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Cholesterol enhances amyloid β deposition in mouse retina by modulating the activities of Aβ-regulating enzymes in retinal pigment epithelial cells

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ABSTRACT

Subretinally-deposited amyloid β (Aβ) is a main contributor of developing age-related macular degeneration (AMD). However, the mechanism causing Aβ deposition in AMD eyes is unknown. Hypercholesterolemia is a significant risk for developing AMD. Thus, we investigated the effects of cholesterol on Aβ production in retinal pigment epithelial (RPE) cells *in vitro* and in the mouse retina *in vivo*. RPE cells isolated from senescent (12-month-old) C57BL/6 mice were treated with 10 μg/ml cholesterol for 48 h. Aβ amounts in culture supernatants were measured by ELISA. Activity and expression of enzymes and proteins that regulate Aβ production were examined by activity assay and real time PCR. The retina of mice fed cholesterol-enriched diet was examined by transmission electron microscopy. Cholesterol significantly increased Aβ production in cultured RPE cells. Activities of Aβ degradation enzyme; neprilysin (NEP) and anti-amyloidogenic secretase; α-secretase were significantly decreased in cell lysates of cholesterol-treated RPE cells compared to non-treated cells, but there was no change in the activities of β- or γ-secretase. mRNA levels of NEP and α-secretase (ADAM10 and ADAM17) were significantly lower in cholesterol-treated RPE cells than non-treated cells. Senescent (12-month-old) mice fed cholesterol-enriched chow developed subRPE deposits containing Aβ, whereas age-matched mice fed standard rodent chow diet did not. Activities and mRNA levels of NEP and α-secretase were significantly lower in native RPE cells freshly isolated from cholesterol-enriched chow fed mice compared to standard rodent chow fed mice. These findings suggest that cholesterol enhances subretinal Aβ accumulation by modulating the activities of enzymes degrading and processing Aβ in RPE cells in senescent subjects.

1. Introduction

Aβ peptides vary in length from 39 to 43 amino residues and have been well identified as a major component of senile plaques in the brains of patients with Alzheimer's disease (AD) [1]. Aβ is produced by the sequential proteolytic processing of amyloid precursor protein (APP). The steady state levels of Aβ peptides are maintained by metabolic balance between synthesis and degradation [2,3]. The amyloidogenic pathway of APP cleavage leads to Aβ synthesis by sequential cleavage of APP by β- and γ-secretase [4,5]. APP cleavage by α-secretase leads to a non-amyloidogenic pathway, thus precludes Aβ synthesis [6,7]. Synthesized Aβ can be degraded by various proteases such as insulin degrading enzyme [8], neprilysin (NEP) [3] and cathepsin B [9].

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population in the western world [10]. Earlier studies have shown that the presence of drusen, identified as grayish-yellow deposits beneath the retinal pigment epithelial (RPE) cells, represents a significant risk for the development of AMD [11]. Aβ is an important component of drusen deposits in AMD [12,13]. We previously reported that senescent NEP gene-disrupted mice developed several features of human eyes with AMD in accordance of the accumulation of Aβ in subretinal space [14]. This suggests that Aβ accumulation in drusen may be a key contributor to the development of AMD. However, the mechanisms that cause subretinal Aβ accumulation in AMD eyes are largely unknown.

Several lines of evidence suggest that genetic predisposition and environmental as well as dietary factors may contribute to the pathogenesis of AMD and AD. Cholesterol (free and esterified) is highly distributed in the human drusen and high intake of cholesterol and saturated fat is associated with an increased risk of AMD [15–19]. Previous studies have also shown that cholesterol-enriched diet dramatically exacerbated Aβ pathology and

Abbreviations: Aβ, amyloid β; APP, amyloid precursor protein; RPE, retinal pigment epithelium; AMD, age-related macular degeneration; AD, Alzheimer's disease; NEP, neprilysin.

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cholesterol-lowering drugs decrease the amounts of Aβ and AD pathology in several animal models of AD [20–24].

The mechanism of cholesterol-induced Aβ accumulation in the brain has not been fully clarified, however, some previous studies [25–29] provided evidence that cholesterol had capacity to modulate activities of Aβ synthesis and degradation enzymes. Xiong et al. [25] reported that cholesterol significantly enhanced activities of β- and γ-secretase in tissue samples of both AD and normal aging brain. Bodovitz and Klein [26] demonstrated that cholesterol decreased α-secretase cleavage of APP in APP-transfected HEK293 cells. NEP activity in the plasma of cholesterol-enriched chow fed rabbits was enhanced compared to that of normal chow-fed rabbits [29]. In addition, the transporter of cholesterol, apolipoprotein E (apoE), its ε4 allele is associated with decreased Aβ clearance in the brain [30,31]. NEP activity was reported to be related to the apoE genotypes, being the highest in ε2-positive and the lowest in ε4-positive brains [32]. Cholesterol and apoE are major constituents of drusen of human AMD [15,16] and apoE ε4 allele has been shown strongly related to pathogenesis of AMD [33]. All of these data strongly indicate that cholesterol might be involved in triggering subretinal Aβ accumulation in eyes with AMD. Thus, the purpose of the present study was to examine the effect of cholesterol on Aβ production as well as the expression and activity of Aβ synthesis and degradation enzymes in RPE cells from senescent mice. We also investigated the retinal phenotype in senescent mice fed cholesterol-enriched diet.

2. Materials and methods

Details for materials, animals, RPE primary culture, mRNA and cell lysates isolation, PCR analysis and electron microscopic examination are available in Supplemental materials.

2.1. ELISA measurements of $A\beta_{1-40}$ and $A\beta_{1-42}$

$A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations in culture supernatants of RPE cells treated with/without cholesterol were determined with commercial ELISA kits (Wako, Osaka, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm in a Bio-Rad Model 450 microplate reader (Bio-Rad Laboratories, Hercules, CA). All experiments were performed at least six times.

2.2. Activity assay of NEP, α-secretase, β-secretase and γ-secretase

Cultured RPE cells treated with/without 10 μg/ml cholesterol and native RPE cells isolated from cholesterol-enriched chow and standard rodent chow fed mice were used to examine the activities of NEP, α-secretase, β-secretase and γ-secretase. One-hundred microliters of DMEM containing 20 μg of total cell lysates of RPE cells was added in duplicate to black CulturPlate-96. Ten micromolar (final concentration) fluorogenic peptide substrate (Mca-RPPGFSAFK[Dnp]-OH; R&D Systems, Cat. No. ES-005) for NEP, 10 μM (final concentration) fluorogenic peptide substrate (Nma-GGVVLATVL[DNP]-D-A-D-A-D-A-NH_2; Calbiochem, Cat. No. 565764) for γ-secretase, 40 μM (final concentration) β-secretase substrate and γ-secretase substrate that provided in β-secretase and γ-secretase activity assay kits were added to each well and were incubated at 37 °C. Fluorescence intensities were read according to the manufacturer's instructions on a fluorescent ELISA plate reader every 30 min of 120 min. The excitation was done at 320 nm for NEP, 355 nm for γ-secretase, 490 nm for α- and β-secretase. The emission was done at 405 nm for NEP, 440 nm for γ-secretase, 520 nm for α- and β-secretase.

Because the substrate for NEP is also an excellent substrate for endothelin-converting enzyme-1 (ECE-1), an inhibition study was performed to determine the specificity of the NEP activity. The RPE cell lysates used for NEP activity assay were preincubated with 100 nM thiorphan; an inhibitor of ECE-1 cleavage of the substrate [34], for 10 min at room temperature before addition of substrate and then processed as described above. All experiments were performed at least four times.

2.3. Statistical analysis

Mann–Whitney *U* test or analysis of variance (ANOVA) was used to determine whether the differences existed between experimental mean values. A P value <0.05 was considered significant. All statistical analysis was done with StatView software (SAS Institute, Cary, NC).

3. Results

3.1. RPE cells treated with cholesterol produced more amounts of Aβ than non-treated cells

The results of ELISA showed that $A\beta_{1-40}$ concentration in the supernatants of cholesterol-treated RPE cells was significantly higher than those of non-treated cells (195.4 ± 25.1 pmol/ml vs 132.6 ± 11.2 pmol/ml, Fig. 1A, $n = 6$, $P = 0.017$). Also, $A\beta_{1-42}$ concentration in the supernatants of cholesterol-treated RPE cells was significantly higher than those of non-treated cells (15.7 ± 1.6 pmol/ml vs 11.8 ± 0.6 pmol/ml, Fig. 1B, $n = 6$, $P = 0.021$).

3.2. Decreased activity of NEP and α-secretase in cultured RPE cells treated with cholesterol and native RPE cells isolated from cholesterol-enriched chow fed mice

Cholesterol-treated/nontreated RPE cell lysates (20 μg diluted in 100 μl DMEM, final volume) were incubated with enzyme-specific fluorogenic substrates. After incubation for 120 min, the increased fluorescence density of NEP-specific substrate was $1.12 \pm 0.14 \times 10^5$ in cholesterol-treated RPE cells, which was significantly lower than $2.54 \pm 0.12 \times 10^5$ in non-treated RPE cells (Fig. 2A, $n = 4$, $P = 5.31 \times 10^{-6}$). The increased fluorescence density of α-secretase-specific substrate was $9.42 \pm 1.01 \times 10^4$ in cholesterol-treated RPE cells, which was significantly lower than $1.85 \pm 0.23 \times 10^5$ in non-treated RPE cells (Fig. 2A, $n = 4$, $P = 3.22 \times 10^{-6}$). Increased fluorescence densities of β-secretase specific substrate or γ-secretase specific substrate did not differ between cholesterol-treated and non-treated RPE cells (Fig. 2C and D, $n = 4$). In agreement with cholesterol-treated RPE cells, increased fluorescence densities of NEP (Supplementary Fig. S1A, $n = 4$, $P = 4.37 \times 10^{-6}$) and α-secretase (Supplementary Fig. S1B, $n = 4$, $P = 1.35 \times 10^{-6}$) were also identified significantly decreased in native RPE cells isolated from cholesterol-enriched chow fed mice compared to standard rodent chow fed mice. Increased fluorescence densities of β-secretase specific substrate or γ-secretase specific substrate did not differ between native RPE cells isolated from cholesterol-enriched chow fed mice and normal chow fed mice (Supplementary Fig. S1C and D, $n = 4$).

3.3. Decreased gene expression of NEP and α-secretase in cultured RPE cells treated with cholesterol and native RPE cells isolated from cholesterol-enriched chow fed mice

The mRNA levels of NEP were significantly lower in cholesterol-treated RPE cells than non-treated cells (Fig. 3A, $n = 6$, $P = 0.0003$). The mRNA levels of α-secretase (ADAM10 and ADAM17) were significantly lower in cholesterol-treated RPE cells than non-treated cells (0.67 ± 0.11 vs 1 ± 0.02 for ADAM10, Fig. 3B, $n = 6$,

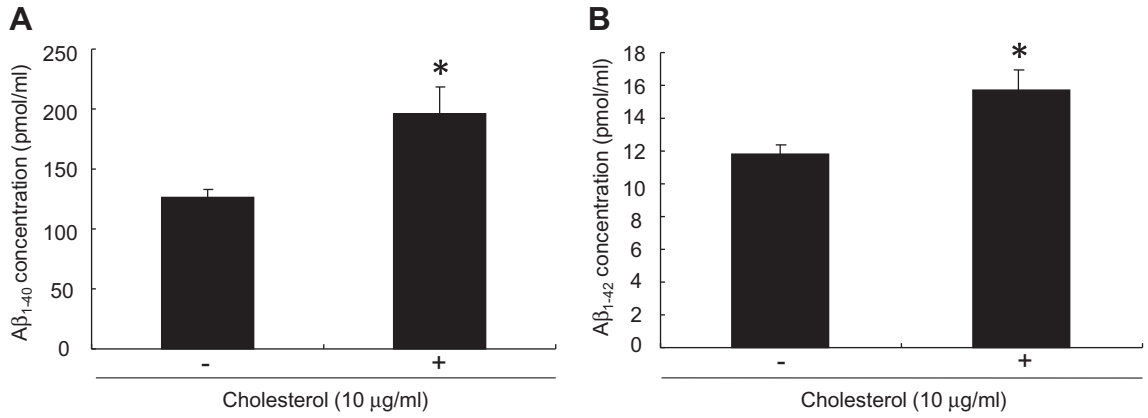

Fig. 1. Concentrations of $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ (B) in culture supernatants of retinal pigment epithelial (RPE) cells in the presence or absence of 10 μg/ml cholesterol. After reaching cellular confluence, the media were changed to serum free DMEM supplemented with or without 10 μg/ml cholesterol and the cells were cultured for another 48 h. Supernatants were then collected and used for ELISA analysis. Values are expressed as the means ± SEMs, $n = 6$, $^*P < 0.05$.

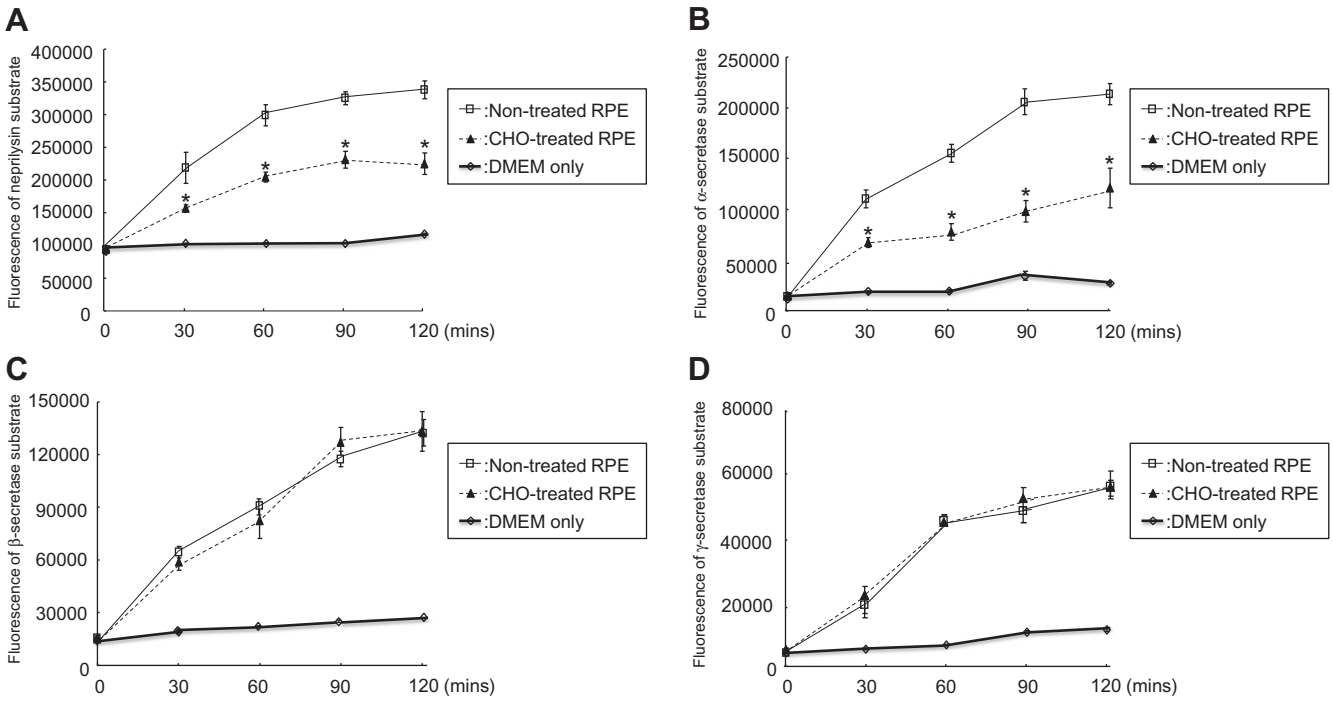

Fig. 2. Activity assay using lysates of retinal pigment epithelial (RPE) cells treated with or without 10 μg/ml cholesterol. Enzyme-specific fluorogenic substrates for neprilysin (A), α-secretase (B), β-secretase (C) and γ-secretase (D) were added to corresponding wells containing 20 μg total cell lysates of 10 μg/ml cholesterol treated or non-treated RPE cells. DMEM without cell lysates (bold lines) was used as negative control. The reaction mixtures were incubated at 37 °C. Fluorescence of catalyzed substrates was measured every 30 min of 120 min. Values are expressed as the means ± SEMs, $n = 4$, $^*P < 0.05$.

$P = 0.0027$; 0.71 ± 0.03 vs 1 ± 0.03 for ADAM17, Fig. 3C, $n = 6$, $P = 0.001$). The mRNA level of APP was not significantly different between cholesterol-treated RPE cells and non-treated cells (Fig. 3D). Significantly decreased mRNA levels of NEP (Supplementary Fig. S2A, $n = 4$, $P = 0.00015$) as well as ADAM10 (Supplementary Fig. S2B, $n = 6$, $P = 0.0055$) and ADAM17 (Supplementary Fig. S2C, $n = 6$, $P = 0.007$) were also observed in native RPE cells isolated from cholesterol-enriched chow fed mice compared to standard rodent chow fed mice. The mRNA level of APP did not differ between native RPE cells isolated from cholesterol-enriched chow fed mice compared to normal chow fed mice (Supplementary Fig. S2D).

3.4. Mice fed cholesterol-enriched chow developed subRPE deposits formation containing Aβ

Electron microscopic observation revealed that senescent mice fed cholesterol-enriched chow developed subRPE deposits (Supplementary Fig. S3A and B), whereas the aged-matched control mice fed standard rodent chow did not (Supplementary Fig. S3C and D). SubRPE deposits of cholesterol fed mice were immunoreactive to anti-Aβ antibody (Fig. 4A and B), whereas Aβ was not detected in subRPE region of age-matched control mice fed standard rodent chow (Fig. 4C and D).

4. Discussion

Lipid rafts are membrane microdomains, enriched in cholesterol and sphingolipids [35]. Lipid rafts have been reported to facilitate the production, aggregation, neuronal binding and toxicity of Aβ oligomers [36]. The Aβ peptides are produced by the lipid raft dependent sequential cleavage of APP, first by β-secretase and then by the γ-secretase complex [27,37,38]. Aβ degradation enzyme, NEP is also partially localized in lipid rafts [39,40], despite its activity does not differ between lipid rafts and nonlipid rafts [41].

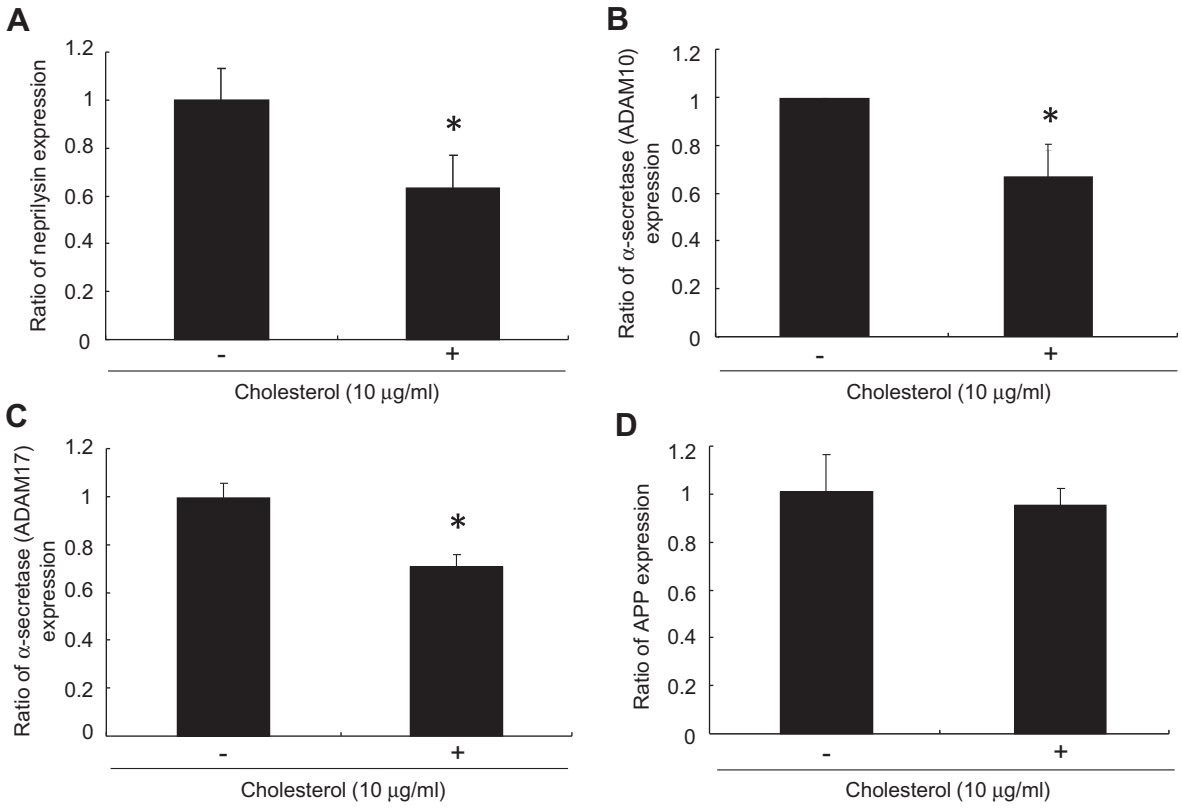

Fig. 3. The mRNA expression of enzymes and proteins that regulate Aβ production between cholesterol-treated or non-treated retinal pigment epithelial (RPE) cells isolated from 12-month-old mice; (A) neprilysin, (B) α-secretase (ADAM10), (C) α-secretase (ADAM17), (D) amyloid precursor protein (APP). The relative changes in gene expression are calculated by subtracting the threshold cycles of the target genes from the internal control gene GAPDH. Values are expressed as the means ± SEMs, $n = 6$, $^{*}P < 0.05$.

Although APP cleavage by α-secretase occurs in nonlipid rafts [7,42], it would be expected that the cholesterol levels can affect the status of Aβ production.

In the present study, we showed that the cholesterol increased Aβ production in RPE cells isolated from senescent mice compared to non-treated cells (Fig. 1), and this was considered to be due to a decreased activity and expression of NEP as well as α-secretase. Although earlier *in vitro* and *in vivo* studies reported that the levels of cholesterol significantly modulated Aβ production in different cell lines and tissues [21–23,29,43–45], PubMed search extracted only three articles reporting the influence of cholesterol on Aβ production in the retina [46–48]. Dasari et al. [47] reported that the cholesterol oxidation metabolite 27-hydroxycholesterol dose-dependently increased Aβ peptide production in human RPE cell line; ARPE-19 cells. They [47] also found an increased accumulation of Aβ within the retina as well as the formation of drusenoid deposits in the rabbit fed with 2% cholesterol-enriched chow for 12 weeks. However, the mechanism of cholesterol-associated alteration of activities of Aβ synthesis and degradation enzymes has never been investigated in the eye. Also, there are some discrepancies in the mechanism of cholesterol-associated alteration of activities among the earlier studies [7,25,26,32,33,49] investigating other tissues than eyes; like brain.

We showed cholesterol decreased the activity and gene expression of NEP and α-secretase in RPE cells from senescent mice (Figs. 2 and 3), whereas cholesterol did not affect the activities of β- or γ-secretase (Fig. 2). We also confirmed this phenomenon in senescent mice that a cholesterol-enriched diet was associated with decreased activity and gene expression of NEP and α-secretase in freshly isolated native RPE cells (Supplementary Figs. S1 and S2). Supporting our data, Bodovitz and Klein [26] demonstrated that cholesterol decreased α-secretase cleavage of APP in APP-transfected HEK293 cells. Kojro et al. [7] and Xiu et al. [49] also showed that cholesterol-lowering drugs like lovastatin could increase α-secretase cleaved soluble APP fragment via nonamyloidogenic pathway and resulted in a decreased Aβ production in astrocytes [49] and APP transfected HEK293 cells [7]. NEP activity in the plasma of cholesterol-fed rabbits was reportedly enhanced compared to that of normal chow-fed rabbits [28]. Xiong et al. [25] showed significantly enhanced the activities of β- and γ-secretase in cholesterol-treated neurons from both AD and normal aging brains. Ghribi et al. [29] also reported that cholesterol increased β-secretase activity in hippocampus of mice fed cholesterol diet. The reason of discrepancies between some of the earlier studies and the present study is difficult to explain and needs to be investigated in the future. RPE cells are the only epithelial cell within the retina and although RPE cells are neural crest origin developmentally. Although earlier studies investigated the effect of cholesterol on various cells and tissues, none of them examined the effect on epithelial cell lines. This study suggests that the regulatory mechanism of Aβ production upon stimulus might be different significantly among difference cell types.

Cholesterol-enriched diet but not standard diet developed sub-RPE deposits containing Aβ in senescent mice (Fig. 4 and Supplementary Fig. S3). This suggests that cholesterol-enriched diet has potential to cause structural and morphological changes in the retina relevant to human AMD. Our data are in accordance with earlier studies reporting the formation of subRPE deposits in cholesterol-enriched diet in senescent rabbits and mice [46,48]. Interestingly, Dithmar et al. [48] found that subRPE deposits were observed only in senescent (8-month-old) mice and not in young (4-month-old) mice even though they were fed with cholesterol-enriched chow. We recently found that the NEP activity was decreased and BACE1 activity was increased in RPE cells from senes-

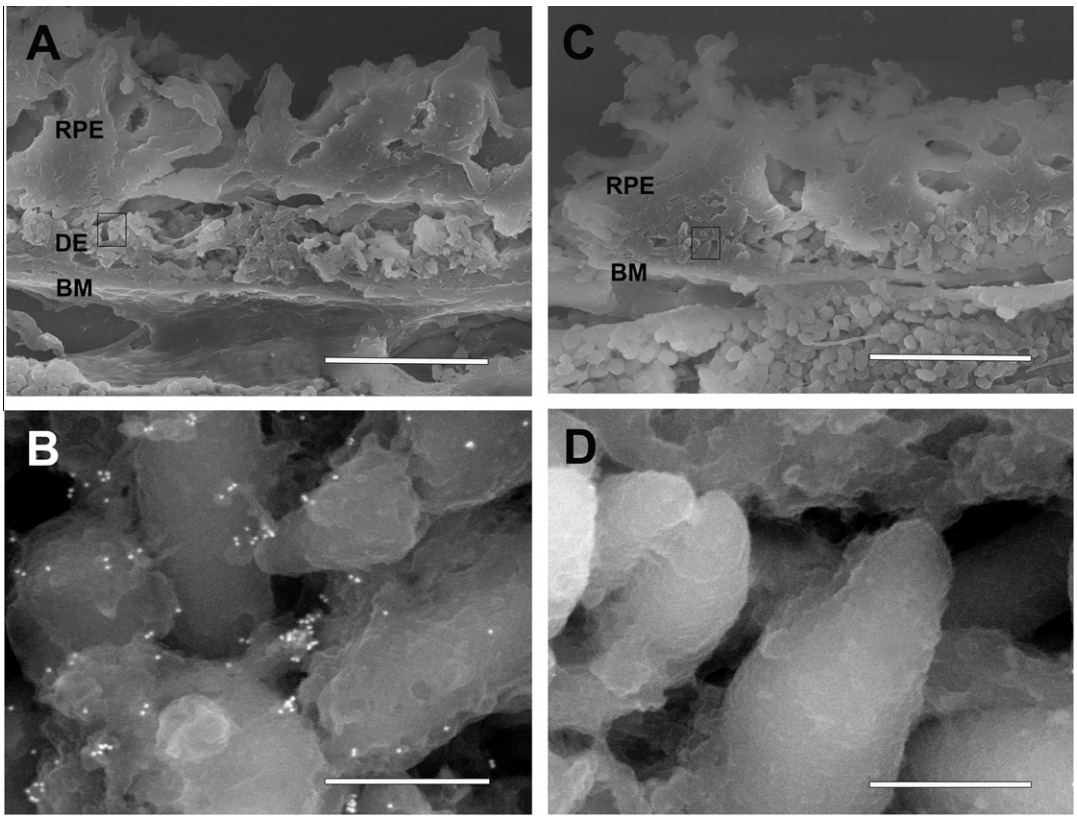

Fig. 4. Immunoelectron microscopic localization of amyloid β (Aβ) in the deposits beneath the retinal pigment epithelium (RPE) in the mice fed with high cholesterol diet. (A and B) Localization of Aβ in sub-RPE deposits of 12-month-old mice fed cholesterol-enriched chow by immunogold labeling. Aβ is present in subRPE deposits shown in a magnified image (B). (C and D) Aβ deposition is not observed in sub-RPE region of 12-month-old mice fed normal rodent chow. (D) shows higher magnification of (C). BM, Bruch's membrane, DE; subRPE deposits Scale bars: (A and C) 10 μm; (B and D) 500 nm.

cent mice compared to young mice [50]. Combining these findings, there might be some associations between aging and high cholesterol in the Aβ accumulation in subRPE space and subsequent development of AMD.

In conclusion, the present study showed that cholesterol increased Aβ production in senescent RPE cells by modulating the expression and activity of NEP and α-secretase. Senescent mice fed cholesterol-enriched chow developed drusen-like subRPE deposits. These findings link three important risks of AMD development; advanced age, hypercholesterolemia and subretinal Aβ accumulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.014.

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