Original Article

CMJ

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The Oligomeric Form of Amyloid Beta Triggers Astrocyte Activation, Independent of Neurons

Bo-Ram Mun[1], Su-been Park[1], and Won-Seok Choi[1,2]*

[1]School of Biological Sciences and Technology, College of Natural Sciences, Chonnam National University, [2]College of Medicine, Chonnam National University, Gwangju, Korea

The most common aging-related neurodegenerative disorder is Alzheimer's disease (AD), of which the main symptom is memory disturbance. Though the mechanism of AD pathogenesis is not fully defined, abnormal aggregation of amyloid beta (Aβ) plaques and tau have been considered as key factors and main histological hallmarks of the disease. Astrocyte is responsible for the control of cells and the environment around brain and spinal cord cells. Astrocytes have been implicated with AD. However, the exact function of astrocytes in AD has not been established. In this study, we investigated the regulation of astrocytes in the AD model using primary cultures. We have demonstrated that oligomerized Aβ is toxic to neurons and can induce cell death in primary cultures. In the primary cultures containing neurons and astrocytes, amyloid beta uptake was observed in both neurons and astrocytes. To verify if the uptake of amyloid beta in astrocytes is dependent on neurons, we separated and cultured primary astrocytes with no neurons. Amyloid uptake was still observed in this pure astrocyte culture, suggesting that the uptake of amyloid beta is a neuron-independent function of astrocytes. Astrocyte activation was observed in both pure and mixed cultures. Taken together, our data suggest that astrocyte is activated by oligomerized Aβ and uptakes it, which is independent of neurons.

Key Words: *Alzheimer's Disease; Amyloid Beta-Peptides; Astrocytes; Neurons; Apoptosis*

Article History:
Received November 5, 2023
Revised December 5, 2023
Accepted December 6, 2023

Corresponding Author:
Won-Seok Choi
School of Biological Sciences and Technology, Chonnam National University, 77 Yongbong-ro, Buk-gu, Gwangju 61186, Korea
Tel: +82-62-530-1912
Fax: +82-62-530-2199
E-mail: choiw@chonnam.ac.kr

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by memory impairment, executive dysfunction, and visuospatial impairment.[1] It is the most common cause of dementia in adults, particularly in those aged 65 and older.[2] AD is a progressive disease that leads to severe cognitive decline and is a leading cause of death. The disease has a prolonged presymptomatic phase followed by a clinical stage with symptoms such as selective short-term memory loss, impairment in problem-solving and judgment, language disorders, and visuospatial skills impairment.[3] Currently, there is no cure for AD, but there are treatments available that may improve some symptoms.

The pathogenesis of AD involves the accumulation of amyloid beta (Aβ) peptides in the form of senile plaques, intracellular neurofibrillary tangles, and neuronal loss.[4] Aβ peptides exist in various conformations, including monomers, oligomers, protofibrils, and fibrils. Among these, oligomers are considered the most neurotoxic.[5] Monoclonal antibodies (mAbs) targeting Aβ have shown promise in modifying the course of AD by decreasing Aβ load in the brain and slowing disease progression.[6] Lecanemab, a specific mAb, has demonstrated a high affinity for large and soluble Aβ protofibrils and is currently being studied in phase 3 clinical trials.[7] Thus, the mechanism of Aβ regulation and Aβ-induced cellular alterations is important for gaining a better understanding of AD pathogenesis.

Astrocytes play a crucial role in the pathogenesis of AD. They are involved in cholesterol metabolism, amyloid β (Aβ) production and clearance, tau phosphorylation, neuroinflammation, and degeneration.[8] Astrocyte biomarkers, such as glial fibrillary acidic protein (GFAP), S100B, and

https://doi.org/10.4068/cmj.2024.60.1.27

chitinase-3-like protein 1 (YKL-40), are consistently altered in AD, suggesting their potential use in diagnostic and research frameworks.[9] Activation of astrocytes by Aβ can lead to neuronal apoptosis and morphological changes in neurons.[10] Cholesterol loading in astrocytes can increase the content of amyloid precursor protein (APP) and enhance its interaction with BACE-1, leading to increased Aβ production.[11] However, the role of neurons in Aβ-induced activation of astrocytes has not been known. Further research is needed to understand the role of astrocytes in AD pathogenesis.

In this study, we investigated the regulation of astrocytes in the AD model and examined the role of neurons in Aβ-induced astrocyte activation using primary cultures.

MATERIALS AND METHODS

1. Primary cortical culture

Primary cells were prepared from the cerebral cortices of C57BL/6 mouse embryos (embryonic day 14). The cortices were dissected and dissociated by trypsinization (0.25% in HBSS) for 30 min in an incubator, followed by the inactivation with 10% FBS in culture medium and trituration using a Pasteur pipette. The cells were plated onto poly-D-lysine (100 μg/mL)-coated Aclar coverslip (Ted Pella Inc., Redding, CA) at a density of 5×10^5 cells per well in a 24-well plate. The cells were maintained in Neurobasal medium supplemented with 200 mM l-glutamine, B-27 supplement, and 1% penicillin/streptomycin (Invitrogen, Waltham, MA, USA) in a humidified 5% CO_2 incubator.

2. Primary astrocyte culture

Neonatal cerebral cortices were dissected from forebrains of 2 or 3-day-old mouse pups and treated with trypsin (0.25%) for 30 min in an incubator. After the inactivation using 10% FBS in culture medium, cells were dissociated with a Pasteur pipette. The cells (2×10^7 cells) were plated onto poly-D-lysine-coated T75 flask and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Half of the medium was changed every 3 days. After 14 days, the T flask was shaken overnight (300 rpm) to harvest detached microglia, and remaining astrocytes were detached using trypsin (0.25%) for 5 min in the incubator. The astrocytes were plated onto Poly-D-lysine-coated coverslips at a density of 3×10^5 cells per well in a 24-well plate and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

3. Aβ oligomerization

Amyloid peptide (Aβ 1-42, LT2460, LifeTein, Somerset, NJ) was dissolved in HFIP and then the HFIP was completely evaporated using a speedvac (1 hr). After drying, DMSO was added to make an Aβ stock solution (5 mM). Right before each treatment, the Aβ solution was diluted with PBS (100 μM).

4. Immunocytochemistry

Following treatment with Aβ for ~72 h, cells were fixed using a solution of 4% paraformaldehyde and 4% sucrose in PBS at room temperature for 20 min, followed by three washes with PBS. The Aclar cover slips were then transferred from the 24-well plate to the staining rack. They were blocked using a solution of 3% BSA and 3% goat serum in PBST (containing 0.15% Tx-100) for half an hour at room temperature. After two washes with PBS, cells were stained with either mouse anti-NeuN antibody (1:1,000; Millipore, Burlington, MA, USA), GFAP antibody (1:4000; Dako, Glostrup, Denmark) or Aβ antibody (6e10, 1:3000; Biolegend, San Diego, CA), and left overnight at 4℃. The cells were then washed twice with PBS and stained with secondary antibodies, goat anti-mouse or goat anti-rabbit (1:500; Invitrogen), at room temperature for 2 h. After two more washes with PBS, cell nuclei were stained with Hoechest (1:1,000). The cells were then mounted on slides and images were captured under a Leica DM LB2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The images were subsequently analyzed using ImageJ (Schneider, Rasband et al., 2012).

5. Quantitation of apoptotic nuclei

Cells were fixed and stained with DAPI (1:1,000). After mounting on slides, images of nuclei were captured under a Leica DM LB2 fluorescence microscope. The number of nuclei with apoptotic morphology was quantified in each image using ImageJ.

6. Data analysis

All data are presented as mean±SEM of at least three independent experiments. The significance of the differences between the groups was determined using a Tukey's post hoc test following one-way analysis of variance (ANOVA, Fig. 1) or an independent t-test (Figs. 2-4). All p-values <0.05 were considered statistically significant.

RESULTS

Among various confirmations, the oligomeric form of Aβ has been known to have potent toxicity for neurons.[12] We tested the effect of oligomerized Aβ on neuronal survival using mouse primary cultures. Cells were stained with a neuronal marker (anti-NeuN antibody) and fluorescence secondary antibody. Cell nuclei were stained with DAPI. The number of NeuN-positive cells were quantified. The number of surviving neurons was decreased after treatment with oligomerized Aβ in a dose-dependent manner (Fig. 1A). This confirms the toxicity of oligomerized Aβ in primary neurons.

Previous studies have suggested that neuronal death in AD could be the result of apoptosis. This hypothesis is supported by the presence of apoptotic markers, such as DNA fragmentation in postmortem AD brains.[13,14] To identify apoptotic modifications of DNA in the nucleus, we stained primary neurons with Hoechst dye and quantified the nu-

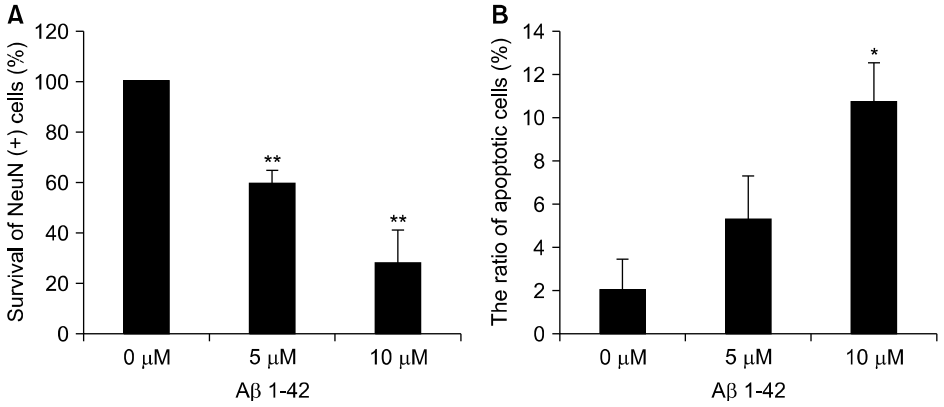

FIG. 1. Amyloid beta (Aβ) induces apoptotic modification of nucleus and neuronal loss in primary culture. Primary cells were cultured from mouse embryonic brain cortex (E14). Cultured primary cells were treated with Aβ oligomer (Aβ 1-42). (A) Aβ oligomer treatment decreased the number of neurons in a dose-dependent manner. (B) The number of apoptotic nuclei were counted, which was increased dose dependently. All data are presented as means and error bars indicate the standard errors of the mean (SEM). Tukey's post hoc test following one-way analysis of variance (ANOVA). *$p < 0.05$, **$p < 0.01$.

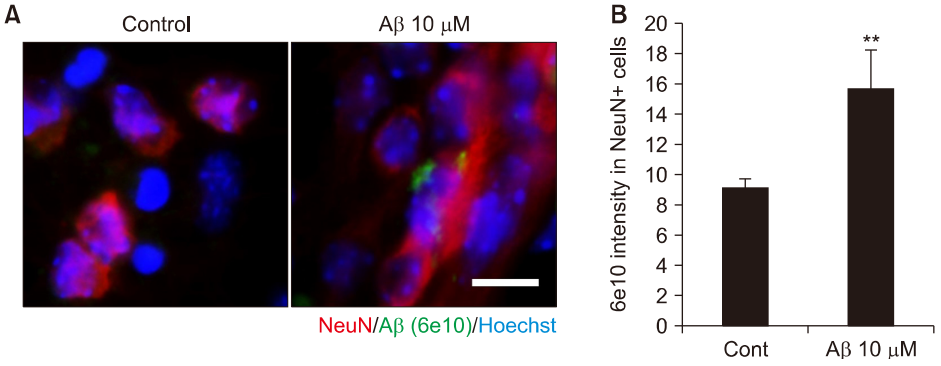

FIG. 2. Primary neurons uptake Aβ. Primary cells cultured from mouse embryonic brain were treated with oligomerized Aβ. Cells were stained with anti-NeuN (red), anti-Aβ (green) antibody and Hoechst (blue). Scale bar: 10 μm. (A) Representative images of stained cells in each group. (B) The intensity of Aβ in NeuN-positive cells was quantitated using ImageJ software. All data are presented as means and error bars indicate the standard errors of the mean (SEM). Student's unpaired t-test. **$p < 0.01$.

clear condensation and fragmentation (apoptotic nuclei) after treating them with oligomerized Aβ. The number of neurons containing apoptotic nuclei increased in a dose-dependent manner, suggesting that oligomerized Aβ induces apoptotic neuronal death (Fig. 1B). We also observed localization of Aβ in the neurons, confirming the toxic accumulation of Aβ in the neurons (Fig. 2). These data suggest that Aβ oligomers are toxic to neurons and can cause apoptotic neuronal death.

Prior reports have suggested that astrocytes are activated by Aβ oligomers and are responsible for the clearance of amyloid in AD models.[15] However, the role of neurons in the activation of astrocytes induced by Aβ oligomer is not fully understood. To investigate whether neurons are necessary for the activation of astrocytes induced by Aβ, we compared astrocytes in embryonic primary culture, which contains neurons, with purified primary astrocytes that do not contain neurons. In the embryonic primary culture containing neurons, the intensity of the astrocyte marker increased after Aβ oligomer treatment, implying astrocyte activation. Furthermore, the intensity of Aβ in astrocytes was increased, suggesting the uptake of Aβ by astrocytes (Fig. 3).

Primary cells were cultured from mouse neonatal cortices and astrocytes were separated from neurons and microglia. In the primary culture of purified astrocytes, Aβ oligomers induced the activation of astrocytes at the similar level to that observed in the embryonic culture (Fig. 4). The level of Aβ in the astrocyte was also increased similarly (Fig. 4). These data suggest that astrocytes can be activated by Aβ oligomers and the activated astrocytes can uptake Aβ, regardless of the presence of neurons.

Taken together, Aβ oligomers induced the loss of neurons through apoptosis and they also activated astrocytes and promoted the uptake of Aβ in astrocytes in a neuron-independent manner. These results suggest that the neuronal effect is minimal on the activation and clearing function of astrocyte induced by Aβ oligomers.

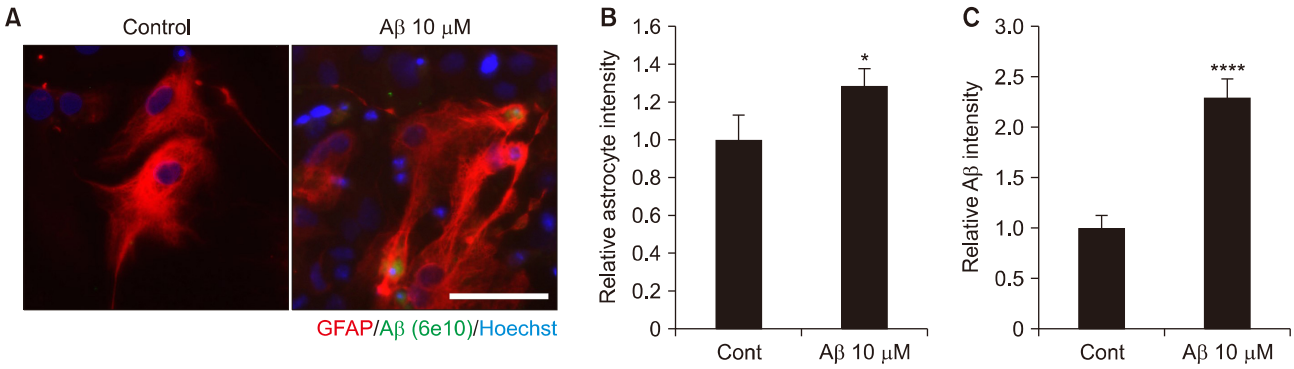

FIG. 3. Astrocytes uptake Aβ. Primary embryonic cortical cells were treated with oligomerized Aβ. Cells were stained with astrocyte marker (anti-GFAP antibody, red). Aβ was stained with anti-Aβ antibody (green). Nuclei were stained with Hoechst (blue). Scale bar: 50 μm. (A) Representative images of stained astrocytes in each group. (B) Relative intensity of GFAP in each group was quantitated using ImageJ software. (C) The intensity of Aβ in GFAP-positive cells. All data are presented as means and error bars indicate the standard errors of the mean (SEM). Student's unpaired t-test. *$p < 0.05$, ****$p < 0.0001$.

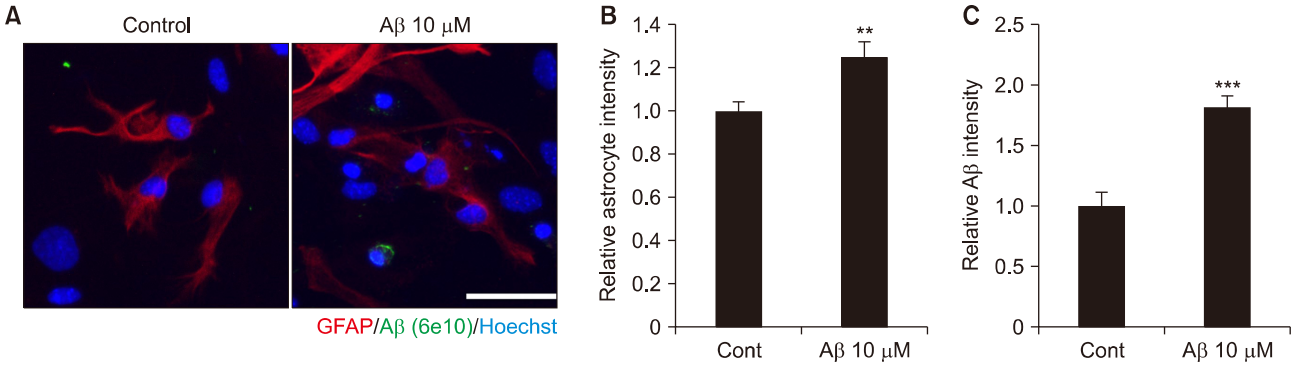

FIG. 4. The internalization of Aβ in astrocyte is independent of neurons. Isolated primary astrocytes were treated with oligomerized Aβ. (A) immunostaining of primary astrocyte for GFAP (red), Aβ (green), and the nuclei marker Hoechst (blue). Scale bar: 50 μm. (B) Relative intensity of GFAP in each group. (C) Total intensity of amyloid beta in GFAP-positive cells. All data are presented as means and error bars indicate the standard errors of the mean (SEM). Student's unpaired t-test. **$p < 0.01$, ***$p < 0.001$.

DISCUSSION

AD is characterized by the presence of extracellular senile plaques and neurofibrillary tangles, along with extensive neuronal loss.[16] Neurons in AD undergo cell death through various mechanisms. Evidence suggests that selective cell death of diseased neurons may play a protective role during the preclinical period of the disease.[17] Apoptosis, the programmed death of neurons, plays a role in the pathogenesis of neurodegenerative diseases such as AD.[18] Caspase-mediated neuronal death, a form of apoptosis, has been observed in cellular and animal models of AD as well as in human brains of affected patients.[19] However, the exact role of apoptosis in AD is still being debated. Modification of the neuronal nucleus, characterized by an abnormal chromosome and a characteristic feature of apoptosis, is increased in preclinical stages of Alzheimer's disease and is associated with decreased viability of neurons.[20] In our data, the number of apoptotic nuclei was increased in Aβ-treated neurons, confirming Aβ neurotoxicity. Thus, understanding the mechanisms and signaling pathways involved in neuronal apoptosis would be important for the understanding of the Aβ-related pathology for AD.

Astrocytes' interactions with neurons plays a crucial role in AD.[8] In AD, astrocytes undergo reactive astrogliosis, a process triggered by inflammatory factors.[21] Astrocytes release pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), contributing to the ongoing inflammatory process in AD.[22] This reactive phenotype of astrocytes is associated with the accumulation of Aβ. Astrocytes in AD exhibit abnormal regulation of various processes, including synaptic activity, neuronal metabolism, and regional blood supply, suggesting the importance of neuron-astrocyte interaction.[23] Though the effect of astrocytes on neurons in AD has been extensively studied, the effect of neurons on astrocyte activation induced by oligomerized Aβ has not been fully defined. In this study, we determined that Aβ can activate astrocytes even in the absence of neurons, suggesting a direct mechanism of Aβ in astrocyte activation.

Overall, the significant role of the interaction between neurons and astrocytes in the pathogenesis of AD neces-

sitates further research for a comprehensive understanding.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2019 R1A2C1004575, WSC) and supported by a grant of the Korea Dementia Research Project through the Korea Dementia Research Center (KDRC), funded by the Ministry of Health & Welfare and Ministry of Science and ICT, Republic of Korea (grant number: HU23C0199, WSC).

CONFLICT OF INTEREST STATEMENT

None declared.

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