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Protective Role of Microglia on Neuronal Survival after Exposure to Amyloid Beta

Sunjun Lee and Won-Seok Choi*

School of Biological Sciences and Technology, College of Natural Sciences, Chonnam National University, Gwangju, Korea

Alzheimer's disease (AD) is the most common cause of neurodegeneration. It is characterized by deposits of amyloid beta (Aβ) plaques and impaired memory. Microglia are associated with AD. They are activated in the AD brain and AD models. However, the exact role of microglia has not been established. We thus investigated the role of microglia in AD models using a primary culture and an ex-vivo assay. We showed that oligomerized Aβ is toxic to neurons in the primary culture. In the ex-vivo assay, a microglial cell line removed amyloid plaques in the brain of 5XFAD (AD model) mice. To verify if microglia can be protective for the neuron, we co-cultured neurons with primary microglia and treated them with Aβ. The loss of neurons, induced by amyloid toxicity, was attenuated by co-cultured microglia. Taken together, our data suggest that microglia promote neuronal survival by phagocytic clearance of Aβ in AD models.

Key Words: *Alzheimer Disease; Amyloid beta-Peptides; Microglia; Phagocytosis*

INTRODUCTION

Alzheimer's disease (AD) is one of the main causes of dementia among elderly individuals and leads to neurodegeneration in various areas of the brain, resulting in memory impairment and loss of cognitive function.[1] Although the exact pathology is unknown, the aggregation of amyloid beta (Aβ), which is toxic, has been hypothesized as the primary cause of AD pathology.[2] Aβ is generated from the cleavage of the amyloid precursor protein (APP). The precise physiological function of APP is not fully known; however, APP overexpression causes neuronal death and has been verified in transgenic mice that overexpress wild-type APP.[3] Several genetic mutations in humans are associated with familial AD, and mouse models expressing those mutants develop pathology similar to that in AD, including amyloid plaques, neuronal loss, and memory deficiency.[4-6]

Microglia are the immunocompetent glial cells of the central nervous system (CNS), which slowly proliferate.[7] Microglial activation has been associated with Aβ deposition and the progression of AD.[8] Microglia are responsible for phagocytosis in the CNS. They are found in an activated state in the AD brain, often in association with senile plaques.[9] Though the exact role of microglia in AD is not fully known, prior studies have demonstrated that microglia become activated in the presence of Aβ and secrete neurotoxic molecules that are thought to contribute to neurodegeneration by mediating a chronic inflammatory reaction.[10] On the other hand, other studies have suggested a protective role of microglia in AD.[11]

In this study, we examined the role of microglia in Aβ clearance and neuronal survival in AD models.

MATERIALS AND METHODS

1. Primary embryonic cortical culture

Primary cortical cells were prepared from E14 C57BL/6 mouse embryos (Daehan Biolink, Daejeon, Korea). Cerebral cortices were removed from the embryos, collected in 15 mL conical tubes containing PBS, and placed on ice. The tissue was incubated with 0.25% trypsin in HBSS for 20 min at 37℃. The trypsin was inactivated by adding a culture medium containing 10% FBS (Gibco, Gaithersburg, MD, USA). Tissue was triturated with a sterile constricted Pasteur pipette and a P-1000 pipette. Cortical neurons were seeded at 1×10^5 cells/aclar coverslip, pre-coated with 100 µg/mL poly-D-lysine in 24-well plates.

Cortical neurons were maintained in Neurobasal media (Invitrogen, Waltham, MA, USA) supplemented with B27 Supplement (Invitrogen), 2 mM glutamine at 37℃ in a humidified 5% CO_2 atmosphere.

2. Primary neonatal cortical culture

Primary neonatal cells were cultured from forebrains of 2 or 3-day-old mouse pups. Each forebrain tissue was dissected and placed in PBS-5% buffer on ice and meninges were removed. Forebrain tissues were minced with small scissors, placed in conical tubes and dissociated by various rounds of pipetting with a P-1000 pipet tip and fire-polished Pasteur pipettes. Dissociated cells were passed through a BD Nylon-strainer (Cat. #352340) into a new 50 mL Falcon tube. Cells were separated by centrifugation and supernatant was removed. Cells were resuspended in culture medium: DMEM-10% FBS+antibiotics and antimycotics. Cortical neurons were seeded at 1×10^5 cells/aclar coverslip, pre-coated with 100 µg/mL poly-D-lysine well in 24-well plates. Cells were incubated in a humidified 5% CO_2 atmosphere.

3. Primary microglia culture

Primary cortical cells were prepared from a neonatal brain as described above. After counting, cells were seeded in 75-cm T flasks ($62,500/cm^2$). Cells were incubated at 37℃ and 5% CO_2. After 4-5 d, we changed culture medium and supplemented 5 ng/mL MCSF or GM-CSF. Culture medium was changed every 4-5 d. At 12-15 day in vitro (DIV), floating microglia were collected from the culture by shaking flasks at 125 rpm for 4-5 h. The cells were counted and seeded for the experiments.

4. Aβ oligomerization

Amyloid powder (62-0-80B, American Peptide Company, Sunnyvale, CA) was dissolved in HFIP completely. All traces of HFIP were evaporated using a speedvac (10-30 min). Aβ stock solution was made in DMSO (5 mM). Before treatment, a 100-µM Aβ solution was made by adding DPBS.

5. Immunocytochemistry

After Aβ treatment (∼72 h), cells were fixed using 4% paraformaldehyde/4% sucrose at room temperature for 20 min and washed three times with PBS. Aclar cover slips were moved from the 24-well plate onto the staining rack and blocked in 1% BSA and 1% goat serum in PBS for 1 h at room temperature. Cells were washed twice using PBS and stained with mouse anti-NeuN antibody (1:1,000; Millipore, Burlington, MA, USA) at 4℃ overnight. Cells were washed twice with PBS and stained with secondary goat anti-mouse 488 (1:500; Invitrogen) for 2 h at room temperature. Cells were washed twice using 0.01M PBS. Cell nuclei were stained with DAPI (1:1,000). Cells were mounted on coverslips and images were captured using a Leica DM LB2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Images were analyzed using ImageJ.[12]

6. Ex-vivo assay

Mice (5XFAD transgenic mice overexpressing human APP mutants from familial Alzheimer's disease [FAD] cases from Sweden [K670N, M671L], Florida [I716V], and London [V717I]) were anesthetized with sevoflurane and perfused transcardially with saline. Brains were removed and snap-frozen in cooled isopentane. Frozen brains were cleaved into 18 mm-thick mid-sagittal sections using a
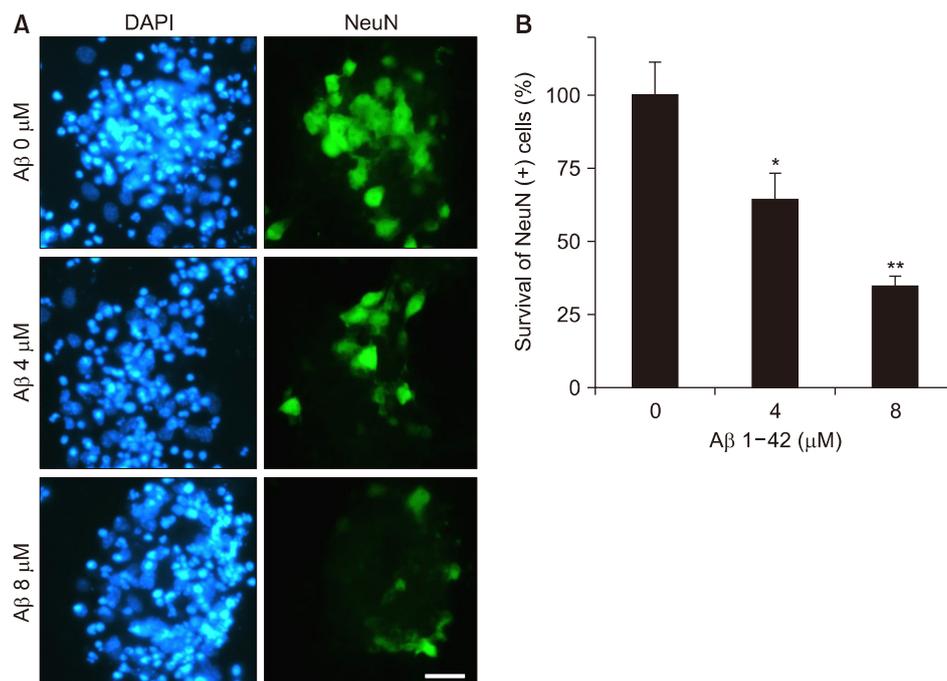

FIG. 1. Dose-dependent toxicity of amyloid beta (Aβ) in primary culture. Primary cells were cultured from neonatal brain cortex. Cultured cortical cells were treated with oligomerized Aβ (Aβ 1-42). (A) Cells were stained with mouse anti-NeuN antibody and secondary fluorescence antibody. Cell nuclei were stained with DAPI. Scale bar: 50 µm. (B) NeuN positive cells were counted using Image J software. The number of neurons decreased in a dose-dependent manner. All data are presented as means and error bars indicate the standard errors of the mean (SEM). *p<0.05, **p<0.01.

cryostat, and mounted onto poly-D-lysine-coated glass cover slips. Brain sections were allowed to dry for at least 2 h at room temperature and then washed with Hank's Balanced Salt Solution (HBSS). The sections were then incubated with microglial cells (2×10^5 BV2 cells per section) in DMEM containing 10% FBS and 1×penicillin/streptomycin for 60 h at 37℃ with 5% CO_2. The sections were washed twice with PBS and once with distilled water. They were then stained with hematoxylin for 5 min at room temperature and then washed with distilled water for 10 min. They were then stained with 1% thioflavin T for 5 min, then rinsed with EtOH for 5 min and with distilled water for 5 min. The sections were mounted and images were captured using a Leica DM LB2 fluorescence microscope (Leica Microsystems). Images were analyzed using ImageJ.

7. Quantitation of apoptotic nuclei

Cells were stained with DAPI (1:1,000). After mounting on coverslips, nuclei were observed, and images were captured using a Leica DM LB2 fluorescence microscope (Leica Microsystems). The number of apoptotic nuclei was quantitated in each microscopic image using ImageJ.

8. Data analysis

All data are shown as mean±SEM of at least three independent experiments. The significance of differences between the groups was determined using a Tukey's post hoc test following one-way analysis of variance (ANOVA). All p values <0.05 were considered statistically significant.

RESULTS

We used neonatal primary cells, which contain neurons and glial cells including microglia, to examine the effect of amyloid plaque accumulation. After the treatment with amyloid peptide or vehicle, cells were labeled with NeuN, a neuronal marker, and counted to quantify neuronal survival. The number of neurons decreased after Aβ treatment for 72 h (Fig. 1). The severity of neuronal loss was dose-dependent for Aβ peptide.

Prior studies have suggested that microglia are responsible for the phagocytosis of amyloid plaques in AD models. To confirm the role of phagocytic cells in the removal of amyloid plaques, we used 5XFAD mouse brain slices, which contain amyloid plaques, for the ex-vivo assay. Brain slices containing hippocampus were obtained from 5XFAD mice and incubated with or without BV2 cells (a microglial cell line). The number of amyloid plaques decreased in brain slices incubated with BV2 compared with those slices without BV2 cells (Fig. 2). This suggests that the phagocytic activity of microglial cells could remove amyloid plaques in the brain during AD pathogenesis.

To test whether the amyloid eliminator, microglia, can promote neuronal survival in living cells, we cultured primary neurons from embryonic brains and incubated these with and without primary microglia cultured from newborn pup brains. When treated with Aβ, the number of neurons (NeuN+) decreased in a dose-dependent manner compared with untreated controls (Fig. 3). However, the loss of neurons was attenuated by microglia in the neuron-microglia co-culture (Fig. 3A, B). This confirmed that microglia can promote neuronal survival amid Aβ-induced toxicity.

Aβ induces neuronal apoptosis.[13] We tested if the protective function of microglia attenuates neuronal apoptosis by analyzing apoptotic nuclei. The number of apoptotic nuclei was increased by Aβ, but microglial co-culture attenuated the elevation of the number of apoptotic nuclei (Fig. 3C). These results suggest that microglia promote neuronal survival against Aβ-toxicity by attenuating neuronal apoptosis.
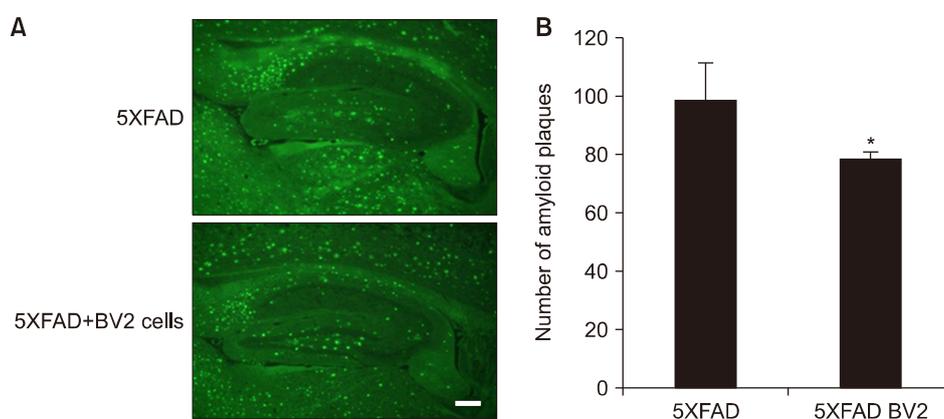

FIG. 2. The phagocytosis of amyloid-plaque in 5XFAD mouse brain tissue. (A) Sagittal brain sections containing hippocampus were obtained from 5XFAD mice. Brain sections were incubated with a microglial cell line, BV2, or medium (control) and stained with thioflavin T, which visualizes amyloid plaques in the brain sections of 5XFAD mice. Scale bar: 200 μm. (B) The number of amyloid plaques in hippocampus was counted using ImageJ. All data are presented as means and error bars indicate the standard errors of the mean (SEM). *p<0.05.
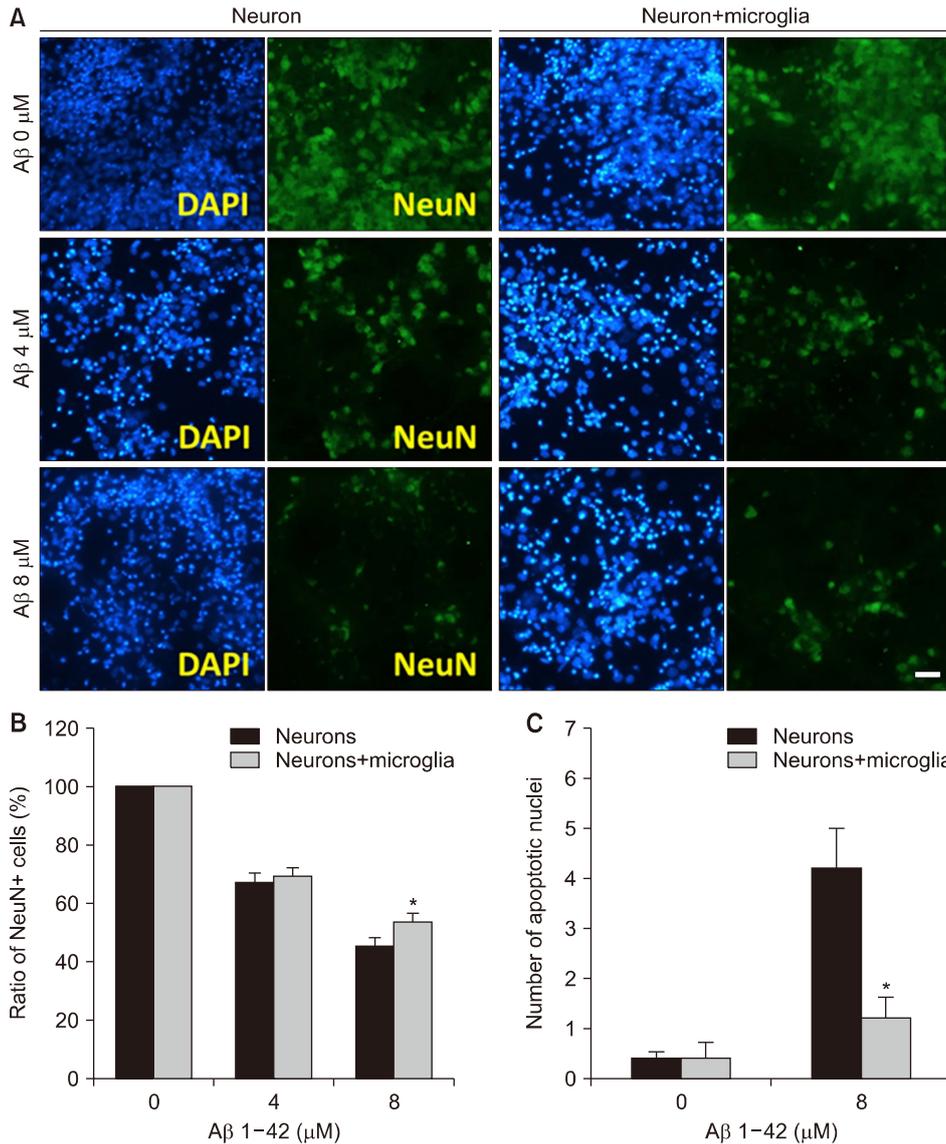
FIG. 3. The protective effect of microglia and neuronal survival was confirmed using primary neuron-microglia co-cultures. Primary cells (E14) were co-cultured with microglia from neonatal primary culture. (A) Neurons were stained with NeuN antibody and nuclei were stained with DAPI. Scale bar: 50 μm. (B) The ratios of neuronal number (NeuN-positive cells in each condition/NeuN-positive cells in vehicle control) were calculated using ImageJ. (C) The number of apoptotic nuclei was quantitated using imageJ. All data are presented as means, and error bars indicate the standard errors of the mean (SEM). *p<0.05.

DISCUSSION

Although the involvement of microglia in AD has been suggested for decades, the precise role of microglia in AD models is not entirely understood. Several studies have suggested that microglial activation could lead to phagocytosis of Aβ and thereby reduce toxicity. However, slow chronic activation of microglia may trigger inflammation that could be neurotoxic.[14] In this study, we investigated the function of microglia using primary culture and an ex-vivo tissue assay. First, we confirmed the toxicity of oligomerized Aβ in primary cortical culture from neonatal mouse brain, which is a neuron-glia mixed environment containing microglia.

Although microglia have many functions, the main role that has been associated with protection in AD is phagocytosis. The removal of amyloid plaques by microglial phagocytosis could reduce the toxic burden on the neurons and could be protective. To determine if microglial phagocytosis could remove amyloid plaques, we used an ex-vivo assay in which brain slices containing amyloid plaques were incubated with a microglial cell line, BV2. After incubation, amyloid plaques were removed by BV2 cells, confirming that the phagocytic function of microglia could reduce the toxic burden of Aβ.

To determine if microglia could be directly protective to the neurons, we used primary cells from the embryonic cortex, which mainly contains neurons, and co-cultured neurons with primary microglia from neonatal mouse brain. After treatment with Aβ, the loss of primary neurons was attenuated by the co-culture of primary microglia, which showed that microglia can be directly protective when Aβ toxicity kills neurons. However, the marginal protection rate raised the possibility of multiple functions of microglia in the mixed culture condition. Activated microglia have been recently shown to be protective or neurotoxic depending on the activation processes or the environment.[14] Microglia can aggravate tau pathology and mediate syn-

aptic loss which are both harmful to neurons.[15,16] This toxic function of microglia might affect neuronal health in our culture condition. However, the protective role of removing toxic Aβ and/or toxic debris of dead cells would represent a stronger effect than the harmful ones, because Aβ toxicity is the main pathogenic factor in our in vitro culture model. Therefore, neuronal apoptosis, which was probably caused by toxic stimulations from Aβ and/or dead cell debris, was attenuated by microglia. Furthermore, synaptic loss would have minimal effect in the primary culture model, where neurons are incubated in an enriched medium containing sufficient growth factors and neuronal survival would not significantly rely on extensive synapses with other neurons. Regarding tau pathology which might interfere with axonal transportation and synaptic communication, it could be secondary to the Aβ toxicity, but neuronal survival would be minimally affected in the enriched culture condition. Our data suggest that a larger fraction of microglia become protective after Aβ-induced activation, though a smaller fraction might have been toxic in our co-culture environment.

Herein, we have provided evidence that Aβ deposits and their toxicity can be alleviated by microglia. Further research on the regulation of microglia could uncover potential drug targets for the treatment of AD.

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CONFLICT OF INTEREST STATEMENT

None declared.

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