**Subretinal injection of amyloid-β peptide accelerates RPE cell senescence and retinal degeneration**

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**Abstract.** Drusen are considered a hallmark characteristic of age-related macular degeneration (AMD). In our previous study, we found that amyloid-β (Aβ) peptide, a component of drusen, induced the cells of the retinal pigment epithelium (RPE; RPE cells) to enter senescence; however, its effects *in vivo* remain unknown. Thus, the present study was carried out to explore the *in vivo* effects of Aβ peptide on RPE cell senescence and senescence-associated inflammation in C57BL/6 mice. C57BL/6 mice received a subretinal injection of Aβ(1-42) peptide; on day 7 post-injection, the mice were anesthetized and subjected to whole-body perfusion with 4% paraformaldehyde (PFA) in PBS and the whole eyes were then enucleated. Retinal function was assessed by electroretinography (ERG), and the morphological characteristics of the retina were examined by light and electron microscopy. Fundus autofluorescence (FAF) was examined by confocal scanning laser ophthalmoscopy (cSLO). The expression of p16INK4a, a marker of cellular senescence, was examined by immunofluorescence staining and western blot analysis. The RPE-choroid was analyzed for cytokine expression by RT-PCR. In Aβ(1-42)-injected mice, scotopic ERG responses declined. Degenerative alterations, including the disruption of the inner segment (IS)/outer segment (OS) junction and extensive vacuolation and thickness of Bruch’s membrane (BrM) were observed under a light microscope. The accumulation of vacuoles and the loss of basal infoldings in the RPE were identified using an electron microscope. FAF and p16INK4a expression increased in Aβ(1-42)-injected mice. In addition, Aβ(1-42) upregulated interleukin (IL)-6 and IL-8 gene expression in the RPE-choroid. In conclusion, our results confirm the effects of Aβ(1-42) peptide on RPE senescence *in vivo*. The Aβ-injected mice developed AMD-like ocular pathology. It is thus suggested that RPE cell senescence is a potential mechanistic link between inflammation and retinal degeneration.

**Introduction**

Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment among the elderly in western countries. AMD is primarily characterized by the formation of drusen, which are extracellular deposits between the retinal pigment epithelium (RPE) and Bruch’s membrane (BrM), containing glycolipids, proteins and cellular debris. The appearance of drusen is strongly correlated with the development of AMD (1,2).

Amyloid-β (Aβ) peptide, a component of drusen, is considered to contribute to neurodegenerative events and/or chronic inflammation in the retina of patients with AMD. Aβ deposition within the vesicles is particularly observed in the eyes of patients with advanced AMD (3,4). It has been postulated that the presence of Aβ triggers a local inflammatory response that activates the immune system. Several *in vitro* studies have demonstrated that inflammation pathways and immune response are significantly upregulated in Aβ-stimulated cells of the RPE (RPE cells) (5,6). A single intravitreal injection of Aβ peptide has been shown to induce inflammasome activation in the retinas of rats (7). A recent clinical trial reported that RN6G, a humanized monoclonal antibody against Aβ, reduced the Aβ deposits in the retina, thus preserving the retinal function of the animals and maintaining normal RPE morphology (8).

Chronic inflammation is associated with aging and plays an important role in AMD (9); however, the source of sterile inflammation that fuels retinal degeneration in AMD remains unknown. Age-related chronic inflammation may be derived from two sources: i) an age-related decline in homeostatic immune function, or ii) senescent cells. Cellular senescence may cause chronic inflammation through the senescence-associated secretory phenotype (SASP). SASP proteins include a wide range of chemokines and cytokines [interleukin (IL)-6, IL-8, IL-1, granulocyte-macrophage colony-stimulating factor, monocyte chemotactic protein (MCP) and matrix...
metalloproteinases (MMPs) that are known to stimulate inflammation (10,11). Cellular senescence is defined as a state of irreversible proliferative arrest caused by a wide range of stimuli, including severe DNA damage, the expression of oncoproteins, oxidative stress and strong mitogenic signals (12-14). Aβ has been recently been shown to be involved in the senescence of neuronal cells, astrocytes and endothelial cells (15-18). Our previous in vitro study demonstrated that Aβ peptide caused RPE cells to enter senescence (19). However, the in vivo effects of Aβ peptide on RPE cell senescence and senescence-associated inflammation remain unclear.

The deposition of Aβ peptide, astrocyte senescence and altered SASP expression have been observed in the brains of patients with Alzheimer's disease (AD) (17). This finding suggests the existence of a correlation between Aβ peptide and senescence-associated neuronal inflammation (17). Previous in vivo studies have demonstrated that the intravitreal or subretinal injection of Aβ peptide may cause retinal degeneration and inflammation (7,20). However, to the best of our knowledge, the effects of the subretinal injection of Aβ peptide on RPE cell senescence have not been investigated to date. Thus, in the present study, we aimed to explore the role of cell senescence and inflammation in the retinal degeneration induced by Aβ(1-42) peptide.

Materials and methods

Aβ(1-42) oligomerization. Aβ(1-42) peptide and its inactive reverse control peptide Aβ(42-1) were prepared as described in our previous study (19). Briefly, 0.5 mg of lyophilized Aβ peptide (Sigma-Aldrich, Shanghai, China) was dissolved in 140 μl hexafluorosopropanol (HFIP) and incubated for 20 min, followed by the addition of 900 μl distilled H₂O and a 20-min incubation in the HFIP/water mixture. Subsequently, the solvent was evaporated from the resulting supernatant under constant stirring at room temperature for 5 days. Oligomerization was verified by dot blot assay as previously described (19).

Animals and animal treatments. To elucidate the in vivo effects of Aβ peptide on the retina, C57BL/6 mice received a single unilateral subretinal injection of Aβ peptide (Fig. 1). Briefly, 5-month-old C57BL/6 mice were anesthetized by an intraperitoneal injection of 300 mg/kg chloral hydrate (Sangon Biotech). Following pupillary dilation and the topical anesthesia of the cornea, gold loop electrodes were placed on the cornea with a drop of 2.5% hydroxypropyl methylcellulose. Subsequently, the reference electrode was placed into the mouth of the animal underneath the tongue, and a ground electrode was subcutaneously inserted into the midwrist of the tail. The ERG waveforms were then recorded in response to a flash at 1.125 cd/s/m². The amplitude of the a-wave was measured from the baseline to the trough of the a-wave. The amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave. The amplitude of the c-wave was measured from the baseline to the peak of the c-wave.

Retinal tissue immunohistochemistry. On day 7 following surgery, the mice were terminally anesthetized and subjected to whole-body perfusion with 4% paraformaldehyde (PFA) in PBS and the whole eyes were then enucleated and fixed in 4% PFA overnight. Only sections including the optic nerve were used for histological and immunohistochemical analyses. Sections (5-μm-thick) of paraaffin-embedded specimens were stained with hematoxylin and eosin (H&E). The slides were dehydrated and placed on a coverslip.

For immunohistochemical analysis, antibody staining was performed on sections of paraaffin-embedded eyes. Antibodies to RPE65 (ab67042) and p16INK4a (ab54210) were obtained from Abcam (Hong Kong, China) and used at a dilution of 1:100. The paraaffin-embedded sections were heated to 60°C for 30 min, and then deparaffinized and rehydrated in graded alcohol series. The paraaffin-embedded sections were incubated for 1 h in PBS containing 5% FCS to reduce non-specific binding, and then overnight at 4°C with the p16INK4a and RPE65 primary antibodies. After washing, the sections were incubated in a solution of 1:200 of goat anti-mouse 594-conjugated secondary antibody or goat anti-rabbit 488-conjugated secondary antibody (Abcam). The sections were then washed, stained for 5 min with DAPI, and washed again in PBS. The sections were then mounted and examined under a fluorescence microscope (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany).

Transmission electron microscopy (TEM). For ultrastructural analysis, the enucleated eyes were dissected at the equator immediately and the posterior eye cups were fixed in 2.5% glutaraldehyde in phosphate buffer at 4°C overnight. The central 2x2 mm tissue temporal to the optic nerve was post-fixed with 2% osmium tetroxide and alcohol dehydrated and embedded in epoxy resin. The ultra-thin sections were stained with lead
citrate and uranyl acetate, and examined under an electron microscope (CM120; Philips, Eindhoven, The Netherlands).

Fundus photography. For the funduscopic examination of the mice, confocal scanning laser ophthalmoscopy (cSLO) was performed using a device available for human fundus imaging (Spectralis HRA; Heidelberg Engineering, Heidelberg, Germany). Fundus images were captured using a 790-nm diode laser for infrared (IR) and 488 nm for autofluorescence (AF).

Western blot analysis. RPE-choroid preparations for western blot analysis and RT-PCR were dissected from the freshly harvested eyes and preserved at -80˚C until further processing. The RPE-choroid complex was sonicated in radioimmunoprecipitation assay (RIPA) buffer containing proteinase inhibitors for 15 min. Protein content was determined by a bicinchoninic acid assay (Pierce, Rockford, IL, USA). Aliquots (50 µg of total protein) of each sample from the RPE-choroid were loaded per lane onto 10% SDS-PAGE gels for electrophoresis and then transferred onto PVDF membranes. The membranes were blocked and incubated with the primary antibody, rabbit polyclonal antibodies against p16INK4a (1:500; Abcam) and rabbit polyclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase [GAPDH (Cat. no. ab37168), 1:1,000] overnight. The membranes were then washed and incubated with horseradish peroxidase-coupled secondary antibodies for 2 h. The blots were washed and developed with chemiluminescence reagent. The membranes were exposed to ImageQuant LAS 4000 imaging, and densitometric analysis was performed using Photoshop CS4.0 software.

RT-PCR and real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was then reverse transcribed into cDNA with RT Master Mix (Takara Biotechnology, Dalian, China). The expression levels of IL-8 and IL-6 were measured using quantitative PCR mix (Takara Biotechnology). The primers used for PC were as follows: IL-6 sense, 5’-TTCCATCCAGTGTCTCTTCTT-3’ and antisense, 5’-CATTCCACGGATCCAGAGA-3; IL-8 sense, 5’-CTTGCCTCTGCTTGTA-3’ and antisense, 5’-CTCGTTGTTCTCAGACAT-3’. Real-time PCR was performed in a ABI7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH (sense, 5’-AGCAGTCCCGTACCTGGCAAAC-3’ and antisense), 5’-TCTGTGGTGATGTAAATGTCCTCT-3’ was used for normalization and the relative gene expression was expressed as the relative ‘fold change’ calculated using the ΔΔCt method.

Results

Aβ peptide leads to impairment of visual function in animals. To elucidate the in vivo effects of Aβ peptide on visual function, C57BL/6 mice received a subretinal injection of Aβ peptide, and visual function was evaluated by ERG on day 7 post-injection. Photoreceptors are the source of the negative-going a-wave (21,22). Rod bipolar cells are the source of the b-wave in the dark-adapted retina (23-25). RPE cells are the source of the positive-going c-wave (26). Representative waveforms of the maximal ERG scotopic response evoked by a single flash at 1.125 cd’s/m² are illustrated in Fig. 2A. No significant differences were observed between the Aβ(42-1)-injected mice and the PBS control mice. However, a moderate but significant decrease in a-, b- and c-wave amplitude was detected in the Aβ(1-42)-injected mice (Fig. 2A and B). These results indicated that the subretinal injection of Aβ(1-42) induced an impairment of visual function in the animals.
Aβ induces retinal degeneration and ultrastructural alterations in the outer retina. To demonstrate the deleterious effects of Aβ(1-42) on the retina, we compared its effects on retinal histology and RPE structure with those of PBS and the inactive reverse peptide Aβ(42-1). The H&E-stained sections of RPE/neural retina were examined under a microscope (Fig. 3A). The mice injected with PBS or the reverse peptide showed a normal appearance of retinal sections (Fig. 3A, left and middle panels). By contrast, degenerative alterations, including extensive vacuolation (V symbol), hyperpigmentation (arrow), hypopigmentation (black asterisk) and thickness of Bruch's membrane (BrM), were observed in the Aβ(1-42)-injected mice. ONL, outer nuclear layer; IS, inner segment; OS, outer segment. (B) Transmission electron microscopy (TEM) of the interface between the RPE and BrM in mice. RPE basal infoldings (black star) and BrM thickness appear normal in the Aβ(42-1)-injected eye (middle panel) compared with the phosphate-buffered saline (PBS)-injected control mice (left panel). By contrast, in the Aβ(1-42)-injected mice, the loss of pigmented granules, obvious thickening of the BrM, the loss of normal basal infoldings and the formation of autophagic vacuoles were observed. White asterisk, pigmented granules; black star, normal basal infoldings; V, vacuoles.
autofluorescent spots were observed by AF-cSLO in almost the same areas (Fig. 4B, right panel). By contrast, no significant distribution of fluorophores was observed in the ocular fundus of the PBS- or Aβ(42-1)-injected mice (Fig. 4A and B). The analysis of cell senescence on RPE/neural retina sections by p16INK4a staining confirmed that Aβ(1-42) induced RPE cell senescence in the mice on day 7 post-injection (Fig. 4C, right panel). However, in the retinal sections of the PBS- or Aβ(42-1)-injected mice, no p16INK4a-positive RPE cells were detected (Fig. 4C, left and middle panels). Subsequently, the expression level of p16INK4a in RPE cells was determined by western blot analysis; the injection of Aβ(1-42) significantly increased p16INK4a expression by (3.12±0.46)-fold compared with the injection of the reverse peptide or PBS (Fig. 4D). Taken together, these data strongly suggest that Aβ(1-42) significantly induces RPE cell senescence in the retinas of mice.

mRNA expression of IL-6 and IL-8 is upregulated following the subretinal injection of Aβ(1-42) peptide. Hallmarks of inflammation, including elevated IL-6 and IL-8 expression, are significantly associated with the severity of AMD. Recent evidence indicates that cellular senescence is accompanied by a marked increase in the secretion of 40-80 factors, which has been termed the SASP (42). The key SASP factors, such as IL-6 and IL-8, are known to stimulate chronic inflammation in age-related diseases (42). Thus, to determine whether the subretinal injection of Aβ alters SASP gene expression in RPE cells, the RPE-choroid layer was isolated and IL-6 and IL-8 mRNA expression was examined by RT-PCR. Aβ(1-42) injection significantly increased the mRNA expression of IL-6 (6.8±0.8-fold, P<0.05) and IL-8 (3.7±1.2-fold, P<0.05), compared with PBS-injected group (Fig. 5), whereas the injection of Aβ(42-1) did not induce any significant changes in the mRNA expression of IL-6 and IL-8, compared with the PBS-injected group (Fig. 5).

Discussion

The main results of the present study were as follows: i) the subretinal injection of Aβ(1-42) impaired the visual function of mice (Fig. 2); ii) Aβ(1-42)-injected mice developed some degenerative alterations in the retina and RPE cells (Fig. 3); iii) signs of RPE cell senescence, including increased FAF and the expression of the senescence-associated marker, p16INK4a, were observed in the Aβ(1-42)-injected mice (Fig. 4); iv) the major SASP factors, such as IL-6 and IL-8, were significantly upregulated in the RPE-choroid of Aβ(1-42)-injected mice (Fig. 5). Taken together, our results demonstrate a causal
connection between Aβ peptide deposition and the appearance of senescent RPE cells, and suggest that the subretinal injection of Aβ induces senescence-associated chronic inflammation. Our data also suggest that Aβ-injected mice represent a useful animal model of AMD.

The electroretinogram is a non-invasive method to evaluate the function of specific layers of the retina. The negative-going a-wave and the positive-going b-wave originate from photoreceptors and rod bipolar cells, respectively (21-25), whereas the sources of the positive-going c-wave are the RPE and retinal glial cells (26). In our study, in Aβ(1-42)-injected mice, there was a statistically significant decrease in the a-, b- and c-wave amplitude compared to the control group (Fig. 2). Similar findings of decreased ERG response have been reported in APP/PS1 transgenic mice, which presented with accumulated Aβ deposition in the retina (28). Therefore, the decreased ERG response or the impaired visual function may be secondary to the retinal degenerative alterations induced by the subretinal injection of Aβ(1-42).

Consistent with the decreased ERG response, microscopic examination revealed severe degenerative alterations in the retinas of the Aβ(1-42)-injected mice, including the loss of inner and outer segments, extensive vacuolation, RPE hypopigmentation and thickness of the BrM (Fig. 3A). These data are consistent with those of other in vivo studies, which demonstrated that the subretinal or intravitreal injection of Aβ peptide induced progressive retinal degeneration and the disorganization of RPE cells (7,20,29).

In Aβ(1-42)-injected mice, ultrastructural analysis by TEM revealed that the RPE cells had multiple vacuoles in the cytoplasm, and that basal infoldings were fewer or absent (Fig. 3B). The RPE appears to be a specific target of Aβ. The subretinal injection of Aβ(1-42) induced a loss of pigmentation and RPE hypertrophy (Fig. 2A). Intravitreal injections of Aβ peptide have been shown to induce a comparable magnitude of gene expression changes in the RPE-choroid compared with the neuroretina (7), indicating that the RPE plays a major role in response to Aβ peptide. The most obvious ultrastructural sign of RPE injury was the loss of basolateral infoldings, which are an established marker of epithelial cell injury (30). Vacuole formation was used as a second sign of RPE injury, as cytoplasmic vacuoles have been identified in the RPE that overlie drusen deposits (31). Of note, in a study on colon cancer cells, Sox2-induced autophagy inhibited cell proliferation and enhanced cellular senescence, suggesting that the formation of autophagic vacuoles is involved in cellular senescence (32). To the best of our knowledge, our study is the first to demonstrate that the subretinal injection of Aβ peptide induced two marked ultrastructural alterations, including the loss of basal infoldings and the formation of intracellular vacuoles, in RPE cells (Fig. 3B).

It has been suggested that Aβ(1-42) plays a central role in the pathogenesis of AD as a mediator of oxidative stress (33). A previous study investigated the in vitro effects of Aβ(1-42) on RPE cells and found that it induced an increase in reactive oxygen species (ROS) production and caused mitochondrial dysfunction (29). Considering that persistent and sublethal oxidative stress accelerates cellular senescence (34), the effects of Aβ(1-42) on RPE cell senescence were evaluated in the present study. First, the increased granular autofluorescent spots were observed by cSLO in the fundus of Aβ(1-42)-injected mice (Fig. 4A and B). During cell senescence, autofluorescent lysosomal storage bodies known as lipofuscins or age-pigments accumulate in many post-mitotic types of cells (35,36). It is well known that the RPE accumulates massive amounts of autofluorescent lysosomal storage bodies (lipofuscins) during cell senescence (37-39). The formation of fundus autofluorescence in human eyes is associated with RPE atrophy and the progression to advanced AMD (40). Lipofuscins contained in the RPE are the main source of FAF; it has been reported that FAF detected at 488 nm excitation with a cSLO is largely attributable to RPE lipofuscins (40,41). Therefore, increased fundus AF detected by cSLO may be a signal of RPE cell senescence (Fig. 4A and B). Subsequently, the increase in p16INK4a expression was proved by immunostaining (Fig. 4C) and western blot analysis (Fig. 4D), indicating that Aβ(1-42)-induced RPE senescence is regulated by p16INK4a. This finding is consistent with our previous in vitro study, indicating that Aβ peptide is involved in the senescence of RPE cells (19). It is also consistent with the results of other studies, suggesting that Aβ peptides induce endothelial cells, astrocytes and neurons to enter senescence (15,17,18).

We observed a significant overexpression of IL-6 and IL-8 in the RPE-choroid of Aβ(1-42)-injected mouse eyes (Fig. 5). Senescent cells contribute to aging and age-related disease by generating a low-grade inflammatory state (42). Our results suggested that cellular senescence may promote inflammation, which is consistent with the findings of other studies demonstrating an increased production of IL-6 and IL-8 by senescent fibroblasts and epithelial cells (43,44). IL-6 and IL-8 may stimulate angiogenesis, enforce macrophage function and induce innate immune responses (45). Moreover, it has been reported that IL-6 and IL-8 reinforce senescent growth arrest (46,47). Kuilman et al (47) verified that the suppression of IL-8, IL-6 or its cognate receptor, IL-6R, was sufficient to allow these cells to re-enter the cell cycle and proliferate, supporting the possibility that these cytokines function in vivo to promote cellular senescence. In addition, the aqueous humor of patients with AMD contains higher concentrations of IL-6 and IL-8 (48). We focused our attention on these cytokines as they can act not only as pro-inflammatory cytokines, but also as potent inducers of growth arrest (49). Overall, we conclude...
that Aβ-induced senescent RPE cells may produce high levels of IL-6 and IL-8, which are both required to amplify and sustain the inflammatory network and senescence.

AMD is an age-related chronic inflammatory disease (50,51). Our previous study (19) and the present study indicate that Aβ-induced senescent RPE cells may constitute a link between chronic inflammation and neuroretinal degeneration. Senescence-causing inducers, such as DNA damage, protein aggregation and increased ROS may activate the p53 and p16INK4a pathways that initiate a senescence response (52-56). Once initiated, senescence becomes fully established and is irreversible. Subsequently, the senescent cells affect the tissue microenvironment by secreting pro-inflammatory cytokines, chemokines and proteases (12,57). It has been reported that the clearance of p16INK4a-positive senescent cells prevents age-related disorders and maximizes a healthy lifespan (58). Thus, the modulation of cellular senescence, including the elimination of selected senescent cells, the clearance of senesence inducers, such as Aβ peptide deposition, the prevention of cellular senescence or affecting the secretory phenotype may reduce age-related sterile chronic inflammation in AMD.

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