15. Ikehara, S. 2008. A novel method of bone marrow transplantation (BMT) for intractable autoimmune diseases. *J. Autoimmun.* **30:**108–115.
16. Ikebukuro, K., Y. Adachi, Y. Suzuki, *et al.* 2006. Synergistic effects of induction in transplantation of allogeneic pancreatic islets. *Bone Marrow Transplant.* **38:** 657–664.
17. Esumi, T., M. Inaba, N. Ichioka, *et al.* 2003. Successful allogeneic leg transplantation in rats by combination of intra-bone marrow (IBM) injection of donor bone marrow cells. *Transplantation* **76:** 1543–1548.
18. Kaneda, H., Y. Adachi, Y. Saiyo, *et al.* 2005. Long-term observation after simultaneous lung and intra-bone marrow-bone marrow transplantation. *J. Heart Lung Transplant.* **24:** 1415–1423.
19. Guo, K., M. Inaba, M. Li, *et al.* 2008. Long-term donor-specific tolerance in rat cardiac allografts by intra-bone marrow injection of donor bone marrow cells. *Transplantation* **85:** 93–101.
20. Ichioka, N., M. Inaba, T. Kushida, *et al.* 2002. Prevention of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells. *Stem Cells* **20:** 542–51.
21. Takada, K., M. Inaba, N. Ichioka, *et al.* 2006. Treatment of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells. *Stem Cells* **24:** 399–405.
22. Ueda, Y., M. Inaba, K. Takada, *et al.* 2007. Induction of senile osteoporosis in normal mice by intra-bone marrow-bone marrow transplantation from osteoporosis-prone mice. *Stem Cells* **25:** 1356–1363.
23. Adachi, Y., H. Oyaizu, S. Taketani, *et al.* 2006. Treatment and transfer of emphysema by a new bone marrow transplantation method from normal mice to Tsk mice and vice versa. *Stem Cells* **24:** 2071–2077.
24. Wang, X., S. Ge, G. McNamara, *et al.* 2003. Albumin expressing hepatocyte-like cells develop in the livers of immune-deficient mice transmitted with highly purified human hematopoietic stem cells. *Blood,* **101:** 4201–4208.
25. Spees, J.L., S.D. Olson, J. Ylostalo, *et al.* 2003. Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma. *Proc. Natl. Acad. Sci. USA* **100:** 2397–2402.
26. Mezey, E., S. Key, G. Vogelsang, *et al.* 2003. Transplanted bone marrow generates new neurons in human brains. *Proc. Natl. Acad. Sci. USA* **100:** 1364–1369.
27. Tomita, M., Y. Adachi, H. Yamada, *et al.* 2002. Bone marrow-derived stem cells can differentiate into retinal cells in injured rat retina. *Stem Cells* **20:** 279–283.
28. Inaba, M., Y. Adachi, H. Hisha, *et al.* 2007. Extensive studies on perfusion metgod plus itra-bone marrow-bone marrow transplantation using cynomolgus monkeys. *Stem Cells* **25:** 2098–2103.
29. Kushida, T., M. Inaba, K. Ikebukuro, *et al.* 2002. Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys of various ages by perfusion or aspiration methods: A preclinical study for human BMT. *Stem Cells* **20:** 155–162.
30. Petrini, M., S. Pacini, L. Trombi, *et al.* 2008. Identification and purificatiopn of mesodermal progenitor cells (MPCs) from human adult bone marrow. *Stem Cells Dev.*, in press.
31. Ikehara, S. 2002. Autoimmune diseases as stem cell disorders: Treatment by allogeneic bone marrow transplantation. In *Molecular Pathology of Autoimmune Diseases.* A.N. Theofilopoulos & C.A. Bona, Eds.: 566–572. Taylor & Francis. USA.
32. Ikehara, S. 2003. A new concept of stem cell disorders and their new therapy. *J. Hematother. Stem Cell Res.* **12:** 643–653.
33. Ikehara, S. 2005. Intra-bone marrow-bone marrow transplantation. *Ann. N.Y. Acad. Sci.* **1051:** 1–9.
34. Fernandes, G., D.R., Alonso, T. Tanaka, *et al.* 1983. Influence of diet on vascular lesions in autoimmune-prone B/W mice. *Proc. Natl. Acad. Sci. USA* **80:** 874–877.
35. Rose, N., M. Afanasyeva. 2003. Autoimmunity: busting the atherosclerotic plaque. *Nat. Med.* **9:** 641–642.
36. Baron, R., I. Harpaz, A. Nemirovsky, *et al.* 2007. Immunity and neuronal repair in the progression of Alzheimer's disease: brief overview. *Exp. Gerontol.* **42:** 64–69.
37. Ratajczak, M.Z., M. Kucia, R. Reca, *et al.* 2004. Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cell "hide out" in the bone marrow. *Leukemia* **18:** 29–40.
38. Houghton, J., C. Stoicov, S. Nomura, *et al.* 2004. Gastric cancer originating from bone marrow-derived cells. *Science* **306:** 1568–1571.

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Amelioration of cognitive ability in senescence-accelerated mouse prone 8 (SAMP8) by intra-bone marrow-bone marrow transplantation

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ABSTRACT

Bone marrow cells (BMCs) can increase the number of activated microglias, which play a central role in the inflammatory response in Alzheimer's disease (AD). Senescence-accelerated mouse (SAM) prone 8 (SAMP8) are widely used in various experiments because of cognitive deficits observed with age. In the present study, 4-month-old SAMP8 were reconstituted with BMCs of C57BL/6 mice by intra-bone marrow-bone marrow transplantation (IBM-BMT), which can reconstitute both donor-derived hemopoietic stem cells and mesenchymal stem cells. Three months after IBM-BMT, the impairment of spatial memory in SAMP8 was found to be ameliorated after analyzing the results of the water maze test. Although IL-1β, IL-6 and iNOS increased and TGF-β decreased in 7 M SAMP8, IL-1β, IL-6 and iNOS decreased while TGF-β increased after IBM-BMT by RT-PCR. Moreover, oxidative stress-related heme oxygenase-1 (HO-1) increased in 7 M SAMP8, but significantly decreased after IBM-BMT. In conclusion, this is the first report suggesting that the impaired cognitive ability of SAMP8 is ameliorated by IBM-BMT. It seems likely that decreases in IL-1β, IL-6, iNOS and HO-1 are a result of the development of donor-derived BMCs.

Aging is the progressive accumulation of changes associated with or responsible for the increasing susceptibility to infections, which results in a degraded quality of life. According to the oxidative stress theory of aging, the free radicals generated through normal metabolic activity cause DNA crosslinking, strand breaks and base lesions. With successive cell divisions, DNA can become increasingly susceptible to degradation due to the shortening of telomeres, which are regions of repetitive DNA sequence at the ends of chromosomes [37,10]. HO-1 is a very sensitive marker of oxidative stress; chronic over-expression of HO-1 in the Alzheimer's disease (AD) brain, possibly in response to excessive amyloid provocation, may account for the (transferrin receptor-independent) iron overload and mitochondrial insufficiency observed in this disorder [27]. The higher oxidative stress status is observed to be partly caused by mitochondrial dysfunction in the senescence-accelerated mouse (SAM), resulting in the excessive production of reactive oxygen species and neurodegeneration [9]. The SAMP8 is an acceptable rodent model for cognitive deficits observed with aging such as AD, and is found to have age-related deficits in learning and memory that cannot be explained in terms of differences in sensorimotor or motivational capabilities [13,22,41].

Many studies have provided evidence that microglias are attracted to and surround senile plaques both in human samples and in rodent transgenic models that develop AD.

In vitro studies of cultured primary microglias have demonstrated that they secrete high levels of cytokines when stimulated with β-amyloid peptides [21,39,38]. On the other hand, some reports support the view that activated microglias favor the release of many neurotrophic molecules that have clear beneficial properties for central nervous system (CNS) elements, including neurons and oligodendrocytes [24]. Since bone marrow stem cells can infiltrate the CNS, give rise to new microglias, and nearly all of the donor-derived microglias are closely associated with blood vessels, it is generally accepted that perivascular microglias are indeed BMCs and do not result from resident brain cell division [23,11,36,40]. BMCs can increase the number of activated microglias, which supports the view that BMCs might be an effective therapeutic vehicle to reduce amyloid deposits in AD patients [20].

Abbreviations: IBM-BMT, intra-bone marrow-bone marrow transplantation; SAMP8, senescence-accelerated mouse prone 8; SAMR1, senescence-accelerated mouse resistant 1; AD, Alzheimer's disease; HSCs, hemopoietic stem cells; MSCs, mesenchymal stem cells; HO-1, heme oxygenase-1; BMCs, bone marrow-derived cells; CNS, central nervous system.

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This is the first report suggesting that the impaired cognitive ability of SAMP8 is ameliorated by IBM-BMT. IL-1β, iNOS and HO-1 decreased as a result of the development of donor-derived BMCs. This might prevent the early onset of AD.

Male SAMP8 and senescence-accelerated mouse resistant 1 (SAMR1) were purchased from Kyoto University (Kyoto, Japan) and maintained in animal facilities under conventional conditions. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee at Kansai Medical University. Two-, 4-, and 7-month-old SAMP8, and 4- and 7-month-old SAMR1 were used for the present study.

Seven-month-old SAMP8 treated with IBM-BMT (3 months after treatment), age-matched SAPM8, and SAMR1 ($n=6$ in each group) were tested by water maze. Behavioural testing took place in a water maze and an exploratory area. We modified the water maze measuring method [8,17]. Briefly, the water maze equipment consisted of a circular pool (1.2 m in diameter) and an escape platform (10 cm in diameter). The pool was filled with water (22–24 °C), and an experimenter monitored the mouse behaviour during each trial by a closed circuit video system. The maximum trial duration was 90 s, with 10 s on the platform at the end of the trails. The mice were placed into the water at and facing the side wall. Swim paths were monitored using an automated tracking system (water maze system (Panasonic WV-BP334 digital camera) and a PC computer running water maze (version 2.6) Actimetrics Software, USA).

On the first day of pre-training, each mouse was placed by hand on a hidden platform situated in the centre of the pool for 30 s (first trial). In the following trials (2 and 3 – with a cued platform, 4 – with a hidden platform), the mice were released into the water close to (about 10 cm away) and facing the platform, providing the experience of a short swim in water and then climbing onto the platform. If the mice failed to reach the platform or stay on it, they were placed on the platform by hand and allowed to remain there for 30 s. The tests started with three consecutive days with a visible platform. Each animal was trained for up to three trials per day from the three different start locations (locations 1–3). From the fourth day, a hidden platform was set up in the same place. The mice were released into the water facing the wall of the pool from the three different start locations, with three trials per day (inter-trial interval = 30 min). Each mouse was given 90 s of swimming in the pool per trial. The mice were then tested for three consecutive days with a hidden platform. Behaviour was recorded by the video tracking system. All tests were performed between 1:00 p.m. and 4:00 p.m.

The 4-month-old SAMP8 mice received fractionated irradiation twice a day (4.5 Gy × 2, with a 4-h interval). One day after the irradiation, whole bone marrow cells from 8-week-old C57BL/6 mice were injected into the recipient mice (1×10^7/mouse) using IBM-BMT, which has proven to be an advantageous strategy for allogeneic BMT when compared with conventional intravenous BMT [19].

The peripheral blood mononuclear cells were obtained from the recipients 1 M after transplantation. These cells were stained with FITC-H-2k^k(553592), PE-H-2k^b(553566), FITC-CD4(553729),

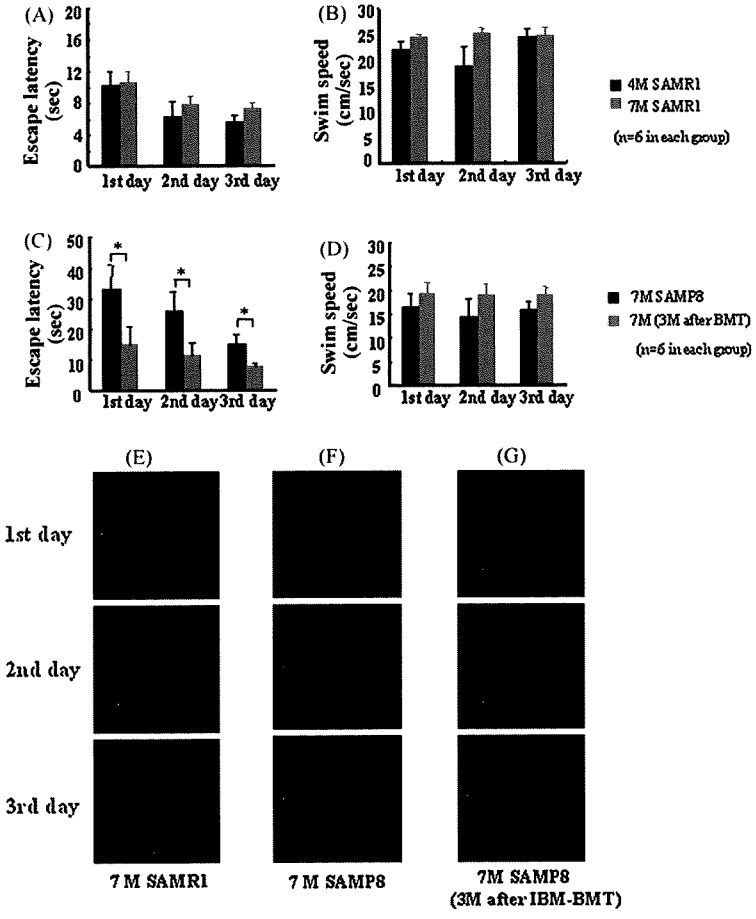

Fig. 1. (A–G) Escape latency and speed of SAMR1 and 4-month-old SAMP8 mice treated with IBM-BMT. Escape latency and swim speed were recorded for 3 days using the hidden platform conditions. Escape latency time was significantly decreased in the 7-month-old SAMP8 treated with IBM-BMT compared to age-matched SAMP8 ((C) $^*p<0.05$). There was no significant difference in swim speed (D). There were no significant differences between 4- and 7-month-old SAMR1 (A and B). The typical paths of SAMP8 and SAMR1 are shown in (F), (G) and (E).

FITC-CD8a(553030) and FITC-CD11b(553310) antibodies (BD Pharmingen, San Diego, CA), FITC-CD45R (Caltag Laboratories, Burlingame, CA) for 30 min on ice. After washing twice with 2% FCS/PBS, the 10,000 events acquired were analyzed by a FACScan (BD, Mountain View, CA). Isotype-matched immunoglobulins (553930, 349051 from BD, 11-4031-81 from eBioscience) were used as controls.

The brains of the recipient, SAMR1, were removed 3 months after the transplantation. After the tissues were fixed in 10% formalin for 24 h at room temperature, they were embedded in paraffin. The sections (6-μm thickness) were stained with hematoxylin and eosin.

Total RNA was isolated from the brains of 2- and 7-month-old SAMP8, and recipients ($n=3$ in each group) by RNA STAT-60™ reagent (TEL-TEST, Inc., Friendswood, TX). cDNA was synthesized from purified mRNA using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The expression of some cytokines was detected by RT-PCR. Primers and product size are shown: iNOS forward: 5′-tgggagccacagccaatata-3′, iNOS reverse: 5′-acagtttggtgtggtgtagg-3′, 293 bp; IL-1β, forward: 5′-tctccatgagctttgtacaagga-3′, reverse: 5′-cttggccgaggactaaggaggt-3′, 320 bp; IL-6 forward: 5′-ccaggagcccagctatgaac-3′, reverse: 5′-cccagggagaaggcaactg-3′, 310 bp; TGF-β forward: 5′-caagtgtggagcaacatgtg-3′, reverse: 5′-cacagcagttcttctctgtg-3′, 399 bp; GAPDH forward: 5′-accacagtccatgccatcac-3′, reverse: 5′-tccaccaccctgttggctgta-3′, 452 bp. All oligonucleotides were purchased from Research and Development Center Nisshinbo Industries, Inc. (Chiba, Japan). PCR amplification was performed as follows: an initial denaturation at 95 °C for 5 min with one cycle, 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. PCR products were separated by electrophoresis in 2% agarose gel.

Brains were frozen at −80 °C until needed for protein measurements. The frozen brains were pulverized and placed in a homogenization buffer, as previously described, and were used to measure protein [3]. Brain proteins were prepared using a lysis buffer supplemented with a protease inhibitor cocktail (Sigma–Aldrich). Protein levels were visualized by immunoblotting with mice anti-HO-1 monoclonal antibody (Stressgen Biotechnologies Corp., Victoria, BC). Approximately 30 μg of lysate supernatant was separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). After the membranes were incubated with 5% milk in 10 mM Tris–HCl (pH 7.4) 150 mM NaCl, 0.05% Tween-20 (TBST) buffer for 1 h, they were incubated with a 1:1000 dilution of anti-HO-1 at 4 °C overnight, and subsequently probed with horseradish peroxidase-conjugated sheep anti-mice IgG and donkey anti-rabbit IgG (Amersham) at a dilution of 1:2000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions. The ratio was according to band density of HO-1 to actin which was measured by image J 1.41 (Wayne Rasband, NIH).

A statistical analysis was performed using Student's t test between two groups. A P value of smaller than 0.05 ($P<0.05$) was considered to be significant.

Since the Morris water maze test is thought to be a sensitive assay for brain abnormalities, especially in the hippocampus [30], we used it for examining the effects of IBM-BMT on spatial learning and memory ability. There was no significant difference

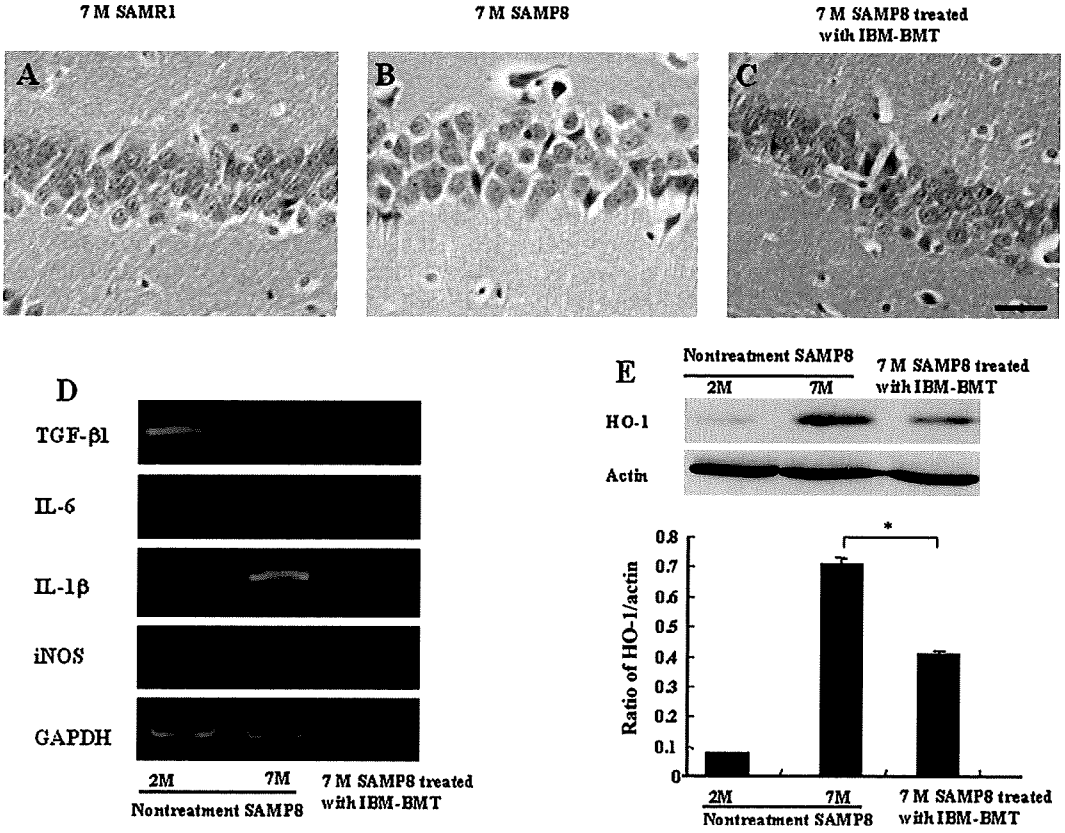

Fig. 2. (A–E) HE staining (A–C), expression of cytokines by RT-PCR (D) and expression of HO-1 by Western blotting. There was no significant difference in the CA1 region of SAMR1 (A) and 7-month-old SAMP8 treated with IBM-BMT (C) and age-matched SAMP8 (B). Scale bar = 125 μm in (A–C). Expression of TGF-β decreased while the expression of IL-1β and iNOS increased in 7 M SAMP8. TGF-β increased and IL-1β and iNOS decreased in the SAMP8 treated with IBM-BMT (D). Expression of HO-1 increased in 7 M SAMP8, while the ratio of HO-1 was significantly decreased (*$p<0.05$) in SAMP8 treated with IBM-BMT (E).

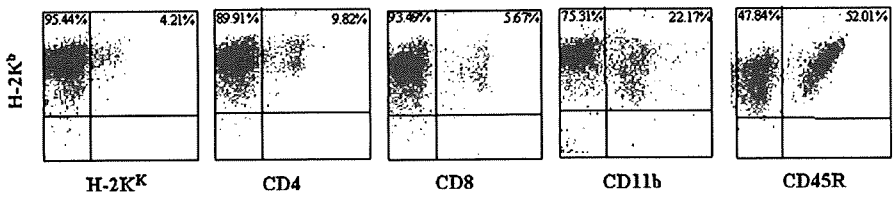

Fig. 3. Analyses of donor-derived cells after the IBM-BMT. The mononuclear cells were obtained from the peripheral blood of the recipients 1 M after the transplantation. These cells were stained with mAbs against H-2K^b, CD4, CD8, CD11b or CD45R. Approximately 99% of cells were of donor-origin, and differentiated into cells of lymphoid or myeloid lineage.

between the escape latency and the swim speed of 4- and 7-month-old SAMR1 (Fig. 1A and B). The escape latency of SAMP8 treated with IBM-BMT was significantly shorter than that of age-matched SAMP8 (Fig. 1C), although there was no difference in swim speed (Fig. 1D). The swim paths of the 7-month-old SAMR1 (positive control) are shown in Fig. 1E. The swim paths of SAMP8 treated with IBM-BMT (Fig. 1G) were more directly toward the hidden platform than those of SAMP8 (Fig. 1F). This suggests that the impairment in spatial learning and memory of the SAMP8 was ameliorated by IBM-BMT.

There was no significant difference in the histological findings (H-E staining) in the hippocampus of SAMR1 (Fig. 2A), SAMP8 (Fig. 2B) and SAMP8 treated with IBM-BMT (Fig. 2C).

It has been reported that proinflammatory cytokines are involved in the formation of neuritic plaques in Alzheimer's disease (AD) [32,14,34]. Therefore, we next used RT-PCR and Western blot to analyze the brains of three mice per group. RT-PCR results showed that the expression of IL-6, IL-1β and iNOS decreased, while TGF-β increased in the SAMP8 treated with IBM-BMT, compared to age-matched SAMP8 (Fig. 2D).

It is known that HO-1 is a 32 kDa protein of the stress protein superfamily, which is upregulated by oxidative stress, metal ions, amino acid analogues, proinflammatory cytokines and hyperthermia [5]. Therefore, we next carried out Western blot analysis to examine the HO-1 expression and found a significant decrease in the ratio of HO-1 to actin in the SAMP8 treated with IBM-BMT, compared to age-matched SAMP8 (Fig. 2E).

Approximately 95% of hematolymphoid cells were of donor-origin (H-2K^b) in the peripheral blood of the recipients treated with IBM-BMT 1-month after BMT. The analyses of cell surface antigens (CD4, CD8, CD11b and CD45R) on donor-derived cells in the recipient mice are shown in Fig. 3; donor-derived cells with mature lineage markers were clearly observed when assayed 1-month after the treatment with IBM-BMT.

It is well known that, after BMT, the number of donor-derived activated microglias increases in the recipient brain. Based on this finding, it has been proposed that BMT might be an effective therapeutic strategy to reduce amyloid deposits in AD patients [20]. Microglias originating from BMCs express higher levels of MHC-II than their residential counterparts [26,28]. This suggests that infiltrated microglias play a central role as APC; bone marrow-derived microglias reduce the amyloid deposit via phagocytosis of β-amyloid and they prevent the progression of AD [29].

We have found IBM-BMT is the best strategy for allogeneic BMT because (i) no GVHD develops even when whole BMCs (including a small number (<6%) of T cells) are injected, and (ii) hemopoietic recovery is rapid. Both HSCs and MSCs can proliferate and differentiate inside the marrow after IBM-BMT without becoming trapped in the lung [15].

It has been suggested that oxidative stress contributes to the impairment of learning and memory observed in the SAMP8 [7,25], and that alterations in the control of oxidative stress are responsible for this accelerated age [12]. Oxidative stress-related enzymes may lead to mitochondrial alterations and activation of protease, which enhances cdk5/Gsk3b, resulting in an increased activation of tau phosphorylation, altered microtubule function and synapse loss during aging [33].

In the present study, the cognitive ability of 7-month-old SAMP8 was ameliorated 3 months after treating with IBM-BMT, compared to age-matched SAMP8. HO-1, iNOS, IL-1β and IL-6 decreased after IBM-BMT, a clear indication of a reduction in oxidative stress. The decrease in iNOS was associated with a decrease in HO-1, as HO-1 responds to oxidative stress [1]. Thus IBM-BMT appears to normalize the environment in SAMP8 by the restoration of antioxidant levels resulting in a decrease in the levels of HO-1. It seems likely that the donor-derived bone marrow stem cells, including HSCs and MSCs, provided an anti-inflammation function. HO-1 is upregulated by oxidative stress and proinflammatory cytokines [2], and some proinflammatory cytokines were changed in the brain [35]. SAMP8 has also shown cytological and molecular alterations indicative of neurodegeneration in the cerebral cortex at the age of 5 months [33]. Furthermore, bone marrow-derived microglias might generate cytokines and might eliminate or prevent the formation of amyloid deposits. Initial clinical trials involving the treatment of patients with non-steroidal anti-inflammatory drugs prior to the development of AD have suggested that inhibiting the immune response reduces the chance of developing the disease [31,4,16,42]. It has been reported that amyloid is implicated in the pathogenesis of age-associated brain dysfunction [6], and amyloid-like immunoreactivity was observed in the form of granular structures in various regions, including the medial septum, cerebral cortex, hippocampus, cerebellum, and some cranial nerve roots [18]. However, in the present study, there were no significant changes in the CA1 and CA3 in the hippocampus of 7-month-old SAMP8, compared to that of 7-month-old SAMR1, and no amyloid deposits were observed on the hippocampus of 7-month-old mice (data not shown) by histochemical studies. We think that the impaired cognitive ability in the early stage might result from neuron dysfunction and that amyloid deposits might be found in the SAMP8 older than 7 months. We are examining cognitive ability and amyloid deposit after IBM-BMT using old mice (older than 7 months) to confirm the existence of donor-derived microglias in the brains of the recipients and the expression of HO-1 and inflammatory cytokines.

In conclusion, this is the first report suggesting that the impaired cognitive ability of SAMP8 mice could be ameliorated by IBM-BMT. IL-6, IL-1β, iNOS, and HO-1 decreased as a result of the development of donor-derived BMCs. This might prevent the early onset of AD.

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References

[1] N.G. Abraham, A. Kappas, Pharmacological and clinical aspects of heme oxygenase, Pharmacol. Rev. 60 (2008) 79–127.
[2] N.G. Abraham, Y. Lavrovsky, M.L. Schwartzman, R.A. Stoltz, R.D. Levere, M.E. Gerritsen, S. Shibahara, A. Kappas, Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 6798–6802.
[3] N.G. Abraham, R. Rezzani, L. Rodella, A. Kruger, D. Taller, V.G. Li, A.I. Goodman, A. Kappas, Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes, Am. J. Physiol Heart Circ. Physiol. 287 (2004) H2468–H2477.
[4] J.C. Anthony, J.C. Breitner, P.P. Zandi, M.R. Meyer, I. Jurasova, M.C. Norton, S.V. Stone, Reduced prevalence of AD in users of NSAIDs and H2 receptor antagonists: the Cache County study, Neurology 54 (2000) 2066–2071.
[5] L.A. Applegate, P. Luscher, R.M. Tyrrell, Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells, Cancer Res. 51 (1991) 974–978.
[6] W.A. Banks, S.A. Farr, J.E. Morley, K.M. Wolf, V. Geylis, M. Steinitz, Anti-amyloid beta protein antibody passage across the blood-brain barrier in the SAMP8 mouse model of Alzheimer's disease: an age-related selective uptake with reversal of learning impairment, Exp. Neurol. 206 (2007) 248–256.
[7] D.A. Butterfield, B.J. Howard, S. Yatin, K.L. Allen, J.M. Carney, Free radical oxidation of brain proteins in accelerated senescence and its modulation by N-tert-butyl-alpha-phenylnitrone, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 674–678.
[8] G. Chen, K.S. Chen, J. Knox, J. Inglis, A. Bernard, S.J. Martin, A. Justice, L. McConlogue, D. Games, S.B. Freedman, R.G. Morris, A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease, Nature 408 (2000) 975–979.
[9] Y. Chiba, A. Shimada, N. Kumagai, K. Yoshikawa, S. Ishii, A. Furukawa, S. Takei, M. Sakura, N. Kawamura, M. Hosokawa, The Senescence-accelerated Mouse (SAM): A Higher Oxidative Stress and Age-dependent Degenerative Diseases Model, Neurochem. Res. (2008).
[10] M. Collado, M.A. Blasco, M. Serrano, Cellular senescence in cancer and aging, Cell 130 (2007) 223–233.
[11] S. Corti, F. Locatelli, C. Donadoni, S. Strazzer, S. Salani, B.R. Del, M. Caccialanza, N. Bresolin, G. Scarlato, G.P. Comi, Neuroectodermal and microglial differentiation of bone marrow cells in the mouse spinal cord and sensory ganglia, J. Neurosci. Res. 70 (2002) 721–733.
[12] J.F. Ewing, M.D. Maines, Rapid induction of heme oxygenase 1 mRNA and protein by hyperthermia in rat brain: heme oxygenase 2 is not a heat shock protein, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 5364–5368.
[13] J.F. Flood, J.E. Morley, Early onset of age-related impairment of aversive and appetitive learning in the SAM-P/8 mouse, J. Gerontol. 47 (1992) B52–B59.
[14] M. Hosokawa, A higher oxidative status accelerates senescence and aggravates age-dependent disorders in SAMP strains of mice, Mech. Ageing Dev. 123 (2002) 1553–1561.
[15] S. Ikehara, A novel strategy for allogeneic stem cell transplantation: perfusion method plus intra-bone marrow injection of stem cells, Exp. Hematol. 31 (2003) 1142–1146.
[16] B.A. in t' Veld, A. Ruitenberg, A. Hofman, L.J. Launer, C.M. van Duijn, T. Stijnen, M.M. Breteler, B.H. Stricker, Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease, N. Engl. J. Med. 345 (2001) 1515–1521.
[17] C. Janus, J. Pearson, J. McLaurin, P.M. Mathews, Y. Jiang, S.D. Schmidt, M.A. Chishti, P. Horne, D. Heslin, J. French, H.T. Mount, R.A. Nixon, M. Mercken, C. Bergeron, P.E. Fraser, P. St George-Hyslop, D. Westaway, A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease, Nature 408 (2000) 979–982.
[18] T. Kagawa, M. Takamura, K. Moritake, A. Tsutsumi, T. Yamasaki, A case of sellar chordoma mimicking a non-functioning pituitary adenoma with survival of more than 10 years, Noshuyo Byori 10 (1993) 103–106.
[19] T. Kushida, M. Inaba, H. Hisha, N. Ichioka, T. Esumi, R. Ogawa, H. Iida, S. Ikehara, Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice, Blood 97 (2001) 3292–3299.
[20] J.K. Lee, H.K. Jin, J.S. Bae, Bone marrow-derived mesenchymal stem cells reduce brain amyloid-beta deposition and accelerate the activation of microglia in an acutely induced Alzheimer's disease mouse model, Neurosci. Lett. 450 (2009) 136–141.
[21] T.M. Malm, M. Koistinaho, M. Parepalo, T. Vatanen, A. Ooka, S. Karlsson, J. Koistinaho, Bone-marrow-derived cells contribute to the recruitment of microglial cells in response to beta-amyloid deposition in APP/PS1 double transgenic Alzheimer mice, Neurobiol. Dis. 18 (2005) 134–142.
[22] M. Miyamoto, Y. Kiyota, N. Yamazaki, A. Nagaoka, T. Matsuo, Y. Nagawa, T. Takeda, Age-related changes in learning and memory in the senescence-accelerated mouse (SAM), Physiol. Behav. 38 (1986) 399–406.
[23] K. Nakano, M. Migita, H. Mochizuki, T. Shimada, Differentiation of transplanted bone marrow cells in the adult mouse brain, Transplantation 71 (2001) 1735–1740.
[24] M.D. Nguyen, J.P. Julien, S. Rivest, Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat. Rev. Neurosci. 3 (2002) 216–227.
[25] Y. Okatani, A. Wakatsuki, R.J. Reiter, Y. Miyahara, Melatonin reduces oxidative damage of neural lipids and proteins in senescence-accelerated mouse, Neurobiol. Aging 23 (2002) 639–644.
[26] J. Priller, A. Flugel, T. Wehner, M. Boentert, C.A. Haas, M. Prinz, F. Fernandez-Klett, K. Prass, I. Bechmann, B.A. de Boer, M. Frotscher, G.W. Kreutzberg, D.A. Persons, U. Dirnagl, Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment, Nat. Med. 7 (2001) 1356–1361.
[27] H.M. Schipper, Heme oxygenase-1: role in brain aging and neurodegeneration, Exp. Gerontol. 35 (2000) 821–830.
[28] A.R. Simard, S. Rivest, Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia, FASEB J. 18 (2004) 998–1000.
[29] A.R. Simard, D. Soulet, G. Gowing, J.P. Julien, S. Rivest, Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease, Neuron 49 (2006) 489–502.
[30] R.W. Skelton, R.K. McNamara, Bilateral knife cuts to the perforant path disrupt spatial learning in the Morris water maze, Hippocampus 2 (1992) 73–80.
[31] W.F. Stewart, C. Kawas, M. Corrada, E.J. Metter, Risk of Alzheimer's disease and duration of NSAID use, Neurology 48 (1997) 626–632.
[32] S. Strauss, J. Bauer, U. Ganter, U. Jonas, M. Berger, B. Volk, Detection of interleukin-6 and alpha 2-macroglobulin immunoreactivity in cortex and hippocampus of Alzheimer's disease patients, Lab. Invest. 66 (1992) 223–230.
[33] F.X. Sureda, J. Gutierrez-Cuesta, M. Romeu, M. Mulero, A.M. Canudas, A. Camins, J. Mallol, M. Pallas, Changes in oxidative stress parameters and neurodegeneration markers in the brain of the senescence-accelerated mice SAMP-8, Exp. Gerontol. 41 (2006) 360–367.
[34] R. Tenhunen, H.S. Marver, R. Schmid, Microsomal heme oxygenase. Characterization of the enzyme, J. Biol. Chem. 244 (1969) 6388–6394.
[35] K.K. Tha, Y. Okuma, H. Miyazaki, T. Murayama, T. Uehara, R. Hatakeyama, Y. Hayashi, Y. Nomura, Changes in expressions of proinflammatory cytokines IL-1beta, TNF-alpha and IL-6 in the brain of senescence accelerated mouse (SAM) P8, Brain Res. 885 (2000) 25–31.
[36] L. Vallieres, P.E. Sawchenko, Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity, J. Neurosci. 23 (2003) 5197–5207.
[37] J. Vijg, J. Campisi, Puzzles, promises and a cure for ageing, Nature 454 (2008) 1065–1071.
[38] D.G. Walker, L.F. Lue, Investigations with cultured human microglia on pathogenic mechanisms of Alzheimer's disease and other neurodegenerative diseases, J. Neurosci. Res. 81 (2005) 412–425.
[39] J. Wegiel, H. Imaki, K.C. Wang, J. Wegiel, R. Rubenstein, Cells of monocyte/microglial lineage are involved in both microvessel amyloidosis and fibrillar plaque formation in APPsw tg mice, Brain Res. 1022 (2004) 19–29.
[40] J.M. Weimann, C.B. Johansson, A. Trejo, H.M. Blau, Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant, Nat. Cell Biol. 5 (2003) 959–966.
[41] H. Yagi, S. Katoh, I. Akiguchi, T. Takeda, Age-related deterioration of ability of acquisition in memory and learning in senescence accelerated mouse: SAM-P/8 as an animal model of disturbances in recent memory, Brain Res. 474 (1988) 86–93.
[42] A.G. Yip, R.C. Green, M. Huyck, L.A. Cupples, L.A. Farrer, Nonsteroidal anti-inflammatory drug use and Alzheimer's disease risk: the MIRAGE Study, BMC Geriatr. 5 (2005) 2.

Prevention of Premature Ovarian Failure and Osteoporosis Induced by Irradiation Using Allogeneic Ovarian/Bone Marrow Transplantation

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Background. Two side effects of irradiation are premature ovarian failure (POF) and osteoporosis, both of which are concerns not only clinically, for patients, but also experimentally, for animals. We examine whether bone marrow transplantation (BMT) can correct the POF induced by radiation and also address whether allogeneic ovarian transplantation (OT) can modulate the adverse effects of radiotherapy.

Methods. Eight-week-old female C57BL/6 mice were lethally irradiated with 6 Gy ×2, and then injected with allogeneic bone marrow cells into their bone marrow cavity using our previously described intrabone marrow (IBM)-BMT technique. Allogeneic ovaries were simultaneously transplanted under the renal capsules of the mice.

Results. Three months after the transplantation, we noted that hematopoietic and lymphoid cells had been successfully reconstituted. The ovaries transplanted under the renal capsules demonstrated signs of development with a large number of differentiating follicles at different stages of development. Importantly, the total bone mineral density of the tibia in the "IBM-BMT+OT" (BMT/OT) group remained normal. However, the reproductive function of the recipient mice was not restored, despite the presence of many immature oocytes in the host ovaries in the BMT/OT group. In the BMT group, no oocytes were found in the host ovaries.

Conclusions. These findings suggest that IBM-BMT with ovarian allografts can be advantageous for young women with POF and osteopenia or osteoporosis that is due to chemotherapy and radiotherapy for malignant diseases.

Keywords: Allogeneic ovarian transplantation, Intra-bone marrow-bone marrow transplantation, Osteoporosis, Oocyte renewal, Premature ovarian failure.

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Allogeneic BMT has commonly been used to treat patients with recurrent or aggressive leukemia and lymphoma or both (*1*). Unfortunately, however, aggressive chemotherapy and radiotherapy as preconditioning regimens lead to premature ovarian failure (POF) and bone disease (*2–4*). In addition, chemotherapy commonly damages oocytes and granulosa cells in a dose-dependent manner (*5*). In fact, total body irradiation (TBI), which is required before BMT, produces a great risk of POF and osteoporosis (*6*).

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The near universally accepted dogma, in which oocytes are endowed as a fixed and nonrenewing stockpile at birth and the pathologic destruction of oocytes is irreversible, has been recently challenged (*7–10*). Earlier work has demonstrated that oocyte manufacturing continues into adult life in mice and that germ cells may originate in the bone marrow. Hence, we hypothesized that BMT would have the potential to preserve and resurrect ovarian function and fertility after drug- or radiation-induced POF.

We, therefore, used a model for allogeneic tolerance induction using ovarian transplantation (OT) with BMT. Our laboratory has emphasized the use of intrabone marrow (IBM)-BMT as a more focused strategy for allogeneic BMT. IBM-BMT creates an appropriate hemopoietic environment for the early recovery of hemopoiesis and donor cell engraftment (*11*). IBM-BMT allows us to replace not only hematopoietic stem cells but also mesenchymal stem cells (MSCs) with donor-derived hematopoietic stem cells and MSCs (*12*). In contrast, intravenous (IV)-BMT permits us to replace only hemopoietic cells. To examine whether mature ovulated eggs are derived from the bone marrow-derived MSCs and also whether osteoporosis induced by irradiation can be prevented, we selected IBM-BMT (instead of IV-BMT) in this study, because we have recently demonstrated that IBM-BMT with OT can be used to prevent bone loss in ovariectomy mice (*13*).

Importantly, germ cells are present in human and rat bone marrow samples; spermatogonia are derived from the bone marrow of adult male mice and men (*14,15*). However, the possibility that germ cells could be derived from the bone marrow of postnatal female mice has been met with skepticism (*16–18*). Recent data (*19*) using female mice have shown that mature ovulated eggs are not derived from the bone marrow or circulating (blood) cells. We report herein that oocytes can self-renew even in postnatal and adult mice that have received BMT/OT. In addition, we demonstrate that BMT/OT can be used to prevent and treat bone disease induced by irradiation.

MATERIALS AND METHODS

Animals

Eight-week-old female C57BL/6 (B6: H-2K^b) mice and BALB/c mice (H-2K^d) were purchased from SLC (Shizuoka, Japan, http://www.jslc.co.jp). These mice were maintained in our animal facilities under specific pathogen-free conditions until use; the mice had ad libitum access to water and commercial standard food. All animal use was approved by the Animal Care Committee of Kansai Medical University.

Experimental Protocols

Female C57BL/6 mice (H-2K^b) were used throughout. Animals were divided into four groups, containing eight mice per group. These groups included (1) a normal control group; (2) ovariectomy (OvX) group (estrogen deficiency and osteoporosis-positive control group); (3) BMT group; and (4) BMT/OT group. The mice were all randomized on entry based on body weight as a selection parameter. After 3 months, two groups of mice requiring IBM-BMT were lethally irradiated at 6 Gy ×2; and 1 day after the irradiation, the mice were transplanted with whole bone marrow cells (BMCs; 1×10^7/10 μL/mouse) from female BALB/c mice (H-2K^d, female 8-weeks old) via IBM injection. Allogeneic BMCs were then injected into the left tibia bone cavity, and each mouse in the BMT/OT group simultaneously received a transplanted allogeneic ovary under its renal capsule. Another group served as an "only BMT" control group. After 3 months of treatment, their uterus and body weights were measured, and the blood was removed by cardiac puncture. The mice were killed by cervical dislocation, and sera were stored at −80°C for further analysis.

Preparation and Inoculation of BMCs

BMCs were collected from the femurs and tibias of BALB/c mice. In brief, donor BMCs from female BALB/c mice were flushed from tibiae, femora, and humeri using Roswell Park Memorial Institute culture medium 1640 (Niken CM1101, Japan) supplemented with 2% heat-inactivated fetal calf serum (PAA.A15-001; Austria) on ice. The BMCs were filtered through a sterile nylon mesh, and then resuspended in sterile phosphate-buffered saline. IBM-BMT injection was carried out according to the method described previously (*13*). In brief, the knee was flexed to 90°, and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the left tibia through the patellar tendon and then inserted into the bone marrow cavity of the left tibia. Using a microsyringe (50 μL; Hamilton Company, Reno, NV, http://www.hamiltoncompany.com), the donor BMCs (1×10^7/10 μL/mouse) were injected into the bone marrow cavity.

Flow Cytometry

BMCs, spleen cells, and peripheral blood cells were prepared from the recipient mice 3 months after the bone marrow transplantation (BMT), followed by red blood cell lysis with ammonium chloride (8.3 g/mL; Sigma-Aldrich, St-Louis, MO). To detect donor- or residual recipient-derived cells, the cells were stained with fluorescein isothiocyanate-conjugated anti-H-2K^d and phycoerythrin-conjugated anti-H-2K^b monoclonal antibodies (mAbs) (PharMingen, San Diego, CA, http://www.bdbiosciences.com/pharmingen). The cells were analyzed using a FACScan (Becton, Dickinson and Company, Mountain View, CA, http://www.bd.com).

Histology of Bone

Vertebrae were fixed in 10% formalin and then decalcified and embedded in paraffin. The lumbar vertebrae were sectioned to obtain a longitudinal midline section through the vertebral body, and the sections were then stained with hematoxylin-eosin. The soft tissues were removed from the right tibiae of the mice and stored in 70% ethanol for peripheral quantitative computed tomography (pQCT) analysis. A small animal pQCT (XCT Research SA, Stratec Medizjntechnik, Pforzheim, Germany) was used for the measurements. When detected, bone was fixed in a plastic tube (8 mm diameter) with a spring and scanned with pQCT equipment (XCT 540; Stratec). To measure levels in the tibia, the reference line was placed at the proximal end of the bone. Three cross-sections, at 0.3 mm intervals, were analyzed 1.8 mm from the reference line. Measurements were also taken from two sections separated by 1 mm, starting 2.5 mm above a reference line at the tibiofibular junction. Special Software version 5.40

(Stratec) was used to analyze the images of each section, with a voxel size of 0.10 mm. The total bone mineral densities (BMD) of the proximal tibia were applied for BMD analyses.

Histology of Ovary and Uterus

Three months after IBM-BMT, the uteri and the ovaries, including the allogeneic ovary transplanted under the renal capsules, were removed, weighed, and then fixed in 10% formalin. The sections were stained with hematoxylin-eosin to observe the ovarian and uterine morphology. All sections were observed by an unbiased observer.

Serum Estradiol and TRACP Levels

Serum specimens were collected from the treated and nontreated B6 mice, separated by centrifugation, and stored at −80°C until used for measurements. Serum estradiol was quantified by an enzyme-linked immunosorbent assay kit (IBL-Hamburg GmbH Corp., Hamburg, Germany, http://www.ibl-hamberg.com). The serum tartrate-resistant acid phosphatase (TRACP) was quantified by an ELISA kit (SB-TR103) (Immunodiagnostic System Ltd., UK, http://www.idsltd.com), to evaluate the osteoclast function and bone resorption indirectly.

Imaging

All bright-field images were taken on an Olympus BH-2 microscope (Olympus Optical, Tokyo, Japan) with a FUJIFILM HC-2500 digital camera (FUJIFILM, Tokyo, Japan) and photograb-2500 software.

Statistical Analyses

All data were presented as mean±SD. Significance of the results was determined by two-way analysis of variance. Differences were calculated by Students *t* test. A *P* value of less than 0.01 was considered statistically significant.

RESULTS

In the study described herein, we performed IBM-BMT instead of conventional IV-BMT. We submit that IBM-BMT is superior from the following points of view: (1) early hemopoietic recovery (*11, 20*); (2) replacement with donor MSCs (*21*); and (3) long-term tolerance induction (*11,22*). In humans, there have been several reports of successful pregnancy and delivery with oocyte donation after BMT including TBI (*1*). In this study, we used a lethal irradiation dose (6 Gy ×2) to destroy the host's ovarian function, and then examined the effects of allogeneic IBM-BMT (abbreviated in this article as BMT) and OT on the renewal of oocytes and bone metabolism.

Cell Surface Antigens

Three months after BMT, we carried out flow cytometrical analyses using peripheral blood cells, spleen cells and BMCs obtained from the recipient mice, and examined the engraftment of donor-derived cells. As demonstrated in Figure 1, hemopoietic cells had been reconstituted by donor-type (H-2k^d) cells in the recipients.

Histology and Weight of Ovary and Uterus

Three months after BMT/OT, the allogeneic ovaries had been accepted under the renal capsules of the B6 mice (Fig. 2). There were a large number of corpora lutea, and follicles at different stages of growth, including primordial follicles, primary follicles, and mature follicles. The uteri demonstrated normal endometrium including endometrial glands (Fig. 3A). However, in the BMT (without OT) group, the uteri demonstrated atrophic endometrium and few endometrial glands (Fig. 3C). Uterus weight had significantly increased in the BMT/OT group in comparison with the BMT group or the OvX group (Table 1). These results indicate that OT leads to the secretion of estrogen and restores the function of the uterus after radiotherapy. There were many immature oocytes in the host ovaries in the BMT/OT group, but no immature oocytes in the host ovaries in the BMT group. The host ovaries demonstrated atrophy both in the BMT group and the BMT/OT group. Although the uterus weight decreased noticeably in the

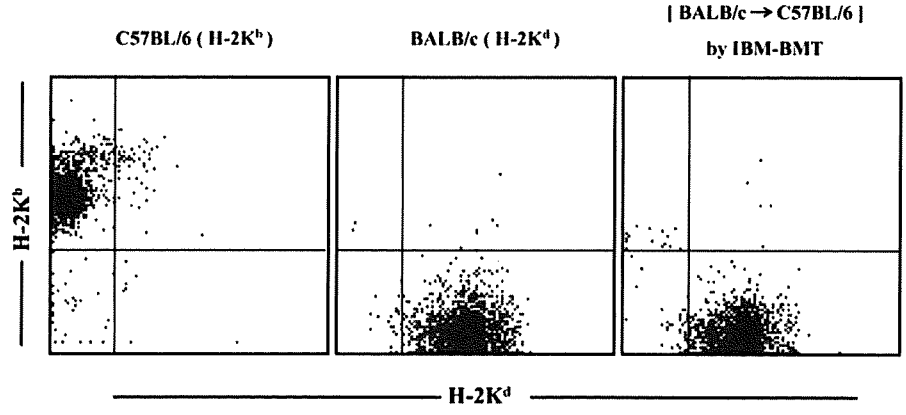

FIGURE 1. Reconstitution of donor-derived hemopoietic cells after IBM-BMT. B6 mice at the age of 8 weeks were irradiated with a lethal dose (6 Gy ×2), and BMCs from normal BALB/c mice were injected directly into the bone marrow cavity (IBM-BMT) of the left tibia. After 3 months, cells from the peripheral blood (PB) of chimeric mice were stained with fluorescein isothiocyanate-conjugated anti-H-2K^b mAb (recipient type) or anti-H-2K^d mAb (donor type). The cells of BALB/c (H-2K^d) PB (A) and C57BL/6J (H-2K^b) PB (B) mice were used as controls. Cells from C57BL/6 mice treated with IBM-BMT from BALB/c mice were of donor origin (H-2K^d) (C). These findings indicate that the hemopoietic cells were reconstituted with donor-type cells after IBM-BMT. IBM, intrabone marrow; BMC, bone marrow cells; BMT, bone marrow transplantation.

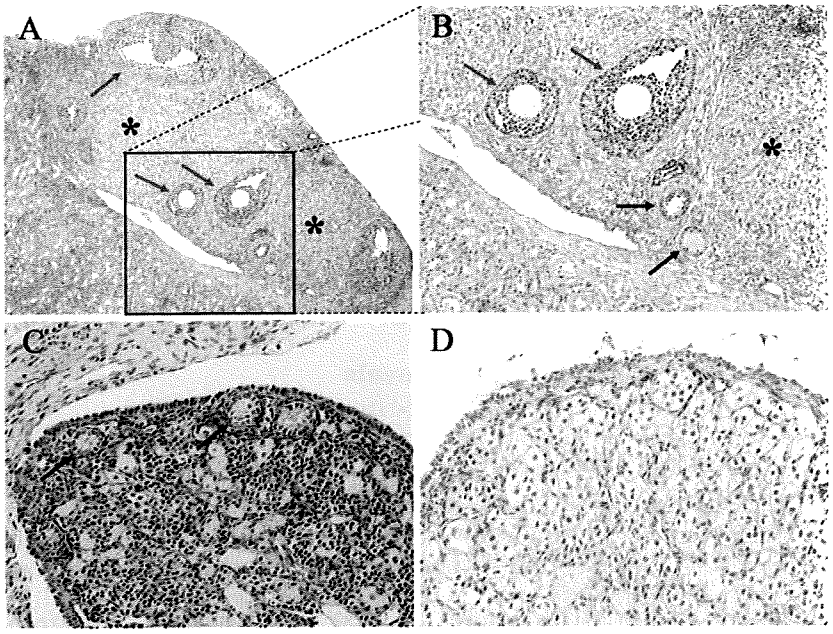

FIGURE 2. Histology of transplanted ovary and host ovary. Three months after bone marrow transplantation/ovarian transplantation (BMT/OT), the ovaries were accepted (original magnification ×100, A) with a large number of mature follicles (*red arrows*), primary follicles (*black arrows*), and corpus luteum (*asterisks*) (original magnification ×200, B) under the renal capsule; the recipients' ovaries demonstrated atrophy both in the BMT/OT group (original magnification ×400, C) and in the BMT group (original magnification ×400, D). There were a few immature follicles (*black arrows*) in the BMT/OT group (×400, C).

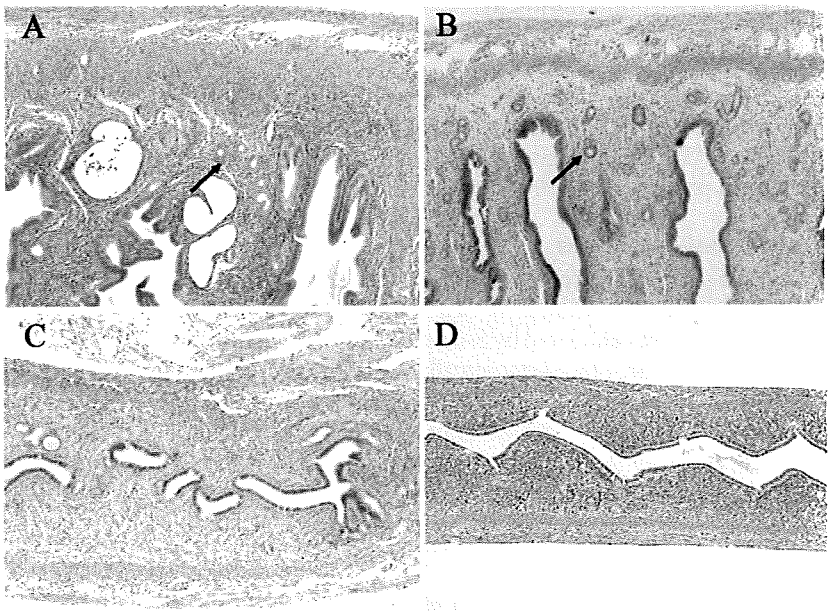

FIGURE 3. Effects of IBM-BMT with OT on uterus. Three months after bone marrow transplantation (BMT), the uteri of the four groups, including the normal control (nontreated) group (A), the BMT/ovarian transplantation (OT) group (B), the BMT group (C), and the OvX group (D), were stained with hematoxylin-eosin. The BMT/OT group's uteri revealed normal endometrial glands (*black arrows*) (B), but the other two experimental groups' uteri (C and D), especially the BMT group's uteri, revealed atrophic endometrium with a few endometrial glands (original magnification ×100 for all panels).

OvX and BMT groups, the body weight markedly increased only in the OvX group.

These findings suggest that irradiation injures the reproductive tissue, resulting in estrogen deficiency, and that BMT/OT is able to heal the damage.

Bone Histology and BMD

In the BMT/OT group (Fig. 4B), the lumbar vertebral-4 (L4) body demonstrated increases in trabeculae number, thickness, and length, whereas in the BMT group, the trabeculae were thin and the number decreased (Fig. 4C). Bone mineral densitometry was next used to assess the bone mass of the tibiae. The total BMD of the proximal tibia was determined with pQCT. After BMT, the mice in the OT group maintained their mass, whereas the bone mass in the BMT (without OT) group rapidly decreased (Table 1); there was a significant difference between the OvX group and the BMT/OT group. These results indicate that TBI as a conditioning regimen for transplantation has toxic effects on the bone, but that bone mass was maintained by, and even increased after, allogeneic OT (Table 1).

Levels of Serum Estradiol

There was no statistical difference between the normal control group and the BMT/OT group in serum estrogen levels (Table 2). This suggests that the allogeneic ovaries transplanted under the renal capsules were accepted and could secrete estrogen, resulting in the maintenance of normal estrogen levels even in the mice treated with lethal irradiation. The estrogen levels in the BMT group decreased in the same

TABLE 1. Body weight, uterus weight, and proximal tibia BMD in mice treated with BMT or OT or both

Group	Proximal tibia BMC (mg/cm^3)	Body weight (g)	Uterus weight (g)
Normal control	$433.62\pm21.75^{a,b}$	24.12 ± 1.89^{a}.	$0.157\pm0.029^{a,b}$
OvX	$350.69\pm25.11^{b,c,d}$	$30.22\pm1.46^{b,c,d}$	$0.023\pm0.003^{c,d}$
BMT/OT	$461.47\pm37.20^{a,b}$	21.27 ± 1.37^{a}	$0.132\pm0.053^{a,b}$
BMT	$400.24\pm18.75^{a,c,d}$	21.37 ± 2.14^{a}	$0.029\pm0.002^{c,d}$

Body weight and uterus weight were measured in the normal control group, BMT/OT group, BMT group, and OvX group.The proximal tibia's BMD was measured by pQCT. The proximal tibia's BMD in the BMT/OT group increased in comparison with the normal control group, indicating that bone mass was maintained.

Data are expressed as mean±SD, n=8.

[a] $P<0.01$ vs. OvX group.

[b] $P<0.01$ vs. BMT group.

[c] $P<0.01$ vs. normal control group.

[d] $P<0.01$ vs. BMT/OT group.

BMC, bone marrow cells; BMD, bone mineral density; BMT, bone marrow transplantation; OT, ovarian transplantation; pQCT, peripheral quantitative computed tomography.

manner as in the OvX group; there was no significant difference between these two groups.

Levels of TRACP

It has been demonstrated that the levels of TRACP are expressed by bone-resorbing osteoclasts and activated macrophages (*23*). The TRACP levels in the OvX group increased markedly, whereas in the BMT/OT group, bone resorption remained at normal levels after allogeneic OT. In the BMT group, the TRACP levels were high, although there was no statistical difference in comparison with the normal control group (non-treated group) or BMT/OT group (Table 2).

DISCUSSION

There has been increased emphasis on improving the quality of life in long-term survivors of radiochemotherapy and BMT. The quality of life of cancer survivors after chemotherapy or radiotherapy is a growing concern because POF and bone disease have a strong impact on self-esteem and quality of life (*24–27*). The pathogenesis of bone loss, such as osteoporosis and fractures following BMT, or organ transplantation, produces substantial morbidity, particularly during the early posttransplant period (*28,29*). POF affects present and future health, especially through estrogen deficiency symptoms, and increases the risk of osteoporosis. Therefore, the lasting adverse effects of these modalities are receiving increasing attention.

OT has been extensively used in experimental endocrinology for over a century. Studies demonstrate that OT restores reproductive power, reinitiates menstrual cycles, and even offers the possibility of natural conception (*30–32*). However, because the ovary is not an immunologically privileged organ (*33*), it is important to determine how to achieve a specific tolerance for the allogeneic ovary transplantation.

IBM-BMT is a new BMT method (*12*) that can lead to the rapid hemopoietic and immune recovery of recipients, inducing donor-specific tolerance in allogeneic organ or tissue transplantation, and promoting the survival rate of recipients. Recently, we have proven that IBM-BMT can induce tolerance in OvX mice, and can, to a certain extent, prevent bone loss (*13*).

In this study, we carried out allogeneic BMT/OT on female mice after radiotherapy to investigate the effects of allogeneic ovary on the recipient's oocyte renewal and bone metabolism. Three months after the radiotherapy and BMT, the hematolymphoid cells were found to be reconstituted with donor-derived cells. The ovarian tissues transplanted under the renal capsules had been accepted without using any immunosuppressants; there were no differences in the levels of endogenous estrogens between the BMT/OT group and the normal control group. It is well known that irradiation in-

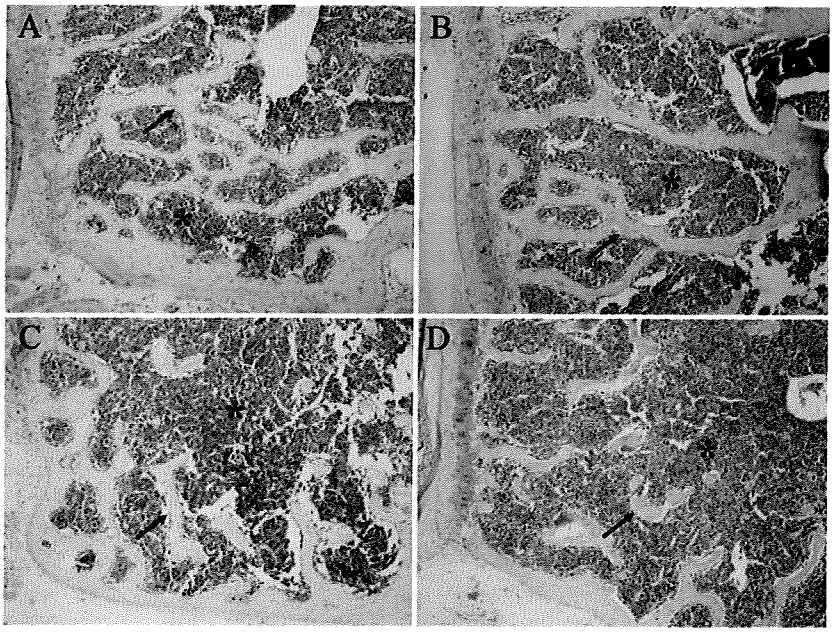

FIGURE 4. Histology of the lumbar vertebrae after intrabone marrow-bone marrow transplantation (IBM-BMT). Three months after IBM-BMT, the 4th lumbar vertebrae of mice in the four groups—normal control group (A), bone marrow transplantation/ovarian transplantation (BMT/OT) group (B), BMT group (C), and OvX group (D)—were stained with hematoxylin-eosin. Significant loss of bone trabeculae (*black arrows*) was observed in the OvX and BMT groups; the bone trabeculae in the BMT group were short and small, whereas they were longer in the BMT/OT group: Original magnification ×40 for all panels (*asterisks*: bone marrow).

TABLE 2. Effects of IBM-BMT With OT on serum estrogen and TRACP

Group	Serum estrogen (pg/mL)	TRACP (U/L)
Normal control	$23.71\pm7.80^{a,b}$	0.62 ± 0.19^{a}
OVX	$11.69\pm4.41^{c,d}$	$1.28\pm0.33^{c,d}$
BMT/OT	$21.47\pm13.44^{a,b}$	0.71 ± 0.39^{a}
BMT	$13.77\pm4.09^{c,d}$	1.08 ± 0.74

Serum estrogen and TRACP were measured using an ELISA kit. There were no significant differences between the normal control group and the BMT/OT group in the serum estrogen assay. The hormonal rise indicated the acceptance of allografts with function. In the TRACP assay, the TRACP level in the BMT/OT group decreased in comparison with the BMT group, indicating that bone resorption had decreased.

Data are expressed as mean±SD, n=8.

[a] $P<0.01$ vs. OvX group.

[b] $P<0.01$ vs. BMT group.

[c] $P<0.01$ vs. normal control group.

[d] $P<0.01$ vs. BMT/OT group.

IBM, intrabone marrow; BMT, bone marrow transplantation; TRACP, tartrate-resistant acid phosphatase; bone marrow transplantation; OT, ovarian transplantation.

duces not only ovarian failure but also uterine dysfunction. However, in this study, after BMT/OT, the weight of the uteri increased in the BMT/OT group. Moreover, the endometrial morphology, including the endometrial glands, was almost normal, although the uterine volume was in the normal range.

It is a doctrine that the mammalian neonatal ovary contains a finite stockpile of nongrowing primordial follicles, each of which encloses an oocyte arrested at the diplotene step in the meiotic prophase. Recent studies of mouse ovaries, however, propose that intra- and extraovarian germline stem cells replenish oocytes and form new primordial follicles after chemotherapy but not after ionizing radiation (*7–10*). Moreover, the notion of oocyte and follicular renewal in the postnatal mouse ovary from within the ovary or external to it has been supported by other authors (*34–38*).

The ovary is radiosensitive tissue, and half of the follicles are lost by 0.1 to 0.3 Gy in mice (*5*). Moreover, low-dose irradiation (0.5 Gy) can sterilize female mice (*19*). In this report, we used a lethal dose of 6 Gy ×2 as a conditioning regimen for IBM-BMT, and there were no oocytes in the host ovaries in the BMT group after 3 months. In the BMT/OT group, however, the host ovaries demonstrated oocytes within primordial and growing immature follicles. However, we have no evidence to support the hypothesis that renewing oocytes are derived from donor-derived bone marrow cells or germline stem cells. Our observations provide qualified support for an as-yet unknown mechanism for follicle renewal in the postnatal and adult mouse ovary, even after ionizing radiation. This is an important area for future study. Interestingly, all of the new oocytes in the host ovaries were observed in immature follicles up to the preantral stage of development, but never in maturing antral or Graafian follicles. Also, 2 weeks after the allogeneic BMT/OT described above, the mice were mated with male mice, but none were fertile.

Others have hypothesized that BMT functions primarily by reactivating host oogenesis (*7*), which becomes impaired in a BMT-reversible manner after chemotherapy but not after ionizing radiation (*4*). Our results demonstrate that BMT therapy after irradiation cannot, on its own, reverse the damage to the ovary. The gonadal environmental factors are essential for the oocyte's self-renewal, and our data indicate that, if a gonadal microenvironment is supplied after irradiation (such as by timely BMT/OT), new follicles may be produced in the host ovary. However, other laboratories have postulated that the frequencies of micronuclei, anaphase-telophase alterations and chromosomal aberrations increase after low-dose irradiation (*39*), and that DNA damage results in genetic instability. We suggest that it is possible that BMT/OT is insufficient to repair the damage induced by irradiation on DNA (*39*), blood vessels (*40*), and granulosa cells (*41*) or fertility genes (*42*) in the host ovaries, which can then no longer support the development of new immature oocytes, such as in the case of primordial germ cells lacking Nanog, which fail to mature on reaching the genital ridge (*43*).

There are direct and indirect toxic effects of irradiation on the bone. Irradiation can directly damage the osteogenic activity of marrow by suppressing osteoblasts, leading to postirradiation osteoporosis (*44*) and postradiation atrophy of mature bone (*45,46*); after irradiation, ovaries cannot secrete estrogen to regulate bone homeostasis (*47*). Estrogen deficiency becomes the main reason for bone loss in young females. The osteoprotective action of estrogen blocks the formation of new osteoclasts, shortens the lifespan of old osteoclasts, and promotes osteoblast proliferation. The data presented in this study report that the bone mass in the BMT/OT group achieved normal levels after 3 months. This finding implies that estrogen secreted by transplanted allogeneic ovaries can efficiently prevent bone loss after heavy irradiation. Hence, we propose that IBM-BMT with ovarian allografts can be advantageous for young women with POF and osteopenia or osteoporosis (due to chemotherapy and radiotherapy for malignant diseases). This is clearly an important area for future study.

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REFERENCES

1. Larsen EC, Loft A, Holm K, et al. Oocyte donation in women cured of cancer with bone marrow transplantation including total body irradiation in adolescence. *Hum Reprod* 2000; 15: 1505.
2. Schimmer AD, Quatermain M, Imrie K, et al. Ovarian function after autologous bone marrow transplantation. *J Clin Oncol* 1998; 16: 2359.
3. Salooja N, Szydlo RM, Socie G, et al. Pregnancy outcomes after peripheral blood or bone marrow transplantation: A retrospective survey. *Lancet* 2001; 358: 271.
4. Donnez J, Dolmans MM, Pirard C, et al. Allograft of ovarian cortex between two genetically non-identical sisters: Case report. *Hum Reprod* 2007; 22: 2653.
5. Gosden RG, Wade JC, Fraser HM, et al. Impact of congenital or experimental hypogonadotrophism on the radiation sensitivity of the mouse ovary. *Hum Reprod* 1997; 12: 2483.
6. Alper MM, Garner PR. Premature ovarian failure: Its relationship to autoimmune disease. *Obstet Gynecol* 1985; 66: 27.
7. Lee HJ, Selesniemi K, Niikura Y, et al. Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. *J Clin Oncol* 2007; 25: 3198.
8. Johnson J, Canning J, Kaneko T, et al. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* 2004; 428: 145.

9. Johnson J, Skaznik-Wikiel M, Lee H-J, et al. Setting the record straight on data supporting postnatal oogenesis in female mammals. *Cell Cycle* 2005; 4: 1471.
10. Johnson J, Bagley J, Skaznik-Wikiel M, et al. Oocyte generation in adult mammalian ovaries by putative germ cells derived from bone marrow and peripheral blood. *Cell* 2005; 122: 303.
11. Kushida T, Inaba M, Hisha H, et al. Intra-bone marrow injection of allogeneic bone marrow cells: A powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. *Blood* 2001; 97: 3292.
12. Ikehara S. Intra-bone marrow-bone marrow transplantation: A new strategy for treatment of stem cell disorders. *Ann NY Acad Sci* 2005; 1051: 626.
13. Feng W, Cui Y, Song C, et al. Prevention of osteoporosis and hypogonadism by allogeneic ovarian transplantation in conjunction with intra-bone marrow-bone marrow transplantation. *Transplantation* 2007; 84: 1459.
14. Nayernia K, Lee J-H, Drusenheimer N, et al. Derivation of male germ cells from bone marrow stem cells. *Lab Invest* 2006; 86: 654.
15. Drusenheimer N, Wulf G, Nolte J, et al. Putative human male germ cells from bone marrow stem cells. *Soc Reprod Fertil Suppl* 2007; 63: 69.
16. Telfer EE, Gosden RG, Byskov AG, et al. On regenerating the ovary and generating controversy. *Cell* 2005; 122: 821.
17. Oktay K, Oktem O. Regeneration of oocytes after chemotherapy: Connecting the evidence from mouse to human. *J Clini Oncol* 2007; 25: 3185.
18. Byskov AG, Faddy MJ, Lemmen JG, et al. Eggs forever? *Differentiation* 2005; 73: 438.
19. Eggan K, Jurga S, Gosden R, et al. Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature* 2006; 441: 1109.
20. Li Q, Hisha H, Yasumizu R, et al. Analyses of very early hemopoietic regeneration after bone marrow transplantation: Comparison of intravenous and intrabone marrow routes. *Stem Cells* 2007; 25: 1186.
21. Takada K, Inaba M, Ichioka N, et al. Treatment of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells. *Stem Cells* 2006; 24: 399.
22. Guo K, Inaba M, Li M, et al. Long-term donor-specific tolerance in rat cardiac allografts by intra-bone marrow injection of donor bone marrow cells. *Transplant* 2008; 85: 93.
23. Willey JS, Lloyd SA, Robbins ME, et al. Early increase in osteoclast number in mice after whole-body irradiation with 2 Gy X rays. *Radiat Res* 2008; 170: 388.
24. Schover L. Sexuality and body image in younger women with breast cancer. *J Natl Cancer Inst Monogr* 1994; 16: 177.
25. Ganz PA, Greendale GA, Petersen L, et al. Breast cancer in younger women: Reproductive and late health effects of treatment. *J Clin Oncol* 2003; 21: 4184.
26. Ganz PA, Rowland JH, Desmond K, et al. Life after breast cancer: Understanding women's health-related quality of life and sexual functioning. *J Clin Oncol* 1998; 16: 501.
27. Kashyap A, Kandeel F, Yamauchi D, et al. Effects of allogeneic bone marrow transplantation on recipient bone mineral density: A prospective study. *Biol Blood Marrow Transplant* 2000; 6: 344.
28. Cohen A, Sambrook P, Shane E. Management of bone loss after organ transplantation. *J Bone Miner Res* 2004; 19: 1919.
29. Cohen A, Shane E. Osteoporosis after solid organ and bone marrow transplantation. *Osteoporos Int* 2003; 14: 617.
30. Wang X, Chen H, Yin H, et al. Fertility after intact ovary transplantation. *Nature* 2002; 415: 385.
31. Schmidt KL, Andersen CY, Loft A, et al. Follow-up of ovarian function post-chemotherapy following ovarian cryopreservation and transplantation. *Hum Reprod* 2005; 20: 3539.
32. Wang X, Bilolo KK, Qi S, et al. Restoration of fertility in oophorectomized rats after tubo-ovarian transplantation. *Microsurgery* 2002; 22: 30.
33. Gosden RG. Survival of ovarian allografts in an experimental animal model. *Pediatr Transplantation* 2007; 11: 628.
34. Kerr JB, Duckett R, Myers M, et al. Quantification of healthy follicles in the neonatal and adult mouse ovary: Evidence for maintenance of primordial follicle supply. *Reproduction* 2006; 132: 95.
35. Hübner K, Fuhrmann G, Christenson LK, et al. Derivation of oocytes from mouse embryonic stem cells. *Science* 2003; 300: 1251.
36. Toyooka Y, Tsunekawa N, Akasu R, et al. Embryonic stem cells can form germ cells in vitro. *Proc Natl Acad Sci USA* 2003; 100: 1145.
37. Novak I, Lightfoot DA, Wang H, et al. Mouse embryonic stem cells form follicle-like ovarian structures but do not progress through meiosis. *Stem Cells* 2006; 24: 1931.
38. Danner S, Kajahn J, Geismann C, et al. Derivation of oocyte-like cells from a clonal pancreatic stem cell line. *Mol Hum Reprod* 2007; 13: 11.
39. Seoane A, Güerci A, Dulout F. Genetic instability induced by low doses of x-rays in hamster cells. *Int J Radiat Biol* 2007; 83: 81.
40. Milliat F, Francois A, Isoir M, et al. Influence of endothelial cells on vascular smooth muscle cells phenotype after irradiation: Implication in radiation-induced vascular damages. *Am J Pathol* 2006; 169: 1484.
41. Mazaud Guittot S, Guigon CJ, et al. Consequences of fetal irradiation on follicle histogenesis and early follicle development in rat ovaries. *Biol Reprod* 2006; 75: 749.
42. Gallardo TD, John GB, Shirley L, et al. Genomewide discovery and classification of candidate ovarian fertility genes in the mouse. *Genetics* 2007; 177: 179.
43. Chambers I, Silva J, Colby D, et al. Nanog safeguards pluripotency and mediates germline development. *Nature* 2007; 450: 1230.
44. Li J, Kwong DLW, Chan GCF. The effects of various irradiation doses on the growth and differentiation of marrow-derived human mesenchymal stromal cells. *Pediatr Transplant* 2007; 11: 379.
45. Ergun H, Howland WJ. Postradiation atrophy of mature bone. *CRC Crit Rev Diagn Imaging* 1980; 12: 225.
46. Hopewell JW. Radiation-therapy effects on bone density. *Med Pediatr Oncol* 2003; 41: 208.
47. Withold W, Wolf HH, Kollbach S, et al. Monitoring of bone metabolism after bone marrow transplantation by measuring two different markers of bone turnover. *Eur J Clin Chem Clin Biochem* 1996; 3 4: 193.

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Research Report

Intra-bone marrow-bone marrow transplantation slows disease progression and prolongs survival in G93A mutant SOD1 transgenic mice, an animal model mouse for amyotrophic lateral sclerosis

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ABSTRACT

It has been reported that bone marrow transplantation (BMT) has clinical effects on not only hematopoietic diseases and autoimmune diseases but also solid malignant tumors and metabolic diseases. We have found that intra-bone marrow-bone marrow transplantation (IBM-BMT) is superior to conventional intravenous BMT, since IBM-BMT enables rapid recovery of donor hematopoiesis and reduces the extent of graft-versus-host disease (GVHD). In this experiment, we examined the effects of IBM-BMT on symptomatic G93A mutant SOD1 transgenic mice (mSOD1 Tg mice), a model mouse line for amyotrophic lateral sclerosis (ALS). Symptomatic mSOD1 Tg mice (12 weeks old) were irradiated with 6 Gy×2 at a 4-hour interval, one day before IBM-BMT. The mice were transplanted with bone marrow cells (BMCs) from 12-wk-old eGFP-transgenic C57BL/6 mice (eGFP Tg mice) or BMCs from 12-wk-old mSOD1 Tg mice. The ALS model mice transplanted with BMCs from eGFP Tg mice showed longer survival and slower disease progression than those transplanted with BMCs from mSOD1 Tg mice or untreated mSOD1 Tg mice. There was a significantly high number of eGFP$^+$ cells in the anterior horn of the spinal cord of the mSOD1 Tg mice transplanted with BMCs of eGFP Tg mice, some of which expressed Iba-1, a marker of microglia, although they did not differentiate into neural cells. These results suggest that the replacement with normal hematopoietic cells improved the neural cell environment, thereby slowing the progression of the disease.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal and progressive neurodegenerative disease, which selectively affects motor neurons in the spinal cord, lower brainstem and cerebral cortex (Strong and Rosenfeld, 2003). The disease clinically shows progressive skeletal muscle atrophy and paralysis, and finally induces individual death, mainly due to respiratory failure,

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Abbreviations: ALS, amyotrophic lateral sclerosis; SOD1, superoxide dimustase 1; mSOD1 Tg mouse, G93A mutant SOD1 transgenic mouse; GVHD, graft-versus-host disease; BMCs, bone marrow cells; BMT, bone marrow transplantation; IBM-BMT, intra-bone marrow-BMT; eGFP, enhanced green fluorescent; eGFP Tg mouse, eGFP-transgenic mouse

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within a few years after onset. Although most cases are sporadic, approximately 10% of cases are familial type, and the disease in approximately 20% of familial patients is caused by dominantly inherited mutations in the gene encoding the antioxidant enzyme copper- and zinc-dependent superoxide dismutase 1 (SOD1) (Rosen et al., 1993). The G93A mutant SOD1 transgenic mouse (mSOD1 Tg mouse) is a model mouse line of familial ALS, and exhibits progressive degeneration of lower motor neurons coincident with the onset of limb tremors and muscle wasting. The mouse eventually dies due to its inability to feed itself (Gurney et al., 1994). Therefore, the mSOD1 Tg mouse is now widely used as a convenient tool not only for understanding the pathogenesis of ALS but also for the development of new therapeutic strategies for this disease.

Until now, investigations of ALS patients and the mSOD1 Tg mouse models have suggested the existence of several pathogenic mechanisms underlying the degenerative processes of ALS, including oxidative stress, excitotoxicity, chronic inflammation, mitochondrial and neurofilamental dysfunction, and ultimately activation of a programmed cell death pathway (Bruijn et al., 2004; Shaw, 2005). Based on these results, several therapeutic approaches have been attempted with ALS model animals, such as anti-oxidants, anti-excitotoxics, anti-inflammatories, immunomodulators, and neurotrophics, and some amelioration of the symptoms in these models has been reported (Benatar, 2007). Nevertheless, clinical trials using these therapies with human patients have been extremely limited. There has been no effective therapy for ALS except for riluzole, which is the only FDA-approved therapeutic agent for ALS.

The transplantation of bone marrow (BM) stem cells has been found to be effective for the treatment of neurological disorders, including stroke (Li et al., 2002), spinal cord injury (Chopp et al., 2000), multiple sclerosis (Saccardi et al., 2005; Van Wijmeersch et al., 2007), Parkinson's disease (Rodríguez-Gómez et al., 2007), Alzheimer's disease (Yamasaki et al., 2007), and Huntington's disease (Keene et al., 2007; Kim et al., 2008). For the treatment of ALS, recent reports have demonstrated that the intra-peritoneal or intravenous injections of murine BM cells (Corti et al., 2004) or human umbilical cord blood cells (Ende et al., 2000; Garbuzova-Davis et al., 2003) into the mSOD1 Tg mice ameliorates the symptoms and prolongs survival.

In the bone marrow transplantation study from normal mice to the mSOD1 Tg mice, Corti et al. used 4-wk-old asymptomatic mSOD1 Tg mice as recipients, and they induced mixed chimerism of hematopoietic cells by transplanting donor bone marrow cells (BMCs) into the peritoneal cavity. They were able to show that the BMT helped retard the onset of the disease and slow its progression.

We have shown that complete chimerism is superior to mixed chimerism, since mixed chimerism is not sufficiently stable to maintain donor hematopoietic cells in the recipients (Hayashi et al., 1997). Recently, we established a new method of bone marrow transplantation (BMT); intra-bone marrow-BMT (IBM-BMT), which enables a reduction in the pretreatment for BMT, rapid recovery of donor hematopoietic cells, and also reduces the extent of graft-versus-host disease (GVHD) (Ikehara, 2002, 2003; Kushida et al., 2001).

In this study, we prepared complete chimerism of the hematopoietic cells from enhanced green fluorescence protein (eGFP)-transgenic C57BL/6 mice (eGFP Tg mice) to symptomatic ALS model mice at an earlier stage of ALS by the combination of split irradiation of 6 Gy×2 and IBM-BMT, and examined the effects of the complete chimerism of hematopoietic cells on the symptomatic mSOD1 Tg mice.

2. Results

2.1. *IBM-BMT from eGFP Tg mice to symptomatic mSOD1 Tg mice slows disease progression and prolongs survival of the mice*

First, we examined whether the IBM-BMT could ameliorate the symptoms of the ALS-like disease in the mSOD1 Tg mice. The recipient mSOD1 Tg mice (12-wk-old female) at an early stage of the disease were irradiated with 6 Gy×2 (4-hour interval) and were then transplanted with BMCs from 12-wk-old eGFP Tg mice or 12-wk-old male mSOD1 Tg mice the following day. In this experiment, we prepared 3 groups: 1) non-treated female mSOD1 Tg mice (non-transplanted group), 2) female mSOD1 Tg mice transplanted with BMCs of male mSOD1 Tg mice using IBM-BMT (mSOD1-BM-transplanted group), and 3) female mSOD1 Tg mice transplanted with BMCs of eGFP Tg mice using IBM-BMT (eGFP-BM-transplanted group). None of the recipient mice ($n=25$) suffered from adverse effects, and our transplantation protocol was successfully carried out. When the recipient and donor mSOD1 Tg mice showed the symptoms, we carried out the IBM-BMT. That is, on the day we identified the onset of the disease in each mouse, we irradiated that mouse with 6.0 Gy×2. The mean ages of onset in the eGFP-BM-transplanted group, the mSOD1-BM-transplanted group, and the non-transplanted group were 89.0±1.1, 88.0±0.5, and 87.7±0.8 days, respectively. There were no significant differences between these three groups. Flow cytometric analyses revealed that all of the recipient mice transplanted with BMCs from eGFP Tg mice showed more than 90% donor-derived cells in the peripheral blood (95.2±1.2%) at 2 weeks after IBM-BMT.

The survival (time period from birth to end-stage sacrifice) and survival interval (time period from onset to end-stage sacrifice) of the eGFP-BM-transplanted group were 145.7±2.8 days and 56.7±3.1 days, being significantly longer than those of the mSOD1-BM-transplanted group (survival 135.9±2.4 days; $p<0.01$, survival interval 47.9±2.1 days; $p<0.05$) and non-transplanted group (survival 132.4±1.8 days; $p<0.01$, survival interval 44.7±1.8 days; $p<0.01$) (Fig. 1A). On the other hand, there were no significant differences in either the survival or survival interval between the mSOD1-BM-transplanted group and the non-transplanted group. In addition, the mean time period from onset to the day when the mice reached a 50% decrease in their grip strength in the eGFP-BM-transplanted, mSOD1-BM-transplanted, and non-transplanted groups was 51.6±3.9, 40.6±2.3, and 41.0±2.3 days, respectively. Similarly, the mean time period from onset to the day of a 10% decrease in body weight in each group was 46.4±3.9, 46.6±2.1, and 43.9±2.4 days, respectively (Figs. 1B,C). The results of log-rank tests demonstrated significant differences in the grip strength between the eGFP-BM-transplanted and the mSOD1-BM-transplanted groups ($p=0.0351$) or eGFP-BM-transplanted and the non-transplanted groups ($p=0.0081$). On the other hand, there

were no significant differences between the mSOD1-BM-transplanted and non-transplanted groups ($p=0.5277$). In contrast, statistical analyses demonstrated no significant differences in loss of body weight between the 3 groups.

2.2. *Histological examination of spinal cord in recipient mice*

To clarify the mechanisms underlying the effects induced by the IBM-BMT, we examined the spinal cord histologically. We found donor-derived eGFP$^+$ cells in the lumbar spinal cord parenchyma of the recipient mice, mostly in the ventral grey matter (Fig. 2A). All of the eGFP$^+$ cells expressed CD45, a marker for hematopoietic cells (data not shown). H&E staining after immunofluorescent investigation using the same specimens revealed that some of the migrated eGFP$^+$ cells showed the morphological feature of ramified and ameboid microglia accompanied by several apophyses (Figs. 2B and C).

The double immunofluorescence technique for eGFP and for Iba-1, GFAP, O4, or NeuN revealed the colocalization of the expression of eGFP and the expression of Iba-1 in the migrated cells (Figs. 2D–F). We could not identify any eGFP$^+$ cells labeled with the anti-GFAP, anti-NeuN, or anti-O4 antibodies (data not shown). H&E staining after the immunofluorescent study revealed that the Iba-1$^+$ eGFP$^+$ cells showed the morphological feature of ramified and ameboid microglia (Fig. 2G).

2.3. *Numbers of axons, motor neurons and microglia in spinal cord*

It has been reported that the number of axons and motor neurons decrease in mSOD1 mice with the progression of the disease, and that the number of microglia increases (Corti et al., 2004; Kang and Rivest, 2007; Gowing et al., 2008). Therefore, we counted the number of axons, motor neurons and microglia in the specimens obtained at the end point of the observation: namely, when the mice were sacrificed at the end point of the experiment, the spinal cords were removed and fixed with 4% paraformaldehyde. We detected 3.4±1.9 motor neurons per sciatic motor pool area in the mSOD1-BM-transplanted groups and 3.3±2.5 in the eGFP-BM-transplanted group, and 11.0±2.7 in wild type (Wt). There was no significant difference in the number of motor neurons between the mSOD1-BM-transplanted group and the eGFP-BM-transplanted group. We also calculated the mean number of axons in three randomly selected areas of 100 μm × 100 μm in the horizontal cross-section of the L5 ventral root. There was no significant difference in the numbers of axons between the mSOD1-BM-transplanted group (32.0±8.3) and the eGFP-BM-transplanted group (33.3±7.5). However, they were much lower than in the Wt (70±3.4) ($p<0.05$). The numbers of Iba-1 positive microglia were 52.6±5.8 in the mSOD1-BM-transplanted group and 54.5±6.8 in the eGFP-BM-transplanted group, and there were no significant

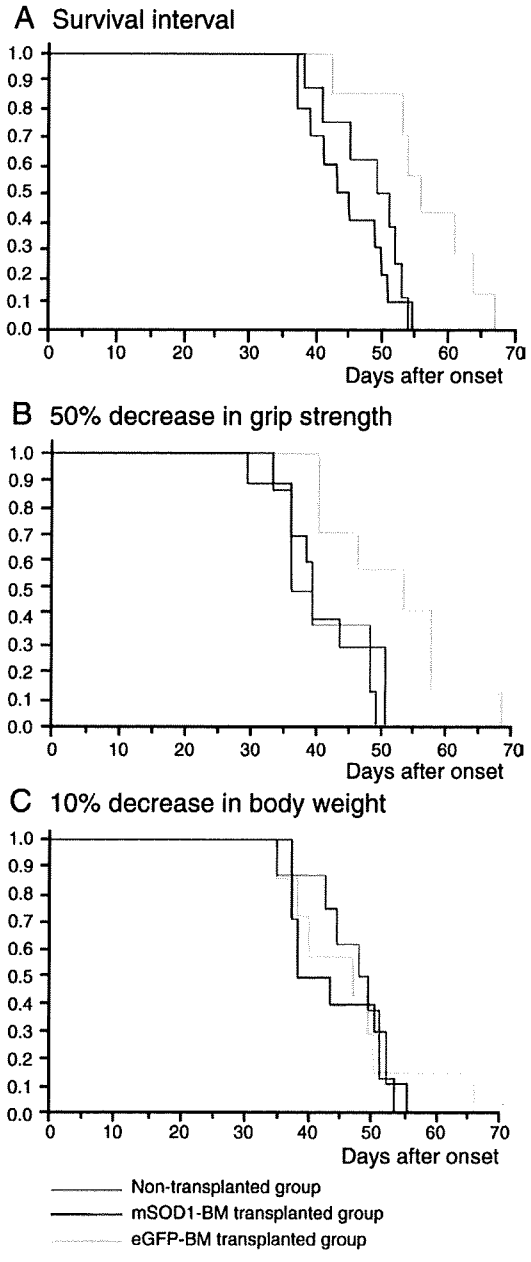

Fig. 1 – IBM-BMT can prolong the survival interval and slow the development of ALS in mSOD1 Tg mice. The mSOD1 Tg mice were randomly assigned into the following experimental groups: 1) non-treated female mSOD1 Tg mice (non-transplanted group) ($n=10$), 2) female mSOD1 Tg mice transplanted with BMCs of male mSOD1 Tg mice using IBM-BMT (mSOD1-BM-transplanted mice group) ($n=8$), and 3) female mSOD1 Tg mice transplanted with BMCs of male eGFP Tg mice using IBM-BMT (eGFP-BM-transplanted mice group) ($n=7$). All the donor mice were 12-wk-old symptomatic males. Each night, examiners who were blind to the information, weighed each mouse and measured the grip strength of their forelimbs using a Digital Grip Strength Meter. Each measurement was repeated 4 times and the maximum value was adopted. When each mouse lost the ability to autofeed or to right itself within 30 s. after having been placed on its side, it was sacrificed under deep anesthesia. "Survival interval" means the term from onset of the disease to the day that the mouse was sacrificed.

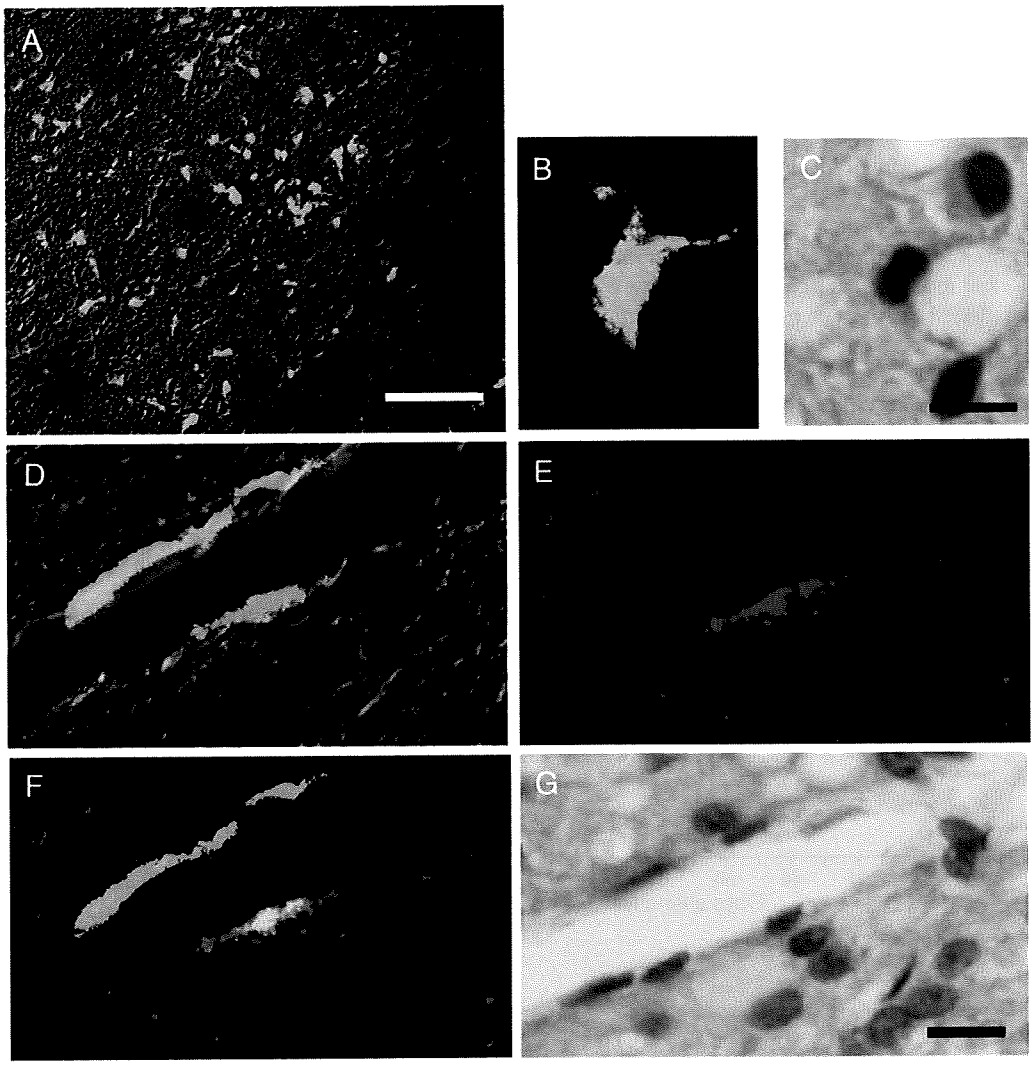

Fig. 2 – eGFP$^+$ cells infiltrated the anterior horn of the spinal cord in the recipient mice transplanted with BMCs of eGFP Tg mice by IBM-BMT. Symptomatic female mSOD1 Tg mice (12-wk-old) were irradiated with 6 Gy×2 (4 h interval) 1 day before IBM-BMT. The recipient mice were transplanted with BMCs of 12-wk-old eGFP Tg mice by IBM-BMT. When the recipient mice could no longer autofeed, they were sacrificed, and specimens were prepared as described in the "Experimental procedures", followed by observation using a confocal microscope. A. shows eGFP$^+$ cells expressing green fluorescence in the anterior horn of the spinal cord in a low-power field, while B. shows a photograph of it in a high-power field. C. shows the same cell as shown in B. stained with H&E. D, E and F show an eGFP$^+$ cell in the anterior horn of the spinal cord expressing Iba-1. D. shows eGFP$^+$ cells (green), E. shows Iba-1$^+$ cells (red), while F. shows a merged figure of eGFP and Iba-1. The single cell expresses both eGFP and Iba-1 (yellow). G. shows the same cell stained with H&E as the cell shown in D,E and F. White scale bar: 100 μm. Black scale bar: 10 μm.

differences between them. However, these numbers were significantly more than that of Wt (13.5±3.7).

2.4. *Effects of chimerism of donor hematopoietic cells on number of infiltrating donor cells in spinal cord, and on survival*

Corti et al. reported that BMT from wild-type to asymptomatic mSDO1 Tg mice ameliorated the symptoms and prolonged the survival of the Tg mice (Corti et al., 2004). Our results are thus consistent with their results. However, Corti et al. prepared mixed chimera by combining 8 Gy-irradiation with the injection of donor BMCs into the recipient's peritoneal cavity. Therefore, we examined the effects of chimerism of hematopoietic cells from eGFP Tg mice on the status of mSOD1 Tg mice. We used an irradiation dose of 5.5 Gy×2 or 6.0 Gy×2 and the recipient mSOD1 Tg mice were transplanted with BMCs from 12-wk-old eGFP Tg mice using IBM-BMT. We then compared the results with those from mSOD1 Tg mice irradiated at 6.0 Gy×2 followed by IBM-BMT with BMCs from eGFP Tg mice. The mean

percentage of hematopoietic engraftment of the mSOD1 Tg mice irradiated with 5.5 Gy×2 was 87.5%, which was lower than the 95.2% in the mSOD1 Tg mice irradiated with 6.0 Gy×2.

On the other hand, the mean survival and survival interval in the 5.5 Gy×2 irradiated mice were 140 days and 52 days, being shorter than the 6 Gy×2 irradiated Group (145.7 days and 56.7 days). The mean number of migrated eGFP$^+$ cells in one recipient lumbar anterior horn of the spinal cord in one slice was 32.1 cells in 5.5 Gy×2 mSOD1 Tg mice and 58.8 cells in 6.0 Gy×2 mSOD1 Tg mice (Figs. 3A and B). We also examined the number of Iba-1$^+$/eGFP$^+$ cells in one recipient anterior horn of the spinal cord in the 5.5 Gy×2 irradiated mSOD1 Tg mice and 6.0 Gy×2 irradiated mSOD1 Tg mice. The mean number of Iba-1$^+$/eGFP$^+$ cells in the recipient anterior horn in one slice was 2.5 cells in the 5.5 Gy×2 irradiated mice and 4.1 cells in 6.0 Gy×2 irradiated mice.

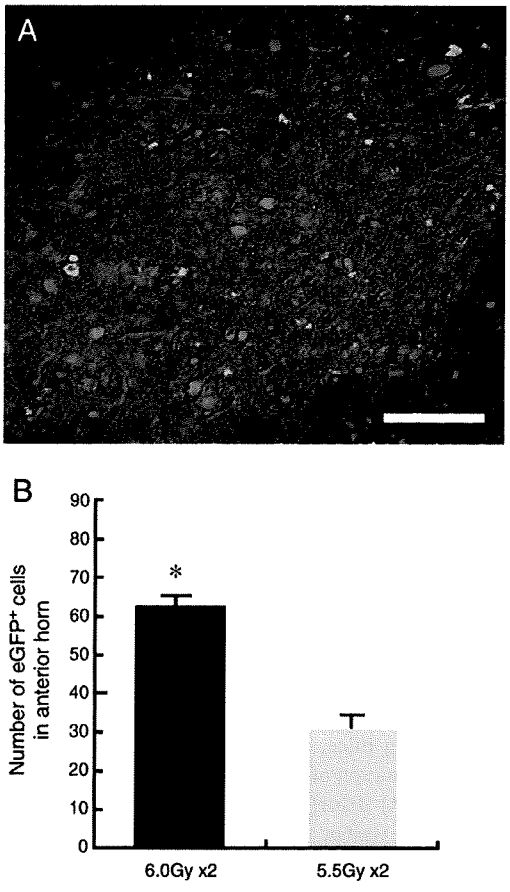

Fig. 3 – Reduced reconstitution rate of hematopoietic cells reduces the effect of BMT. Female 12-wk-old symptomatic mSOD1 Tg mice were irradiated with 5.5 Gy×2 one day before IBM-BMT. The mSOD1 Tg mice were transplanted with BMCs of eGFP Tg mice using IBM-BMT. When the mice could no longer autofeed, they were sacrificed and eGFP$^+$ cells in the spinal cord were histologically examined (A). eGFP$^+$ cells in the anterior horn in one slice were counted and compared with the 6 Gy×2 irradiated mSOD1 Tg mice. Representative data are shown in panel B. *: significant difference to 5.5 Gy×2 irradiated mSOD1 Tg mice.

3. Discussion

In this paper, we have examined the effects of allogeneic IBM-BMT on the symptomatic mSOD1 Tg mice. IBM-BMT slowed the disease progression and prolonged the survival in these mice. In the recipient mice, some microglia in the spinal cord expressed eGFP, which is a marker of donor cells, suggesting that the replacement with normal hematopoietic cells, including microglia, slowed the progression of the disease. As far as we can ascertain, this is the first report of the effects of BMT on symptomatic mSOD1 Tg mice.

Corti et al. transplanted BMCs from normal mice into mSOD1 Tg to induce mixed chimerism of hematopoietic cells, and retarded the onset of the disease and prolonged survival (Corti et al., 2004). In their experiment, they used 4-wk-old mice that still did not show any symptoms. However, it is very difficult to predict which human patients will develop ALS and it is also probably unethical to carry out BMT on asymptomatic patients even if they are carrying an abnormal gene. In this experiment, we used symptomatic 12-wk-old mSOD1 Tg mice, and induced significantly donor-predominant chimerism of the hematopoietic cells of normal mice, thereby slowing disease progression and prolonging survival.

It has been reported that IBM-BMT is superior to conventional BMT in the suppression of GVHD and tolerance induction (Kushida et al., 2001). Therefore, we carried out IBM-BMT, with the result that more than 90% of the peripheral blood nuclear cells were reconstituted into donor type without GVHD. For the pretreatment of recipient mice, we irradiated mSOD1Tg mice at "6.0 Gy×2" with a 4-hour interval, since we have found that fractionated irradiation provides better results than single irradiation (Cui et al., 2002; Kushida et al., 2001; Takeuchi et al., 1998), and that 4h is the optimal interval for fractionated irradiation in mice (Cui et al., 2002).

It has been reported that autoimmunity is related to the development of ALS (Appel et al., 1996). We have treated several model mouse strains for autoimmune diseases by BMT (Ikehara et al., 1994; Ikehara, 1998, 2002; Kushida et al., 2001). In our experiments, we showed that BMT had significant effects on the treatment of the autoimmune diseases in model mice: the autoimmune diseases disappeared and the transplanted mice survived as long as normal mice. However, in this study, we showed that the effects of BMT on mSOD1Tg mice were limited. Therefore, even if the development of ALS is associated with an autoimmune mechanism, it is unlikely that the autoimmune mechanism is the main factor in its development.

To investigate the efficacy of stem cell therapy for ALS, a variety of cell sources have been applied for mouse models for ALS, including human umbilical cord blood stem (HuCB) cells (Chen and Ende, 2000; Ende et al., 2000; Garbuzova-Davis et al., 2003; Habisch et al., 2007), human neural stem cells (Klein et al., 2005; Xu et al., 2006), rodent BM stem cells (Corti et al., 2004; Solomon et al., 2006), human BM stem cells (Hermann et al., 2004), and human neuron-like teratoma cells (hNT cells) (Garbuzova-Davis et al., 2001, 2002, 2006; Willing et al., 2001). These cells have been administered to ALS model mice intravenously (Ende et al., 2000; Garbuzova-Davis et al., 2001; Hermann et al., 2004; Solomon et al., 2006;), intraperitoneally (Corti et al., 2004), lumbar subcutaneously (Corti et al., 2007;

Garbuzova-Davis et al., 2001, 2002, 2006), or intrathecally (Habisch et al., 2007). There are also some reports indicating that neural stem cells improved neurological symptoms in rat models of the same phenotype (Klein et al., 2005; Xu et al., 2006).

Recently, the effects of stem cell therapy on mSOD1 Tg mice have been reported to possibly be due not to neurogenesis but neuroprotection as a result of the secretion of neurotrophic factor(s) derived from donor-derived cells (Beers et al., 2006; Corti et al., 2004; Kang and Rivest, 2007). The mutant SOD1 expressed in neurons alone or astrocytes alone failed to induce a significant motor neuron loss in G37R mutant SOD1 Tg or G86R mutant SOD1 Tg mice (Lino et al., 2002; Pamatarova et al., 2001; Gong et al., 2000). Clement et al. also reported that nonneuronal cells not expressing mutant SOD1 delay degeneration and significantly extend the survival of motor neurons expressing mutant SOD1, suggesting that an improvement in the environment of neural cells is crucial for the treatment of ALS (Clement et al., 2003). In our study, we found no donor-derived neural cells but we did find donor-derived microglia in the recipient spinal cord. Our results are consistent with the results of Corti et al. (2004) Beers et al. (2006) and Boillée et al. (2006). They show that the delayed onset, delayed progression of the symptoms and prolonged survival of mSOD1 Tg mice and $PU^{-/-}$ mSOD1 Tg mice were induced by BMT from normal mice to the mSOD1 Tg mice and $PU^{-/-}$ mSOD1 Tg mice in the preclinical stage.

It has been reported that the numbers of motor neurons and axons decrease after onset in the mSOD1 Tg mice (Corti et al., 2004), and that the number of microglia increases (Gowing et al., 2008). It has also been reported that BMT from normal mice to mSOD1 Tg mice retards the decrease in the numbers of motor neurons and axons. Therefore, we calculated the numbers of motor neurons, axons and microglia. The numbers of motor neurons and axons decreased in comparison with Wt mice, and the number of microglia increased. However, there were no significant differences in the numbers of motor neurons, axons and microglia between the mSOD1-BM-transplanted group and the eGFP-BM-transplanted group. In our experiment, the mice were sacrificed at the end point of the disease, when the mice could no longer feed by themselves. Namely, we sacrificed the mice at different ages. Corti et al. sacrificed the mice at the same age, 100 days after birth, and showed significant differences in the numbers of the motor neurons and axons between eGFP-BM-transplanted mice and mSOD1-BM-transplanted mice. Therefore, the differences between their results and ours could be attributable to the timing of sacrifice. It has been reported that BMT from mSOD1 Tg mice into $PU^{-/-}$ mSOD1Tg mice failed to ameliorate the symptoms of ALS-like disease but that BMT from eGFP Tg mice into $PU^{-/-}$ mSOD1Tg mice had some effects (Beers et al., 2006). They found donor-derived $eGFP^+$ microglia in the recipients' spinal cord. We also found donor-derived $eGFP^+$ microglia in the recipients' spinal cord. However, the number of microglia was too small (4.1 cells per lumbar anterior horn) in comparison with $eGFP^+$ cells (58.8 cells). Recently, it has been reported that T cells are also important in retarding the disease progression (Beers et al., 2008). Therefore, not only microglia but also other types of hematopoietic cells are associated with the retardation of the disease progression. Somehow, these results suggest that the infiltration of normal hematopoietic cells, including microglia (not carrying mutant SOD1), into the recipients' spinal cord is crucial for the retardation of the ALS-like disease. Further examinations are warranted with other types of mSOD1 Tg mice and with appropriate model animals to clarify the mechanisms underlying the effects of BMT on the amelioration of ALS.

As we previously described, the greater the predominancy of the donor hematopoietic cells induced, the more stable was the tolerance to donor antigens (Hayashi et al., 1997). Therefore, we examined the effects of the reduction of the irradiation dose from 6.0 Gy×2 to 5.5 Gy×2, on the status of ALS-like disease. The reduction in the irradiation induced a reduction in the numbers of donor-derived $eGFP^+$ cells and $Iba\text{-}1^+/eGFP^+$ cells infiltrating the spinal cord and a shortening of survival. These results suggest that the replacement of recipient hematopoietic cells carrying mutant SOD1 with donor hematopoietic cells carrying the normal genotype is necessary for an improvement in the disease.

In the present study, we have shown that IBM-BMT can also slow the progression of the symptoms of mSOD1 Tg mice and prolong survival, even after onset, and that the migration of donor hematopoietic cells, including microglia, into the recipient spinal cord is the most plausible explanation for the improvement.

4. Conclusions

We have shown the effects of allogeneic IBM-BMT on mice expressing the mutant SOD1, the ALS model mice. The effects are possibly due to an improvement in the environment of the spinal cord resulting from the replacement of hematopoietic cells, including microglia carrying the mutant gene with normal hematopoietic cells. However, the effect was not as pronounced as with our previous BMT therapy using autoimmune model mice. Therefore, we believe there may be merit in attempting other approaches, including a combination of new medicine(s) combined with IBM-BMT.

5. Experimental procedures

5.1. *Animals*

Male mSOD1 Tg mice, which are heterozygous for the ALS-linked G93A mutation of the human gene for SOD1 (TgN [B6SJL-Tg (SOD1-G93A) 1Gur]), were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type female B6SJLF1/J mice were bred and paired with the heterozygous mSOD1 Tg male mice. The mSOD1 Tg mice were identified using an ASTEC research thermal cycler for polymerase chain reaction amplification of mouse DNA extracted from tail snips (Rosen et al., 1993). The detailed protocol was described previously (Wate et al., 2005).

Heterozygous female mSOD1 Tg mice were used as recipients, since there are some differences in disease progression between genders in the mice (Heiman-Patterson et al., 2005).

As donors of therapeutic BM stem cells, eGFP Tg mice (C57BL/6 background) were kindly donated by Dr. Okabe (Okabe et al., 1997). Procedures involving animals and their care were conducted in conformity with our institutional

guidelines, which are in compliance with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

5.2. *Intra-bone marrow-bone marrow transplantation (IBM-BMT)*

We carried out IBM-BMT on 12-wk-old mSOD1 Tg mice at an early stage of the disease. We determined the onset of the symptom in each mouse by the manifestation of a leg tremor on 2 consecutive days. The symptomatic recipient mice were exposed to a total radiation dose of 5.5 Gy×2 or 6.0 Gy×2 (1.0 Gy/min with a 4 h interval) from a ^{137}Cs source (Gamma cell 40 Exactor; MDS Nordion International Inc., Ottawa, Ontario, Canada). The day after the total body irradiation, bone marrow cells (BMCs) from the eGFP Tg mice (male, 12-wk-old) or symptomatic mSOD1 Tg mice (male, 12-wk-old) were transplanted into the recipient female mice using the IBM-BMT technique.

Donor BMCs were transplanted into the bone marrow cavity of both tibias of the recipients (3.0×10^7 cells in each bone, in total 6.0×10^7 cells per mouse) using the IBM-BMT method, as previously described (Kushida et al., 2001). The mice were observed until they could no longer feed by themselves.

5.3. *Flow cytometric analysis of hematopoietic engraftment*

For analysis of the percentages of hematopoietic engraftment, peripheral blood was obtained from the tail vein of the recipient mice 2 weeks after transplantation. Red blood cells were lysed with 0.15 M NH_3Cl and the samples were stained with a PE-conjugated anti-CD45 antibody (BD Bioscience Pharmingen, San Jose, CA). The percentage of donor cells in the peripheral blood in each transplanted mouse was calculated as (percentage of both eGFP$^+$ and CD45$^+$ cells)×100 / (percentage of CD45$^+$ cells). Flow cytometric analysis was performed using a FACScan (Becton Dickinson; Mountain View, CA, USA).

5.4. *Histological examination*

When the recipient mice became unable to feed by themselves, they were anaesthetized using diethyl ether and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS as a fixative. The spinal cord at the level of L3 to L4 was sampled from each mouse, embedded in paraffin, and sectioned sequentially throughout into sections of 7-micrometer thickness for histological study.

In order to avoid counting the same cell twice in consecutive sections, every 5th section from each mouse was deparaffinized, washed in PBS, incubated in 3% H_2O_2 in methanol for 15 min to inhibit endogenous peroxidase activity, washed in PBS, and blocked in PBS containing 3% normal bovine serum albumin (PBS-BSA) for 1 h at room temperature. The sections were then incubated with the rabbit polyclonal antibody against Iba-1 (Wako Pure Chemical Industries, Osaka, Japan; diluted 1:100 with PBS-BSA, with the rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP: Millipore Corporation, Billerica, MA, USA; 1:200), with the mouse monoclonal antibody against oligodendrocyte marker O4 (Millipore Corporation, 1:150), or with the mouse monoclonal antibody against neuronal nuclei (NeuN: Millipore Corporation; 1:200) overnight at 4 °C. After washing in PBS, the sections were incubated with the secondary antibody (Alexa Fluor 647 goat anti-rabbit or anti-mouse IgG (H+L) high cross-adsorbed: Invitrogen Corporation, Carlsbad, CA, USA; 1:500) for 1 h at room temperature. Alternatively, the samples were stained with PE-labeled anti-CD45 antibody. More than 10 samples were prepared for each Ab.

Using a laser scanning confocal microscope (Olympus FluoView™ FV300 Version 4.3, Olympus Corporation, Tokyo, Japan), neuropathologists unaware of the identity of sections counted the numbers of eGFP$^+$, eGFP$^+$/Iba-1$^+$, eGFP$^+$/GFAP$^+$, eGFP$^+$/O4$^+$, or eGFP$^+$/NeuN$^+$ cells within the anterior horn region.

After examination using a confocal microscope, some sections were rinsed and then stained with hematoxylin and eosin (H&E) to identify the morphological features of the eGFP$^+$ cells.

The staining specificity was assessed by replacing the primary antibody with PBS-BSA. No deposits of reaction products were seen in the sections thus treated.

5.5. *Evaluation of the effects of IBM-BMT on grip strength and body weight*

Each night, examiners who were blind to the information weighed each mouse, and measured the grip strength of their forelimbs using a Digital Grip Strength Meter (Columbus Instruments, OH, USA). Each measurement was repeated 4 times and the maximum value was adopted.

When each mouse had lost the ability to autofeed or to right itself within 30 s. after having been placed on its side, it was sacrificed under deep anesthesia. This provided us with the "survival day" and "survival interval" for each mouse. The "survival interval" was defined as the time period from onset of the disease to the day when the mouse was sacrificed.

5.6. *Statistical analyses*

All of the data were expressed as the mean±standard error of the mean (SEM). Statistical analyses were performed using JMP statistical discovery software version 7 (SAS Institute, Cary, NC, USA). The data concerning the numbers of the GFP$^+$ cells in the anterior horn of the mSOD1 Tg mice irradiated with 6 Gy×2 and 5.5 Gy×2 were analyzed by the Wilcoxon rank sum.

The data of survival interval, days for reduction of the grip strength by more than 50% of that at onset, and days for decrease in body weight by more than 10% of that at onset were analyzed by the Kaplan–Meyer method with Wilcoxon rank sum test.

Differences between groups were analyzed using Wilcoxon rank sum test and $p<0.05$ was considered to be significant.

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