J. Microbiol. Biotechnol.

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Human Apolipoprotein E2 Transgenic Mice Show Lipid Accumulation in Retinal Pigment Epithelium and Altered Expression of VEGF and bFGF in the Eyes

LEE, SUNG-JOON, JEONG HUN KIM[1], JIN HYOUNG KIM[2], MI JA CHUNG, QINGCHENG WEN, HUM CHUNG[1], KYU-WON KIM[2], AND YOUNG SUK YU[1]*

Division of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Institute of Biomedical Science and Safety, Korea University, Seoul 136-713, Korea
[1]Department of Ophthalmology, College of Medicine, Seoul National University & Clinical Research Institute, Seoul National University Hospital, Seoul 110-744, Korea
[2]Division of Pharmaceutical Bioscience, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Korea

Received:
Accepted:

Abstract We investigated the human apolipoprotein E2 (apoE2) transgenic mouse as an animal model system for age-related macular degeneration (AMD). Transgenic mice expressing human apoE2 and C57BL/6J mice were fed normal chow or a high-fat diet for 4 weeks. Eyes were collected from the mice and lipid deposits in retinal pigment epithelium (RPE) were assessed using electron microscopy. The expressions of apoE, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and pigment-epithelium derived factor (PEDF), which are molecular markers for angiogenesis, were assessed with immunohistochemistry. Eyes from apoE2 mice, regardless of diet, contained lipid accumulation in RPE under electron microscopy, whereas control C57BL/6J eyes did not. Lipid accumulation was found predominantly in the RPE and the Bruch's membrane and increased in the eyes of apoE2 mice after one month of a high-fat diet (8±2 per 50 μm^2 for normal chow and 11±2 per 50 μm^2, $p<0.05$). ApoE expression was similar in the apoE2 and control mice; however, VEGF and bFGF were overexpressed in the retinal pigment epithelium of apoE2 eyes compared with control eyes, and PEDF expression was slightly decreased. These expression patterns of VEGF, bFGF, and PEDF suggest angiogenesis is progressing in apoE2 eyes. In conclusion, the eyes of apoE2 mice develop typical lipid accumulations, a common characteristic of AMD, making them a suitable animal model for AMD. The expression profile of VEGF and bFGF on the retinal pigment epithelium suggests that apoE2 may induce neovascularization by altering angiogenic cytokines.

*Corresponding author
Phone: 82-2-2072-3492; Fax: 82-2-741-3187;
E-mail: ysyu@snu.ac.kr

Key words: Age-related macular degeneration, apolipoprotein E2, VEGF, bFGF

Age-related macular degeneration (AMD) is a blinding disease that affects the elderly. The disease is characterized by degeneration of the macular retina and choroid by atrophy or detachment and scarring caused by choroidal neovascularization [16]. Early signs of AMD include pigmentary changes at the level of the retinal pigment epithelium (RPE) and abnormal accumulations of extracellular lipids, called drusen, adjacent to the basal surface of the RPE [1, 8]. Histologically, drusens are located between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane. The drusen size, number, and degree of confluence are significant risk factors for the development of AMD [13, 20]. It has been proposed that drusen, as well as other age-related changes that can occur in the vicinity of Bruch's membrane, may lead to dysfunction and/or degeneration of the RPE and retina by inducing ischemia and/or by restricting the exchange of nutrients and waste products between the neural retina and the choroid.

Vascular endothelial growth factor (VEGF) is a major vascular growth factor [23, 37] that plays a key role in the pathogenesis of choroidal neovascularization [15]. Because of its high degree of selectivity for endothelial cells and abundant expression in the subfoveal membrane in AMD patients, selective inhibition of VEGF is being developed as a potential therapy for AMD [5]. Pigment epithelium-derived factor (PEDF) is a glycoprotein produced in abundance in RPE that inhibits angiogenesis and proliferation of

several cell types [4]. PDGF has been shown to block neovascularization in an experimental murine model [12, 29,30], and its anti-angiogenic action may stem from its promotion of endothelial apoptosis [7]. Vascular endothelial growth factor (VEGF) and pigment-epithelium derived factor (PEDF) expression are both regulated by oxygen availability, and the regulation of PEDF by oxygen is reciprocal to that of VEGF [2, 11, 41]. Basic fibroblast growth factor (bFGF) is an angiogenic heparin-binding growth factor that can modulate endothelial cell proliferation [34]. The expression of bFGF is elevated both in human maculae of AMD and cultured RPE cell lines [21, 32]. Since these three cytokines are abundantly expressed in the RPE, the crucial junction between Bruch's membrane and the neurosensory retina, changes in their expression levels may be used as biomarkers for detecting AMD.

The etiology of AMD is thought to involve extracellular lipid deposits, whose composition is similar to that of the lipid plaques that accumulate in atherosclerotic lesions [24, 28]. Because of the potential involvement of abnormal lipid deposits, genes that cause lipid disorders have received attention as potential candidates for initiators of AMD development.

Apolipoprotein E (apoE) plays a central role in lipid transport and distribution. The presence of dysfunctional alleles of apoE, such as apoE2, can cause triglyceride and cholesterol to accumulate in the plasma and subsequently in peripheral tissues by damaging the normal removal pathway for lipoprotein particles [25]. This mechanism possibly contributes to extracellular lipid accumulation in the RPE. ApoE is present in AMD-related tissues, such as soft drusen, basal laminar deposit, and RPE cells [31]. Small-scale genetic epidemiologic studies have shown the association of the apoE genotype with the prevalence of AMD [36]. Although the results have not been consistent, two case-control studies in the literature suggested a modestly increased risk for AMD due to the presence of the APOE-2 allele [19, 38]. When the results from four case-control studies were combined, evidence for an increased risk of AMD due to the APOE-2 allele was found for men, but not for women (OR for men 1.54, 95% CI 0.97-2.45) [36]. Using an animal model will help to clarify the effect of human apoE2 on the development of AMD and will help to identify the molecular and genetic mechanisms that could cause AMD.

To study whether apoE2 is associated with the development of AMD, we investigated the lipid accumulation profile in the eyes of transgenic mice expressing human apoE2 but not the mouse counterpart. We found that lipid deposits in RPE were uniformly present in the eyes of transgenic mice, whereas control eyes did not display this phenotype. In addition, the upregulation of vascular VEGF and bFGF and the slight downregulation of PEDF in the eyes of these apoE2 mice suggest the presence of angiogenesis, which is a characteristic of AMD. Our results show that apoE2 transgenic mice may be a useful animal model for AMD research.

MATERIALS AND METHODS

Animal and Tissue Preparations

Human apolipoprotein E2 mice (B6.129P2-Apoe$^{tm1(APOE2)Mae}$ N8) and C57BL/6J mice were purchased from Taconic (Germantown, NY, U.S.A.) and Jackson Laboratory (Bar Harbor, ME, U.S.A.), respectively. The human apoE2 transgenic mice specifically express human apoE2 but not mouse apoE (mapoe$^{-/-}$/hapoe2$^{+/+}$) [39]. The animals were kept in a specific-pathogen-free room at 21–25°C with a 12-h day/night cycle, and water and standard chow were provided *ad libitum*. Eight mice per each group, aged 30–36 weeks, were fed normal chow or chow containing high fat (high-fat diet: 15% cacao butter, 0.5% cholate, 1% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil, and 4.7% cellulose) for four weeks. At the end of this period, each mouse was anesthetized with an intraperitoneal injection of avertin (0.4 mg/g), and both eyes were removed and fixed overnight in 4% paraformaldehyde in PBS buffer (pH 7.4). One eye from each mouse was used for an electron microscopic histological study and the central retina of the other eye was embedded in paraffin for immunohistochemistry. For electron microscopy, ultrathin sections were contrasted with uranyl acetate and lead citrate. Tissue sections were analyzed using a Zeiss EM 9 S-2 electron microscope.

Immunohistochemistry

Sections of eyes and epithelial sheets were cut at 7 μm, mounted on poly-L-lysine coated slides, and deparaffinized, and the sections were treated with 100% ethanol. For immunostaining, the eye sections were first incubated with 3% hydrogen peroxide for 20 min to remove endogenous peroxidase activity, and then rehydrated with 70% ethanol and washed in PBS. The specimens were treated with normal rabbit serum for 30 min to block the nonspecific binding of antibodies and then incubated overnight at 4°C with primary antibody. Four primary antibodies were used in the experiments: goat anti-human apolipoprotein E antibody (Santa Cruz Biotechnology, goat polyclonal IgG stock diluted 1:500), goat anti-mouse VEGF antibody (Santa Cruz Biotechnology), goat anti-mouse PEDF antibody (Santa Cruz Biotechnology), and goat anti-mouse bFGF antibody (Santa Cruz Biotechnology). FITC-conjugated IgG and TRITC-conjugated IgG (Jackson ImmunoResearch West Grove, PA, U.S.A.) were used as secondary antibodies. The slides were mounted with Faramount Aqueous mounting medium (DAKO, CA, U.S.A.) and observed under a fluorescence microscope.

Cell Culture and Lipoprotein Treatments

The ARPE-19 cell line was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). Cells were grown in a 1:1 (v/v) mixture of Dulbecco's Modified Eagle's Medium and HAM's F-12 (Join Bio-Innovation, Seoul, Korea) supplemented with 10% fetal calf serum and 2.5 mM L-glutamine. The cells were seeded at high density (100,000 cells/cm^2) and maintained in culture by feeding weekly. Very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) were purchased from Sigma (St. Louis, MO, U.S.A.). Each lipoprotein was treated with confluent ARPE-19 cells for 8 h and then the expressions of VEGF, bFGF, and PEDF were examined by standard Western blotting methods [17, 18, 26]. The protein concentration in the cytosolic fraction was measured using a BCA protein assay kit (Pierce, Rockford, IL, U.S.A.). Anti-VEGF antibody, anti-bFGF antibody, and anti-PEDF antibody were purchased from Santa Cruz Biotechnology. To ensure the equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against β-actin.

Data Analysis

The data are expressed as the mean±SD unless otherwise indicated. Student's *t*-test was performed for two-group comparisons. Values were considered statistically significant at $p<0.05$.

Table 1. Plasma lipids and glucose concentrations in human apolipoprotein E2 transgenic and C57BL/6J mice.

Mice	Total cholesterol	Triglyceride	Glucose
Human apoE2	527±100*	188±21*	98±8
C57BL/6J	90±6	52±3	104±7

Values are milligram per deciliter (mg/dl). Data are means±standard error. * indicates $p<0.0001$.

RESULTS

Plasma Lipid and Electron Microscopy

Plasma lipid and glucose concentrations are shown in Table 1. In line with previously published data, human apoE2 transgenic mice had severe hyperlipidemia. Plasma cholesterol and triglyceride concentrations were elevated 5.9- and 3.6-fold, respectively, compared with control C57BL/6J mice. Ultrastructures of mice eyes were examined by electron microscopy. All 12 eyes of the apoE2 mice contained extracellular lipid accumulation, regardless of

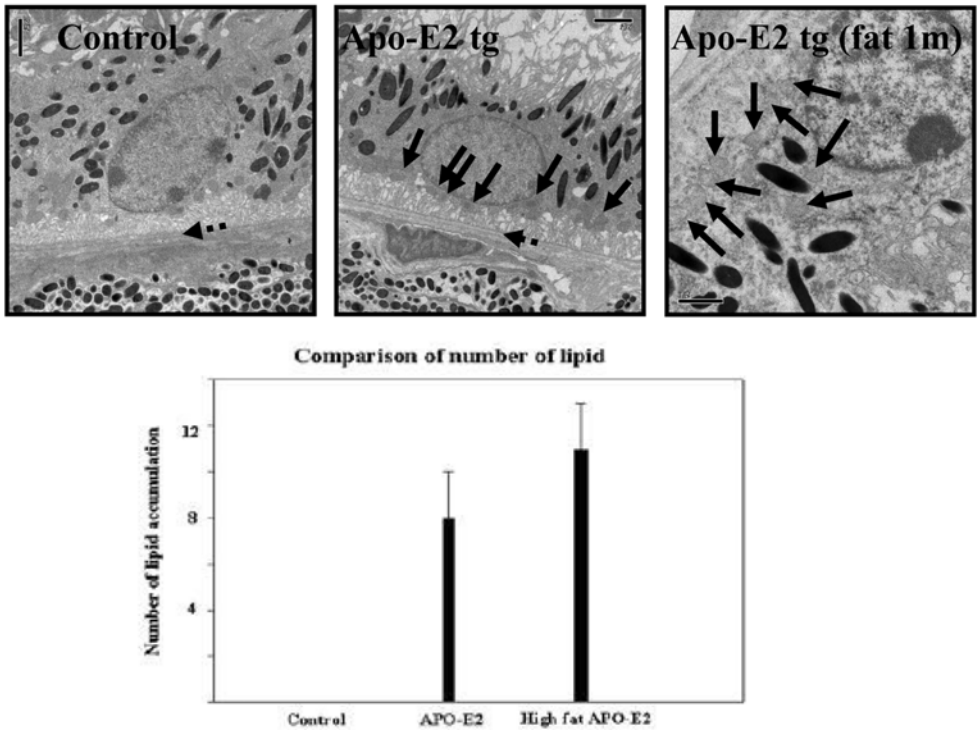

Fig. 1. Electron micrographs of the RPE of control and apoE2 transgenic mice.
Ultrastructure of eyes in the control and human apoE2 mice was examined by electron microscopy. Eyes from human apoE2 mice on either a normal or high-fat diet showed intracellular lipid accumulation of RPE (arrows). The number of lipid deposits in the eyes of apoE2 mice was 8±2 per 50 μm^2 for the normal chow group and 11±2 per 50 μm^2 for the high-fat diet group ($p<0.05$). In addition, basement membrane of RPE was thickened in apoE2 mice (dotted arrows). Control, eyes in C57BL/6J mice; ApoE2-tg, eyes in human apolipoprotein E2 transgenic mice; ApoE2-tg (fat-1m), eyes in human apoE2 mice after 1 month of high-fat diet.

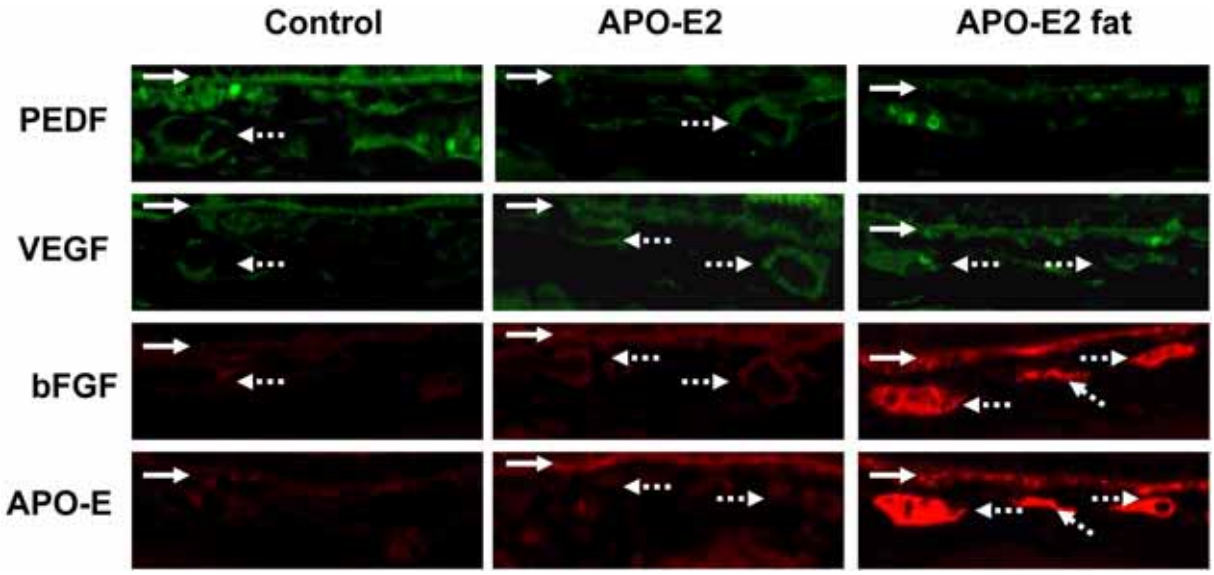

Fig. 2. Immunohistochemistry of PEDF, VEGF, bFGF, and ApoE in RPE of control and apoE2 transgenic mice.
In the figure, arrows indicate the RPE, and dotted arrows the choriocapillaris. PEDF expression was decreased in the RPE (arrows) and choriocapillaris (dotted arrows) of apoE2 eyes. In apoE2 eyes on high-fat diet, PEDF expression on the choriocapillaris was nearly extinguished (the high fluorescence of the figure indicates blood cells, not vessels). VEGF and bFGF expression were markedly increased in the RPE (arrows) and choriocapillaris (dotted arrows) of apoE2 eyes compared with control eyes (arrows). ApoE expression was increased in the RPE (arrows) and choriocapillaris (dotted arrows) of apoE2 eyes. Magnification 400×. Legends are the same as in Fig. 1.

diet. Typical images are shown in Fig. 1. Extracellular lipid deposits appeared as homogeneous basement membrane-like material, and basal laminar deposit was located in Bruch's membrane in apoE2 transgenic mice. In addition, increased lipid accumulation was consistently observed in RPE cells (Fig. 1, short arrow). ApoE2 eyes from mice on the high-fat diet showed more lipid accumulations than apoE2 eyes from mice on normal chow. Strikingly, no lipid accumulations were found in the C57BL/6J mice even after 1 month on the high-fat diet. Eyes from apoE mice fed normal chow contained 8±2 lipid deposits per 50 μm^2, and eyes from apoE mice fed the high-fat diet contained 11±2 lipid deposits per 50 μm^2 ($p<0.05$).

Immunohistochemical Localization of apoE, bFGF, PEDF, and VEGF

The expression pattern of apoE was immunohistochemically localized to RPE cells and choroids in both control and apoE2 eyes, and the expression levels were increased in apoE2 eyes, especially after high-fat diet, compared with control eyes. The localization patterns of apoE in eyes were in accordance with previously reported RT-PCR data. All apoE2 eyes were found to express a high level of apoE in the area of lipid accumulation (data not shown). The expression levels of VEGF, bFGF, and PEDF were also assessed. VEGF and bFGF are growth factors whose expressions are upregulated, whereas PEDF expression is known to be downregulated during angiogenesis. Increased VEGF and decreased PEDF levels have been found in the eyes of patients with AMD. VEGF,

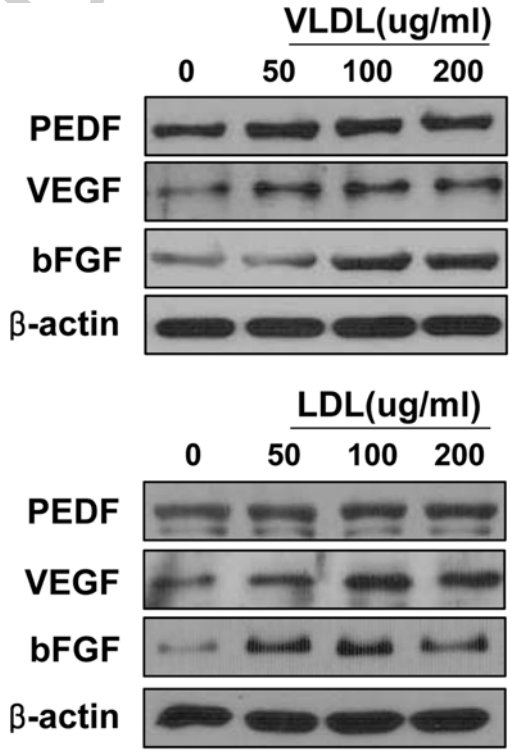

Fig. 3. Effect of LDL or VLDL treatment in ARPE-19 on expression of PEDF, VEGF, and bFGF.
With the treatment of LDL or VLDL, VEGF and bFGF expression increased in a dose-dependent manner. However, PEDF expression did not change by LDL or VLDL treatment. β-Actin served as the loading control. Each test was performed in triplicate.

bFGF, and PEDF were all expressed in both control and apoE2 eyes, but VEGF and bFGF expression was increased in the choriocapillaris of apoE2 eyes compared with control eyes, and bFGF expression was further elevated after high-fat diet. PEDF expression appeared to be slightly decreased in apoE2 eyes compared with control eyes.

Effects of Lipoprotein Treatments in ARPE-16 Cell Line
To examine whether lipoproteins carrying plasma lipids could affect the expression of angiogenesis-related molecular markers in the retina, we incubated cultured retinal pigment epithelium with isolated lipoprotein particles including VLDL and LDL. The VEGF induction was not affected at 50 μg/ml but was elevated at 100 to 200 μg/ml concentrations of LDL particles. VEGF expression was mild, but dose dependently induced after VLDL incubation. bFGF was dramatically induced at high levels of VLDL concentrations but bFGF was mildly induced at 50 and 100 μg/ml LDL. Both VLDL and LDL incubation did not appear to change PEDF expression.

DISCUSSION

We have shown that human apoE2 transgenic mice display drusen-like extracellular lipid accumulations and increased levels of VEGF and bFGF in eyes, which are typical traits of AMD. Our results showed that human apoE2 transgenic mice could be used as a mouse model of AMD, and suggest the significance of the apoE allele in the etiology of AMD. Additionally, in cultured retina pigmented epithelial cells, VEGF and bFGF expressions were increased by both VLDL and LDL incubation.

ApoE is a surface component of lipoprotein particles including VLDL and LDL and is a ligand for lipoprotein uptake by hepatic receptors (LDL receptor and LDL receptor-related protein) [6]. ApoE produced in the liver accounts for approximately two-thirds of plasma apoE, although apoE is produced in most other organs, including eyes. ApoE is a polymorphic protein that has three common alleles (ε2, ε3, ε4) at a single gene locus. These alleles are responsible for six phenotypes, three homozygous (E2/2, E3/3, E4/4) and three heterozygous (E3/2, E4/2, E4/3). The apoE3 allele is the most common allele in the general population. Molecular epidemiologic studies have consistently found that ApoE4 has a mild protective effect against AMD; however, the role of the apoE2 allele in the development of AMD is less clear from the epidemiologic data. Combined results from four case-control studies found evidence that the apoE2 allele increases the risk of AMD in men, but not women (OR for men 1.54, 95% CI 0.97-2.45; OR for women 0.74, 95% CI 0.52-1.06) [36], and two case-control studies reported moderately increased risk of AMD in individuals with the apoE2 allele [19, 38]. These mixed results may result from the complicated lipid profile in persons with the apoE2 allele, and more studies are required to elucidate the relationship between the apoE2 allele and AMD prevalence.

The lipid profile of individuals with the apoE2 allele is interesting. In humans, individuals either homozygous or heterozygous for apoE2, in general, show lower levels of total and LDL cholesterol levels than the average population [42]. Accordingly, these persons have a low incidence of coronary heart disease. However, approximately 5% of apoE2 homozygotes develop type III hyperlipidemia, accumulating chylomicron remnants in the circulation, and these patients are at high risk for developing atherosclerosis and coronary heart disease [27]. Since AMD prevalence is correlated with atherosclerosis and hyperlipidemia, it is possible that only individuals homozygous for apoE2 with type III hyperlipidemia are at high risk for AMD. In fact, epidemiologic studies have not controlled for hyperlipidemia status, which might confound the true association of the apoE2 allele with AMD prevalence.

In mice, when mouse apoE is replaced with human apoE2, nearly 100% of the mice develop type III hyperlipidemia with impaired removal of hepatic chylomicron remnants [25, 39]. As a result, their total cholesterol and triglyceride levels are approximately five times higher than the levels of control mice [25], and these mice are highly prone to develop atherosclerosis and have a short lifespan.

Based on evidence from the epidemiologic studies and the apparent hyperlipidemic characteristics of apoE2 transgenic mice, we reasoned that apoE2 transgenic mice could be a useful animal model for AMD. To test this supposition, we investigated the presence of basal lipid accumulation in the retinas of control and apoE2 mice using electron microscopy. The lipid accumulation was found exclusively in apoE2 eyes, and not in C57BL/6J control eyes, and the number of lipid accumulations was further increased in apoE2 mice after four weeks of a high-fat diet. Lipid accumulations were found in RPE cells, choroid capillaries, and Bruch's membrane, a distribution pattern that suggests that the lipid accumulations in our results are comparable to the extracellular drusen deposits often found in human AMD.

Interestingly, apoE expression levels were also correlated with lipid accumulation in our data, and apoE levels were elevated, especially after high-fat diet. This suggests that the lipid accumulation could be due to an altered expression of apoE, whose major function is as a ligand for the LDL receptor for lipoprotein clearance. In the coronary vessel, lipid accumulation is correlated with apoE deposition in atherosclerotic fatty streak lesions [3]. Considering that the apoE2 allele has lost almost all binding affinity for the LDL receptor, the extracellular lipid accumulation in apoE2 eyes might have a close connection with the dysfunctional binding of apoE2 to the LDL receptor. *In vitro*, ApoE2 retains only 2–3% of the wild-type apoE binding affinity for the LDL receptor [9, 10, 40]. Others have also reported that transgenic mice expressing human apoE3-Leiden,

which also has markedly reduced binding affinity for the LDL receptor [14], and LDL receptor-deficient mice show lipid accumulations in the eyes [22, 35]; the similarity to our data suggests the importance of the apoE-LDL receptor-mediated lipoprotein clearance pathway for this process. However, intriguingly, lipid accumulation was not observed in apoE-deficient mice, although these mice are severely hyperlipidemic and lack the apoE-LDL receptor lipoprotein clearance pathway [22]. We do not have a clear explanation for the phenotype of the apoE-deficient mice; however, it might be associated with the blunted angiogenic response in apoE-deficient mice [33]. One study showed that apoE-deficient mice did not upregulate VEGF in response to ischemic conditions, the physiological stimulus for angiogenesis. It is possible that the reduced angiogenic sensitivity of apoE-deficient mice might reduce the lipid accumulation in eyes that is associated with AMD.

In many AMD cases, growing blood vessels are found in the central retina, and the expression of neovascularization biomarkers are altered in the eyes. Thus, VEGF and bFGF expressions are elevated in the eyes of AMD patients as they were in our apoE2 transgenic mice. Although the presence and distribution of lipid accumulation were distinctive and bFGF/VEGF expression was upregulated in the apoE2 eyes, PEDF expression was only slightly downregulated in the apoE2 eyes compared with controls. In the current study, some mice consumed a high-fat diet for one month, and the excess lipoproteins present following the prolonged high-fat treatment may augment the angiogenic signals in the apoE2 eyes compared with controls.

In conclusion, the present study demonstrated that the eyes of apoE2 mice show typical AMD-like extracellular lipid accumulations, making these mice suitable as an animal model of AMD. Lipid accumulation may be due to the impaired binding affinity of apoE2 to the LDL receptor, in line with other suggested AMD animal models. The expression profile of VEGF and bFGF on RPE cells suggests that apoE2 may induce neovascularization by altering angiogenic cytokines.

Acknowledgments

This work was supported by the Seoul National University Hospital Research Fund (21-2005-017), the Basic Research Program of the Korea Science and Engineering Foundation (R01-2004-000-10212-0), and a grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A050376).

REFERENCES

1. Algvere, P. V. and S. Seregard. 2003. Drusen maculopathy: A risk factor for AMD. Can we prevent visual loss? *Acta Ophthalmol. Scand.* **81:** 427–429.
2. Amano, S., S. Yamagishi, Y. Inagaki, K. Nakamura, M. Takeuchi, H. Inoue, and T. Imaizumi. 2005. Pigment epithelium-derived factor inhibits oxidative stress-induced apoptosis and dysfunction of cultured retinal pericytes. *Microvasc. Res.* **69:** 45–55.
3. Badimon, J. J., B. A. Kottke, T. C. Chen, L. Chan, and S. J. Mao. 1986. Quantification and immunolocalization of apolipoprotein E in experimental atherosclerosis. *Atherosclerosis* **61:** 57–66.
4. Bhutto, I. A., D. S. McLeod, T. Hasegawa, S. Y. Kim, C. Merges, P. Tong, and G. A. Lutty. 2006. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. *Exp. Eye Res.* **82:** 99–110.
5. Browning, A. C., W. M. Amoaku, and H. S. Dua. 2004. Treatment of age-related macular degeneration. *J. Soc. Med.* **97:** 166–169.
6. Cooper, A. D. 1997. Hepatic uptake of chylomicron remnants. *J. Lipid Res.* **38:** 2173–2192.
7. Dawson, D. W., O. V. Volpert, P. Gillis, S. E. Crawford, H. Xu, W. Benedict, and N. P. Bouck. 1999. Pigment epithelium-derived factor: A potent inhibitor of angiogenesis. *Science* **285:** 245–248.
8. De Jong, P. T. 2004. Risk profiles for ageing macular disease. *Ophthalmologica* **218 Suppl 1:** 5–16; discussion 39–41.
9. de Knijff, P., A. M. van den Maagdenberg, A. F. Stalenhoef, J. A. Leuven, P. N. Demacker, L. P. Kuyt, R. R. Frants, and L. M. Havekes. 1991. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *J. Clin. Invest.* **88:** 643–655.
10. de Man, F. H., F. de Beer, A. van de Laarse, A. H. Smelt, J. A. Leuven, and L. M. Havekes. 1998. Effect of apolipoprotein E variants on lipolysis of very low density lipoproteins by heparan sulphate proteoglycan-bound lipoprotein lipase. *Atherosclerosis* **136:** 255–262.
11. Duh, E. J., H. S. Yang, I. Suzuma, M. Miyagi, E. Youngman, K. Mori, M. Katai, L. Yan, K. Suzuma, K. West, S. Davarya, P. Tong, P. Gehlbach, J. Pearlman, J. W. Crabb, L. P. Aiello, P. A. Campochiaro, and D. J. Zack. 2002. Pigment epithelium-derived factor suppresses ischemia-induced retinal neovascularization and VEGF-induced migration and growth. *Invest. Ophthalmol. Vis. Sci.* **43:** 821–829.
12. Gehlbach, P., A. M. Demetriades, S. Yamamoto, T. Deering, E. J. Duh, H. S. Yang, C. Cingolani, H. Lai, L. Wei, and P. A. Campochiaro. 2003. Periocular injection of an adenoviral vector encoding pigment epithelium-derived factor inhibits choroidal neovascularization. *Gene Ther.* **10:** 637–646.
13. Hageman, G. S., P. J. Luthert, N. H. Victor Chong, L. V. Johnson, D. H. Anderson, and R. F. Mullins. 2001. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog. Retin. Eye Res.* **20:** 705–732.
14. Havekes, L., E. de Wit, J. G. Leuven, E. Klasen, G. Utermann, W. Weber, and U. Beisiegel. 1986. Apolipoprotein E3-Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. *Hum. Genet.* **73:** 157–163.

15. Hera, R., M. Keramidas, M. Peoc'h, M. Mouillon, J. P. Romanet, and J. J. Feige. 2005. Expression of VEGF and angiopoietins in subfoveal membranes from patients with age-related macular degeneration. *Am. J. Ophthalmol.* **139:** 589–596.
16. Howes, K. A., J. M. Frederick, A. Marks, and W. Baehr. 2003. Progressive pathways in age-related macular degeneration. *Adv. Exp. Med. Biol.* **533:** 91–96.
17. Kang, K. Y., C. H. Choi, J. Y. Oh, H. Kim, G. R. Kweon, and J. C. Lee. 2005. Chloramphenicol arrests transition of cell cycle and induces apoptotic cell death in myelogenous leukemia cells. *J. Microbiol. Biotechnol.* **15:** 913–918.
18. Kim, S.-J., D. Y. Jun, C. H. Yang, and Y. H. Kim. 2006. Cloning and expression of *hpaA* gene of Korean strain *Helicobacter pylori* K51 in oral vaccine delivery vehicle *Lactococcus lactis* subsp. *lactis* MG1363. *J. Microbiol. Biotechnol.* **16:** 318–324.
19. Klaver, C. C., M. Kliffen, C. M. van Duijn, A. Hofman, M. Cruts, D. E. Grobbee, C. van Broeckhoven, and P. T. de Jong. 1998. Genetic association of apolipoprotein E with age-related macular degeneration. *Am. J. Hum. Genet.* **63:** 200–206.
20. Klein, R., T. Peto, A. Bird, and M. R. Vannewkirk. 2004. The epidemiology of age-related macular degeneration. *Am. J. Ophthalmol.* **137:** 486–495.
21. Kliffen, M., H. S. Sharma, C. M. Mooy, S. Kerkvliet, and P. T. de Jong. 1997. Increased expression of angiogenic growth factors in age-related maculopathy. *Br. J. Ophthalmol.* **81:** 154–162.
22. Kliffen, M., E. Lutgens, M. J. Daemen, E. D. de Muinck, C. M. Mooy, and P. T. de Jong. 2000. The APO(*)E3-Leiden mouse as an animal model for basal laminar deposit. *Br. J. Ophthalmol.* **84:** 1415–1419.
23. Ko, H.-R., H. Kakeya, A. Yoshida, R. Onose, M. Ueki, M. Muroi, A. Takatsuki, H. Matsuzaki, and H. Osada. 2002. PC-766B' and PC 766B, 16-membered macrolide angiogenesis inhibitors produced by *Nocardia* sp. RK-97-56. *J. Microbiol. Biotechnol.* **12:** 829–833.
24. Kopitz, J., F. G. Holz, E. Kaemmerer, and F. Schutt. 2004. Lipids and lipid peroxidation products in the pathogenesis of age-related macular degeneration. *Biochimie* **86:** 825–831.
25. Lee, S. J., S. Kadambi, K. C. Yu, C. David, S. Azhar, A. D. Cooper, and S. Y. Choi. 2005. Removal of chylomicron remnants in transgenic mice overexpressing normal and membrane-anchored hepatic lipase. *J. Lipid Res.* **46:** 27–35.
26. Lim, J. K., H. J. Seo, E. O. Kim, M. Meydani, and J. D. Kim. 2006. Identification of anti-angiogenic and anti-cell adhesion materials from halophilic enterobacteria of the *Trachurus japonicus*. *J. Microbiol. Biotechnol.* **16:** 1544–1553.
27. Mahley, R. W., Y. Huang, and S. C. Rall Jr. 1999. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia). Questions, quandaries, and paradoxes. *J. Lipid Res.* **40:** 1933–1949.
28. Malek, G., C. M. Li, C. Guidry, N. E. Medeiros, and C. A. Curcio. 2003. Apolipoprotein B in cholesterol-containing drusen and basal deposits of human eyes with age-related maculopathy. *Am. J. Pathol.* **162:** 413–425.
29. Mori, K., E. Duh, P. Gehlbach, A. Ando, K. Takahashi, J. Pearlman, K. Mori, H. S. Yang, D. J. Zack, D. Ettyreddy, D. E. Brough, L. L. Wei, and P. A. Campochiaro. 2001. Pigment epithelium-derived factor inhibits retinal and choroidal neovascularization. *J. Cell Physiol.* **188:** 253–263.
30. Mori, K., P. Gehlbach, A. Ando, D. McVey, L. Wei, and P. A. Campochiaro. 2002. Regression of ocular neovascularization in response to increased expression of pigment epithelium-derived factor. *Invest. Ophthalmol. Vis. Sci.* **43:** 2428–2434.
31. Mullins, R. F., S. R. Russell, D. H. Anderson, and G. S. Hageman. 2000. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *FASEB J.* **14:** 835–846.
32. Ogata, N., A. Ando, M. Uyama, and M. Matsumura. 2001. Expression of cytokines and transcription factors in photocoagulated human retinal pigment epithelial cells. *Graefe's Arch. Clin. Exp. Ophthalmol.* **239:** 87–95.
33. Pola, R., E. Gaetani, A. Flex, T. Aprahamian, A. S. Proia, M. Bosch-Marce, R. C. Smith, and P. Pola. 2003. Peripheral nerve ischemia: Apolipoprotein E deficiency results in impaired functional recovery and reduction of associated intraneural angiogenic response. *Exp. Neurol.* **184:** 264–273.
34. Presta, M., P. Dell'Era, S. Mitola, E. Moroni, R. Ronca, and M. Rusnati. 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev.* **16:** 159–178.
35. Rudolf, M., B. Ivandic, J. Winkler, and U. Schmidt-Erfurth. 2004. Accumulation of lipid particles in Bruch's membrane of LDL receptor knockout mice as a model of age-related macular degeneration. *Ophthalmologe* **101:** 715–719.
36. Schmidt, S., C. Klaver, A. Saunders, E. Postel, M. De La Paz, A. Agarwal, K. Small, N. Udar, J. Ong, M. Chalukya, A. Nesburn, C. Kenney, R. Domurath, M. Hogan, T. Mah, Y. Conley, R. Ferrell, D. Weeks, P. T. de Jong, C. van Duijn, J. Haines, M. Pericak-Vance, and M. Gorin. 2002. A pooled case-control study of the apolipoprotein E (APOE) gene in age-related maculopathy. *Ophthalmic Genet.* **23:** 209–223.
37. Scrofani, S. D. B. and A. D. Nash. 2001. Toward a structure-function relationship for vascular endothelial growth factor-B (VEGF-B). *J. Microbiol. Biotechnol.* **11:** 543–551.
38. Simonelli, F., M. Margaglione, F. Testa, G. Cappucci, M. P. Manitto, R. Brancato, and E. Rinaldi. 2001. Apolipoprotein E polymorphisms in age-related macular degeneration in an Italian population. *Ophthalmic Res.* **33:** 325–328.
39. Sullivan, P. M., H. Mezdour, S. H. Quarfordt, and N. Maeda. 1998. Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse Apoe with human Apoe*2. *J. Clin. Invest.* **102:** 130–135.
40. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257:** 2518–2521.
41. Werdich, X. Q., G. W. McCollum, V. S. Rajaratnam, and J. S. Penn. 2004. Variable oxygen and retinal VEGF levels: Correlation with incidence and severity of pathology in a rat model of oxygen-induced retinopathy. *Exp. Eye Res.* **79:** 623–630.
42. Yamamura, T., L. M. Dong, and A. Yamamoto. 1992. Apolipoprotein E polymorphism and coronary heart disease. *Chin. Med. J. (Engl)* **105:** 738–741.