Effects of Simvastatin on Retinal Structure and Function of a High-Fat Atherogenic Mouse Model of Thickened Bruch’s Membrane

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PURPOSE. To determine the effect of a statin (simvastatin) on the ultrastructure and function of the RPE, Bruch’s membrane (BM), and photoreceptor interface in a high-fat atherogenic mouse model of thickened BM.

METHODS. Wild-type C57BL/6 mice (6-weeks old) were divided into three study groups according to their diet and treatment given; Group 1, normal chow diet-fed mice; Group 2, high fat diet (HFD) fed mice; and Group 3, HFD-fed mice treated with simvastatin daily for 30 weeks. All mice were followed-up for 30 weeks. The retinal morphology and function was examined in vivo using fundus imaging and electroretinography at 15- and 30-weeks follow-up. At the end of the study, at 36 weeks of age, eye tissues were collected and retinal sections were examined using light microscopy and transmission electron microscopy.

RESULTS. Fundus images of the HFD-fed mice showed the presence of discrete, multiple white spots, which was significantly reduced by approximately 73% in the simvastatin-treated animals. In the HFD-fed mice, there was an increase in the empty cytoplasmic vacuoles of the RPE, presence of lipid droplets in the BM, thickening and fragmentation of the elastic lamina of the BM, and a reduction in retinal function; these ultrastructural and functional changes were significantly improved in the simvastatin-treated group.

CONCLUSIONS. Chronic administration of simvastatin significantly improves the ultrastructure and function of the RPE, BM, and photoreceptor in a high-fat atherogenic mouse model of thickened BM.

Keywords: high-fat atherogenic mouse model, simvastatin, Bruch’s membrane, retina, electroretinography

Age-related macular degeneration is the leading cause of irreversible blindness in individuals over 50 years of age in developed countries. Approximately 25 to 30 million people worldwide have the condition, and the incidence of AMD is expected to continue to increase exponentially over the next 40 years.1 Currently, there is no proven, preventative treatment to slow the progression of early to late AMD.

Bruch’s membrane (BM) consists of five sublayers containing basement membranes of RPE and choriocapillary endothelium, outer and inner collagenous layers, and a central elastic layer.2,3 It separates the outer retina and RPE from their choroidal blood supply. Bruch’s membrane is responsible for many transport and support functions. Transport of nutrients to the retina/RPE and removal of waste material is vital across the BM to ensure the health and normal function of both the RPE and photoreceptors.4,5 The permeability of BM is altered with age, in part, due to the constant perfusion of cholesterol-rich lipoproteins causing a decrease in hydraulic conductivity in extracellular matrix with age, and the increase in the accumulation of extracellular material resulting in an increase in BM thickness.6–7 While the exact pathogenesis of AMD is not known, these changes in BM are considered central to any hypothesis. Alteration in the regulation of cholesterol pathway8 may increase accumulation of lipoproteins over time,9 which is thought to increase in diffusion barrier and decrease in hydraulic permeability10 leading to a lack of metabolic supply to the retina,11,12 which in turn causes retinal hypoxia, photoreceptor dysfunction,13 increased expression of retinal growth factors, and ultimately the development of late stages of AMD, including choroidal neovascularization or geographic atrophy.14

Some have postulated that the changes in BM are reminiscent of the changes seen in walls of arterioles in subjects with vascular disease,15 suggesting that some common pathogenic mechanisms may exist between cardiovascular disease and AMD. Furthermore, associations between cardiovascular disease, dyslipidemia, and AMD have been reported in several large epidemiology studies.16–21 Similar risk factors exist for both diseases, including smoking, inflammation, and increased dietary fat intake.22–24 While diets low in trans unsaturated fat and rich in omega-3 fatty acids and olive oil have been reported to reduce risk,25

Many studies, including a recent Cochrane review,26 have investigated the potential benefit effects of cholesterol-lower-
ing medications, especially 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins), on AMD prevalence, incidence, and progression with inconclusive results.\(^{27-34}\) To date, there is still lack of data on the possible effects of the HMG CoA reductase inhibitor medications on the RPE/choroid/photoreceptor interface. The purpose of this study was to determine the effect of one of the family of HMG CoA reductase, (simvastatin; Merck, Whitehouse Station, NJ) has on the structure and function of this interface in a high-fat atherogenic mouse model, which develops a thickened BM.

**Materials and Methods**

**Experimental Groups**

It has been shown that mice fed with a high-fat atherogenic diet developed significant ultrastructural changes in RPE and BM that reproduces some of the characteristics of human AMD. For example, ultrastructural changes observed in mice receiving a high-fat diet at 30 weeks of age include RPE atrophy, BM breaks, vesicular and amorphous deposits in BM thickened BM, and an accumulation of lipid droplets in the RPE cytoplasm.\(^{35}\) These changes were not observable in the normal chow–fed mice, even at 45 weeks of age.

In this study, wild-type C57BL/6 mice (6 weeks old) purchased from National University of Singapore were used for the study. Mice were divided into three experimental groups of (1) normal chow diet–fed mice (control group), (2) high-fat diet (HFD)–fed mice, and (3) HFD with simvastatin (treatment group)–fed mice \((n = 18\) mice/group). The normal chow diet–fed contains 19.6% protein, 4.6% total fat, 4.5% crude fiber, and 14.5 MJ/kg digestible energy (Specialty Feeds, Glen Forrest, Western Australia). The HFD contains 20% protein, 20% carbohydrate, and 60% fat (Research Diets, New Brunswick, NJ). In the treatment group, animals were given in addition to HFD, oral medication of simvastatin (10 mg/Kg/BW). The images were used for examining fundus photographs at 30 to 80 kV under an electron microscope.

**Histopathology**

The whole mouse eye was embedded in optimal cutting temperature compound at \(-20^\circ C\) for 1 hour. Six micrometer sections were cut with a cryostat (Hyrax C50; Carl Zeiss Microm Engineering GmbH, Jena, Germany). Sections were air dried at room temperature for 1 hour. A standard procedure for hematoxylin and cosin (H&E) staining was performed (hema-toxylin [=GHS380], eosin [=HT1101128]; Sigma-Aldrich, St. Louis, MO).

**Oil Red O (ORO) Staining**

Oil red O (0.7 g; O-0625; Sigma-Aldrich) was dissolved in 100 mL of 100% propylene glycol (PEG) (P3640; Sigma-Aldrich) and this was heated to 100°C for 1 to 2 minutes with constant stir. The solution was then filtered with a filter paper (Whatman #4; VWR International, Radnor, PA), and then left over night at room temperature (RT). The solution was filtered again before use. Six-micrometer frozen sections were air dried at RT and rinsed with distilled water for 2 minutes. The sections were then stained with ORO solution for 10 minutes and were rinsed in 85% PEG for 2 minutes. The sections were then stained with distilled water and stained with hematoxylin. Then, the slides were washed thoroughly in running water for 5 minutes and dehydrated with distilled water for two changes. The slides were air dried and mounted with a water-base preservative (Crystal/Mount; Sigma-Aldrich).

**Transmission Electron Microscopy (TEM)**

Eyes from each group (four per group) were enucleated and fixed in a combination of 2% paraformaldehyde/2.5% glutaraldehyde in PBS for 4 hours. The eyeballs were dissected at the level of the limbus and removed the lens, and each posterior eyecup was dissected in two parts. Samples were postfixed in 1% osmium tetroxide in cacodylate buffer (0.1 M, pH 7.4), and dehydrated in solutions of increasing ethanol concentration (25%, 50%, 75%, 95%, and 100%). The samples were then stained with toluidine blue and the morphology of the retina/RPE/choroid was examined under an Axiosplan, Zeiss Light Microscope (Carl Zeiss). Ultrathin sections (0.01-0.090 mm) were contrasted by using uranyl acetate and lead citrate and were viewed and photographed at 50 to 80 kV under an electron microscope.
Philips EM 208S Transmission Electron Microscope; FEI Electron Optics BV, Eindhoven, The Netherlands). Ultrathin sections were used to quantify the BM thickness in all three groups. Measurements of BM thickness without the intercapillary pillars were performed on six eyes of each studied group using Adobe Photoshop (Adobe, San Jose, CA). A random sample was selected for the measurements. Six measurements at different locations on a single image were obtained for each eye and the group average was calculated.

Statistical Analysis

Quantitative data such as ERG responses and BM thickness were assessed using the ANOVA and post hoc Bonferroni tests. Data are expressed as mean, SD, and 95% confidence interval (CI). $P$ less than 0.05 was considered statistically significant.

RESULTS

Fundus Appearance

A typical finding from color fundus images of the study groups at various time points are shown in Figure 1. There were multiple white dots in the retina, which accumulated over time, detected in the HFD group but not in the control group. The white dots were significantly reduced in HFD mice treated with simvastatin, especially at 30 weeks after treatment. Asterisks (*) indicate the optic nerve head.
Retinal Function

Representative ERG response waveforms and the group average ERG data are shown in Figure 2. On average, the combined rod-cone bright flash a- and b-wave amplitudes of the HFD group were significantly reduced compared with the control (Fig. 2). There were also reductions in the combined rod-cone bright flash a- and b-wave amplitudes of the HFD+simvastatin group, however, these reductions were not statistically significant.

Light Microscopy

Retinal sections were stained with H&E and demonstrated changes in the RPE/BM/choriocapillaris interface in the HFD-fed mice (Fig. 3E) as compared with control mice (Fig. 3D). Oil red O staining, which stain for lipids showed the presence of lipid droplets within this interface of the HFD mice (Fig. 3H). High-fat diet mice treated with simvastatin also developed changes of the RPE/BM/choriocapillaris interface (Fig. 3F), but to a lesser extent compared with HFD mice without simvastatin treatment. No lipid droplets were detected in the HFD+simvastatin mice (Fig. 3I).

Retinal Ultrastructural Changes

Electron micrograph displayed normal-appearing RPE/BM interface in the control and HFD+simvastatin mice. In the HFD-fed mice, increases in the empty cytoplasmic vacuoles and RPE basal infoldings were seen. The BM thickening due to deposition of amorphous material and fragmentation of the elastic lamina was found in HFD group. These changes were not seen in normal diets group or HFD+simvastatin group (Fig. 4). The average BM thicknesses of the three groups are presented in Figure 5.

DISCUSSION

In this study, we examined the effects of one HMG CoA reductase inhibitor, simvastatin on the RPE/BM/photoreceptor
interface in a high-fat atherogenic mouse model of thickened BM. We found that chronic administration of simvastatin improved retinal function and reduced the BM thickness in HFD-fed animals. This novel finding provides some evidence to suggest that this class of cholesterol-lowering drug, the statins, may have potential benefits on the development and progression of human AMD.

Statin or HMG CoA reductase inhibitors are the most commonly used lipid-lowering drugs in patients with atherosclerotic disease or hyperlipidemia.6,7 Their pharmacologic effect is generally regarded as being pleiotropic and may be divided broadly into lipid- and nonlipid-related categories.37,38 The lipid-related effects include reduced serum levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride, and an increased level of high-density lipoprotein (HDL) cholesterol.39 These effects are thought to be protective against cardiovascular disease by directly modifying one of the major atherosclerosis risk factors, namely, dyslipidemia.40,41 More recently, however, statins have been thought to exert an expanded profile of nonlipid-related therapeutic effects, which include improved endothelial function and decreased LDL oxidation, foam cell formation, smooth muscle cell proliferation, plaque rupture, and thrombus formation.42-44 Furthermore, statins have also been found to exert both anti-inflammatory and anti-angiogenic effects, both of which are relevant to vascular disease and in the pathogenesis of AMD.45-47 Several clinical studies have also suggested that protective effect of statins on the development and progression of AMD is via their antioxidant activity.48 It has been shown that statins lead to promoter activation of the antioxidative defense protein (heme oxygenase-1, HO-1), which might explain their pleiotropic antioxidative, anti-inflammatory actions.49 A recent study using cultured human RPE cells showed that HO-1 is a target site and an antioxidant mediator of simvastatin and that simvastatin-dependent upregulation of HO-1 is mainly via the PI3K/Akt-dependent signaling pathways.50 Simvastatin increased the HO-1 mRNA and protein levels, which subsequently leads to the inhibition of free radical formation.50

It has been shown that the human neural retina and RPE express most of the cholesterol-related genes to maintain their cholesterol homeostasis.8 In the RPE, cholesterol could be directed into several different pathways and the apolipoprotein B (apoB)-mediated transport has been suggested to play an

Figure 3. Histopathologic findings of the whole retina in control (A, D, G), HFD (B, E, H), and HFD+simvastatin mice (C, F, I). Digital magnification of the RPE/BM/choriocapillaris interface are shown in (D–F) and ORO staining for presence of lipids are presented in (G–I). There was an increase in the thickness of RPE/BM/choriocapillaris interface in both the HFD and HFD+simvastatin mice, however, the thickening of this interface in the HFD mice was to a much greater extent. Accumulation of lipids at the layers of RPE/BM/choriocapillaris interface was also detected but only in the HFD mice. White arrows indicate RPE/BM/choriocapillaris interface. Black arrows indicate oil ORO staining of lipids. H&E magnification: all ×400.
Figure 4. Electron microscopic findings of the RPE/BM interface. Representative electron microscopic images of RPE/BM of normal diet-fed (A), HFD (B), and HFD+simvastatin (C) mice at 30 weeks. Higher magnification images are shown in (D–F). Normal appearance of the RPE and BM are shown in the control group. Mice fed with HFD showed an increase of vacuoles adjacent to the RPE basal infoldings (*), the empty vacuoles in the RPE cytoplasm (v), consist of lipofuscin material (lip), and thickened BM due to deposition of amorphous material (arrow). The deposition of heterogeneous debris in BM and vacuoles were not seen in simvastatin-treated mice (C, F). Also note the presence of phagosomes (ph), lipofuscin material (lip), and well-defined choriocapillaris basement membrane (arrowhead). The BM thickness (double-headed arrow) was increased in the HFD (E) and HFD+simvastatin (F) groups. Magnification: ×5000 (A–C); ×20,000 (D–F).
important role in the pathogenesis of AMD. The apoB-containing particles accumulate in BM with age and form deposits rich in esterified cholesterol and unesterified cholesterol, which represents the clinical hallmark of drusen. It has been suggested that an imbalance in protein expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and low density lipoproteins receptor (LDLR) determining cholesterol input, and a cholesterol efflux transporter (ABCA1) and cholesterol-catabolizing CYPs mediating cholesterol removal leading to increased RPE cholesterol levels. As such, apoB particle secretion would be increased, and more lipids would be trapped in BM with age, thereby increasing predisposition to AMD. Indeed, two recent genome wide scans identified HDL-related genes as risk factors for AMD.\(^{51,52}\) Recently, it has been shown that human RPE cells synthesize and secrete apoB100 lipoproteins, and that this secretion is driven by cellular cholesterol content.\(^9\) It has also been demonstrated that statins inhibit HMG-CoA reductase reduces cellular cholesterol, which in turn reduces apoB100 synthesis and secretion.\(^9\) It is believe that reduction of apoB100 lipoprotein secretion induced by simvastatin, how-ever, the mechanism need to be confirmed in future studies with details analysis of lipid components and cholesterol pathway.

Our findings provide experimental evidence to support the clinical and epidemiologic observations. In human AMD, the thickening of the BM, which creates a diffusion barrier, is thought to lead to a lack of metabolic supply to the outer retina resulting in photoreceptor dysfunction. In this study, a similar observation was noted with a reduction of retinal function being associated with thickening of BM in HFD mice. More importantly, animals treated with simvastatin, in the HFD model, demonstrated similar retinal function and BM thickness as the control animals that were not fed a HFD. Our results indicate that simvastatin has protective effects on the changes in structure and function of this important photoreceptor/RPE/BM/choriocapillaris interface in this high-fat model. Whether simvastatin would have a similar effect if it was given at a later stage when BM had already become thickened, and changes in the RPE had already occurred is unknown and will required further investigation. However, given the current understanding on the mechanisms of how statin reduces lipoprotein accumulation in BM,\(^9\) it is unlikely that a similar effect will be observed if simvastatin was given when thick BM has already developed. The findings from this study highlight the importance of further studies to determine the possible beneficial effects that statins may have in AMD. As there is no mouse model currently available that is consistent with all aspects of AMD in humans because of the complex and unique nature of human macula, the potential therapeutic benefit of statin observed in this study cannot be directly translated to human AMD.

There are numerous white dots detected on fundus photographs of the HFD-fed animals. These white dots may represent retinal microglia similar to those reported by Xu and associates,\(^53\) though the white dots observed in our study were much smaller compared with those reported by Xu and colleges.\(^53\) The presence of the white dots and reduced retinal function observed in this study may also be related to the presence of the rd8 mutation in the studied animals. Although, we did not screen for this mutation and it was a limitation of the study, the presence of the rd8 mutation was unlikely since the retinal phenotype did not occur in the control group. Furthermore, the typical photoreceptor rosettes associated with this mutation\(^54\) was not observed on histologic slides in any of the animals.

In conclusion, chronic administration of a statin (simvastatin) improved retinal function and reduced the BM thickness in HFD-fed mice. These findings highlight the importance of further studies on determining the role of dyslipidemia in the pathogenesis of AMD.

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References


Figure 5. Measurements of BM thickness were performed by two independent observers using ultrathin sections. Data points in the figures represent means of six measurements at different points in the measuring field. The average BM thickness of the HFD mice was significantly increased compared with that of the naive- and statin-treated mice. Error bars represent the SD.
Simvastatin and Bruch's Membrane


