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Oxidized low density lipoprotein-induced senescence of retinal pigment epithelial cells is followed by outer blood–retinal barrier dysfunction

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ABSTRACT

Age-related macular degeneration is the most common cause of vision loss in the elderly, which starts from aging processes of retinal pigment epithelial cells. Among variable risk factors in occurrence and progression of age-related macular degeneration, oxidized low density lipoprotein could be causally involved in pathobiological changes of RPE cells. Herein we showed that oxidized low density lipoprotein-induced senescence of retinal pigment epithelial cells is followed by outer blood–retinal barrier dysfunction. Under sub-lethal concentration, oxidized low density lipoprotein could promote advanced senescence of retinal pigment epithelial cells. Interestingly expression of CRALBP and RPE 65, indicators of retinal pigment epithelial cell differentiation, was decreased by oxidized low density lipoprotein. In addition, oxidized low density lipoprotein induced reactive oxygen species production and up-regulated inflammatory factors such as tumor necrosis factor-α and vascular endothelial growth factor, when β-catenin, a critical mediator of the canonical Wnt pathway, was also elevated. Oxidized low density lipoprotein increased paracellular permeability of retinal pigment epithelial cells, when zonula occludens-1 at intercellular junctions markedly decreased as well. Furthermore, in retinal pigment epithelial cells and choriocapillaris of human apolipoprotein E2 transgenic mouse eye, increased vascular endothelial growth factor and decreased zonula occludens-1 expression was observed. Therefore, our results suggest that oxidized low density lipoprotein could promote senescence of retinal pigment epithelial cells which leads to induce outer blood–retinal barrier dysfunction as an early pathogenesis of age-related macular degeneration.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of visual impairment in elderly people (Klein et al., 2004). Depending on clinico-pathological features AMD may be grouped as two types of dry form and wet form. The dry form is demonstrated by the progressive degeneration of retinal pigment epithelial (RPE) cells and the subsequent loss of photoreceptors whereas the wet form is characterized by choroidal neovascularization which may lead to the sudden vision loss from subretinal hemorrhage or edema (Jager et al., 2008). Regardless of different clinico-pathological features, AMD is assumed to start from common age-related pathological processes of RPE cells including drusen and pigmentary changes limited in RPE cells and adjacent structures (Roth et al., 2004; Feher et al., 2006). Accordingly, an approach to intervene the senescence-associated changes of RPE cells would be helpful for preventing development or progression of AMD. Although the cause of AMD remains to be elucidated, it has been proposed that variable risk factors including nutritional, medical, genetic factors as well as lifestyles may play a role in occurrence and progression of AMD (Van Leeuwen et al., 2003; Clemons et al., 2005; DeWan et al., 2007; Chakravarthy et al., 2010). In particular, cardiovascular risk factors are closely linked to AMD development and progression. In addition to previous history of cardiovascular diseases, increased low-density lipoprotein (LDL) in serum was related to the increased risk of AMD whereas increased high-density lipoprotein (HDL) was related to decreased risk (Tan et al., 2007; Chakravarthy et al., 2010). We also reported that the eyes of human apolipoprotein E2 transgenic mouse (apoE2) with high serum cholesterol develop lipid accumulation in RPE cells, a typical characteristic of AMD (Lee et al., 2007). Furthermore, native LDL up-regulated expression of

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vascular endothelial growth factor (VEGF), a major angiogenic and inflammatory factor in RPE cells (Lee et al., 2007).

RPE cells in the retina form a monolayer between photoreceptors and choroidal vessels which is a barrier to maintain the normal structural and functional integrity of the retina. The blood–retinal barrier (BRB) is composed of inner BRB of retinal microvascular endothelial cells and outer BRB of RPE cells (Cunha-Vaz, 1976). As the BRB is essential to serve functions in the eye, the BRB breakdown could lead to serious visual impairment. For example, AMD is closely related to outer BRB breakdown whereas diabetic retinopathy is a common cause of inner BRB breakdown (Kim et al., 2006; Jo et al., 2010). Physiologically outer BRB could be maintained by intercellular tight junction between RPE cells, which is however disrupted with the redistribution of tight junction proteins and increased paracellular permeability under pathological conditions (Kim et al., 2006, 2010a).

Oxidized LDL has been known to be relevant to pathobiological changes of variable human diseases including artherosclerosis, which is mediated by oxidative stress (Steinberg, 1997). Interestingly, the oxidative stress has been thought to play a critical role in the pathogenesis of AMD, for RPE cells are basically prone to oxidative stress from high oxygen tension of high metabolic activity, physiological phagocytosis as well as life-long light illumination (Roth et al., 2004; Feher et al., 2006; Jo et al., 2010). Although transient fluctuations of reactive oxygen species from normal oxidative condition could play some regulatory roles in cellular physiology, abnormally increased and sustained oxidative stress could lead to pathobiological changes including outer BRB breakdown and senescence of RPE cells (Martindale and Holbrook, 2002; Bailey et al., 2004). Therefore, based on our and other researchers' reports that high cholesterol could link to AMD development and progression (Tan et al., 2007; Chakravarthy et al., 2010), oxidized LDL could be causally involved in pathobiological changes of RPE cells (Lee et al., 2007; Kamei et al., 2007; Yu et al., 2009).

In the present study, we for the first time demonstrated that oxidized LDL-induced senescence of RPE cells is followed by outer BRB dysfunction. Interestingly, oxidized LDL promoted advanced senescence of RPE cells and inhibited differentiation of RPE cells. With treatment of oxidized LDL, ROS production as well as expression of inflammatory factors such as tumor necrosis factor (TNF)-α and VEGF was significantly increased, when β-catenin was also up-regulated. Surprisingly, oxidized LDL increased paracellular permeability of RPE cells, when ZO-1 at intercellular junctions markedly decreased. Furthermore, VEGF expression was increased in RPE cells and choriocapillaris of apoE2 eyes compared to control whereas ZO-1 expression was significantly decreased in apoE2 eyes. Taken together, oxidized LDL may promote senescence of RPE cells which would lead to outer BRB dysfunction via oxidative stress and inflammation.

2. Materials and methods

2.1. Cell culture

ARPE-19 cells were used for human RPE cells. ARPE-19 cell was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). According to the standard procedure, the cells were kept in Dulbecco's modified Eagle's medium (Invitrogen, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 100 U/ml of penicillin (Sigma–Aldrich, St. Louis, MO, USA), 100 μg/ml of streptomycin (Invitrogen, Gibco, Carlsbad, CA, USA), and 1 mM of sodium pyruvate (Sigma–Aldrich, St. Louis, MO, USA). ARPE-19 cells used in this study were taken from passages 4 to 6.

2.2. Mouse

Human apolipoprotein E2 transgenic mouse (B6.129P2-$^{Apoetm1(APOE2)Mae}$ N8) and C57BL/6J mice were purchased from Taconic (Germantown, NY, USA) and Samtako (Seoul, Korea), respectively. The apoE2 specifically express human apoE2 but not mouse apoE (mapoe$^{-/-}$/hapoe2$^{+/+}$) (Sullivan et al., 1998). Care, use, and treatment of all animals in this study were in strict agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Mice were kept in standard 12-h dark–light cycles and approximately 23 °C room temperature. Ten 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, the enucleated eyes for imunohistochemistry were fixed in 4% paraformaldehyde and subsequently embedded in paraffin.

2.3. Preparation of oxidized LDL

With modification from a previous report (Chen et al., 2003), native LDL (Sigma, St. Louis, MO, USA) was oxidized by exposure to 10 mM $CuSO_4$ in phosphate-buffered saline (PBS) at 37 °C for 24 h. After the oxidation was terminated by adding 0.3 mM EDTA, the preparation was dialyzed and preserved in nitrogen-filled tubes. Protein concentration of the prepared LDL was quantified by a modification of Lowry's method.

2.4. Cell viability assay

Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. ARPE-19 cells (1×10^5 cells) were incubated with variable concentrations of oxidized LDL (10–100 μg/ml) for 24 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h. After incubation, the medium was carefully removed from the plate and dimethyl sulfoxide was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Senescence-associated β-galactosidase (SA-β-gal) staining

According to a previous report (Dimri et al., 1995), ARPE-19 cells (1×10^5 cells) were incubated with variable concentrations of oxidized LDL (10–50 μg/ml) and fixed with 2% formaldehyde/0.2% glutaraldehyde and incubated under light protection at 37 °C for 8 h with fresh SA-β-gal stain solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside, 40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$. The RPE cells with SA-β-gal staining were evaluated by two masked and independent observers (Kim JH and Lee SJ) on randomly selected 10 fields at a ×400 magnification under light microscopy (Carl Zeiss, Chester, VA, USA).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

For mRNA measurement of *CRALBP* and *RPE 65*, ARPE-19 cells (1×10^5 cells) were maintained in culture for 1 week or 5 weeks after seeding, and then incubated with 50 μg/ml oxidized LDL. For mRNA measurement of *TNF-α* and *VEGF*, ARPE-19 cells (1×10^5 cells) were incubated with 50 μg/ml oxidized LDL. Total RNA from cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-stranded cDNA was synthesized with 3 μg each of DNA-free total

RNA and oligo-(dT) 16 primer by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Equal amounts of cDNA were subsequently amplified by PCR in a 50-μl reaction volume containing 1 × PCR buffer; 200 μM of dNTPs; 10 μM of specific primer for *CRALBP* (5′-TGGCAAAGTCAAGAAATCACC-3′ and 5′-CGTGGACAAAGACCCTCTCA-3′), *RPE 65* (5′-GTGTAGTT-CTGAGTGTGGTG-3′ and 5′-CACAGAGGAAGTATGATTAT-3′), TNF-α (5′-CCAAACGATGTTGTACCCGA-3′ and 5′-CAGTTGGAGG-AGAGACGGTA-3′) *VEGF* (5′-TTGCCTTGCTGCTCTACCTC-3′ and 5′-AAATGCTTTCTCCGCTCTGA-3′) and *GAPDH* (5′-TCCCTCAAG-ATTGTCAGCAA-3′ and 5′-AGATCCACAACGGATACATT-3′) and 1.25 U of Taq DNA polymerase (TaKaRa, Tokyo, Japan). Amplification was performed for a total of 25–35 cycles. To ensure the equal loading of mRNA in each lane, *GAPDH* expression was measured.

2.7. Intracellular ROS measurement

ARPE-19 cells (1×10^5 cells) were incubated with 50 μg/ml oxidized LDL and were then labeled with 20 μM of 2′,7′-dichlorofluorescein-diacetate (2′,7′-DCFH-DA; Sigma–Aldrich Co., St. Louis, MO, USA) for 30 min at 37 °C. DCF fluorescence was measured with excitation and emission settings of 495 and 525 nm, respectively. Nonspecific fluorescence values without cells were subtracted from the fluorescence values with cells.

2.8. Western blot analysis

ARPE-19 cells (1×10^5 cells) were incubated with 50 μg/ml oxidized LDL or 20 mM lithium chloride (Sigma–Aldrich Co., St. Louis, MO, USA). Western blotting was performed using standard western blotting methods. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by electrophoresis on 5–10% SDS-PAGE and transferred electrophoretically on nitrocellulose membrane (Amersham, Little Chalfont, UK). The membranes were blocked for 30 min in 5% non-fat milk. The membranes after blocking were incubated overnight with antibodies against β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. To ensure the equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against β-actin. Intensity values were normalized relative to control values. The blots were scanned using a flatbed scanner and the band intensity analyzed using the TINA software program (Raytest, Staubenhardt, Germany).

2.9. Permeability assay

ARPE-19 cells (1×10^5 cells) were incubated on to 0.4 μM pore polyester membrane filters (Corning Costar, Acton, MA, USA) and then treated with 50 μg/ml oxidized LDL. Rhodamine isothiocyanate conjugated dextran was added to the seeded cells. The fluorescence was measured with a fluorometer at an excitation wavelength of 570 nm and emission wavelength of 595 nm (Tecan Group Ltd, Mannedorf, Switzerland).

2.10. Immunocytochemistry

As our previous report, ZO-1 expression at intercellular junction was examined by an immunocytochemical method. ARPE cells treated with 50 μg/ml oxidized LDL were fixed with 2% paraformaldehyde, blocked with 3% bovine serum albumin, and incubated with an antibody against ZO-1 (1:100, Zymed, San Francisco, CA, USA). The slides were viewed by a fluorescence microscopy (BX50, OLYMPUS, Japan).

2.11. Immunohistochemistry

Four mm-thick serial sections were prepared from paraffin blocks. Sections were deparaffinized, hydrated by sequential immersion in xylene and graded alcohol solutions, treated with proteinase K. Slides were incubated overnight at 4 °C with antibodies against VEGF (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ZO-1 (1:100, Zymed, San Francisco, CA, USA) and anti-ZO-2 (1:100, Zymed, San Francisco, CA, USA), followed by a biotinylated goat anti-mouse antibody (Dako, Glostrup, Denmark), revealed by the avidin–biotin complex (Vectastain kit; Vector, Burlingame, CA) and the 3-amino-9-ethyl-carbazole chromogen. The slides were mounted Faramount Aqueous mounting medium (DAKO, Glostrup, Denmark) and observed under a light microscopy (Carl Zeiss, Chester, VA, USA).

2.12. Statistical analysis

Statistical differences between groups were evaluated using Student's paired *t*-test. All statistical tests were completed using SPSS for Windows, version 12.0 (SPSS, Chicago, IL, USA). Figures are depicted as means ± SD. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Oxidized LDL promotes senescence of RPE cells.

To examine the cytotoxicity of oxidized LDL on human RPE cells, MTT assay was carried out with variable concentrations of oxidized LDL (10–100 μg/ml). Interestingly, up to 50 μg/ml of oxidized LDL, incubation of RPE cells with oxidized LDL showed no significant change in the cellular viability (Fig. 1A).

Based on clinical and experimental evidences of increased risk of AMD with high serum LDL (Lee et al., 2007; Tan et al., 2007; Chakravarthy et al., 2010), we next investigated whether oxidized LDL promotes the senescence of RPE cells. When RPE cells were treated with sub-lethal doses of oxidized LDL, SA-β-gal staining positive cells increased in dose-dependent manner (Fig. 1B).

3.2. Oxidized LDL inhibits differentiation of RPE cells.

To assess whether oxidized LDL to promote senescence of RPE cells could affect to differentiation status of RPE cells, mRNA expression of CRALBP and RPE 65 was measured in differentiated RPE cells with treatment of oxidized LDL. As expression of CRALBP and RPE 65 correlate with differentiation of RPE cells (Bunt-Milam and Saari, 1983; Hamel et al., 1993), RPE cells cultured for 5 weeks after seeding demonstrated dramatically increased levels of CRALBP and RPE 65 compared to 1 week culture, which was however significantly suppressed by treatment of 50 μg/ml oxidized LDL (Fig. 2).

3.3. Oxidized LDL increases ROS production as well as expression of inflammatory factors in RPE cells.

Given that cumulative oxidative stress caused by ROS contributes to incidence and progression of AMD (Bailey et al., 2004; Clemons et al., 2005; Kim et al., 2010a), we investigated the effect of oxidized LDL on ROS production in RPE using the cell permeable fluorescence dye. As demonstrated in Fig. 3A, the intensity of DCF peak was increased 2-fold compared to control after 50 μg/ml oxidized LDL treatment in RPE cells.

In addition to oxidative stress, inflammation in RPE cells is regarded as a major stimulus for the development of AMD. Based on accumulating evidence of inflammation of RPE cells in AMD pathogenesis (Roth et al., 2004; Jo et al., 2010), the expression of

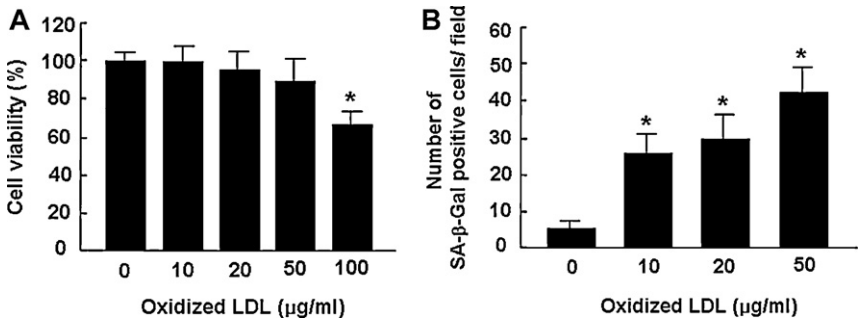

Fig. 1. Oxidized LDL promotes senescence of RPE cells. (A) The cellular viability was measured in ARPE-19 cells treated with different concentrations of oxidized LDL (10–100 μg/ml) by MTT assay. Each value represents means ± SE from three independent experiments (*$P < 0.05$). (B) The cellular senescence was evaluated in ARPE-19 cells treated with different concentrations of oxidized LDL (10–50 μg/ml) by SA-β-Gal activity. Quantitative analysis for SA-β-Gal activity was quantitatively measured by counting SA-β-Gal positive cells in 10 representative fields. Each value represents means ± SE from three independent experiments (*$P < 0.05$).

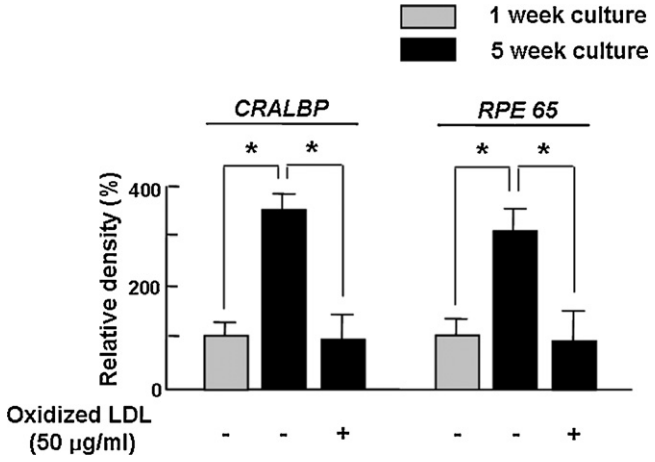

Fig. 2. Oxidized LDL inhibits differentiation of RPE cells. ARPE-19 cells were cultured for 1 week or 5 weeks after seeding before treatment with 50 μg/ml oxidized LDL. Using total mRNAs isolated from ARPE-cells RT-PCR was performed with specific primers for CRALBP and RPE 65. GAPDH served as an internal control. Quantitative analysis was performed by measuring mRNA expression relative to the control. Each value represents means ± SE from three independent experiments (*$P < 0.05$).

inflammatory factors such as TNF-α and VEGF induced by oxidized LDL was assessed by RT-PCR. In addition to increased ROS production, a significant up-regulation of TNF-α (2.3-fold) and VEGF (2.2-fold) mRNA was observed with treatment of oxidized LDL (50 μg/ml) (Fig. 3B).

3.4. Oxidized LDL activates Wnt pathway, which could regulate VEGF expression in RPE cells

Oxidative stress and Inflammation in RPE cells interdependently play roles in the development of AMD (Jo et al., 2010). Based on recent reports that activation of the canonical Wnt pathway is responsible for regulation of retinal inflammation as well as oxidative stress in RPE cells (Steindl-Kuscher et al., 2009; Zhou et al., 2010), we assessed whether oxidized LDL induces activation of the canonical Wnt pathway by measuring total levels of β-catenin, as a critical mediator of the canonical Wnt pathway. As demonstrated in Fig. 4A, 50 μg/ml oxidized LDL significantly increased β-catenin level.

Next, to investigate whether increased VEGF expression induced by oxidized LDL could be mediated by the canonical Wnt pathway, RPE cells were incubated with lithium chloride, a GSK 3 inhibitor, or oxidized LDL. As expected, VEGF expression was significantly increased with lithium chloride treatment, which was comparable to oxidized LDL-induced VEGF expression (Fig. 4B).

3.5. Oxidized LDL increases paracellular permeability of RPE cells which is accompanied by disruption of tight junction between RPE cells

Outer BRB is determined by tight junction in differentiated RPE cells, which maintains paracellular permeability of RPE cells (Kim et al., 2006, 2010a; Jo et al., 2010). Therefore, based on our result that oxidized LDL inhibits differentiation of RPE cells, we investigated whether oxidized LDL disrupts the integrity of outer BRB by measuring the fluorescence flux through RPE cells and by evaluating the localization of tight junction proteins between RPE cells. With treatment of oxidized LDL, paracellular permeability of RPE cells was significantly increased compared to control (Fig. 5A). As shown in Fig. 5B, ZO-1, a protein located on a cytoplasmic membrane surface of intercellular tight junctions, was well arranged along intercellular junctions in confluent RPE cells. However, ZO-1 arrangement at intercellular junction were markedly disrupted (Fig. 5B)

3.6. VEGF expression in RPE cells of apoE2 eyes was increased whereas ZO-1 expression was decreased

Using apoE2 with high serum cholesterol to demonstrate pathological characteristics of AMD in RPE cells (Lee et al., 2007), VEGF and ZO-1 expression was assessed by immunohistochemistry. Similar to increased expression of VEGF in the eye of patients with AMD (Jaeger et al., 2008), VEGF expression was increased in RPE cells and choriocapillaris of apoE2 eyes compared to control, which appeared to be slightly elevated after high-fat diet (Fig. 6). However, apoE2 eyes with and without high fat-diet showed decreased ZO-1 expression in RPE cells and choriocapillaris, which indicates weakening of the tight junction of outer BRB (Fig. 6).

4. Discussion

In the current study, we for the first time demonstrated that oxidized LDL promotes senescence of RPE cells, which leads to outer BRB disruption through oxidative stress and inflammation in RPE cells mediated by the canonical Wnt pathway.

Herein we showed that under sub-lethal concentration, oxidized LDL could promote senescence of RPE cells and inhibited differentiation of RPE cells. Based on epidemiological studies to provide that age is the most definite risk factor for AMD (Van Leeuwen et al., 2003; Clemons et al., 2005; DeWan et al., 2007; Chakravarthy et al., 2010), senescent changes in RPE cells and their adjacent structures might result in AMD development (Roth et al., 2004; Feher et al., 2006). However molecular mechanisms underlying incidence and progression of AMD that is conferred by aging should be still elucidated. Although senescent changes are composed of variable

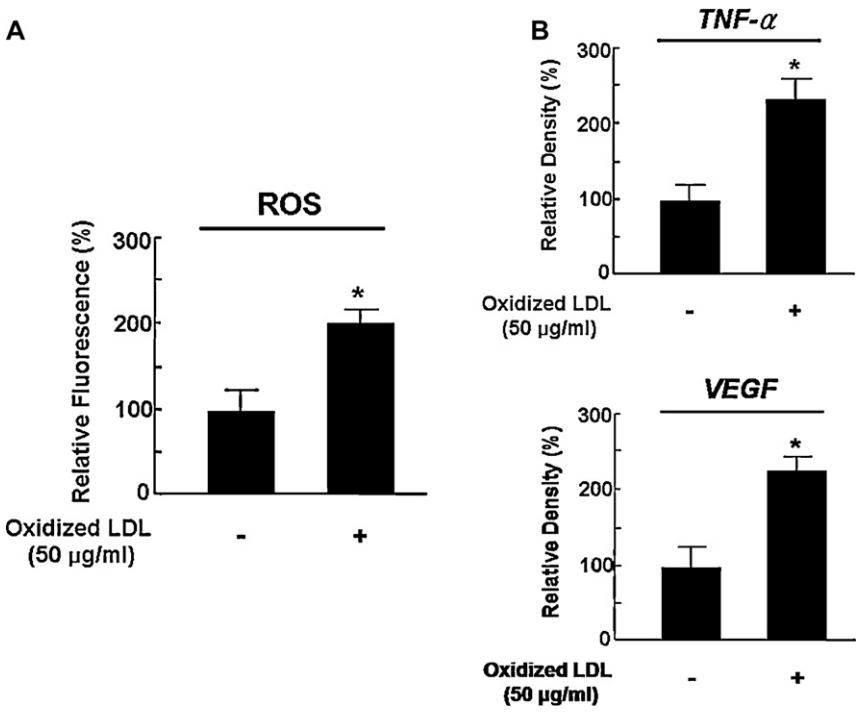

Fig. 3. Oxidized LDL increases ROS production as well as expression of inflammatory factors in RPE cells. ARPE-19 cells were treated with 50 μg/ml oxidized LDL. (A) For measuring ROS production, ARPE-19 cells were labeled with DCFH-DA. Quantitative analysis was performed by measuring the fluorescence intensity relative to the control. Each value represents means ± SE from three independent experiments (*$P < 0.05$). (B) For evaluating TNF-α and VEGF expression, total mRNAs isolated from ARPE-cells, and RT-PCR was performed with specific primers for TNF-α and VEGF. GAPDH served as an internal control. Quantitative analysis was performed by measuring mRNA expression relative to the control. Each value represents means ± SE from three independent experiments (*$P < 0.05$).

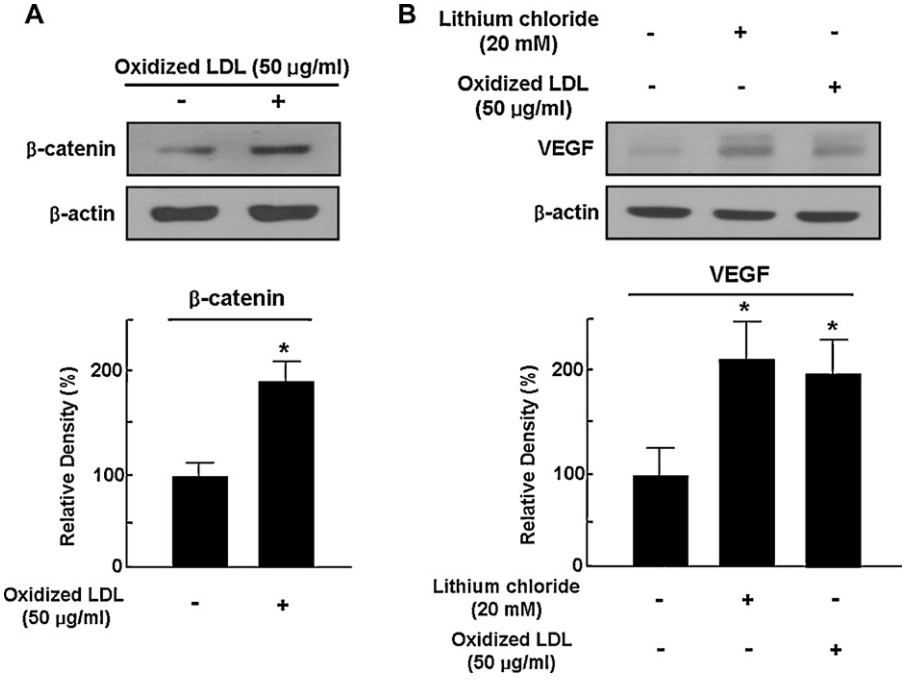

Fig. 4. Oxidized LDL activates Wnt pathway, which could regulate VEGF expression in RPE cells. (A) ARPE-19 cells were treated with 50 μg/ml oxidized LDL. Activation of the canonical Wnt pathway was evaluated by measuring β-catenin level using Western blot analysis. β-Actin was served as the loading control. Figures were selected as representative data from three independent experiments. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents means ± SE from three independent experiments (*$P < 0.05$). (B) ARPE-19 cells were treated with 50 μg/ml oxidized LDL or 20 mM lithium chloride. VEGF expression was measured by Western blot analysis. β-Actin was served as the loading control. Figures were selected as representative data from three independent experiments. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents means ± SE from three independent experiments (*$P < 0.05$).

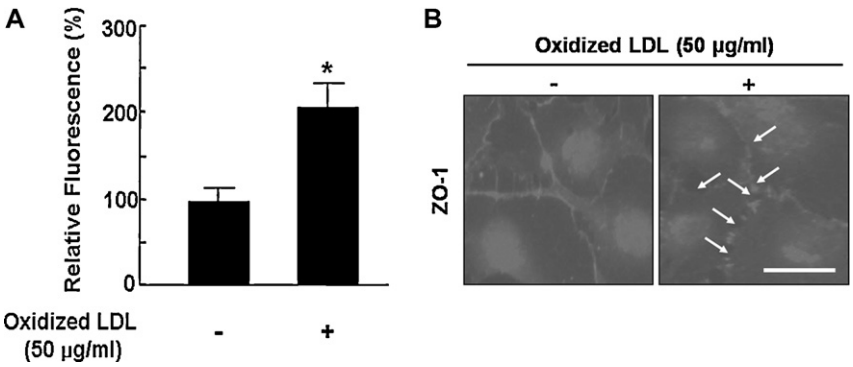

Fig. 5. Oxidized LDL increases paracellular permeability of RPE cells which is accompanied by disruption of tight junction between RPE cells. Confluent ARPE-19 cells were treated with 50 μg/ml oxidized LDL. (A) Permeability of fluorescence dye was quantified in a fluorescence spectrofluorophotometer. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents means ± SE from three independent experiments (*$P < 0.05$). (B) ZO-1 expression in intercellular junction of ARPE-19 cells was examined by an immunocytochemistry. Arrows indicate points where intercellular junction was disrupted. Figures were selected as representative data from three independent experiments. Scale bar: 4 μm.

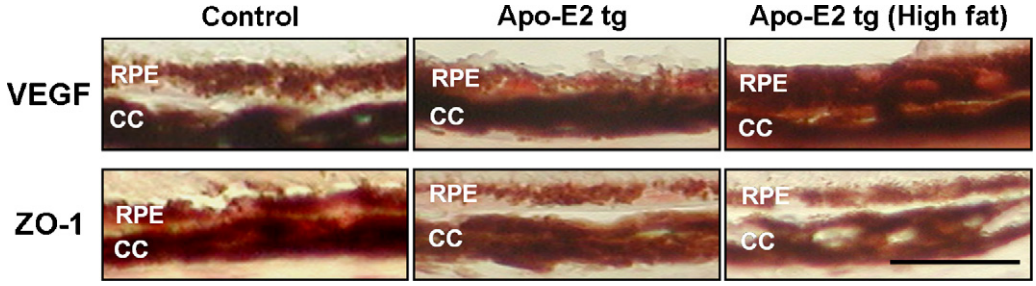

Fig. 6. VEGF expression in RPE cells of apoE2 eyes was increased whereas ZO-1 expression was decreased. Immunohistochemistry for VEGF and ZO-1 was performed in the apoE2 eyes with or without high-fat diet. Figures were selected as representative data from three independent experiments. Scale bars: 100 μm. RPE, retinal pigment epithelial cell layer; CC, choriocapillary layer.

biological processes not to be attributed to a certain molecule or signaling pathway, oxidative stress and inflammation in RPE cells has been proposed as important contributors to AMD (Bailey et al., 2004; Roth et al., 2004; Clemons et al., 2005), which actually induce advanced senescence of RPE cells (Nilsson et al., 2003; Kamei et al., 2007; Jo et al., 2010).

Furthermore, we found out that oxidized LDL increases expression of inflammatory factors as well as ROS production, which could be regulated by activation of the canonical pathway. Considering a recent report that oxidative stress and retinal inflammation are directly and indirectly mediated by activation of the canonical Wnt pathway (Zhou et al., 2010), ROS production and inflammatory factors such as TNF-α and VEGF induced by oxidized LDL could be regulated through the canonical Wnt pathway. Although VEGF expression is known to be directly induced by β-catenin (Zhang et al., 2001), TNF-α is not known to be a target gene regulated by the canonical pathway. However, in addition to ROS production, activation of the canonical Wnt pathway could finally result in elevated expression of inflammatory factors including TNF-α and VEGF (Chen et al., 2009). Therefore, oxidized LDL-mediated activation of β-catenin signaling pathway would be sufficient to induce pro-inflammatory factors, in particular VEGF.

In addition to phagocytosis and visual cycle regulation, RPE cells function as an outer BRB. Oxidative stress as well as inflammation increase RPE permeability, and induce redistribution of tight junction proteins, which results in dysfunction of the outer BRB (Ho et al., 2006). Increased permeability in RPE cells is accompanied by decrease of tight junction proteins including occludin and ZO-family (Bailey et al., 2004). As our previous reports, ZO family could play critical roles in changes of permeability in BRB, which directly linked of occludin to actin to form tight junction (Kim et al., 2009, 2010b). As the dysfunction of RPE cell is one of early pathogenic changes in AMD development (Kim et al., 2006; Jo et al., 2010), oxidized LDL-induced oxidative stress and inflammation in RPE cells could lead to outer BRB dysfunction. Similarly our results clearly provided that oxidized LDL increases paracellular permeability and disruption of tight junction in RPE cells, which was supported by increased VEGF and decreased ZO-1, a tight junction protein in apoE2 eyes. Taken together, oxidized LDL could induce the outer BRB dysfunction in RPE cells which would lead to AMD development.

In summary, oxidized LDL promotes senescence of RPE cells via oxidative stress and inflammation, which would be regulated by the canonical Wnt pathway. Furthermore, oxidized LDL-induced oxidative stress and inflammation could induce the outer BRB dysfunction. Based on these available evidences, we suggest that oxidized LDL-induced senescence of RPE cells could lead to dysfunction of the outer BRB, which would be closely related to incidence and progression of AMD. Therefore, blockade of oxidized LDL-induced pathological cascades in RPE cells should be considered for the therapeutic approach to AMD.

Contribution

Jeong Hun Kim designed the research; Jin Hyoung Kim and Sung-Joon Lee performed the research; Sung-Joon Lee, Kyu-Won Kim and Young Suk Yu contributed materials and established methods; Jin Hyoung Kim and Sung-Joon Lee collected data; Jeong Hun Kim, Jin Hyoung Kim, and Sung-Joon Lee analyzed data; and Jeong Hun Kim, Jin Hyoung Kim, and Sung-Joon Lee wrote the paper.

Conflict of interest

The authors declare no competing financial interests.

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References

Bailey TA, Kanuga N, Romero IA, Greenwood J, Luthert PJ, Cheetham ME. Oxidative stress affects the junctional integrity of retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2004;45:675–84.

Bunt-Milam AH, Saari JC. Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. J Cell Biol 1983;97:703–12.

Chakravarthy U, Wong TY, Fletcher A, Piault E, Evans C, Zlateva G, et al. Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. BMC Ophthalmol 2010;10:31.

Chen CH, Jiang T, Yang JH, Jiang W, Lu J, Marathe GK, et al. Low-density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. Circulation 2003;107:2102–8.

Chen Y, Hu Y, Moiseyev G, Zhou KK, Chen D, Ma JX. Photoreceptor degeneration and retinal inflammation induced by very low-density lipoprotein receptor deficiency. Microvasc Res 2009;78:119–27.

Clemons TE, Milton RC, Klein R, Seddon JM, Ferris 3rd FL, Age-Related Eye Disease Study Research Group. Risk factors for the incidence of Advanced Age-Related Macular Degeneration in the Age-Related Eye Disease Study (AREDS) AREDS report no. 19. Ophthalmology 2005;112:533–9.

Cunha-Vaz JG. The blood–retinal barriers. Doc Ophthalmol 1976;41: 287–327.

DeWan A, Bracken MB, Hoh J. Two genetic pathways for age-related macular degeneration. Curr Opin Genet Dev 2007;17:228–33.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995;92:9363–7.

Feher J, Kovacs I, Artico M, Cavallotti C, Papale A, Balacco Gabrieli C. Mitochondrial alterations of retinal pigment epithelium in age-related macular degeneration. Neurobiol Aging 2006;27:983–93.

Hamel CP, Tsilou E, Harris E, Pfeffer BA, Hooks JJ, Detrick B, et al. A developmentally regulated microsomal protein specific for the pigment epithelium of the vertebrate retina. J Neurosci Res 1993;34: 414–25.

Ho TC, Yang YC, Cheng HC, Wu AC, Chen SL, Tsao YP. Pigment epithelium-derived factor protects retinal pigment epithelium from oxidant-mediated barrier dysfunction. Biochem Biophys Res Commun 2006;342:372–8.

Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. N Engl J Med 2008;358:2606–17.

Jo DH, Kim JH, Kim JH. How to overcome retinal neuropathy: the fight against angiogenesis-related blindness. Arch Pharm Res 2010;33:1557–65.

Kamei M, Yoneda K, Kume N, Suzuki M, Itabe H, Matsuda K, et al. Scavenger receptors for oxidized lipoprotein in age-related macular degeneration. Invest Ophthalmol Vis Sci 2007;48:1801–7.

Nilsson SE, Sundelin SP, Wihlmark U, Brunk UT. Aging of cultured retinal pigment epithelial cells: oxidative reactions, lipofuscin formation and blue light damage. Doc Ophthalmol 2003;106:13–6.

Kim JH, Kim JH, Jun HO, Yu YS, Min BH, Park KH, et al. Protective effect of clusterin from oxidative stress-induced apoptosis in human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2010a;51:561–6.

Kim JH, Kim JH, Jun HO, Yu YS, Kim KW. Inhibition of protein kinase C delta attenuates blood–retinal barrier breakdown in diabetic retinopathy. Am J Pathol 2010b;176:1517–24.

Kim JH, Kim JH, Yu YS, Kim DH, Kim KW. Recruitment of pericytes and astrocytes is closely related to the formation of tight junction in developing retinal vessels. J Neurosci Res 2009;87:653–9.

Kim JH, Kim JH, Park JA, Lee SW, Kim WJ, Yu YS, et al. Blood–neural barrier: intercellular communication at glio-vascular interface. J Biochem Mol Biol 2006;39:339–45.

Klein R, Peto T, Bird A, Vannewkirk MR. The epidemiology of age-related macular degeneration. Am J Ophthalmol 2004;137:486–95.

Lee SJ, Kim JH, Kim JH, Chung MJ, Wen Q, Chung H, et al. Human apolipoprotein E2 transgenic mice show lipid accumulation in retinal pigment epithelium and altered expression of VEGF and bFGF in the eyes. J Microbiol Biotechnol 2007;17:1024–30.

Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 2002;192:1–15.

Roth F, Bindewald A, Holz FG. Keypathophysiologic pathways in age-related macular disease. Graefes Arch Clin Exp Ophthalmol 2004;242:710–6.

Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 1997;272:20963–6.

Steindl-Kuscher K, Krugluger W, Boulton ME, Haas P, Schrattbauer K, Feichtinger H, et al. Activation of the beta-catenin signaling pathway and its impact on RPE cell cycle. Invest Ophthalmol Vis Sci 2009;50:4471–6.

Sullivan PM, Mezdour H, Quarfordt SH, Maeda N. Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse Apoe with human Apoe*2. J Clin Invest 1998;102:130–5.

Tan JS, Mitchell P, Smith W, Wang JJ. Cardiovascular risk factors and the long-term incidence of age-related macular degeneration: the Blue Mountains Eye Study. Ophthalmology 2007;114:1143–50.

Van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, De Jong PT. The risk and natural course of age-related maculopathy: follow-up at 6 1/2 years in the Rotterdam study. Arch Ophthalmol 2003;121:519–26.

Yu AL, Lorenz RL, Haritoglou C, Kampik A, Welge-Lussen U. Biological effects of native and oxidized low-density lipoproteins in cultured human retinal pigment epithelial cells. Exp Eye Res 2009;88:495–503.

Zhang X, Gaspard JP, Chung DC. Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. Cancer Res 2001;61:6050–4.

Zhou T, Hu Y, Chen Y, Zhou KK, Zhang B, Gao G, et al. The pathogenic role of the canonical Wnt pathway in age-related macular degeneration. Invest Ophthalmol Vis Sci 2010;51:4371–9.