A mesenchymal stem cell line transplantation improves neurological function and angiogenesis in intraventricular amyloid β infusion rats

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Abstract

Mesenchymal stem cell transplantation is demonstrated to improve neurological performance in neurodegenerative diseases including Alzheimer's disease. To understand the underlying mechanism, we infused amyloid β (Aβ) peptide in the lateral ventricle of adult Wister rats using osmotic pump. After 15 days of continuous infusion, a mesenchymal stem cell line (B10) was transplanted in the lateral ventricle. Learning related behavior was evaluated by 2-way shuttle avoidance test. The results showed that Aβ infusion significantly decreased learning related behavior, whereas that was not decreased in B10 transplanted group. Nissl staining results demonstrated that hippocampal pyramidal neurons in CA1 area was decreased in Aβ-infused rats, whereas that was not decreased in B10 transplanted group. Immunohistochemical examination showed that Aβ mainly deposited in the vessels of the brains of Aβ-infused rats, which was decreased by B10 transplantation. Further analysis showed that B10 transplantation increased vessel density, as revealed by immunofluorescence staining. Moreover, the number of astrocyte and microglia was decreased by Aβ infusion, which was returned to the level of sham animals by B10 transplantation. Real time PCR and immunostaining results showed that B10 transplantation significantly increased IL-1β mRNA and protein expression. Thus, our result showed that MSC transplantation effectively decreased Aβ deposition in cerebral vessel and increased angiogenesis, which could be possible cause of improved neurological performance in Aβ-infused rats.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory disturbance and cognitive impairment. The main pathological feature of AD is the presence of extracellular amyloid-β (Aβ) peptide deposits, and intra-neuronal fibrillary tangles composed mainly of phosphorylated tau protein (Lee et al., 2001; Selkoe, 1991). Several studies have suggested Aβ peptide deposition as a causative factor of AD. Aβ is shown to induce neurotoxicity directly, or through neuroinflammation, oxidative stress

and synaptic changes (Lambert et al., 1998; Schubert et al., 1995; Townsend and Pratico, 2005). This peptide is produced from amyloid precursor protein by the enzymatic action of γ- and β-secretase complex. After formation, Aβ peptide is usually clear up from the brain through perivascular pathway. Increased production of Aβ is a cause of its deposition in brain and found in familial AD pathology. Also, impaired clearance of Aβ due to vascular dysfunction could result its deposition in brain. Such mechanism of Aβ deposition is thought to be important especially in late onset sporadic AD (Gupta and Iadecola, 2015; Zekry et al., 2002).

There are several animal models have been developed to explore the pathology of AD, such as APP and APP-processing enzymes transgenic mice with various AD related mutations (Mullan et al., 1992; Sturchler-Pierrat et al., 1997). In these mouse models, Aβ production is increased, resulting deposition of aggregated peptide in hippocampal and cortical areas and neurodegeneration. Additionally, direct infusion of Aβ into the ventricle of rat brains shows deposition of the peptide along with neurodegeneration in the hippocampal and cortical areas, and impairment of the memory similar like AD models (Hashimoto et al., 2002; Hashimoto et al., 2006). The advantage of this model is that this is cost effective and less time consuming than transgenic AD model. In the transgenic models, Aβ deposition is time sensitive and can only be found in relatively older animals. Since, vascular function is altered during aging process; it is difficult to distinguish the role of increased production of the peptide from decreased clearance as a cause deposition in transgenic AD models. But in infusion model, the peptide can be infused in animals of any age, thereby vascular dysfunction as a possible cause of deposition can be eliminated. Hence, in this study we prepared AD animal model by continuous infusion of Aβ peptide into the lateral ventricle of 8-weeks old rats.

Recently stem cell transplantation-based strategy is gaining much interest for the therapy of neurodegenerative diseases because of their ability to replace damaged neurons, neuroprotection, immunomodulation and angiogenesis (Chen et al., 2003; Chen et al., 2004; Sheikh et al., 2011b). Several types of stem cells including embryonic stem cells, neuronal stem cells, induced

pluripotent cells and mesenchymal stem cells (MSCs) are being used. Among them, MSCs are useful because they are easy to culture in vitro, readily available from various source including adult tissues, and for their differentiation and immunomodulatory and neuroprotective properties. Accordingly, numerous studies have demonstrated the effectiveness of the transplantation of MSCs in animal models of central nervous system diseases such as cerebral infarction, spinal cord injury and autoimmune encephalomyelitis (Gharibi et al., 2015; Jung et al., 2009; Wakabayashi et al., 2010b).Transplanted MSCs are expected to differentiate and be integrated in the tissues. However, MSCs also have immunomodulatory functions over lymphocytes, immune cells, endothelial cells, microglia and others by regulating specific antigens and releasing cytokines and trophic factors (Aggarwal and Pittenger, 2005; Lin et al., 2015; Wang et al., 2013), which would be a main mechanism of its therapeutic effects. To investigate the possible effects of MSC transplantation in AD, the cells were transplanted in 6-7 months old transgenic mouse models, and immunomodulation, neo-vascularization and inhibition of neuroinflammation were studied (Garcia et al., 2014; Kim et al., 2013; Lee et al., 2012). Since these animals are older, it might not be possible to understand whether the observed beneficial effects of MSC transplantation are due to their effect on improvement of vascular pathology, or reduction of Aβ mediated neurodegeneration or neuroinflammation. Hence, in this study we investigated the effect of MSC (B10) intraventricular transplantation on 2-months old AD model rats generated by continuous Aβ infusion (Hashimoto et al., 2002; Hashimoto et al., 2008; Nitta et al., 1994).

Previously, we have studied the effects of B10 cells transplantation on cerebral infarction model rats and demonstrated the pathological and molecular mechanism of the improvement. B10 cells showed neuroprotective effects by releasing various kinds of cytokines and neurotrophic factor and affect glial cells to improve pathology by those factors and direct contact (Sheikh et al., 2011b; Wakabayashi et al., 2010b; Wang et al., 2013). Since, neuroprotective function and neuroinflammation play important roles in AD pathology, we hypothesized that B10 might modulate the neuropathological changes related to the disease.

Our results showed that MSC transplantation improved vessel integrity and clear up deposited Aβ from vessel wall.

Materials and methods

Generation and culture of a human MSC line

A human mesenchymal stem cell line (HM3.B10 (B10)) was established by v-*myc* gene transfer into primary human fetal bone marrow cell culture, as described previously (Nagai et al., 2007). Briefly, bone marrow cells were collected from human fetal spinal vertebrae of 15 weeks' gestation, cultured and transfected with a viral vector encoding v-myc. One clone (B10) showed similar morphology, along with expressional profile of neurotrophic factors and differentiation potentials of primary human bone marrow MSCs (Nagai et al., 2007).

Animal model of Alzheimer disease

The experimental protocol is shown in Fig. 1. Adult male Wister rats of 8-weeks old (Charles River, Kyoto, Japan), weighing 250- 300 g were used to prepare AD model. AD rat model was prepared as previously described (Hashimoto et al., 2002). In a brief, 8.5 nmol of $A\beta_{1-42}$ peptide (Peptide Inst., Osaka, Japan) was dissolved in 100 µl of 35% acetonitrile containing 0.1% trifluroacetic acid (pH 2.0), and infused into left lateral ventricle for 14 days at a rate of 0.25 µl/ h for 14 days. For pump insertion, the rats were anesthetized, skull was exposed by midline incision and both lateral ventricles (right and left, relative to bregma; 0.8 mm posterior, 1.4 mm lateral) were pointed out according to the atlas of Paxinos and Watson(Paxinos and Watson, 1986). Left lateral ventricle was drilled and the other point was left for B10 injection. Then, 0.5 µg $AlCl_3$ (in 5 µl, i.c.v., 1 µl/min) was injected initially into the left ventricle of all rats except sham group using Hamilton syringe. The cannula of a mini-osmotic pump (alzet 1002; durect Co., Cupertino, CA, USA) containing $A\beta_{1-42}$ peptide solution was inserted through the hole to the depth of lateral ventricle (3.5 mm) quickly, and the pump was implanted subcutaneously at the back of

the rats (n=10). The rats were placed inside a heating chamber (37^0C ± 0.5^0C) to recover from anesthesia.

B10 cell transplantation

2 weeks after $A\beta_{1-42}$ infusion, another hole was drilled on the right side of the skull according to the atlas of Paxinos and Watson (1986) using a stereotaxic frame (Narishige, Tokyo, Japan). Twenty µl B10 cells (2×10^5 cells) was injected into the right lateral ventricle (n=4) as a single shot by intraventricular injection.

Behavior study

Learning related behavior was assessed for all the rats every week after AD model preparation. This test was continued for 4 weeks till the end of the experiment (figure 1). Two-way shuttle avoidance test apparatus was used to measure the learning capabilities of experimental rats (Hashimoto et al., 2002). 3 sessions were continued for each rat per day having 3 hours gap between each session. Each rat was given 10 minutes exploration period at the beginning of the day. Each session consisted of 10 trials and each trial was 23 seconds long. Conditioned stimulus (CS) and unconditioned stimulus (UCS) were applied in all the trials with 10 seconds inter trial interval. CS was combined stimulus of light (DC 24 V, 36 mA) and sound (80 dB) for 10 seconds whereas UCS was only 3 second electric foot shock (0.5 mA) into the grid floor. CS was followed by UCS. Rats could avoid the shock by changing the compartment during CS and the trial stopped automatically. Rat received electric shock when remain in the same compartment after CS. The greater number of shock avoidance the higher learning capability.

Tissue preparation

4 weeks after $A\beta_{1-42}$ infusion, all the rats were deeply anesthetized with isoflurane and perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH-7.4). The rat

brains (n=14) were removed and post-fixed into the same fixative for 6 hours to overnight. Then the brain samples were cryoprotected with 30% sucrose in 0.1M PBS for 48 hours. The frozen tissues were serially sectioned coronally into tissue blocks of 2 mm thickness, and 10 µm thickness tissue slices were prepared for staining using a cryostat (Leica, Wetzlar, Germany). For real-time PCR the rats were anesthetized, transcardially perfused with normal saline, hippocampus was dissected out from the brain and stored at -70°C until total RNA isolation.

Immunofluorescence staining

Rat brain tissue sections were incubated in blocking reagent containing 0.1% TritonX 100 and 5% goat or horse serum for 30 minutes. The tissue was incubated with anti-Iba1 IgG (rabbit polyclonal, 1:200, Abcam), anti-IL-1β IgG (goat polyclonal, 1:200, SantaCruz) or anti-GFAP IgG (rabbit polyclonal, 1:200, Dako) for overnight at 4^{o}C. Then the tissue was incubated in FITC conjugated anti-goat or Texas red conjugated anti-rabbit IgG at room temperature for 1 h, and immunoreactive proteins were analyzed using a fluorescence microscope. Hoechst staining (10 mg/ml) was used to identify nuclei. Immunoreactive cells were counted in the pyramidal cell layer of hippocampal CA1 area in a blinded manner (x 20 magnification).

Aβ staining was done using a mouse monoclonal anti-Aβ IgG (B4, SantaCruz, 1:200), where the tissue was incubated in IgG for overnight at 4^{o}C. Then, the tissue was incubated with biotin conjugated anti-mouse IgG for 1 h at room temperature. After treating the tissue with avidin biotin complex for 30 min, the Immunoreactive protein was visualized by reaction with DAB (3,3-Di amino Benzidine), and analyzed with a light miscoscope.

Nissl staining

Tissue sections over slides were hydrated with different concentrations of ethanol serially and incubated in 0.1% cresyl violet solution in water for 30 minutes. Excess stain was removed by brief immersion into solutions of 95%

ethanol and 2% acetic acid (1:1), followed by 95% ethanol and chloroform (1:1), and finally into 95% ethanol. Tissue sections were dehydrated in 100% ethanol, cleared alcohol from the tissue using two different xylene successively and mounted in oil based solution (Canada balsam). A light microscope (80IT-RFL-4, Nikon, Tokyo, Japan) was used to count the neuron in pyramidal cell layer of hippocampal CA1 area at 20× magnification.

Lectin staining

Vessels in the brain were visualized by lectin staining. Briefly, the tissue sections were incubated in fluorescin conjugated Solanum tuberosum lectin (Vector, 1:200) at room temperature for 1 h. Nuclei were counterstained with Hoechst, and the tissue was mounted with water based mounting medium (Dako). Then the tissue was examined under a fluorescence microscope.

Cell counting:

After microglia immunostaining over, Photos (20x magnification) were taken for microglia and nucleus (Hoechst) in the pyramidal cell layer of CA1 area at the same plane of all the experimental rats. Three pictures were taken successively in CA1 area of each hippocampus in same tissue. We considered this for two consecutive tissues. Microglia and Hoechst were merged and Image J software was used to count the total number of cells considering the nucleus of the cell in each three fields separately. Then we consider the average cell number per sample.

After Nissl stain, photos (20x magnification) were taken similarly in the pyramidal cell layer of CA1 area. One picture was taken in CA1 area from each hippocampus of same tissue. Image J software was used to count the neuronal cell number separately. Then we consider the average cell number per sample for analysis.

After astrocyte stain, photos (20x magnification) were taken for astrocyte and nucleus (Hoechst) similarly in the same location in pyramidal cell layer. One picture was taken from each hippocampus of same tissue. Astrocyte and

Hoechst were merged and Image J software was used to count the astrocyte cell number separately. Finally, we took the average astrocyte cell number per sample for analysis.

Real-time PCR

The rats were deeply anesthetized with isoflurane and transcardially perfused with normal saline. The hippocampus was dissected out from the brains. Total RNA was isolated from hippocampal tissue using high pure RNA tissue kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Two μg of total RNA was reverse transcribed with reverse transcriptase (ReverTraAce, Toyobo, Osaka, Japan) and oligo dT primers in a 20 μl reaction mixture. To determine the mRNA levels of target genes, real-time PCR was performed with gene specific primers and SyBr Green PCR master mix (Power SyBr Green, Applied Biosystems, Foster City, USA), using a ABI Prism 7900 Sequence Detector system (Applied Biosystems). GAPDH mRNA was used as an internal control and the target gene mRNA level in a sample was determined by means of a relative quantification method; results were expressed as fold induction relative to 1 sample of sham group. The following primers were used for qPCR: BDNF (Forward: 5´-GCCCAACGAAGAAAACCATAAG-3´, Reverse: 5´-AAGAGCAGAGGAGGCTCCAAA-3´), GDNF (Forward: 5´-CAAGCCACCATCAAAAGACTGA-3´, Reverse: 5´-TTGCCGGTTCCTCTCTCTTC-3´), NGF (Forward: 5´-GAAGCCCACTGGACTAAACTTCA-3´, Reverse: 5´-CACACGGGCAGCTATTGGTT-3´), EGF (Forward: 5´-AAAGAGGTGGCATCGTTGGA-3´, Reverse: 5´-CACCATGATCTCAGCCACTAGTG-3´), bFGF (Forward: 5´-CTACAGCTCCAAGCAGAAGAGAGA-3´, Reverse: 5´-GCAGCCGTCCATCTTCCTT-3´), VEGF (Forward: 5´-GAGGAAAGGGAAAGGGTCAAAA-3´, Reverse: 5´-CACAGTGAACGCTCCAGGATT-3´), IL-1β (Forward: 5´-

GCTTTCGACAGTGAGGAGAATGA-3´, Reverse: 5´-TCAAGGGCTTGGAAGCAATC-3´), IL-6 (Forward: 5´-TGGATGCTTCCAAACTGGATATAA-3´, Reverse: 5´-GGTAGAAACGGAACTCCAGAAGAC -3´), IL-10 (Forward: 5´-CAGAGAACCATGGCCCAGAA-3´, Reverse: 5´-9-CAGCTGTATCCAGAGGGTCTTCA -3´), TNFα (Forward: 5´-CCCAGACCCTCACACTCAGATC-3´, Reverse: 5´-TGCTTGGTGGTTTGCTACGA -3´).

Statistical analysis:

All numerical data are expressed as mean ± SD. Statistical analysis to compare mean values was performed using one-way ANOVA, followed by Scheffe's post hoc test, or Student's *t* test. Statistical significance was denoted as $p < 0.05$.

Results

Effects of B10 transplantation on neurological performance and neurodegeneration in AD model rats.

Memory related neurological performance was evaluated by two-way shuttle avoidance test (Hashimoto et al., 2002; Hashimoto et al., 2005). Compared to sham group, the mean of shock avoidance responses in AD model rats was lower starting from 1 week, which became statistically significant at 4 week ($p < 0.05$) (Fig. 2A). Although B10 transplanted AD model rats showed an improvement in shock avoidance, result was not statistically significant compared to AD model rats (Fig. 2A).

Next, the number of neurons in hippocampal CA1 pyramidal cell layer was evaluated by Nissl staining. The results showed that pyramidal neurons in hippocampal CA1 area was decreased AD model rats (Fig. 2B and 2C). On the other hand, B10 transplantation significantly increased neuronal cell number in CA1 area of AD model rats, and the number was similar like sham group (Fig. 2B and 2C).

Deposition of Aβ in AD model rats

The deposition of Aβ in AD model rat brains was evaluated by Aβ immunostaining. The results showed that Aβ was deposited mainly in the vessel wall of AD model rats. Rats of sham group did not show such staining pattern. B10 transplantation decreased Aβ deposition in the vessel wall of AD model rats brains (Fig. 3).

Evaluation of vessel in AD model rats.

Since the main area of Aβ deposition in this model is blood vessels, we sought to investigate further about vessel density in this AD model rat. Staining with STL showed that vessel density was not decreased in AD model rats compared to sham group. However, B10 transplantation significantly increased vessel density in AD model rats, even compared to sham group (Fig. 4B). Further examination revealed that the number small sized vessel was increased in B10 transplanted AD model rat cortex and hippocampus areas (Fig. 4A).

Glial changes in hippocampus with B-10 cell transplantation

Next, we evaluated microglia and astrocytes by immunofluorescence staining of ionized calcium binding adapter molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP) respectively. The number of microglia in the hippocampal CA1 area per field was decreased in AD model rats, which was significantly ($p < 0.05$) increased after B10 transplantation (Fig. 5A and 5B). `Also, the number of astrocyte in hippocampal CA1 pyramidal cell layer per field was decreased in AD model rats, which was significantly ($p < 0.05$) increased in B10 transplanted AD model rats (Fig. 5C and 5D).

Expression levels of growth factors and cytokines

Since glial cells are important source of cytokines and growth factors, we investigated their expression at mRNA level in AD model rats after B10

transplantation. The real time PCR results demonstrated that the mRNA levels of growth factors including EGF and VEGF was decreased in AD model rats compared to Sham group, and transplantation of B10 did not affect their expression (Fig. 6). In the case of cytokines, IL-6 mRNA was decreased in AD model rats, which was returned to the level of Sham group by B10 transplantation. Interestingly, IL-1β mRNA level in AD model rats was similar like Sham group, but significantly increased in B10 transplanted AD model rats (Fig. 6).

Effects of B10 transplantation on IL-1β and VEGF protein levels in AD model rats.

To validate our real time PCR results, we checked the levels of IL-1β and VEGF in AD model rat brains by immunohistochemistry. The result showed that IL-1β was weakly positive in the cell body of hippocampal CA1 area of Sham group, which was not changed in AD model rats (Fig.7A). However, IL-1β level was increased in those cells in B10 transplanted AD model rats (Fig.7A). VEGF was also found to be positive in the cell body of hippocampal CA1 area of Sham group (Fig. 7B). VEGF level was decreased in AD model rats, which was not changed by B10 transplantation (Fig. ‘B).

Discussion

Recent several studies have demonstrated that MSC transplantation improves memory-related behavior in amyloid precursor protein (APP) transgenic mice models and can modulate immune system along with enhancement of Aβ clearance (Garcia et al., 2014; Kim et al., 2013; Shin et al., 2014). In this study, we have found that intra-ventricular infusion of Aβ induces deposition of the peptide in the vessel walls in brain parenchyma. Transplantation of B10 cells inhibited such deposition along with an induction of angiogenesis. Moreover, B10 protected hippocampal neurons and improved memory-related neurological performance. Since vascular dysfunction and consequent Aβ accumulation-induced neurodegeneration is considered as a

cause of AD, improvement of vascular function by B10 transplantation could be the reason for the improvement that we have seen in this AD model rats.

In this AD model rats, Aβ peptide deposition was mainly found around cerebral arteries of cortex and hippocampal areas. To prepare the model, the peptide was infused in the ventricle. CSF is considered to be absorbed through arachnoid villi. However, experimental findings demonstrated that it can also be absorbed through the adventitial layer of cerebral arteries (Carare et al., 2008; Morris et al., 2016). On the other hand, perivascular pathway, which is demonstrated to be important for drainage of interstitial fluid, plays an important role of Aβ clearance (Iliff et al., 2012). Hence, the deposition of Aβ peptide around the blood vessel might occur either during its absorption from CSF or during perivascular clearance. However, we found that Aβ was deposited in the arteries of both superficial and deep cortical areas, as well as in the hippocampus. Consequently, incomplete clearance of Aβ peptide from the interstitial space might have the major role for arterial deposition in this model. We also found decreased cell number in hippocampal pyramidal cell layer. Such findings also suggested that Aβ peptide absorbed from the ventricle, moved through the interstitial space to exert its neurodegenerative effects on pyramidal cell layer, and not just trapped in the vessel wall on its way to interstitial space.

In the AD model rats, B10 transplantation decreased Aβ peptide deposition around vessels, suggesting that the cells might induce a mechanism that increased the peptide clearance processes. Deposited Aβ can be cleared by enzyme-mediated degradation, cells-mediated clearance, and clearance through perivascular pathways. Although we did not check enzyme-mediated Aβ degradation, we found that both astrocytes and microglia number was increased in B10 transplanted AD model rats. Since microglial is known to clear up Aβ from brains (Czirr et al., 2017; Zhang et al., 2018), it is conceivable that transplanted B10 cells were interacted with resident microglia to induce them to clear up deposited the peptide. While not known in this animal model, previously we have found an interaction of microglia and B10 cells in a cerebral ischemia animal model that modulate the inflammatory condition (Narantuya et

al., 2010). Indeed, we have found a few number of transplanted B10 cells still present in brain parenchyma 15 days after transplantation (data not shown). Both microglia and astrocytes are known to produce growth factors that have neuroprotective effects (Bsibsi et al., 2006; Lalancette-Hebert et al., 2007). However, we did not find an increase of growth factors mRNA expression 15 days after transplantation. Nevertheless, their regulation at an earlier time point might be important since the growth factor gene expression is upstream of effects like neuroprotection which is certainly seen in B10 cell transplanted AD model rats.

Another important finding of this study is that B10 transplantation increases small sized vessel number in this model of AD, implicating its effects on the angiogenesis. Angiogenesis is an important process for forming new blood vessels, manifested by extensive interactions between a wide variety of molecules including growth factors, cytokines, adhesion molecules, chemokines, enzymes and activators and inhibitors of angiogenesis (Carmeliet and Jain, 2011). Moreover, angiogenesis is intimately related to neural proliferation and neuroprotection. To investigate about the underlying mechanism of increased neural cell number and angiogenesis, we checked the mRNA expression of various cytokines and growth factors. Most of the growth factors and cytokines we have checked were not changed by B10 transplantation. Only IL-1β and IL-6 mRNA was found to be increased by B10 transplantation. IL-6 is known to have angiogenic and neuroproliferative properties. In a report, it has been demonstrated that its astrocyte-specific expression is beneficial in cerebral injury animal model (Penkowa et al., 2003; Ziebell and Morganti-Kossmann, 2010). IL-1β is an important inducer of IL-6 expression in various cell types, suggesting that its increased expression in B10 transplanted models could have an indirect angiogenic and neuroprotective effect through IL-6. IL-1β is also shown to induce VEGF directly. However, both real time PCR results and immunostaining results showed that VEGF expression was not increased in B10 transplanted group. In our previous study, we found that B10 transplantation transiently increased VEGF expression at an earlier time point than other growth factors in MCAO rat brains, which returned

to basal level afterwards. Since we have checked the level only at 1 time point of 15 days after transplantation, it is possible that B10 could increase VEGF mRNA expression at an earlier time point. Interestingly both VEGF and IL-1β were expressed at protein level in the hippocampal pyramidal cell layers, suggesting there might be a functional relation of IL-1β with VEGF expression.

In our previous study, we have found that B10 transplantation provides neuroprotection along with increased growth factors expression in a MCAO rat model (Wakabayashi et al., 2010a). Moreover, it showed neuroprotection and angiogenesis properties in that model. These properties of B10 are also established in this AD model rats. Since Aβ peptide deposition occurs around blood vessels in this model, angiogenic properties of B10 are important (Sheikh et al., 2011a). Such angiogenesis may induce supply of microglia to Aβ deposited areas in the brain, leads phagocytosis and clearance of Aβ (Lee et al., 2009; Lee et al., 2010; Simard et al., 2006) and may also be involved in clearance of Aβ through blood brain barrier (Zlokovic, 2005). Since Aβ deposition was not seen in the brain parenchyma even in Aβ infusion model without MSC transplantation, it is speculated that upregulated angiogenesis and activation of endothelial cells mainly accelerate the clearance of Aβ without increase of microglial phagocytosis.

In conclusion, we have shown in this study that transplantation of B10 in AD model rats provide neuroprotection, prevent Aβ deposition and improve neurological function probably through angiogenesis. Therefore, angiogenesis related therapy could be a novel strategy for the management of neurodegenerative disease like AD.

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Figure Legends

Figure 1. Experimental design for B10 cell transplantation in AD model rats. AD model was generated by continuous infusion of $A\beta_{1-42}$ peptide into the ventricle using osmotic pumps for 15 days. B10 cells were intraventricularly transplanted 2 weeks after starting $A\beta_{1-42}$ infusion. The rats were sacrificed and pathologically evaluated after 4 weeks. Memory tests were done weekly starting 1 week before Aβ infusion and continued during the experimental period.

Figure 2. Effects of B10 transplantation on neurological performance and neurodegeneration in AD model rats. To evaluate the neurological performance, 2-way shuttle avoidance test was carried out (A). Pyramidal neurons in hippocampal CA1 area were counted in Nissl stained tissue sections at X400 magnification. Representative photomicrographs Nissl stained CA1 neurons are shown in (B), and the cell number in (C). The numerical data are

presented here as average ± SD, and statistical significance is denoted as follows, *p< 0.05.

Figure 3. Effects of B10 transplantation on Aβ deposition in AD model rat brains. To evaluate Aβ deposition, immunohistochemistry was done using anti-Aβ IgG, as described in the Materials and Methods. Representative photomicrographs of CA1 hippocampal area of sham, Aβ-treated AD rats and MSC-treated AD rats are shown here. Scale bar = 50 μm.

Figure 4: Effects of B10 transplantation on vessel density in AD model rats. To visualize vessels, brain tissue sections were stained with FITC conjugated Solanum tuberosum lectin, as described in the Materials and Methods. Representative photomicrographs of cortex (a, b and c) and hippocampal areas (e, f and g) sham (a and e), Aβ-treated (b and f) and MSC-treated (C and g) AD rat brains are shown in (A). The magnified pictures of the boxed areas in c and g are shown in d and h, respectively. (B) The density of the vessel was evaluated using ImageJ, and calculated as % of total area of a microscopic field. The data of vessel density was expressed as average ± SD. Statistical significance was denoted as follows, *p< 0.05.

Figure 5. Effects of B10 transplantation on glial reaction in the CA1 region of AD model rat brains. To evaluate the effects on glial reaction, the brain tissues were immunostained for Iba1 (microglia marker) and GFAP (astrocyte marker). Representative photomicrographs of Iba1 and GFAP immunostaing of CA1 area are shown in (A) and (C), respectively. Hoechst staining was done to identify the nuclei. Immuno-positive cells were counted at X400 magnification. The number of Iba1 positive and GFAP positive cells are shown in (B) and (D), respectively. The data are presented as average ± SD, and statistical significance was denoted as *p< 0.05. Scale bar = 20 μm.

Figure 6. Effects of B10 transplantation on mRNA expression of cytokines and growth factors in the hippocampus of AD model rats. Total RNA was

isolated from the hippocampal tissues of AD model rats, cDNA was prepared, and mRNA levels of growth factors (BDNF, GDNF, NGF, EGF, bFGF and VEGF) and cytokines (IL-1β, IL-6, IL-10 and TNF-α) were quantified by real time PCR. After normalizing with corresponding GAPDH mRNA, the target mRNA level of a sample was calculated relatively using a sample of sham rat as a calibrator. Data presented here as average ± SD. Statistical significance is denoted as follows; *p< 0.05, between indicated two groups.

Figure 7. Effects of B10 transplantation on IL-1β and VEGF level in the hippocampus of AD model rats. To evaluate the level of IL-1β and VEGF in the hippocampal area of AD model rats, immunofluorescence staining was done, as described in the Materials and Methods. IL-1β was visualized with FITC conjugated, and VEGF with Texas red conjugated secondary antibodies. Hoechst staining was done to identify nuclei. Representative photomicrographs of IL-1β and VEGF in the hippocampal CA1 area are shown in (A) and (B), respectively. Scale bar = 100 μm.