alone or in control B6 mice, but significantly higher than in the mice treated with IBM-BMT + DLI. The low number of CD4 T cells in mice treated with IBM-BMT + DLI may result from the progression of GVHD. Much evidence has recently accumulated that Tregs are involved in the regulation of GVHD in mice,[42,43] and that, in humans, the reduced frequency of Tregs is also observed in patients with chronic GVHD and is negatively correlated with its severity.[44] This was the case in the present study, in which severe GVHD was observed in recipients with few Tregs after IBM-BMT + DLI, while only mild GVHD was observed in recipients possessing relatively high numbers of Tregs in the spleen after IBM-BMT + ATT. IBM-BMT alone produced no GVHD and a high proportion of Tregs, comparable to that in non-treated mice. These findings strongly suggest the participation of Tregs in the inhibition or negative regulation of GVHD.

Next, we investigated GVT effects associated with IBM-BMT + ATT. Interestingly, the growth of Meth-A tumours was most significantly inhibited by IBM-BMT + ATT, compared with non-treatment. IBM-BMT alone also produced significant regression, compared with non-treatment. This may be partially attributable to the effects of irradiation. In addition, IBM-BMT plus a high dose of DLI also induced strong tumour regression, but death from severe GVHD, whereas IBM-BMT plus a low dose of DLI elicited less tumour regression but mild GVHD with a long survival time. In histological analysis, IBM-BMT + ATT produced marked lymphocyte infiltration inside the tumour involving significant high numbers of donor-derived CD8 T cells compared with IBM-BMT alone. In addition, IFN-γ, which is a T helper type 1 (Th1) cytokine, also showed significantly high production in the mice with IBM-BMT + ATT, compared with the non-treated mice and the mice treated with IBM-BMT alone. Because IFN-γ itself has a GVT effect, it may not only promote an immune response with GVH effects, but also facilitate tumour regression. The mice treated with IBM-BMT alone also showed higher IFN-γ production than did the non-treated mice, suggesting that not only irradiation but also IFN-γ may play a role in tumour regression in these mice, too. As a result, the tumour cells also displayed significantly increased apoptosis. Although other tumour cell lines should be examined, these findings indicate that IBM-BMT + ATT induces strong GVT effects with donor cytotoxic $CD8^+$ T lymphocytes against the tumour.

In the MLR analyses, T cells showed a low anti-host response in the mice treated with IBM-BMT + ATT. As the mice treated with IBM-BMT alone showed no response to host cells, the difference is probably derived from ATT. Notably, the anti-host response was lower than in the mice treated with IBM-BMT + DLI. In addition, there was a positive correlation with the degree of GVHD and a negative correlation with the percentage of Tregs.

We could thus induce GVT effects without inducing severe GVHD by treatment with allogeneic IBM-BMT + ATT. Although the details of the mechanisms are still unknown, there are a number of possibilities. One is that Tregs also function to create the GVT effect in recipients treated with IBM-BMT + ATT, as it has been reported that Tregs suppress GVHR induced by CD4 T cells, but do not reduce GVT effects induced by CD8 T cells;[29,45] Tregs suppress the peripheral proliferation of CD8 T cells but do not inhibit cytotoxic T lymphocyte (CTL) activity. Thus, the intermediate proportion of $CD4^+$ $FoxP3^+$ Tregs induced by IBM-BMT + ATT can only achieve incomplete – but continuous – inhibition of GVHR but can maintain CTL activity, which leads to strong GVT effects. The other possibility, from the viewpoint of effector T-cell development, is that, in the case of IBM-BMT + DLI, the mature T cells with allo-MHC reactivity that were present easily induced severe GVHD. In the case of IBM-BMT + ATT, the reactivity of allo-specific T cells derived from the grafted thymus might be insufficient as a result of incomplete repertoire formation, as the thymic dendritic cells in the engrafted B6 thymus may present BALB/c-derived molecules (including their MHC) in negative selection during thymopoiesis. However, tumour-specific antigens are difficult to detect in the transplanted thymus because the tumour itself is located far from the thymus. As a result, the small number of allo-specific T cells (but not Tregs) may have proliferated peripherally and led to the low ratio of Tregs in CD4 T cells. To confirm these findings, further studies are required of the expression of CD4, CD8 and Foxp3 staining in GVHD and GVT sites. In addition, direct transfer and/or deletion experiments using Tregs should be carried out.

The continuous supplementation of T cells from the allogeneic ATT may induce mild GVHD and strong GVT effects with well-balanced effector and regulatory T cells. Alternatively, the transplanted thymus itself may regulate homeostasis of the cells. Thymus transplantation thus initially appears to be a simple method, but may prove to be an effective approach in that it supplies the organ in which T cells are differentiated, produced and functionally regulated. The method may be adequate to cure slow progressive diseases such as cancers, whereas the direct transfer of T cells, including Tregs, may be adequate for acute diseases such as infection and in the case of acute rejection.

Overall, we have found that allogeneic IBM-BMT + ATT induces high thymopoiesis with a mild GVH reaction and elicits strong GVT effects. Although it may clinically be difficult to obtain adequate thymus ethically and technically (with problems including donor age), grafts could be obtained from patients with congenital heart diseases or from aborted fetuses, as previously utilized.[33] In this respect, we have recently found that, even if the thymus

donor is different from the donor of BMCs, the effect is comparable to that seen with transplantation from the same donor using triple chimeric mice.[46] In addition, a method of regenerating the thymus has also been developed dramatically.[47] We thus believe that IBM-BMT + ATT could become a viable strategy for the treatment of malignant tumours in humans.

Acknowledgements

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References

1 Thomas ED. Landmarks in the development of hematopoietic cell transplantation. *World J Surg* 2000; **24**:815–8.

2 Bacigalupo A, Oneto R, Bruno B *et al.* Current results of bone marrow transplantation in patients with acquired severe aplastic anemia. Report of the European Group for Blood and Marrow transplantation. On behalf of the Working Party on Severe Aplastic Anemia of the European Group for Blood and Marrow Transplantation. *Acta Haematol* 2000; **103**:19–25.

3 Chen RL, Hou JW, Chang PY, Tsai FJ, Wang PJ. Matched unrelated bone marrow transplantation without splenectomy for a child with Gaucher disease caused by homozygosity of the L444P mutation, who also suffered from schizencephaly. *J Pediatr Hematol Oncol* 2007; **29**:57–9.

4 Sato T, Kobayashi R, Toita N, Kaneda M, Hatano N, Iguchi A, Kawamura N, Ariga T. Stem cell transplantation in primary immunodeficiency disease patients. *J Pediatr Hematol Oncol* 2007; **29**:57–9.

5 Kanamori H, Tanaka M, Kawaguchi H, Yamaji S, Fujimaki K, Tomita N, Fujisawa S, Ishigatsubo Y. Resolution of psoriasis following allogeneic bone marrow transplantation for chronic myelogenous leukemia: case report and review of the literature. *Am J Hematol* 2002; **71**:41–4.

6 Saba N, Flaig T. Bone marrow transplantation for nonmalignant diseases. *J Hematother Stem Cell Res* 2002; **11**:377–87.

7 Ueno NT, Rizzo JD, Demirer T *et al.* Allogeneic hematopoietic cell transplantation for metastatic breast cancer. *Bone Marrow Transplant* 2008; **41**:537–45.

8 Kolb HJ, Schattenberg A, Goldman JM *et al.* European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 1995; **86**: 2041–50.

9 Saito TI, Rubio MT, Sykes M. Clinical relevance of recipient leukocyte infusion as antitumor therapy following nonmyeloablative allogeneic hematopoietic cell transplantation. *Exp Hematol* 2006; **34**:1271–7.

10 Ohmori I, Hayamizu K, Oishi K, Yoshimitsu M, Itamoto T, Asahara T. Inhibition of tumor growth in immunocompromised hosts by restoring type-2 immunity using infusion of G-CSF-treated allogeneic CD8+ leukocytes. *Cytokine* 2005; **32**:255–62.

11 Russell NH, Byrne JL, Faulkner RD, Gilyead M, Das-Gupta EP, Haynes AP. Donor lymphocyte infusions can result in sustained remissions in patients with residual or relapsed lymphoid malignancy following allogeneic haemopoietic stem cell transplantation. *Bone Marrow Transplant* 2005; **36**:437–41.

12 Chao NJ. Graft-versus-host disease. The view point from the donor T cell. *Biol Blood Marrow Transplant* 1997; **3**:1–10.

13 Goulmy E, Schipper R, Blokland E, Falkenburg F. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host-disease after bone marrow transplantation. *N Engl J Med* 1996; **334**: 281–5.

14 Murphy WJ, Blazar BR. New strategies for preventing graft-versus-host disease. *Curr Opin Immunol* 1999; **11**:509–15.

15 Hosaka N, Nose M, Kyogoku M, Nagata N, Miyashima S, Good RA, Ikehara S. Thymus transplantation, a critical factor for correction of autoimmune disease in aging MRL/+mice. *Proc Natl Acad Sci USA* 1996; **93**:8558–62.

16 Kushida T, Inaba M, Takeuchi K, Sugiura K, Ogawa R, Ikehara Y. Treatment of intractable autoimmune disease in MRL/lpr mice using a new strategy for allogeneic bone marrow cells transplantation. *Blood* 2000; **95**:1862–8.

17 Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, Iida H, Ikehara S. 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**:3292–9.

18 Nakamura K, Inaba M, Sugiura K, Yoshimura T, Kwon AH, Kamiyama Y, Ikehara S. Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of graft-versus-host diseases (GvHD) by intra-bone marrow-bone marrow transplantation plus donor lymphocyte infusion. *Stem Cells* 2004; **22**:125–34.

19 Hashimoto F, Sugiura K, Inoue K, Ikehara S. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. *Blood* 1997; **89**:49–54.

20 Sugiura K, Hisha H, Ishikawa J, Adachi Y, Taketani S, Lee S, Nagahama T, Ikehara S. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. *Stem Cells* 2001; **19**:46–58.

21 Ikehara S. A novel strategy for allogeneic stem cell transplantation: perfusion method plus intra-bone marrow injection of stem cells. *Exp Hematol* 2003; **31**:1142–6.

22 Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; **99**:3838–43.

23 Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, Ringdén O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; **363**:1439–41.

24 Moore NC, Anderson G, Smith CA, Owen JJ, Jenkinson EJ. Analysis of cytokine gene expression in subpopulations of freshly isolated thymocytes and thymic stromal cells using semiquantitative polymerase chain reaction. *Eur J Immunol* 1993; **23**:922–7.

25 Bodey B, Bodey B Jr, Siegel SE, Kaiser HE. Review of thymic hormones in cancer diagnosis and treatment. *Int J Immunopharmacol* 2000; **22**:261–73.

26 Ziegler SF, Liu YJ. Thymic stromal lymphopoietin in normal and pathogenic T cell development and function. *Nat Immunol* 2006; **7**:709–14.

27 Sakaguchi S, Ono M, Setoguchi R *et al.* Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 2006; **212**:8–27.

28 Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, Cohen JL. Recipient-type specific CD4+ CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest* 2003; **112**:1688–96.

29 Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, Negrin RS. CD4+ CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 2003; **9**:1144–50.

30 Hosaka N, Ryu T, Miyake T, Cui W, Nishida T, Takaki T, Inaba M, Ikehara S. Treatment of autoimmune diseases in MRL/lpr mice by allogeneic bone marrow transplantation plus adult thymus transplantation. *Clin Exp Immunol* 2007; **147**:555–63.

31 Ryu T, Hosaka N, Miyake T *et al.* Transplantation of newborn thymus plus hematopoietic stem cells can rescue supralethal irradiated mice. *Bone Marrow Transplant* 2008; **41**:659–66.

32 van den Brink MR, Alpdogan O, Boyd RL. Strategies to enhance T-cell reconstitution in immunocompromised patients. *Nat Rev Immunol* 2004; **4**:856–67.

33 Markert ML, Boeck A, Hale LP, Kloster AL, McLaughlin TM. Transplantation of thymus tissue in complete DiGeorge syndrome. *N Engl J Med* 1999; **341**:1180–9.

34 Markert ML, Hicks CB, Bartlett JA, Harmon JL. Hale Effect of highly active antiretroviral therapy and thymic transplantation on immunoreconstitution in HIV infection. *AIDS Res Hum Retroviruses* 2000; **16**:403–13.

35 Hill GR, Cooke KR, Teshima T, Crawford JM, Keith JC Jr, Brinson YS, Bungard D, Ferrara JL. Interleukin-11 promotes T cell polarization and prevents acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Clin Invest* 1998; **102**:115–23.

36 Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* 1997; **90**:3204–13.

37 Hosaka N, Minato T, Yoshida S, Toki J, Yang G, Hisha H, Ikehara S. Kimura's disease with unusual eosinophilic epithelioid granulomatous reaction: a finding possibly related to eosinophil apoptosis. *Hum Pathol* 2002; **33**:561–4.

38 Hosaka N, Kitajiri S, Hiraumi H, Nogaki H, Toki J, Yang G, Hisha H, Ikehara S. Ectopic pituitary adenoma with malignant transformation. *Am J Surg Pathol* 2002; **26**:1078–82.

39 Sempowski GD, Gooding ME, Liao HX, Le PT, Haynes BF. T cell receptor excision circle assessment of thymopoiesis in aging mice. *Mol Immunol* 2002; **38**:841–8.

40 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**:399–405.

41 Suzuki Y, Adachi Y, Minamino K, Zhang Y, Iwasaki M, Nakano K, Koike Y, Ikehara S. A new strategy for treatment of malignant tumor: intra-bone marrow-bone marrow transplantation plus $CD4^-$ donor lymphocyte infusion. *Stem Cells* 2005; **23**: 365–70.

42 Miura Y, Thoburn CJ, Bright EC, Arai S, Hess AD. Association of Foxp3 regulatory gene expression with graft-versus-host disease. *Blood* 2004; **104**:2187–93.

43 Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* 2002; **196**:389–99.

44 Hoffmann P, Edinger M. CD4+ CD25+ regulatory T cells and graft-versus-host disease. *Semin Hematol* 2006; **43**:62–9.

45 Sakaguchi S. Taming transplantation with T cells. *Nat Med* 2003; **9**:1117–8.

46 Cui W, Hosaka N, Miyake T *et al.* Analysis of tolerance induction using triple chimeric mice: MHC-disparate thymus, hemopoietic cells and microenvironment. *Transplantation* 2008; **85**:1151–8.

47 Zhang L, Sun L, Zhao Y. Thymic epithelial progenitor cells and thymus regeneration: an update. *Cell Res* 2007; **17**:50.

A New Bone Marrow Transplantation Method for Stem Cell Disorders

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Using animal models for autoimmune diseases, we have previously shown that allogeneic bone marrow transplantation (BMT) can be used to treat autoimmune diseases and that autoimmune diseases are hemopoietic stem cell (HSC) disorders. We have recently developed a new BMT method. The method consists of a perfusion method (PM) plus intrabone marrow (IBM)-BMT. The PM, in comparison with conventional aspiration, can minimize the contamination of BMCs with T cells from peripheral blood, and consequently no graft-versus-host disease develops. Because bone marrow cells collected by the PM contain not only HSCs but also mesenchymal stem cells (MSCs), the injection of both cells directly into the bone marrow cavity facilitates the engraftment of donor hemopoietic cells. Using this new method, we show that most age-associated diseases, such as osteoporosis and emphysema, are MSC disorders and, on the basis of this evidence, propose a new concept of stem cell disorders, including HSC and MSC disorders. We believe that the advent of IBM-BMT and our proposed concept herald a revolution in the field of transplantation (BMT and organ transplantation) and regeneration medicine.

Key words: **bone marrow transplantation; stem cell disorder; hemopoietic stem cell; mesenchymal stem cell; perfusion method; intrabone marrow; autoimmune disease; osteoporosis; emphysema**

Introduction

In 1985, we found that allogeneic bone marrow transplantation (BMT) (but not autologous BMT) could be used to prevent and treat autoimmune diseases in autoimmune-prone mice.[1,2] In addition, we succeeded in inducing autoimmune diseases in normal mice by the transplantation of T cell-depleted bone marrow cells (BMCs) or partially purified hemopoietic stem cells (HSCs) from autoimmune-prone mice to normal mice.[3,4]

Based on these findings, we have proposed that autoimmune diseases originate from defects in HSCs[3–7] and have also found that abnormal HSCs of autoimmune-prone mice are more resilient than normal HSCs;[4,8,9] abnormal HSCs can proliferate even in allogeneic microenvironments, while normal HSCs can proliferate in collaboration with major histocompatibility complex (MHC)-compatible stromal cells (mesenchymal stem cells; MSCs) but not MHC-incompatible MSCs.

From these findings, we realized that, in the case of BMT across MHC barriers, we would have to transplant both donor-derived HSCs and MSCs to ensure that the donor-derived normal HSCs grow and survive in the allogeneic environments.

Recently we discovered that the injection of whole BMCs directly into the bone cavity (intrabone marrow-BMT; IBM-BMT) provides distinct advantages, as IBM-BMT can efficiently recruit not only donor-derived HSCs but also MSCs. We here review our data

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regarding IBM-BMT plus the perfusion method (PM), which is capable of efficiently collecting MSCs, and also propose a novel concept of stem cell disorders (SCDs).

Strategies for Recruitment of Donor Stromal Cells

Using radiosensitive and chimeric-resistant MRL/lpr mice, we have found that the recruitment of donor stromal cells is essential for successful allogeneic BMT, as described above. We have found that three methods are effective in replacing recipient stromal cells with donor-derived stromal cells: (1) conventional intravenous BMT (IV-BMT) plus bone grafts;[10,11] (2) BMT from the portal vein (PV-BMT),[12] because tolerance can be easily induced when the antigen is portal venously injected into the liver; and finally (3) IBM-BMT.[13] IBM-BMT was found to be most effective as IBM-BMT allows us to use a mild conditioning regimen (5 Gy × 2), as shown in Figure 1. We therefore used IBM-BMT instead of the conventional IV-BMT for the following experiments.

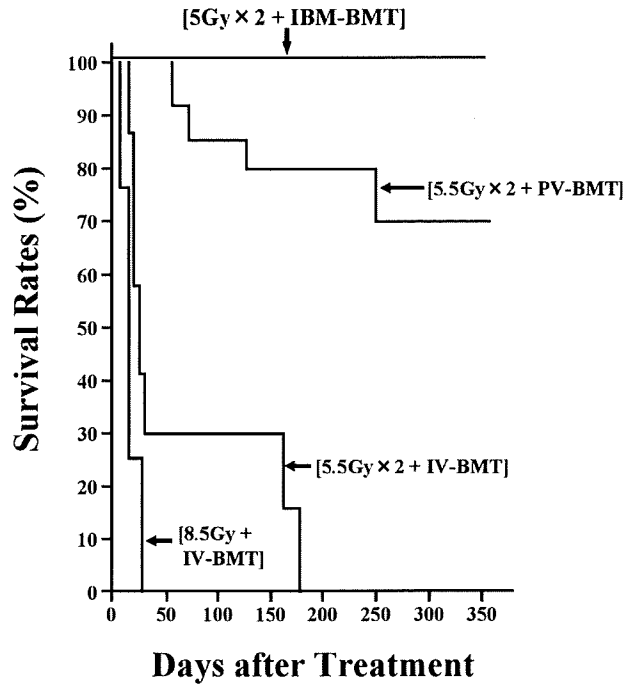

Figure 1. Treatment of autoimmune diseases in MRL/lpr mice by intrabone marrow–bone marrow transplantation (IBM-BMT) (5 Gy × 2). IBM-BMT can be used to treat autoimmune diseases in MRL/lpr mice even when the radiation dose is reduced to 5 Gy × 2.

IBM-BMT for Organ Transplantation

We previously found that the combination of organ allografts and conventional IV-BMT from the same donors prevents the rejection of organ allografts,[14] and we, therefore, attempted to apply IBM-BMT to organ allografts. IBM-BMT was the most effective strategy because the radiation dose could be reduced to 4.0 Gy × 2 in mouse skin allografts.[15] In addition, we found that IBM-BMT is applicable to allografts of other organs and tissues, such as pancreas islet[16] and leg transplantion,[17] lung,[18] and heart[19] in rats.

IBM-BMT for Regeneration Therapy

As our experiments showed that donor stromal cells could be effectively recruited by IBM-BMT, we next attempted to prevent osteoporosis in senescence-accelerated mouse pront 6 (SAMP6) mice. The SAMP6 mouse (a substrain of senescence-accelerated mice) spontaneously develops osteoporosis early in life and is therefore a useful model for examining the mechanisms underlying osteoporosis. After IBM-BMT, the hematolymphoid system was completely reconstituted with donor-type cells. Thus-treated SAMP6 mice showed marked increases in trabecular bone, even at 12 months of age (Fig. 2), and the bone mineral density (BMD) remained similar to that of normal B6 mice. Bone marrow stromal cells in IBM-BMT-treated SAMP6 mice were replaced by donor stromal cells.[20] Thus, we succeeded in preventing osteoporosis in SAMP6 mice by IBM-BMT, which can recruit not only donor HSCs but also MSCs.

We have also recently succeeded in curing osteoporosis in mice by IBM-BMT (Fig. 2).[21] We, therefore, believe that IBM-BMT could well become a powerful strategy in regeneration therapy.

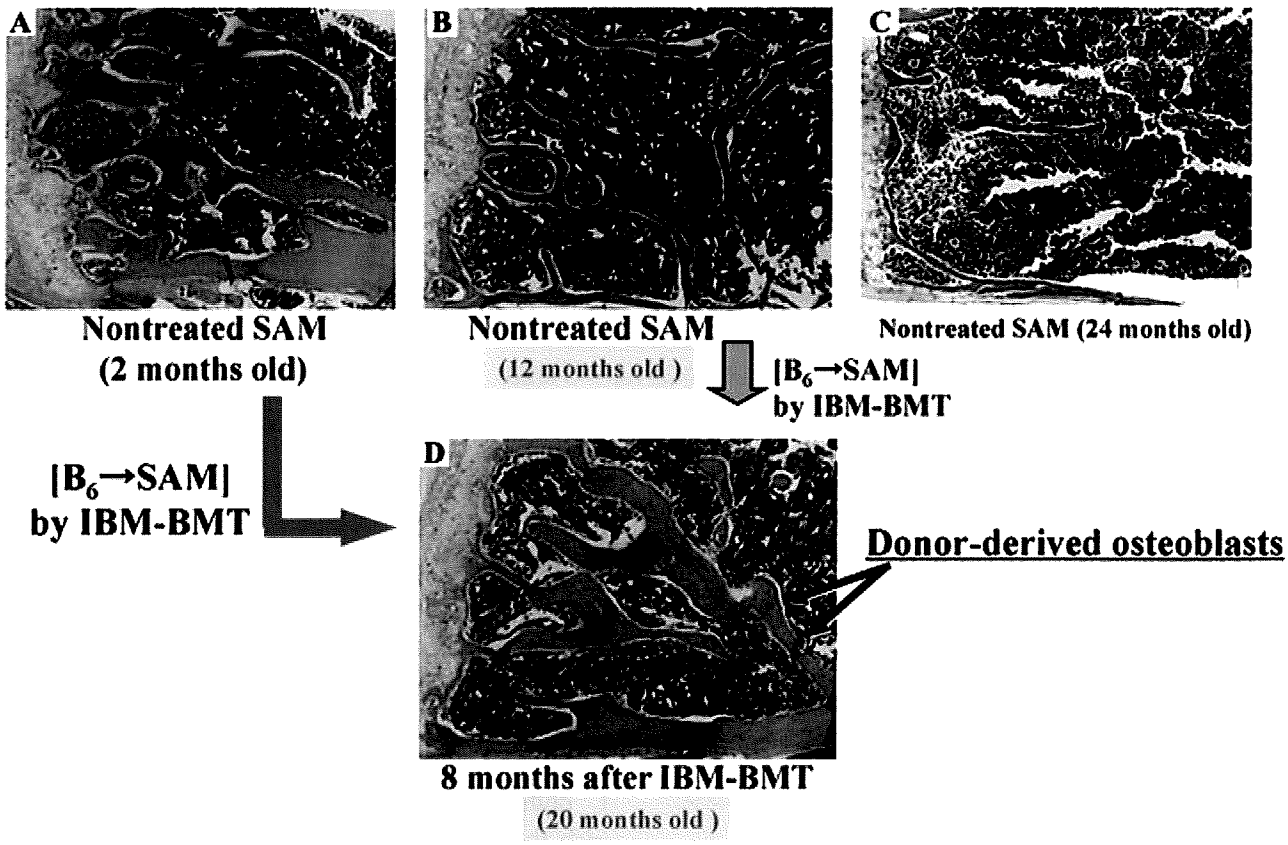

Figure 2. The lumbar spinal vertebral body of the nontreated senescence-accelerated mouse (SAM) at 2 months (**A**), 12 months (**B**), and 24 months (**C**) after birth. Significant loss of trabecular bone and cortical bone thickness was observed at 12 months of age or older. SAM treated with IBM-BMT from B6 mice [B6→SAM] showed an increase in trabecular bone at 12 months of age (8 months after the treatment) (**D**).

We next examined whether senile osteoporosis is attributable to SCDs. One month after IBM-BMT from SAM to B6 mice [SAM→B6], the hematolymphoid cells had been completely reconstituted by donor-derived cells, and bone marrow stromal cells that could differentiate into osteocytes were also found to be of donor origin. In addition, the recipient C57BL/6 mice showed the features of osteoporosis in the trabecular bone at 12 months of age. Decreases in BMD and increases in urinary deoxypyridinoline were also observed.[22] These findings indicate that not only hemopoietic cells but also bone marrow stromal cells in the [SAM→B6] mice are replaced by SAM cells as a result of IBM-BMT. The findings also suggest that the development of senile osteoporosis might be attributable to SCDs, probably MSC disorders because IV-BMT from SAM to B6 could not induce osteoporosis; the stromal cells of the [SAM→B6] mice treated with IV-BMT were host derived.

We have also found that IBM-BMT can be used to treat emphysema in Tsk mice and that the emphysema in these mice can be transferred to normal mice by IBM-BMT. This suggests that emphysema in Tsk mice originates from defects in stem cells in the bone marrow.[23] Thus, IBM-BMT can efficiently reconstitute recipients with both donor-derived normal HSCs and MSCs, which can differentiate into all cells, including hepatocytes,[24] epithelial cells,[25] nerve cells,[26] and retinal cells.[27] The use of this method, therefore, might become a valuable strategy not only for the treatment of intractable diseases, such as autoimmune diseases, but also in regeneration therapy for injured organs and tissues, such as myocardial infarction, cerebral infarction, and Alzheimer's disease.

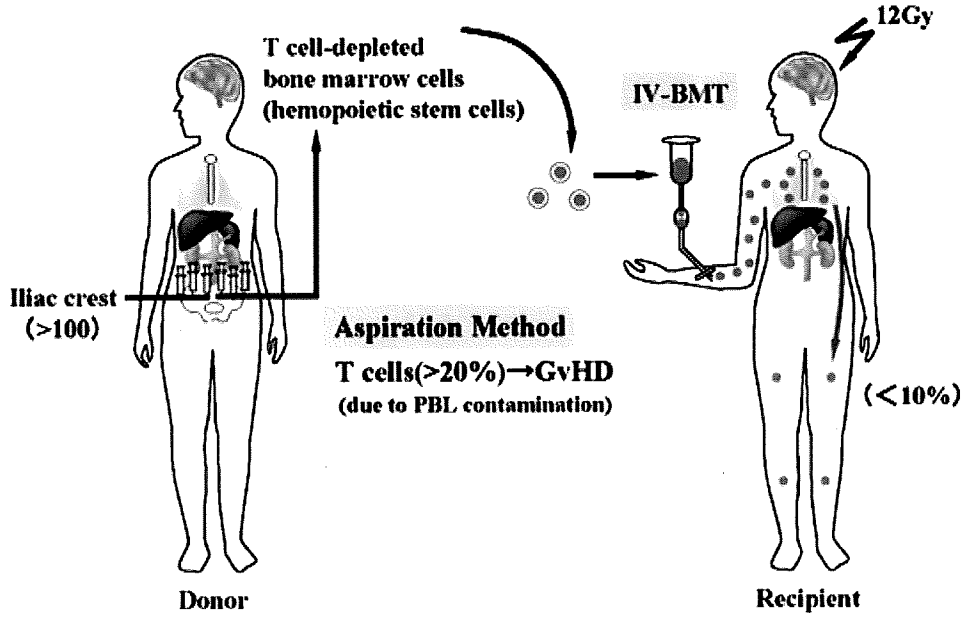

Figure 3. Conventional BMT for allogeneic BMT. Conventional BMT is carried out using an aspiration method followed by the intravenous injection of BMCs (IV-BMT).

Conventional BMT versus Novel BMT

Conventional BMT is carried out as follows: bone marrow needles are inserted into the iliac bones more than 100 times and the BMCs are collected by the aspiration method (AM) (Fig. 3). As a result, contamination with peripheral blood (particularly T cells) is inevitable. When these collected cells are intravenously injected, most cells become trapped in the lung and only a few cells migrate into the bone marrow (Fig. 3).

To apply our new BMT methods to humans, we established, using cynomolgus monkeys, a perfusion method (PM), which minimizes the contamination of BMCs with T cells. As shown in Figure 4, two needles are inserted into a long bone, such as the humerus, femur, or tibia. The end of the extension tube is connected to a needle, and the other end is placed in a syringe containing 0.5 mL heparin. The other needle is connected to a syringe containing 30 mL of saline, and the saline is then pushed gently from the syringe into the medullary cavity to flush out the bone marrow (BM). The saline containing the BM fluid is then collected.

There is significantly less contamination with T cells when using the PM (<10%) than with the conventional AM (>20%). Therefore, T cell depletion is unnecessary with the PM, and whole BMCs can be used. However, in the case of conventional AM, T cell depletion is necessary, and the loss of some important cells, such as MSCs during the process of T cell depletion, is inevitable. Furthermore, the number and progenitor activities of the cells harvested using the PM are greater than when using the conventional AM.[28,29]

We have also found that the PM is applicable to the iliac bones as well as the long bones not only in monkeys but also in humans.

A New Concept of Stem Cell Disorders

Finally, I would like to present a new concept of SCDs after a minor modification of my previous proposal.[7,15] As shown in Figure 5, various types of stem cells exist. In the bone marrow, HSCs, MSCs, and organ-specific stem cells (OSSCs) should be differentiated from embryonic stem (ES)-like cells. We have recently found that ES-like cells are present in the bone marrow of even human adults.[30]

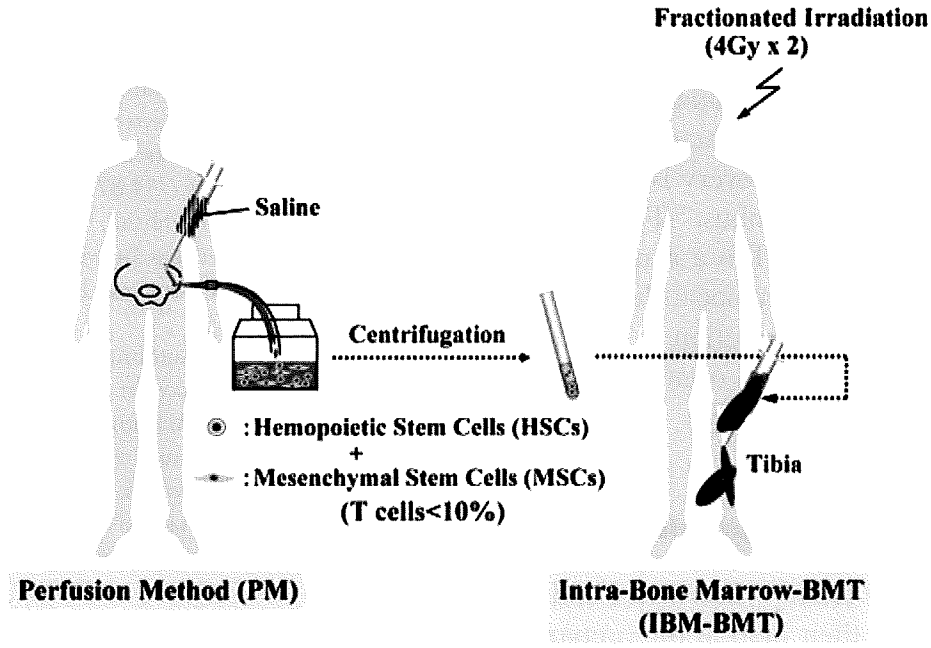

Figure 4. A new BMT method for allogeneic BMT. The new method consists of the perfusion method plus IBM-BMT.

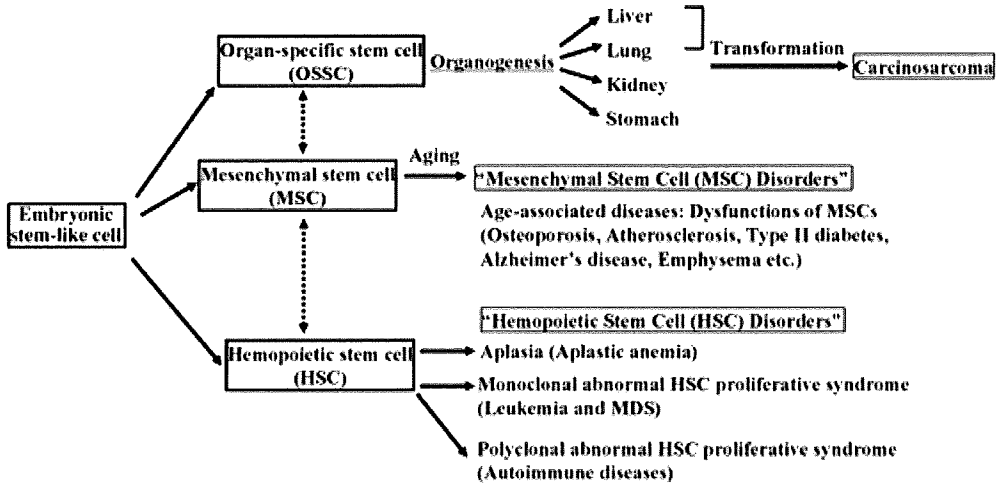

Figure 5. A novel concept of stem cell disorders.

HSC disorders are recognized as falling into the following categories: (1) aplasia of HSCs (aplastic anemia), (2) monoclonal or oligoclonal abnormal HSC proliferative syndromes (leukemias and myelodysplastic syndrome), and (3) polyclonal abnormal HSC proliferative syndromes (autoimmune diseases).[31,32]

On the other hand, there are mesenchymal stem cell disorders, which include age-associated diseases, such as osteoporosis[22] and emphysema.[23] It has also been proposed that autoimmune mechanisms are involved in the development of atherosclerosis[32–35] and Alzheimer's disease.[36]

Recently the existence of OSSCs or tissue-committed stem cells has been proposed,[32,37] and we would also like to propose that carcinosarcoma (in the liver, lung, and kidney) is a result of the malignant transformation of OSSCs. From the findings to date, it is conceivable that all the body's cells originate in the bone marrow and that all diseases might therefore originate from defects in the bone marrow. One paper already suggests that gastric cancer originates from bone marrow-derived cells.[38]

Because most intractable diseases are not only HSC disorders but also MSC disorders, we believe that the use of our new BMT

methods (PM + IBM-BMT), which can efficiently collect both HSCs and MSCs and transplant both, will become a valuable strategy for the treatment of various intractable diseases.

In conclusion, this discovery is, in many respects, an "Egg of Columbus." The combination of PM + IBM-BMT is a simple solution that seems obvious in retrospect. It is also a solution that heralds a revolution in the field of transplantation (BMT and organ transplantation) and regeneration therapy.

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Conflicts of Interest

The author declares no conflicts of interest.

References

1. Ikehara, S., R.A. Good, T. Nakamura, *et al.* 1985. Rationale for bone marrow transplantation in the treatment of autoimmune diseases. *Proc. Natl. Acad. Sci. USA* **82:** 2483–2487.
2. Ikehara, S., H. Ohtsuki, R.A. Good, *et al.* 1985. Prevention of type I diabetes in nonobese diabetic mice by allogeneic bone marrow transplantation. *Proc. Natl. Acad. Sci. USA* **82:** 7743–7747.
3. Ikehara, S., M. Kawamura, F. Takao, *et al.* 1990. Organ-specific and systemic autoimmune diseases originate from defects in hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **87:** 8341–8344.
4. Kawamura, M., H. Hisha, Y. Li, *et al.* 1997. Distinct qualitative differences between normal and abnormal hemopoietic stem cells in vivo and in vitro. *Stem Cells* **15:** 56–62.
5. Ikehara, S. 1997. Autoimmune diseases as "stem cell disorders." *Acta Histochem. Cytochem.* **30:** 3–12.
6. Ikehara, S. 1998. Autoimmune diseases as stem cell disorders: Normal stem cell transplant for their treatment. *Int. J. Mol. Med.* **1:** 5–16.
7. Ikehara, S. 2003. State-of-the-Art review. A new concept of stem cell disorders and their new therapy. *J. Hematother. Stem Cell Res.* **12:** 643–653.
8. Hashimoto, F., K. Sugiura, K. Inoue, *et al.* 1997. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. *Blood* **89:** 49–54.
9. Sugiura, K., H. Hisha, J. Ishikawa, *et al.* 2001. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. *Stem Cells* **19:** 46–58.
10. Ikehara, S., M. Inaba, S. Ishida, *et al.* 1991. Rationale for transplantation of both allogeneic bone marrow and stromal cells in the treatment of autoimmune diseases. In *New Strategies in Bone Marrow Transplantation. UCLA Symposia on Molecular and Cellular Biology, New Series*, Vol. 137. R.E. Champlin & R.P. Gale, Eds.: 251–257. Wiley-Liss, Inc. New York, Chichester, Brisbane, Toronto, Singapore.
11. Ishida, T., M. Inaba, H. Hisha, *et al.* 1994. Requirement of donor-derived stromal cells in the bone marrow for successful allogeneic bone marrow transplantation: complete prevention of recurrence of autoimmune diseases in MRL/MP-lpr/lpr mice by transplantation of bone marrow plus bones (stromal cells) from the same donor. *J. Immunol.* **152:** 3119–3127.
12. Kushida, T., M. Inaba, K. Takeuchi, *et al.* 2000. Treatment of intractable autoimmune diseases in MRL/lpr mice using a new strategy for allogeneic bone marrow transplantation. *Blood* **95:** 1862–1868.
13. Kushida, T., M. Inaba, H. Hisha, *et al.* 2001. Intrabone marrow injection of allogeneic bone marrow cells: A powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. *Blood* **97:** 3292–3299.
14. Nakamura, T., R.A. Good, S. Inoue, *et al.* Successful liver allografts in mice by combination with allogeneic bone marrow transplantation. *Proc. Natl. Acad. Sci. USA* **83:** 4529–4532.

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),

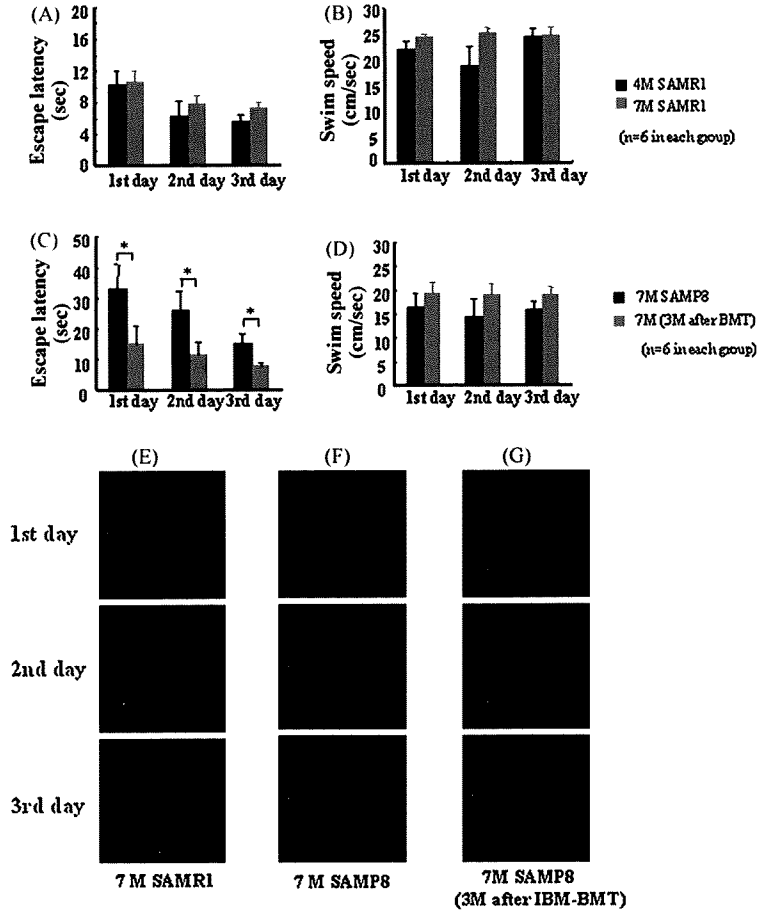

Fig. 1. (A–G) Escape latency and speed of SAMR1 and 4-month-old SAMP8 mice treated with IBM-BMT. Escape latency and swim speed were recorded for 3 days using the hidden platform conditions. Escape latency time was significantly decreased in the 7-month-old SAMP8 treated with IBM-BMT compared to age-matched 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

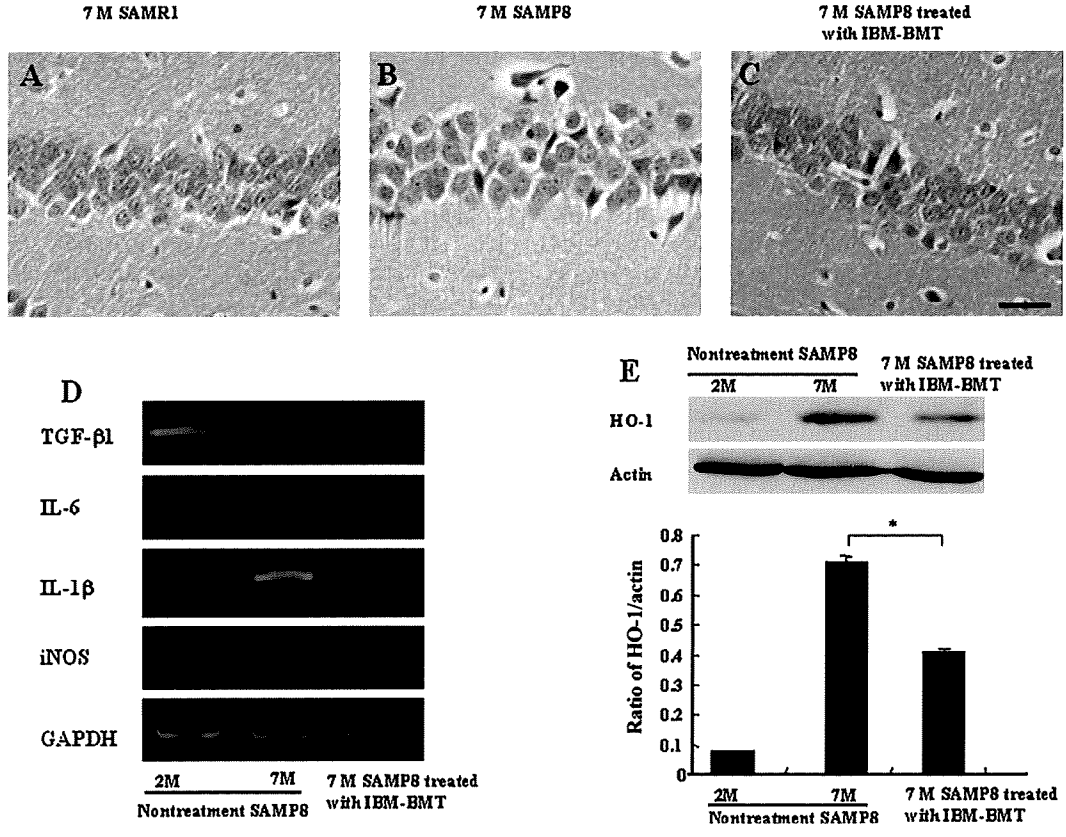

Fig. 2. (A–E) HE staining (A–C), expression of cytokines by RT-PCR (D) and expression of HO-1 by Western blotting. There was no significant difference in the CA1 region of SAMR1 (A) and 7-month-old SAMP8 treated with IBM-BMT (C) and age-matched SAMP8 (B). Scale bar = 125 μm in (A–C). Expression of TGF-β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

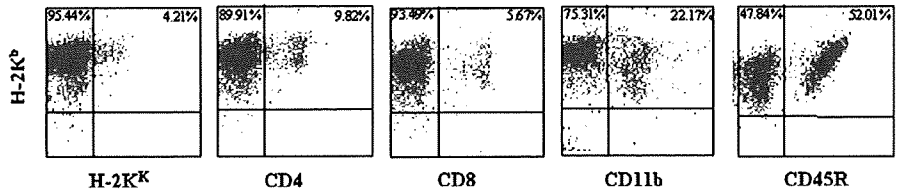

Fig. 3. Analyses of donor-derived cells after the IBM-BMT. The mononuclear cells were obtained from the peripheral blood of the recipients 1 M after the transplantation. These cells were stained with mAbs against H-2K^b, CD4, CD8, CD11b or CD45R. Approximately 99% of cells were of donor-origin, and differentiated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

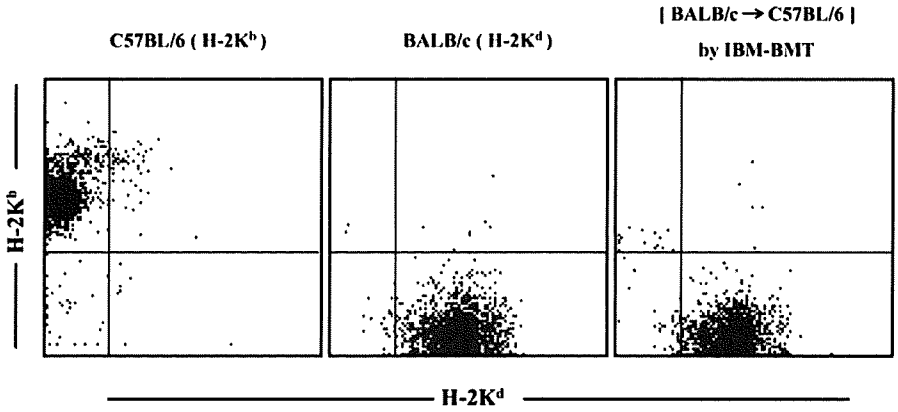

FIGURE 1. Reconstitution of donor-derived hemopoietic cells after IBM-BMT. B6 mice at the age of 8 weeks were irradiated with a lethal dose (6 Gy ×2), and BMCs from normal BALB/c mice were injected directly into the bone marrow cavity (IBM-BMT) of the left tibia. After 3 months, cells from the peripheral 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.

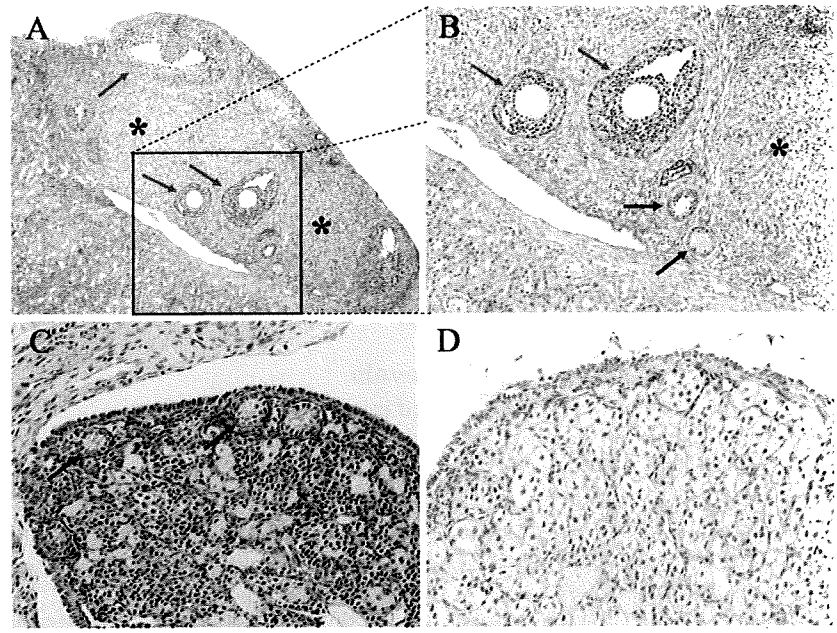

FIGURE 2. Histology of transplanted ovary and host ovary. Three months after bone marrow transplantation/ovarian transplantation (BMT/OT), the ovaries were accepted (original magnification ×100, A) with a large number of mature follicles (*red arrows*), primary follicles (*black arrows*), and corpus 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).

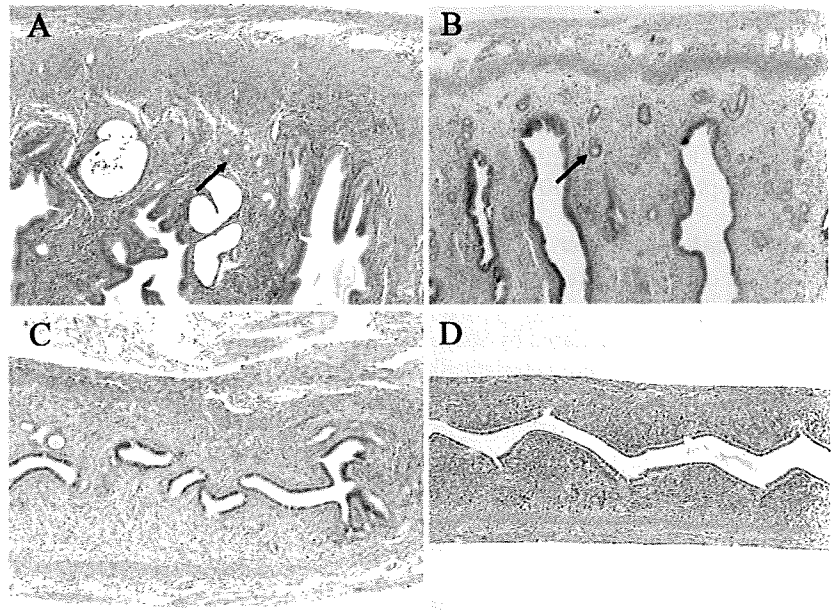

FIGURE 3. Effects of IBM-BMT with OT on uterus. Three months after bone marrow transplantation (BMT), the uteri of the four groups, including the normal control (nontreated) group (A), the BMT/ovarian transplantation (OT) group (B), the BMT group (C), and the OvX group (D), were stained with hematoxylin-eosin. 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±21.75^{a,b}	24.12±1.89^{a}.	0.157±0.029^{a,b}
OvX	350.69±25.11^{b,c,d}	30.22±1.46^{b,c,d}	0.023±0.003^{c,d}
BMT/OT	461.47±37.20^{a,b}	21.27±1.37^{a}	0.132±0.053^{a,b}
BMT	400.24±18.75^{a,c,d}	21.37±2.14^{a}	0.029±0.002^{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-

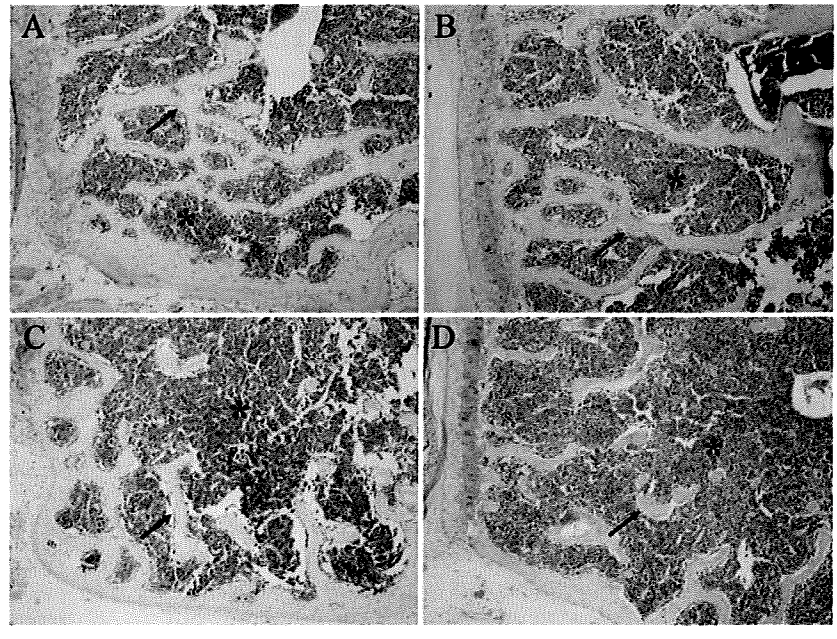

FIGURE 4. Histology of the lumbar vertebrae after intrabone marrow-bone marrow transplantation (IBM-BMT). Three months after IBM-BMT, the 4th lumbar vertebrae of mice in the four groups—normal control group (A), bone marrow transplantation/ovarian transplantation (BMT/OT) group (B), BMT group (C), and 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).