Diabetic retinopathy (DR) is the most frequent and consequential complication of diabetes mellitus and is the main cause of reduced vision in the working age population. The number of patients with diabetes will increase by 70% in developing countries and by 20% in industrialized countries by 2050.1 Therefore, the number of patients with DR and DME indicates that ApoE2 and ApoE3 are involved in the development of retinal neovascularization in eyes. Keywords: apolipoprotein E, retinal angiogenesis, human retinal microvascular endothelial cells, proliferative diabetic retinopathy, oxygen-induced retinopathy

**Apolipoprotein E2 and E3, but Not E4, Promote Retinal Pathologic Neovascularization**

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**PURPOSE.** To determine the relationship between the different isoforms of apolipoprotein E (ApoE) and retinal neovascularization.

**METHODS.** The concentrations of ApoE and VEGF in vitreous humor samples with either a macular hole (MH), or diabetic macular edema (DME), or proliferative diabetic retinopathy (PDR) with or without intravitreal injection of bevacizumab (IVB) were measured by ELISA. The effects of each isoform of ApoE on human retinal microvascular endothelial cells (HRMECs) in culture or on the retina of oxygen-induced retinopathy (OIR) mice were investigated.

**RESULTS.** The concentrations of ApoE and VEGF were significantly higher in the vitreous humor of patients with PDR and DME than in patients with an MH. There was a significant positive correlation between the concentrations of ApoE and VEGF in vitreous humor of patients. In vitro assays showed that ApoE2 and ApoE3, but not ApoE4, promoted the VEGF-induced cell proliferation and migration. In vivo assays showed that intravitreal injections of ApoE2 and ApoE3 increased the number and area of nodes in the retina of OIR mice. Moreover, ApoE was expressed in the vascular endothelial cell in both normal and OIR retinas, but their expression levels were different at postnatal day (P) 12 and P17.

**CONCLUSIONS.** These results demonstrate that ApoE2 and ApoE3, but not ApoE4, have proangiogenic effects, and the increased expression of ApoE in the vitreous humor of patients with PDR and DME indicates that ApoE2 and ApoE3 are involved in the development of retinal neovascularization in eyes.

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cells, pericytes, RPE cells, and Müller cells. Recently, Yu’s group reported that diabetes confers susceptibility to retinal injury imposed by intravitreal injection of modified LDL. These findings suggest that extravasated, modified plasma lipoproteins contribute to the propagation of DR.

Apolipoprotein E (ApoE) is a 34-kDa glycoprotein and exists in three common isoforms: ApoE2, ApoE3, and ApoE4. The ApoEs are produced mainly in the liver and serve as ligands for the LDL receptor and its family members. The ApoEs are involved in lipid metabolism and are expressed in the photoreceptor outer segments, the retinal ganglion layer, and Bruch’s membrane. It is also secreted by RPE cells, and is synthesized by Müller glial cells and secreted into the vitreous.

Apolipoprotein E2 transgenic targeted replacement mice have an accumulation and deposits of lipids in the RPE, which are typical features of AMD in humans. In ApoE4 targeted replacement transgenic mice, the laser-induced choroidal neovascularization was more extensive than in transgenic ApoE3 targeted replacement mice. It has been reported that the VEGF level is significantly and positively correlated with the ApoE levels in the retina and choroid of murine eyes and also in the human brain. Because VEGF has been shown to be associated with neovascularization, these findings suggest that ApoE may be related to retinal neovascularization. However, the effects of the ApoEs (e.g., ApoE2, ApoE3, and ApoE4) on retinal neovascularization have not been fully investigated. Thus, the aim of this study was to evaluate the effects of the ApoEs on retinal pathologic neovascularization focusing on the differences among the ApoE2, ApoE3, and ApoE4 isoforms.

**Materials and Methods**

**Animals**

Eight-week-old male and female C57BL/6J mice (Japan SLC, Hamamatsu, Japan) were used. The mice weighed 20 to 30 g and were kept under 12/12-hour light/dark cycle. They had access to food and water ad libitum. All of the experimental procedures were approved by the Animal Experimental Committee of the Gifu Pharmaceutical University, and they were performed in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

**Preparation of Vitreous Samples**

The clinical procedures conformed to the tenets of the Declaration of the Helsinki. After explaining the purpose and procedures of the study, informed consent was obtained from all patients. The study was approved by the Ethics Committee of the Gifu Pharmaceutical University and the Gifu University Graduate School of Medicine (application number 23-84). Vitreous humor was collected from 40 eyes with a macular hole (MH), 8 eyes with diabetic macular edema (DME), and 39 eyes with PDR just before pars plana vitrectomy. For the eyes with PDR, 15 had an intravitreal injection of bevacizumab (IVB-i) and 24 eyes did not have an IVB (IVB--). The samples of the vitreous humor were diluted by 10, 50, and 100 times. The concentration of ApoE in the vitreous humor of the patients was measured with Human Apolipoprotein E/ApoE Quantikine ELISA Kit (DAPE00; R&D Systems, Inc., Boston, MA, USA), and the VEGF with the Human VEGF Quantikine ELISA Kit (DVE00; R&D Systems, Inc.). The procedures were performed according to the manufacturer’s protocol. In these assays, assay diluent, standard, control, or sample solution were added to each well. The absorbance was measured at 450 nm and 540 nm (as wave length correction) using VARIOSKAN FLASH (Thermo Fisher Scientific, Waltham, MA, USA) within 30 minutes of adding the stop solution.

**Cell Cultures**

Primary human retinal microvascular endothelial cells (HRMECs) obtained from DS Pharma Biomedical (Osaka, Japan) were cultured in Cell Systems Corporation (CSC) complete recombinant medium with 10% fetal bovine serum (FBS) but without hormones, antibiotics, and phenol red (DS Pharma Biomedical). The medium contained culture boost growth factor (DS Pharma Biomedical), attachment factor (DS Pharma Biomedical), 100 U/mL penicillin (Meiji Seika, Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika). The cultures were grown at 37°C in a humidified atmosphere with 5.0% CO2. The cells were released by trypsinization and passaged every 3 to 4 days. Human retinal microvascular endothelial cells from passages 3 to 8 were used for the experiments.

**Cell Proliferation Assay**

Cell proliferation was assayed as described in detail with some modification. In brief, HRMECs were seeded at a density of 2.0 × 10^4 cells per well in 96-well plates and incubated at 37°C in a humidified atmosphere of 5.0% CO2 for 24 hours. After replacing the culture medium by CSC medium (DS Pharma Biomedical) with 10% FBS but without growth factor, the cells were further incubated for 24 hours. Apolipoprotein E2, ApoE3, or ApoE4 (Perotech, Inc., Rocky Hill, NJ, USA) was added to a final concentration of 0.05 to 3 μM and incubated for 1 hour before adding VEGF(165) (R&D Systems, Inc.) at a final concentration of 10 ng/mL. This VEGF concentration is the best for proliferation assay using HRMECs. After 24 hours of incubation with VEGF(165), the medium was replaced by fresh CSC medium with 10% FBS to remove the ApoEs and VEGF. The number of living cells was determined by the WST-8 assay (Cell Counting Kit-8, Dojindo Kagaku, Kumamoto, Japan). The absorbance at 450 and 650 nm was measured with a microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, USA). In this assay, WST-8 is turned orange by the dehydrogenase in living cells. The change in the differences in the absorption at 450 and 650 nm at 3 hours was taken to be the number of living cells.

**Migration Assays**

Cell migration assays were performed as described earlier with some modification. In brief, HRMECs were seeded at a
density of \(4.0 \times 10^6\) cells per well in 12-well plates and incubated at 37°C in a humidified atmosphere of 5.0% CO\(_2\) for 24 hours. After replacing the culture medium by CSC medium with 1% FBS and incubation for 6 hours, the HRMEC monolayer at the center of the well was scraped with a 1.0 \(\times\) 10\(^3\) μL pipette tip. To remove the floating cells, the culture medium was replaced by CSC medium with 1% FBS again. Then, ApoE2, ApoE3, or ApoE4 was added to a final concentration 0.03 to 3 μM and incubated for 1 hour before adding VEGF\(_{165}\) at a final concentration of 10 ng/mL. After adding the reagents, the cells were incubated at 37°C and 5.0% CO\(_2\) for 24 hours. To determine the degree of migration, four photographs covering an area of 3.6 mm\(^2\) each well were taken with a charge-coupled device camera (Olympus, Tokyo, Japan). The photographs were taken before and after the cell migration. The number of cells that migrated into the scraped area was counted.

**Oxygen-Induced Retinopathy (OIR) Model**

Mice with OIR were created as described in detail with some modification.\(^2\) Neonatal mice and their mothers were transferred to a custom-built chamber on postnatal day (P7) and exposed to an atmosphere of 75% O\(_2\) for 5 days with the oxygen level controlled by an oxygen controller (PRO-OX 110; Rerning Bioinstruments Co., Redfield, SD, USA). The pups were returned to room air on P12, and then human ApoE was injected intravitreally at a final concentration of 3 lM and incubated for 1 hour before adding VEGF\(_{165}\) at a final concentration of 10 ng/mL. After adding the reagents, the cells were incubated at 37°C and 5.0% CO\(_2\) for 24 hours. To determine the degree of migration, four photographs covering an area of 3.6 mm\(^2\) each well were taken with a charge-coupled device camera (Olympus, Tokyo, Japan). The photographs were taken before and after the cell migration. The number of cells that migrated into the scraped area was counted.

**Imaging and Quantification of Retinal Neovascularization**

Photographs of the entire flat-mounted retina were taken with the MetaMorph Microscopy Automation and Image Analysis Systems (Universal Imaging Corp., Downingtown, PA, USA). To evaluate the degree of pathologic neovascularization, the number and area of the nodes in the retina were determined with the Angiogenesis Tube Formation module of the MetaMorph Microscopy Automation and Image Analysis Systems (Universal Imaging Corp.). A node is a region containing connected blobs and is determined by the thickness and maximum width of the vessels.

**Immunohistochemistry**

Eyes of OIR mice were enucleated on P17, and fixed in 4% PFA at 4°C for 24 hours. After fixation, the eyes were placed in optimum cutting temperature compound (Sakura Finetechical Co., Ltd., Tokyo, Japan), frozen with liquid nitrogen, and stored at -80°C. For the analysis, transverse 10-μm serial sections were cut on a cryostat and mounted on microscope slides (MAS COAT; Matsunami Glass Ind. Ltd., Osaka, Japan). The sections were washed twice in 0.01 M PBS for 10 minutes each, followed by incubation in mouse-on-mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA, USA) for 1 hour. Mouse anti-CD31 antibody (1:500 dilution; AnaSpec, Inc., San Jose, CA, USA) and rabbit anti-ApoE antibody (1:100 dilution; Bioworld Technology, St. Louis Park, MN, USA) were used. Anti-CD31 antibody was applied to the sections overnight at 4°C. The sections were blocked with 5% goat serum in PBS, and anti-ApoE antibody was applied overnight at 4°C. The immunoreactivity was made visible by incubating the sections with goat anti-mouse antibody conjugated with Alexa 546 (1:2000; Thermo Fisher Scientific) as the secondary antibody for 1 hour at room temperature. Secondary goat anti-rabbit antibody conjugated with Alexa 488 (1:2000; Thermo Fisher Scientific) was applied for 1 hour at room temperature. Finally, Hoechst 33342 (1:2000) was applied to the samples for 30 minutes. The fluorescent images were obtained by the Metamorph system. The tissue that was incubated without primary antibody and followed by incubation with secondary antibodies and detection reagents was treated as a negative control.

**Western Blotting**

Eyes of normal and OIR mice were enucleated on P17, and the retinas isolated and quickly frozen in liquid nitrogen. To extract the proteins, the retinas were homogenized in RIPA buffer with cocktails 2 and 3 protease and phosphatase inhibitors (Sigma-Aldrich Corp.). Lysates of the retina were centrifuged at 12,000g for 20 minutes. The protein concentration of the supernatant was measured using BSA and a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA). Five micrograms of the samples of normal and OIR retinas were dissolved in 20% 2-mercaptoethanol (Nacalai Tesque, Inc., Kyoto, Japan) for the Western blot analysis. The solution containing the samples was subjected to electrophoresis on 5% to 20% SDS-PAGE. Protein bands were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corporation, Billerica, MA, USA), and the membranes were blocked for 30 minutes at room temperature with Block One-P (Nacalai Tesque, Inc.). The membranes were then incubated overnight at 4°C with rabbit anti-ApoE primary polyclonal antibody (1:1000 dilution) and mouse anti-β-actin antibody (1:10000 dilution; Sigma-Aldrich Corp.) as a loading control. Goat anti-rabbit horseradish peroxidase-conjugated antibody (1:2000; Thermo Fisher Scientific) was used as a secondary antibody. The bands were made visible by Immuno Star LD (Wako Pure Chemical, Osaka, Japan), and Lumino Imaging Analyzer (LAS-4000 Mini; Fuji Film, Tokyo, Japan) and quantified.

**Statistical Analyses**

Data are presented as the means ± SEMs. Statistical comparisons were performed by Student’s t-tests, Dunnett’s multiple comparison tests, or Mann-Whitney U tests with Bonferroni correction using statistical software SPSS (version 16.0J; IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA). A significant difference between groups was set at \(P < 0.05\).

**RESULTS**

**Concentration of ApoE and VEGF in Vitreous Humor of Patients With DME, PDR, and an MH**

We determined the concentrations of ApoE and VEGF in the vitreous humor of patients with DME, PDR, and an MH with ELISA kits. The expressions of both ApoE and VEGF were increased by approximately 3-fold in the patients with DME and PDR compared with that in eyes with an MH (Figs. 1A, 1B). In eyes with IVB, the concentration of VEGF was significantly lower (Fig. 1B). There was a positive and significant correlation between the concentrations of ApoE and VEGF in eyes of all patients (\(r = 0.530, P < 0.01\); Spearman’s rank correlation
The relationship between ApoE and VEGF can be described by the equation,

$$y = 0.3052x + 2.7132,$$

in which $y$ is the logarithm level of ApoE in ng/mL and $x$ is the logarithm level of VEGF in pg/mL.

Effect of ApoE on VEGF-induced Cell Proliferation and Migration

We determined the effect of ApoE on VEGF-induced cell proliferation and migration in HRMECs in culture. Both ApoE2 and ApoE3 increased the VEGF-induced proliferation of HRMECs (Figs. 2A, 2B). Apolipoprotein E2 and ApoE3 also significantly enhanced the VEGF-induced cell migration (Figs. 3A, 3B). On the other hand, ApoE4 tended to decrease VEGF-induced cell proliferation (Fig. 2C), and had no effect on the VEGF-induced cell migration (Fig. 3C).
Effect of ApoE on Retinal Neovascularization in OIR Mice

To investigate the effects of ApoE on retinal neovascularization, the number and size of the areas of the nodes were determined in OIR and control mice. The number and sizes were both significantly increased in the ApoE2-treated group compared with the vehicle-treated group (Figs. 4A, 4C). In addition, the number and area of nodes were also significantly increased in the ApoE3-treated group (Figs. 4B, 4D).

Expression and Localization of ApoE and CD31 in Retinas of OIR Mice

Apolipoprotein E and CD31, a marker for endothelial cells, were colocalized in the vascular endothelial cells in both normal and OIR retinas (Fig. 5A). The expression of ApoE in the OIR retina was upregulated at P12 but was downregulated at P17 (Fig. 5B).

DISCUSSION

Our results showed that the expressions of ApoE and VEGF were upregulated in the vitreous of eyes with DME and PDR, but not eyes with an MH, and there was a positive and significant correlation between the levels of ApoE and VEGF in eyes of all patients. In addition, we confirmed that ApoE2 and ApoE3 had proangiogenic effects in vitro and in vivo, and the ApoEs were partially expressed in vascular endothelial cell retina of normal and OIR mice. Interestingly, the expression of the retinal ApoEs was increased at P12 just after returning the pups to normal oxygen levels and was decreased at P17 compared with that in normal mice.

Human ApoEs have three major isoforms, ApoE2, ApoE3, and ApoE4, that are encoded by ε2, ε3, and ε4 alleles. These isoforms have different amino acid residues that determine the sites of the receptor-binding domain, positions 112/158; ApoE2 at Cys/Cys, ApoE3 at Cys/Arg, and ApoE4 at Arg/Arg.

**Figure 3.** Effects of ApoE on VEGF-induced cell migration. The effects of ApoE2 (A), ApoE3 (B), and ApoE4 (C) on the VEGF-induced migration of HRMECs. Representative images are shown in (A[a]), (B[a]), and (C[a]). Scale bar represents 100 μm. The values are the means ± SEM (n = 6). **P < 0.01 versus control (Student’s t-test); *P < 0.01 versus control group (Student’s tests); *P < 0.01 versus vehicle-treated group (Dunnett’s test); **P < 0.01 versus control group (Dunnett’s test).
These polymorphisms control the receptor and lipid-binding affinities of the three isoforms of ApoE, and they differ for different cellular responses. Apolipoprotein E binds to the LDL receptor family members, including the LDL receptor, LDL receptor-related protein 1 (LRP1), VLDL receptor, and ApoE receptor 2 (ApoER2, LRP8). The expression of LDLR, VLDLR, and LRP1 in endothelial cells has been reported by many researchers. Moreover, we confirmed the expression of ApoER2 in HRMECs (Supplementary Fig. S1). These findings suggest that there is a possibility that the interaction of ApoE and each receptor is included in proangiogenic effects.

Assay by ELISA showed that amount of ApoE remained at high concentrations (Fig. 1). Moreover, in an in vitro assay, ApoE2 and ApoE3 promoted cell proliferation and migration even without VEGF. These findings indicate that ApoE has a proangiogenic mechanism not only dependent but also independent of VEGF.

It was recently reported that ApoE3 stimulates endothelial nitric oxide synthase (eNOS) and endothelial cell migration by ApoER2 and kinase/Akt on PI3. On the other hand, ApoE4 antagonizes ApoE3/ApoER2 binding and does not stimulate eNOS or endothelial cell migration. In addition, intracellular
NO production was increased in human umbilical vein endothelial cells by cell-derived human ApoE2 and also in EA.hy926 human endothelial cells by cell-derived human ApoE2 and ApoE3.42,43 Because eNOS plays an important role in angiogenesis, 44–47 the proangiogenic effects of ApoE2 and ApoE3 may act by activating eNOS. We performed the cell proliferation assay with NOS antagonist N omega-nitro-L-arginine methyl ester (L-NAME). As same as data in Figure 1, ApoE2 and ApoE3 increased the cell proliferation and L-NAME pretreatment inhibited it (Supplementary Fig. S2). Moreover, Ulrich et al.41 demonstrated that L-NAME inhibited the ApoE3-induced cell migration. These data indicate that eNOS is partly involved in ApoE-induced cell proliferation.

Apolipoprotein E interacts with HDL, whereas ApoE2 and ApoE3 bind with HDL more than ApoE4. 48,49 It was recently reported that HDL interacts with the scavenger receptor, SR-B1, which then activates the PI3K pathway and HIF-1α/VEGF pathway, resulting in hypoxia-induced angiogenesis.50,51 Thus, under hypoxic conditions, HDL enhances the expressions of HIF-1α, VEGF, and VEGFR2. Indeed, the expressions of VEGF and BFGF, both potent proangiogenic factors, were also stronger in eyes of human ApoE2 transfused replacement transgenic mice than in control C57BL/6J mice.53 Furthermore, the retinal VEGF level is lower in human ApoE4 transgenic mice than human ApoE3 transgenic mice,24,27 and ApoE3 treatment increases the level of VEGF in the RPE cell culture medium.56 These findings indicate that ApoE2 and ApoE3, but not ApoE4, have the potential of increasing the level of ocular VEGF. Earlier, Antes et al.24 reported that the laser-induced choroidal neovascularization was more exacerbated in ApoE4 transgenic mice than in ApoE3 transgenic mice. However, neonatal ApoE4 transgenic mice (P4–P7) have low levels of ApoE and an increase in the vascular density, buds, and branching compared with ApoE3 transgenic mice.27 These findings are present because ApoE4 may have protective effects, and its expression is increased to protect the retina from laser-induced choroidal neovascularization. Indeed, ApoE4 blocked the ApoE3-induced NOS activity, cell migration, and phosphorylation of Akt. Moreover, the anti-VEGF treatment outcome is better in neovascular AMD patients who have ApoE e4 allele compared with those who have the ApoE e2 allele. It has been reported that HDL activates the PI3K/Akt/NO pathway to promote the differentiation of peripheral blood monocytes into endothelial progenitor cells.57 Based on these findings, the proangiogenic effects of ApoE2 and ApoE3 may control eNOS activation by binding to HDL; however, it is known that the HDL level of humans is different from that of mice: human HDL is much lower. Therefore, the hypothesis including HDL might be not applicable.
ApoE2 and ApoE3 Promote Retinal Angiogenesis

Another possibility as the mechanism is heparin binding. In the study by Yamauchi et al.,58 they demonstrated that ApoE4-heparin binding was greater than ApoE2 and ApoE3-heparin binding in both lipid-free and lipilated states. There is a possibility that ApoE2 and ApoE3 have poor bonding to heparin sulfate proteoglycan (HSPG) and great binding to cell surface receptor, and then these exhibited additive effects with VEGF. On the other hand, ApoE4 has great binding to HSPG (including free HSPG) competing with VEGF and poor bonding to cell surface receptor or binds different receptor from ApoE2 and ApoE3 binding receptor. However, this hypothesis is completely conjecture, and therefore further study will be needed.

Under normal conditions, the expression of ApoE in the murine retina begins to increase on P7.22 In contrast, our studies focusing on alleles or isoforms of patients are needed to reveal the role of ApoE in DR completely. Further study will be needed.

In the present study, we demonstrated promotive effects of ApoE2 and ApoE3 on angiogenesis. These findings suggest that there is a possibility that retinal angiogenesis in the eyes of patients who have e2 allele or e3 allele might be promoted by ApoE, which remains a high concentration in the eyes even given anti-VEGF treatment. On the other hand, there is a possibility that patients who have e4 allele obtain the maximum benefits of anti-VEGF treatment. Indeed, it has been reported that in wet-type AMD therapy; prognosis of patients with e4 allele is better than that of patients with the e2 allele.59 However, there are some differences in ApoE expression, receptor expression, and lipid metabolism between humans and mice, and between human ApoE targeted replacement mice used in a previous report.24,27,60-63 Therefore, further studies focusing on alleles or isoforms of patients are needed to reveal the role of ApoE in DR completely.

In conclusion, ApoE2 and ApoE3, but not ApoE4, have proangiogenic effects, which is in keeping with the increased expression of ApoE in the vitreous of patients with PDR and DME. These findings indicate that ApoE2 and ApoE3 most likely play important roles in the pathogenesis for PDR, which are characterized by retinal neovascularization.

Acknowledgments

The authors thank the patients and families who participated in this study.

Disclosure: T. Masuda, None; M. Shimazawa, None; Y. Hashimoto, None; A. Kojima, None; S. Nakamura, None; S. Suemori, None; K. Mochizuki, None; H. Kawakami, None; K. Kawase, None; H. Hara, None

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