Long-term immunomodulatory effect of amniotic stem cells in an Alzheimer’s disease model

Kyung-Sul Kim a,b,1, Hyun Sook Kim c,1, Ji-Min Park b, Han Wool Kim b, Mi-kyung Park b, Hyun-Seob Lee b, Dae Seog Lim a, Tae Hee Lee d, Michael Chopp e, Jisook Moon a,b,1

a Department of Bioengineering, College of Life Science, CHA University, Seoul, Korea
b General Research Institute, Gangnam CHA General Hospital, Seoul, Korea
c Department of Neurology, CHA Bundang Medical Center, CHA University, Gyeonggi, Korea
d Formulae Pharmacology Dept. School of Oriental Medicine, Gachon University, Gyeonggi, Korea
e Department of Neurology, Henry Ford Hospital, Detroit, MI, USA

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ABSTRACT
Amyloid beta (Aβ) plays a major role in Alzheimer’s disease (AD), and neuroinflammatory processes mediated by Aβ plaque-induced microglial cells and astrocytes contribute to AD pathogenesis. The present study examined human placenta amniotic membrane-derived mesenchymal stem cells (AMSCs), which have potent immunomodulatory and paracrine effects in a Tg2576 (APPswe) transgenic mouse model of AD. AMSCs secreted high levels of transforming growth factor-β and, under in vitro inflammatory environment conditions. Six weeks after the intravenous injection of AMSCs, APPswe mice showed evidence of improved spatial learning, which significantly correlated with the observation of fewer Aβ plaques in brain. The number of ED1-positive phagocytic microglial cells associated with Aβ plaques was higher in AMSC-injected mice than in phosphate-buffered saline-injected mice, and the level of Aβ-degrading enzymes (matrix metalloproteinase-9 and insulin-degrading enzyme) was also significantly higher. Furthermore, the level of pro-inflammatory cytokines, interleukin-1 and tumor necrosis factor-α, was lower and that of anti-inflammatory cytokines, interleukin-10 and transforming growth factor-β, was higher in AMSC-injected mice than phosphate-buffered saline-injected mice. These effects lasted until 12 weeks after AMSC injection. Taken together, these results collectively suggest that injection of AMSCs might show significant long-lasting improvement in AD pathology and memory function via immunomodulatory and paracrine mechanisms.

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1. Introduction
Alzheimer’s disease (AD) is the most common form of dementia; however, practical treatment of AD is limited to symptomatic approaches. The β-amyloid (Aβ) peptide is considered to be the root cause of AD; however, the neuroinflammatory process mediated by Aβ plaque-induced microglial cells and astrocytes also contributes to AD pathogenesis (Salloway et al., 2008).

The main functions of microglial cells are to remove damaged tissues, to promote neuronal cell survival and, in the mature brain, to facilitate repair by guiding the migration of neuroprogenitor cells to sites of inflammation and injury (Ekdahl et al., 2009); however, chronically-activated microglia showing evidence of overactivation and dysregulation are thought to be active contributors to neuronal damage because of excessive production of cytotoxic factors such as superoxide, nitric oxide (NO), and tumor necrosis factor (TNF)-α (Heneka and O’Banion, 2007).

Recent studies in a mouse model of AD suggest that alternatively-activated microglia (not chronically-activated microglia) might provide a positive feed-forward loop of decreasing pro-inflammatory reactions and increasing phagocytosis of Aβ fibrils, which results in cognitive improvement (Lee et al., 2012; Nikolic et al., 2008; Ohtaki et al., 2008; Simard et al., 2006). Activated microglial cells cluster around aggregated Aβ plaques and penetrate the neuritic plaques, either by infiltration of the peripheral macrophages into the brain (Simard et al., 2006) or by transformation of brain-resident microglia (Parese et al., 1997; Simard et al., 2006). Prostaglandin E2 receptor subtype 2-null bone marrow (BM)-derived microglia, which influence microglial phagocytosis and the production of neurotoxic cytokines, showed a reduced Aβ burden in an APPswe-PS1ΔE9 double-transgenic
AD mouse model (Keene et al., 2010). Also, microglia produce a growth factor such as insulin-like growth factor 1 and brain-derived neurotrophic factor and show enhanced uptake of fibrillar Aβ peptides in the presence of the anti-inflammatory cytokine, interleukin (IL)-4 (Butovsky et al., 2006; Koenigsknecht-Talboo and Landreth, 2005). An important role played by microglial cells is adhesion to, and phagocytosis of, fibrillar Aβ or aggregated amyloid via multiple pattern recognition receptors, including Toll-like receptor 2, scavenger receptor class A and scavenger receptor class B, CD36, macrophage receptor with collagenous domain, and receptor for advanced glycosylation end products (Block et al., 2007). However, the role of microglial cells in the pathogenesis of AD remains undefined.

Microglial activation starts before the development of AD symptoms, and the degree of activation increases as the disease progresses (Henecka and O’Banion, 2007; Nunomura et al., 2001). Microglia activated by Aβ produce cytokines and neurotoxins that promote neurodegeneration (Coraci et al., 2002); therefore, in an attempt to ameliorate the amyloid burden and/or manipulate the neuroinflammatory cascade, research has focused on the effects of mesenchymal stem cells (MSCs) on AD pathology (Lee et al., 2012).

Amniotic MSCs (AMSCs) derived from human term placenta have enormous potential for use in stem cell therapy; there are no ethical concerns, they are immune privileged, easily accessible and abundant, and have potential for neurogenic differentiation (Chang et al., 2010; Portmann-Lanz et al., 2010; Sakuragawa et al., 2004). Although transplanted BM-MSCs show immunomodulatory effects and cognitive benefits in an AD mouse model (Lee et al., 2009a), BM harvesting is a painful procedure and the cells show limited survival after injection into the brain (Bergwerf et al., 2011). More importantly, AMSCs exert more potent immunomodulatory and paracrine effects than BM-MSCs (Chang et al., 2006). Unlike BM-MSCs, human AMSCs express human leukocyte antigen (HLA)-A, HLA-B, HLA-C (major histocompatibility complex-I), but not HLA-DR (major histocompatibility complex-II) and HLA-G, on the cell surface (Chang et al., 2010; Portmann-Lanz et al., 2010); thus, placenta-derived cells are immune privileged and are less likely to be rejected after transplantation (Ilancheran et al., 2007).

Moreover, human AMSCs have the potential to modulate T-cell proliferation, at least in vitro (Magatti et al., 2008). They might also inhibit T-cell proliferation and show dose-dependent inhibition of peripheral blood mononuclear cell-mediated immune responses (Chang et al., 2010; Wolbank et al., 2007). Mechanistically, suppression of lymphocyte activity by placental cells results from decreased cell proliferation and an increase in the number of regulatory T cells. Immunomodulation is thought to be mediated via IL-10 and transforming growth factor (TGF)-β (Chang et al., 2006). Also, recent studies showed that placental trophoblasts and placental sources of mesenchymal stem cells express the tolerance signaling molecule, CD200 (Clark et al., 2003; Witkowska-Zimny and Wrobel, 2011). This might have important therapeutic implications for the survival of AMSCs transplanted across HLA barriers and for modulation of host immune-related inflammatory responses. The aim of the present study was to examine the beneficial effects of AMSCs derived from human term placenta in an APPswe transgenic mouse model of AD.

2. Methods

2.1. Animals

A transgenic mouse model of AD was used to evaluate the effects of AMSC transplantation. APPswe (Tg2576) mice (Hsiao et al., 1996) were originally obtained from Taconic Laboratory (Germantown, NY, USA). Adult female APPswe mice (15-16 months old; 20 g; n = 8 per group) were used for the behavioral experiments and for pathological analysis at 12 weeks after transplantation. Also, adult male APPswe mice (12-13 months old; 20 g; n = 8 per group) were used for additional pathological analysis at 1 week after transplantation. Wild type littermate mice (normal group, n = 10) were compared with the APPswe groups. To evaluate the immunomodulatory effects of AMSCs on AD pathology, 3xTg-AD mice (6-7 months old; 3 female mice at each time point) purchased from Jackson Laboratory (Bar Harbor, ME, USA) were used to save breeding time (Clifton et al., 2007). All experimental animals were housed in specific pathogen-free conditions (CHA Laboratory Animal Research Center) and handled in accordance with an animal protocol approved by the CHA University Institutional Animal Care and Use Committee.

2.2. Preparation and injection of AMSCs

Normal human placenta (>37 gestational weeks) showing no evidence of medical, obstetrical, or surgical complications were obtained after Cesarean section. All donors provided written, informed consent. Sample collection and use for research purposes were approved by the Institutional Review Board (IRB) of CHA General Hospital, Seoul, Korea. Each placenta was carefully dissected and the tissue washed several times in phosphate-buffered saline (PBS), and then mechanically minced and digested with 0.5% collagenase IV (Sigma, St. Louis, MO, USA) for 30 minutes at 37 °C. Harvested cells were cultured in a T-25 flask (Nunc, Rochester, NY, USA) in alpha-MEM supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 μg/mL each penicillin and streptomycin (Gibco), 25 ng/mL FGF4 (R&D System, Minneapolis, MN, USA), and 1 μg/mL heparin (Sigma) (Han et al., 2008).

Cells were cultured in dishes coated with 15 g/mL polyornithine (Sigma) and 4 μg/mL fibronectin (Sigma) at a density of 1 × 10^4 cells/cm². Complete medium containing 25 ng/mL FGF4 and 1 μg/mL heparin was then added, and the cells incubated for 6 days at 37 °C in an atmosphere of 3% CO₂. For intravenous injection, 200 μL of cell suspension (approximately 2 × 10^6 cells) were injected into the tail vein (AMSC-injected group). For the control group, 200 μL of PBS was injected into the tail vein (PBS-injected group).

2.3. In vivo fluorescence imaging study

Fluorescence images were obtained using a FOBI Imaging System (Neoscience, Kyunggi-do, Korea) for data acquisition. Before imaging, AMSCs were labeled with 20 nM Vybrant Cell Labeling DiD solution (Molecular Probes, Paisley, UK) following the manufacturer’s instruction and injected through the tail vein (n = 2). Images of organs were acquired 7 days after cell injection. Identical illumination settings were used for all image acquisitions.

2.4. Microglial cells

The BV2 mouse microglial cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine, and penicillin-streptomycin (Gibco) at 37 °C in an incubator with 5% CO₂. Synthetic Aβ_{25–35} (Sigma) was dissolved in sterile, distilled water at a concentration of 0.4 μM and were used. AMSCs (2 × 10^6 cells per well) were seeded in the transwell inserts and BV2 cells (2 × 10^5 cells per well) were
plated in the 6-well dishes and allowed to attach overnight. The plates were left to incubate for 24 hours before Aβ25–35 treatment, and BV2 cells were treated for 24 hours with 50 μM amyloid peptides.

2.5. Nitrite quantification

Nitrite (NO2\textsuperscript{−}) in culture supernatants was measured to assess NO production in BV2 cells after Aβ25–35 treatment. Fifty microliters of sample aliquots were mixed with 50 μL of Griess reagent (Promega, Madison, WI, USA) in a 96-well plate and incubated at 25 °C for 10 minutes. The absorbance at 550 nm was measured using a microplate reader. NaNO\textsubscript{2} was used as the standard to calculate NO2\textsuperscript{−} concentrations.

2.6. Behavioral evaluation

Exploration-based tasks such as the elevated plus-maze (Ouagazzal et al., 1999), the light/dark transition test (Crawley and Goodwin, 1980), and the locomotor and open field tests (Bolivar et al., 2000) were used to evaluate basal locomotor and anxiety-like behaviors, with some modifications (see later in text). Finally, cognitive changes were measured using the water maze test (WMT) (Vorhees and Williams, 2006). All behavioral tests were performed 6 weeks after AMSC transplantation.

2.6.1. WMT

A tank (1.1 m in diameter) containing a platform (10 cm in diameter) was used for this test. Mice were subjected to 36 trials over the initial 5 consecutive days. Trial blocks, 1, 2, 3, and 4 comprised 8 trials and the 5th block constituted 4 trials. On Day 6, a single probe trial was included in 1 of the trial blocks. On Day 7, all mice performed 6 cued learning tests, which served as a sensorimotor index for each mouse (Ha et al., 2010).

2.6.2. Locomotion test

Assessment took place in a 40-cm square. Two areas were differentiated within the open field: the periphery and the central area. Mice were placed in the center of the open field and allowed to freely move for 60 minutes while being tracked by an automated tracking system.

2.6.3. Open field test

Assessment took place in a 40-cm square with a black background and an inner circular platform 20 cm in diameter, which was illuminated by a lamp. Mice were placed in the center of the open field arena and allowed to freely move for 60 minutes while being tracked by a video-recording system.

2.6.4. Light/dark transition test

A cage (21 x 42 x 25 cm) was divided into 2 sections of equal size by a partition containing a door. One chamber was brightly illuminated by white diodes (390 lux), and the other chamber was dark (2 lux). Mice were allowed to move freely between the 2 chambers for 5 minutes. The total numbers of transitions, the time spent in each chamber, and the latency to enter the light chamber were recorded.

2.6.5. Elevated plus maze

The maze comprised 4 arms (45 cm in length x 10 cm in width) in the form of a “+”: 2 arms were open arms and 2 arms (of the same size) were enclosed by roof and walls. The maze was set up in the test room 50 cm above the floor. For each trial, mice were placed on the central platform facing an open arm and allowed to move freely for 5 minutes. The total number of transitions, the time spent in the each chamber, and the latency to enter the light chamber were recorded.

2.7. Video monitoring

Behavior was assessed using the WMT, locomotion test, open field test, and elevated plus maze as described and recorded using a charge-coupled device camera connected to a video monitor and a computer. The tests were conducted using a video-tracking system (Ethovision; Noldus, Wageningen, Netherlands).

2.8. Tissue preparation and immunohistological analysis

2.8.1. Tissue preparation and thioflavin S staining

ARDS plaque-free brain areas were selected for morphological analysis of activated microglial cells around Aβ plaques made it impossible to accurately assess the morphology of a single microglia. Immunofluorescence staining for microglial cells (Iba-1; Wako, Osaka, Japan) and CD68 (ED1; Serotec, Washington, DC, USA) was performed by incubating sections with antibodies to Iba-1 (1:500) and ED1 (1:500) overnight followed by incubation with Alexa Fluor 488-, or 594-conjugated secondary antibodies for 1 hour at room temperature. Double immunofluorescence staining of microglia, CD68, and astrocytes was performed using an antibody to human nuclei (HuNu) (1:100; Millipore, Billerica, MA, USA). Normal goat serum (isotype control), or PBS (0.1 M, pH 7.4) were used instead of primary antibody for the negative controls. HuNu staining was performed using the horseradish peroxidase (HRP)-diaminobenzidine (DAB) method. Images were acquired using a microscope fitted with a digital camera system (Nikon) and a confocal microscope (Zeiss, Oberkochen, Germany), and routed to a Windows PC for quantitative analyses using Adobe Photoshop CS5 software.

Aβ plaques were classified according to their size, and counted. Large (>100 μm in diameter), middle-sized (50–100 μm in diameter), and small (<50 μm in diameter) plaques were detected and counted in the cortex and hippocampus using confocal microscopy.

2.9. In vitro analysis of cytokine levels after stimulation with interferon (IFN)-γ and TNF-α

2.9.1. In vitro stimulation of AMSCs

AMSCs were cultured in 24-well plates in 1 mL Dulbecco’s modified Eagle’s medium medium (2 x 10^5 cells per mL) and stimulated with TNF-α (10 ng/mL; R&D Systems) and interferon-γ (100 ng/mL; Peprotech) for 6 hours. The cytokine concentrations and reaction time were carefully optimized (data not shown). Cells were harvested after 24 hours and the supernatants collected and
assayed using commercial enzyme-linked immunosorbent assays (ELISAs) for TGF-β (R&D Systems).

2.9.2. Cytokine measurement

The concentration of cytokines in the AMSC or culture supernatants was measured at 24 hours after stimulation using standard sandwich ELISAs for TGF-β and matrix metalloproteinase (MMP)-9 (R&D Systems) according to the manufacturer’s instructions. Cytokine concentrations were calculated by comparison with known standards. All measurements were made in triplicate and the results presented as the mean ± standard error of the mean.

2.10. RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted from the cells (AMSC and BV2 cells) and right hemispheres using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. We converted 1 μg of purified total RNA from samples into cDNA by using SuperScript III (Invitrogen) and stored all cDNA samples at −80°C. Real-time polymerase chain reaction (PCR) was performed using the SYBR-Green reaction kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions in a LightCycler (Roche), and the relative expression of a housekeeping gene (GAPDH) and the genes for TGF-β, IL-10, TNF-α, IFN-γ, IL-1, insulin-degrading enzyme (IDE), and MMP-9 were determined. The primers used for real-time PCR are listed in Supplementary Table 1. The values for target gene expression were normalized against GAPDH. We assigned an expression value of control in the data set as standard and calculate relative expression levels for all other samples.

2.11. Immunophenotyping

2.11.1. Immunophenotyping of AMSCs

For the phenotypic analysis, AMSCs were stained in Fluorescence Activated Cell Sorting (FACS) buffer for 20 minutes at 4°C with the appropriate concentrations of the following antibodies: fluorescein isothiocyanate (FITC)-labeled anti- SSEA4, TRA-1-81, CD34, and phycoerythrin (PE)-labeled anti- TRA-1-60, CD90, CD9, and CD13 (BD Pharmingen, San Diego, CA, USA); PE-labeled CD200 (R&D Systems). After washing the cells, they were analyzed using a FACS Caliber flow cytometer (BD) and Cell Quest software (version 4.0.2).

2.11.2. Isolation and immunophenotyping of lymphocytes

Mouse lymphocytes were isolated from spleens as described by Zhao et al. (2007). Briefly, single-cell suspensions of the spleen were obtained by disruption followed by ficoll-Conray density gradient centrifugation to remove dead cells and red blood cells. Mouse lymphocytes were stained in the FACS buffer for 30 minutes at 4°C with FITC-labeled anti-CD4 and PE-labeled anti-CD25. Relative immunofluorescence of cells was analyzed using a FACS Calibur flow cytometer (BD).

2.12. Statistical analysis

Statistical analyses were conducted using a CHA University mainframe computer using the Statistical Analysis System (SAS, version Enterprise 4.0; SAS Korea, Inc, Seoul, Korea). Basic motor function analysis, data of image intensity, and real-time PCR data were analyzed using either a t-test or 2-way variance analysis followed by Fisher least significant difference post hoc tests. All other measures were analyzed using a mixed model analysis of variance procedure (SAS, PROC MIXED) to account for the random effect of mice. The correlation between the WMT and plaque counting was analyzed using the Pearson correlation coefficient. Data are presented as the mean ± standard error of the mean and a p value of < 0.05 was considered significant.

3. Results

3.1. Placental stem cells

Fig. 1A shows the morphology of AMSCs harvested at passage 4. Flow cytometry analysis of the immunophenotypic surface profile of the AMSCs showed that they were negative for CD34 (a hematopoietic and endothelial cell marker), SSEA4, TRA-1-81, and TRA-1-81 (embryonic stem cell markers), and positive for CD9 (a nontrophoblast marker), and CD13 and CD90 (MSC markers).

Fig. 1. Placental stem cells. Morphology of amniotic mesenchymal stem cells (AMSCs) obtained from passage 4 (A). Scale bar, 20 μm. Fluorescence Activated Cell Sorting (FACS) analysis of the immunophenotypic surface profile of AMSCs. AMSCs were negative for CD34 (hematopoietic and endothelial cell markers) and SSEA-4, TRA-1-81 (embryonic stem cell markers), and positive for CD9 (nontrophoblast marker) and CD13 and CD90 (mesenchymal stem cell markers). Moreover, AMSCs were positive for a cell surface antigen, CD200 which is a membrane glycoprotein that suppresses immune activity (B).
Moreover, AMSCs were positive for the cell surface antigens CD200 (Fig. 1B). The recently identified transmembrane glycoprotein, CD200, has been shown to be an important player in immunoregulation, tolerance via its receptor, CD200R (Holmannova et al., 2012).

3.2. AMSCs reduce activation of microglial cells induced by \(\text{A}\beta_{25-35}\)

To investigate whether AMSCs have an immunomodulatory effect on amyloid-activated microglia, BV2 cells, an immortalized murine microglial cell line was challenged with 50 \(\mu\text{M} \text{A}\beta_{25-35}\) peptide, the neurotoxic domain of the full-length \(\text{A}\beta_{1-42}\) peptide and was cocultured with AMSCs. After stimulation with \(\text{A}\beta_{25-35}\) for 24 hours, activated microglia showed a densely packed cell clumps, whereas microglia in coculture with AMSCs did not cause clumping (Fig. 2A). In addition, AMSCs influenced \(\text{A}\beta_{25-35}\) mediated NO production in microglia. Normal microglia produced a very little amount of nitrite (2.7 ± 0.1 \(\mu\text{M}\)). After stimulation of \(\text{A}\beta_{25-35}\) for 24 hours, NO production was dramatically increased (21.4 ± 1.2 \(\mu\text{M}\); \(p < 0.001\)). In contrast, coculture with AMSCs exhibited a significant decrease in nitrite accumulation (13.4 ± 2.2 \(\mu\text{M}\)) (Fig. 2B). Next, we investigated the influence of AMSCs on the messenger RNA (mRNA) expression of TNF-\(\alpha\) and IL-1 in activated microglia. As shown in Fig. 2C, \(\text{A}\beta_{25-35}\) caused a marked upregulation of TNF-\(\alpha\) and IL-1 in microglia. Compared with vehicle-treated microglia, cocultures with AMSCs significantly inhibited \(\text{A}\beta_{25-35}\)-induced overproduction of TNF-\(\alpha\) and IL-1β.

3.3. Transplantation of AMSCs improves memory function

The WMT was performed 6 weeks after transplantation and measured the mean escape latencies required to reach a hidden platform from each starting point (east, north, northwest, and southeast; Fig. 3A). There was no statistically significant interaction between the groups at any of the starting points (\(F(6,66) = 0.50; p = 0.80\)); however, there was a significant interaction between the trial block and test groups (\(F(8,88) = 2.73; p = 0.009\); Fig. 3B). In trial block 1, the normal control mice tended to find the hidden platform more quickly than the PBS- and AMSC-injected groups (\(p = 0.06\); Fig. 3B), whereas in trial block 2, the mean escape latencies of the 3 groups were not significantly different (Fig. 3B). The latency in the normal control group was significantly faster in trial block 3 than the PBS-injected and AMSC-injected groups (\(p < 0.0001\) and \(p < 0.0002\), respectively; Fig. 3B). Interestingly, from trial block 3 to trial block 5, the AMSC-injected group showed a dramatic change in mean escape latency, indicating that impaired memory function was reversed by stem cell injection. Moreover, though there was no statistically significant difference between the AMSC-injected group and the normal control mice in trial blocks 4 and 5, there was a significant difference between the

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![Fig. 2. Effect of amniotic mesenchymal stem cells (AMSC) on microglial cells stimulated by amyloid beta (\(\text{A}\beta_{25-35}\)). \(\text{A}\beta_{25-35}\)-activated microglia showed densely packed cell clumps, whereas microglia in coculture with AMSCs did not show massive clumps (A). Significantly decreased nitrite was detected in coculture with AMSCs compared with the control (B). Expression of proinflammatory cytokines, tumor necrosis factor (TNF)-\(\alpha\) and interleukin (IL)-1 was significantly decreased in cocultured microglia compared with the control. Scale bar, 100 \(\mu\text{m}\). *** \(p < 0.001\); ** \(p < 0.01\); * \(p < 0.05\) for each comparison. Data are expressed as mean ± standard error of the mean.](image-url)
AMSC-injected group and the PBS-injected group ($p < 0.06$ for trial block 4 and $p < 0.002$ for trial block 5; Fig. 3B). The results for the normal control mice were also significantly different from those of the PBS-injected group in trial blocks 4 and 5 ($p < 0.002$ and $p < 0.0003$, respectively; Fig. 3B). Representative swimming path at trial block 5 from each group exhibited increased spatial learning acquisition in the AMSC-injected group relative to the PBS-injected group (Fig. 3C).

Next, a probe test was performed on Day 5, 24 hours after the last training trial. The probe trial involved removing the platform and recording the length of time each mouse spent within the zone previously occupied by the platform over a period of 60 seconds. The results were $2.41 \pm 0.35$ seconds for the normal control group, $0.92 \pm 0.31$ seconds for the PBS-injected group, and $2.61 \pm 0.75$ seconds for the AMSC-injected group ($F(2,21) = 4.32; p = 0.03$; Fig. 3D). The time spent in the zone was significantly less for the PBS-injected group than for the normal control ($p < 0.008$) and AMSC-injected groups ($p < 0.02$). In terms of total swimming distance, there was a significant interaction between the trial block and test groups ($F(8,88) = 2.68; p < 0.01$; Supplementary Fig. 2A). There was no difference between the normal control group and the AMSC-injected group in terms of swimming velocity (interaction between trial block and test group; $F(8,88) = 2.40; p < 0.02$) throughout the first 4 trial blocks, but there was a significant difference between the normal control group and the PBS-injected group ($p < 0.05$; Supplementary Fig. 2B).

There was no difference between wild type mice (normal control group), APPswe mice injected with PBS (PBS-injected group), or APPswe mice injected with AMSCs (AMSC-injected group) in terms of locomotion tests, the elevated plus-maze test, light/dark transition tests, or open field tests (Supplementary Fig. 1).

### 3.4. AMSC treatment ameliorates Aβ plaques in the brains of APPswe mice

Every sixth section from brain tissue excised from mice treated with AMSCs for 12 weeks was stained with cresyl violet (Fig. 4A) and thioflavin S (Fig. 4B–G). The number of Aβ plaques in 2 brain areas, the cortex and the hippocampus, was much lower in the AMSC-injected group (8.72 ± 1.22) than in the PBS-injected group (19.84 ± 3.45) (Fig. 4H; $p < 0.02$). Plaques were analyzed according to subcategories based on the size of the individual Aβ plaques. The results showed that the number of small (<50 μm in diameter) and intermediate (50–100 μm in diameter) plaques were significantly lower in the AMSC-injected group than in the PBS-injected group (Fig. 4H; $p < 0.01$ and $p < 0.03$, respectively), whereas the number of large plaques (>100 μm in diameter) was not different between the groups.
This was not the case 1 week after AMSC injection (data not shown). The AMSC-injected group tended to show a positive correlation between mean escape latency in the WMT and the number of amyloid plaques ($r = 0.85$, $p < 0.06$), whereas no such correlation was found in the PBS-injected group ($p = 0.83$; Supplementary Fig. 3).

3.5. AMSC transplantation tends to regulate the recruitment of microglia, and increases the proportion of alternatively-activated, phagocytic microglia

To investigate the mechanism(s) by which the number of Aβ plaques decreased in the AMSC-injected group, the number of resident microglia was counted. At Week 1 postinjection, the number of Iba-1-positive microglia significantly increased in the AMSC-injected group (67.58 cells per mm$^2$) compared with the PBS-injected group (58.36 cells per mm$^2$; $p < 0.02$; Fig. 5A and B), whereas the number of Iba-1-positive microglia was not different from each group at Week 12 postinjection ($p = 0.95$; Fig. 5A and B). Further quantitative image analysis revealed that the density of Iba-1-positive microglia was higher in the AMSC-injected group than in the PBS-injected group at Week 1 postinjection ($p < 0.04$; Supplementary Fig. 3), but lower at Week 12 postinjection ($p < 0.05$; Fig. 5C). These findings suggest that injected AMSCs were able to recruit microglial cells during the initial acute stage after transplantation in the AD mouse model. And after the initial stage AMSCs maintained a lower number of resident microglial cells, despite the proinflammatory environment. This supports an immunosuppressive role for AMSCs.

The density of activated microglia within the Iba-1-positive population that was also positive for ED1 was analyzed. Fig. 6A shows that ED1 negative resting microglia and Fig. 6B shows ED1-positive microglia in the AMSCs-injected group were mainly scattered around the Aβ plaques, and not found in brain areas where there were no Aβ plaques (Supplementary Fig. 4). Next, Aβ plaques and a surrounding area of microglial cells of a similar size were selected for more precise analysis. Most Iba-1-positive

![Figure 4](https://example.com/figure4.png)  
**Fig. 4.** Effect of amniotic mesenchymal stem cell (AMSC) administration on cerebral amyloid beta (Aβ) plaque deposition. Brain histology of the hippocampal and cortical areas, inserts reveal magnified area for analysis shown in (B–G) (A). Mouse frozen brain section from the cortex and hippocampus; low magnification ($\times 40$, B, and C), high magnification ($\times 100$, D, E, F, and G) were stained with thioflavin S (green). Quantitative analysis of Aβ plaques in APPswe mice with phosphate-buffered saline (PBS) injection (Sham-AD) and APPswe mice with AMSC transplantation (AMSCs-AD). There were significantly fewer small (<50 μm), medium-sized (between 50 and 100 μm) Aβ plaques, and total number of Aβ plaques in the AMSCs-AD relative to the Sham-AD in the brain regions examined. Number of large-sized (>100 μm) Aβ plaques was not significantly different between the 2 groups (H). Scale bar, 100 μm. **$p < 0.01$; *$p < 0.05$; for each comparison. Data are expressed as mean ± standard error of the mean. Abbreviation: AD, Alzheimer's disease.
microglial cells in the AMSCs-injected group were ED1-positive, whereas the few Iba-1-positive microglial cells in the PBS-injected group appeared to be co-stained with ED1 (Fig. 6B). Consistent with this result, the ratio of ED1 expression to Iba-1 expression was significantly higher in the AMSCs-injected group than in the PBS-injected group at Week 1 and Week 12 postinjection (p < 0.02 and p < 0.05, respectively; Fig. 6C). These findings also suggested the transplanted AMSCs modulated the phagocytic activity of microglial cells.

Finally, HuNu immunostaining was performed to further define the location of the transplanted AMSCs. No human cells were found in AMSC-injected mouse brains at Week 1 postinjection, but HuNu-positive cells were identified in the spleen (Supplementary Fig. 5A and B) at least until Week 1 postinjection. In addition, an in vivo tracking study was performed using a FOBI Imaging System. DiD-labeled AMSCs were injected through the tail vein and fluoroscence using a primary antibody (Iba-1) was used to label microglia. Consistently, Iba-1 positive area was significantly increased in the AMSCs-injected group compared with the Sham-AD at 1 week after transplantation and the difference is reversed at 12 weeks after transplantation. Scale bar, 20 μm. * p < 0.05 for each comparison. Data are expressed as mean ± standard error of the mean. Abbreviation: AD, Alzheimer’s disease.

The levels of IL-1 and TNF-α, IL-10, and TGF-β, and secrete Aβ-degrading enzymes

The levels of mRNA encoding the proinflammatory cytokines, IL-1 and TNF-α, were lower in the AMSC-injected group than in the normal control group at Week 1 postinjection (p < 0.01 and p < 0.06, respectively). However, the level of mRNA encoding the anti-inflammatory cytokine, IL-10, was higher in the AMSC-injected group than in the normal control group (p < 0.04). The level of TGF-β mRNA was not significantly different between the 2 groups (p < 0.08; Fig. 7A).

By Week 12 postinjection, mRNA levels for IL-10 and TGF-β were significantly higher in the AMSC-injected group than in the normal control group (p < 0.0003 and p < 0.01, respectively), but there was no significant difference in the expression of IL-1 and TNF-α mRNA (Fig. 7B).

The levels of Aβ-degrading enzymes, including IDE and MMP-9, were higher in the AMSC-injected group than in the normal control group at Week 1 postinjection (p < 0.005 and p < 0.06, respectively; Fig. 7C).

3.7. AMSCs express high levels of TGF-β and MMP-9 after stimulation with the proinflammatory cytokines, IFN-γ and TNF-α

To further investigate the immunomodulatory effect of AMSCs, AMSCs were tested with a combination of IFN-γ and TNF-α. The optimized condition of IFN-γ and TNF-α was determined by a preliminary test in that the level of AMSCs mediating suppression of T-cell proliferation was measured (Supplementary Table 2). Coadministration of exogenous IFN-γ and TNF-α stimulated AMSCs in vitro. Quantitative real-time PCR confirmed a significant increase in TGF-β expression after coadministration of IFN-γ and TNF-α (p < 0.001; Fig. 8A). MMP-9 and IDE mRNA levels were significantly higher in AMSCs cultured with proinflammatory cytokines than in AMSCs cultured without (p < 0.0009 and p < 0.05, respectively; Fig. 8A). The level of TGF-β was significantly higher in cell lysates (236.70 pg/mL) and supernatants (155.14 pg/mL) from AMSCs cultured with proinflammatory cytokines than in cell lysates (126.73 pg/mL) and supernatants (117.63 pg/mL) from cells cultured without cytokines (p < 0.03 and p < 0.000006, respectively; Fig. 8B), and the level of MMP-9 was significantly higher in lysates (452.09 pg/mL) and supernatants (261.06 pg/mL) from cells cultured with cytokines than in cell lysates (235.59 pg/mL) and supernatants (95.51 pg/mL) cultured without (p < 0.002 and p < 0.04, respectively). These findings are consistent with an in vivo model, in which AMSCs play a potent immunomodulatory role in AD brains.

4. Discussion

The transgenic mouse model bearing the “Swedish mutation” for human APP (APPSwe, line Tg2576) shows age-related amyloid
plaques with a neuritic pathology (Hsiao et al., 1996; Kawarabayashi et al., 2001), deficits in spatial learning and working memory (Deacon et al., 2008; Hsiao et al., 1996; Ognibene et al., 2005), and intact locomotor activity and anxiety levels (Chapman et al., 1999; Deacon et al., 2008; Ognibene et al., 2005). Consistent with previous findings, the basic locomotor and anxiety-like behaviors observed between normal control, PBS-injected, and AMSC-injected mice in the present study were not statistically different, suggesting that the poor performance of the APPswe mice in the WMT was not because of locomotor disability. The mean escape latencies observed in the in AMSC-injected group improved to the level of that observed in the normal controls, whereas the PBS-injected group continued to show delayed escape latency compared with normal control and AMSC-injected mice. It is interesting to note that the improvement in performance in the AMSC-injected group was observed from the third and fourth trial blocks, indicating that placenta-derived stem cells show delayed, but definite, effects in repeated spatial acquisition trials.

Histological analyses revealed that the number of Aβ plaques was lower in AMSC-treated mice than in PBS-injected mice at Week 12 postinjection. The number of small (<50 μm) and intermediate-sized (50–100 μm) plaques was significantly lower to the level of that observed in the normal controls, whereas the PBS-injected group continued to show delayed escape latency compared with normal control and AMSC-injected mice. It is interesting to note that the improvement in performance in the AMSC-injected group was observed from the third and fourth trial blocks, indicating that placenta-derived stem cells show delayed, but definite, effects in repeated spatial acquisition trials.

Histological analyses revealed that the number of Aβ plaques was lower in AMSC-treated mice than in PBS-injected mice at Week 12 postinjection. The number of small (<50 μm) and intermediate-sized (50–100 μm) plaques was significantly lower
in AMSC-injected mice than that in PBS-injected mice, whereas no difference was observed in the number of large (>100 μm) plaques. Memory function improved significantly as the number of Aβ plaques decreased; a trend not seen in the PBS group. In particular, the difference in the positive slope calculated from the final trial block minus the first trial block, which is an index of functional recovery induced by AMSCs, correlated well (Supplementary Fig. 3) with the reduced number of amyloid plaques observed in the AMSC-injected group. This finding provides further evidence that functional improvements shown by the AMSC-injected group resulted from a reduction in the concentration of soluble Aβ. This is consistent with results from previous studies, which show that a reduction in Aβ levels after stem cell treatment in an AD mouse model is accompanied by an improvement in cognitive function (Lee et al., 2009a, 2012; Scholtzova et al., 2008; Tan et al., 1999).

Microglia can be classified as “resident” or “activated” (Streit and Xue, 2009). In AD, activated microglia play different roles according to the disease stage. Activated microglia with phagocytic activity eliminate all forms of amyloid proteins, particularly during the early phase of AD (Lee et al., 2009a; Nikolic et al., 2008; Parvathy et al., 2009; Simard et al., 2006); however, as AD progresses, the microglia become chronically activated and begin to play a deleterious role within the brain (Hickman et al., 2008; Streit et al., 2009). Therefore, the present study examined the coexpression of Iba-1 (a general microglial marker) and ED1 (expressed by activated phagocytes) on microglial cells (Engelsberg et al., 2004). The numbers of resident and activated microglia with phagocytic activity around visible Aβ plaques increased significantly in the AMSC-injected group compared with those in the PBS-injected group at Week 1 postinjection. Interestingly, the number of

**Fig. 7.** Effect of amniotic mesenchymal stem cell (AMSC) administration on inflammatory cytokines and proteolytic enzyme release. Quantitative mRNA expression level of the inflammatory cytokines and proteolytic enzymes in brain of phosphate-buffered saline (PBS)-injected APPswe mice (Sham-AD) and AMSC-injected APPswe mice (AMSCs-AD) at 1 week after transplantation and 12 weeks after transplantation. There was a significant decrease in RNA expression of proinflammatory cytokines interleukin (IL)-1 and TNF-α, whereas an increase in the anti-inflammatory cytokine, IL-10 in the AMSCs-AD compared with the Sham-AD group at 1 week after transplantation. The increase of the anti-inflammatory cytokine, TGF-β in the AMSCs-AD was not significant (A). After 12 weeks, increased expression of the anti-inflammatory cytokines, IL-10 and TGF-β were observed in the AMSCs-AD compared with Sham-AD (B). Expression of proteolytic enzymes, MMP9 and IDE was significantly increased in the AMSCs-AD compared with the Sham-AD at 1 week after transplantation (C). **p < 0.01; *p < 0.05; #p < 0.06; ##p < 0.08 for each comparison. Data are expressed as mean ± standard error of the mean. Abbreviations: AD, Alzheimer’s disease; IDE, insulin-degrading enzyme; MMP, Matrix metalloproteinase; TGF, transforming growth factor; TNF, tumor necrosis factor.
microglia was the same at Week 12 postinjection, although the microglial density was less in the AMSC-injected group; however, the number of activated microglia with phagocytic activity was still higher in the AMSC-injected group. These results suggest that AMSCs could restore the immune system within the brain by controlling the number of microglia with phagocytic properties and modulate the immune status toward Aβ removal.

BM-derived MSCs play a crucial role in AD by clearing Aβ via phagocytosis (Simard et al., 2006); also, they might enhance the immune response by secreting neuroprotective cytokines (Streit et al., 2009). However, increased levels of proinflammatory cytokines such as TNF-α and IL-1 cause a deterioration in the protective ability of the microglia as AD progresses (Hickman et al., 2008), and therefore the changes of inflammatory cytokine secretion and microglial activation after AMSC injection were investigated. The expression levels of mRNA encoding TNF-α, IL-1, IL-10, and TGF-β were measured in mouse brain tissue. The expression of TNF-α and IL-1 was significantly lower in AMSC-injected mice than in PBS-injected mice 1 week postinjection, whereas the expression of IL-10 and TGF-β (both anti-inflammatory cytokines) was significantly higher in AMSC-injected mice than in PBS-injected mice. The latter was still the case at Week 12 postinjection. In addition, the levels of Aβ-degrading enzymes such as IDE and MMP-9 were significantly higher in AMSC-injected mice than in PBS-injected mice. Taken together, these results suggest that AMSCs exert potent paracrine effects, which promote the production of anti-inflammatory cytokines and degradation of Aβ.

It is arguable whether the short-term survival of injected stem cells is a prerequisite to protect the brain degeneration or recover impaired functions. Surprisingly, our results suggest that despite the short-term survival and limited distribution of AMSCs after the systemic administration, the beneficial effects such as reduction of Aβ formation, stimulation of alternatively activated microglia and an improvement in behavioral functions are long-lasting. These results indicated that AMSCs reverse, at least partially, the impaired brain environment and the beneficial effects last in a long-term fashion. A plausible explanation of long-term effects of injected AMSCs is that although AMSCs survive transiently after administration these effects of AMSCs normalized and/or reconstructed the impaired immune system of the brain. Consequently, the reconstructed immune system restores and maintains the homeostasis in the brain from environmental threats.

The responses of AMSCs to proinflammatory cytokines were evaluated in vitro to support the hypothesis that these cells directly modulate immune reactions. Cells were cultured with TNF-α and IFN-γ to mimic a proinflammatory environment (English et al., 2007; Ryan et al., 2007). Quantitative reverse transcription-PCR showed that AMSCs increased their expression of TGF-β in response to coadministration of TNF-α and IFN-γ; however, they did not increase expression of IL-10. These results are consistent with earlier research showing a neuroprotective role for TGF-β in AD (Wyss-Coray, 2006; Wyss-Coray et al., 2001), and support the hypothesis that TGF-β released by the injected AMSCs in response to proinflammatory factors might modulate microglial activation and regulate brain inflammation in AD.

TGF-β is an immunosuppressive cytokine, which is also involved in immune regulation (Horwitz et al., 1999). TGF-β expression is reported in brain tissue, cerebrospinal fluid, and serum from AD patients (Swardfager et al., 2010). Also, TGF-β mRNA levels are increased in AD brains compared with those in control brains (Wyss-Coray et al., 1997). Although the role of TGF-β in AD is still not fully understood, it might function to organize protective and regenerative responses (Finch et al., 1993). Increased levels of TGF-β mediate the anti-inflammatory effects of BM-MSC observed in an AD model (Nikolic et al., 2008), and are associated with a reduction in the number of plaques and in Aβ levels (Peress and Perillo, 1995). Conversely, decreased levels of TGF-β are associated with increased neuronal death and microgliosis (Briolone et al., 2003). An in vivo study reported that TGF-β causes microglial activation and stimulates the degradation of a synthetic Aβ peptide (Wyss-Coray et al., 2001). Moreover, TGF-β is thought to be associated with other immunosuppressive mechanisms involving regulatory T-cells.

Fig. 8. In vitro response of amniotic mesenchymal stem cells (AMSCs) to inflammatory stimulation. Increased mRNA expression of TGF-β, MMP9, and IDE in AMSCs stimulated with a combination of TNF-α and IFN-γ compared in unstimulated AMSCs (A). Increased TGF-β and MMP9 protein expression measured by enzyme-linked immunosorbent assay in AMSCs stimulated with a combination of TNF-α and IFN-γ compared with unstimulated AMSCs (B). ***p < 0.001; **p < 0.01; *P < 0.05 for each comparison. Data are expressed as mean ± standard error of the mean. Abbreviations: IDE, insulin-degrading enzyme; IFN, interferon; MMP, Matrix metalloproteinase; TGF, transforming growth factor; TNF, tumor necrosis factor.
(Chen and Wahl, 2003). Unlike effector T-cells, regulatory T-cells play a physiologically significant role in the "normal" functioning of the immune system, particularly in terms of immunosuppression (Sakaguchi et al., 2008), as is demonstrated by the fact that AD patients show reduced numbers of regulatory T-cells (Larbi et al., 2009; Saresella et al., 2010). Thus, the data reported in the present study (Supplementary Fig. 6) support the hypothesis that TGF-β released by the injected AMSCs in response to the proinflammatory environment within the AD brain (Kim et al., 2011; Swardfager et al., 2010) might induce microglia and regulatory T-cells to exert a neuroprotective effect; therefore, the immunomodulatory properties of AMSCs make them viable candidates for an efficacious AD treatment modality.

The results reported herein were obtained after intravenous injection of AMSCs, rather than by the intracranial cell transplantation method used in other studies (Lee et al., 2009a, 2012). Therefore, HuNu staining was performed to localize and possibly to relate the observed immunomodulatory effects to the injected cells. No HuNu-positive cells were identified in the brain tissue. However, HuNu-positive cells were observed in the spleen for up to 1 week after intravenous injection, suggesting that the injected cells reside within the peripheral organ rather than the brain tissue, and that paracrine factors and immunomodulatory function of AMSCs were responsible for their observed effects. This paracrine mechanism of action was also reported in a myocardial infarction model treated with MSCs, in which injected MSCs were not detected in the myocardium itself, but in the peripheral tissues; however, they were still associated with increased levels of anti-inflammatory cytokines (Lee et al., 2009b). In addition, AMSCs showed a positive signal for the cell surface antigens CD200. CD200 is a highly conserved member of the immunoglobulin superfamily and is commonly expressed in cells of the myeloid lineage. Recent studies have suggested a role of CD200’s function in attenuating experimental immune responses (Rosenblum et al., 2004). Additionally, CD200–CD200R engagement decreased macrophage-induced cytokine production of IL-13, TNF-α, and IL-17 (Jenmalm et al., 2006). CD200 might induce the proliferation and induction of regulatory T-cells (Gorczyński et al., 2004). These data further support that intravenously injected AMSCs reverse AD pathologic and functional recovery through variable mechanisms of paracrine and immunomodulatory effects.

4.1. Conclusion

Taken together, the results of the present study suggest that AMSCs transplanted into an AD mouse model modulate the properties of microglial cells toward an Aβ plaque-reducing anti-inflammatory response rather than a chronic cytotoxic response. This is supported by the increased numbers of ED1-positive microglia observed around the Aβ plaques in AMSC-injected mice, which correlate with high levels of Aβ-degrading enzymes, reduced levels of proinflammatory cytokines, and increased levels of anti-inflammatory cytokines (TGF-β and IL-10). In addition, injection of AMSCs slowed the progression of Alzheimer’s pathology and improved memory function in the AD mouse model. Another mechanism might involve the direct secretion of TGF-β by AMSCs in response to proinflammatory cytokines. The current data also suggest that TGF-β secreted by AMSCs modulates the activity of microglial cells toward immunosuppressive and neuroprotective effects. As a result, intravenous injection of AMSCs improved spatial learning ability and brain pathology in an AD mouse model by stimulating alternately activated microglia, which suggests that immunomodulation and paracrine effects are the plausible mechanisms of the therapeutic effect of AMSC transplantation on AD.

Disclosure statement

There are no actual or potential conflicts of interest.

All experimental animals were handled in accordance with an animal protocol approved by the CHA University Institutional Animal Care and Use Committee. Normal human placentas were obtained after Cesarean section, and all donors provided written, informed consent. Sample collection and use for research purposes were approved by the Institutional Review Board (IRB) of CHA General Hospital, Seoul, Korea.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2013.03.029.

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