig. 6I). We did not observe any statistically significant difference in RPE thickness between the HFC-fed KO and HFC-fed WT eyes, despite the reproducible observations of RPE ultrastructural abnormalities (Table 3).

We studied electron micrographs of HFC-fed WT (Fig. 7C) and HFC-fed KO mouse retinas: there were major abnormalities in the photoreceptor outer segments (POS) of HFC-fed KO mice, with disorganized and abnormal dilated disc membranes, loss of photoreceptor disks, extracellular electrolucent spaces, and even complete destruction of some POS (Figs. 7A, 7B, asterisks). Large extracellular apparent vacuolization was reproducibly observed near the photoreceptor inner segments (PIS; Figs. 4C, 7D, asterisks). In all the eyes analyzed, cells of unidentified nature were found close to these electrolucent spaces in the PIS. The nuclei of these cells were unambiguously localized in the ONL, whereas their cellular bodies appeared ectopically inserted among the PIS and POS. These cells present vacuolar inclusions that seem to result from putative phagocytosis or ingestion of the large electrolucent vacuolar

**Figure 6.** SR-BI KO mice developed atherosclerotic choriocapillary lesions after 30 weeks’ exposure to HFC diet associated with local inflammation. (A) Representative photomicrograph of ORO-stained ocular cross sections of SR-BI KO mice fed the HFC diet shows local staining in vascular choroidal vessels (arrow). (B, D, E, G, H) Fluorescence immunohistochemistry. (B) Negative control with nonimmune serum buffer. (C) Blue: (anti-α-smooth muscle actin; arrows) accumulation of smooth muscle cells in the endothelium of the choroidal vessels. (D) Red: T-lymphocyte staining (anti-CD3; arrow) in the endothelium of choroidal vessels. (E) Autofluorescence in (D). (F) A 1-μm plastic section stained with toluidine blue showing cell accumulation posterior to sub-RPE deposits (arrow) with intracellular inclusions. (G) Red: immunolabeling of macrophages in the CC (MOMA 2 antibody). (H) Autofluorescence in (G). (I) Transmission electron micrographs showing macrophage infiltration in the CC (★). The RPE had vacuolar inclusions (arrows). (J) Accumulation and hypertrophy of endothelial cells (★) posterior to sub-RPE deposits. (K) Disorganization and duplication of CC basal lamina were visible next to the sub-RPE deposits (arrow). Infiltrations of banded structures resembling fibrous collagen (★) appeared to fill the choriocapillary space and replace the capillaries. (L) Large cell localized in the CC with numerous vacuolar inclusions (electrolucent spaces marked with ★). Scale bar (A, C, F) 10 μm; (B, D, E, G, H) 50 μm; (I, J, L) 2 μm; (K) 1 μm.
spaces nearby (Fig. 7E). We also observed some abnormal extracellular electrolucent spaces in the photoreceptor ONL (Fig. 7F) with abnormally dense and small nuclei. These observations are highly suggestive of photoreceptor cell losses. Cell counts in the central and peripheral regions of the ONL were not significantly different between the HFC-fed WT and HFC-fed KO mice, and there was no sign of photoreceptor degeneration (Table 3).

VEGF Induction in the Outer Retina of SR-BI KO Mice Fed the HFC Diet

We also observed a significant specific VEGF immunostaining in the outer nuclear layer (ONL) in retinas of the HFC-fed KO mice (Figs. 8C, 8D). In contrast, VEGF immunostaining was weak or absent in the WT mice (Figs. 8B, 8E). We did not detect any abnormal choroidal neovascularization in the HFC-fed SR-BI KO mice, despite the abnormal thickness of BM and a continuum of sub-RPE deposits. As expected, we did not detect choroidal neovascularization in the WT mice (Figs. 5A, 5C).

DISCUSSION

Age-related macular degenerations (AMDs) are the leading cause of severe visual loss in patients above the age of 50 in industrialized countries.29,30 Clinical epidemiologic studies have consistently identified cardiovascular risk factors in AMDs.31–33 Moreover, some pathogenic processes seem to be common to both aging of the BM and atherosclerosis of the vascular wall.32,34 The link between atherosclerosis and AMD is further substantiated by the similarities in molecular composition of drusen and atherosclerotic deposits,1,6,35 as well as by the description of mouse models with significantly elevated plasma lipids that exhibit some features characteristic of early AMD in human eyes.13

Herein, we have described a correlation between hypercholesterolemia caused by an HFC diet in SR-BI knockout mice and the development of basal deposits in the neuro-retina-RPE-BM-CC complex. These deposits were associated with an altered ERG response, indicating a functional impairment.
As described before by Krieger's team (Zhang et al.11,12), the combination of SR-BI deficiency and HFC diet resulted in high plasma total cholesterol concentrations and provoked extensive occlusive atherosclerotic lesions in SR-BI KO mice; this was confirmed by our ORO staining results. Lipid and lipoprotein analyses confirmed that SR-BI KO mice fed an HFC diet had an atherogenic profile with a heterogeneous population of enlarged HDL lipoproteins.16 Our analysis of this mouse model exposed to an HFC diet for a long period (30 weeks) revealed substantial alterations throughout the retina and choriocapillar- sary layer correlating with plasma total cholesterol levels.

We found a prominent structural abnormality of the BM, with a statistically significant doubling of its thickness associated with an accumulation of vacuolar deposits (appearing as particles) in the HFC-fed SR-BI KO mice. Heterogeneous materials and sub-RPE basal deposits were detected beneath the RPE in BM by EM, especially in the peripheral retina, and were similar to those reported in other studies.8-12 ORO staining of sub-RPE deposits strongly suggested that the electronlucent vacuoles observed in EM images contained neutral lipids. These sub-RPE deposits show striking morphologic similarities with the basal deposits associated with AMD in the human eye.30 Note that we also detected rare deposits in semithin sections from 10-month-old WT mice fed with a cholestero- containing diet. Although Dithmar et al.9 reported a thickening of Bruch's membrane with age in 8-month-old C57Bl/6 mice with numerous non-membrane-bound vacuolations, Ong et al.17 did not report any subretinal deposits in C57Bl/6 mice fed a cholesterol-rich diet (25 weeks).

Similar to the process observed in the vascular intima of arterial walls, the BM undergoes a diffuse thickening process with aging. There is an age-related exponential accumulation of lipids and debris in the BM, particularly in its outer collagen layer.13,38-40 Lipids in the BM consist of both esterified and unesterified cholesterol, suggesting a vascular origin reminiscent of atherosclerosis.1,6,41 However, this is not consistent with the emerging hypothesis that a large lipoprotein of intracellular origin may be a major molecular building block of an important pathway for constituent retinal lipid processing and the biogenesis of drusen.42 There is circumstantial evidence that ultrastructural changes of the BM contribute to the decreased efficiency of exchange between RPE and choriocapillar- lar vessels and facilitate the increased deposition of material in the BM with age.43-44 Our observations are consistent with these studies. Note that, in our study, mice were fed an HFC diet for 30 weeks, whereas previous studies have mostly exposed mice to high-fat diets for much shorter periods.

The absence of SR-BI from macrophages and smooth muscle cells of the arterial wall may therefore impair the cholesterol efflux capacity and may promote the formation of foam cells.30-32 Possibly SR-BI KO mice fed an HFC diet display accelerated age-related changes in the BM and CC. These changes are likely to result from impaired cholesterol transport in the RPE and the subsequent local, deleterious effects of hypercholesterolemia and very high tissue concentrations of lipoproteins enriched in lipids and cholesterol. The changes that occur with ageing and AMD may impair the required fast access of HDLs to the basal surface of the RPE and the inner BM. It is possible that, with ageing, some of the larger molecular weight HDL species in SR-BI KO mice, especially when exposed to an HFC diet, are not able to cross the BM efficiently. Any resulting decrease in reverse cholesterol transport32 may contribute to the pathologic deposition of lipids and cholesterol observed in the SR-BI KO mice and, possibly, in AMD affected patients.

We also observed abnormalities in the CC of the HFC SR-BI KO mice reminiscent of AMD ocular pathologic alterations: apparent hypertrophy of the endothelial cell bodies, reduplication of the basement membrane, and loss of choriocapillary endothelial cell fenestrations.38-40 Of importance, large lipid droplets and endothelial deposits that stained positive with ORO were also observed to be distributed throughout the CC. These lesions were associated with macrophage infiltration and accumulation of smooth muscle cells and T-lymphocytes in the endothelium of choroidal vessels. These abnormalities of the CC closely resembled the lesions that we observed in the atherosclerotic aortic roots of the HFC-fed SR-BI KO mice. Their detection in our model, involving an HFC diet, provides further evidence of a link between the apparently different phenotypes observed in atherosclerotic and patients with AMD.

We also observed macrophage infiltration in the choroidal layer of HFC SR-BI KO mice. Some of these cells present characteristics similar to the clusters of lipid-charged macrophages surrounded by collagen fibers described by Salazar et al.17 in the suprachoroidal of hypercholesteremic rabbits. Other studies have demonstrated that macrophages accumulate in the ocular tissues of patients affected by wet-type AMD or RPE geographic atrophy, especially in areas close to the damaged RPE in the BM and the choroidal neovascular membranes.48-51 Although we did not observe choroidal neovas- cularization in our model, we found very intense specific VEGF immunostaining of the ONL in the HFC-fed SR-BI KO mice, reminiscent of observations in developing embryonic and fetal retinas in both mice and humans.52 We did not detect such specific and intense VEGF immunostaining in any animals from the other groups studied. These findings are very similar to those reported by studies with LDLR-deficient mice exposed to high-fat diets.12

The HFC diet also caused abnormalities in the RPE and photoreceptors of SR-BI KO mice. In some areas, RPE cells presented vacuolar inclusions that might contain vesicular lipids. There were also regions of expanded infolding of the BM of basal membrane and disorganized apical villi in these mutant mice exposed for a long period to the HFC diet. Photoreceptors were also affected. Indeed, structural, cellular, and functional analyses revealed a variety of alterations in the retina of the SR-BI-deficient mice fed the HFC diet for 30 weeks. EM of the retina revealed the destruction of POS with disorganized normally curved disc membranes, loss of photoreceptor disks, and vacuolar spaces. The nature of the large neuroretinal vacuoles observed is still uncertain. There was aberrant accumu- lation of material in the ONL with abnormal densely stained nuclei suggestive of diet-induced apoptosis. Moreover, unidentified cells were localized within regions of the INL and photoreceptor layers. These cells were very close to abnormal deposits, which may be cellular debris that chemoattracts these cells for possible phagocytosis. The nature of these cells remains to be determined. Photoreceptor damage in AMD is associated with the migration of microglia from the inner to outer retina and altered chemotaxis of microglia is associated with human AMD.52-54 The striking lesions of the RPE, BM, and CC vessels may at least partly explain the highly unusual accu- mulation of lipids within patches of photoreceptors and the destructive lesions in the POS of the HFC-fed SR-BI KO mice. Indeed, all nuclear layers of the retina contain abundant SR-BI mRNA and protein (Provost AC, et al. IOVS 2002;43:ARVO E-Abstract 4562).26-27 It is unsurprising, therefore, that their absence from these layers may have deleterious consequences; however, further work is needed to elucidate the exact nature of these consequences. Moreover, the SR-BI KO mice fed an HFC diet had prolonged a- and b-wave implicit times. Our results unambiguously demonstrate a retinal dysfunction asso- ciated with local outer nuclear loss. However, cell counts did not allow us to characterize morphologically the process of overall retinal degeneration. Hypercholesterolemia seems to play a major role in damaging neuroretinal cells and impairing
retinal function, as reported for other animal models with cholesterol-enriched diets.\textsuperscript{15-37} Disruption of cholesterol homeostasis leads to retinal damage, probably with progressive local retinal degeneration. These observations show morphologic similarities with ultrastructural changes observed in humans with early AMD.\textsuperscript{56} However, these similarities are not in themselves sufficient for considering HFC-fed SR-BI–deficient mice as a genuine animal model of dry AMD.

SR-BI is involved in maintaining a continuous supply of α-tocopherol to the neural retina. α-Tocopherol is an essential micronutrient serving to protect the retina against oxidative stress.\textsuperscript{57} In the present study, we report ultrastructural changes in rod outer segments (ROS), similar to those observed in α-tocopherol transfer protein-null mice fed a vitamin E-deficient diet.\textsuperscript{58} The SR-BI KO mice had impaired α-tocopherol transport and tissue uptake, probably contributing to the reproductive, cardiovascular, and retinal diseases exhibited by these animals.\textsuperscript{18,59} Very old SR-BI KO mice show deficient synaptic plasticity and altered cognitive capabilities (alterations of the recognition memory and of the spatial memory);\textsuperscript{60} thus, SR-BI is involved in neural and cognitive processes, a finding that highlights the contribution of cholesterol and α-tocopherol homeostasis to correct cognitive function.\textsuperscript{59} These observations may explain, at least in part, the alterations observed in our ERG analysis. It has been suggested that fine-tuning of neural cholesterol dynamics is essential for basic synapse function, plasticity, and behavior.\textsuperscript{61} The severely altered cholesterol and α-tocopherol homeostasis in the HFC-fed SR-BI KO mice may result in the abnormal brain and retinal synaptic dysfunctions observed.

HFC-fed SR-BI KO mice are thus a new mouse model with sub-RPE lipid-rich deposits, and morphologic alterations of the RPE, CC, and neural retina possibly due to impaired reverse cholesterol function in the choroidal/RPE area. This model has a peculiarity that may constitute a limitation for its use in studies pertaining either to atherosclerosis and/or AMD: a high mortality rate linked to the unusually prolonged duration of the HFC diet. Most previous studies with other related mouse models used shorter durations of HFC diet. Comparisons of short and long HFC diet durations might be useful in future studies. Our findings lend further support to the hypothesis that AMD shares biological pathways with atherosclerosis.\textsuperscript{62} Our study also opens an avenue for further exploration of synaptic abnormalities associated with imbalanced cholesterol homeostasis in aging mice, as well as in murine models of AMD.

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