

Placenta-derived mesenchymal stem cells improve memory dysfunction in an $A\beta_{1-42}$ -infused mouse model of Alzheimer's disease

H-M Yun^{1,6}, HS Kim^{2,3,6}, K-R Park¹, JM Shin^{2,3}, AR Kang^{2,3}, K il Lee^{2,3}, S Song¹, Y-B Kim⁴, SB Han¹, H-M Chung^{*,5} and JT Hong^{*,1}

Mesenchymal stem cells (MSCs) promote functional recoveries in pathological experimental models of central nervous system (CNS) and are currently being tested in clinical trials for neurological disorders, but preventive mechanisms of placenta-derived MSCs (PD-MSCs) for Alzheimer's disease are poorly understood. Herein, we investigated the inhibitory effect of PD-MSCs on neuronal cell death and memory impairment in $A\beta_{1-42}$ -infused mice. After intracerebroventricular (ICV) infusion of $A\beta_{1-42}$ for 14 days, the cognitive function was assessed by the Morris water maze test and passive avoidance test. Our results showed that the transplantation of PD-MSCs into $A\beta_{1-42}$ -infused mice significantly improved cognitive impairment, and behavioral changes attenuated the expression of APP, BACE1, and $A\beta$, as well as the activity of β -secretase and γ -secretase. In addition, the activation of glia cells and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were inhibited by the transplantation of PD-MSCs. Furthermore, we also found that PD-MSCs downregulated the release of inflammatory cytokines as well as prevented neuronal cell death and promoted neuronal cell differentiation from neuronal progenitor cells in $A\beta_{1-42}$ -infused mice. These data indicate that PD-MSC mediates neuroprotection by regulating neuronal death, neurogenesis, glia cell activation in hippocampus, and altering cytokine expression, suggesting a close link between the therapeutic effects of MSCs and the damaged CNS in Alzheimer's disease.

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Mesenchymal stem cell (MSC) has been recently considered to improve disease progression of neurological disorders.^{1,2} MSC is a multipotent stem cell with capacity for self-renewal and differentiation with broad tissue distribution.³ MSC was first identified and isolated from bone marrow (BM) more than 43 years ago.⁴ However, within the bone marrow, BM-MSC is a rare cell population, resulting in a low MSC yield when isolated.⁵ The progenitor pool may be depleted following extensive proliferation, leading to reducing ability to ensure regeneration after injury.^{6,7} For these reasons, novel sources of MSCs are now being investigated for clinical use in diseases.^{8,9} It was reported that MSCs derived from placenta (PD-MSCs) showed higher proliferative capacity and safety,^{8,10} and also included noninvasive and ethically nonproblematic availability.¹¹ Thus, PD-MSCs can be obtained in large quantities, and the required numbers of these stem cells can be transplanted as an ideal alternative to

adult bone marrow in therapeutic approaches for tissue replacement.

It was reported that BM-MSCs reduced $A\beta$ levels in an acutely amyloid β ($A\beta$)-injected mouse model of Alzheimer's disease,¹² and attenuated $A\beta$ -induced apoptotic cell death in primary cultured hippocampal neurons by the activation of the cell survival signaling pathway.¹³ In amyloid precursor protein (APP) and PS1 double-transgenic mice, BM-MSCs significantly also reduced $A\beta$ level associated with defective microglial function and decreased tau hyperphosphorylation and improved cognitive function.¹⁴ Recently, human umbilical cord blood-(hUCB)-derived MSCs significantly also improved learning and memory decline through the modulation of neuroinflammation in an APP and PS1 double-transgenic mice.¹⁵ Interestingly, no studies are available on the various roles played by PD-MSCs in eliciting the therapeutic effects in Alzheimer's diseases. Therefore, it is important to elucidate

¹College of Pharmacy and Medical Research Center, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea; ²CHA Bio & Diotech Co., Ltd, 606-16 Yeoksam 1 dong, Gangnam gu, Seoul 135-970, Republic of Korea; ³Department of Applied Bioscience, CHA University, Gyeonggi-do 463-836, Republic of Korea; ⁴College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea and ⁵Department of Stem Cell Biology, School of Medicine, Konkuk University, 120 Neungdong-ro Gwangjin-gu, Seoul 143-701, Republic of Korea

*Corresponding author: H-M Chung, Department of Stem Cell Biology, School of Medicine, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea. Tel: +82 2 3468 3398; Fax: +82 2 3468 3373; E-mail: hmchung@cha.ac.kr or JT Hong, College of Pharmacy and Medical Research Center, Chungbuk National University, 12 Gaeshin-dong, Heungduk-gu, Cheongju, Chungbuk 361-463, Republic of Korea. Tel: +82 43 261 2813; Fax: +82 43 268 2732; E-mail: jinthong@chungbuk.ac.kr

⁶These authors contributed equally to this work.

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Abbreviations: $A\beta$, amyloid β ; APP, amyloid precursor protein; BM-MSC, bone marrow-derived MSC; β -secretase or BACE1, β -site APP cleaving enzyme 1; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; GCL, granule cell layer; MSC, mesenchymal stem cell; PD-MSC, placenta-derived MSC; SGZ, subgranular zone; UCB-MSC, umbilical cord blood-derived MSC

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the novel roles and potential effects mediated by PD-MSCs in Alzheimer's diseases.

Alzheimer's disease as one of the most important neurodegenerative disorders is an irreversible and progressive disorder with observable memory impairment.^{16,17} $A\beta$ is generated from APP, a membrane-spanning protein to be digested by β -secretase (β -site APP cleaving enzyme 1 (BACE1)).^{18,19} It was reported that APP metabolism and $A\beta$ generation associated with senile plaques are related to the activation of glia cells.²⁰ $A\beta$ peptide activates glia cells that are found to surround $A\beta$ plaques.^{21,22} The activation of these cells is associated with many inflammation-associated neurodegenerative diseases including Alzheimer's disease.^{23,24} It is also suggested that glia cells could act as a source for $A\beta$ because they overexpress BACE1 in response to chronic stress.²⁵ Thus, the feed-forward vicious cycle by $A\beta$ between amyloidogenesis and neuroinflammation culminates in widespread neuronal dysfunction and cell death, leading to progressive Alzheimer's disease associated with extensive $A\beta$ pathology.

In the present study, we examined whether the transplantation of PD-MSCs could have beneficial effects in $A\beta_{1-42}$ -infused mice model. We found that the transplantation of PD-MSCs rescued cognitive impairment, promoted anti-amyloidogenic and anti-inflammatory response, and also increased neuronal survival and neurogenesis.

Results

PD-MSC reverses memory impairment in $A\beta_{1-42}$ -infused mice. In order to identify whether PD-MSCs could affect memory impairment by $A\beta_{1-42}$ in Alzheimer's diseases, ICR mice were infused with $A\beta_{1-42}$ (300 pmol per day per mouse) for 14 days and then compared memory deficiency between the control mice and mice transplanted with PD-MSCs (1×10^5 , 5×10^5 , and 1×10^6 cells per mouse) via intravenous injection into mouse tail vein. As shown in Figure 1, we performed behavioral tests for a total of 12 days using the Morris water maze and passive avoidance tests. All mice were trained for three trials per day for 7 days. Escape latency (time, s) and swimming distance (distance, cm) that were traveled to reach a platform in water maze were measured to determine memory impairment effect of PD-MSCs. The $A\beta_{1-42}$ -infused mice exhibited significantly delayed escape latency (33.2 ± 17.94 s, $**P < 0.01$; Figure 2a) and swimming distance (851.5 ± 123.5 cm, $**P < 0.01$; Figure 2b) compared with the control mice (escape latency: 17.2 ± 10.03 s; swimming distance: 418.2 ± 75.21 cm); however, mice transplanted with PD-MSCs showed significantly better performance on the water maze test than

$A\beta_{1-42}$ -infused mice (1×10^5 cells: 25.5 ± 12.68 s, 583.3 ± 152.8 cm, $##P < 0.01$; 5×10^5 cells: 28.4 ± 14.38 s, 541.3 ± 138.3 cm, $##P < 0.01$; 1×10^6 cells: 22 ± 6.60 s, 489.3 ± 61.02 cm, $##P < 0.01$; Figures 2a and b).

After the water maze test, a probe test was performed in which the platform was removed to analyze whether the animals used a nonspatial strategy to find the platform. During the probe test, we calculated the time spent in the target quadrant zone during the 60 s test. The memory deficiency in $A\beta_{1-42}$ -infused mice (15.92 ± 10.32 s, $*P < 0.05$) was significantly improved in PD-MSC mice group (1×10^5 cells: $25.89 \pm 7.51\%$; 5×10^5 cells: $28.58 \pm 7.63\%$, $#P < 0.05$; 1×10^6 cells: $30.84 \pm 5.27\%$, $#P < 0.05$; Figure 2c). To determine whether PD-MSC mice improved the contextual memory in $A\beta_{1-42}$ -infused mice, we next evaluated learning and memory capacities by the passive avoidance test using the step-through method. In the passive avoidance test, there was no significant difference on the learning trial. In contrast, PD-MSC mice (1×10^5 cells: 31.58 ± 7.67 s, $#P < 0.05$; 5×10^5 cells: 39.45 ± 8.19 s, $##P < 0.01$; 1×10^6 cells: 41.13 ± 10.27 s, $##P < 0.01$) significantly increased the step-through latency compared with the $A\beta_{1-42}$ -infused mice (20.23 ± 6.54 s, $***P < 0.001$; Figure 2d). Thus, we validated that the beneficial effects of PD-MSCs remained consistent over a prolonged period of time. These results indicate that PD-MSC improves deficits of learning and memory in $A\beta_{1-42}$ -infused mice.

PD-MSC reveals anti-amyloidogenic effects in $A\beta_{1-42}$ -infused mice. We investigated whether PD-MSCs influenced amyloidogenesis in the $A\beta_{1-42}$ -infused mice. The number of $A\beta_{1-42}$ -reactive cells was determined with immunohistochemical analysis and $A\beta_{1-42}$ expression was significantly lowered by the transplantation of PD-MSCs (Figure 3a) and the levels of $A\beta_{1-42}$ were also measured using ELISA (Con: 13.00 ± 8.06 ; $A\beta$: 33.53 ± 5.44 , $*P < 0.05$; $A\beta + MSC$: 19.79 ± 4.01 , $#P < 0.05$; Figure 3e). To clarify how $A\beta_{1-42}$ deposition had occurred, we analyzed the expression level of APP and BACE1. As shown in Figures 3b–d, there was a significant rise in the level of the proteins in the brain after $A\beta_{1-42}$ injection, which was inhibited by PD-MSCs. To confirm the speculation, we next attempted to measure β - and γ -secretase activity. The activity of β - and γ -secretase significantly increased in the brain of $A\beta_{1-42}$ -infused mice (β -secretase: $A\beta$, 420.5 ± 39.77 , $*P < 0.05$; γ -secretase: $A\beta$, 245.2 ± 14.15 , $**P < 0.01$), whereas the alteration in β - and γ -secretase activity was attenuated by the transplantation of PD-MSC (β -secretase: $A\beta + MSC$, 348.0 ± 7.45 , $#P < 0.05$; γ -secretase: $A\beta + MSC$, 141.3 ± 20.71 , $##P < 0.01$; Figures 3f and g). These data suggest that PD-MSC has an anti-amyloidogenic effect in $A\beta_{1-42}$ -infused mice.

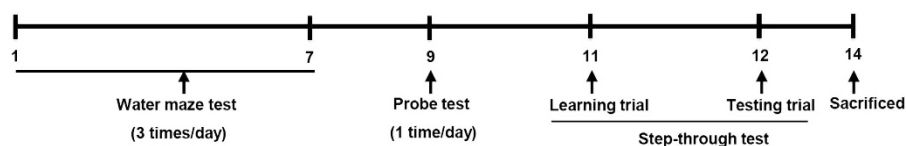


Figure 1 The scheme of experimental study on an $A\beta_{1-42}$ -infused mouse model. After $A\beta$ infusion for 14 days, the Morris water maze test, probe test, and passive avoidance test were conducted as shown

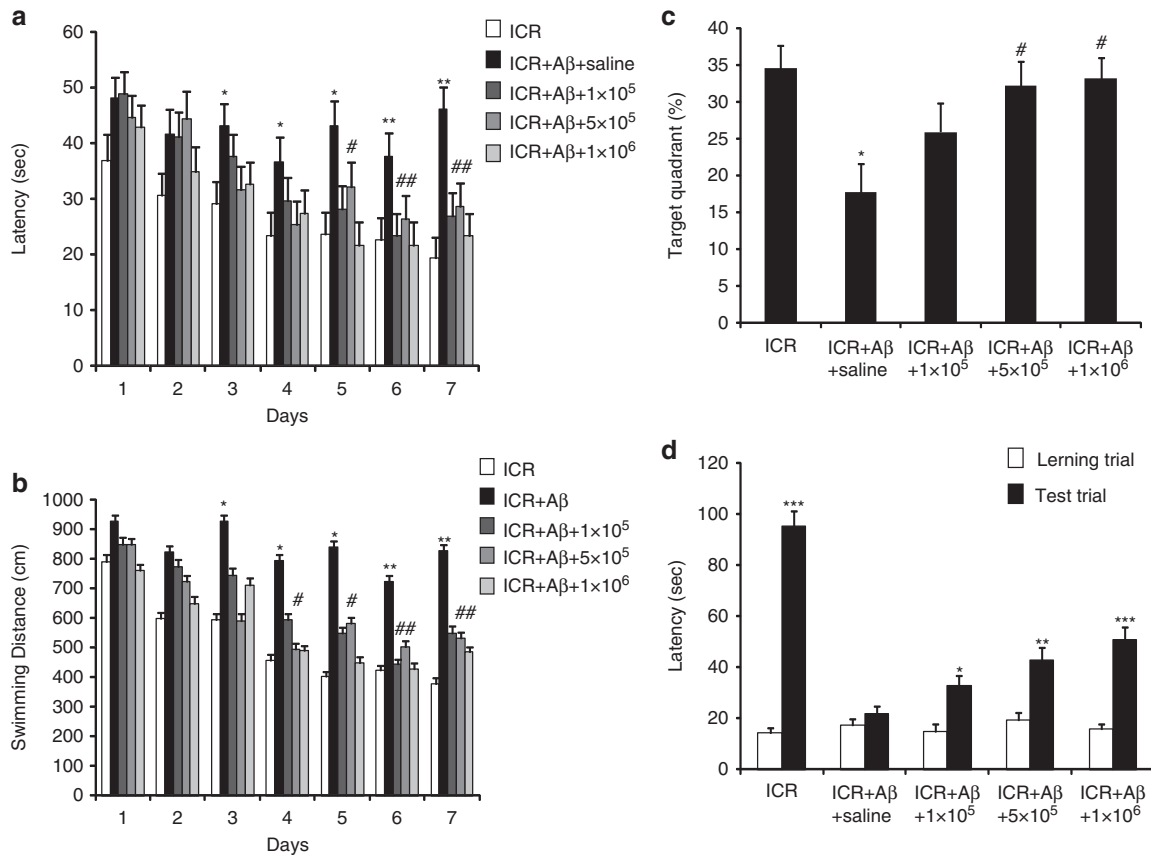


Figure 2 Inhibitory effects of PD-MSCs on memory impairment in $A\beta_{1-42}$ -infused mice model. (a–c) Training trial was performed three times a day for 7 days. Swimming time (a) and swimming distance (b) to arrive at platform were automatically recorded. At 24 h after training trials, a probe test was performed. The time spent in the target quadrant and target site crossing within 60 s was represented (c). Each value is presented as mean \pm S.E.M. from 10 mice. (d) To perform passive avoidance test, the mice were given electric shock when entered into the dark compartment for training on learning day. After 1 day, the retention time in illuminated compartment was recorded. Each value is presented as mean \pm S.E.M. from 10 mice. Significant difference from control mice (* P <0.05, ** P <0.01, and *** P <0.001). Significant difference from $A\beta_{1-42}$ -infused mice (# P <0.05 and ## P <0.01)

PD-MSC reveals antineuroinflammatory effects in $A\beta_{1-42}$ -infused mice. It was also reported that activation of glia cells is one of the characteristic feature in the Alzheimer's disease, and they can produce inflammatory cytokines as well as generate $A\beta$ when they are activated. Therefore, we compared activation of astrocytes and microglia in the brains between $A\beta_{1-42}$ -infused mice and $A\beta_{1-42}$ -infused PD-MSC mice. As shown in Figures 4a and b, GFAP-positive cell numbers (astrocytes) and Iba1-positive cell number (microglia) were much higher in the brains of $A\beta_{1-42}$ -infused mice as compared with the brains of $A\beta_{1-42}$ -infused PD-MSC mice. To verify these results, we investigated the expression of GFAP and Iba1 by western blotting. The results revealed that the GFAP and Iba1 levels were significantly increased in $A\beta_{1-42}$ -infused mice as compared with control mice. However, $A\beta_{1-42}$ -infused PD-MSC mice were significantly attenuated the expression of GFAP and Iba1 in the $A\beta_{1-42}$ -infused mice (Figure 4e). We also demonstrated the effect of the transplantation of PD-MSCs on memory formation and $A\beta$ deposition was mediated by neuroinflammation. The expression of the inflammatory protein such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the brain of $A\beta_{1-42}$ -infused mice were significantly

increased than the control mice, whereas PD-MSC mice revealed a decrease in the expression of iNOS and COX-2 detected by immunohistochemical analysis (Figures 4c and d) and western blotting (Figure 4e). To validate the activation of glia cells and inflammatory response, we performed mouse cytokine array using Proteome profiler arrays. We found that the levels of G-CSF, I-309, sICAM-1, IFN- γ , IL-1 β , IL-7, IL-17, IL-27, IP-10, KC, MIG, CCL5, TIMP-1, and TNF- α by infusion of $A\beta_{1-42}$ were significantly altered, which is inhibited by PD-MSC (Supplementary Figure 1). Our results indicate that PD-MSC regulates glia cells, inflammatory response, and cytokine release to improve the symptom in Alzheimer's diseases.

PD-MSC prevents neuronal cell death and promotes neuronal differentiation. We explored whether PD-MSC was able to improve memory impairment by preventing $A\beta_{1-42}$ -induced neuronal cell death. In the CA1, CA3, and dentate gyrus (DG) region of the hippocampus, $A\beta_{1-42}$ injection significantly increased apoptotic cell death as compared with control mice (CA1: 88.69 ± 9.30 , P <0.001; CA3: 78.64 ± 9.35 , P <0.01; DG: 68.81 ± 13.05 , P <0.05). PD-MSCs inhibited apoptotic cell death in $A\beta_{1-42}$ -infused

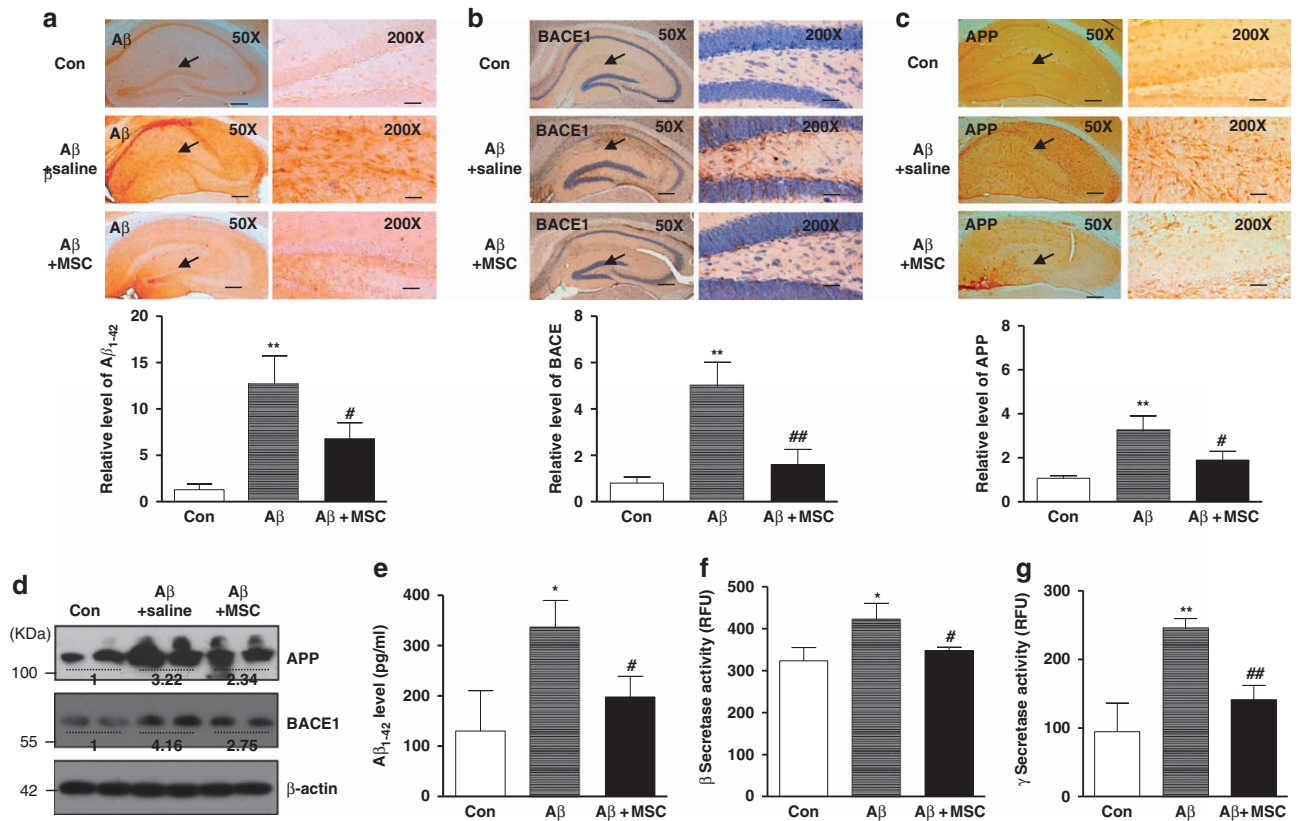


Figure 3 Inhibitory effects of PD-MSCs on A β_{1-42} , BACE1 and APP in the brain of A β_{1-42} -infused mice. (a–c) A β_{1-42} , BACE1, and APP were observed by immunohistochemical analysis as described in the Materials and Methods. The sections of brains were incubated with anti-A β_{1-42} (a), anti-BACE1 (b), and anti-APP (c) antibodies, followed by biotinylated secondary antibody. Immunoperoxidase staining was expressed as brown color. (d) The expression of APP and BACE1 were detected by western blotting using specific antibodies in brain lysates. β -Actin was used as a loading control. (e) A β_{1-42} level was measured in mouse brains by ELISA as described in the Materials and Methods. Values measured from each group of mice were calibrated by amount of protein and expressed as mean \pm S.E.M. from 8 mice. Significant difference from control mice (* $P < 0.05$ and ** $P < 0.01$). Significant difference from A β_{1-42} -infused mice (# $P < 0.05$ and ## $P < 0.01$). The experiments shown in Figure 2 were repeated in triplicate with similar results

mice (CA1: 48.56 ± 5.21 , $P < 0.05$; CA3: 43.23 ± 10.61 , $P < 0.05$; DG: 31.56 ± 4.80 , $P < 0.05$; Figures 5a–d). Moreover, we investigated whether A β_{1-42} infusion or PD-MSC affects neuronal precursor cells to be differentiated into neuron. As shown in Figure 5e, in the subgranular zone (SGZ)–granule cell layer (GCL) of the hippocampus, A β_{1-42} -infused mice revealed that DCX-positive cells (adult–newborn neurons) were diminished when compared with control mice, whereas the implantation of PD-MSC recovered adult–newborn neurons decreased by A β . Ki-67-positive cells were not changed between control, A β_{1-42} -infused, and PD-MSC mice (Figure 5f), implying that PD-MSCs did not affect proliferation of adult neural stem cell. As MSC is a multipotent cell that can be differentiated into neural cell lineages, we therefore investigated whether the protective effects of PD-MSCs were mediated by direct differentiation into neuron or glia cells. As shown in Figure 6, PD-MSCs implanted in A β_{1-42} -infused mice was not colocalized with NeuN (mature neuron) (Figure 6a), DCX (newborn neuron; Figure 6b), or GFAP (astrocyte; Figure 6c) positive cells. Taken together, these data suggest that PD-MSC prevents neuronal cell death and induces newly generated neuron regardless of the neural differentiation potential of PD-MSC.

Discussion

In the present study, we originally reported clear evidences in the role of PD-MSCs using an A β -infused mouse model of Alzheimer's diseases. A β -infused mouse model has been used to directly investigate the toxicity of A β that consisted of senile plaques of Alzheimer's disease.^{12,13,26–28} Also, we continually investigated amyloidogenesis, neuroinflammation, and memory impairment through the injection of A β .^{29–33} Therefore, to investigate the effect on the A β toxicity of PD-MSCs, we used the A β -infused mouse model in the present study. We demonstrated that the transplantation of PD-MSCs in A β_{1-42} -infused mice has anti-amyloidogenic and anti-neuroinflammatory effects to improve neuronal survival and neurogenesis, and prevent memory deficiency.

Amyloidogenesis postulates that gradual changes in the metabolism and aggregation of A β initiate a cascade of neuronal and inflammatory injury that culminates in extensive neuronal dysfunction and cell death in Alzheimer's disease.^{34,35} In these experiments, we demonstrated that PD-MSCs reduced the amyloid cascade in the A β_{1-42} -infused mouse model. The effect of PD-MSC is likely attributable to inhibition in the expression of BACE1 and APP, and the

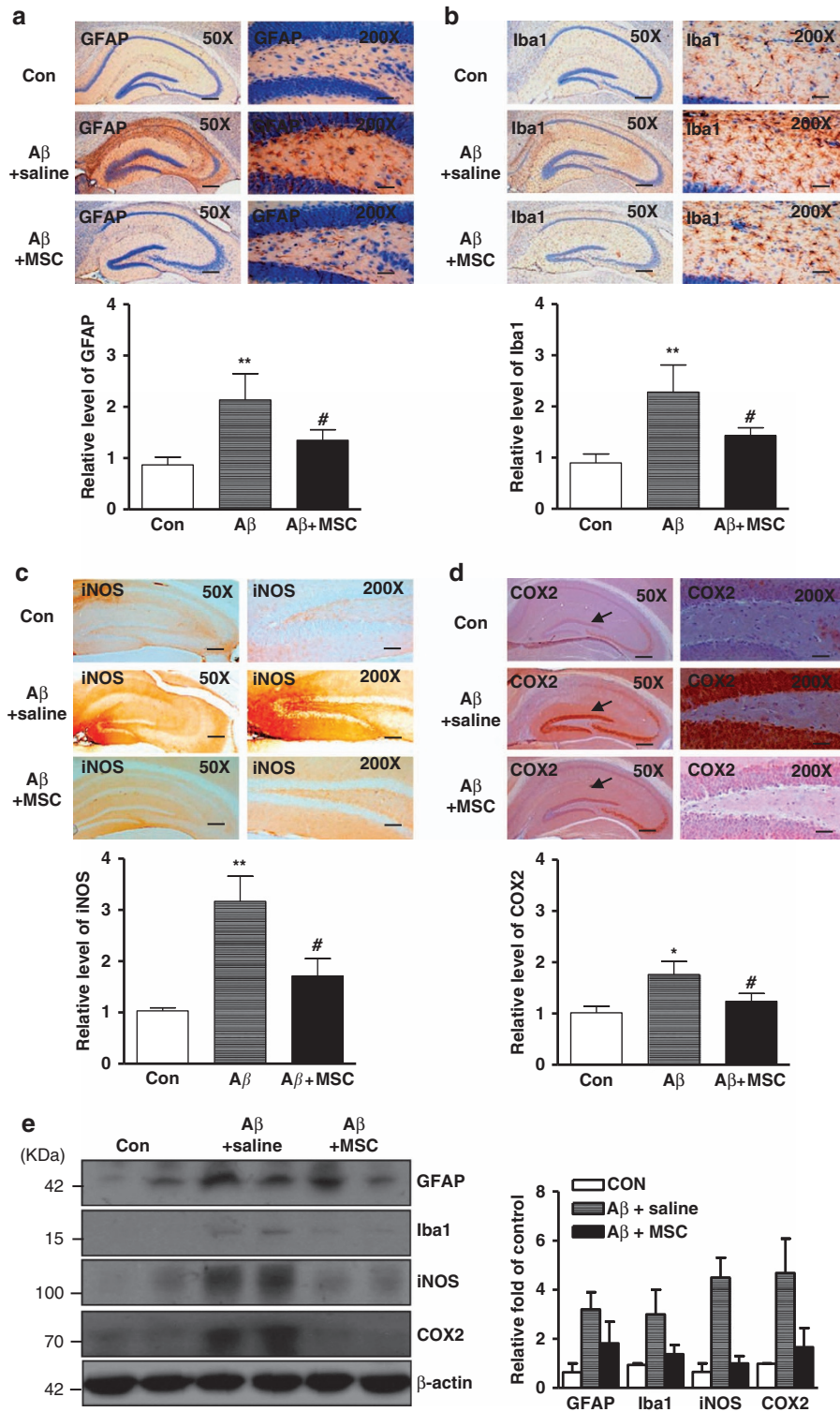


Figure 4 PD-MSC inhibits the activation of astrocytes and microglia, and reduces the expression of COX-2 and iNOS. (a and b) The effect of PD-MSC on reactive astrocytes and activated microglia cells was measured by immunohistochemical analysis. The sections were incubated with antigial fibrillary acidic protein (GFAP) (a) and anti-ionized calcium binding adaptor molecule 1 (Iba1) (b) antibodies, followed by the biotinylated secondary antibody ($n = 8$). Immunoperoxidase staining was expressed as brown color. (c and d) The sections were incubated with anti-iNOS (c) and anti-COX-2 (d) antibodies, and then followed by the biotinylated secondary antibody ($n = 8$). Immunoperoxidase staining was expressed as brown color. (e) The expression of GFAP, Iba1, iNOS, and COX-2 was detected by western blotting using specific antibodies. The signals of GFAP, Iba1, iNOS, and COX2 were normalized using β -actin and the numerical values were expressed as relative fold of control. Significant difference from control mice ($*P < 0.05$ and $**P < 0.01$). Significant difference from A β_{1-42} -infused mice ($#P < 0.05$). The experiments shown in Figure 3 were repeated in triplicate with similar results

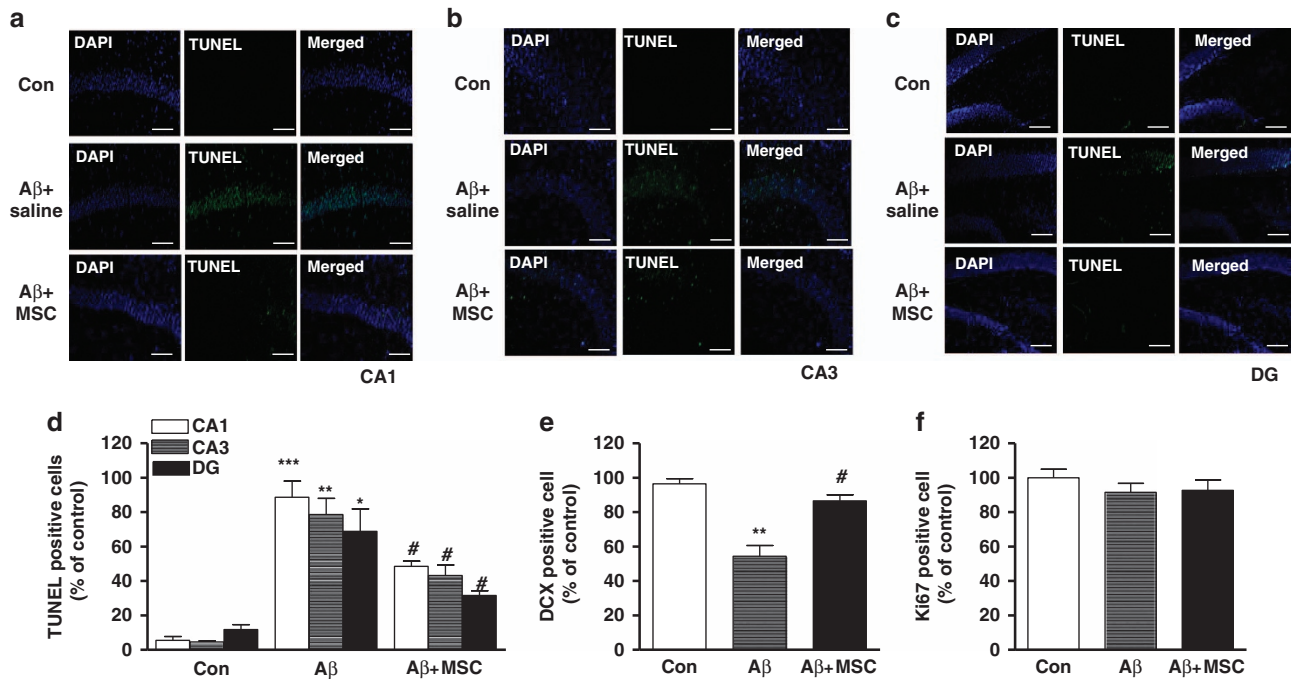


Figure 5 PD-MSC prevents $A\beta_{1-42}$ -induced apoptotic cell death and promotes neuronal differentiation. (a–c) Representative photographs (original magnification $\times 100$) of each region of control mice, $A\beta_{1-42}$ -infused mice, and PD-MSC (1×10^6 cells) mice. Apoptotic cell death in hippocampus CA1 (a), CA3 (b), and DG (c) of mouse brain was determined by DAPI and TUNEL staining as described in the Materials and Methods. (d) DAPI- and TUNEL-positive cells were counted in three separate locations and expressed as % of control. Significant difference from control mice ($*P < 0.05$, $**P < 0.01$, and $***P < 0.001$). Significant difference from $A\beta_{1-42}$ -infused mice ($\#P < 0.05$). (e and f) The effect of PD-MSC on newly generated neurons and proliferation of neural stem cells was measured by immunohistochemical analysis. The sections were incubated with anti-doublecortin (DCX) (e) and anti-antigen Ki-67 (Ki-67) (f) antibodies, followed by the biotinylated secondary antibody ($n = 8$). Significant difference from control mice ($**P < 0.01$). Significant difference from $A\beta_{1-42}$ -infused mice ($\#P < 0.05$). The experiments shown in Figure 4 were repeated in triplicate with similar results.

activity of γ -secretase. Amyloidogenic processing of APP involves sequential cleavages by BACE1 and γ -secretase.³⁵ C-terminal fragment of APP generated by BACE1 cleavage was further processed by γ -secretase to produce $A\beta_{40/42}$. Our results indicated that the expression of BACE1 and APP, and the activity of γ -secretase, were decreased by PD-MSCs in $A\beta_{1-42}$ -infused mice. Previously, it was reported that BM-MSCs significantly reduced $A\beta$ deposition and attenuated $A\beta$ -induced memory impairment and apoptosis by inhibiting neuronal cell death.^{12–14} Adipose tissue-derived MSCs also improved cognitive function in aging mice.³⁶ Thus, we suggest that PD-MSC improves cognitive dysfunction through BACE1 and APP expression, and γ -secretase activities to diminish amyloidogenesis.

APP and BACE1 are upregulated in astrocytes and microglia upon exposure to cytokines influencing APP processing to induce amyloidogenesis, and induced in the proximity of activated astrocytes and microglia.^{37,38} We found that the number of astrocytes and microglia were elevated in $A\beta_{1-42}$ -infused mice that were prevented by PD-MSCs. It was also reported that BACE1 and APP transcriptions are increased by other inflammatory gene expression such as iNOS and COX-2.^{39,40} Several studies have indicated that neuroinflammatory reactions could contribute to BACE1 and APP expression and γ -secretase activity. These studies also support the idea for neuroinflammation as a direct effect on amyloidogenesis. Especially, hUCB-derived MSCs significantly reduced the levels of $A\beta$, BACE1, and Tau

hyperphosphorylation by regulating neuroinflammatory responses including alternatively elevated microglia and decreased proinflammatory cytokines (IL-1 β and TNF α).¹⁵ In present study, we also found that PD-MSCs inhibited neuroinflammatory responses through decreased iNOS and COX-2 expression, decreased proinflammatory cytokines including IL-1 β and TNF α , and inactivated glia cells. These results suggest that the inhibitory effect of PD-MSCs on the activation of astrocytes and microglia might be significant to modulate neuroinflammatory-mediated amyloidogenesis. Based on previous reports in Alzheimer's diseases patients and animal models,^{41–43} we speculated whether activated microglia and astrocytes by $A\beta$ also caused cytokine releases. As a result of mouse cytokine array, the transplantation of PD-MSCs also inhibited cytokine secretion induced in $A\beta_{1-42}$ -infused mice. We found that the levels of cytokines including IL-1 β , IL-17, TNF- α , and IP-100 were significantly altered by $A\beta_{1-42}$ that were inhibited by PD-MSCs. IL-1 β , IL-17, and TNF α have important roles in affected brain regions in association with plaques and tangles in Alzheimer's disease.^{44,45} $A\beta$ induces the production and secretion of IL-1 β ,⁴⁶ and astrocytes induce neuronal death by induction of IL-1 β and IL-17, together with TNF α .⁴⁷ TNF α binds to neuronal Cd120a/b receptors triggering caspase activation through death effector domains.⁴⁸ IP-10 is markedly increased in reactive astrocytes in Alzheimer's disease brains, and IP-10-positive astrocytes are associated with senile plaques and $A\beta$ -induced neurotoxicity.^{49,50} Similar to our observation,

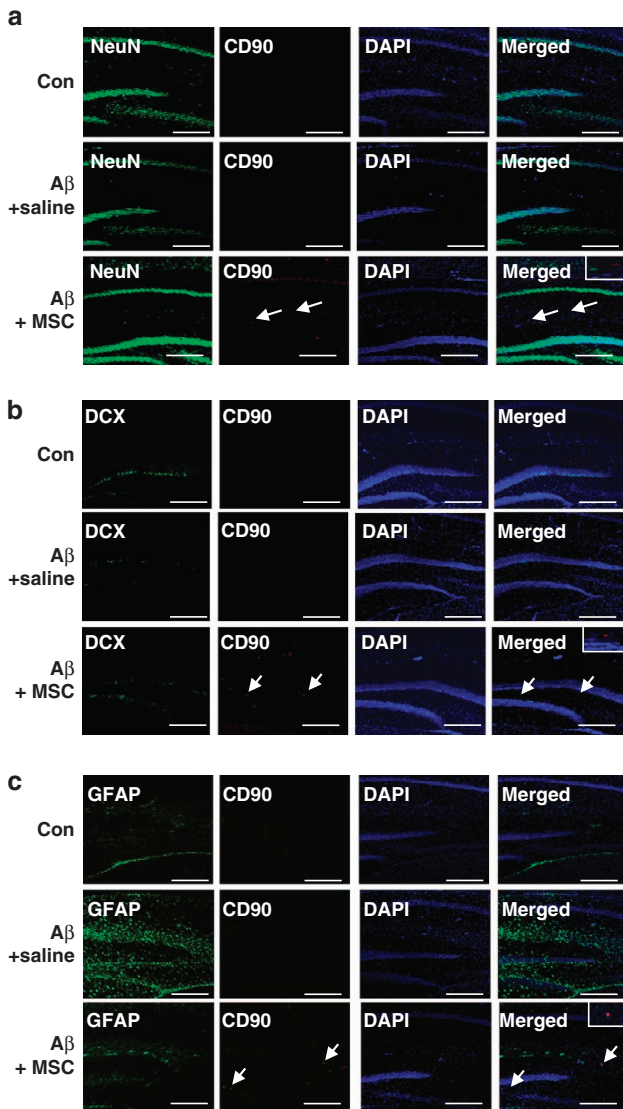


Figure 6 The localization of PD-MSCs with NeuN, DCX, and GFAP in $A\beta_{1-42}$ -infused mice. (a–c) Immunofluorescence analysis using confocal microscope in $A\beta_{1-42}$ -infused mice. The sections were fixed and permeabilized. Mature neuron (a), newborn neuron (b), and astrocytes (c) (green, left) were immunostained with anti-NeuN, anti-DCX, and anti-GFAP antibodies, and then followed by Alexa488-conjugated secondary antibodies. PD-MSC (red, middle) was immunostained with anti-CD90 antibody, and then followed by Alexa555-conjugated secondary antibodies. The right panels of (a–c) show the merged images of the left and middle panels. The experiments shown in Figure 5 were repeated in triplicate with similar results

MSC tends to be migrated and localized to tissues that undergo an inflammatory response, altering the spectrum of local cytokines.⁵¹ MSC is a multipotent cell that can be differentiated into a variety of cell types, including: osteogenic, chondrogenic, adipogenic, and neural cell lineages.⁵² However, in the mouse brain, PD-MSC was not differentiated into neural cell. Therefore, our results implied that PD-MSCs might have a role in the memory improvement in Alzheimer's diseases because of the antineuroinflammatory reaction-associated anti-amyloidogenesis, but not the multipotency of PD-MSCs.

Inflammation is also frequently associated with hippocampal neurogenesis that causes deficits in cognition.⁵³ The cognitive deficits associated with Alzheimer's diseases were ameliorated by stimulation of cell proliferation and survival and integration of newborn cells into neuronal network of the SGZ–GCL of the hippocampus.⁵⁴ The decrease of hippocampal neurogenesis is associated with behavioral deficits and developed with age, in parallel with the development of Alzheimer's diseases.^{55,56} Neural stem cells in the hippocampus are located in the SGZ at the border between the GCL and hilus of the DG.⁵⁷ Some of the daughter cells produced by division of those precursor cells differentiate into neurons and develop the prominent apical dendrite that characterizes dentate granule neurons as they move into the GCL.⁵⁸ Adult-newborn neurons (DCX-positive cells) project axons to the primary target of dentate granule neurons and the stratum lucidum of CA3 area, and then integrated into the hippocampal circuitry.⁵⁹ In $A\beta_{1-42}$ -infused mice model, a decrease in hippocampal neurogenesis was detected on the SGZ at the border between the GCL and hilus of the DG, whereas PD-MSCs recovered neurogenesis of adult neural progenitor cells. Overall, our data indicated that PD-MSCs could also improve memory function through partially enhanced neurogenesis.

In conclusion, our data not only provide novel evidences of PD-MSCs on inflammation-mediated amyloidogenesis, neurogenesis, and neuroprotection in an $A\beta_{1-42}$ infused mice model, but also our findings strongly support the therapeutic potential and clinical use of PD-MSCs in Alzheimer's diseases.

Materials and Methods

Animals. Male ICR mice (20–25 g) were purchased from Samtako (Seoul, Korea) and were maintained in accordance with the National Institute of Toxicological Research of the Korea Food and Drug Administration guideline for the humane care and use of laboratory animals. All experimental procedures in the present study were approved by the IACUC of Chungbuk National University (approval number: CBNUA-144-1001-01). Animals were housed in a room that was automatically maintained at 21–25°C and relative humidity (45–65%) with a controlled light–dark cycle. All animals had free access to food (Samyang Foods, Seoul, Korea) and water.

Isolation and expansion of human PD-MSCs. Normal human placentas with no evidence of medical, obstetrical, or surgical complications were collected from normal full-term pregnancies (≥ 37 weeks of gestational age). All donors provided written informed consent before placenta collection. The protocols for sample isolation and their subsequent use for research purposes were approved by the institutional review board (IRB) of the Bundang CHA General Hospital, Korea. PD-MSC was harvested from the amniotic membrane of placentas obtained after Cesarean section. We isolated PD-MSCs and characterized them (Supplementary Figure 2) as previously described.⁶⁰ Briefly, the chorioamniotic membrane was peeled and separated from the placenta. Subsequently, the amnion and innermost membrane from the chorion and decidua were removed. These tissues were washed several times in phosphate-buffered saline (PBS, Gibco, Grand Island, NY, USA), and then cells of chorionic plate side scraped from the membrane were removed and the remainder membrane was dissected and minced in a Petri dish. The minced tissue was transferred to a 50 ml centrifuge tube and digested for 30 min at 37°C shaking incubator containing Hank's balanced salt solution (HBSS, Gibco) with a combination of 2 mg/ml trypsin (Sigma, St. Louis MO, USA), 20 μ g/ml DNaseI (Sigma), 1.2 U/ml Dispase (Gibco), and 1 mg/ml Collagenase type IV (Sigma). The harvested cells were cultured in T25 flasks (BD Biosciences, San Jose, CA, USA) in MEM- α GlutaMAX supplemented with 10% fetal bovine serum (Gibco) and 25 ng/ml FGF4 (R&D System, Minneapolis, MN, USA), and 1 μ g/ml heparin (Sigma) at 37°C in an

atmosphere of 5% CO₂ and 3% O₂. When cells reached 70–80% confluence, adherent cells were dissociated with TrypLE (Gibco), washed, and replated at a concentration of 4×10^3 cells/cm².

A β _{1–42}-infused mouse model. The infusion mouse model was adapted from previous work on the rat infusion model (Nitta *et al.*²⁶). The anesthetized animals were placed in a stereotaxic instrument and catheters were attached to an osmotic mini-pump (Alzet 1002, ALZA, Mountain View, CA, USA) and brain infusion kit 1 (Alzet kit 3–5 mm, ALZA) that were implanted according to the following coordinates: mouse (unilaterally): – 1.0 mm anterior/posterior, + 0.5 mm medial/lateral, and – 2.5 mm dorsal/ventral. The pump contents were released over a period of 14 days consisting of 300 pmol aggregated A β _{1–42} (Bachem Chemical, Torrance, CA, USA) dissolved in sterile saline (0.9% NaCl) for each pump.

Water maze test. The learning and memory capacity was assessed using two separate tests (water maze and passive avoidance test). The water maze test is a widely accepted method for testing memory. The examination was performed using the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height 35 cm, diameter 100 cm) was filled with water (containing milk) kept at 22–25°C. An escape platform (height 14.5 cm, diameter 4.5 cm) was positioned submerged 0.5–1 cm below the surface of the water. The test was performed three times per day for 7 days. Each trial lasted for 60 s or ended as soon as the mouse reached the submerged platform and was allowed to remain on the platform for 2 s. The mice were allowed to swim until they sought the escape platform. Escape latency, escape distance, swimming speed, and swimming pattern of each mouse were monitored by a camera above the center of the pool connected to a SMART-LD program (Panlab). A quiet environment, consistent lighting, constant water temperature, and fixed spatial frame were maintained throughout the period of the experiment.

Probe test. A probe test to assess memory consolidation was performed 2 days after the 7-day acquisition tests. In this test, the platform was removed from the tank, and the mice were allowed to swim freely. For these tests, the spent time on the target quadrant within 60 s was recorded. The time spent in the target quadrant was taken to indicate the degree of memory consolidation that had taken place after learning. The swimming pattern of each mouse was monitored by a camera above the center of the pool connected to a SMART-LD program as described above.

Passive avoidance performance test. The passive avoidance test is also widely accepted as a simple and rapid method for testing memory. The passive avoidance response was determined using a 'step-through' apparatus (Med Associates, Georgia, VT, USA) that consists of an illuminated and a dark compartment (each 20.3 × 15.9 × 21.3 cm) adjoining each other through a small gate with a grid floor of 3.175 mm stainless steel rods set 8 mm apart. At 1 day after the probe test, the training trial was performed. The mouse was placed in the illuminated compartment facing away from the dark compartment. When the mouse moved completely into the dark compartment, it received an electric shock (1 mA, 3 s duration). Then the mouse was returned to its home cage. After 1 day, the mouse was placed in the illuminated compartment and the latency period to enter the dark compartment, defined as 'retention,' was measured. The time when the mouse entered the dark compartment was recorded and described as step-through latency. The retention trials were set at a limit of 180 s as cutoff time.

Brain tissue collection and preservation. After behavioral tests, animals were perfused with PBS under inhaled diethyl ether anesthetization. The brains were immediately removed from skull, and the cortex and hippocampus were dissected on ice. All the brain tissues were immediately stored at – 80°C until biochemical assays were conducted.

Western blot analysis. Cells and each area of the brain tissue were homogenized and lysed by a 30 min incubation on ice. The lysates were centrifuged at $15\,000 \times g$ for 15 min. An equal amount of total protein (20 μ g) isolated from brain tissues was resolved on a sodium dodecyl sulfate (SDS) 10 or 12% polyacrylamide gel and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were blocked for 1 h at room temperature with 5% (w/v) nonfat dried milk in Tris-buffered saline Tween-20 (TBST: 10 mM Tris (pH 8.0) and a 150 mM NaCl

solution containing 0.05% Tween-20). After a short wash in TBST, membranes were incubated at 4°C with specific antibodies. The blot was then incubated with the HRP-conjugated anti-mouse or anti-rabbit antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive proteins were detected with the enhanced chemiluminescence (ECL) western blotting detection system.

Immunohistochemistry and immunofluorescence. Mice were anesthetized with ether. While under general anesthesia, the mice received intracardiac perfusion with 50 ml of saline. Brains were fixed in formalin and paraffin-enclosed for examination. Tissue sections, 5 μ m thick, were used with immunohistochemistry (also used in immunofluorescence). Paraffin-embedded sections were deparaffinized and rehydrated, washed in distilled water, and then subjected to heat-mediated antigen retrieval treatment. Endogenous peroxidase activity was quenched by incubation in 1% hydrogen peroxide in methanol for 30 min and then cleared in PBS for 5 min. The sections were blocked for 30 min with 3% normal horse/goat serum diluted in PBS. These sections were incubated overnight with appropriate antibodies. After washing in PBS, the sections were incubated in biotinylated goat anti-mouse/rabbit IgG antibody (1:1000 dilution, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were subsequently washed and incubated with avidin-conjugated peroxidase complex (ABC kit, 1:200 dilution, Vector Laboratories) for 30 min followed by PBS washing. The peroxidase reaction was performed in PBS using 3, 3'-diaminobenzidine tetrahydrochloride (DAB, 0.02%) as the chromogen. Finally, sections were dehydrated in ethanol, cleared in xylene, and mounted with Permount (Fisher Scientific, Waltham, MA, USA), and evaluated on a light microscopy (Olympus, Tokyo, Japan). For immunofluorescence, sections were incubated with an anti-rabbit secondary antibody labeled with Alexa-Fluor 488 (1:400 dilution; Invitrogen, Carlsbad, CA, USA) or anti-mouse secondary antibody labeled with Alexa-Fluor 568 (1:400 dilution, Invitrogen) for 2 h at room temperature. Final images were acquired using a confocal laser scanning microscope (TCS SP2, Leica Microsystems AG, Wetzlar, Germany).

Measurement of A β _{1–42}. Lysates of brain tissue were obtained through protein extraction buffer containing protease inhibitor. A β _{1–42} level was determined using each specific enzyme-linked immunosorbent assay (ELISA) Kit (Immuno-Biological Laboratories Co., Ltd, Takasaki-Shi, Gunma, Japan). In brief, 100 μ l of sample was added into the precoated plate and was incubated overnight at 4°C. After washing each well of the precoated plate with washing buffer, 100 μ l of labeled antibody solution was added and the mixture was incubated for 1 h at 4°C in the dark. After washing, chromogen was added and the mixture was incubated for 30 min at room temperature in the dark. Finally, the resulting color was assayed at 450 nm using a microplate absorbance reader (Sunrise™, TECAN, Switzerland) after adding stop solution.

TUNEL assay. DNA fragmentation was examined by terminal deoxynucleotidyl transferase-mediated FITC-dUDP nick-end labeling (TUNEL). TUNEL assays were performed using the *in situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after fixation of 25- μ m cryosections with 4% paraformaldehyde, treatment with 0.1% NaBH₄ and 0.1 Triton X-100, the slides were incubated for at least 1 h with a reaction mix containing deoxynucleotidyl transferase and FITC-dUDP (Roche, Reinach, Switzerland). For 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) staining, slides were incubated for 15 min at room temperature in the dark with mounting medium for fluorescence containing DAPI (Vector Laboratories). The tissues were then observed through a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) and the nuclei were visualized by the DAPI staining.

Proteome profiler arrays. The brain tissues were excised and homogenized in PBS with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and Triton X-100 (final concentration 1%). The samples were frozen at – 70°C, thawed, and centrifuged at $10\,000 \times g$ for 5 min to remove any cellular debris. Proteins (200 μ g) collected from three samples per group were used for mouse cytokine array according to the protocol provided by supplier (Proteome Profiler, ARY006, R&D Systems).

Statistical analysis. The data were analyzed using the GraphPad Prism version 4 program (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as mean \pm S.D. Statistical significance was performed on the data

using one-way analysis of variance (ANOVA) or unpaired Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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