disease (GVHD) [11, 12]. The number of regulatory T cells increases in patients with cancer, and the cells inhibit anti-tumor immunity [13, 14]. These cellular factors have been suggested to play important roles in the immune suppression of patients with cancer.

In humans, auto or allogeneic BMT or peripheral blood stem cell transplantation (PBSCT) are used to treat malignant tumors. Auto BMT or PBSCT is applied to recover hematopoiesis after intensive chemo- or irradiation therapy [15], whereas Allo BMT or PBSCT are used to replace host cells with donor cells to induce the graft-versus-tumor (GVT) effect, although the very harmful GVHD is elicited if the effect is too strong [16, 17]. We have recently developed a new BMT method, intra-bone marrow–bone marrow transplantation (IBM–BMT), in which BMCs are injected directly into the bone marrow cavity [18]. IBM–BMT results in a reduced incidence of GVHD and greater engraftment of donor cells, including mesenchymal stem cells (MSC), than the conventional intravenous (iv) method [19, 20].

Very recently, we have developed a BMT method in conjunction with thymus transplantation (TT). The combination of BMT plus TT is effective in restoring donor-derived T cell function even in aged mice, chimeric-resistant mice, tumor-bearing mice, supralethally irradiated mice, and low-dose irradiated mice, and in mice injected with low numbers of BMCs [21–25]. However, TT has only been applied clinically in patients with DiGeorge syndrome or HIV infection who show hypoplasia of the thymus [26, 27]. The effects of BMT plus TT have not been examined in cases of advanced cancer in relation to the involution of the thymus.

In the present study, we examined Syn or Allo IBM–BMT plus fetal TT (IBM–BMT + TT) in mice with advanced malignant tumors. We also used 3rd-party TT, in which the major histocompatibility complex (MHC) type of the thymus was different from the MHC types of the donor BMCs and of the recipient (microenvironment) [28]. We did this because it is difficult to obtain such immature thymus from the BMC donor. From the results of this study, we propose that IBM–BMT + TT could become a powerful strategy for the treatment of patients with advanced tumors.

Materials and methods

Mice

Eight-week-old female BALB/c (H-2^d) and C57BL/6 (B6) (H-2^b) mice and fetal (day-16) BALB/c, B6, and C3H (H-2^k) mice were purchased from Shimizu Experimental Animal Laboratory (Shizuoka, Japan), and maintained until use in our animal facilities under specific pathogen-free conditions. All animal research was reviewed and approved by the Animal Experimentation Committee of Kansai Medical University.

Cell lines

Meth-A cells (H-2^d) were derived from methylcholanthrene-induced sarcoma in BALB/c mice, as previously used [23]. The cells were kindly provided by Dr. Junko Yoshida of Kanazawa Medical School (Kanazawa, Japan) from the Cell Research Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum with antibiotics.

Inoculation of tumor cells

One day before the transplantation of tumor cells, the recipients (BALB/c mice) underwent total body irradiation (3 Gy) using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International, Ottawa, ON, Canada). The next day, 2×10^6 Meth-A cells were inoculated subcutaneously into the right flank of the mice.

IBM–BMT and TT

When the tumor had reached >4 cm^2 in size (about 3 weeks after transplanting the cells), the tumor-bearing BALB/c mice were lethally irradiated (7 Gy) using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International) 1 day before IBM–BMT. BMCs were flushed from the shafts of donor femora and tibiae, and single-cell suspensions were prepared. Next, 1×10^7 BMCs were injected directly into the bone marrow cavity of the recipient's tibia, as described previously for the IBM–BMT method [18]. Briefly, 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 tibia through the patellar tendon and then inserted into the bone cavity. Simultaneously, a fetal day-16 thymus was grafted under the renal capsule of the left kidney in some mice. We also treated tumor-bearing mice with irradiation only.

Experimental groups

Group 1 consisted of BALB/c mice with advanced tumors (>4 cm^2) without treatment as controls (Non-treated) (Table 1). Group 2 consisted of lethally-irradiated BALB/c mice with advanced tumors transplanted with BMCs from syngeneic BALB/c mice by IBM–BMT (Syn-BMT). Groups 3 and 4 consisted of mice from Group 1 plus TT from syngeneic BALB/c or allogeneic B6 mice

Table 1 Experimental groups and their chimerism

Groups	Description	*n*	Transplantation		Chimerism[a]		
			BMCs	Thymus	H-2K^b	H-2K^d	H-2K^k
1	Non-treated	8	(–)	(–)	0.1 ± 0.1	99.8 ± 0.2	ND
2	Syn-BMT	11	BALB/c	(–)	0.2 ± 0.1	97.8 ± 0.2	ND
3	Syn-BMT + Syn-TT	11	BALB/c	BALB/c	0.7 ± 0.3	98.2 ± 0.3	ND
4	Syn-BMT + Allo-TT	13	BALB/c	B6	0.5 ± 0.5	99.5 ± 0.2	ND
5	Allo-BMT	13	B6	(–)	98.8 ± 0.2	0.2 ± 0.2	ND
6	Allo-BMT + Allo-TT	12	B6	B6	99.6 ± 0.5	0.2 ± 0.3	ND
7	Allo-BMT + 3rd party-TT	12	B6	C3H	96.7 ± 0.7	0.7 ± 0.5	0.9 ± 0.7

The categorizing of each group is described in "Materials and methods"

[a] Chimerism was determined in spleen cells of the mice in the non-treatment group (group 1, $n = 4$) 2–3 weeks after the tumor reached >4 cm^2 in size and in the mice in the BMT groups (groups 2–7, $n = 4$–6) 4–5 weeks after transplantation

(Syn-BMT + Syn-TT and Syn-BMT + Allo-TT, respectively). The lethally irradiated BALB/c mice with advanced tumors transplanted with BMCs from allogeneic B6 by IBM–BMT mice comprised Group 5 (Allo-BMT). The mice in Groups 6 and 7 consisted of Group 5 plus TT from allogeneic B6 or 3rd-party C3H mice (Allo-BMT + Allo-TT and Allo-BMT + 3rd-party TT, respectively).

Histological studies

Several organs, including the small intestine, lung, liver, kidney, and transplanted thymus, were removed from the chimeric mice, fixed in 10% formalin for 48 h, and embedded in paraffin according to standard procedures. Sections of 4 μm thickness were stained using hematoxylin and eosin (HE). The average numbers of metastatic nodules (100× magnification) were calculated from ten blind fields from every five sections of both left and right lungs.

Analyses of mitogen responses

To analyze lymphocyte function, mitogen responses were examined as described previously [28]. The stimulation index (SI) was calculated as the average ^{3}H-TdR incorporation of triplicate samples of responding cells with mitogen/^{3}H-TdR incorporation of responding cells in medium alone.

Analyses of surface markers and intracellular FoxP3 expression and cytokine production by flow cytometry

Surface markers on lymphocytes in the spleen were analyzed under three-color fluorescence staining using a FACScan system (BD Pharmingen, Franklin Lakes, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE) anti-H-2K^b, anti-H-2K^d, and anti-H-2K^k antibodies (BD Pharmingen) were used to determine chimerism. FITC, PE or biotin-conjugated CD4, CD8, B220 (BD Pharmingen), were used to analyze lymphocyte subsets. Avidin-PE-Cy5 (Dako, Kyoto, Japan) was used as the third color in the avidin/biotin system. Intracytoplasmic FoxP3 staining was performed using an FITC-anti mouse/rat FoxP3 staining set (eBioscience, San Diego, CA, USA). The procedure was performed in accordance with the manufacturer's instructions. Intracellular cytokines (IL-2, IL-4, IL-10, IFN-γ and TNF) were detected using an Intracellular Cytokine Staining Kit (BD Pharmingen) in accordance with the manufacturer's instructions.

Statistical analyses

Non-parametric analyses (Mann–Whitney U test and log-rank test) and simple regression were performed using StatView software (Abacus Concepts, Berkley, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Chimerism and survival rates

To examine the effects of thymic function on hosts with advanced tumors, we performed Syn- or Allo-BMT with or without Syn-, Allo-, or 3rd-party TT in mice with Meth-A sarcomas measuring >4 cm^2 (Table 1). In humans, due to the difficulty in performing the combination of Allo-BMT + Allo-TT from the same donor, we also carried out the Allo-BMT + 3rd-party TT, as we described previously [28].

H-2 typing showed full donor chimerism in all groups treated with BMT even in the mice treated with Allo-BMT + 3rd-party TT (Table 1), suggesting that BMT was successfully carried out. With regard to survival, all of the non-treated control mice died within 20 days due to the growth of tumors, and all the mice treated with irradiation

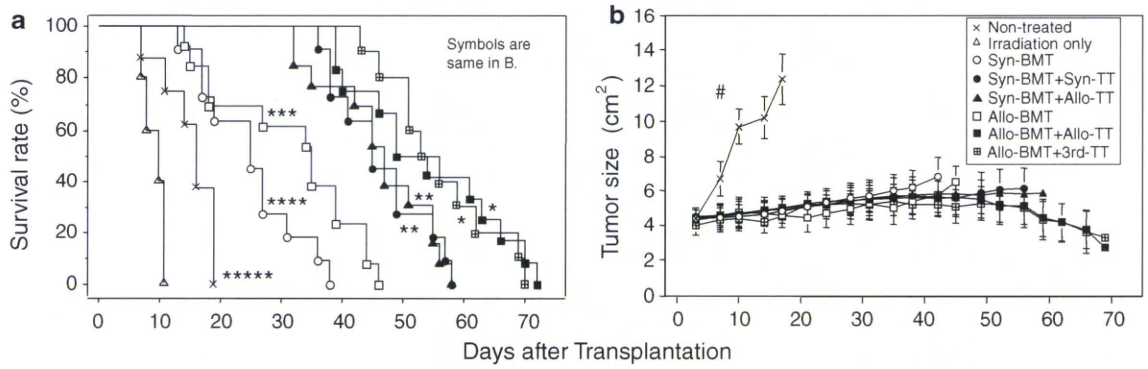

Fig. 1 Survival rate and tumor size in mice with advanced tumors treated with BMT + TT. Survival rate (**a**) and tumor size (**b**) in the mice with advanced tumors are shown. The treatments for the mice are described in Table 1. $^{*}P < 0.05$ compared with the non-treated controls, the mice treated with irradiation only, Syn-BMT, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, and Allo-BMT. $^{**}P < 0.01$ compared with the non-treated controls, the mice treated with irradiation only, Syn-BMT, and Allo-BMT. $^{***}P < 0.05$ compared with the non-treated controls, the mice treated with irradiation only, and Syn-BMT. $^{****}P < 0.005$ compared with the non-treated controls and the mice treated with irradiation only. $^{*****}P < 0.005$ compared with the mice treated with irradiation only. $^{\#}P < 0.0001$ compared with mice treated with irradiation only, Syn-BMT, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, Allo-BMT, Allo-BMT + Allo-TT, and Allo-BMT + 3rd-party TT. Non-treated ($n = 8$), Irradiation only ($n = 5$), Syn-BMT ($n = 11$), Syn-BMT + Syn-TT ($n = 11$), Syn-BMT + Allo-TT ($n = 13$), Allo-BMT ($n = 13$), Allo-BMT + Allo-TT ($n = 12$), and Allo-BMT + 3rd-party TT ($n = 10$)

alone died within 12 days as a result of hematopoietic failure (Fig. 1a). The mice treated with Syn-BMT or Allo-BMT showed a slight improvement in survival rates, compared with the non-treated control, although the mice treated with Allo-BMT fared far better than the mice treated with Syn-BMT. Interestingly, the mice treated with BMT (Syn or Allo) + TT (Syn or Allo) showed significantly prolonged survival rates in comparison with the mice treated with Syn- or Allo-BMT alone. In these combinations, the mice treated with Allo-BMT + Allo-TT showed significantly longer survival than those treated with Syn-BMT + Syn-TT or Syn-BMT + Allo-TT. The survival rate of the mice treated with Syn-BMT + Allo-TT was comparable to the mice treated with Syn-BMT + Syn-TT. Notably, the mice treated with Allo-BMT + 3rd-party TT also showed a comparable survival rate to the mice treated with Allo-BMT + Allo-TT (Fig. 1a). At autopsy, GVHD was seen only minimally in the mice from all groups (data not shown).

Primary tumor size and lung metastasis

Mice in the non-treated control group showed significantly greater tumor growth than mice in the other seven groups (Fig. 1b). The tumor grew very slowly in all the other seven groups, and there were no significant differences between the groups.

Next, we examined lung metastasis of the tumors. We confirmed that there was no lung metastasis in the mice with tumors before BMT. The mice in the non-treated control group showed many metastatic tumor nodules 2–3 weeks after reaching a tumor size of >4 cm^2 (Fig. 2a, b). Although the day of analysis (4–5 weeks after transplantation) was different from the mice in the non-treated control group because of their early death, the mice treated with Syn-BMT showed the greatest numbers of metastatic tumor nodules in all the groups. This strong metastasis was due to the immunosuppressive effect of irradiation. The mice treated with Syn-BMT + Syn-TT and Syn-BMT + Allo-TT showed no significant difference in the number of metastatic tumors compared with those in the non-treated control group. However, the mice treated with Allo-BMT showed a significant inhibition of metastasis compared with non-treated controls. Furthermore, the mice treated with Allo-BMT + Allo-TT or Allo-BMT + 3rd-party TT showed the lowest rates of metastasis.

Host thymus and implanted thymus

We next examined the host and implanted thymus in mice with advanced tumors. The host thymus in mice in the non-treated control group showed marked involution (reduction in size), compared with the thymus from BALB/c mice without tumors (Fig. 3). In addition, the subsets of thymocytes were abnormally regulated in the mice with tumors: the percentage of $CD4^{+}CD8^{+}$ double-positive thymocytes markedly decreased, whereas the percentages of $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, and $CD4^{-}CD8^{-}$ thymocytes increased. The donor-type MHC class I expression of H-2K region in the thymocytes of the mice with tumors increased due to relative maturation of thymocytes, compared with the BALB/c mice without tumors. Although the day of analysis was different from the mice in the non-treated control group because of their early death, the mice treated with either Syn-BMT (data not shown) or Allo-BMT alone showed

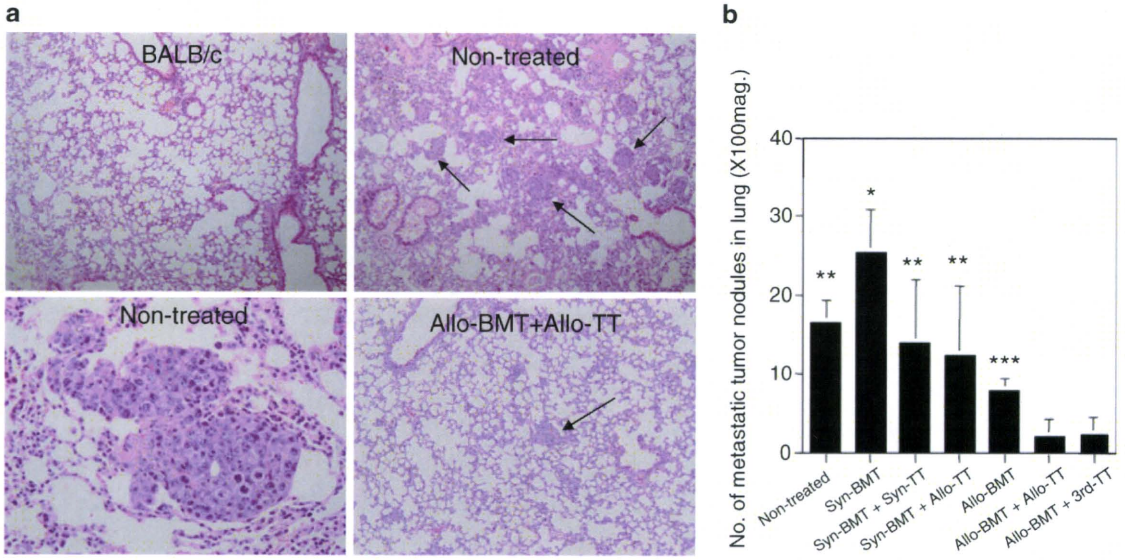

Fig. 2 Lung metastasis in mice with advanced tumors treated with BMT + TT. Lung metastasis in the mice with advanced tumors is shown. Autopsy and analysis for the metastasis were performed in the non-treated mice 2–3 weeks after the tumor reached a size of >4 cm^2 because of early death and in Syn-BMT, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, Allo-BMT, Allo-BMT + Allo-TT, and Allo-BMT + 3rd-party TT groups 4–5 weeks after BMT. Representative histological findings of lung metastasis in mice with advanced tumors are shown (**a**). The non-treated controls showed a number of metastatic tumor nodules (*arrows*) (*upper right* ×100 and *lower left* ×400), whereas no tumor was found in the BALB/c mice (*upper left* ×100). Those treated with Allo-BMT + Allo-TT showed only a few nodules (*arrow*) (*lower right* ×100). The results are summarized in **b**. $*P < 0.02$ compared with non-treated controls, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, Allo-BMT, Allo-BMT + Allo-TT, and Allo-BMT + 3rd-party TT. $**P < 0.02$ compared with Allo-BMT + Allo-TT, and Allo-BMT + 3rd-party TT. $***P < 0.02$ compared with non-treated controls and Syn-BMT. Non-treated ($n = 5$), Syn-BMT ($n = 4$), Syn-BMT + Syn-TT ($n = 4$), Syn-BMT + Allo-TT ($n = 4$), Allo-BMT ($n = 5$), Allo-BMT + Allo-TT ($n = 4$), and Allo-BMT + 3rd-party TT ($n = 6$). Data are shown as mean ± SD

similar results. Interestingly, although the host thymus showed involution in the mice treated with Allo-BMT + Allo-TT, the transplanted thymus grew and engrafted well. The thymocyte subsets of the transplanted thymus were similar to those of normal control mice. The mice treated with Allo-BMT + 3rd-party TT showed results comparable to those of the mice treated with Allo-BMT + Allo-TT (data not shown).

Lymphocyte subsets

We next investigated lymphocyte subsets in the spleens from mice with advanced tumors. The number of $CD4^+$ T cells in the mice in the non-treated control group was significantly reduced, compared with BALB/c mice 2–3 weeks after reaching a tumor size of >4 cm^2 (Fig. 4a). Although the day of analysis was different from the mice in the non-treated control group because of their early death, BALB/c mice treated with Syn-BMT or Allo-BMT showed further reductions in the number of $CD4^+$ T cells, compared with the non-treated control. However, the numbers were significantly elevated in mice treated with Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, Allo-BMT + Allo-TT and Allo-BMT + 3rd-party TT, compared with the mice treated with Syn-BMT or Allo-BMT alone or non-treated controls. However, the number of $CD4^+$ T cells was still lower than that in BALB/c mice.

We next investigated $CD4^+FoxP3^+$Treg cells (Fig. 4b). The highest percentage of $FoxP3^+$Treg cells in $CD4^+$ T cells was observed in the non-treated control group, followed by the mice treated with Syn-BMT, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, and Allo-BMT, and the lowest percentages were observed in the mice treated with Allo-BMT + Allo-TT or Allo-BMT + 3rd-party TT. However, the percentages in all groups were significantly higher than that in BALB/c mice. The results regarding $CD8^+$ T cells were similar to those of $CD4^+$ T cells (Fig. 4c). The numbers of B cells in the mice treated with Syn-BMT or Allo-BMT were lowest (data not shown). The number of $Gr\text{-}1^+/CD11b^+$ myeloid suppressor cells, which increases with tumor progression, was highest in the non-treated control group (Fig. 4d). Interestingly, the mice treated with Allo-BMT + Allo-TT or Allo-BMT + 3rd-party TT showed the lowest numbers of these cells. The cell numbers were lower in the mice treated with Syn-BMT + Syn-TT, Syn-BMT + Allo-TT or Allo-BMT than in the mice treated with Syn-BMT alone. However, the levels were higher in all of these groups than in normal BALB/c mice.

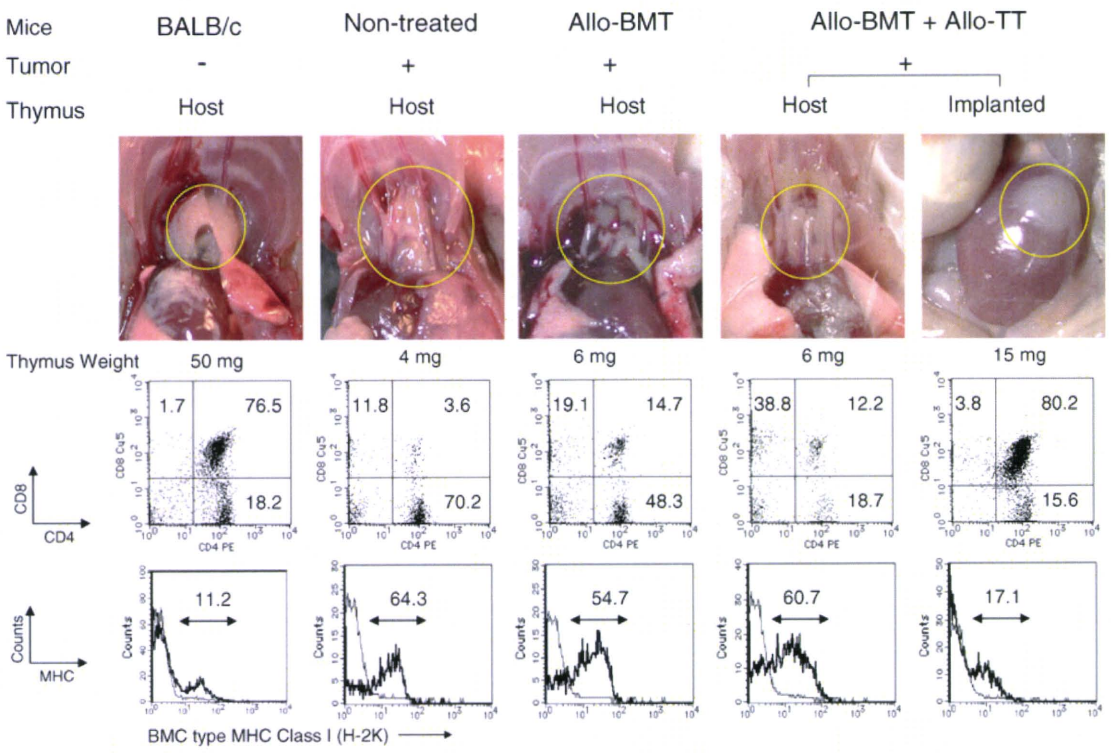

Fig. 3 Findings related to host and transplanted thymus, CD4/CD8 subsets, and MHC expression in thymocytes from mice with advanced tumors treated with BMT + TT. The macroscopic findings (*upper panel* thymus in *yellow circle*), FACS profile (*middle panel*), and MHC class I (H-2K) expression (*lower panel*) in thymocytes from the host and transplanted thymus in BALB/c mice, non-treated controls with advanced tumors, and those treated with Allo-BMT and Allo-BMT + TT are shown. Autopsy and analysis were performed at the same time as those in Fig. 2. H-2K expression *thick line*, BMC type (H-2K^d in BALB/c and non-treated controls, H-2K^b in Allo-BMT and Allo-BMT + Allo-TT), *thin line*, negative control or host type (H-2K^b in BALB/c and non-treated controls, H-2K^d in Allo-BMT and Allo-BMT + Allo-TT). Representative data of 3 or 4 experiments are shown

Mitogen responses and cytokine production

We next analyzed the lymphocyte function of the spleen cells. Con A responsiveness was low in the non-treated control group and in the mice treated with either Syn-BMT or Allo-BMT alone (Fig. 5a). However, it increased significantly in mice treated with either BMT (Syn or Allo) + TT (Syn, Allo, or 3rd-party), although the level did not reach that of the BALB/c mice. LPS responsiveness showed no significant differences between any of the groups.

In the analysis of cytokine production (Fig. 5b), the level of IL-2 was significantly elevated in the mice treated with Allo-BMT + Allo-TT or Allo-BMT + 3rd-party TT, although the level was still lower than that of BALB/c mice. In contrast, IFN-γ was significantly elevated in the mice treated with Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, Allo-BMT, Allo-BMT + Allo-TT or Allo-BMT + 3rd-party TT compared with non-treated control group or those treated with Syn-BMT. The levels were the same as those in BALB/c mice. The levels of the other cytokines (IL-4, IL-10, and TNF) were very low in all the groups in this experiment.

Factors correlated to survival

Finally, we examined the correlations between the above factors and survival rates in all the groups (Table 2). There were positive correlations between the median survival days and the numbers of CD4$^+$ and CD8$^+$ T cells, the Con A response, or the IFN-γ production, whereas there were negative correlations between the survival days and the lung metastasis, the numbers of Gr-1$^+$/CD11b$^+$ cells, or the percentage of FoxP3$^+$ cells in CD4$^+$ T cells. These factors were also correlated with each other (data not shown).

There was no correlation between survival rates and the size of the main tumor, the numbers of B220$^+$ B cells, the LPS response, and the IL-2-, IL-4-, IL-10-, or TNF-production.

Discussion

In the present study, we examined the effects of BMT (Syn or Allo) + TT (Syn, Allo or 3rd-party) in mice with advanced tumors. The mice treated with BMT + TT showed better

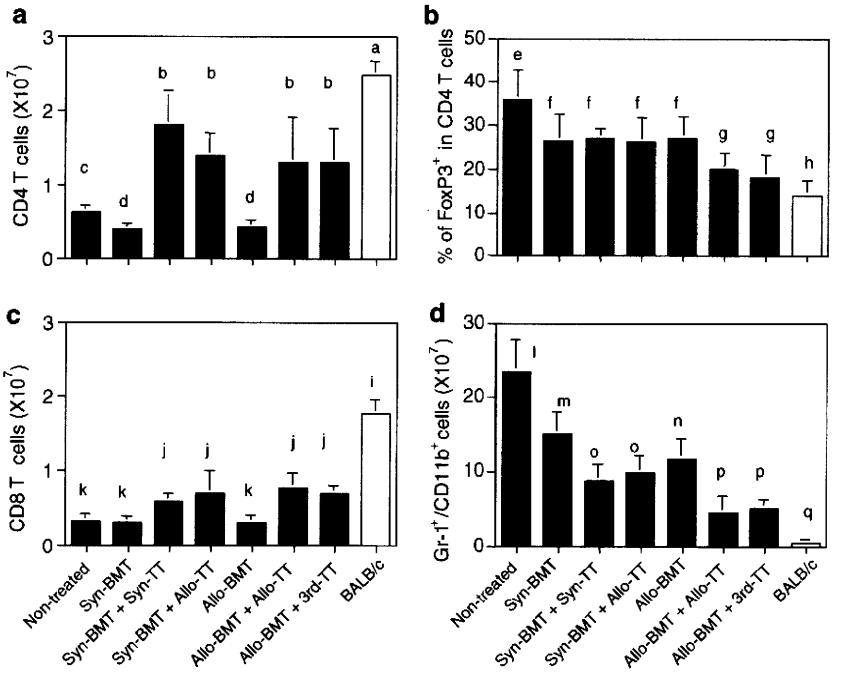

Fig. 4 The numbers of the cells in spleen from the mice with advanced tumors treated with BMT + TT. The numbers of $CD4^+$ T cells (**a**), % of $FoxP3^+$ cells in $CD4^+$ T cells (**b**), the numbers of $CD8^+$ T cells (**c**) and Gr-1/CD11b cells (**d**) in the spleen were evaluated in each group. Analyses were performed at the same time as those in Fig. 2. a versus b, c, and d: $P < 0.03$; b versus c and d: $P < 0.02$; c versus d: $P < 0.02$; e versus f, g, and h: $P < 0.02$; f versus g and h: $P < 0.03$; g versus h: $P < 0.03$; i versus j and k: $P < 0.02$; j versus k: $P < 0.03$; l versus m, n, o, p and q: $P < 0.02$; m versus o, p, and q: $P < 0.03$; n or o versus p and q: $P < 0.03$; p versus q: $P < 0.03$. Non-treated ($n = 5$), Syn-BMT ($n = 4$), Syn-BMT + Syn-TT ($n = 4$), Syn-BMT + Allo-TT ($n = 4$), Allo-BMT ($n = 5$), Allo-BMT + Allo-TT ($n = 4$), and Allo-BMT + 3rd-party TT ($n = 4$). Data are shown as mean ± SD

Table 2 Analysis of correlations with survival period in mice with advanced cancer

Factors	*P* value*
Main tumor	NS
Lung metastasis	0.048#
$CD4^+$ T cells	0.045
$CD8^+$ T cells	0.004
$B220^+$ B cells	NS
Gr-1 +/CD11b + cells	0.004#
% of FoxP3/CD4 T cells	0.018#
Con A	0.010
LPS	NS
IL-2	NS
IL-4	NS
IL-10	NS
IFN-γ	0.011
TNF	NS

NS not significant

* *P* values were calculated for median survival period by simple regression in seven groups

\# A negative correlation was observed

survival rates than the mice treated with BMT only. Interestingly, the mice treated with Allo-BMT + Allo-TT or Allo-BMT + 3rd-party TT showed the longest survival rates. In addition, lung metastasis was significantly inhibited in these mice. T cell number, T cell function, and IFN-γ production significantly increased, whereas the number of $Gr\text{-}1^+/CD11b^+$ cells and the percentage of $FoxP3^+$ cells in $CD4^+$ T cells significantly decreased in these mice, compared with the other groups. These results suggest that BMT + TT, and particularly "Allo-BMT + Allo-TT" or "Allo-BMT + 3rd-party TT", is most effective for hosts with advanced tumors in prolonging survival by restoring T cell function.

First, we have shown that TT plays an important role in BMT for the long-term survival of hosts with advanced cancer (Fig. 1a). A significant inhibition of lung metastasis was observed by Allo-BMT + Allo-TT or 3rd-party TT; there was a negative correlation between metastasis and survival (Fig. 2a, b; Table 2). In contrast, the gradual growth of the main tumor may be the result of the irradiation, because there was no significant difference in size between the mice treated with BMT and those treated with irradiation only, although it should be noted that the duration

of observation in the latter was cut short by the hematopoietic failure. These findings suggest that the inhibition of metastasis is one of the mechanisms promoting survival.

In addition, it should be noted that GVHD was not particularly obvious in the "Allo-BMT + Allo-TT" or "Allo-BMT + 3rd-party TT" mice. This may be due to the relatively high proportion of $FoxP3^+$ regulatory T cells, which inhibit GVHD but preserve the GVT effect [29], and/or the induction of tolerance by BMC-derived and transplanted thymus-derived thymic DCs [28]. Furthermore, the IBM–BMT method itself suppresses GVHD [20]. Thus, the contribution of GVHD to survival may be minimal.

Next, we showed that the thymus in mice with advanced tumors shows marked involution with or without BMT (Fig. 2). In the thymocyte subsets, the number of $CD4^+CD8^+$ thymocytes decreased, whereas that of $CD4^-CD8^-$, $CD4^+CD8^-$, or $CD4^-CD8^+$ thymocytes increased, as previously described [4, 5]. In contrast, the transplanted thymus grew large, showing almost normal subsets in donor-derived thymocytes, although the host thymus showed involution. The high expression of class I of H-2K region, which is low in most normal thymocytes [30], also supports the hypothesis that there is a dysregulation of thymocytes with the relative maturation of those thymocytes. These findings indicate that the transplanted thymus (but not the host thymus) can grow even in the presence of advanced tumors. Although the mechanism is unknown, the high proliferative activity of the transplanted thymus seems to overcome the immunosuppressed status.

We next examined lymphocyte subsets and T cell function in the spleens of mice with advanced tumors. The numbers of both $CD4^+$ T and $CD8^+$ T cells were significantly higher in the mice treated with either "Allo-BMT + TT" or "Syn-BMT + TT" than in the non-treated controls or in the mice treated with either Allo-BMT or Syn-BMT alone, although these numbers were still lower than those of normal BALB/c mice (Fig. 4a, c). The elevated T cell counts were probably from the result of the TT [23], and there was a positive correlation with survival (Table 2), suggesting that T cells may play a significant role in prolonging survival by preventing infection and tumor growth.

We further investigated the $FoxP3^+CD4^+$ regulatory T cells. The percentage of $FoxP3^+$ cells among $CD4^+$ T cells, which reflects the suppressor activity directly by the ratio of regulatory/effector T cells, was highest in the non-treated control group and lowest in the mice treated with Allo-BMT + Allo-TT or 3rd-party TT (Fig. 4b). Since a reduction in the percentage of $FoxP3^+$ cells in $CD4^+$ regulatory T cells enhances GVT activity [31, 32], the strong inhibition of metastasis in these mice may reflect this. It should be noted that the percentage of $FoxP3^+$ cells among $CD4^+$ T cells in these mice was still higher than that in normal BALB/c mice, suggesting that apparent GVHD could not be elicited by these cells, since GVHD is evident with a lower percentage than normal of these cells [23].

The mice treated with "Allo-BMT + Allo-TT" or "Allo-BMT + 3rd-party TT" showed the lowest number of $Gr\text{-}1^+/CD11b^+$ myeloid suppressor cells (Fig. 4d), indicating that the reduction of these cells may also facilitate GVT activity. As the myeloid suppressor cells can be induced by tumor exosomes [33], the inhibition of metastasis by GVT seems to be due to the reduction in the number of these cells. There was a negative correlation between the survival and not only the number of myeloid suppressor cells but also the percentage of $FoxP3^+$ in $CD4^+$ T cells (Table 2). In addition, there was a very strong correlation between the numbers of myeloid cells and the percentage of $FoxP3^+$ cells among $CD4^+$ T cells ($P = 0.0032$). In this respect, $Gr\text{-}1^+/CD11b^+$ myeloid suppressor cells may induce $FoxP3^+CD4^+$ regulatory T cells, as previously reported [34, 35].

We have also found that the mice treated with BMT + TT show a significantly higher Con A response than the mice treated with BMT alone, although the response was still lower than that in normal BALB/c mice (Fig. 5). In the analyses of cytokine production, there was a positive correlation between the survival and the level of IFN-γ (but not IL-2 production), suggesting that the level of IFN-γ may be more related to prolonged survival than that of IL-2.

Based on these results, it is evident that the elevated number of T cells and improved function resulting from TT play a crucial role in prolonging the survival of hosts with advanced cancers. Although T cell number and function did not reach normal levels, several other factors, such as regulatory T cells and myeloid suppressor cells, were synchronously suppressed.

Although, for both technical and ethical reasons (including donor age), it may be clinically difficult to obtain adequate thymic tissue, it is conceivable that grafts could be obtained from patients with congenital heart diseases or from aborted fetuses, as described previously [26, 27]. The combinations of Syn-BMT + Allo-TT or Allo-BMT + 3rd-party TT might then be practical in a clinical setting. Here, the allo-BMT + 3rd-party TT have shown tolerance to all three MHC determinants [28], suggesting a benefit for transplantation of other organs from the two MHC-disparate donors. Alternatively, a method of regenerating the thymus has also been developed [36, 37]. Thus, regenerated thymus could be expected to use all combinations in future.

In summary, we have shown that BMT + TT can prolong survival in hosts with advanced tumors by restoring T cell function. Although our model may be different from the varied immunogenic cancers in humans, the elevation of T cell function itself will be effective for the many complications induced by cancer. We think that BMT + TT could

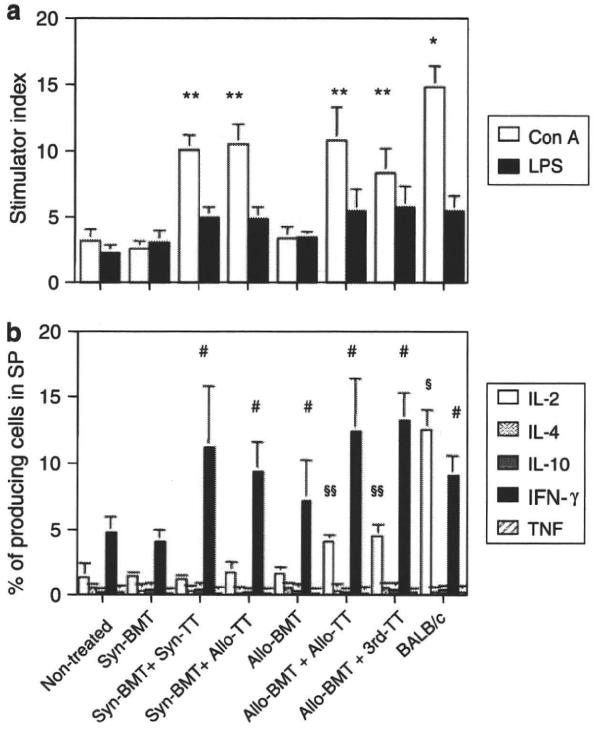

Fig. 5 The mitogen responses and percentage of cytokine-producing cells in the spleens from mice with advanced tumors treated with BMT + TT. Mitogen responses: Con A and LPS (**a**) and percentage of cytokine-producing cells (**b**) in the spleen were evaluated in each group. Analyses were performed at the same time as those in Fig. 2. $^{*}P < 0.03$ compared with the non-treated controls, Syn-BMT, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, Allo-BMT, Allo-BMT + Allo-TT, and Allo-BMT + 3rd-party TT. $^{**}P < 0.03$ compared with the non-treated controls, Syn-BMT, and, Allo-BMT. $^{\#}P < 0.03$ compared with the non-treated controls and Syn-BMT. $^{\S}P < 0.03$ compared with the non-treated controls, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT, Allo-BMT, Allo-BMT + Allo-TT, and Allo-BMT + 3rd-party TT. $^{\S\S}P < 0.03$ compared with the non-treated controls, Syn-BMT, Syn-BMT + Syn-TT, Syn-BMT + Allo-TT and Allo-BMT. Non-treated ($n = 4$), Syn-BMT ($n = 4$), Syn-BMT + Syn-TT ($n = 4$), Syn-BMT + Allo-TT ($n = 4$), Allo-BMT ($n = 4$), Allo-BMT + Allo-TT ($n = 4$), Allo-BMT + 3rd-party TT ($n = 4$), and BALB/c mice ($n = 4$). Data are shown as mean ± SD

become a viable strategy for the treatment of advanced cancer in humans.

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Conflict of interest statement We have no conflict of interest in this manuscript.

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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

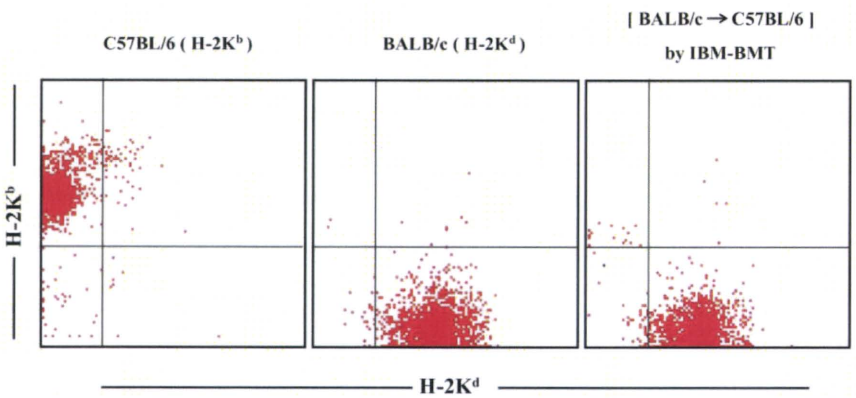

FIGURE 1. Reconstitution of donor-derived hemopoietic cells after IBM-BMT. B6 mice at the age of 8 weeks were irradiated with a lethal dose (6 Gy ×2), and BMCs from normal BALB/c mice were injected directly into the bone marrow cavity (IBM-BMT) of the left tibia. After 3 months, cells from the peripheral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

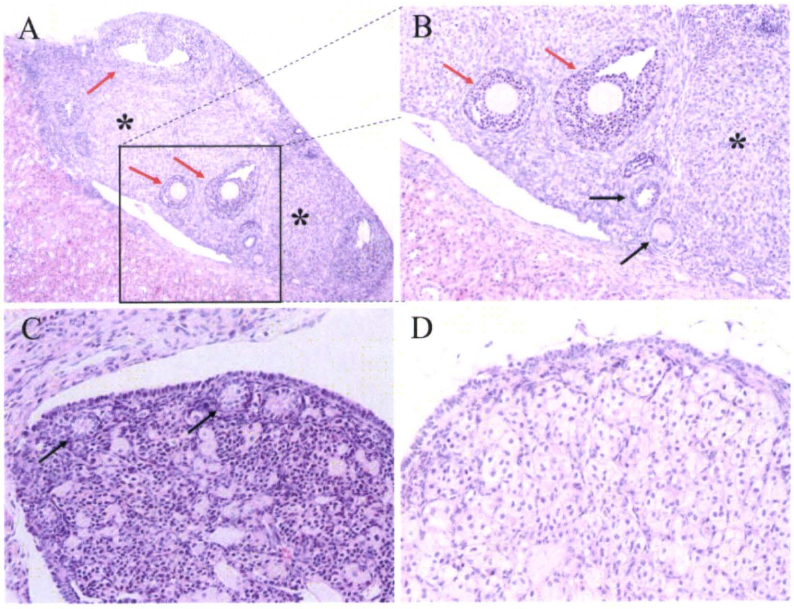

FIGURE 2. Histology of transplanted ovary and host ovary. Three months after bone marrow transplantation/ovarian transplantation (BMT/OT), the ovaries were accepted (original magnification ×100, A) with a large number of mature follicles (*red arrows*), primary follicles (*black arrows*), and corpus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

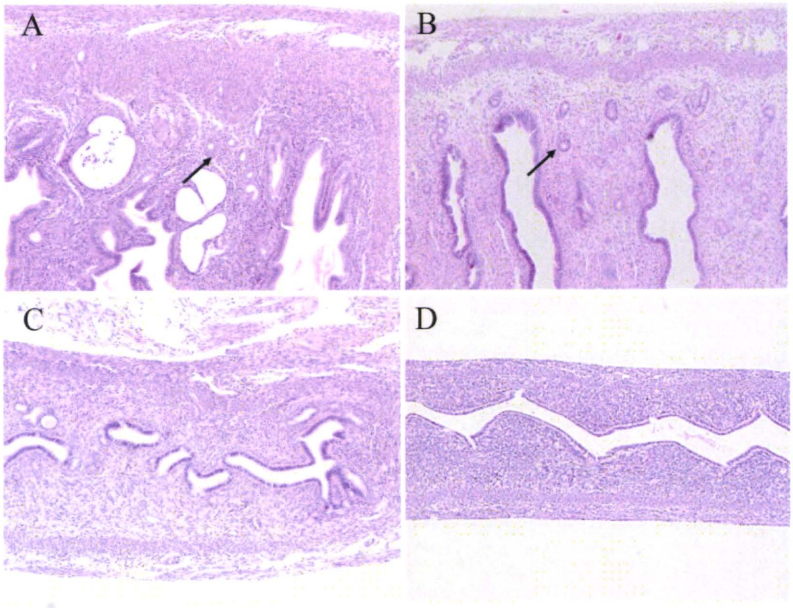

FIGURE 3. Effects of IBM-BMT with OT on uterus. Three months after bone marrow transplantation (BMT), the uteri of the four groups, including the normal control (nontreated) group (A), the BMT/ovarian transplantation (OT) group (B), the BMT group (C), and the OvX group (D), were stained with hematoxylin-eosin. 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-

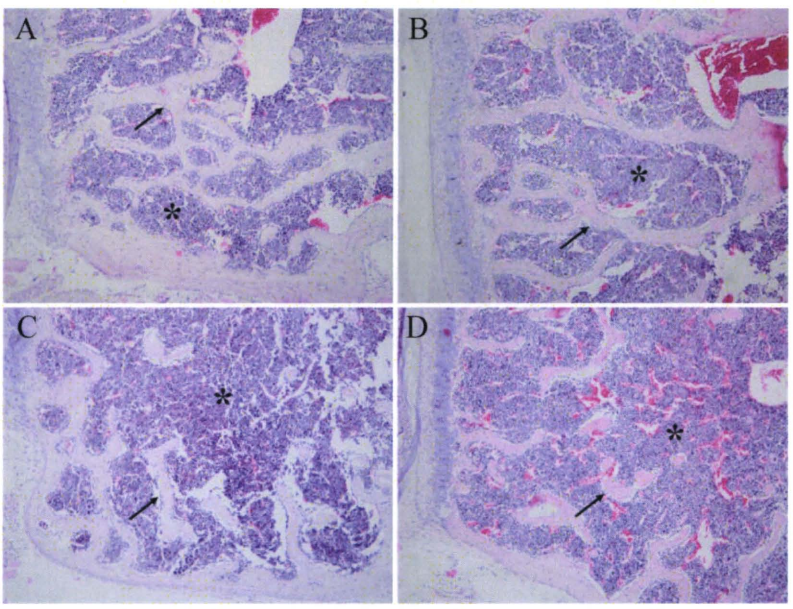

FIGURE 4. Histology of the lumbar vertebrae after intrabone marrow-bone marrow transplantation (IBM-BMT). Three months after IBM-BMT, the 4th lumbar vertebrae of mice in the four groups—normal control group (A), bone marrow transplantation/ovarian transplantation (BMT/OT) group (B), BMT group (C), and 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	$23.71 \pm 7.80^{a,b}$	0.62 ± 0.19^{a}
OVX	$11.69 \pm 4.41^{c,d}$	$1.28 \pm 0.33^{c,d}$
BMT/OT	$21.47 \pm 13.44^{a,b}$	0.71 ± 0.39^{a}
BMT	$13.77 \pm 4.09^{c,d}$	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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Mouse mesenchymal stem cells can support human hematopoiesis both *in vitro* and *in vivo*: the crucial role of neural cell adhesion molecule

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ABSTRACT

Background
We previously established a mesenchymal stem cell line (FMS/PA6-P) from the bone marrow adherent cells of fetal mice. The cell line expresses a higher level of neural cell adhesion molecule and shows greater hematopoiesis-supporting capacity in mice than other murine stromal cell lines.

Design and Methods
Since there is 94% homology between human and murine neural cell adhesion molecule, we examined whether FMS/PA6-P cells support human hematopoiesis and whether neural cell adhesion molecules expressed on FMS/PA6-P cells contribute greatly to the human hematopoiesis-supporting ability of the cell line.

Results
When lineage-negative cord blood mononuclear cells were co-cultured on the FMS/PA6-P cells, a significantly greater hematopoietic stem cell-enriched population ($CD34^+CD38^-$ cells) was obtained than in the culture without the FMS/PA6-P cells. Moreover, when lineage-negative cord blood mononuclear cells were cultured on FMS/PA6-P cells and transplanted into SCID mice, a significantly larger proportion of human $CD45^+$ cells and $CD34^+CD38^-$ cells were detected in the bone marrow of SCID mice than in the bone marrow of SCID mice that had received lineage-negative cord blood mononuclear cells cultured without FMS/PA6-P cells. Furthermore, we found that direct cell-to-cell contact between the lineage-negative cord blood mononuclear cells and the FMS/PA6-P cells was essential for the maximum expansion of the mononuclear cells. The addition of anti-mouse neural cell adhesion molecule antibody to the culture significantly inhibited their contact and the proliferation of lineage-negative cord blood mononuclear cells.

Conclusions
These findings suggest that neural cell adhesion molecules expressed on FMS/PA6-P cells play a crucial role in the human hematopoiesis-supporting ability of the cell line.

Key words: neural cell adhesion molecule, cord blood, human hematopoiesis, mesenchymal stem cells.

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Introduction

Human umbilical cord blood (CB) has been used as an alternative source of hematopoietic stem cells (HSC) for various diseases, such as leukemia, aplastic anemia and autoimmune diseases. The advantages of CB transplantation over bone marrow (BM) or mobilized peripheral blood stem cell transplantation include the ease of stem cell collection, the less stringent requirement on human leukocyte antigen (HLA) matching between donors and recipients, as well as the low severity of graft-*versus*-host diseases.[1-3] However, the low cell content in CB units is a major limiting factor, particularly for adult recipients, which has confined the use of CB transplants mostly to patients with low body weight.[1,2] Some studies have demonstrated that successful engraftment can be achieved in CB transplantation with a cell dose of over 4×10^7 nucleated cells/kg body weight of the recipient.[1-4] When insufficient numbers of cells are grafted, the consequent delay in reconstitution causes a high morbidity and mortality, due to systemic infections, accompanied by high costs due to hospitalization and blood cell transfusions. Thus, efforts are being made to generate large number sof HSC and progenitor cells by *ex vivo* expansion in order to improve the applicability and outcome of CB transplantation. Some clinical improvements have been observed in trials using expanded CB cells,[5] BM cells,[6] and peripheral blood stem cells.[7,8] However, a major disadvantage of culturing HSC in the presence of hematopoietic growth factors is the accelerated differentiation from HSC to lineage cells, possibly at the expense of multipotent HSC with self-renewal and long-term engrafting potential.[9] It has been reported that long-term hematopoiesis can be maintained only by co-culturing HSC with stromal cells in human and mouse hematopoietic systems.[10-15] We have also found that successful BM transplantation depends on the co-transplantation of stromal cells obtained from donor mice;[16-19] stromal cells migrate into the recipient BM and spleen, where they support hematopoiesis. These findings have shaped the view that stromal cell-hematopoietic cell interactions in the marrow microenvironment are crucial for physiological hematopoiesis.

We have recently obtained a mesenchymal stem cell line (FMS/PA6-P) from BM adherent cells of day-16 fetal mice.[20,21] This cell line is highly positive for neural cell adhesion molecules (NCAM) and shows a higher hematopoiesis-supporting capacity in mice than other stromal cell lines (MS-5[12] and PA6).[20] The human cDNA sequence encoding NCAM (145-kDa isoform) was reported by Saito *et al.* in 1994[22] and we found that there is 94% homology between human and murine NCAM. In the present study, therefore, we attempted to examine whether the FMS/PA6-P cells support human hematopoiesis and whether NCAM expressed on the FMS/PA6-P cells contributes greatly to the human hematopoiesis-supporting ability of the cell line.

Design and Methods

Purification of lineage-negative cord blood mononuclear cells from human cord blood

CB samples were collected from cord veins of uncomplicated full-term, vaginal deliveries. The samples were collected into bags containing citrate-phosphate-dextrose (Terumo, Japan) and processed within 24 h. Informed consent was obtained for all CB collections and this study was approved by the Ethics Committee for Clinical Research of Kansai Medical University. Low-density CB mononuclear cells were isolated by Ficoll-Paque PLUS density gradient centrifugation (<1.077g/mL, GE Healthcare, Uppsala, Sweden) and cryopreserved in IMDM medium containing 10% dimethyl sulfoxide and 20% fetal bovine serum (FBS) until use. Dead cells contained in the cryopreserved low-density CB mononuclear cells were depleted using the Ficoll-Paque PLUS density gradient centrifugation. Lineage-positive cells, expressing CD3, CD9, CD11b, CD14, CD15, CD16, CD19, CD20 and CD235a (glycophorin A) molecules, were then removed using a magnetic bead separation system; the low-density CB mononuclear cells were incubated with monoclonal antibody (mouse IgG class; BD Biosciences Pharmingen, San Diego, CA, USA) cocktails against the above-mentioned lineage markers, and then incubated twice with sheep anti-mouse IgG-conjugated immunobeads (#110.31; Dynal Inc., Oslo, Norway) with gentle agitation at 5:1 and 3:1 bead/cell ratios. The immunobead-rosetted cells were removed using a magnetic particle concentrator. The thus-prepared lineage-negative CB mononuclear cells (L CBMC) were considered as a partially-HSC-enriched population. The L CBMC were stained with fluorescent isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies against human CD34 (#348053), CD38 (#555459) and CD56 (#556647) (BD Biosciences Pharmingen). Cells stained with isotype-matched IgG served as a negative control. The stained cells were analyzed by a FACScan (BD, Mountain View, CA, USA).

Co-culture of lineage-negative cord blood mononuclear cells on FMS/PA6-P cells

The FMS/PA6-P cells were cultured in 12-well plates containing DMEM (low glucose) supplemented with 10% FBS at 37°C in 5% CO_2 in air and confluent monolayers were prepared. After irradiation (20 Gy) of the FMS/PA6-P monolayers, the L CBMC (7×10^3/well in 1 mL IMDM supplemented with 10% FBS and human cytokines [SCF, Flt3-L and TPO (20 ng/mL)] were inoculated. Flt3-L (#300-19) was purchased from PeproTech (Rocky Hill, NJ, USA). TPO and SCF were kindly donated by the Kirin Brewery Co. Ltd. (Tokyo, Japan). The L CBMC were also cultured without the FMS/PA6-P cells. Weekly, half of the medium in the wells containing non-adherent cells was removed and replaced with fresh medium. At 1 and 2 weeks, all non-adherent cells in the well were collected, and the adherent cells (FMS/PA6-P cells + hematopoietic cells which adhered to or under the FMS/PA6-P cells) were collected by trypsin-EDTA treatment. The adherent and non-adherent cells obtained from the same well were mixed, and the number of hematopoietic cells was counted (the FMS/PA6-P cells are larger than hematopoietic cells and, therefore, easily distinguished from these latter).

The collected cells were stained with FITC- or PE-labeled monoclonal antibodies against human CD34 (#CD34-581-04) (Caltag), CD11b (#555388), CD38 (#555459), CD235a (#555570) (BD Biosciences Pharmingen), CD15 (#IM1954), CD14 (#IM0650) and CD41 (#IM1416) (Beckman Coulter, Fullerton, CA, USA). The stained cells were analyzed by a FACScan.

The number of colony-forming cells (CFU-C), including colony-forming unit-granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M), colony-forming unit-granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), were assessed in clonal cell culture using a methylcellulose assay (Methocult GF H3434, Stem Cell Technologies Inc., Vancouver, BC, Canada).

The collected cells were also spread on a glass slide using a cyto-

centrifuge and stained with May-Giemsa reagent to observe their morphology.

The L⁻CBMC were also co-cultured on a monolayer of MS-5 cells[12] (kindly donated by the Kirin Brewery Co. Ltd.) and the hematopoiesis-supporting ability of the MS-5 cells was analyzed using the methods described above.

Engraftment of culture-expanded cells into SCID mice

Seven- to 8-week-old Icr-Scid (SCID) mice were purchased from Japan Clear Experimental Animal Laboratory (Tokyo, Japan). All mice were maintained in a pathogen-free environment and were kept for at least 2 weeks before the initiation of experiments. Experiments using mice were conducted in accordance with protocols approved by the university's committee for animal research. The L⁻CBMC (5×10^4/well) were cultured in the presence of the FMS/PA6-P cells for 2 weeks as described above. All the cells, including the FMS/PA6-P cells from one well, were collected using trypsin-EDTA and injected into 3 Gy-irradiated SCID mice (n=6) via the intravenous route (total 6 mice). The L⁻CBMC were also cultured in the absence of the FMS/PA6-P cells, and the culture-expanded cells were collected without trypsin-EDTA treatment and transplanted into SCID mice (n=6).

For the assessment of human CD45⁺ cells and subsets, the mice were sacrificed by cervical dislocation 8 weeks post-transplantation, and the femora and tibiae were removed and cleaned of all connective tissue. BM cells were collected by flushing the femora and tibiae with 2% FBS/PBS using a 26-gauge needle, filtered, and washed twice. Peripheral blood cells were collected by heart puncture. For flow cytometric analyses, contaminating red blood cells were lysed with BD Pharm Lyse™ Lysing Buffer (BD Biosciences Pharmingen) and washed with 2% FBS/PBS. These cells were then double-stained with anti-human CD45 (#0452 or #0454, Exalpha Biologicals, Inc., Watertown, MA, USA) and anti-human CD14, CD19 (#302205, BioLegend, San Diego, CA, USA), CD34, CD41 or CD235a monoclonal antibodies or with anti-human CD34 and anti-human CD38 monoclonal antibodies. The stained cells were analyzed by a FACScan.

Polymerase chain reaction (PCR) analysis using primers targeted to human specific DNA 17-α satellite gene was also performed to confirm the engraftment of human cells into the BM of the SCID mice. The sequences of the human-specific DNA 17-α satellite gene are as follows: Forward, gggATAATTTCAgCTgAC TAAACAg; Reverse, TTCCgTTTAgTTAggTgCAgTTATC.

Non-contact culture of lineage-negative cord blood mononuclear cells on FMS/PA6-P cells

Confluent FMS/PA6-P monolayers were prepared in a 24-well plate and irradiated at a dose of 20 Gy. The L⁻CBMC (2.5×10^4/well) were loaded directly on the stromal layer (contact culture) or loaded into a culture chamber insert (pore size: 0.45 μm, Intercell, Kurabo, Osaka, Japan) placed above the stromal layer (non-contact). The same number of L⁻CBMC were cultured without the stromal layer. The culture medium consisted of 10% FBS/IMDM containing human cytokines [SCF, Flt3-L and TPO (20 ng/mL)]. After 2 weeks, all cells in the well were harvested, and flow cytometric analyses and clonal cell cultures were performed.

Addition of anti-neural cell adhesion molecule antibodies to co-culture of lineage-negative cord blood mononuclear cells and FMS/PA6-P cells

Confluent FMS/PA6-P monolayers prepared in a 24-well plate were irradiated at a dose of 20 Gy and the culture medium was replaced with fresh IMDM supplemented with 10% FBS and anti-mouse NCAM monoclonal antibody recognizing the three major isoforms (120, 140 and 180 kDa) (1 μg/mL, #556324, Clone: N-CAM 13, BD Bioscience Pharmingen) or corresponding isotype (mouse IgG2a)-matched monoclonal antibody. After 2 h, 3.4×10^3 L⁻CBMC were added to the wells and co-cultured. On day 7, half of the medium in the wells containing non-adherent cells was removed and replaced with fresh medium with anti-NCAM or isotype monoclonal antibodies. Two weeks later, all cells were collected and analyzed as described above.

Statistics

The engraftment experiment was carried out twice and the *in vitro* culture experiments three or more times. Reproducible results were obtained. Representative data are shown in the figures. Statistical differences in all experiments were analyzed by a Student's two-tailed t test.

Results

Expansion of human hematopoietic stem cells and progenitor cells on FMS/PA6-P cells in vitro

To examine the human hematopoiesis-supporting ability of the FMS/PA6-P cells, we purified a human HSC-enriched population (L⁻CBMC) from a CB sample. May-Giemsa staining revealed that the L⁻CBMC showed HSC-like features (Figure 1A). The proportions of $CD34^+CD38^-$ and $CD34^+CD56(NCAM)^+$ cells in the population were 23.8±3.8% and 4.2±0.6%, respectively. When the L⁻CBMC were inoculated on the FMS/PA6-P cell layer (7×10^3/well), the L⁻CBMC adhered rapidly to the stromal layer, and 2196.1±190.9 cells/well had adhered to the layer by 4 h after the inoculation. These cells then began to "crawl" under the stromal layer and 1880.2±242.6 cells/well showed pseudoemperipolesis to the FMS/PA6-P cells 18 h later. Cell division under the stromal layer was seen at and after 32 h. The proliferating cells demonstrated a cobblestone-like appearance on day 2-3 of culture and were referred to as "cobblestone area-forming" cells (CAFC) (Figure 1A).

After 1 or 2 weeks of culture, adherent and non-adherent cells in the co-culture with the FMS/PA6-P cells were collected (the former by typsin-EDTA treatment) and assessed for cellularity and differentiation capacity. There were only non-adherent cells in the culture without the FMS/PA6-P cells, and these cells were, therefore, collected without trypsin-EDTA treatment. In the co-culture of L⁻CBMC with FMS/PA6-P cells, a significantly higher production of hematopoietic progenitor cells ($CD34^+$ cells) as well as HSC-enriched population ($CD34^+CD38^-$ cells) was observed at both 1 and 2 weeks (Figure 1B) than in the culture without FMS/PA6-P cells. The numbers of total hematopoietic cells, $CD34^+$ cells and $CD34^+CD38^-$ cells increased by 51-, 27-, and 25-fold of the original cell input in the co-cultures with the FMS/PA6-P cells at 1 week, whereas only 19-, 9- or 8-fold increases were found in the culture without FMS/PA6-P cells. An even more remarkable difference was observed at 2 weeks; total cells, $CD34^+$ cells and $CD34^+CD38^-$ cells increased by 526-, 205- and 189-fold, respectively, in the co-cultures with FMS/PA6-P cells and by 107-, 30- and 26-fold in the cultures without FMS/PA6-P cells.

The culture-expanded cells were then examined for their ability to form clonal hematopoietic colonies (CFU-C) using MethoCult GF H3434. Significantly higher CFU-C counts were observed at 1 week in co-cultures with

FMS/PA6-P cells than in the cultures without FMS/PA6-P cells (Figure 1B). Although there was no evident difference in the total CFU-C counts between the two culture conditions at 2 weeks, the cell components of the CFU-C differed; a higher number of lineage-committed CFU-C (CFU-G, CFU-M and BFU-E, but not CFU-GM and CFU-GEMM) was detected in the cultures without the FMS/PA6-P cells than in the co-cultures with the FMS/PA6-P cells. Adherent cells in the co-cultures were collected using trypsin-EDTA treatment. Our preliminary experiments showed that trypsin-EDTA treatment of freshly-isolated and cultured L$^-$CBMC did not affect the expression of cell surface markers (CD11b, CD14, CD15, CD34, CD38, CD41 and CD235a) or cell viability. In contrast, the total CFU-C counts were reduced to 73.2% in freshly-isolated L$^-$CBMC and 74.5% in the cultured L$^-$CBMC after the trypsin-EDTA treatment. There was no evident decrease in the CFU-M counts, but CFU-G, BFU-E, CFU-GM and CFU-GEMM counts were markedly reduced after the trypsin-EDTA treatment. Thus, the trypsin-EDTA treatment affects not only the total CFU-C count but also the cell components of CFU-C. It is, therefore, possible that the actual CFU-C counts in the co-culture with the FMS/PA6-P cells would have been much higher than the values shown in the present experiments. These findings suggest that FMS/PA6-P cells can support the proliferation of human HSC and progenitors *in vitro*.

Lineage-positive cells expressing CD11b, CD14, CD15, CD41 or CD235a molecules were also detected by flow-cytometric analysis in the co-culture of L$^-$CBMC with FMS/PA6-P cells (*data not shown*). The production of mature hematopoietic cells was further confirmed by May-Giemsa staining of the non-adherent cells recovered from the co-culture; normoblasts and megakaryocytes were seen in addition to many granulocytes and macrophages (Figure 1C). These findings suggest that the FMS/PA6-P cells facilitate the proliferation of human HSC and progenitors, resulting in the production of mature myeloid, erythroid and megakaryocytic cells. A slightly, but not statistically significantly higher percentage of lineage-positive (CD11b, CD14, CD15, CD41 or CD235a-positive) cells was also seen in the culture without FMS/PA6-P cells than in the culture with FMS/PA6-P cells (*data not shown*).

It is known that the murine BM stromal cell line MS-5 has hematopoiesis-supporting ability for human cells.[15] We previously showed that NCAM is expressed at lower levels on MS-5 than on FMS/PA6-P cells.[20] Here we co-cultured L$^-$CBMC on MS-5 cells and the hematopoiesis-supporting ability of these latter was compared with that of the FMS/PA6-P cells. At 1 week of culture, there was no significant difference in the number of total cells, CD34$^+$ cells, CD34$^+$CD38$^-$ cells or CFU-C per well between the two cultures. At 2 weeks, however, the number of CD34$^+$ cells and CD34$^+$CD38$^-$ cells per well was, respectively, 1.40 and 1.38 times higher in the culture on the FMS/PA6-P cells than in that on the MS-5 (both $P<0.05$). This finding indicates that the FMS/PA6-P cells have greater hematopoiesis-supporting ability than the MS-5 cells in the present experimental system.

Engraftment of ex vivo-expanded human hematopoietic stem cells and progenitor cells into SCID mice

To investigate whether the HSC and progenitor cells produced in the co-culture system with FMS/PA6-P cells are able to proliferate and differentiate *in vivo*, we injected the culture-expanded cells into sublethally-irradiated SCID mice. All cells in each culture well, including the expanded cells and FMS/PA6-P cells, were harvested using trypsin-EDTA treatment and injected intravenously into a single SCID mouse. Eight weeks after the transplantation, there was no significant difference in survival rates between the mice which had received the cells co-cultured with the FMS/PA6-P cells (hereafter described as the co-cultured group) (5/6) and those which had received cells

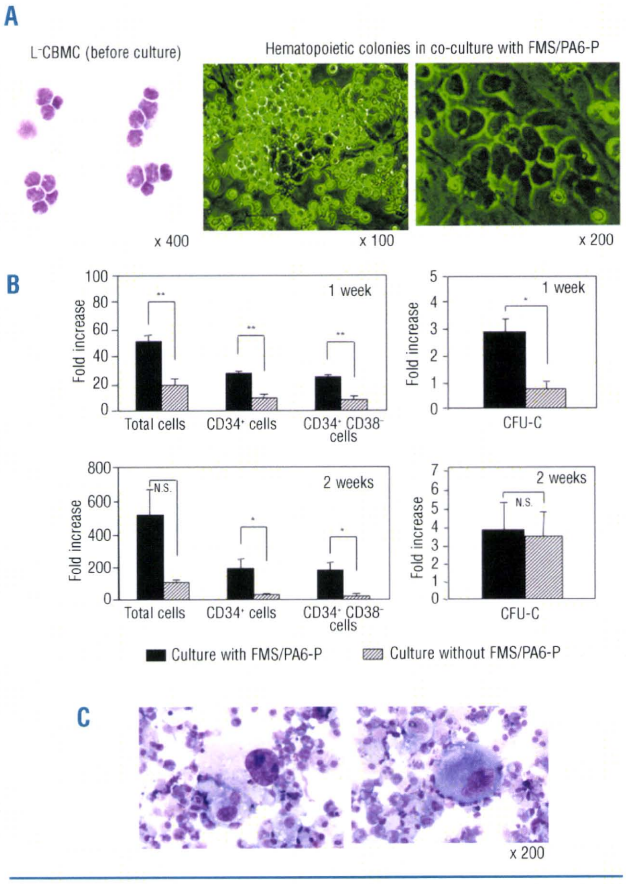

Figure 1. Human hemopoiesis-supporting capacity of FMS/PA6-P cells *in vitro*. (A) Morphology of L$^-$CBMC and formation of cobblestone colonies in the co-culture of L$^-$CBMC with FMS/PA6-P cells. The purified L$^-$CBMC were stained with May-Giemsa reagent (left photograph). The L$^-$CBMC ($7x10^3$/well) were inoculated to the monolayers of confluent FMS/PA6-P cells (20 Gy-irradiated) and the formation of cobblestone areas was observed on day 2-3 of culture (middle and right photographs). Phase-contrast images (B) Expansion of L$^-$CBMC on the monolayer of FMS/PA6-P cells. The L$^-$CBMC ($7x10^3$/well) were cultured with or without the FMS/PA6-P cells (20 Gy-irradiated). At 1 and 2 weeks, all cells in the well were collected by trypsin-EDTA treatment and the number of hematopoietic cells was counted. The number of CD34$^+$ cells and CD34$^+$CD38$^-$ cells per well was calculated from the hematopoietic cell number per well and the percentages of these populations obtained by flow cytometric analyses. The number of CFU-C per well was also calculated from the hematopoietic cell number per well and the number of CFU-C/10^4 cells obtained by clonal cell culture assay. Fold Increase = the number of hematopoietic cells per well after culture / the number of hematopoietic cells per well before culture. The fold increases of CD34$^+$ cells, CD34$^+$CD38$^-$ cells and CFU-C counts were also calculated from the number of these populations before and after culture, respectively. Each sample was run in triplicate. Representative data from three independent experiments. $^{**}P<0.01$; $^{*}P<0.05$. (C) Multi-lineage differentiation of L$^-$CBMC in co-culture with FMS/PA6-P cells. At 2 weeks of culture, non-adherent cells were collected from the co-culture of the L$^-$CBMC and FMS/PA6-P cells, and were stained with May-Giemsa reagent.