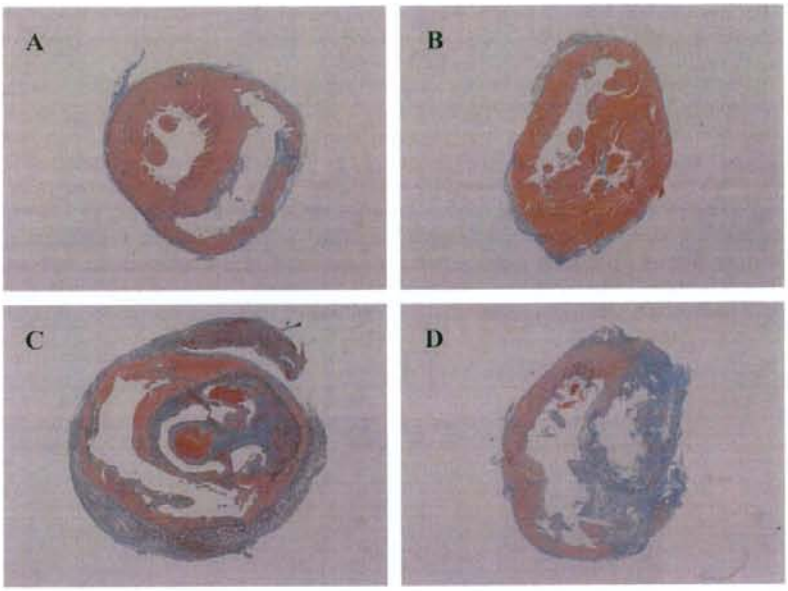

FIGURE 3. Macroscopic appearance in cardiac allografts after IBM-BMT or IV-BMT. The cardiac allografts from IBM-BMT (A, B) and IV-BMT groups (C, D) were stained with MT and coronal sections examined (×5) at the time of rejection. Allografts examined after IBM-BMT and irradiation with 3.5 Gy×2 (A) or 4.0 Gy×2 (B) showed mild interstitial fibrosis at 180 days. Allografts after the IV-BMT showed extensive interstitial fibrosis and abnormal cardiac remodeling when rejected on day 28 (3.5 Gy×2) (C) or day 86 (4.0 Gy×2) (D).

terstitial fibrosis and myocyte atrophy in the allografts with IV-BMT were significant and obvious when compared with those observed with IBM-BMT.

Analyses of Donor-Derived DCs in the Recipient Thymus

Donor-derived DCs stained with FITC-conjugated donor-specific mAb were clearly detected in the thymus of the recipient rats treated with IBM-BMT (3.5 Gy×2, and 4.0 Gy×2; Fig. 4A-C). By contrast, the presence of donor-derived DCs in the recipient thymus could not be detected in the rats treated with IV-BMT (3.5 Gy×2, or 4.0 Gy×2; Fig. 4 D-F). Therefore, regarding the long-term macrochimerism and chronic rejection-free cardiac allograft acceptance after IBM-BMT, the donor-derived DCs might interfere with the induction of donor-specific tolerance of both donor and recipient major histocompatibility complex (MHC) molecules.

Analyses of Immunological Functions

Regardless of the conditioning regimens with fludarabine plus irradiation of 3.5 Gy×2 or 4.0 Gy×2 prior to IBM-BMT, newly developed T cells were tolerant of both host-type (BN rat) and donor-type (F344 rat) MHC determinants, whereas they showed a significant response to the third-party (PVG rat) MHC determinants in MLRs (Fig. 5). In contrast, tolerance failed to be induced in the rats that had lost allografts within 3 months and also in the rat that had been sacrificed at 180 days with a weakly functioning allograft. This was further confirmed by skin grafting. The donor skin grafts were accepted (>24 weeks), whereas the third-party skin grafts from PVG rats were rejected (mean survival time: 8.4±1.5 days, n=5) in the rats treated with IBM-BMT. In contrast, the mean survival time of skin allografts in the rats treated with IV-BMT was 9.2±1.6 days (n=5) and 11.6±3.4 days (n=5) with irradiation of 3.5 Gy×2 or 4.0 Gy×2, respectively. These findings indicate that successful cooperation can be achieved among newly-developed T cells, B cells, and antigen-presenting cells in the rats treated with IBM-BMT.

Analyses of Development of GvHD

None of the rats treated with IBM-BMT or IV-BMT had any apparent body weight loss after transplantation, compared with age-matched nontreated BN rats. None of the animals showed clinical or histological evidence of GvHD throughout the period of observation (data not shown).

DISCUSSION

The major obstacles that remain in the current clinical transplantation setting include chronic rejection and side effects due to lifelong usage of nonspecific immunosuppressants (*1*). The induction of stable hematopoietic chimerism has been an attractive approach for the depletion of donor-reactive T cells in the thymus while preserving immunoreactivity against third-party antigens (*2, 3*). The toxicity from conditioning regimens and the risk of graft failure restrict the use of conventional BMT (IV-BMT) to clinical trials for the induction of transplantation tolerance (*4*). In the present study, we demonstrated that the robust donor-specific tolerance in cardiac allografts could be readily established by simultaneously performing IBM-BMT, with less myelotoxic conditioning regimens than with IV-BMT, and free of GvHD or of the need for immunosuppressive agents after transplantation.

Similar to our previous findings in the transplantation of the leg (*12*), lung (*13*), and pancreatic islets (*14*), IBM-BMT facilitated the rapid reconstitution of donor multilineage hematolymphoid cells even when the rats were conditioned with less myelotoxic regimens than with IV-BMT. The mechanisms underlying the optimal outcome with IBM-BMT (but not with IV-BMT) may be presumed to be as follows: 1) direct injection

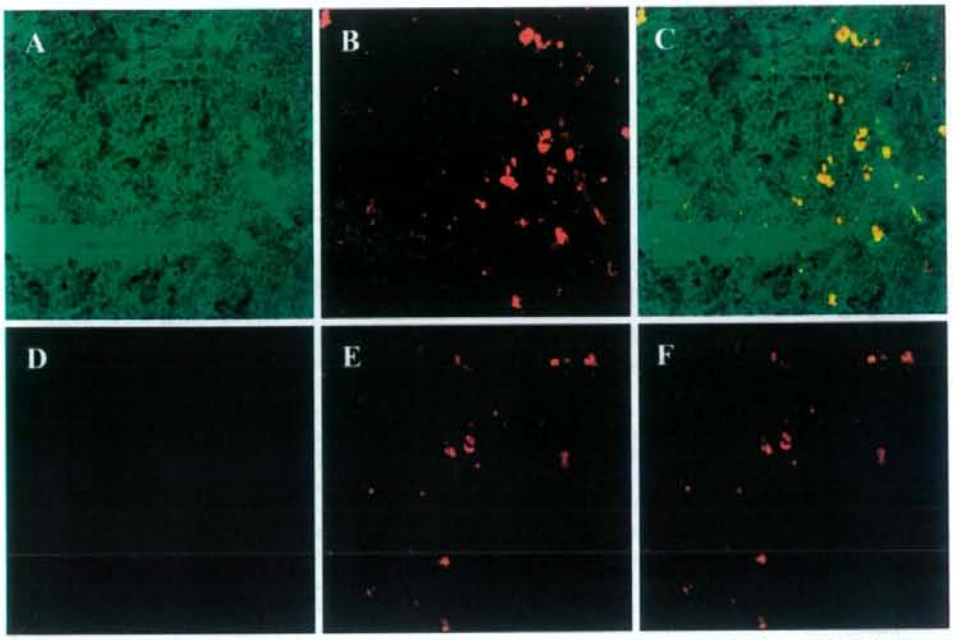

FIGURE 4. Presence of donor-derived DCs in the thymus of the rats treated with IBM-BMT or IV-BMT. Thymic sections were double-stained with FITC-antiRT1[1] mAb (A and D, cells colored green) and purified OX-62 mAb followed by PE-conjugated goat antimouse immunoglobulin G (B and E, cells colored red) for DCs and then analyzed by confocal microscopy (×200). Donor-derived DCs from the recipient rats treated with IBM-BMT were double-positive for RT1[1] and OX-62 (C, cells colored yellow); however, DCs from the recipient rats treated with IV-BMT cannot be recognized with donor-specific mAb (D, no cells colored).

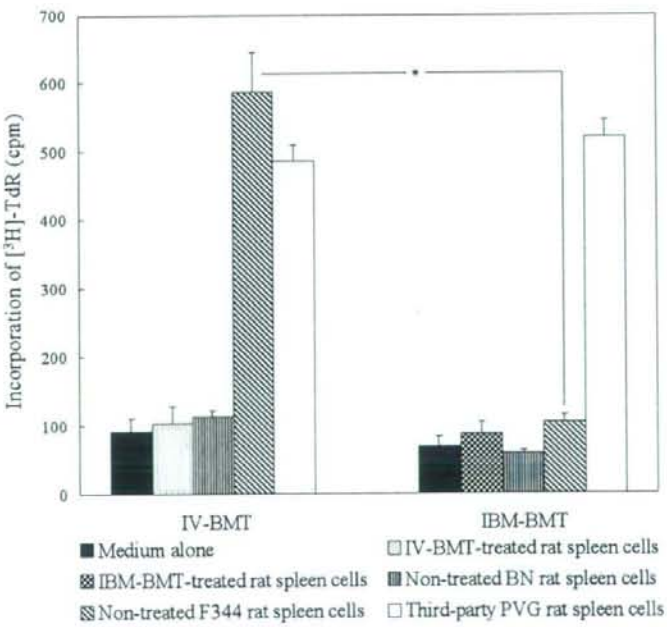

FIGURE 5. MLRs in recipient rats treated with heart transplantation plus IBM-BMT or IV-BMT. MLRs were performed 180 days after the treatment. The recipient rats treated with IBM-BMT or IV-BMT were assessed using untreated BN, F344 and PVG rats (the third party). Responder T cells (2×10^5) from the rat treated with IBM-BMT or IV-BMT were mixed with 2×10^5 stimulator cells. The results are expressed as the mean ± SD of five rats. The spleen cells from the rats treated with fludarabine+3.5 Gy×2+IBM-BMT showed tolerance of both donor-type (F344) and recipient-type (BN) MHC determinants, but showed a significant response to the third party (PVG) MHC determinants. In contrast, the spleen cells from the rats treated with fludarabine+3.5 Gy×2+IV-BMT showed significant response to donor-type (F344) MHC determinants. *$P<0.001$.

of the donor stem cells to engraftment niches without the homing process (*10*); 2) avoidance of the depletion of the antigen-disparate donor cells by the host immune system during systemic circulation (*19*); 3) donor-derived stromal cells injected into the bone marrow cavity being capable of supporting MHC-matched donor hemopoietic stem cells (HSCs) (*10, 20, 21*); and 4) injection of the donor BMCs directly into the bone marrow induces a local megadose effect, which improves the efficiency of the donor HSC engraftment, particularly under nonmyeloablative conditioning regimens (*19*). In addition, the high-dose pulse administration of fludarabine (50 mg/kg) can also facilitate the engraftment even if modest levels of irradiation are used.

The underlying mechanism by which our protocol induced hematopoietic macrochimerism and tolerance appears to involve the deletion of host alloreactive cells in both the thymus and the periphery of chimeric rats (*3*). With respect to the induction of tolerance, both the quality and quantity of the chimeric hematopoietic cells are presumed to be determinative factors (*22*). Microchimerism was first detected in some patients after solid organ transplantation due to the movement of passenger leukocytes transplanted with the graft (*23*) and was thought to be an epiphenomenon (*24*). Furthermore, acute rejection- or chronic rejection-associated graft loss has been observed in spite of microchimerism (*25*). Although many protocols achieved the acceptance of allografts via microchimerism, they often failed to sustain the long-term survival of the allografts (*26*) or failed to accept permanently fully MHC-mismatched donor skins (*9, 27*) owing to a lack of the continuous presence of bone marrow-derived donor DCs in the thymus (*28*). Therefore, the macrochimerism approach seems to be a prime strategy for the induction of transplantation tolerance, though requiring relatively stringent conditioning. In our experiments, IBM-BMT proved to be of higher efficacy in inducing persistent stable high levels of chimerism (>70%) than IV-BMT, under the mild conditioning regimens (3.5 Gy×2). Furthermore, donor-derived DCs were clearly observed in the thymus of the recipient rats after IBM-BMT but not IV-BMT (Fig. 4), indicating that bone marrow-derived donor DCs may migrate into the thymus of the chimeric rats and be involved in the induction of donor-specific tolerance. It has been reported that microchimerism does not correlate with the survival of murine cardiac allografts (*26*), and at least 30% of donor lymphocyte chimerism was found to be required to prevent rejection of allogeneic islet cells in nonobese diabetic mice (*29*). In our study, the cardiac allografts with low levels of transient chimerism were completely lost because of no use of immunosuppressants after transplantation. We hypothesize that microchimerism or low levels of chimerism may be sufficient to induce tolerance but insufficient to maintain it, particularly in the absence of immunosuppression.

Despite improvements in short-term and mid-term survival, long-term survival remains far from satisfactory in clinical heart transplantation (*30, 31*). CAV associated with chronic rejection accounts for the majority of these graft losses after operation (*32*). This lesion is known as an irreversible progressive pathogenesis and, unfortunately, the traditional immunosuppressive agents have proven to have a very limited effect except for retransplantation (*31*). At present, the only definitive treatment is known as prevention against the pathogenesis through the induction of donor-specific tolerance. Therefore, the rapid reconstitution and high levels of donor-origin hematopoietic chimerism induced by IBM-BMT might play a determinative role against CAV pathogenesis. In contrast, moderate to severe CAV and interstitial fibrosis were assessed by immunohistochemistry in the allografts treated with IV-BMT, notwithstanding the transient chimerism in the early stages.

Some recent protocols have attempted to reduce the incidence of GvHD using T-cell-depleted bone marrow. However, the chronic GvHD, engraftment failure, or opportunistic infections associated with these protocols still need to be addressed (*33*). From our previous studies, facilitating cells in the bone marrow, including $CD8^+$ T cells and stromal cells, have proven to be required for the engraftment of HSCs, especially under nonmyeloablative conditioning regimens (*34*). Martin (*35*) reported similar data where donor-derived $CD8^+$ T cells were necessary for the engraftment in autoimmune MRL/lpr mice, and the addition of a small number of donor $CD8^+$ T cells to T cell-depleted donor BMCs was capable of reconstituting recipients with donor hemopoietic cells. The graft-enhancing effect of $CD8^+$ T cells in the BMCs might be attributed to their cytotoxic or suppressive activity against host $CD8^+$ and/or $CD4^+$ T cells responsible for causing graft rejection (*36, 37*). Therefore, we injected the whole BMCs (including the facilitating cells) directly into the BM cavity. No notable differences in inducing hematopoietic chimerism between IBM-BMT and IV-BMT were observed with the injection of high doses of BMCs ($\geq 1\times10^8$) and with high doses of radiation ($\geq$4.5 Gy×2). However, using lower doses of BMCs (3×10^7) under less myelotoxic conditioning regimens ($\leq$4.0 Gy×2), stable high levels of macrochimerism were readily established and immunocompetence was well preserved by IBM-BMT but not by IV-BMT (Table 1). Furthermore, none of the animals that underwent IBM-BMT had clinical or histological appearance of GvHD throughout the period of observation (>10 months), which is consistent with our previous findings (*10–14*). To be of value in clinical application, IBM-BMT has proven its validity in the induction of tolerance in various immunogenic organs in rodents (*10–14*).

In conclusion, we have shown that IBM-BMT is an optimal strategy for the induction of permanent donor-specific tolerance for: 1) facilitating the establishment of stable macrochimerism with low doses of BMCs even under less myelotoxic conditioning regimens than with conventional IV-BMT; 2) inducing persistent donor-specific tolerance to allografts without acute/chronic rejection even in the absence of immunosuppressants after transplantation; and 3) reducing the incidence of GvHD even after long-term observation (>10 months). This strategy would therefore be applicable to humans.

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Prevention of Osteoporosis and Hypogonadism by Allogeneic Ovarian Transplantation in Conjunction With Intra-Bone Marrow–Bone Marrow Transplantation

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Background. We investigated the effects of ovarian allograft in conjunction with intra-bone marrow–bone marrow transplantation (IBM-BMT) on estrogen deficiency in mice.

Methods. Female C57BL/6 mice underwent ovariectomy (OvX). After 3 months, the mice were irradiated at 9.5 Gy, and the bone marrow cells (BMCs) of female BALB/c mice (8 weeks old) were then injected into the bone cavity of the B6 mice. Simultaneously, allogeneic ovaries from BALB/c mice were transplanted under the renal capsules of the B6 mice.

Results. Three months after the transplantation, the hematolymphoid cells were found to be completely reconstituted with donor-derived cells. The transplanted ovary tissues under the renal capsules were accepted without using immunosuppressants; there were a large number of growing follicles at different stages of development. Atrophic endometrium and its glands were also recovered by ovarian transplantation (OT). The transplanted allogeneic ovaries secreted estrogen at normal levels. Furthermore, bone loss was prevented to a certain extent.

Conclusions. These findings suggest that IBM-BMT + OT will become a valuable strategy for young women with malignant tumors to prevent premature senescence, including hypogonadism and osteoporosis, after radiochemotherapy.

Keywords: Allogeneic ovary transplantation, Intra-bone marrow–bone marrow transplantation, Ovariectomy, Osteoporosis, Hypogonadism.

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Intensive uses of radiochemotherapy and stem cell transplantation have greatly improved the prognosis of intractable diseases such as malignant tumors in young women. Allogeneic bone marrow transplantation (BMT) has commonly been recommended in the treatment of children with relapsed or very high-risk leukemias and lymphomas today: the 10-year-survival rate after BMT is close to 50% (*1*). As the population of transplant recipients has grown, new challenges have arisen in the management of long-term complications of transplantation. Especially, the improvement of vital prognosis is frequently associated with premature ovarian failure and bone diseases. Schimmer et al. reported that all women became menopausal after BMT (*2*). A retrospective survey found that only 232 (0.6%) patients conceived after stem cell transplantation relating to 19,412 allogeneic and 17,950 autologous transplant patients (*3*). Premature ovarian failure is one of the major risk factors associated with the development of osteoporosis (*4*).

Estrogen replacement therapy is used clinically, but its risks and benefits need to be carefully weighed because of its side effects, such as the development of breast, uterine and ovarian cancers, and heart diseases, especially in young women with premature ovary failure (POF) who

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need to use exogenous estrogen for a long time (*5–7*). If endogenous estrogen can be provided, it may solve the problem of side effects resulting from long-term estrogen replacement therapy.

We have recently found that intra–bone marrow BMT (IBM-BMT) is so far the best strategy for allogeneic BMT (*8*). IBM-BMT creates an appropriate hemopoietic environment for the early recovery of hemopoiesis and donor cell engraftment, since BMCs collected by the perfusion method contain not only hemopoietic stem cells (HSCs) but also mesenchymal stem cells (MSCs) (*9*), and IBM-BMT can efficiently recruit both (*8*). We have also shown that IBM-BMT can be used for organ transplantation because it allows us to reduce irradiation doses as the conditioning regimen (*10–12*).

In the present study, we attempt to treat secondary hypogonadism and osteoporosis by IBM-BMT plus ovarian transplantation (OT). We here show that IBM-BMT+OT can be used to treat patients with secondary ovarian failure and osteoporosis.

MATERIALS AND METHODS

Animals

Female 8-week-old C57BL/6 (B6: H-2K^b) mice, BALB/c mice (H-2K^d), and C3H/HeN mice (as third party) were purchased from SLC (Shizuoka, Japan). These mice were maintained in our animal facilities under specific pathogen-free conditions until use. Mice had ad libitum access to water and commercial standard food.

Experimental Protocols

The female C57BL/6 (B6: H-2K^b) mice were divided into four groups with eight mice per group. In brief, there was a sham-operated group (Sham-operated), OvX group, OvX+IBM-BMT group and OvX+IBM-BMT+OT group, according to a randomized block design using body weight as a selection parameter. At the begining of the experiment, three groups underwent OvX (*13*) bilaterally under diethyl ether anesthesia, and the remaining group (intact) was sham-operated. After 3 months, two groups of OvX mice were lethally irradiated at 9.5 Gy, and 1 day after the irradiation, the mice were transplanted with whole BMCs (1×10^7 /10 μL/mouse) from female BALB/c mice (H-2K^d, female 8 weeks old) via IBM injection. Allogeneic bone marrow cells were then injected into the left tibia bone cavity, and each mouse in one group was simultaneously transplanted an allogeneic ovary under its renal capsule. Another group served as an only IBM-BMT control group. After 3 months of treatment, mice were killed by cervical dislocation, weighted their uterus and body, and blood was removed by cardiac puncture. Serum was stored at −80°C for further analysis (Fig. 1A).

All of the 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.

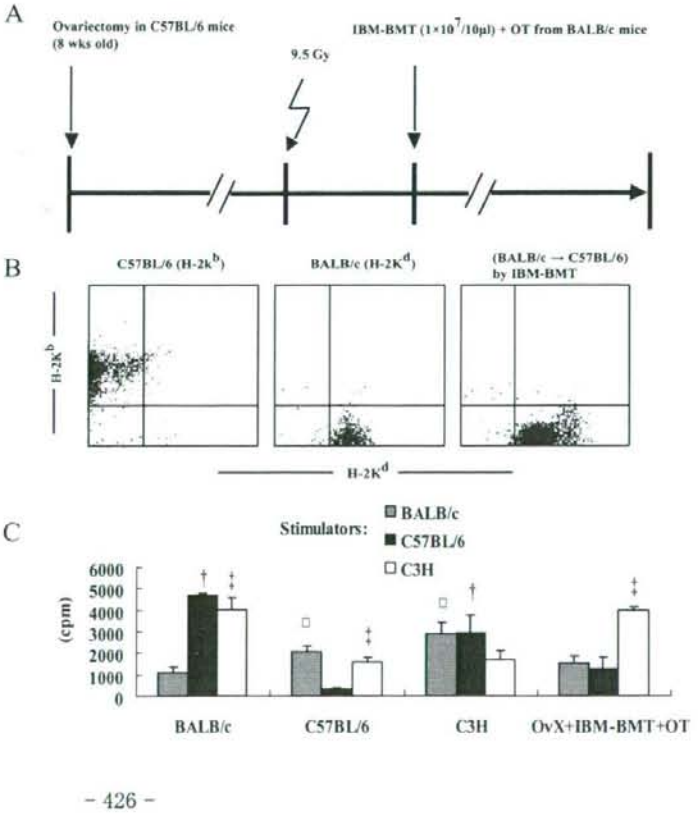

FIGURE 1. OvX mice treated with IBM-BMT. B6 mice at the age of 8 weeks were OvX. After 3 months, the mice were irradiated with a lethal dose (9.5 Gy), and BMCs from normal BALB/c mice were injected directly into the bone marrow cavity (IBM-BMT) of the left tibia (A). Three months after IBM-BMT and OT, cells from the peripheral blood of chimeric mice were stained with FITC-conjugated anti-H-2K^b mAb (recipient type) or anti-H-2K^d mAb (donor type). Cells from C57BL/6 mice treated with IBM-BMT from BALB/c mice were donor origin (H-2K^d). These findings indicate that the hemopoietic cells were reconstituted with donor-type cells after IBM-BMT (B). Mixed leukocyte reactions: Responder cells (4×10^5) were cultured with 3×10^5 irradiated (15 Gy) stimulator cells for 96 hr and pulsed with 0.5 μCi of [^{3}H]-thymidine for the last 18 hr of the culturing period (C). Cultures were performed in triplicate. Data are expressed as mean±SD, n=3. □$P<0.01$ vs. BALB/c; †$P<0.01$ vs. C57BL/6; ‡$P<0.01$ vs. C3H.

Preparation and Inoculation of BMCs

Bone marrow cells were collected from the femurs and tibias of BALB/c mice. In brief, Donor BMCs from female BALB/c mice were flushed from tibiae, femurs, and humeri using Roswell Park Memorial Institute (RPMI) 1640 medium (Niken CM1101, Japan) supplemented with 2% heat-inactivated fetal calf serum (PAA.A15–001; Austria) on ice. BMCs were filtered through a sterile nylon mesh, resuspended in sterile phosphate-buffered saline. IBM-BMT injection was carried out according to the method described previously (9). In brief, the knee was flexed to 90 degrees 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 (BM) cavity of the left tibia. Using a microsyringe (50 μL; Hamilton Company, Reno, NV), the donor BMCs (1×10^7/10 μL/mouse) were injected into the BM cavity.

Flow Cytometry

BMCs, spleen cells, and peripheral blood cells were prepared from the recipient mice after three months with bone marrow transplantation, 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 (FITC)–conjugated anti–H-$2K^d$ and phycoerythrin (PE)–conjugated anti-H-$2K^b$ monoclonal antibodies (mAbs; PharMingen, San Diego, CA). The cells were analyzed using a FACScan (Becton, Dickinson and Company, Mountain View, CA).

Mixed Leukocyte Reaction

Mixed leukocyte reaction (MLR) was performed as follows: splenic T cells (derived from normal C57BL/6, BALB/c, C3H mice and OvX+IBM-BMT+OT mice) were isolated by mechanical dissociation using microscope slides followed by red blood cell lysis with ammonium chloride (8.3 g/ml; Sigma-Aldrich). The 4×10^5 responder T cells were cultured with 4×10^5 or 3×10^5 irradiated (15 Gy) stimulator spleen cells for 96 hr in 10% FBS RPMI with 50 μM 2-ME, then pulsed with 0.5 μCi of [^{3}H]-thymidine for the last 18 hr of the culturing period.

Histology of Bone

Vertebras were fixed in 10% formalin and then decalcified and paraffin-embedded. The lumbar vertebra was sectioned to obtain a longitudinal midline section through the vertebral body, and then the sections were stained with hematoxylin and eosin (H&E). Left tibias of mice were removed the soft tissues, stored in 70% ethanol for peripheral quantitative computed tomography (pQCT) analysis. Bone histomorphometry and pQCT were used to evaluate bone mass. Percentage of trabecular bone area (B.Ar/T.Ar) was used to evaluate bone mass in histomorphometry: the fourth lumbar vertebra of every sample was cut into five consecutive sections, and these sections were measured by image analysis software (Lumina vision 1 image analysis system, Japan). A small animal pQCT (XCT Research SA, Stratec Medizintechnik, Pforzheim, Germany) was used for the measurements. When detected, bone was fixed in plastic tube (8-mm diameter) with a spring and scanned with pQCT equipment (XCT 540; Stratec). For the measurement levels in 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 their ovary, including the allogeneic ovary transplanted under the renal capsules were removed, weighed and then fixed in 10% formalin. The sections were stained with H&E to observe ovarian and uterine morphology.

Serum Estradiol Levels

Serum specimens were collected from the treated and nontreated B6 mice, Serum samples were separated by centrifugation and stored at −80°C until used for measurements. Serum estradiol was quantified by an enzyme-linked immunosorbent assay (ELISA) kit (IBL-Hamburg GmbH Corp., Hamburg, Germany).

Urine Deoxypyridinoline (DPD) Analyses

Urine specimens were collected from the treated and nontreated B6 mice, stored at −80°C until used for measurements. The urinary DPD was quantified by an ELISA kit (Quidel Corp., San Diego, CA) to evaluate the bone loss.

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 <0.01 was considered statistically significant.

RESULTS

In our preliminary experiments, we compared the survival rates of chimeric mice (BALB/c→B6) treated with conventional BMT (intravenous injection of bone marrow cells: IV-BMT) with those treated with IBM-BMT. IV-BMT-treated chimeric mice showed significantly shorter survival than IBM-BMT-treated chimeric mice, although those mice in the IV-BMT-treated group that did survive showed full chimerism (>98% donor type); we used a lethal irradiation dose (9.5 Gy) in this experiment to examine the effects of irradiation on osteoporosis and ovarian dysfunction. Therefore, in the present study (as shown in Fig. 1A), we first ovariectomized B6 mice (8 weeks old) and, 3 months later, irradiated them with 9.5 Gy. They received IBM-BMT (instead of IV-BMT) the day after the irradiation.

Cell Surface Antigens

Three months after IBM-BMT, we carried out flow cytometrical analyses using BMCs, spleen cells and peripheral blood cells obtained from the recipient mice and examined the engraftment of donor-derived cells. The percentages of

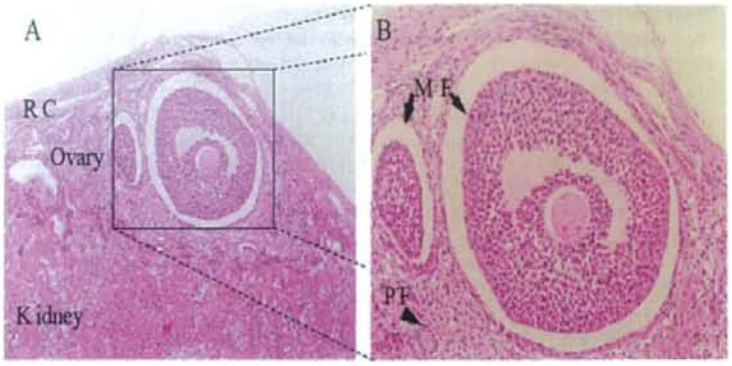

FIGURE 2. Histology of transplanted ovary after IBM-BMT. Three months after IBM-BMT and OT, the ovary was accepted (original magnification ×100, A) with a large number of mature follicles (MF) and primary follicles (PF) (original magnification×200, B) under the renal capsule (RC).

donor (BALB/c) – derived cells (H-2k^d) in the peripheral blood (Fig. 1B), BM, and spleen are 99.23%, 99.69%, and 99.54%, respectively.

Immunological Functions

We performed mixed lymphocyte reaction (MLR) using T cells as a responder obtained from the spleen of IBM-BMT-treated mice. Newly-developed T cells, as shown in Figure 1C, were tolerant of both host (C57BL/6)-type and donor (BALB/c)-type major histocompatibility complex (MHC) determinants, whereas they showed normal responses to the third-party (C3H) cells.

Histology and Weight of Ovary and Uterus

Three months after IBM-BMT+OT, the mice were sacrificed and confirmed that allogeneic ovaries had been accepted under the renal capsules of B6 mice (Fig. 2). In the mice, there were a large number of growing follicles in different stages of development, such as mature follicles, primary follicles and primordial follicles. The uteri showed normal endometrium including endometrial glands. However, in the OvX+IBM-BMT group (without OT), the uteri showed atrophic endometrium and few endometrial glands (Fig. 3A). Uterus/body weight ratios significantly increased in the OvX+IBM-BMT+OT group, compared with the OvX+IBM-BMT group or the OvX group (Fig. 3B). Uterine weight also increased in the OvX+IBM-BMT+OT group, compared with the OvX+IBM-BMT group. These results indicate that OT leads to the secretion of estrogen and restores uterine growth in the OvX mice.

Bone Histology

In the OvX+IBM-BMT+OT group, the sections of a lumbar vertebral-4 (L4) body showed that trabeculae number, thickness, and longness increased, in comparison with the OvX+IBM-BMT group, indicating that bone loss was prevented, while, in the OvX+IBM-BMT group, trabeculae were thin, and trabecular numbers decreased in comparison with the OvX group, the OvX+IBM-BMT+OT group, and the sham-operated group (Fig. 4). Histomorphometry and bone mineral densitometry were utilized to assess the bone mass of lumbar vertebrae and tibiae, respectively. The percentages of trabecular bone area (B.Ar/T.Ar) of lumbar vertebrae in the OvX+IBM-BMT+OT group increased significantly, compared with the OvX+IBM-BMT group. The total BMD of the proximal tibia are determined with pQCT. After IBM-BMT, the OT group maintained their mass, while the bone mass in the OvX+IBM-BMT group rapidly decreased; there were significant differences between the OvX group and the OvX+IBM-BMT+OT group. These results indicated that bone mass was maintained and increased after allogeneic OT, and that total body irradiation as a conditioning regimen for transplantation has toxic effects on the bone (Table 1).

Levels of Serum Estradiol and Urine DPD

There were no statistical differences between the sham-operated group and the OvX+IBM-BMT+OT group in the serum estrogen levels, suggesting that allogeneic ovaries transplanted under the kidney capsules were accepted and could secrete estrogen, resulting in maintaining normal estrogen levels in the OvX mice. The estrogen levels in the OvX+IBM-BMT group were the lowest in the three experimental groups; the OvX group was similar to the OvX+IBM-BMT group, while there was a significant difference between the OvX group and the OvX+ IBM-BMT+OT group. The levels of DPD released from the breakdown of collagen were measured in the urine to estimate osteoclast activity. The DPD levels in the OvX+IBM-BMT+OT group decreased, indicating that bone resorption decreased and bone turnover rate by OvX was suppressed after allogeniec OT. In the OvX+IBM-BMT group, however, the DPD levels were high, indicating that bone loss could not be prevented without OT (Table 2).

DISCUSSION

More and more cancer patients will be long-term survivors of radiochemotherapy and BMT, but a long lifespan does not necessarily imply a normal life. Especially in young women, ovarian failure and premature menopause have a strong impact on self-esteem and quality of life (*14–17*). Therefore, the lasting adverse effects of these modalities are receiving increasing attention.

Research during the last decade has revealed that estrogen regulates bone homeostasis through direct effects on bone cells. Estrogen deficiency results in an uncoupling of bone remodeling with a marked increase in bone resorption compared with bone formation.

Initiation of estrogen replacement therapy (ERT) in experimental animals and humans decreases erosion depth and

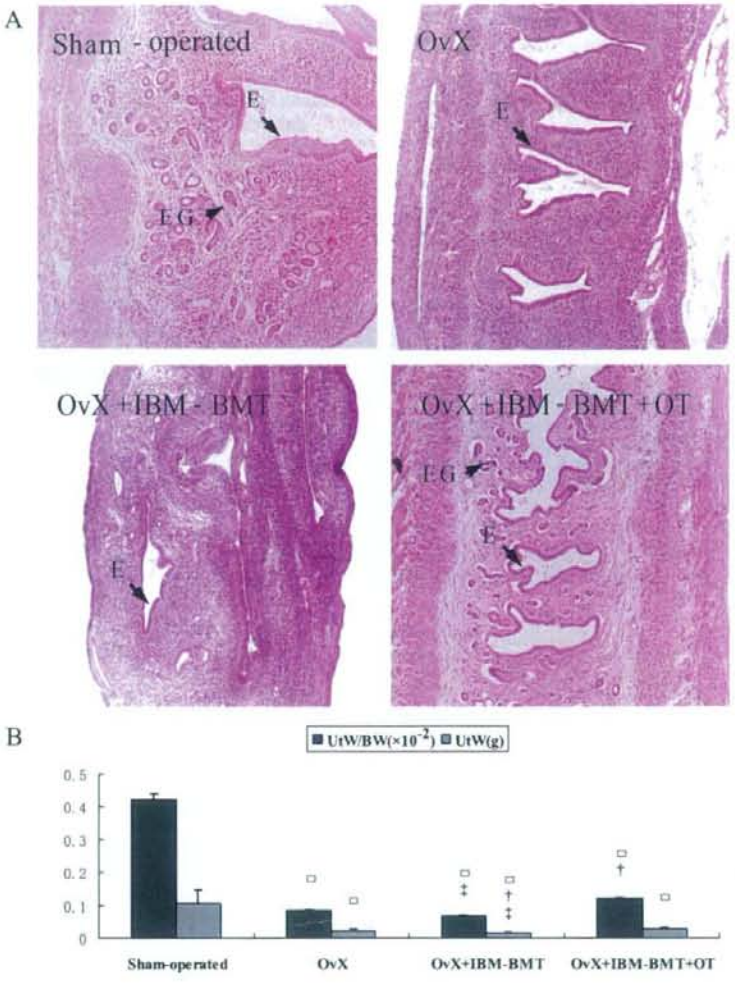

FIGURE 3. FEffects of IBM-BMT with OT on uterus. Three months after IBM-BMT, uteri of four groups (sham-operated group, OvX group, OvX+IBM-BMT group, and OvX+IBM-BMT+OT group) were sectioned and stained with hematoxylin and eosin. The OvX+IBM-BMT+OT group's uteri revealed normal endometrial glands (EG) and endometrium (E) morphology, but the other two experimental groups' uteri, especially the OvX+IBM-BMT group's uteri, revealed atrophic endometrium and few endometrial glands. Original magnification: ×100 for all panels (A). Uterus/body weight ratio and uterus weight were measured in the sham-operated group, OvX group, OvX+IBM-BMT+OT group, and OvX+IBM-BMT group. Data are expressed as mean ±SD, n=8. □$P<0.01$ vs. sham-operated group; †$P<0.01$ vs. OvX group; ‡$P<0.01$ vs. OvX+IBM-BMT+OT group (B).

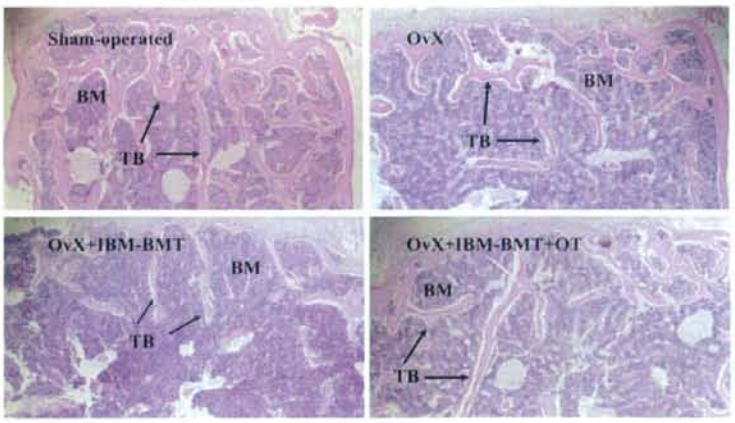

FIGURE 4. Histology of the lumbar vertebrae after IBM-BMT. Three months after IBM-BMT, the 4th lumbar vertebrae of mice in each of the four groups (sham-operated group, OvX group, OvX+IBM-BMT group, and OvX+IBM-BMT+OT group) were sectioned and stained with hematoxylin and eosin. Significant loss of trabecular bone (TB) was observed in the OvX group and the OvX+IBM-BMT groups; the trabeculars in the OvX+IBM-BMT group were short and small, but there were longer trabecular bones in the OvX+IBM-BMT+OT group. Original magnification: ×40 for all panels. BM, bone marrow.

TABLE 1. Effects of IBM-BMT with OT on L4 trabecular bone area and proximal tibia BMD in OvX mice

Group	L4 trabecular bone area percent (B.Ar/T.Ar)	Proximal tibia BMD (mg/cm^3)
Sham-operated	28.03±4.68	512.75±21.87
OvX	10.64±2.41[a]	445.58±27.61[a]
OvX+IBM-BMT	7.53±1.72 [a,b,c]	336.97±71.45[a,b,c]
OvX+IBM-BMT+OT	15.18±1.83[a,b]	442.10±29.34[a]

The OvX mice were treated for 3 months, and the L4 trabecular bone area and proximal tibia BMD were then measured by histomorphometry and pQCT, respectively. The trabecular bone area and proximal tibia BMD of the OvX+IBM-BMT+OT group increased, compared with the OvX+IBM-BMT group, indicating that bone formation increased. Data are expressed as means± SD, n=8.

[a] $P < 0.01$ vs. sham-operated group.

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

[c] $P < 0.01$ vs. OvX+ IBM-BMT+OT group.

B.Ar/T.Ar, bone area/total area.

TABLE 2. Effects of IBM-BMT with OT on serum estrogen and urinary deoxypiridinoline (DPD) in OvX mice

Group	Serum estrogen (pg/mL)	Urinary DPD (nM)
Sham-operated	7.88±2.09	8.57±3.42
OvX	4.59±1.66[a]	14.01±6.28[a]
OvX+IBM-BMT	1.77±1.19[a,b,c]	18.95±6.16[a,b,c]
OvX+IBM-BMT+OT	7.69±1.71[b]	11.41±3.91[a]

Serum estrogen and urinary DPD were measured using ELISA kit. There were no significant differences between the sham-operated group and the OvX +IBM-BMT+OT group in serum estrogen assay. The hormonal rise indicated functioning allografts. In the DPD assay, the DPD level of the OvX+IBM-BMT+OT group declined compared with the OvX+IBM-BMT group, indicating that bone resorption decreased. Data are expressed as means± SD, n=8.

[a] $P < 0.01$ vs. sham-operated group.

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

[c] $P < 0.01$ vs. OvX+IBM-BMT+OT group.

osteoclast activation frequency by stimulating apoptosis and blocking osteoclastogenesis (*18*). Estrogen therapy may decrease the rate of bone turnover by about 50% in the adult, resulting in fracture reduction at the spine, hip, and other sites of between 30% and 50% (*19, 20*). However estrogen needs to be given for as long as the effect is required, within a few years of stopping hormone replacement therapy (HRT), the antifracture effect is no longer present (*21*).

The limitations in the use of estrogen are a consequence of the adverse effects. The Women's Health Initiative and The Million Women Study found an increased risk of stroke, coronary heart disease, and breast cancer associated with long-term treatment with HRT (*19*). Young women with premature ovarian failure need estrogen therapy for longer than older women, with the result that the adverse effects of exogenous estrogen will be more severe.

In this study, we carried out allogeneic IBM-BMT+OT on OvX mice to investigate the effects of endogenous estrogen secreted by allogeneic ovary, since we have recently proven in many animal experiments that different kinds of donor cells including HSCs could be efficiently recruited into the bone marrow by IBM-BMT, which leads to the rapid hemopoietic and immune recovery of recipients, inducing donor-specific tolerance in allogeneic organ transplantation, and promoting the survival rate of recipients (*8, 9*).

In the present study, three months after the transplantation, the hematolymphoid cells were found to be completely reconstituted with donor-derived cells. The transplanted ovary tissues under the renal capsules were accepted without using immunosuppressants; even after 10 months, mature follicles were found in the allogeneic ovary engrafted after IBM-BMT without using any immunosupressants. The levels of endogenous estrogen were no different between the OvX+IBM-BMT+OT group and normal control group (sham-operated group). We know that irradiation can result not only in ovarian failure, but also in uterine dysfunction. However, in the present study, after IBM-BMT+OT, the ratio of uterus/body weight and uteri weight increased in the OvX+IBM-BMT+OT group. Moreover, endometrial morphology including endometrial glands was almost normal, although the uterine volume was still below the normal range. In another experiment, female mice without OvX received allogeneic IBM-BMT and allogeneic OT under the renal capsule after lethal irradiation. After 3 months, many immature follicles remained in the recipients' ovaries. It is well known that gonads are radiosensitive; low-dose irradiation can completely sterilize female mice. The immature follicles that remained in the recipients' ovaries had probably developed from germline stem cells (GSCs) residing in the recipients' ovaries (*22*) or from bone marrow (*23*) after IBM-BMT+OT. Also, after allogeneic IBM-BMT+OT as described above, in one group of mice we used the Johnson protocol (*22*) to transplant the allogeneic ovaries into the recipient's bursal cavity in contact with the remaining host ovarian. These mice were then mated with male mice. Two of 8 mice achieved pregnancy (data not shown). We therefore believe that IBM-BMT + OT may help promote the reconstitution of uterine functions.

The dominant acute effect of estrogen is the blockade of new osteoclasts formation. Osteoclasts arise by cytokine-driven proliferation and differentiation of monocyte precursors that circulate within the hematopoietic cell pool (*24*). This process is facilitated by bone marrow stem cells, which provide physical support for nascent osteoclasts and produce soluble and membrane-associated factors essential for the proliferation and differentiation of osteoclast precursors.

IBM-BMT can facilitate the early engraftment of hematopoietic cells of donor origin (*8, 9*), indicating that it can generate normal osteoclasts in the bone marrow. As has been recently reported, BMCs contain not only HSCs (including osteoclast precursors), but also MSCs (including osteoblasts) (*25*). BM stromal cells can differentiate into osteoblasts, chondrocytes, adipocytes, cardiomyocytes, and even neurons (*26–28*). By IBM-BMT, not only donor-derived HSCs but also donor-derived MSCs can be recruited into the bone marrow site and can proliferate and differentiate. Estrogen can directly modulate the differentiation of bone marrow stromal cells into osteoblasts and increase the deposition and mineralization of matrix (*29, 30*).

In the present study, mice were first ovariectomized to precipitate a marked reduction in endogenous estrogen con-

centrations and to induce bone remodeling abnormalities that augment bone loss and increase the risk of developing osteopenia or osteoporosis. We found that the bone mass in the OvX+IBM-BMT+OT group showed a significant increase after 3 months, while DPD as a biochemical bone resorption marker decreased compared with the OvX+IBM-BMT group, although the BMD did not regain the normal levels, in contrast to the sham-operated group. In another experiment, we noted that the bone mineral density of the OvX+IBM-BMT+OT group did not increase even after 6 months, compared with 3 months in the present experiment (data not shown). Lee youngwon et al. showed that the differentiation of bone marrow stromal cells into osteoblasts was impaired after BMT (*31*). On the other hand, Wang et al. reported that MSC transplantation can help to strengthen osteoporotic bone (*32*) without using irradiation as the conditioning regimen. Thus, the toxic effect of total body irradiation (TBI) on the bone is so severe and complicated (*33, 34*) that we think both antiosteoporotic effects of endogenous estrogen secreted by the transplanted ovary and donor-derived MSCs were insufficient to establish normal BMD in 3 months; nonmyeloablative conditioning regimens may be substituted for TBI in future. We suggest that antiosteoporotic drugs are used as soon as possible after ovarian failure, and before and after BMT in clinical practice to prevent bone loss.

Many antiosteoporotic drugs are used to prevent and treat postmenopausal osteoporosis, but they do not have curative effects on symptoms such as hot flashes and the urogenital complaints induced by endogenous estrogen deficiency besides HRT (*35, 36*).

From our results, we believe that IBM-BMT plus ovarian allografts would be advantageous for young women with primary or secondary estrogen deficiency (due to chemotherapy and radiation therapy in malignant diseases). Transplantation surgery is limited by the supply of fresh donor organs. It has been reported that cryopreserved intact ovary grafts succeeded in syngeneic rats (*37*). We believe that, if this new strategy can be shown to be safe for application to humans, IBM-BMT+OT could not only be used to treat cancer and prevent bone loss, but could also help promote the patient's self-esteem and quality of life.

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Successful Acceptance of Adult Liver Allografts by Intra-Bone Marrow–Bone Marrow Transplantation

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Previously, we have shown that liver allografts obtained from the fetus or young mice are accepted when bone marrow cells (BMCs) from adult mice of the same strain are co-grafted. However, for practical clinical use, it is more convenient to obtain both BMCs and liver from the same adult donors. C57BL/6 mice were irradiated with a single high-dose irradiation or two low-dose irradiations and injected with donor BALB/c (8 weeks old) BMCs intravenously (IV-BMT) or directly into the recipient BM cavity (IBM-BMT). Liver tissues taken from the same donor were, on the same day, engrafted under the kidney capsules. Higher survival rates and more complete reconstitution of donor cells were achieved in the IBM-BMT group than in the IV-BMT group, and this was the case in both irradiation protocols. The acceptance of donor liver tissue was seen in all mice in which hematolymphoid cells were replaced by donor-type cells. The liver grafts of the reconstituted mice showed normal morphology and stained positively with anti-albumin antibody and Periodic Acid Schiff (PAs) staining, indicating that the grafted livers were accepted, had grown, and were functioning. These results demonstrate that the acceptance of allogeneic liver can be achieved by cografting donor BMCs via the IBM route.

Introduction

ORTHOTOPIC LIVER TRANSPLANTATION is a well-established routine treatment for end-stage liver diseases [1]. The success of this procedure is related, in large part, to the availability of potent immunosuppressive agents for preventing and treating acute and chronic rejection [2,3]. These agents, however, require continuous administration and induce a considerable risk of renal dysfunction, opportunistic infections, and so on [4–6]. Without the use of such immunosuppression, all allogeneic hepatocytes are rejected within a few days after the transplantation [7,8]. Therefore, the development of a new strategy that does not involve the long-term use of immunosuppressants has been eagerly awaited.

Twenty years ago, we showed that liver allografts obtained from young (1–4 weeks old) mice were accepted in recipient mice that had received bone marrow cells (BMCs) from adult (8 weeks old) mice of the same strain by the conventional intravenous (IV) route 2–3 months beforehand [9]. Moreover, we found that hepatosplenomegaly could be prevented in an animal model of Niemann–Pick disease (C57BL/Ks] spm/spm) when allogeneic adult BMCs and fetal liver transplantations (to replace the defective enzyme) were performed on the same day [10]. In these recipient mice, the newly developed T cells had acquired tolerance to both recipient- and donor-type cells. Thus, the co-administration of allogeneic BMCs gives great advantages for the acceptance of liver grafts from the same strain. For practical clinical use, however, it is more convenient to obtain both BMCs and liver from the same adult donor and to engraft the BMCs and liver in the patients on the same day. In addition, to prevent the potentially unfavorable side effects caused by the irradiation conditioning, lower radiation doses are necessary for its routine use, particularly in clinical solid organ transplantations.

Recently, there has been an accumulation of clinical data showing the facilitating effects of bone marrow transplantation (BMT) on liver allografts; the liver grafts are accepted in patients that have received allogeneic BMT from the same donor [11–14]. An attempt at allogeneic liver transplantation, followed by BMT from the same donor by the conventional IV route, was made, and mixed donor–recipient

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hematopoietic chimerism was achieved without severe acute graft-ersus-host disease (GVHD) [15]. Five months later, however, the patient developed progressive pneumonia and died. These results indicate that the co-administration of donor BMCs induces tolerance and leads to the effective acceptance of the allografts, although further research is needed.

Recently, we have found that the injection of donor BMCs directly into the bone marrow cavity—intra-bone marrow (IBM)-BMT—induces the persistent donor-specific tolerance and the rapid reconstitution of the hematolymphoid system of donor origin in a chimerism-resistant combination [normal → MRL/lpr], even if the radiation doses are reduced to sublethal levels (5.5 × 2 Gy with a 4-h interval) [16]. Moreover, we have found that donor stromal cells injected into the BM cavity offer a suitable environment for MHC-matched hematopoietic stem cells (HSCs) in IBM-BMT, whereas there is little migration of donor stromal cells or HSCs into the recipient BM cavity when they entered into systemic circulation by intravenous injection (IV-BMT) [16–18]. It is of interest that the mice receiving the IBM-BMT show no histopathological signs of GVHD, even in refractory BMT settings [16,19]. Moreover, we have shown that more rapid and complete acceptance of organ allografts (islets and legs) can be achieved by the IBM-BMT of donor BMCs than IV-BMT [20,21]. Very recently, we have also found that heart allografts can be accepted without either acute or chronic rejection for more than 10 months by co-administration of donor BMCs by the IBM route [22].

These findings prompted us to examine whether IBM-BMT might have significant advantages over IV-BMT in the allografts of livers obtained from adult mice, even if the recipient mice only receive conditioning with low doses of irradiation. Moreover, to find the optimal conditioning regimen in allografts, we investigated whether fractionated low doses of irradiation induce more complete reconstitution of donor cells and acceptance of liver grafts than a single high dose of irradiation.

Materials and Methods

Animals

C57BL/6 (B6, H-2^b), BALB/c (H-2^d), and C3H (H-2^k) mice were obtained from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan), and maintained in pathogen-free conditions in our animal facility. All of the mice 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.

Irradiation

Recipient mice (B6, 8 weeks old) were placed in an acrylic container in a continuous airflow between two opposing ^{137}Cs γ-ray sources (Gamma cell 40; Nordion International, Kanata, Ontario, Canada). The mice received a single dose (7.5, 8.5, or 9.5 Gy) or fractionated doses of irradiation with a 4-h interval (4.5 × 2, 5 × 2, 5.5 × 2, or 6 × 2 Gy).

Liver and BMT

Liver tissue taken from 8-week-old BALB/c mice was cut into about rice grain-size fragments in RPMI-1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) and incubated in medium containing 0.01% collagenase (Type 1; Sigma St. Louis, MO) for 30 min at 37°C. The liver fragments were then transferred into 2% fetal bovine serum/phosphate-buffered saline (FBS/PBS) and kept in the solution until their engraftment (within 1 h). The liver fragments were grafted into the left kidney capsule of each mouse (B6) that had received irradiation 1 day before. The kidney capsules were selected as the sites of liver grafts because we had previously shown that these well-vascularized tissues could easily induce the rapid growth of various grafted tissues (liver, bone marrow, thymus and pancreas) [9,10,23–25]. A small incision was made in the kidney capsule and a single liver fragment was inserted into the subcapsular space.

Donor (BALB/c, 8 weeks old) BMCs were collected from the femoral and tibial bones using PBS containing 2% (FBS). Soon after the liver grafts, the recipient mice were injected with donor BMCs (1×10^7 or 5×10^6) IV or directly into the BM cavity (IBM injection). The IBM injection was carried out according to the method described previously [16]. In brief, the region from the thigh to the knee joint 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 marrow cavity. Using a microsyringe (50 μl; Hamilton Co; Reno, NV), the donor BMCs were injected into the BM cavity.

Surface marker analyses

Peripheral blood was collected from the recipient mice and peripheral blood mononuclear cells (PBMCs) were prepared using Lymphplyte-M (Cedarlane, Ontario Canada). To distinguish between donor- and recipient-derived cells, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-H-$2K^b$ and phycoerythrin (PE)-conjugated anti-H-$2K^d$ monoclonal antibodies (mAbs) (BD Pharmingen, San Diego, CA). In some experiments, BMCs, spleen cells, PBMCs, and thymic cells were collected from the recipient mice and were double stained for lineage markers (FITC- or PE-conjugated mAbs against CD4, CD8, Mac-1, Gr-1 and B220) and anti-H-2 mAbs (BD Pharmingen). The stained cells were analyzed using a FACScan fluorescence-activated cell sorter (Becton Dickinson & Co.; Mountain View, CA).

Histological examinations

The recipient mice were sacrificed at 2, 4, and 8 weeks after the transplantation. The kidneys with engrafted donor liver fragments were removed and fixed with 10% neutral formalin. The 4 μm sections were stained with Hematoxylin & Eosin (H&E) or Masson and Gitter reagents to analyze whether the engrafted liver tissues were intact or had changed to fibrous tissues. To examine the functions of the engrafted liver, the sections were reacted with Periodic Acid

Schiff (PAS) reagents to detect glycogens. Moreover, the sections were analyzed for the production of albumin. Briefly, the sections were deparaffinized with xylene and methanol and incubated with 0.3% hydrogen peroxide in methanol for 10 min to block the reactivity of endogenous peroxidase. They were washed in PBS and incubated with 1% gelatin for 30 min and then incubated with goat antimouse albumin Ab (dilution of 1:5,000; Bethyl Laboratories Inc. Montgomery, TX) for 1 h. After being washed with PBS, they were incubated with biotinylated anti-goat immunoglobulin G (IgG) Ab (Vector Laboratories, CA) at room temperature for 30 min. They were incubated with peroxidase-labeled streptavidin and detected using metal-enhanced 3,3′ -diaminobenzidine (DAB).

Electron microscopic examinations

The kidneys with engrafted liver fragments were fixed with buffered PBS containing 2.5% glutaraldehyde, pH 7.4, at 4°C for 2 h. After being rinsed with PBS, the tissues were post-fixed with 2% osmium tetroxide at 4°C for 2 h. Thereafter, the samples were routinely processed, dehydrated with ethanol, and embedded in epoxy resin. Semithin sections (0.5 μm) of the kidneys were stained with Toluidine Blue. Ultrathin sections of the selected area were cut on a copper grid, stained with uranyl acetate and lead citrate, and observed using a Hitachi H-7000 electron microscope (Hitachi, Ibaragi, Japan).

Mixed lymphocyte reaction

Spleen cells from the chimeric mice were observed to assess whether the mice had acquired tolerance to donor cells but still had the ability to react with allogeneic spleen cells. The spleen cells (responder) obtained from the chimeric mice, untreated (age-matched to the chimeric mice) B6 or BALB/c mice were incubated with 15 Gy-irradiated spleen cells (stimulator) from untreated B6, BALB/c and C3H mice (8 weeks old) for 3 days in 96-well flat-bottomed culture plates (responder, 3×10^5/well; stimulator, 4×10^5/well) (6 wells/sample). The culture medium (RPMI-1640) was supplemented with 10% FBS and 50 μM 2-mercaptoethanol. As a control, wells containing only responder cells were prepared. The incubated cells were pulsed with [^{3}H]thymidine (TdR) for the last 18 h of the culture period.

Intracellular cytokine staining

Three weeks after the transplantation, spleen cells were collected from the chimeric mice and incubated with a leukocyte activation cocktail (intracellular cytokine staining starter kit mouse, 559311, BD Pharmingen). After 4 h of incubation, the cells were stained by an anti-CD4 mAb, permeabilized, and further stained with mAbs against interleukin-2 (IL-2), IL-4, or IL-10. The double-stained cells were analyzed using a FACScan, and the percentages of the cells having intracellular cytokines were measured.

Statistical analyses

Log-rank test and the Student two-tailed *t*-test were used to determine the statistical significances in survival rates and in other experiments, respectively. A p value of less than 0.05 was considered statistically significant.

Results

Survival and reconstitution in mice that received liver allografts plus IBM-BMT or IV-BMT

Previously, we have shown that allogeneic BMT, followed by liver allografts from the same mouse strain (2–3 months after BMT), results in the acceptance of the liver allografts [9]. In addition, using an animal model for Niemann–Pick disease, we have shown that the combination of allogeneic BMT (IV-BMT) + fetal liver allografts leads to the acceptance of the liver tissue, resulting in the recruitment of sphingomyelinase to the recipients [10].

On the basis of these findings, we examined in the present study whether IBM-BMT has advantages over IV-BMT for the acceptance of adult (not fetal) liver allografts, even if the liver grafts were carried out on the same day that donor whole BMCs were injected into the recipients. Whole BMCs (1×10^7) obtained from adult BALB/c mice were injected via the IV- or IBM-route into recipient B6 mice that had received 7.5, 8.5, or 9.5 Gy of irradiation, and liver fragments obtained from the adult BALB/c mice were then engrafted under the renal capsules of the recipient B6 mice on the same day. At all irradiation doses, higher survival rates were observed in the chimeric mice that had received BMCs from the IBM-route than from the IV route (not significant) (Fig. 1A). To investigate the levels of chimerism, PBMCs were collected from the chimeric mice 8 weeks after the transplantation and were double-stained with anti-H-2K^b (recipient type) and H-2K^d mAbs (donor type). This time point (8 weeks) was selected because the relative percentage of donor- and recipient-type cells in each chimeric mouse remained at a similar level thereafter (even 8–12 months after the transplantation). The chimeric mice with more than 90% of donor-type cells in their PBMCs were considered to be reconstituted mice. The percentages of the reconstituted mice were calculated using the surviving mice 8 weeks after the transplantation. In the chimeric mice that had received 8.5 Gy of irradiation (Fig. 1B), significantly higher engraftment of donor-type cells was observed in the IBM-BMT group than in the IV-BMT group. In the 9.5 Gy-irradiated group (Fig. 1B), there was a tendency for the engraftment in the IBM-BMT group to be higher than in the IV-BMT group, but the difference was not significant. When the recipient mice were irradiated at a dose of 7.5 Gy, 100% survival was achieved in the IBM-BMT group and 92% in the IV-BMT group (Fig. 1A). No reconstitution of donor-type cells, however, was observed in PBMCs obtained from the chimeric mice in the IBM- or the IV-BMT groups (Fig. 1B), indicating that such a low dose of irradiation resulted in less side effects in the recipient mice but could not completely remove the recipient's immunocompetent cells and HSCs. The chimeric mice that received higher doses of irradiation showed lower survival rates. This suggests that the higher-dose single irradiation caused acute side effects in the mice and that overall condition of the mice became worse than in the mice that received lower doses of single irradiation (Fig. 1A).

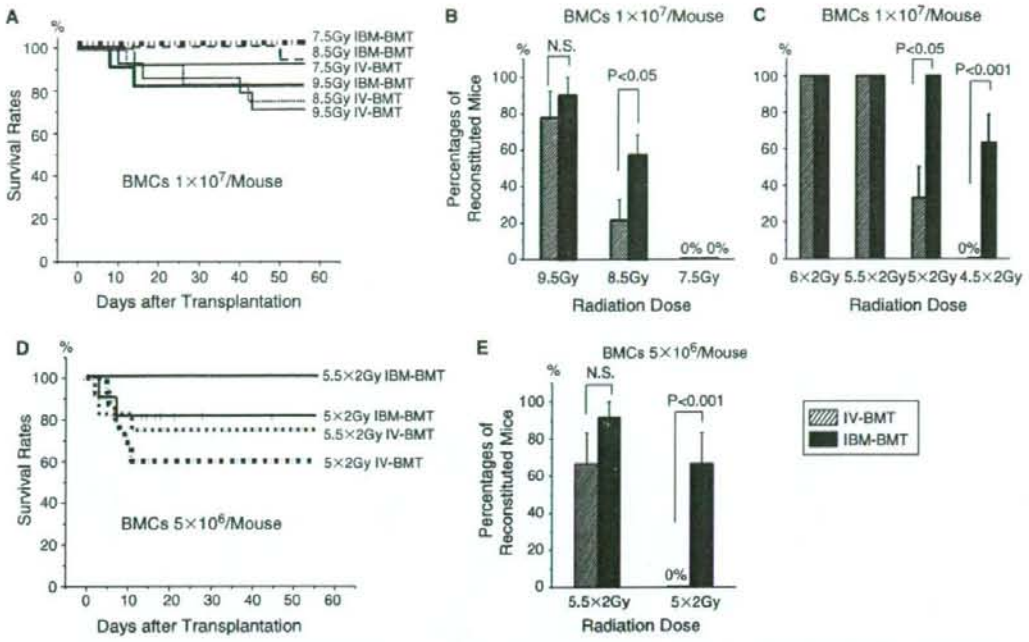

FIG. 1. Survival and reconstitution of chimeric mice. **(A)** Survival rate in chimeric mice that were exposed to a single dose of irradiation and then received liver allograft and intravenous bone marrow transplantation (IV-BMT) or intra-bone marrow (IBM)-BMT. B6 mice were irradiated with a single dose of 7.5, 8.5, or 9.5 Gy. BALB/c bone marrow cells (BMCs) (1×10^7) were transplanted into the irradiated B6 mice via the IV or IBM pathway 1 day later, and BALB/c liver fragments were grafted under the kidney capsules of the recipient mice on the same day. The survival rate up to 60 days post-transplantation is shown here, because no death due to graft failure was observed thereafter. A summary of three independent experiments (18 chimeric mice per group) is shown. There was no significant difference between the IBM-BMT and IV-BMT groups at each irradiation dose. **(B)** Percentages of reconstituted mice among chimeric mice that were exposed to a single dose of irradiation and then received liver allograft and IV-BMT or IBM-BMT. BALB/c BMCs (1×10^7) were transplanted into the single dose-irradiated B6 mice via the IV or IBM pathway in conjunction with BALB/c liver fragments. Eight weeks later, PBMCs were collected from the chimeric mice and double-stained with anti-H-2K^b and H-2K^d mAbs. The chimeric mice having more than 90% of donor-type cells in their PBMCs were considered to be reconstituted mice. Mean ± SD of three independent experiments (11–18 chimeric mice per group). N.S., Not significant. **(C)** Percentages of reconstituted mice among chimeric mice that were exposed to fractionated irradiation and then received liver allograft IV-BMT or IBM-BMT. B6 mice were irradiated with two doses of 4.5, 5, 5.5, or 6 Gy (with a 4-h interval). BALB/c BMCs (1×10^7) were transplanted into the irradiated B6 mice via IV or IBM pathway in conjunction with BALB/c liver fragments. Eight weeks later, PBMCs were collected from the chimeric mice and double-stained with anti-H-2K^b and H-2K^d mAbs. Mean ± SD of three independent experiments (18 chimeric mice per group). **(D)** Survival rate in chimeric mice that were exposed to fractionated irradiation and then received liver allograft + IV-BMT or IBM-BMT. B6 mice were irradiated with two doses of 5 or 5.5 Gy (with a 4-h interval). BALB/c BMCs (5×10^6) were transplanted into the irradiated B6 mice via IV or IBM pathway at the same time as BALB/c liver fragments. A summary of three independent experiments (18 chimeric mice per group). There was no significant difference between the IBM-BMT and IV-BMT groups at each irradiation dose. **(E)** Percentages of reconstituted mice among chimeric mice that were exposed to fractionated irradiation and then received liver allograft + IV-BMT or IBM-BMT. B6 mice were irradiated with two doses of 5 or 5.5 Gy (with a 4-h interval). BALB/c BMCs (5×10^6) were transplanted into the irradiated B6 mice via the IV or IBM pathway in conjunction with BALB/c liver fragments. Two months later, PBMCs were collected from the chimeric mice and double-stained with anti-H-2K^b and H-2K^d mAbs. Mean ± SD of three independent experiments (10–18 chimeric mice per group). N.S., Not significant.

Previously, we have shown in various donor/recipient combinations that fractionated low doses of irradiation with an interval of 4 h are more effective in promoting the survival and engraftment of donor cells than a single high dose of irradiation [26]. Therefore, the recipient mice were irradiated at doses of 4.5×2 to 6×2 Gy with an interval of 4 h, and 1×10^7 of donor BMCs were injected in conjunction with liver allografts into the recipient mice. As expected, all

of the chimeric mice survived more than 60 days at all doses of fractionated irradiation (data not shown). At the irradiation dose of 4.5 × 2 or 5 × 2 Gy, significantly higher reconstitution was achieved in the IBM-BMT group than in the IV-BMT group ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 1C). When the recipient mice were irradiated at a dose of 5.5 × 2 or 6 × 2 Gy, all the recipient mice were reconstituted with donor cells, and therefore there was no significant difference in the engraftment between the IBM-BMT group and the IV-BMT group.

Next, we examined whether the injection of half the number of donor BMCs (5×10^6) could reconstitute mice that had received the fractionated irradiation. As shown in Fig. 1D, all of the chimeric mice that had received 5.5 × 2 Gy of irradiation, IBM-BMT, and liver graft survived more than 60 days, and 92% of the thustreated mice were reconstituted with donor cells (Fig. 1E). In contrast, only a 76% survival rate was obtained in the chimeric mice that had received 5.5 × 2 Gy of irradiation, IV-BMT, and liver grafts, and the reconstitution rate was also lower than the IBM-BMT group (not significant) (Fig. 1 D,E). Poor survival rates were observed in the mice that received the lower dose of irradiation (5 × 2 Gy) than in the mice that received the higher dose of irradiation (5.5 × 2 Gy). This discrepancy can be explained as follows: immunocompetent cells of the recipient mice remaining after the insufficient irradiation (5 × 2 Gy) attacked the donor cells, resulting in a failure to reconstitute the hematolymphoid system and the death of the mice. Although a small number of HSCs likely remained in the recipient mice after insufficient irradiation, they may have no capacity to effect reconstitution. In the mice that received 5.5 × 2 Gy, a much smaller number of immunocompetent cells remained and a higher reconstitution of donor cells was achieved, especially in the IBM-BMT group. At a dose of 5 × 2 Gy, there was a significant difference in the engraftment of donor-type cells between the IBM-BMT and the IV-BMT groups ($p < 0.001$).

Collectively, these results indicate that higher survival rates and more complete reconstitution of donor-type cells in PBMCs can be achieved by IBM-BMT than by IV-BMT, and that this is so with both the single high-dose and also the fractionated low-dose irradiation protocols. Particularly, the most distinct difference in the reconstitution of PBMCs was observed between the IBM- and the IV-BMT groups of chimeric mice that had received the 4.5 × 2 Gy of irradiation plus the administration of 1×10^7 BMCs (Fig. 1C); 63% of reconstitution in the IBM-BMT group versus 0% in the IV-BMT group. Such a clear difference was also seen in the chimeric mice that had received 5 × 2 Gy of irradiation plus the administration of 5×10^6 BMCs (Fig. 1E). Therefore, to analyze the facilitating effects of IBM-BMT further, the following experiments were performed using the chimeric mice that had received the 4.5 × 2 Gy of irradiation plus the administration of 1×10^7 BMCs (Fig. 1C).

Hematolymphoid reconstitution in various tissues obtained from mice that had received liver allografts plus IBM-BMT or IV-BMT

Next, we examined whether complete multilineage reconstitution was achieved in the various lymphohematopoietic tissues of the chimeric mice that had received 4.5 × 2 Gy of irradiation, IBM-BMT, and liver allograft 10 weeks after the transplantation in comparison with the chimeric mice that had received 4.5 × 2 Gy of irradiation, IV-BMT, and liver allograft (Fig. 2). The collected cells were double-stained with a panel of mAbs against mature lymphoid, myeloid or erythroid cells and anti-H-$2K^d$ mAbs (donor-type). Figure 2 shows representative data of fluorescence-activated cell sorting (FACS) analyses. The phenotypic profiles of cells in the IBM-BMT group showed the reconstitution of donor-derived cells in analyzed tissues, whereas those of cells in the IV-BMT group were similar to those of normal B6 mice, and no reconstitution of donor cells was observed.

Histological findings of liver allografts

Figure 3A shows the time course of histological changes in the liver tissues engrafted under the renal capsules of the recipient mice. As stated above, the mice that had received 4.5 × 2 Gy of irradiation, IV-BMT, and liver allograft did not show any sign of the acceptance of donor cells in any of the analyzed tissues (Fig. 2). This was the case with the engrafted liver. Severe tissue damage with hemorrhaging was observed 2 weeks after the grafting, and the infiltration of leukocytes into the engrafted liver was evident at 4 weeks. Finally, the engrafted liver tissue was rejected and replaced by fibrous tissue at 8 weeks. On the other hand, no infiltration of leukocytes was observed in the engrafted liver tissue from the mice that had received 4.5 × 2 Gy of irradiation, IBM-BMT, and liver graft at any time after the grafting (Fig. 3A), and the proliferation of hepatocytes was evident around 8 weeks. Indeed, significant outgrowth exceeding the original rice-grain size was observed after 8 weeks (Fig. 3B). These findings indicate that 8 weeks after the transplantation is an adequate time point for the evaluation of acceptance of the engrafted liver tissue.

It should be noted that the acceptance of donor liver tissue was seen in all of the mice in which hematolymphoid cells were replaced by donor-type cells, whereas all liver allografts were rejected in the mice in which no donor-type hematolymphoid cells were seen. In addition, we observed that liver allografts were rejected in all the recipient mice, which received only 4.5 × 2 Gy of irradiation (without BMT) (5 of 5 examined mice), and that all liver allografts to untreated recipient mice (without irradiation and BMT) were rejected (5 of 5 examined mice). These results indicate that the reconstitution with donor cells is absolutely necessary for the acceptance of liver grafts.

Masson and Gitter staining was performed to detect fibrous changes in the liver tissues, as shown in Fig. 3C. The rejected liver tissues showed collagen fibers, because the tissues were stained blue in Masson staining and stained silver in Gitter staining. In contrast, such findings were not observed in the accepted liver tissues.

Next, it was important to investigate whether the accepted liver tissues were functioning. Therefore, the production of albumin and glycogen were examined using immunostaining by anti-albumin antibody and PAS staining, respectively (Fig. 3D). In the rejected liver tissues, no cells stained positively in either stainings, whereas every cell showed positive staining in the accepted liver tissues. This observation was confirmed by electron microscopy findings of the accepted liver tissues, glycogenesis being observed in all the cells

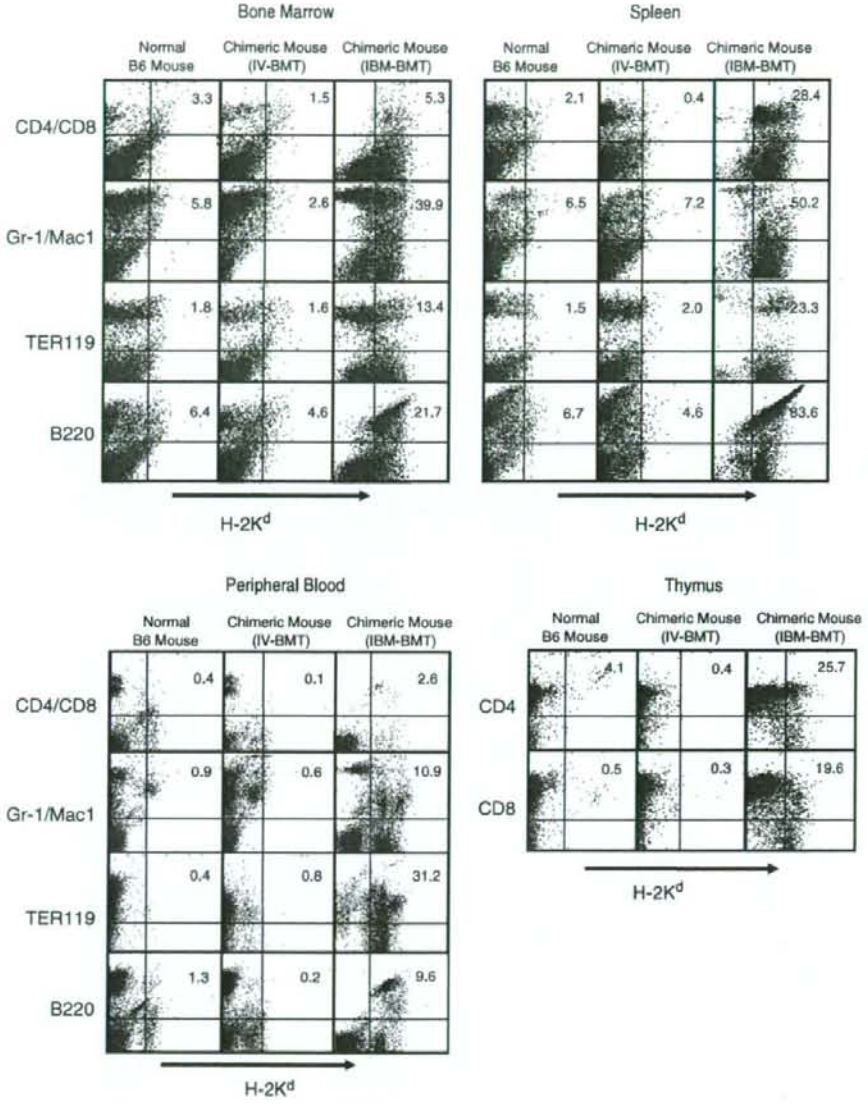

FIG. 2. FACS analyses of lineage-positive cells in bone marrow, spleen, thymus, and peripheral blood obtained from chimeric mice that received 4.5 × 2 Gy of irradiation, IV- or IBM-BMT, and liver allograft. (10 weeks after transplantation). Representative staining patterns of three independent experiments.

(Fig. 4B). In addition, the morphology was comparable to normal liver tissue obtained from an untreated 8-week-old BALB/c mouse (Fig. 4A); every cell had a round nucleus, mitochondria, and Golgi apparatus, and micro-bile ducts were also found among the liver cells (Fig. 4B).

Analyses of immunological functions in mice that received liver allografts plus IBM-BMT or IV-BMT

It was important to investigate whether lymphocytes in the chimeric mice could function normally and had

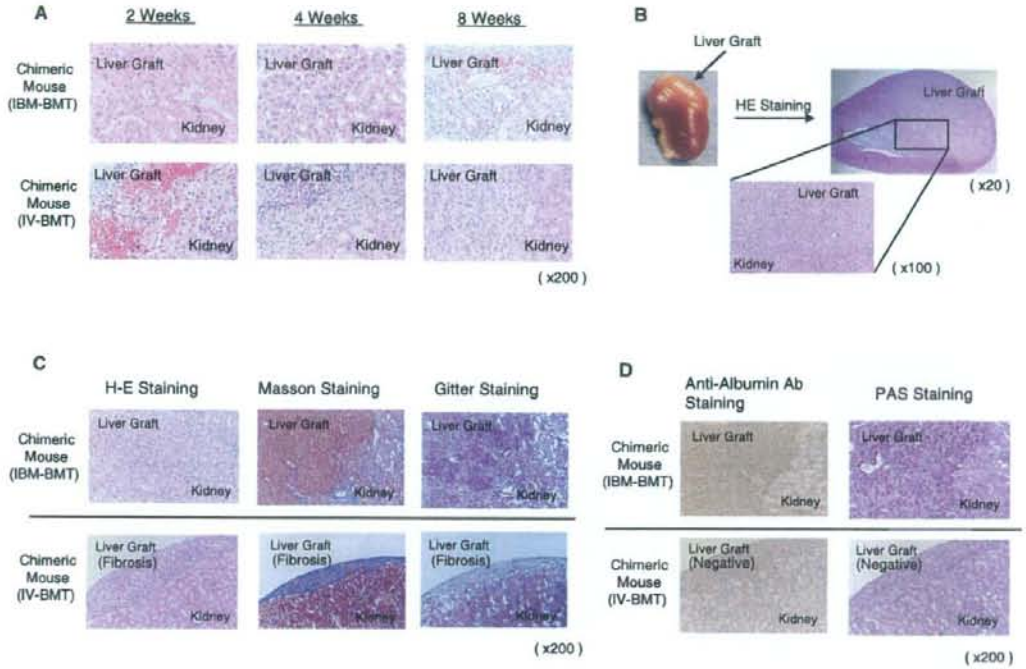

FIG. 3. Histological analyses of liver tissues grafted into kidney capsules of chimeric mice that received 4.5 × 2 Gy-irradiation/intravenous (IV) or intra-bone marrow (IBM) bone marrow transplantation (BMT). (A) Kinetics of histology (2, 4, and 8 weeks after transplantation) The liver tissues engrafted in the IBM-BMT group showed a normal architecture, and no lymphocyte infiltrations were detected at any time analyzed, showing that the grafts were accepted. However, remarkable lymphocyte infiltrations were observed, particularly at 4 weeks after the transplantation, in the liver tissues engrafted in the IV-BMT group, and the rejected liver tissues were finally replaced by fibrous tissues 8 weeks after the transplantation. Hematoxylin and eosin (H&E) staining. Representative photographs of six independent experiments. (B) Macroscopic and microscopic observations of engrafted liver fragment (8 weeks after transplantation). The liver tissues engrafted in the IBM-BMT group enlarged in size and cell number. H&E staining. Representative photographs of more than 10 independent experiments. (C) Fibrous changes in engrafted liver tissues in chimeric mice that received IV-BMT (8 weeks after transplantation). The liver tissues engrafted in the IBM group did not show any signs of fibrosis, whereas fibrous changes were evident in the chimeric mice in the IV-BMT group. Representative photographs of three independent experiments. (D) Production of albumin and glycogen in engrafted liver tissues of chimeric mice that received IBM-BMT (8 weeks after transplantation). Immunostaining using antialbumin antibody and PAS staining showed that the liver tissues engrafted in the IBM group produced albumin and glycogen, respectively. However, no positive staining was observed in the liver tissues engrafted in the IV group. Representative photographs of 10 independent experiments.

acquired tolerance to donor-type cells. To address this question, we employed a mixed lymphocyte reaction (MLR) assay using spleen cells from the chimeric mice that had received 4.5 × 2 Gy of irradiation, IV- or IBM-BMT, and liver graft. As shown in Fig. 5, the spleen cells of the mice that received IBM-BMT showed no response to donor (BALB/c) or recipient (B6) cells, whereas they responded well to the third-party (C3H) cells. This finding indicates that the lymphocytes had acquired immunotolerance to donor cells, but not to third-party cells. In contrast, the spleen cells of the mice that had received IV-BMT showed a response to donor cells.

Finally, to analyze the mechanism by which the IBM-BMT facilitated the acceptance of donor cells, cytokine production from $CD4^+$ spleen cells obtained from the mice that had received 4.5 × 2 Gy of irradiation, IV- or IBM-BMT, and liver graft was investigated 3 weeks after the transplantation. As shown in Fig. 6, the percentages of the cells producing IL-4 and IL-10 [cytokines produced from helper T (Th) 2 cells] were significantly higher in the IBM group than in the IV group. In contrast, the production of IL-2 (a Th1-type cytokine) was lower in the IBM group than in the IV group (not significant). Thus, the relative balance of Th1 and Th2 cells skewed into Th2 type in the IBM group, and this

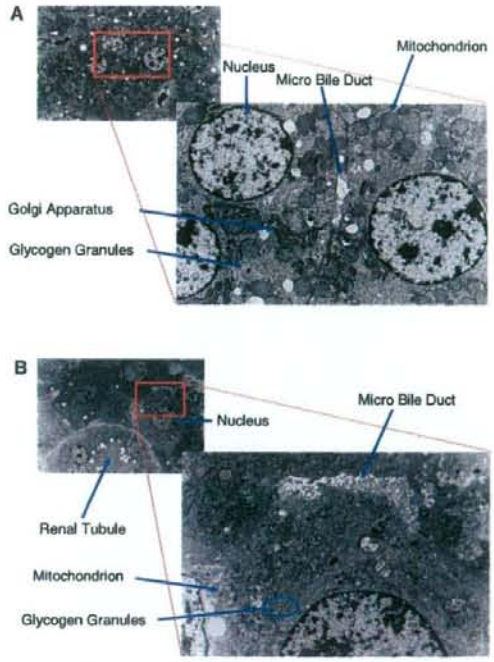

FIG. 4. Ultrastructural analyses of liver tissues. (A) Hepatocytes of normal BALB/c mouse. (B) Liver tissue engrafted into kidney capsules of chimeric mice that received 4.5 × 2 Gy of irradiation and intra-bone marrow–bone marrow transplantation (8 weeks after transplantation). The cells in the grafts showed normal hepatocyte features: large round nuclei, many mitochondria, micro-bile ducts, and glycogen granules. Representative photographs of two independent experiments.

may be one of the reasons why donor-type cells were easily accepted in the IBM-BMT group.

Discussion

In the present study, using the combination of low-dose irradiation and IBM-BMT, successful liver transplantation was achieved without using any immunosuppressants. It is evident that the engrafted liver tissues were accepted, had grown, and were functioning, because of not only the positive stainings with anti-albumin Ab and PAS-staining (Fig. 3D) but also the observation of glycogenesis by electron microscopy findings (Fig. 4B). Indeed, hepatocytes in all the liver grafts showed normal morphology; the mitochondria, nuclei, and micro-bile ducts were histologically normal (Fig. 4 B). FACS analyses revealed that the hematolymphoid cells from the mice that accepted the liver allografts had been completely replaced by donor-derived hematolymphoid cells (>90%) (Fig. 2). Newly developed T cells in the chimeric mice acquired the tolerance to the donor-type cells (but not third-party cells), as shown in the MLR assay (Fig. 5). It is of importance that T cells from the chimeric mice responded well to foreign antigens, indicating that the immune system and the hematopoietic system in the mice had been completely reconstituted, and that the chimeric mice could prevent the invasion of infectious microorganisms. Moreover, we found that the percentages of IL-4 and IL-10-producing cells in $CD4^+$ spleen cells were significantly higher in the IBM-BMT group than the IV-BMT group 3 weeks after transplantation (Fig. 6). However, such differences were not observed longer than 8 weeks after transplantation (data not shown). Therefore, it can be speculated that the polarization to Th2 type in the early phase (<3 week after BMT) is very important for the acceptance of donor cells.

Our earlier study [9] has demonstrated that the induction of tolerance to donor-type MHC by allogeneic BMT is the indispensable condition for the acceptance of the donor-type liver tissues from the two experimental results. First, liver allografts alone (without irradiation and allogeneic BMT) resulted in the aggressive rejection of the livers. Second, the chimeric mice that had received 8.5 Gy of irradiation and allogeneic BMT accepted both BM donor-type and recipient-type liver tissues, but not third-party liver tissues. This was the case in the present experiments, because the liver allografts were rejected in all the recipient mice that had not been reconstituted with donor cells. It has been reported that the majority of orthotopic whole liver allografts are spontaneously accepted in untreated recipient mice [27] and rats [28], in contrast to other tissues (kidneys, hearts, and intestines). This phenomenon can be explained by the liver allograft-induced tolerance: the suppression of recipient immune responses by passenger leukocytes and immature dendritic cells contained in the liver allografts [28,29], the neutralization of rejection by a large amount of donor soluble MHC antigen produced by the liver allografts [30], and so on. In the present study, rice grain-size fragments (not whole) of donor livers were transplanted, and the fragments were treated with collagenase before transplantation. Therefore, it is unlikely that the liver allograft-induced tolerance played a key role in the acceptance of liver in the present experimental settings.

Immunosuppressive cytokines are known to be key mediators for alloimmune responsiveness [31], and IL-4 and IL-10 are the most potent immunosuppressive cytokines, because they have a wide immunosuppressive spectrum [32]. Recently, Schmidt-Weber et al. reported that IL-4 enhanced the IL-10 gene expression in vitro [33]. Another in vitro study also showed that IL-10 acts synergistically with IL-4 to inhibit nitric oxide (NO) production from interferon-γ (IFN-γ)-treated macrophages and suppress their killing activity [34]. In fact, there are many reports showing that IL-4 and/or IL-10 are important cytokines to induce and/or maintain the acceptance of allogeneic liver grafts in mice [35] and in humans [36].

In accordance with our previous data [26], the two low-dose irradiations (fractionated irradiation) with a 4-h interval were more effective in the survival rates (Fig. 1D), the reconstitution to donor-type cells (Fig. 1C,E), and the acceptance of liver grafts than a single high dose of irradiation (Fig. 1A,B). Such advantages can be explained as follows: (1) the low-dose irradiation reduces the acute side effects on the