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Elevated amyloid β production in senescent retinal pigment epithelium, a possible mechanism of subretinal deposition of amyloid β in age-related macular degeneration

Jiying Wang [a], Kyoko Ohno-Matsui [a,*], Ikuo Morita [b]

[a] Department of Ophthalmology and Visual Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[b] Section of Cellular Physiological Chemistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

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ABSTRACT

Age-related macular degeneration (AMD) is the most common cause of legal blindness in the elderly individuals in developed countries. Subretinally-deposited amyloid β (Aβ) is a main contributor of developing AMD. However, the mechanism causing Aβ deposition in AMD eyes is unknown. Aging is the most significant risk of AMD, thus, we examined the effect of aging on subretinal Aβ deposition. mRNAs and cell lysates were isolated from retinal pigment epithelial (RPE) cells derived from 24-month-old (24M RPE) and 2-month-old (2M RPE) C57BL/6 mice. Aβ concentration in culture supernatants was measured by ELISA. Activity and expression of proteins that regulate Aβ level were examined by activity assay and real time PCR. Effect of β-secretase (BACE) on Aβ production was examined by siRNA silencing. Aβ amounts in supernatants of 24M RPE were significantly higher than 2M RPE. Activity and mRNA levels of neprilysin, an Aβ degrading enzyme, were significantly decreased in 24M RPE compared to 2M RPE. PCR analysis found that BACE2 was significantly more abundantly expressed than BACE1 in RPE cells, however, inactivation of BACE2 gene did not affect Aβ production. BACE1 protein amounts did not differ between 24M and 2M RPE, however, BACE1 activity was significantly higher in 24M RPE compared to 2M RPE. There were no significant changes in the activities of α- or γ-secretase between 2M and 24M RPE. In conclusion, RPE cells produce more amounts of Aβ when they are senescent, and this is probably caused by a decrease in Aβ degradation due to a reduction in the expression and activity of neprilysin and an increase in Aβ synthesis due to increased activity of BACE1.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of legal blindness and central vision decrease in the elderly individuals in developed countries. Advanced AMD can be classified into geographic atrophy and exudative AMD [1,2]. Geographic atrophy is characterized by the retinal degeneration involving the photoreceptors and retinal pigment epithelial (RPE) cells. Exudative AMD is characterized by the growth of new blood vessels from the choroid into the subRPE and subretinal spaces, eventually resulting in the formation of disciform scars with loss of photoreceptors. Drusen, grayish-yellow deposits beneath RPE cells, have been identified as a significant risk for developing AMD [3]. Amyloid β (Aβ) has been found in drusen [4,5] and is a major component of senile plaques in the brains of patients with Alzheimer's disease (AD) [6]. The results of our earlier study on senescent neprilysin (NEP) gene-disrupted mice, which lack the Aβ-degrading enzyme, demonstrated that there was a significant increase in the deposition of Aβ in the subretinal space, and those mice developed several features of human eyes with AMD [7]. These results suggest that Aβ deposition in drusen may be a key contributor to the development of AMD. However, the mechanism that causes subretinal deposition of Aβ in eyes with AMD has yet been clarified.

Aβ peptides vary in length from 39 to 43 amino residues and are 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 [8–11]. Activation of the amyloidogenic pathway leads to Aβ synthesis by sequential cleavage of APP by β-secretase (BACE) and γ-secretase [12,13]. Two isoforms of BACE exist; BACE1 and BACE2. BACE1 is the main isoform in the brain and BACE2 is dominant in peripheral tissues like colon, kidney and pancreas [14]. A

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

* Corresponding author. Fax: +81 3 3818 7188.
E-mail address: k.ohno.oph@tmd.ac.jp (K. Ohno-Matsui).

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cleavage of APP by α-secretase leads to a non-amyloidogenic pathway, thus precludes Aβ synthesis [15]. Synthesized Aβ can be degraded by various proteases such as insulin degrading enzyme [16], NEP [10] and cathepsin B [17].

RPE is a monolayer which lies beneath the neural retina and plays critical role in maintaining the homeostasis of the photoreceptors [18,19]. RPE cells constitutively expressed APP [7], α-secretase [20], BACE [7], γ-secretase [21] and NEP [7]. Activity and expression alterations of Aβ synthesis and degradation enzymes have been reported in the brain of both normal aging individuals and AD patients and contribute to Aβ deposition [22–27]. Recent studies suggested that Aβ deposition is not exclusive to AMD and AD but also occurs during normal aging, and most humans and mice accumulate Aβ in their brains and retinas as they age [28]. These findings suggest the possibility that the alterations in the activity and the expression of Aβ synthesis and degradation enzymes in senescent RPE cells may trigger subretinal deposition of Aβ in AMD. Thus, the purpose of the present study was to examine the effect of aging on Aβ production, expression and activity of Aβ synthesis and degradation enzymes in RPE cells isolated from senescent and young mice.

2. Materials and methods

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

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

The concentrations of $A\beta_{1-40}$ and $A\beta_{1-42}$ in the supernatants of cultured RPE cells isolated from 24-month-old (24M RPE) and 2-month-old (2M RPE) mice were determined with a commercial ELISA kit (Wako, Osaka, Japan). 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, BACE and γ-secretase

Hundred microliters DMEM containing 20 μg total cell lysates of freshly isolated 2M RPE and 24M RPE were respectively added in duplicate to black CulturPlate-96. Ten micrometers (final concentration) fluorogenic peptide substrate (Mca-RPPGF-SAFK[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-NH2; Calbiochem, Cat. No. 565764) for γ-secretase, 40 μM (final concentration) BACE1 substrate and γ-secretase substrate that provided in BACE1 and γ-secretase activity assay kits were respectively added to each corresponding well and incubated at 37 °C. Fluorescence intensities were read on a fluorescent ELISA plate reader every 30 min of 120 min. The excitations were done at 320 nm for NEP, 355 nm for γ-secretase, 490 nm for α-secretase and BACE. The emissions were done at 405 nm for NEP, 440 nm for γ-secretase, 520 nm for α-secretase and BACE. Because the substrate for NEP is also an excellent substrate for endothelin-converting enzyme-1 (ECE-1), to determine the specificity of the NEP activity, an inhibition study was performed. The RPE cell lysates used for NEP activity assay were preincubated with 100 nM thiorphan; an inhibitor of ECE-1 cleavage of the substrate [29], 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. SiRNA inactivation of BACE2 gene expression

Predesigned siRNA specific for mouse BACE2 was purchased from Dharmacon (Lafayette, CO). Transfection of the siRNA into cultured RPE cells was done with lipofectamine 2000 and OPTI-MEM according to the manufacturer's instructions. In brief, primary RPE cells were allowed to reach 40% confluence on 10 cm plate till the day of transfection. One milliliter of OPTI-MEM containing 10 μM siRNA was incubated with lipofectamine 2000 for 20 min at room temperature. This mixture was then added to the medium of the RPE cells which contained 4 ml OPTI-MEM. Cells were incubated for 24 h at 37° C prior to changing the medium to DMEM supplemented with 10% FCS. A second transfection was carried out after dividing the transfected RPE cells into 12 well plates and after the cells had reached 40% confluence. After the second transfection, the cells were allowed to reach confluence in the DMEM supplemented with 10% FCS. The medium was then changed to serum free DMEM, and incubated for another 48 h before the supernatants were collected for ELISA analysis of Aβ. The inhibition of BACE2 gene expression was confirmed by real time PCR. Non-target siRNA was used as negative control. All the experiments were performed at least six times.

2.4. Measurements of BACE1 by ELISA

The level of BACE1 protein expressed by 24M RPE and 2M RPE was determined by a commercial ELISA kit (Wuhan, China). Twenty micrograms total cell lysates of freshly isolated RPE cells, diluted in standard diluent to a final volume 100 μl, was added to wells pre-coated with the BACE1 antibody. The absorbance was measured at 450 nm in Bio-Rad Model 450 microplate reader (Bio-Rad Laboratories, Hercules, CA). All experiments were performed at least six times.

2.5. 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 isolated from senescent mice produce more Aβ than young mice

The concentration of $A\beta_{1-40}$ was 65.5 ± 10.8 pmol/ml in the supernatants of 24M RPE which was significantly higher than the 33.6 ± 6.5 pmol/ml in the supernatants of 2M RPE (Fig. 1A, $n = 6$, $P = 0.012$). The concentration $A\beta_{1-42}$ was 8.4 ± 0.8 pmol/ml in 24M RPE which was significantly higher than the 4.2 ± 0.3 pmol/ml in the 2M RPE (Fig. 1B, $n = 6$, $P = 0.008$).

3.2. Activity and mRNA levels of NEP decreased in RPE cells isolated from senescent mice than young mice

RT-PCR results showed that mRNAs of APP, NEP, α-secretase, BACE (BACE1 and BACE2) and γ-secretase (PS1 subunit) were constitutively expressed in primary mouse RPE cells (Fig. S1). The mRNA levels of APP were not significantly different between 2M RPE and 24M RPE (Fig. S2). However, the mRNA levels of NEP were significantly decreased to 0.06 ± 0.02 in 24M RPE compared to 1.00 ± 0.17 in 2M RPE (Fig. 2A, $n = 6$, $P = 2.35 \times 10^{-7}$). Total RPE cell lysates (20 μg) diluted in 100 μl DMEM was incubated with