mice were kept for at least 2 weeks before the initiation of the experiments. B6 mice (15–16 weeks old) were used for recipients and both C3H (4–5 weeks old) and B/c (4–5 weeks old) mice for donors of BMCs and bones. Two- to three-day-old C3H mice were obtained by mating in our animal facility and used for donors of bones. A.SW (H-2^s) mice were kindly supplied by Dr. Hashimoto (Kanazawa University). The university's committee for animal research approved all experiments.

Proliferation ability and cellularity of BM adherent cells

BMCs were flushed from the humeri, femora and tibiae of 2–3-day-old (suckling) or 4–5-week-old (adult) B6 mice using a 27 G needle attached to a syringe into phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS). After gentle dissociation, a BMC suspension was prepared. The BMCs were collected from four or five suckling or adult mice. It is known that many BM stromal cells lodge near bones. Therefore, the bones, from which BMCs had been flushed out, were cut into pieces in 2% FBS/PBS, and BM suspensions containing many BM stromal cells was prepared. The two kinds of the BMC suspensions were then mixed and filtered through a nylon mesh in order to obtain single cells and remove bone pieces, and then the cell number was counted. Various numbers ($0.1–5 \times 10^4$ cells/well) of BMCs were put in a 96-well plate containing 10% FBS/IMDM. More than 15 wells were prepared per each cell number. Two weeks later, the medium was removed and the adherent cells on the 96-well plate were stained by May–Giemsa solution. The number of wells having fibroblastic cell colonies was counted as positive wells.

In some experiments, the cellularity of BM adherent cells was compared between suckling and adult mice. BMCs and bone pieces from suckling or adult mice were cultured in flasks and BM adherent cells were obtained 2 weeks later. The BM adherent cells were detached from the flasks via trypsin/EDTA treatment and double stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b and phycoerythrin (PE)-conjugated anti-CD45 monoclonal antibodies (mAbs) (BD-Pharmingen, San Diego, CA). The stained cells were then quantified with a FACScan (Becton Dickinson, Mountain View, CA).

Hemopoiesis-supporting ability of BM adherent cells

BMCs and bone pieces collected from suckling or adult B6 mice were cultured in flasks containing 10% FBS/IMDM for 3 weeks in order to obtain adherent cells. The adherent cells were collected by trypsinization and then re-cultured in a 96-well plate (10^4 cells/well). After 3 days, the adherent cells were irradiated (20 Gy) and then partially purified HSCs (the purification procedure is shown below) from 4 to 5-week-old B6 mice were inoculated on the adherent cell layer (5000 cells/well). These cells were cultured for 5 days and ^{3}H-thymidine (TdR) (0.5 μCi) was introduced during the last 24 h of the culture period. The uptake of ^{3}H-TdR was measured using 1450 MicroBeta TRILUX (Perkin-Elmer, Wellesley, MA, USA).

The partially purified HSCs were purified from whole BMCs as follows: low-density ($\rho < 1.077 g/cm^3$) (LD) BMCs were obtained by discontinuous density gradient centrifugation using Lymphoprep (Axis-Schield PoC AS, Oslo, Norway). The LD cells were incubated with mAb (rat IgG class) cocktails against lineage makers (Mac-1, Gr-1, B220, CD4, CD8, and TER119) (BD-Pharmingen) for 30 min on ice, and then incubated twice with sheep anti-rat IgG-conjugated immunobeads (Ibs) (Dynal Inc. Oslo, Norway) at 4 °C for 20 min with gentle agitation at a 2:1 bead/cell ratio. Ibs-rosetted cells were removed using a magnetic particle concentrator. Non-rosetted cells were recovered and reincubated with the same number of beads mentioned above. The remaining non-rosetted cells (LD and lineage-negative cells) were used as partially purified HSCs.

Bone marrow transplantation and bone graft

Recipient B6 mice received a fractionated irradiation (5.5 + 5.5 Gy, 4 h interval) of gamma-ray from a ^{137}Cs source (Gammacell 40 Exactor; Nordion International Inc.; Kanata, Ontario, Canada; http://www.mdsnordion.com) 1 day before transplantation. Injection of 5×10^6 BMCs from C3H or B/c mice (5–6 weeks old) into the tibia cavity (IBM–BMT) or the tail vein (IV-BMT) of recipient mice was performed as described previously. Briefly, for IBM–BMT, the mice were anesthetized with pentobarbital sodium (0.05 g/g body weight), and the left tibia was gently drilled with a 26 G needle through the patellar tendon. The donor BMCs in 10 μl were injected into the bone cavity through the hole in the tibia using a Hamilton microsyringe.

On the same day, C3H or B/c bones (femora, tibiae, and humeri) from suckling or adult mice were engrafted subcutaneously and under the renal capsules. The subcutaneous tissues and renal capsules were selected as the sites of bone grafts because it was previously shown that hemopoietic regeneration could be easily achieved in the bones implanted into these well-vascularized tissues (Tavassoli and Crosby, 1968; Fan et al., 1990). For the transplantation under the renal capsule, a small incision was made in the capsule and bones were inserted into the subcapsular space. For the subcutaneous transplantation, subcutaneous pouches

were prepared on the lateral side of the recipient mice using forceps and the bones were grafted in the pouches. Before the bone grafts, all the bones had been flushed of their BMCs to avoid necrotic tissue damage by poor circulation of blood and body fluid, and the bones from adult mice were cut into 3–4 pieces to adjust their length to the size of the bones from suckling mice. The bones collected from two suckling mice were grafted to a single recipient mouse, and the bones collected from one adult mouse were grafted to a single recipient mouse.

Analysis of chimerism

Twelve weeks after the IBM injection, the peripheral blood mononuclear cells (PBMCs), BMCs, spleen cells and thymic cells were phenotyped for recipient/donor cells by flow cytometry. The PBMCs were obtained from the recipient blood by discontinuous density gradient centrifugation using Lymphocyte-mammal (Cedarlane; Ontario, Canada). These cells were incubated with FITC-conjugated anti-H-2K mAb and PE-conjugated anti- Mac-1, Gr-1, B220, TER119, CD4, or CD8 mAbs (BD-Pharmingen). The stained cells were then quantified with a FACScan.

Mixed lymphocyte reaction (MLR)

Spleen cells (responder cells) were collected from the single-chimeric, untreated (age-matched to the chimeric mice) B6 or C3H mouse and put in flat-bottomed 96-well micro-well trays containing a total of 0.2 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, and 50 μM 2-mercaptoethanol (2-ME) (Wako; Osaka, Japan) (2×10^5 cells/well). Spleen cells (stimulator cells) from untreated A.SW, C3H and B6 mice (7–8 weeks old) were irradiated with 15 Gy and added to the responder cells. The cells (triplicate) were incubated for 4 days in a humidified 5% CO_2 atmosphere. ^{3}H-TdR (0.5 μCi) was introduced during the last 18 h of the culture period and the uptake of ^{3}H-TdR was measured.

Plaque-forming cell (PFC) assay

T cells were enriched from the spleen cells of the single-chimeric mouse by depleting B cells and APCs using mAbs against I-A^b (clone: AF6-120.1) and I-A^k (clone: 11-5.2) (BD-Pharmingen) plus sheep anti-mouse IgG-conjugated immunobeads (Dynal Inc., Oslo, Norway). T-cell-depleted spleen cells were prepared by treating the spleen cells from normal C3H and B6 mice with anti-CD4 (clone: RM4-5) and anti-CD8 (clone: 53-6.7) mAbs (BD-Pharmingen) plus sheep anti-rat IgG-conjugated immunobeads. The T cells (3×10^6) were co-cultured in a 24-well plate containing 2 ml of RPMI supplemented with 10% FCS and 50 μM 2-ME with T-cell-depleted spleen cells (9×10^6) in the presence or absence of sheep red blood cells (4×10^6) (SRBCs). The anti-SRBC antibody-producing cells in the cultured cells were counted by direct PFC assay (triplicate) 5 days later.

Analyses for donor-derived adherent cells

To prepare the BM-derived adherent cells, the femora and tibiae were obtained from contralateral sites (donor BMC-non-injected sites) of the chimeric mice. The BMCs and bone pieces were cultured in a flask containing IMDM with 10% FBS at 37 °C in 5% CO_2 in air. The spleen cells and thymic cells were also collected from the chimeric mice and cultured. The medium in the culture flask was replaced with the same volume of fresh medium weekly. Three weeks later, non-adherent cells, if any, were extensively removed, and the adherent cells were then collected from the surface of the flask using trypsin–EDTA solution (Sigma; St. Louis, MO). To detect donor-derived stromal cells, the cultured cells were stained with anti-PA6 mAb (rat IgG class), which is specific for BM stromal cells (Izumi-Hisha et al., 1991) followed by PE-anti-rat IgG (BD-Pharmingen, San Diego, CA). After blocking with normal rat IgG (BD-Pharmingen), the cells were further stained with FITC-anti-H-2K^b or anti-H-2K^k mAb, and analyzed by a FACScan. The cultured cells stained with the isotype-matched immunoglobulins served as a negative control.

Confocal microscopy

The BM, spleen and thymus were collected from the chimeric mice and frozen using OCT compound (Tissue-Tek; Miles Inc., USA). The tissue sections (3 μm, without any fixation) were incubated for 20 min at room temperature with anti-PA6 mAb, and FITC-labeled anti-rat IgG Ab (Serotec; Sapporo, Japan). These sections were further stained using PE-labeled anti–H-2K^k mAbs (BD-Pharmingen). The negative control was stained with anti–H-2K^d (third-party) and anti-PA6 mAb + FITC-labeled anti-rat IgG Ab. The stained samples were examined on a confocal laser scanning microscope (LSM-GB200; Olympus, Tokyo, Japan) equipped with a $\times 40$ objective lens. The samples were visualized using a band pass F490-560 filter after excitation at 488 nm for FITC and a high pass TR 610 after excitation at 568 nm for PE.

Statistics

Statistical differences in survival rates were analyzed by a log-rank test and those in other experiments were

analyzed by Student's 2-tailed t-test. Each experiment was carried out five or more times. Reproducible results were obtained, and therefore representative data are shown in the figures.

Results

Comparison of proliferative and hemopoiesis-supporting capacities between BM adherent cells derived from bones of suckling and adult mice

In our previous report (Li et al., 2000), we have shown that the engraftment of donor cells can be facilitated by the co-administration of donor (8–10 weeks old) bones, and that donor BM stromal cells migrate into the recipient thymus. To determine the suitable age for the donor bones, we investigated both the proliferative ability and the HSC-supporting ability of BM adherent cells obtained from suckling (2–3 days old) or adult (4–5 weeks old) mice.

BMCs obtained from suckling or adult mice were put in a 96-well plate and cultured for 2 weeks. The number of wells having fibroblastic cell colonies (stromal cell colonies) was counted as positive wells. Fig. 1A shows the percentages of wells having stromal cell colonies. The BMCs from suckling mice had a significantly higher proliferation activity of stromal cells than the BMCs from adult mice; only 50% of wells showed stromal cell colonies in the adult BMCs even if 5×10^4 BMCs/well were cultured. This result indicates that the BMCs from suckling mice contain higher amounts of stromal cells and/or stromal progenitor cells than adult BMCs. Indeed, when all BM adherent cells were stained with anti-CD11b and anti-CD45 mAbs, a higher percentage ($40.2 \pm 9.3\%$) of double-negative cells (which are considered as stromal cells) was observed in the BM adherent cells from suckling mice in comparison with those ($14.3 \pm 6.3\%$) of adult mice, indicating that BM adherent cells from bones of suckling mice (hereafter described as suckling bones) contain almost three times more stromal cells than those from adult bones. In contrast, the whole BM adherent cells of adult mice contained double-positive cells (considered as macrophages) at the percentage of $77.0 \pm 8.8\%$, whereas suckling mice contained macrophages at the low percentage of $56.6 \pm 7.8\%$.

The above results show that suckling bones contain about three times more stromal cells than adult bones. However, adult donor bones are quite large in size, compared with suckling bones, and therefore the total BM adherent cell number obtained from one mouse is much higher in an adult than in a suckling: $4.02 \pm 0.58 \times 10^4$ cells per adult mouse versus $0.65 \pm 0.87 \times 10^4$ cells per suckling mouse. In other words, an adult mouse has about six times more BM adherent cells than a suckling mouse. Taking these results into consideration, we determined to graft the bones obtained from two suckling mice into one recipient mouse, but the bones obtained from one adult mouse into one recipient mouse, because the amount of BM stromal cells in the grafted bones is almost equivalent in such combination of bone grafts.

Next, the supporting capacity of the BM adherent cells was examined. Partially purified HSCs derived from adult (4–5 weeks old) mice were cultured on the BM adherent cells derived from suckling or adult mice. About six times higher supporting ability was observed in the adherent cells from suckling mice than in those of adult mice (Fig. 1B). These results clearly indicate that the BM adherent cells of suckling mice are superior to those of adult mice in proliferative ability and supporting of HSCs.

Survival and reconstitution of chimeric mice

The aim of the present study is to examine the possibility that the co-administration of donor bones in conjunction with IBM–BMT can induce a complete reconstitution (containing T-cell functions), even if relatively low numbers of donor BMCs are injected into aged mice. Therefore, aged (more than 15 weeks old) B6 mice were used for recipients and suboptimal doses (5×10^6) of BMCs were injected into the recipient mice. Facilitating effects of co-grafted bones on hematolymphoid recovery were compared between the mice that received suckling or adult bones. All the data shown below are the experimental results of the chimeric [C3H → B6] mice. Experiments of the combination of [B/c → B6] were also performed and the efficiency of suckling bone grafts plus IBM–BMT was confirmed (data not shown).

As demonstrated in Exp. 1 of Fig. 2, a higher (not significant) survival rate was observed in the mice that received IBM–BMT + suckling bones (the bones obtained from two suckling mice were grafted into one recipient mouse), compared with the mice that received IBM–BMT + adult bones (the bones obtained from one adult mouse were grafted into one recipient mouse). When BMCs were injected into the recipient mice via the IBM route without co-administration of bones, the survival rates were lower (not significant) than the mice that received IBM–BMT + adult bones. All mice grafted with BMC alone via the IV route died within 16 weeks. Co-administration of suckling bones is not effective in the case of IV-BMT, because no improvement in survival rates was observed in the IV-BMT + suckling bone group (Exp. 2 in Fig. 2). All mice that received suckling bones alone died soon after the transplantation. These results indicate that suckling bones have a

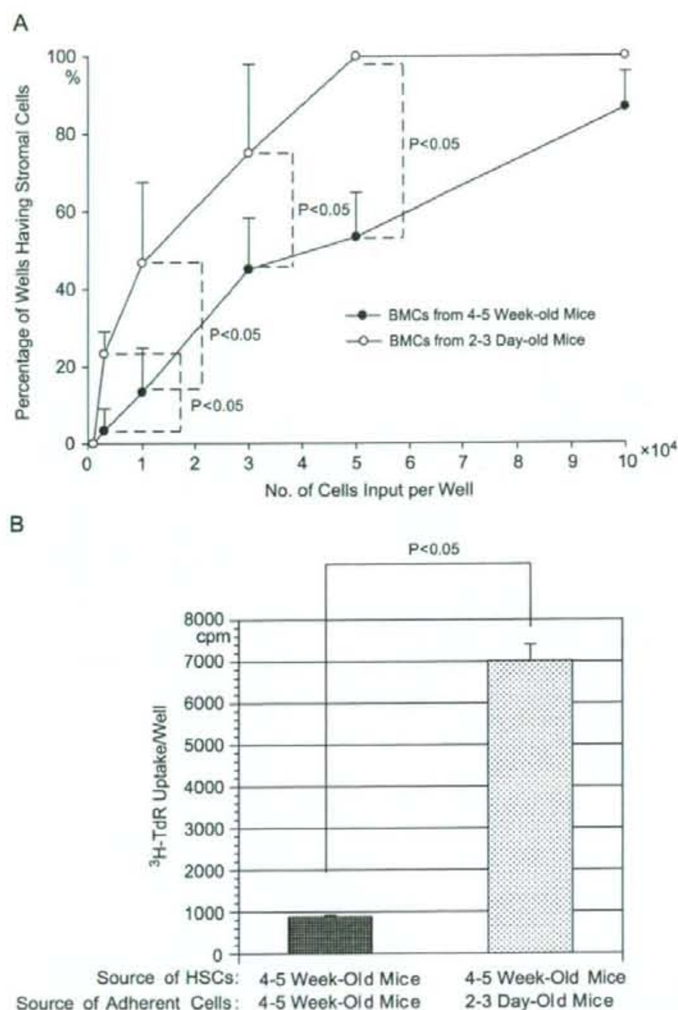

Fig. 1. Comparison of BM adherent cells derived from suckling or adult mice. (A) Higher frequency of BM stromal cells derived from suckling mice. BMCs were collected from four or five suckling (2–3 days old) or adult (4–5 weeks old) C3H mice and cultured in various cell numbers using a 96-well plate for 2 weeks. The wells having fibroblastic cells, which are considered as stromal cells, were counted as positive wells. Mean ± SE of five independent experiments. (B) Higher supporting ability of BM adherent cells derived from suckling mice on HSC-proliferation assay. BMCs were collected from four or five suckling or adult C3H mice and cultured for 3 weeks in order to obtain adherent cells. The adherent cells were collected by trypsinization and then cultured in a 96-well plate. After 3 days, LD, Lin- HSCs from adult C^3H mice were inoculated on the adherent cell layer (5000 cells/well) (quadruplicate). These cells were cultured for 5 days and the uptake of ^{3}H-TdR was measured. Mean ± SE of quadruplicates. Representative data of six independent experiments.

greater ability to rescue the lethally irradiated mice, when co-grafted with IBM–BMT. The survival rate up to 16 or 20 weeks post-transplantation is shown here, because no death due to graft failure was observed thereafter.

Fig. 3 shows a typical FACS pattern of various hematolymphoid tissues in the mice that received IBM–BMT alone or IBM–BMT + suckling bone grafts, 3 months after BMT. All the tissues from the mice of the IBM–BMT alone group showed recipient-type hematolymphoid cells. In contrast, almost complete reconstitution with donor-type cells was achieved in the IBM–BMT + suckling bone graft group. Table 1 shows the percentages of donor- and recipient-type cells in peripheral blood (PB) obtained from chimeric mice 6 weeks after BMT. We selected this time point because

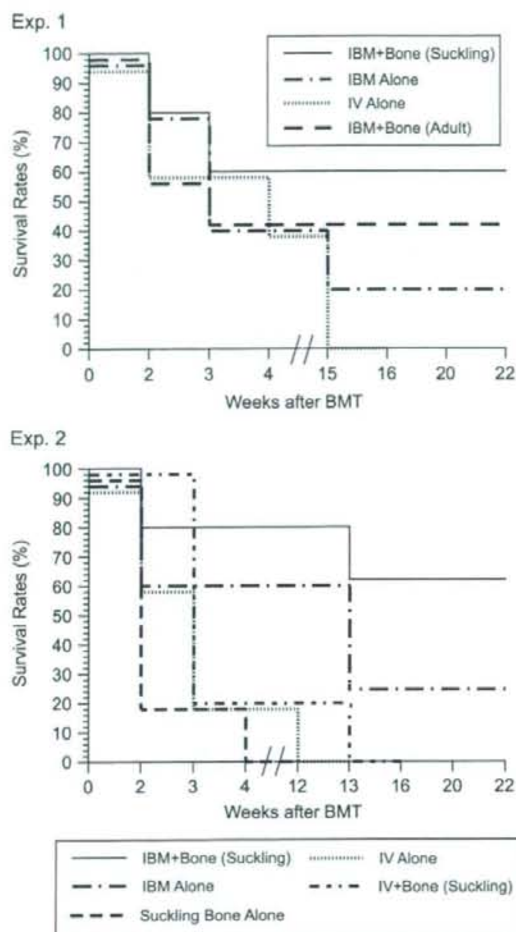

Fig. 2. Higher survival rate in chimeric mice that received IBM–BMT+suckling bone graft. C3H BMCs were injected into the irradiated B6 mice via IV or IBM pathway with or without bone graft (5–6 recipient mice per each group). Two representative survival rates (Exps.1 and 2) of 10 independent experiments are shown.

the relative balance between donor- and recipient-type cells in each chimeric mouse remained at a similar level thereafter, and the number of surviving chimeric mice in each group was sufficient (more than five mice) at the 6th week after BMT but subsequently declined, particularly in the chimeric mice of IV-BMT groups. The reconstitution to donor type was highest (93.7%) in the mice that received IBM–BMT+suckling bone grafts compared with the other five groups. Thereafter, stable reconstitution to donor type could be maintained in the mice, because $98.1 \pm 0.9\%$ ($n = 6$) of PBMCs were donor type even 8 months after BMT. A similar percentage (87.7%) of donor-type cells was also observed in the mice that received IBM–BMT+adult bone grafts (not significantly different from the mice of BMT+suckling bone grafts), but recipient-type cells still remained at 6.7% [significantly ($P<0.05$) higher than the mice that received BMT+suckling bone grafts]. The mice that received IBM–BMT alone showed much lower engraftment of donor cells compared with the mice that received IBM–BMT+bone grafts. The reconstitution to donor type was very low in all the IV-BMT-treated groups. Therefore, it is obvious that IBM–BMT can induce a higher engraftment of donor cells than IV-BMT.

Analyses of allo-reactive proliferation and MHC restriction of T cells in chimeric mice

To examine the functional recovery of lymphocytes, we employed MLR assay using spleen cells from the chimeric mice. As shown in Fig. 4A, the spleen cells of the mice that received IBM–BMT+suckling bones showed no responses against donor (C3H) and recipient (B6) cells, whereas they responded well against the third-party (A.SW) cells. This finding indicates that the lymphocytes have acquired immunotolerance to both donor and recipient cells, but not to third-party cells. In contrast, the spleen cells of the mice that received IBM–BMT alone showed a response against donor cells.

MHC restriction of T cells in chimeric mice was analyzed using the PFC assay. In this assay, B cells can differentiate into anti-SRBC antibody-producing cells in collaboration with MHC-matched T cells and APCs. T cells from normal or chimeric mice were co-cultured with SRBCs and APCs+B cells of either C3H or B6 mice. The PFCs per well were counted (Fig. 4B). Higher PFC counts were observed when T cells of normal B6 mice were cultured with APCs+B cells from the same strain, whereas MHC incompatibility between T cells and APCs+B cells resulted in a substantially lower production of PFC. It is obvious that T cells of the chimeric mouse that received BMT+suckling bone grafts have acquired MHC restrictions to the donor types, because higher PFC counts were obtained when the T cells were co-cultured with APCs+B cells of C3H mice. In the chimeric mice without bone grafts, however, T cells are still restricted to the recipient (B6) type, not the donor type.

Detection of donor-derived BM stromal cells in BM, splenic and thymic adherent cells of chimeric mice

BM adherent cells were prepared by a 3–4-week culture of BMCs obtained from the chimeric mice. The adherent cells were double-stained with anti-PA6

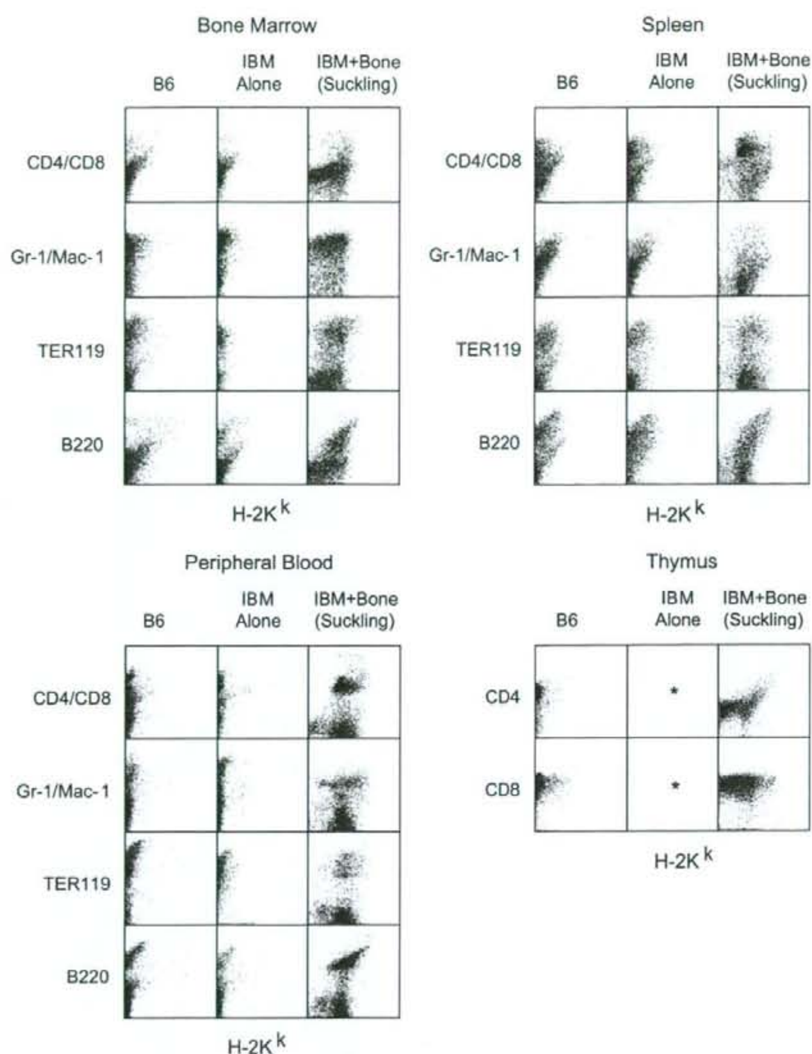

Fig. 3. FACS analysis of lineage-positive cells in BM, peripheral blood, spleen and thymus of chimeric mice that received IBM–BMT alone or IBM–BMT + suckling bone graft (12 weeks after BMT). Twelve weeks after BMT, the PBMCs, BMCs, spleen cells and thymic cells were collected from chimeric mice and stained with FITC-conjugated anti-H-$2K^k$ mAb and PE-conjugated anti- Mac-1, Gr-1, B220, TER119, CD4, or CD8 mAbs. As a control, the cells collected from untreated B6 were also stained as mentioned above. The stained cells were then analyzed using a FACScan. A representative staining pattern of 10 independent experiments is shown. *Thymi of B6 mice that received IBM–BMT alone were very small. They showed fibrosis, and insufficient thymic cells were obtained.

(specific for BM stromal cells) and donor-type anti-H-$2K^k$ mAbs. As shown in Fig. 5A, H-2, expression was examined in the PA6-positive fraction of BM adherent cells obtained from the chimeric mice. In the IBM–BMT + suckling bone graft group, most cells in the PA6-positive fraction were donor type. In contrast, host-type cells were predominant in the mice that received IBM–BMT alone or IV-BMT alone (Fig. 5A).

Splenic and thymic adherent cells of the mice that received BMT + suckling bone grafts were also double-stained with anti-PA6 and donor-type anti-H-$2K^k$ mAbs. The adherent cell population, recognized by the anti-PA6 mAb, contained more donor-type cells than host-type cells (Fig. 5B and C).

Table 1. Higher percentage of donor-type cells in PBMCs from chimeric mice that received IBM–BMT + suckling bone graft

Treatment	Percentage of	
	Donor-type cells ($H\text{-}2^k$)	Recipient-type cells ($H\text{-}2^b$)
IBM + bone (suckling)	93.7 ± 6.5 ($n = 6$)	0.9 ± 0.8 ($n = 6$)
IBM + bone (adult)	87.7 ± 11.6 ($n = 6$)*	6.7 ± 12.3 ($n = 6$)$^{\#}$
IBM alone	11.4 ± 15.8 ($n = 5$)**	84.8 ± 11.6 ($n = 5$)$^{\#\#}$
IV + bone (suckling)	11.3 ± 21.0 ($n = 5$)$^{+}$	86.1 ± 20.3 ($n = 5$)‡
IV + bone (adult)	9.0 ± 12.3 ($n = 5$)$^{++}$	87.3 ± 10.6 ($n = 5$)‡‡
IV alone	1.1 ± 2.0 ($n = 5$)$^{+++}$	95.1 ± 2.3 ($n = 5$)‡‡‡

PBMCs were obtained from the chimeric mice 6 weeks after BMT. The cells were double stained with anti-H-$2K^k$ and anti-H-$2K^b$ mAbs and analyzed using FAC Scan.

Versus donor-type cells in IBM + bone (suckling): *NS, $^{**}P<0.001$, $^{+}P<0.001$, $^{++}P<0.001$, $^{+++}P<0.001$.

Versus recipient-type cells in IBM + bone (suckling): $^{\#}P<0.05$, $^{\#\#}P<0.001$, $^{\ddagger}P<0.001$, $^{\ddagger\ddagger}P<0.001$, $^{\ddagger\ddagger\ddagger}P<0.001$.

Detection of donor-derived BM stromal cells in BM, splenic and thymic sections of chimeric mice using confocal microscopy

To examine whether donor-type BM stromal cells really exist in the BM, spleen and thymus of the chimeric mice that received IBM–BMT + suckling bone grafts, these tissue sections were double-stained with anti-PA6 and anti-H-$2K^k$ mAbs and observed using confocal microscopy (Fig. 6). In these chimeric mice, hematolymphoid cells in various tissues were reconstituted by donor-type cells, and the T cells showed donor-type MHC restriction (Figs. 3 and 4). All the BM and spleen cells showed donor-type H-$2K^k$ (Panel 1 in Fig. 6A and B) and a small population in the BM and spleen was stained with anti-PA6 mAb (Panel 2 in Fig. 6A and B). Some cells in Panel 3 showed yellow fluorescence, indicating that H-$2K^k$-positive cells have molecules detected by the anti-PA6 mAb. This result shows that donor-derived BM stromal cells exist in the BM and spleen of the chimeric mice.

Similar to the results of the BM and spleen, most thymic cells were stained positively by donor-type H-$2K^k$ (Panel 1 in Fig. 6C) and a few cells were recognized with anti-PA6 mAb (Panel 2 in Fig. 6C). Some cells showing yellow fluorescence were found in Panel 3 of Fig. 6C. These results indicate that donor-derived BM stromal cells also migrate into the thymus of the chimeric mice and take crucial roles in the maturation process of thymic cells. As a negative control, the staining of the thymic section with anti-H-$2K^d$ (a third party) and anti-PA6 mAb was also performed (Fig. 6D).

Discussion

It is well known that in mice and humans the recovery of T-cell functions is incomplete after BMT across MHC barriers. In the present study, we have shown that the highest survival rate and complete hematolymphoid reconstitution (including T-cell functions) can be achieved only when donor (suckling) bones are co-grafted in conjunction with IBM–BMT. In the treated recipient mice, the MLR showed tolerance to both recipient-type and donor-type MHC determinants (Fig. 4A). Moreover, the PFC assay also indicated that the T cells of such mice show donor-type major MHC restriction (Fig. 4B). Notably, the migration of donor-type BM stromal cells was observed in the recipient thymi (Figs. 5 and 6). Our previous report (Li et al., 2000) has demonstrated that complete reconstitution including T-cell functions can be achieved in adult recipient mice when 2×10^6 BMCs, from which T cells, macrophages and stromal cells have been depleted using Sephadex G-10 column and anti-Thy 1.2 Ab + complement, are administered by IV route, suggesting that BM stromal cells derived from the grafted donor bones play a crucial role in the regeneration of donor BMCs. The present study demonstrated that IBM–BMT + suckling bone grafts is superior to the previous BMT protocol (IV-BMT + adult bone grafts) in several ways. First, in the present study, a suboptimal number of donor cells (5×10^6 BMCs/recipient mouse) could reconstitute effectively the recipient mice when suckling bones were co-grafted. In the previous experiments, 2×10^6 BMCs, from which T cells, macrophages and stromal cells had been depleted, were administered by the IV route. During such depletion procedure, the number of BMCs declined greatly and, therefore, about 3.3×10^7 whole BMCs are needed to get thus-depleted 2×10^6 BMCs. In contrast, the present study shows that IBM–BMT + suckling bone grafts need only 5×10^6 whole BMCs to reconstitute the recipients completely. Second, suckling bones obtained from only two mice are sufficient to reconstitute the recipient mice, whereas adult bones from three mice were used in the previous experiments. Third, the IBM–BMT + suckling bone grafts can reconstitute even adult (more than 15 weeks old) mice. In the adult mice that received IBM–BMT + suckling bone grafts, the thymi contained a nearly normal number of thymic cells and showed normal architecture (Fig. 6) and T cells of the mice had acquired MHC restriction to donor type (Fig. 4B).

In the present study, we also compared the reconstitution of donor cells (Table 1) between the two groups

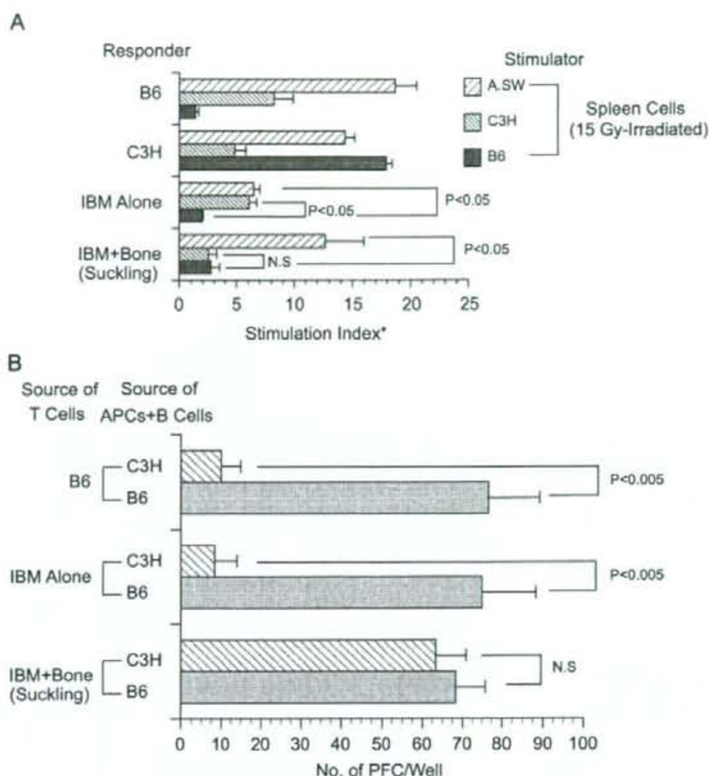

Fig. 4. Analyses of lymphocyte function in chimeric mice. (A) Mixed lymphocyte reaction of spleen cells in chimeric mice that received IBM–BMT alone or IBM–BMT + suckling bone grafts. Spleen cells were collected from a single chimeric mouse 15 weeks after BMT. Spleen cells were also obtained from a single-normal B6 or C3H mouse. The cells were cultured in the presence of stimulator cells (triplicate). Four days later, ^{3}H-TdR uptake was measured. Tolerance of both donor and recipient types was observed in the chimeric mice that received IBM–BMT + suckling bone grafts. Mean ± SD of triplicates. Representative data of five independent experiments. *Stimulation index = ^{3}H-TdR uptake on sample well (responder + stimulator)/^{3}H-TdR uptake on control well (responder alone). (B) MHC restriction of T cells obtained from chimeric mice to donor-type cells in anti-SRBC PFC response. T cells enriched from spleen cells, obtained from a single chimeric mouse (15 weeks after BMT) or a single normal B6 mouse, were cultured with SRBC and APCs + B cells of either C3H or B6. Five days later, direct PFC assay was performed (triplicate). Mean ± SD of triplicates. Representative data of seven independent experiments.

(IBM–BMT + suckling bone grafts versus IV-BMT + adult bone grafts). The mice that received IBM–BMT + suckling bone grafts showed a significantly higher engraftment of donor cells than those that received IV-BMT + adult bone grafts. In these experiments, the bones obtained from two suckling mice were grafted into one recipient mouse, whereas the bones obtained from one adult mouse were grafted into one recipient mouse. The grafted bone numbers per recipient mouse was determined, as shown in the "Results", by both the total BM adherent cell number obtained from one suckling or adult mouse and the frequency of BM stromal cells among the BM adherent cells and, therefore, we believe that the facilitating effect of adult and suckling bones was evaluated precisely.

It is generally accepted that BM stromal cells obtained from younger mice have a higher hemopoiesis-supporting ability than those obtained from aged mice (Hotta et al., 1980). Recently, there have been several reports showing that rat BM-derived mesenchymal stem cells (MSCs) lower the chondrogenic potential with age (Zheng et al., 2007), and that telomere length of human MSCs is shortened rapidly following *in vitro* expansion (Baxter et al., 2004). However, an opposite observation, showing that age does not influence the adipogenic and myogenic differentiation of human MSCs, was also reported (Roura et al., 2006). MSCs have some phenotypes (surface molecule expression, growth factor secretion and hemopoiesis-supporting ability, etc.) similar to traditional BM stromal cells.

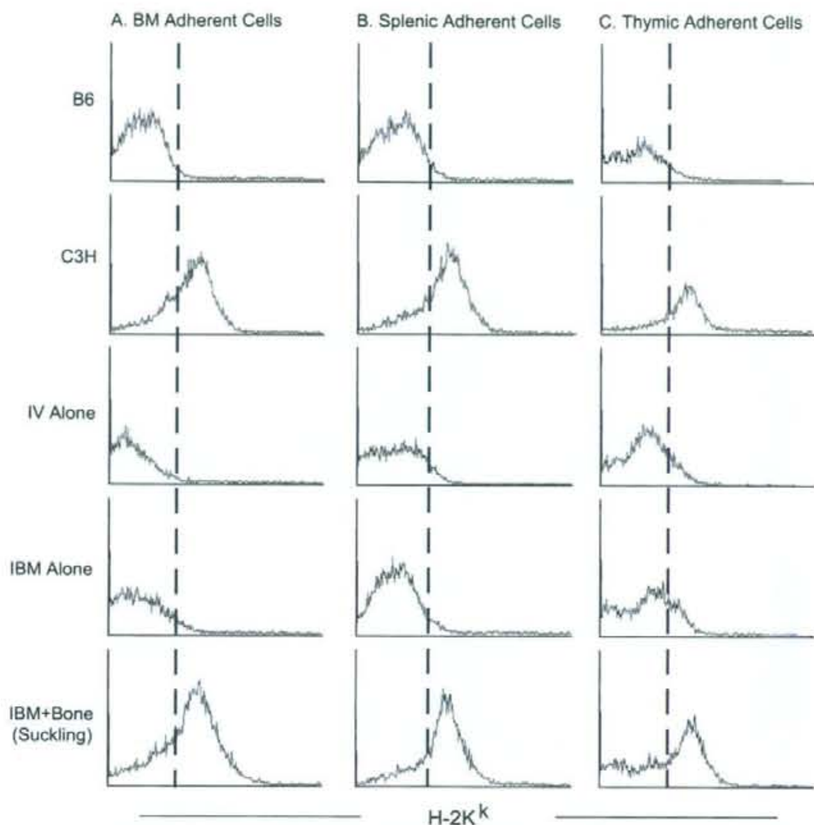

Fig. 5. H-2 expression of BM stromal cell population among BM, splenic and thymic adherent cells from normal or chimeric mice that received IBM–BMT alone or IBM–BMT + suckling bone grafts. BMCs, spleen cells and thymus cells were collected from a single chimeric mouse 14 weeks after BMT and cultured for 3–4 weeks in order to obtain adherent cells. The adherent cells were collected by trypsinization and double stained with anti-PA6 and anti-H-$2K^k$ mAbs. As a control, the adherent cells derived from a single normal B6 or C3H mouse were also double-stained. PA6-positive populations were considered as BM stromal cells. H-$2K^k$ expression in the PA6-positive populations was compared. A representative staining pattern of five independent experiments.

In this sense, we can consider that there is no obvious difference between MSCs and BM stromal cells. Our present study showed clearly that the BM adherent cells derived from suckling bones contained CD45- and CD11b-double-negative cells (BM stromal cells) at a higher level than those derived from adult bones. Moreover, the BM adherent cells from suckling bones have a higher proliferation and supporting ability, in contrast to those from adult mice (Fig. 1). Taking our present results and other laboratories' reports (Hotta et al., 1980; Zheng et al., 2007; Baxter et al., 2004) into consideration, it is conceivable that BM stromal cells from younger animals have much higher hemopoiesis-supporting, proliferating and differentiating ability than those from older animals. We are now investigating whether BM adherent cells obtained from fetal mice possess much higher hemopoiesis-supporting and proliferating ability, in comparison with those obtained from suckling and adult mice.

The PA6 mAb reacts with BM adherent cells but not with peritoneal macrophages (Izumi-Hisha et al., 1991) and thymic dendritic cells. This mAb does not react with any cells in the spleen, lymph nodes, thymus or liver of normal mice (Izumi-Hisha et al., 1991). Moreover, our previous reports show that this mAb also inhibits the pseudoemperipolesis of PA6 (a stromal cell line) to HSCs for at least 24 h, and that the proliferation of HSCs is greatly reduced when HSCs are cultured for 8–12 days in the presence of this mAb, suggesting that it binds to the molecules important for the interaction between stromal cells and HSCs. Very recently, we have found that the protein recognized with the anti-PA6 mAb is a neural cell adhesion molecule (NCAM) (Wang et al., 2005), and that a BM stromal cell line

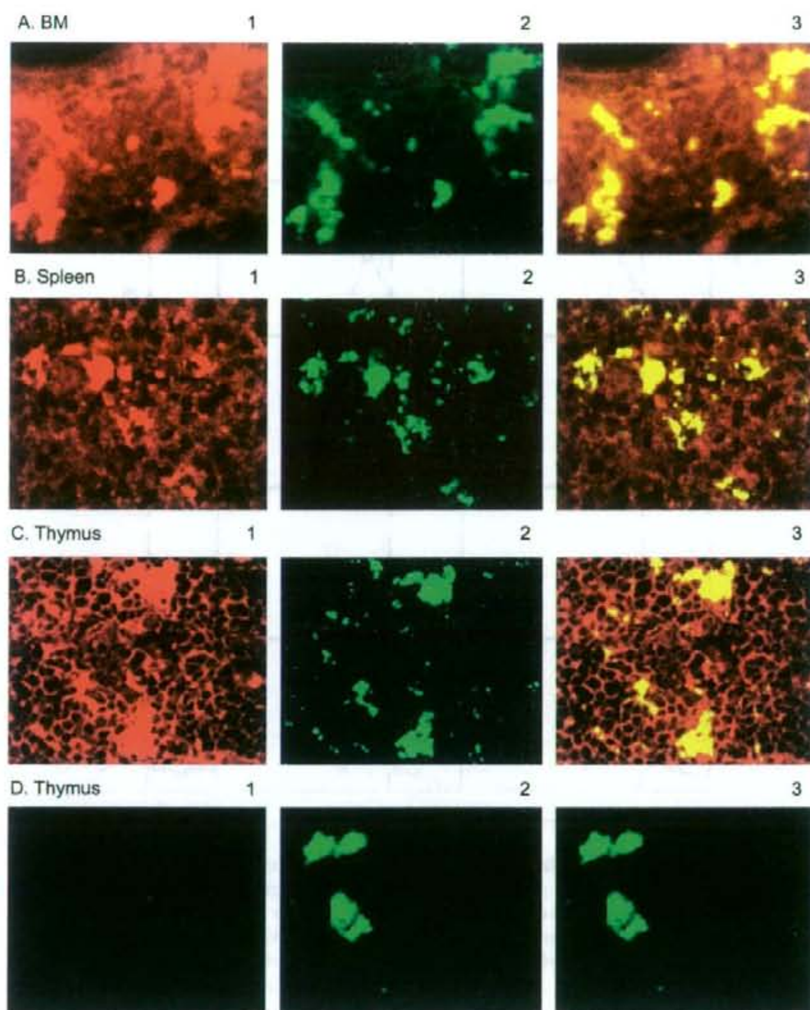

Fig. 6. Presence of donor-type BM stromal cells in BM, spleen and thymus of chimeric mice that received IBM–BMT + suckling bone grafts. BM, splenic and thymic sections, obtained from chimeric mice 15 weeks after BMT, were double stained with PE-anti-H-2K^k and FITC-anti-PA6 mAbs and then analyzed by confocal microscopy. The images are displayed in three panels with red (PE), green (FITC) and yellow signals (both) (Panels 1–3 in A–C). Panels 1–3 in (D) shows the staining of the thymic section with PE-anti-H-2K^d (a third party) and FITC-anti-PA6 mAbs. ×400. A representative staining pattern of five independent experiments.

(FMS/PA6-P) established from fetal mouse BMCs using the anti-PA6 mAb have characteristics of MSCs (Wang et al., 2006). Indeed, we have found very recently that NCAM molecules are expressed on human and monkey BM-derived MSCs (manuscript in preparation). Therefore, it seems likely that the PA6-positive populations found in the recipient thymi (Figs. 5 and 6) represent BM-derived stromal cells and/or MSCs. Our previous study (Li et al., 2000), demonstrated that the population stained with both anti-PA6 and H-2 mAbs was very small in normal mice (2.3–3.2%), whereas 9.3–34.4% of thymic adherent cells were stained with both anti-PA6 and donor-type H-2 mAbs in the chimeric mice that received BMT + bone grafts. In the present experiments, a similar increase of the anti-PA6 and donor-type H-2 mAb-positive population was observed in the chimeric mice that received IBM–BMT + suckling bone grafts, suggesting that donor-type stromal cells migrate from the engrafted bones into the recipient thymus.

In conclusion, the successful engraftment was achieved by co-administration of donor suckling bones even in aged mice that received a low number of donor

BMCs by the IBM route. Although further studies are required, this study might provide a valuable strategy for older patients to whom BMT has not been applied so far.

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TRANSLATIONAL AND CLINICAL RESEARCH

Intra-Bone Marrow Injection of Donor Bone Marrow Cells Suspended in Collagen Gel Retains Injected Cells in Bone Marrow, Resulting in Rapid Hemopoietic Recovery in Mice

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Key Words. Intra-bone marrow bone marrow transplantation • Collagen gel • Colony-forming units of spleen • Reconstitution • Enhanced green fluorescent protein

ABSTRACT

We have recently developed an innovative bone marrow transplantation (BMT) method, intra-bone marrow (IBM)-BMT, in which donor bone marrow cells (BMCs) are injected directly into the recipient bone marrow (BM), resulting in the rapid recovery of donor hemopoiesis and permitting a reduction in radiation doses as a pretreatment for BMT. However, even with this IBM injection, some of the injected BMCs were found to enter into circulation. Therefore, we attempted to modify the method to allow the efficient retention of injected BMCs in the donor BM. The BMCs of enhanced green fluorescent protein transgenic mice (C57BL/6 background) were suspended in collagen gel (CG) or phosphate-buffered saline (PBS), and these cells were then injected into the BM of irradiated C57BL/6 mice. The numbers of retained donor cells in the injected BM, the day 12 colony-forming units of spleen (CFU-S) counts, and the reconstitution of donor cells after IBM-BMT were compared between the CG and PBS groups. The number of transplanted cells detected in the injected BM in the CG group was significantly higher than that in the PBS group. We next carried out CFU-S assays. The spleens of mice in the CG group showed heavier spleen weight and considerably higher CFU-S counts than in the PBS group. Excellent reconstitution of donor hemopoietic cells in the CG group was observed in the long term (>100 days). These results suggest that the IBM injection of BMCs suspended in CG is superior to the injection of BMCs suspended in PBS. STEM CELLS *2008;26:2211–2216*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow transplantation (BMT) was originally developed to treat congenital immunodeficiencies and hematologic disorders [1, 2]. Recently, BMT-related methods have been improved, because of the discoveries of more effective immunosuppressants, powerful antibiotics, antithymocyte globulin, and fractionated irradiation, all of which add up to a better prognosis [3, 4]. BMT has, at this stage, been carried out for the treatment not only of immunodeficiencies and hemopoietic diseases but also of autoimmune diseases and solid malignant tumors [5–10]. However, BMT is still a difficult procedure because of the risk of lethal side effects, such as infection, graft-versus-host disease (GVHD), graft failure, and so on [11–13]. Recently, we developed a new and powerful BMT method: intra-bone marrow (IBM)-BMT [14]. In this method, donor bone marrow cells (BMCs) are injected directly into the recipient bone marrow (BM), and a much greater number of donor hemopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) can therefore be inoculated into the recipient BM than by conventional i.v. BMT, resulting in the rapid reconstitution of donor hemopoietic cells and permitting a reduction in radiation doses as a pretreatment for BMT [14, 15]. In addition, we have shown that the IBM-BMT can be used for organ transplantation, with the engrafted organs surviving long-term without the use of immunosuppressants [16, 17]. Collagen gel (CG) was originally used for the three-dimensional cell culture systems for isolated cells [18]. Cellmatrix (Nitta Gelatin, Inc., Yao, Japan, http://www.nitta-gelatin.com) is an acidic soluble type I collagen that is liquid on ice but forms a gel when warm. In this paper, we show that the CG helps retain more injected donor BMCs in the

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recipient BM upon IBM-BMT than phosphate-buffered saline (PBS), resulting in excellent reconstitution of donor cells and permitting a reduction in radiation doses as a pretreatment for BMT.

Materials and Methods

Mice

C57BL/6 mice (B6) were purchased from SLC (Shizuoka, Japan, http://www.jslc.co.jp) for use as recipients, and enhanced green fluorescent protein (eGFP) transgenic (tg) mice, for use as donors, were kindly donated by Dr. Okabe (Osaka University, Osaka, Japan) [19]. These mice were used at 8–10 weeks of age. The university's committee for animal research approved all experiments.

Reagents

The antibodies (Abs) used in this study were as follows: phycoerythrin (PE)-labeled anti-mouse CD3 Ab, anti-mouse B220 Ab, anti-mouse Gr-1 Ab, peridinin chlorophyll protein (PerCP)-Cy5.5-labeled anti-mouse CD45 Ab, biotin-labeled anti-CD3 Ab, biotin-labeled anti-B220 Ab, biotin-labeled anti-CD11c Ab, biotin-labeled anti-Mac-1 Ab, biotin-labeled anti-NK1.1 Ab, biotin-labeled TER119 Ab, and biotin-labeled anti-Gr1 Ab (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml). Cellmatrix, a purified collagen solution for cell culture, was purchased from Nitta Gelatin, Inc.

Whole-Body Irradiation of Recipient Mice

Gamma radiation was delivered by a Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada, http://www.mds.nordion.com) with two ^{137}Cs sources. Recipient mice were irradiated with 7, 8, 9 or 10 Gy, the day before BMT.

Preparation of the Collagen Gel Matrix

The collagen gel matrix, Cellmatrix, was prepared following the manufacturer's instructions. That is, solutions A, B, and C were mixed at a ratio of 8:1:1 and were kept on ice to prevent gel formation until use. This is because the mixture is liquid on ice but becomes a gel when it is warmed. Hereafter, this mixture is referred to as collagen gel.

Treatment of Donor BMCs

BMCs were flushed from the medullary cavities of the femurs and tibias of donor mice with PBS. After gentle dissociation, the BMC suspension was filtered through a 70-μm nylon mesh (Becton Falcon, Franklin Lakes, NJ, http://www.bd.com). The BMCs were counted.

The BMCs were then divided into two groups: the CG group and the PBS group. The BMC suspension was centrifuged, and the supernatant was aspirated. The BMCs were suspended in 4°C PBS for the PBS group and ice-cold CG for the CG group and adjusted to 10^7, 10^8, or 10^9 cells per milliliter.

Bone Marrow Transplantation

One day after irradiation, the BMCs of eGFP tg mice were transplanted into recipient mice directly into the bone cavity via the intra-bone marrow route (IBM-BMT), as previously described [14]. Briefly, the mice were anesthetized, and the area from the inguinal region to the knee joint was shaved. The tibia was gently drilled with a 26-gauge needle through the patellar tendon into the BM cavity. BMCs suspended in PBS or CG were aspirated into a microsyringe (50 μl; Ito, Fuji, Shizuoka, Japan, http://www.ito-ex.co.jp) and then kept at room temperature. The BMCs (10^5, 10^6, or 10^7 in 10 μl) were then injected into the BM cavity using the microsyringe.

Analysis of Donor Cells in Recipient BM, Peripheral Blood, or Spleen

To detect injected donor BMCs in the recipient BM, the BMCs of eGFP tg mice (10^7 in 10 μl) or cultured MSCs (5×10^5 in 10 μl) were transplanted into the BM of B6 mice the day after 10-Gy irradiation. One hour after IBM-BMT, the B6 mice were sacrificed and BMC-injected bones were flushed to obtain the BMCs. The BMCs were then stained with biotin-labeled lineage Abs (anti-Gr1, anti-Mac-1, anti-TER119, anti-B220, anti-NK1.1, anti-CD3, and anti-CD11c Abs), PE-labeled anti-Sca-1 Ab, and PerCP-Cy5.5 labeled anti-CD45 Ab, followed by incubation with allophycocyanin (APC)-coupled streptavidin. The cells were analyzed using a FACSCalibur instrument (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com).

To detect donor-lineage$^-$ Sca-1$^+$ c-kit$^+$ cells (KSL cells) in the recipient mice after BMT, the BMCs of eGFP tg mice (1×10^6) were injected into 9-Gy-irradiated recipient B6 mice intravenously or into the left tibia (l-tibia). Spleen cells and BMCs from the BMC-injected l-tibia and noninjected right tibia (r-tibia) were obtained from the recipient mice, independently, at 5, 7, or 9 days after BMT. We calculated the numbers of nuclear cells using an SF-3000 autoanalyzer (Sysmex, Kobe, Japan, http://www.sysmex.co.jp/en) and stained the cells with APC-labeled anti-c-kit Ab (BD Pharmingen), PE-labeled anti-Sca-1 Ab (BD Pharmingen), and biotin-labeled lineage Abs followed by staining with PerCP-Cy5.5-coupled streptavidin (BD Pharmingen). The percentages of KSL cells in the spleen and the BM were analyzed using the FACSCalibur. The total numbers of donor KSL cells, which are eGFP$^+$, in the spleen and the BM were calculated by using the total cell numbers and percentage of donor KSL cells in the cells.

To detect donor-derived peripheral blood nuclear cells, the peripheral blood (PB) of the recipient mice was examined at 2 weeks, 1 month, 2 months, and 100 days after BMT. The PB of each mouse was divided into three lots, and samples from each mouse were stained with PE-conjugated CD3 and PerCP-Cy5.5-conjugated CD45, PE-conjugated B220 and PerCP-Cy5.5-conjugated CD45, and PE-conjugated Gr1 and PerCP-Cy5.5-conjugated CD45. To examine the cells retained in the injected bone, the recipient mice were sacrificed 1 hour after IBM-BMT. The BMCs were flushed from the tibia that had been previously injected and were then suspended in ice-cold PBS. This step was carried out on ice. The number of BMCs was counted, and 10^6 BMCs were stained with PerCP-Cy5.5-conjugated CD45. The stained cells were analyzed using a FACSCalibur instrument equipped with CellQuest software.

Percentages of Donor Cells by FACS Analyses

Leukocytes were first gated by CD45$^+$ cells, which were estimated as nuclear cells. The percentage of donor T lymphocytes was estimated as eGFP$^+$/CD3$^+$cells. The percentages of donor B lymphocytes and granulocytes were estimated as eGFP$^+$/B220$^+$ cells and eGFP$^+$/Gr-1$^+$ cells, respectively.

Statistical Analysis

The results are represented as means ± SD. The Student t test was used to determine a statistical significance. A p value $<.05$ was considered a significant difference.

Results

CG Retains Injected Donor BMCs in Recipient BM

First, we examined whether CG can retain more injected cells in the injected BM than PBS. We obtained BMCs from eGFP tg mice and suspended the BMCs in ice-cold CG (Cellmatrix) or PBS. These were then warmed to room temperature (>20°C) in a syringe, since Cellmatrix is liquid at 4°C but changes into the gel state when warm. The BMCs suspended in CG or PBS were injected directly into the BM, as previously described [14]. The mice were sacrificed 1 hour after

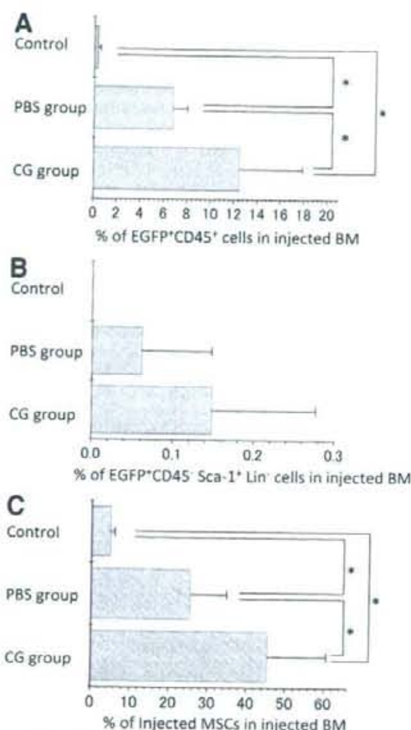

Figure 1. CG efficiently helps retain donor cells in recipient BM. Recipient (B6) mice were irradiated at 10 Gy 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs were obtained from eGFP transgenic (tg) mice (donor mice). The BMCs ($1 \times 10^7/10\ \mu l$) suspended in PBS (PBS group) or CG (CG group) were injected into the BM of the B6 mice. Control mice were injected with only PBS into the BM. One hour after IBM-BMT, the mice were sacrificed, and percentage of $CD45^+$ donor cells **(A)** and percentage of $CD45^-$lineage$^-$Sca-1$^+$ donor cells **(B)** in the BM injected with donor BMCs were analyzed by FACScan. $n = 3$ (control; mice injected with only PBS), 6 (PBS group), and 6 (CG group). The cells from control mice show autofluorescence. *, $p < .05$. **(C):** B6 mice were irradiated at 10 Gy 1 day before IBM-BMT. Cultured MSCs (5×10^5 cells per 10 μl) from eGFP tg mice suspended in PBS (PBS group) or CG (CG group) were injected into the BM of the B6 mice. Control mice were injected with only PBS into the BM. One hour after IBM-BMT, the mice were sacrificed, and percentages of donor cells were analyzed by FACScan. $n = 3$ (control; mice injected with only PBS), 5 (PBS group), and 6 (CG group). The cells from control mice show autofluorescence. *, $p < .05$. Abbreviations: BM, bone marrow; CG, collagen gel; EGFP, enhanced green fluorescent protein; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline.

IBM-BMT, and the percentages of donor cells in the injected BM were examined. As shown in Figure 1A, many more donor BMCs remained in the injected BM cavity in the CG group than in the PBS group. Next, we examined whether IBM-BMT using CG helps more mesenchymal stem cells to be retained in the injected recipient BM. As shown in Figure 1B, CG helped more MSCs to be retained in the recipient BM, but there was no significant difference between the groups, possibly because of the large SDs. Therefore, we transplanted cultured MSCs of eGFP tg mice into the BM of B6 mice and analyzed the percentages of donor MSCs in the recipient BM. As shown in Figure 1C, a significantly larger number of donor MSCs existed in the recipient BM in the CG group than in the PBS group ($p < .05$). These results suggest that CG can help more injected cells (both HSCs and MSCs) be retained in the injected BM than PBS.

CG Retains KSL Cells in the Injected Site, Followed by Helping Proliferation and Migration of KSL Cells into the Spleen and Noninjected Sites

To examine the kinetics of the KSL cells in the recipients, we carried out IBM-BMT or i.v. BMT using BMCs suspended in PBS or CG from eGFP tg mice into B6 mice. As shown in Figure 2, on day 5, KSL cells were found only in the BM of the BMC-injected l-tibia in both PBS and CG groups; the number of KSL cells in the BMC-injected l-tibia was significantly higher in the CG group than in the PBS group. However, we could not detect KSL cells in the BM in the case of the i.v. group. In contrast, a greater number of KSL cells were detected in the spleen in the case of i.v. BMT. On day 7, we could still detect a high number of KSL cells in the BMC-injected l-tibia in the CG group. The KSL cells also appeared even in the noninjected r-tibia in the CG group, although no KSL cells were detected in the noninjected r-tibia in the PBS group in the case of IBM-BMT. On day 9, we still detected many KSL cells in the BMC-injected l-tibia in the CG group. There were no significant differences between the other groups due to the increases in the numbers of KSL cells. These findings suggest that immature hemopoietic progenitor cells (KSL cells) proliferate inside the BMC-injected bone cavity, followed by an acceleration of the migration of the KSL cells into the noninjected bone and the spleen in the CG group.

CG Group Shows Significantly Higher Day 12 Colony-Forming Units of Spleen Counts After IBM-BMT Than PBS Group

Next, we carried out day 12 colony-forming units of spleen (CFU-S) assays to examine the hemopoietic stem cell/immature hemopoietic progenitor activity after IBM-BMT using CG or PBS (Fig. 3). In the CG group, 16.1 ± 4.4 colonies per spleen (106.8 ± 35.9 mg of spleen weight) were observed on day 12, whereas 6.6 ± 3.4 colonies per spleen (68.2 ± 15.5 mg of spleen weight) were observed in the PBS group ($p < .05$). These results suggest that hemopoietic stem cell/progenitor cells can be more effectively retained in the CG group than in the PBS group.

Reduced Radiation Doses As Pretreatment for BMT

As described above, we have shown that CG can effectively help donor BMCs be retained in the recipient BM after IBM-BMT, resulting in the formation of significantly higher CFU-S counts. Next, we examined whether radiation doses could be reduced by the use of IBM-BMT with CG (Fig. 4). First, we irradiated recipient B6 mice at 8 Gy and reconstituted them with eGFP mouse BMCs by IBM-BMT. In this experiment, both the PBS and CG groups showed good reconstitution of donor hemopoietic cells 1 month after BMT. There was no significant difference between the two groups: the mean and SDs of the percentage of donor cells in the PBS group and the CG group were 89.3% ± 2.0% and 85.6% ± 5.0%, respectively, at 1 month after IBM-BMT. We therefore reduced the radiation dose (8 to 7 Gy). With 7 Gy of radiation, 1 month after BMT, significantly higher percentages of reconstitution with donor cells were observed in the CG group than in the PBS group. These results suggest that the IBM injection of BMCs suspended in CG is effective in reducing irradiation doses as a pretreatment.

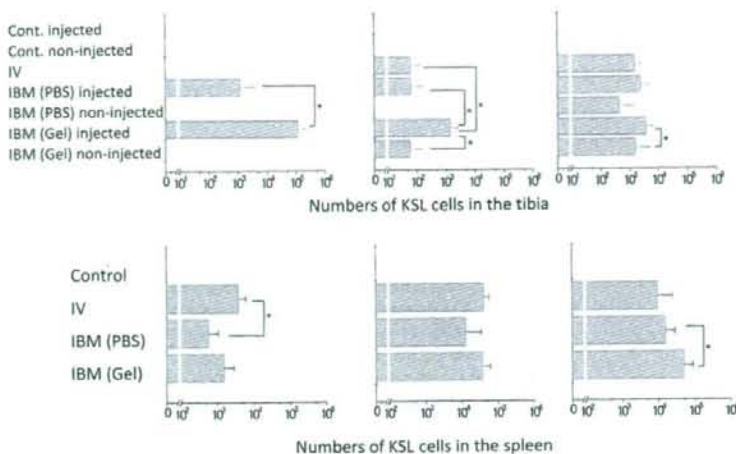

Figure 2. Collagen gel (CG) augments increment of KSL cells in injected bone marrow (BM) followed by increment of KSL cells in noninjected BM and spleen. B6 mice were irradiated at 9 Gy 1 day before bone marrow transplantation (BMT). BMCs from enhanced green fluorescent protein (eGFP) transgenic (tg) mice (1×10^6 cells per 10 μl) suspended in CG were injected into the left tibia. In the IV group, 1×10^6 BMCs from eGFP tg mice suspended in PBS were injected intravenously. As a control, the mice were injected with only PBS into the left tibia. The mice were sacrificed 5, 7, or 9 days after BMT. Total cell numbers of the spleen, the left tibia (injected tibia), and right tibia (noninjected tibia) were calculated with Sysmex, and percentages of donor-derived KSL cells were examined with FACSCalibur. Next, we calculated the total number of eGFP-positive KSL cells in the spleen, the BMC-injected tibia, and the noninjected tibia. n = 5–11. *, $p < .05$. Abbreviations: IBM, intra-bone marrow; IV, intravenous; KSL, donor-lineage$^-$ Sca-1$^+$ c-kit$^+$; PBS, phosphate-buffered saline.

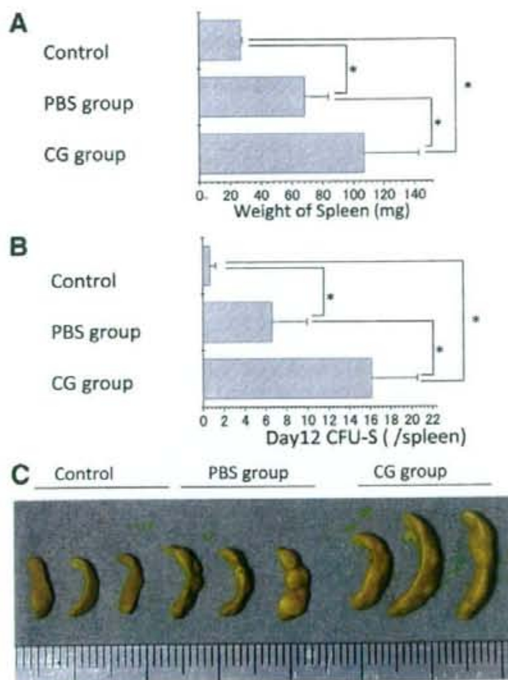

Figure 3. Heavier spleen weight and higher day 12 CFU-S counts in the CG group than in the PBS group. Recipient (B6) mice were irradiated at 9 Gy, 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs (1×10^5 cells per 10 μl) from enhanced green fluorescent protein transgenic mice were injected into the bone marrow of the B6 mice. Twelve days after IBM-BMT, the spleens of the B6 mice were weighed, and numbers of CFU-S were counted. **(A):** Means and SDs of the weight of spleens. **(B):** Means and SDs of CFU-S. **(C):** Representative photograph of spleens. There were three mice in the control group (mice injected with only PBS), five in the PBS group, and eight in the CG group. *, $p < .05$. Abbreviations: CFU-S, colony-forming units of spleen; CG, collagen gel; PBS, phosphate-buffered saline.

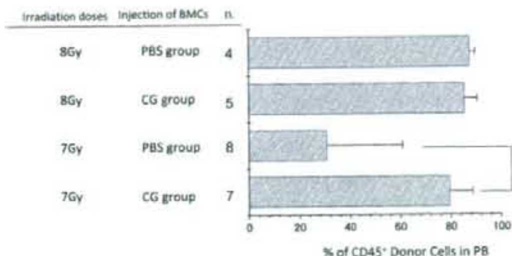

Figure 4. Better reconstitution of donor hemopoietic cells in the CG group than the PBS group. Recipient (B6) mice were irradiated at 7 or 8 Gy, 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs (1×10^6 cells per 10 μl) from enhanced green fluorescent protein mice were injected into the bone marrow of the B6 mice. One month after IBM-BMT, nuclear cells in the PB were obtained from the mice, and percentages of donor cells were analyzed by FACScan. *, $p < .05$. Abbreviations: BMC, bone marrow cell; CG, collagen gel; PB, peripheral blood; PBS, phosphate-buffered saline.

Next, we examined whether cells of various lineages can differentiate in the CG group. As shown in Table 1, donor BMCs had differentiated into multilineage hemopoietic cells in both the CG and PBS groups. The percentages of donor cells (all lineage cells) in the CG group were higher than those in the PBS group even on day 100 after IBM-BMT (Table). We also calculated the percentage of donor cells in various lineages at 6 months after IBM-BMT and obtained results similar to those at 100 days after IBM-BMT (data not shown). These results suggest that CG is effective in reconstituting recipients with donor cells by IBM-BMT not only in short-term observation but also in long-term observation after IBM-BMT.

Discussion

In the present study, we have shown that IBM-BMT using CG helps transplanted BMCs to be retained in the recipient BM upon IBM-BMT and permits a reduction in radiation doses as a pretreatment for BMT. In 2001, we reported that IBM-BMT promotes the rapid recovery of donor hemopoiesis in BMT [14] and that IBM-BMT can be used to treat intractable autoimmune diseases in MRL/Mp-lpr/lpr mice, which are resistant to conventional BMT

Table 1. Short-term (1 month) and long-term (100 days) reconstitution of donor-derived hemopoietic cells between the PBS group and the CG group

	n	% Of donor cells			
		CD45	CD3	B220	Gr-1
1 Month after IBM-BMT					
PBS group	8	30.8 ± 30	22.6 ± 16.2	51.0 ± 35.1	34.1 ± 35.0
CG group	7	79.5 ± 9.3[a]	52.5 ± 17.5[a]	91.4 ± 4.2[a]	85.8 ± 7.0[a]
100 Days after IBM-BMT					
PBS group	8	31.3 ± 40.3	16.8 ± 23.3	35.9 ± 43.3	26.6 ± 38.0
CG group	7	87.3 ± 8.7[a]	57.1 ± 17.4[a]	94.7 ± 3.3[a]	80.1 ± 20.8[a]

[a] $p < .05$ versus PBS group.
Abbreviations: CG, collagen gel; IBM-BMT, intra-bone marrow bone marrow transplantation; PBS, phosphate-buffered saline.

therapy [14]. Since then, we have shown the advantages of IBM-BMT not only in the treatment of hemopoietic diseases but also in organ transplantations and solid malignant tumors [20–24]. In these papers, we have shown that IBM-BMT induces rapid recovery of donor hemopoietic cells, easily induces tolerance in the recipients, reduces the severity of GVHD, and permits a reduction in radiation doses as a pretreatment for BMT. The mechanisms underlying the effects of IBM-BMT are (a) the efficacy of injection of donor BMCs (the direct injection of HSCs into the recipient BM) and (b) the injection of donor MSCs into the recipient BM. However, as there is an abundance of blood vessels in the BM, we have found that some of the injected BMCs get into the blood vessels, and thereby into peripheral circulation, even with IBM-BMT. Therefore, there was a need to modify the method of IBM-BMT to promote a greater retention of the injected BMCs at the site of injection in the BM. In this paper, we used CG (Cellmatrix) to help retain the donor BMCs in the recipient BM. Cellmatrix is liquid on ice but becomes a gel when warmer [18]. Therefore, we expected that the warmed CG containing BMCs would prevent the BMCs entering into circulation. As shown in Figure 1, we found a significantly higher number of donor BMCs in the injected bone of the recipients when donor BMCs were suspended in CG. As we expected, higher CFU-S counts were found in the CG group than in the PBS group, and the peripheral blood cells showed better reconstitution with donor hemopoietic cells. These results suggest that the more BMCs are injected and are retained in the recipient BM upon IBM-BMT, the better the reconstitution of the recipient mice. It has been reported that the interaction of stromal cells (particularly MSCs) and HSCs is crucial for the differentiation of hemopoietic cells and the maintenance of HSCs [16, 25, 26]. Therefore, it is necessary that the injected HSCs migrate to the BM and interact with stromal cells, including MSCs, for the production of mature hematopoietic cells and the maintenance of the HSCs. Recently, we have found that unique HSCs exist in the human cord blood [27]. These HSCs cannot migrate to the BM when they are injected into the vein. However, they can differentiate into mature hematopoietic cells and produce HSCs, resulting in long-term hematopoiesis. Therefore, we assume that some HSCs cannot participate in hematopoiesis if they are injected into the vein, possibly because of a lack of some important receptor(s) for migration to the bone marrow niche. These results and concepts suggest that BMT via the vein results in the loss of some multipotent HSCs and that IBM-BMT is effective in retaining the injected HSCs in the BM. However, in the present study, some of the HSCs were found to have entered into peripheral circulation even after IBM-BMT. IBM-BMT using CG could help retain more HSCs in the BM than conventional IBM-BMT. As shown in Figure 2, it is likely that KSL cells (immature hemopoietic progenitor cells) of the CG group proliferate more rapidly in the injected bone than those of the PBS and IV groups even 5 days after BMT, and that they migrate to the other bones and the spleen more effectively, resulting in the expansion of hemopoiesis followed by high CFU-S counts. Thus, CG is a candidate for improving conventional IBM-BMT, since more donor BMCs can be retained in the recipient BM upon IBM-BMT and since CG itself does not induce severe inflammation or foreign body granuloma when it is injected into mice (data not shown).

Conclusion

Collectively, our results show that CG helps retain more injected donor BMCs in the recipient BM upon IBM-BMT than PBS, resulting in excellent reconstitution of donor cells and permitting a reduction dose as a pretreatment for BMT in mice.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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ORIGINAL ARTICLE

Contribution of neural cell adhesion molecule (NCAM) to hemopoietic system in monkeys

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Abstract Neural cell adhesion molecules (CD56) are important adhesion molecules that are mainly expressed on neural cells and natural killer cells. Although freshly isolated cynomolgus monkey bone marrow cells (BMCs) contained only a few CD56-positive cells, almost all the BM adherent cells (obtained after a 2- to 3-week culture of the BMCs) were stained positively with anti-CD56 monoclonal antibody (mAb). The BM adherent cells showed uniformly fibroblastic morphology and were negative for hematolymphoid markers (CD4, CD8, CD11b, CD14, CD34, and CD45). Adipogenesis and osteogenesis were observed under inductive culture conditions. The BM adherent cells had the ability to support hemopoiesis of hemopoietic stem cells (HSCs) in vitro, and the proliferation of HSCs was significantly inhibited by the addition of anti-CD56 mAb to the coculture system. CD56 molecules were also expressed on HSCs because about 20% of an HSC-enriched population (lineage-negative and blast-gated cells) was positive for CD56. In addition, the immunostaining of monkey BM sections revealed that many stromal cells were CD56-positive, and some CD56-positive stromal cells came into direct contact with CD56-positive hemopoietic cells. These results indicate that the CD56 molecule is expressed on both HSCs and BM stromal cells (containing MSCs) in monkeys, and therefore it can be speculated that CD56 also contributes to the human hematopoietic system.

Keywords NCAM · MSCs · Bone marrow stromal cells · Cynomolgus monkey

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Introduction

The bone marrow (BM) long-term culture system, established by Dexter et al. [1] and Whitlock and Witte [2], has demonstrated the importance of direct interactions between hemopoietic cells and BM stromal cells in order to induce active proliferation and differentiation of hemopoietic progenitor–stem cells and to maintain long-term hemopoiesis. The stromal cells provide niches for hemopoietic stem cells (HSCs) by expressing adhesion molecules and secreting cell matrix molecules and growth factors. Several important adhesion molecules, such as VLA-4, VLA-5, VCAM-1, ICAM-1, and CD44, have been found and their functions have been well elucidated [3–5]. To find other

important interaction molecules for hemopoiesis, we previously established a monoclonal antibody (mAb; anti-PA6) against a mouse BM stromal cell line (PA6) [6]. The mAb inhibits pseudoemperipolesis and suppresses the proliferation of HSCs, suggesting that it reacts with molecules responsible for the interaction between HSCs and stromal cells. Affinity chromatography and mass peptide fingerprinting revealed that the PA6 protein is a neural cell adhesion molecule (NCAM, CD56) [7]. We also established a stromal cell line (FMS/PA6-P) from fetal mouse bone marrow cells (BMCs) using the anti-PA6 mAb. The cell line is highly positive for CD56 and has a higher ability to support hemopoiesis than other stromal cell lines. Moreover, the cell line has characteristics of mesenchymal stem cells (MSCs) because it can differentiate into adipocytes, osteoblasts, and vascular endothelial cells [8]. These results indicate that CD56 is expressed not only on stromal cells but also on MSCs in the murine system.

The CD56 molecule has been identified in retinal tissues of chick embryos by Thiery et al. [9] in 1977 and was shown to be an important adhesion molecule involved in the morphogenesis of neural cells in embryonic development [9, 10]. The CD56 molecule belongs to the immunoglobulin superfamily, and three isoforms (NCAM-120, NCAM-140, and NCAM-180), differing in molecular size of the intracellular domain, are known. NCAM-180 is expressed mainly on neural cells, whereas NCAM-120 and NCAM-140 are expressed on cardiac muscle, skeletal muscle, some T cell populations, and natural killer (NK) cells. However, the expression of the CD56 molecule on hematolymphoid cells, except for NK cells, has not thus far been well elucidated, although there are a few reports showing that CD56 is highly expressed on some malignant cells: plasma cells in multiple myeloma [11] and leukemic cells in acute myeloid leukemia [12]. As for BM stromal cells, it has been reported that human BM endosteal cells express CD56 [13] and that a human osteoblastic cell line U2-OS, derived from osteoblastoma, has the capacity to support hemopoiesis and is highly positive for CD56 [14].

Recently, we have established a new method for harvesting monkey BMCs (the perfusion method) [15–17]. By this method, the contamination of the collected BMCs with peripheral blood was reduced to a minimum, compared with the conventional aspiration method. To examine whether the contribution of CD56 to hemopoiesis is a feature common to all species, we attempted to investigate the expression of CD56 on monkey BM adherent cells that were obtained by the culture of BMCs collected from cynomolgus monkeys using the perfusion method. It is known that CD56 molecules regulate cell migration, homing, proliferation, and maturation by homophilic (CD56 binding to CD56) as well as heterophilic (CD56 binding to a number of proteins and extracellular matrix molecules) interactions. Therefore, we examined whether CD56 molecules were also expressed on HSC-enriched populations and whether the interaction between HSCs and stromal cells through CD56 molecules is important for the proliferation and differentiation of HSCs. Recent research has demonstrated that human and murine BMCs contain MSCs and the MSCs can be obtained easily by the culture of BMCs. In the present study, we also investigate whether the monkey BM adherent cells, expressing CD56 molecules, have characteristics of MSCs.

Materials and methods

Monkeys

Normal cynomolgus monkeys (3 to 6 years old; 3- to 6-kg body weight) were obtained from Keari (Osaka, Japan). The monkeys were free of intestinal parasites and were seronegative for tuberculosis, herpes B, hepatitis A, and hepatitis B viruses. All surgical procedures and postoperative care of animals were carried out in accordance with the guidelines of the National Institutes of Health for care and use of primates. Experiments using these monkeys were conducted in accordance with protocols approved by the university's committee for animal research.

Harvesting of monkey BMCs

Monkeys were anesthetized using Ketalar (5 mg; Sankyo Co. Ltd.; Tokyo Japan) and an analgesic agent, Pentagin (Sankyo Co. Ltd.), and BMCs were then collected from the long bones (humerus and femur) and the ilium by the perfusion methods established in our laboratory [15–17]. In brief, one BM puncture needle was inserted into the proximal side of the long bone and the other was inserted into the distal side. In the case of the ilium, one needle was inserted into the end of the iliac crest and the other into the edge of the iliac crest. A syringe containing 0.5 ml of heparin was connected to the needle and a 30-ml syringe containing 30 ml of phosphate-buffered saline (PBS) was connected to the other needle. The PBS was gently pushed from the syringe into the BM cavity, and the PBS containing BMCs was collected in the syringe containing heparin.

Adherent cell culture of monkey BMCs

Monkey whole BMCs (1×10^7) were cultured in flasks (25 cm^2, #35-3014; BD Falcon, Franklin Lakes, NJ, USA) containing IMDM (Gibco, Grand Island, NY, USA)