平成20年度厚生労働省免疫アレルギー疾患等予防・治療研究事業
「同種末梢血幹細胞移植を非血縁者間で行う場合等の医学、医療、社会的基盤に関する研究」班（宮村班）

非血縁末梢血幹細胞移植施設基準

北海道大学大学院医学研究科血液内科学
田中淳司

現状(1)

非血縁骨髄移植年間1,000例以上

JMDP認定移植施設 160施設

DLI採取認定施設 51施設

新規非血縁骨髄採取申請施設に対しては
ドナー安全委員会が施設査察後に認定

現状(2)

血縁末梢血幹細胞移植施設 233施設

血縁末梢血幹細胞移植(2006) 474例

移植種類別報告件数ー造血細胞移植学会

	2001	2002	2003	2004	2005	2006
血縁BMT	365	317	375	397	475	441
血縁PBSCT	672	777	655	470	526	474
非血縁BMT	721	731	704	781	892	884

非血縁骨髄移植ーJMDPー

平成（年度）	14	15	16	17	18	19
開始	11,409	13,143	14,152	16,843	18,200	19,620
確認検査	4,244	4,693	4,954	5,315	5,325	5,677
非血縁BMT	739	737	851	908	963	1027

DLIーJMDPー

平成（年度）	14	15	16	17	18	19	20
採取施設	37	37	37	39	43	48	51
採取回数	25	36	42	42	37	40	

将来予測(1)

非血縁末梢血幹細胞移植が血縁並みに行われたら

2002年　777/1094　71%
2006年　474/915　52%

年間約500例？
(少なくて300?–多いと700?)

将来予測(2)

非血縁末梢血幹細胞移植が血縁並みに
行われたらとしたら一年間約500例？
(300?–700?)

全てのJMDP認定移植施設で
採取を行わなければならない

移植施設＝採取施設

施設認定手順

施設認定基準の作成

施設側からの認定申請

書類審査の後、施設査察

施設認定手順

日本造血細胞移植学会　ガイドライン委員会
「同種末梢血幹細胞移植のための健常人ドナーからの
末梢血幹細胞動員・採取に関するガイドライン」改訂
ならびに「施設認定基準」作成小委員会
（平成21年1月11日）

ガイドラインに基づき
認定基準とチェックリスト作成

厚生労働省免疫アレルギー疾患等予防・治療研究事業
「同種末梢血幹細胞移植を非血縁者間で行う場合等の医学、医療、社会的基盤に関する研究」班

「非血縁者末梢血幹細胞採取の最適化に関する研究」

長藤宏司
〒830-0011　福岡県久留米市旭町 67　久留米大学医学部　内科学講座　血液内科部門

同種末梢血幹細胞採取をより多くのドナーから安全にかつ効率的に採取するため、その最適化を検討する。そのため九州大学病院で行った同種末梢血幹細胞採取を後方視的に解析した。

方法　1999 年 10 月より 2008 年 7 月まで九州大学病院・輸血センターにて施行した血縁者間同種末梢血幹細胞採取 244 例のなかで，日本骨髄移植推進財団の骨髄バンクドナー年齢条件である 18 歳以上，55 歳以下に該当する症例を対象とする。

ドナー症例　199 例　年齢　中央値　38 歳（18-55）　男：女　93：106

体重　59.6 kg（36.8-87.9）

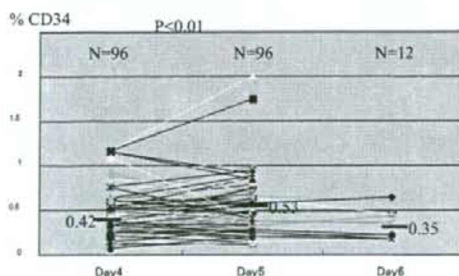
G-CSF 10μg/kg または 400μg/sqm を連日 1 回皮下注．day4 から採取を開始。

採取した CD34 陽性細胞数が充分量に達するまで day5,6 と随時採取を行う。

結果

Apheresis 1回目　(day4)	199 例	処理血液量/ドナー体重	121-295 (205) mL/kg
Apheresis 2回目(days4&5)	96 例	処理血液量/ドナー体重	94-292 (196) mL/kg
Apheresis 3回目(days4-6)	12 例	処理血液量/ドナー体重	114-293 (187) mL/kg

2回以上apheresisを施行した患者でのCD34 percentage比較



199 例の同種末梢血幹細胞採取を行った。Apheresis　1 回のみで充分 148 例（74.3%）、2 回必要であったのは 49 例（24.6%）、3 回必要であったのは 1 例、poor mobilizer 1 例であった。

考察

G-CSF 投与後、day4 に採取を行い $2x10^6$/kg 以上の CD34 陽性細胞を 70%以上のドナーで採取できる。複数回採取が必要な場合は、CD34 陽性細胞の%は、Day4 より Day5 の方が有意に多かった。以上のデータから、単回で充分量の末梢血幹細胞採取を行うためには、Day5 に採取することが妥当と考えられた。

平成 20 年度厚生労働科学研究免疫アレルギー疾患等予防・治療研究事業「同種末梢血幹細胞移植を非血縁間で行う場合等の医学、医療、社会的基盤に関する研究」班(宮村班)
班会議資料(2009 年 1 月 18 日)

非血縁者間末梢血幹細胞移植法の臨床試験体制確立に関する研究

金 成元 (国立がんセンター中央病院)

【目的】日本で初めて導入される非血縁者間 PBSCT の安全性を後方視的に検証する。

【安全性を前向きに検証するのが困難な背景】前向き臨床試験で非血縁者間 PBSCT を受けた患者の安全性(生着生存、GVHD など)を検証するには、原疾患の状態、HLA 適合度、移植前処置、GVHD 予防など、適格規準やプロトコル治療を定める必要がある。しかし、保険承認前に非血縁者間 PBSCT の臨床試験を実施する必要がなく(新薬治験とは大きく異なる)、保険承認後は、様々な状態の患者に対する様々な移植前処置を用いた非血縁者間 PBSCT が全国一斉に実施されることが予想されるため、国内最初の数例~100 例を前向き臨床試験に登録して安全性を検証することはほぼ不可能である。したがって、後方視的な調査研究が主体とならざるを得ない。

【研究デザイン案】

1. 移植前情報および移植後早期アウトカムに関する調査研究(非血縁者間 PBSCT 最初の 30 例)
2. Matched-cohort study (非血縁者間 PBSCT 最初の 100 例 vs 非血縁者間 BMT200 例)
3. 非血縁ドナーに対する QOL 調査 (PBSC ドナー全例 vs BM ドナー全例、研究期間 1 年)
4. BM 採取と PBSC 採取の両方を経験した非血縁ドナーに対する QOL 調査

【移植前情報および移植後早期アウトカムに関する調査研究】

✧ 目的:非血縁者間 PBSCT の安全性を少数例の段階で公表する。
✧ 対象:非血縁者間 PBSCT 最初の 30 例。患者年齢、HLA 適合度、移植前処置、GVHD 予防などは問わない。
✧ データベース:TRUMP
✧ 解析法:記述統計
✧ 主要項目:移植後 100 日時点での生着生存
✧ 副次的項目:年齢、性別、HLA 血清型、HLA 遺伝子型、ABO 血液型、疾患名・組織型、移植時病期、移植前処置、GVHD 予防、採取 CD34 陽性細胞数、生着不全、急性 GVHD、感染症、その他の合併症、再発、死因

【Matched-cohort study】

- ✧ 対象：非血縁者間 PBSCT 最初の 100 例 vs 非血縁者間 BMT200 例
- ✧ データベース：TRUMP および JMDP
- ✧ 解析法：Matched-cohort analysis
- ✧ 主要評価項目：移植後 100 日時点での生着生存割合
- ✧ 副次的評価項目：移植後 1 年での全生存および無増悪生存割合、移植後 1 年での非再発死亡割合、一次性および二次性生着不全発症割合、急性および慢性 GVHD の発症頻度・重症度、再発割合、細菌・真菌・ウイルス感染症発症割合

【非血縁ドナーに対する QOL 調査】

- ✧ 対象：PB ドナー全例 vs BM ドナー全例（非血縁者間 PBSCT1 例目実施から 1 年間）
- ✧ 方法：郵送によるアンケート調査。採取直前、退院 1 週後、採取 3 ヶ月後の計 3 回。SF-36 を用いて身体的および心理社会的負担について両群間で比較検討する。

【BM 採取と PBSC 採取の両方を経験した非血縁ドナーに対する QOL 調査】

- ✧ 対象：BM 採取と PBSC 採取の両方を経験したドナー（数十例）
- ✧ 方法：郵送によるアンケート調査。PBSC 採取直前、退院 1 週後、採取 3 ヶ月後の計 3 回。過去の BM 採取時と直近の PBSC 採取時の身体的および心理社会的負担について同一個体内で比較する。

VIII. 研究成果の刊行物・別刷

STEM CELLS AND DEVELOPMENT 17:291-302 (2008)
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Original Research Report

Long-Term Maintenance of Donor-Derived Hematopoiesis by Intra-Bone Marrow–Bone Marrow Transplantation

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ABSTRACT

The long-term maintenance of hematopoietic stem cells (HSCs) is assessed by serial bone marrow transplantation (BMT), in which HSCs are injected intravenously. Recently, we have found that intra-bone marrow (IBM)–BMT can efficiently reconstitute the hematopoietic system with cells of donor origin, in contrast to conventional intravenous (IV) BMT. In the present study, we have compared the long-term maintenance of HSCs using multiple rounds of serial IV-BMT and IBM-BMT. The frequencies of donor-derived progenitor cells (Lin^-/c-kit^+ cells) and more primitive progenitors (Lin^-/c-kit^+/$CD34^+$/Sca-1^+ cells) were higher in the tertiary recipients by serial IBM-BMT than in those that had received bone marrow cells by serial IV-BMT. Furthermore, neither donor-derived progenitor cells nor mature hematolymphoid cells were detected in ~25% of the tertiary recipients after serial IV-BMT, indicating that progenitor cells can be efficiently maintained by IBM-BMT but not by IV-BMT. Finally, we confirmed that the recipients treated with the primary IBM-BMT (without carrying out serial BMT) showed a significantly higher survival rate than those treated with IV-BMT. These findings clearly show that IBM-BMT efficiently promotes the long-term maintenance of donor-derived hematopoiesis.

INTRODUCTION

In general, bone marrow transplantation (BMT) is a useful strategy for the treatment not only of hematologic disorders such as leukemia and aplastic anemia but also of malignant tumors. Furthermore, it has been shown that BMT can be used efficiently to treat various types of autoimmune diseases in animal models and also in humans [1]. In our previous papers, we have shown that the injection of donor bone marrow cells (BMCs) directly into the bone marrow cavity (intra-bone marrow–bone marrow transplantation, IBM-BMT) enhances the rapid recovery or reconstitution of the hematolymphoid system (including bone marrow stromal cells) with cells of donor origin, resulting in the amelioration of autoimmune diseases in MRL/lpr mice, in which conventional intravenous (IV) BMT had been unsuccessful [2]. IBM-BMT also facilitates the maintenance of hematopoietic stem cells (HSCs) and progenitor cells [3].

HSCs are defined by their ability to self-renew and to give rise to hematolymphoid cells of all lineages. The continuous supply of hematolymphoid cells and their precursors by HSCs is sustained in the bone marrow (BM) throughout life. It has been widely accepted that the multipotent progenitor pool is heterogeneous and can be divided into long-term self-renewing HSCs (LT-HSCs), transiently self-renewing (short-term, ST) HSCs (ST-HSCs), and non-self-renewing multipotent progenitors [4]. Hematopoietic recovery after myeloablation is dependent on the number as well as quality of these HSCs [5–8]. To examine the long-term maintenance of hematopoiesis in vivo, the serial BMT assay has convention-

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ally been used. In this assay method, donor BMCs containing HSCs are retransplanted into secondary and even tertiary recipients while retaining both their self-renewal and multilineage differentiation capacities.

In most clinical and experimental stem cell transplantation protocols, it has been usual to employ the IV injection of HSCs into recipients after myeloablative conditioning, such as total body irradiation and/or chemotherapy. After BMT, the presence of donor-type hematopoietic cells in the BM is the outcome of both homing and engraftment/repopulation [9]. It has been shown that most donor HSCs are trapped and retained in the nonhematopoietic organs such as the liver [10] when they are injected either IV or via the portal vein (PV). On the basis of these findings, we have established a new method for BMT called IBM-BMT. In addition to the benefits mentioned above [2,3], we previously reported that IBM-BMT induces persistent donor-specific tolerance in mice even if the radiation doses are reduced to sublethal levels. This was confirmed by the transplantation of allogeneic legs [11] and pancreatic islets [12] in rats in combination with IBM-BMT. It is of interest that the recipients treated with IBM-BMT show no clinical or histopathological signs of graft-versus-host diseases (GVHD) or graft failure [13].

Thus, IBM-BMT seems to have significant advantages in inducing the early, complete, and persistent reconstitution of the hematolymphoid system with cells of donor origin. In our previous paper, we have also determined the frequencies of the respective dendritic cells (DCs) subsets (cDCs and pDCs) after serial BMT using MHC-matched/CD45-disparate combinations and have concluded that IBM-BMT can reconstitute both subsets to normal levels [3].

In the present study, using two serial BMT assays, we show that IBM-BMT can fully reconstitute the hematopoietic system efficiently with cells of donor origin, even in complete MHC-disparate mouse combinations, in contrast to conventional IV-BMT.

MATERIALS AND METHODS

Mice

Female BALB/c Cr (BALB, H-2^d) mice, C3H/HeN (C3H, H-2^k) mice, and C57BL/6 Cr (B6, H-2^b) mice were purchased from SLC (Shizuoka, Japan) and maintained in our animal facility until use. All mice were used at 6–12 weeks of age. All animal research was reviewed and approved by the Animal Experimentation Committee, Kansai Medical University.

Preparation of donor BMCs and transplantation

The BMCs were collected from both sides of the femora and tibiae of donor (BALB) mice. Recipient (C3H or B6) mice were irradiated in fractionated doses (5.5Gy × 2 = 11Gy; 4-h interval). One day after the irradiation, the mice were transplanted with the whole BMCs (2×10^7 cells) by IBM injections (IBM-BMT) and IV injections (IV-BMT).

The IBM injection was carried out as described in a previous report [3]. In brief, after anesthetizing recipient mice with diethyl ether (Nacalai Tesque, Inc., Kyoto, Japan), the hair on the region from the inguen to the knee joint was shaved with a razor. The knee was flexed to 90°, and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the bone marrow cavity. Using a microsyringe (50 μl, Hamilton Co., Reno, NV) containing the donor BMCs ($2 \times 10^7/10$ μl), the cells were injected from the bone hole into the bone marrow cavity. IV injections were carried out via the tail vein.

Serial BMT

Serial bone marrow transplantation was carried out to examine the marrow repopulating ability of donor (BALB) BMCs (Fig. 1). Two months after the IBM-BMT or IV-BMT, the primary recipients were sacrificed, and the BMCs were transplanted to the secondary recipients (C3H or B6) that had been irradiated in fractionated doses (5.5Gy × 2 = 11Gy). The BMCs (5×10^6 cells) from the primary recipients treated with IBM-BMT were transplanted to the secondary recipients by IBM-BMT, and those (5×10^6 cells) from the primary recipients treated with IV-BMT were transplanted to the secondary recipients by IV-BMT. The entire procedure was repeated with transplantations in lethally irradiated tertiary recipients; the BMCs (5×10^6 cells) from the secondary recipients of BMCs by IBM-BMT were transplanted into the tertiary recipients by IBM-BMT, and the BMCs (5×10^6 cells) from the secondary recipients of BMCs by IV-BMT were transplanted into the tertiary recipients by IV-BMT. The primary recipients treated with IBM-BMT and those with IV-BMT are referred to as $[BALB \rightarrow C3H]^{1\text{-}IBM}$, and $[BALB \rightarrow C3H]^{1\text{-}IV}$, respectively, the secondary and the tertiary recipients treated with serial IBM-BMT or serial IV-BMT are referred to as $[BALB \rightarrow C3H]^{2\text{-}IBM}$ or $[BALB \rightarrow C3H]^{2\text{-}IV}$, and $[BALB \rightarrow C3H]^{3\text{-}IBM}$ or $[BALB \rightarrow C3H]^{3\text{-}IV}$. As previously reported, IBM-BMT can facilitate not only the development of cells with mature lineage markers but also the early engraftment of $Lin^-/c\text{-}kit^+$ progenitor-enriched cells [3]. Therefore, to clearly examine the efficacy of IBM-BMT and IV-BMT in the maintenance of donor cell engraftment, we performed serial BMT at a 2-month interval.

Some of the primary recipients treated with IBM-BMT and IV-BMT were maintained without carrying out serial BMT to examine long-term survival rates and the maintenance of donor cell engraftment until 12 months after BMT.

Analyses for surface marker antigens

Peripheral blood cells, spleen cells, and BMCs were prepared from the recipient mice. Spleen cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-H-2^d monoclonal antibodies (mAbs) (purchased from BD Pharmingen, San Diego, CA) to identify the donor-derived cells. The cells were also stained with phycoerythrin (PE)-anti-H-2^k or -H-2^b mAb (BD Pharmingen) to detect the cells of recipient origin. The

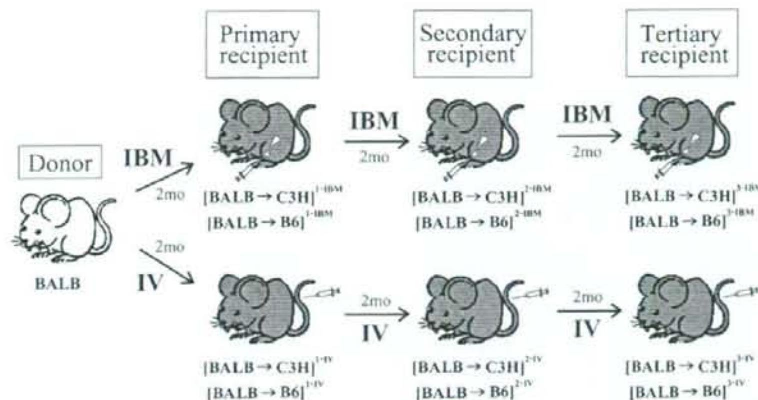

FIG. 1. Protocol for serial BMT. The BMCs were collected from femora and tibiae of BALB mice. Using a microsyringe, the donor BMCs ($2 \times 10^7/10\ \mu l$) were injected into the bone marrow cavity of recipient (C3H or B6) mice that had been irradiated in fractionated doses ($5.5Gy \times 2 = 11$ Gy; 4-h interval) (IBM-BMT, referred to as $[BALB \rightarrow C3H]^{1\text{-}IBM}$ or $[BALB \rightarrow B6]^{1\text{-}IBM}$). BMCs ($2 \times 10^7$) were injected via the tail vein of the recipient mice (IV-BMT, $[BALB \rightarrow C3H]^{1\text{-}IV}$ or $[BALB \rightarrow B6]^{1\text{-}IV}$). Following $5.5Gy \times 2$ irradiation, the secondary recipients (C3H or B6) were transplanted with (5×10^6) BMCs from the primary recipients by IBM-BMT or IV-BMT, 2 months after the first transplantation ($[BALB \rightarrow C3H]^{2\text{-}IBM}$ or $[BALB \rightarrow B6]^{2\text{-}IBM}$, $[BALB \rightarrow C3H]^{2\text{-}IV}$ or $[BALB \rightarrow B6]^{2\text{-}IV}$). A tertiary transplantation was carried out in the same way as the secondary transplantation ($[BALB \rightarrow C3H]^{3\text{-}IBM}$ or $[BALB \rightarrow B6]^{3\text{-}IBM}$, $[BALB \rightarrow C3H]^{3\text{-}IV}$, or $[BALB \rightarrow B6]^{3\text{-}IV}$).

cell-surface phenotypes were also analyzed by PE-conjugated mAbs against CD4, CD8, CD11b, B220, and Gr-1 (BD Pharmingen and eBiosciences, Sakyo, Kyoto, Japan).

BMCs were stained with biotinylated mAbs against lineage (Lin) markers (anti-CD4, anti-CD8, anti-B220, anti-Gr-1, anti-CD11b, and anti-CD11c) followed by streptavidin-RPE-Cy5-conjugated mAbs, then further stained with PE-anti-H-2^d mAb and FITC-anti-c-kit mAb (BD Pharmingen). The cells with the Lin^-/c-kit^+/H-2^{d+} immunophenotype were categorized as donor-derived hematopoietic progenitors [3]. The stained cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

In some experiments, Lin^-/c-kit^+/H-2^{d+} donor-derived hematopoietic progenitor cells at the injected and noninjected (contralateral) sites were analyzed serially at 1, 2, 7, 10, and 14 days to examine the localization of the hematopoietic cells immediately after the injection into the bone marrow (IBM-BMT). The recipients treated with IV-BMT served as controls.

Histological examination

The right femora, where BMCs had not been injected, and spleens were removed from the tertiary recipients or the long-surviving recipients and fixed in 4% neutral-buffered paraformaldehyde. The femora were then decalcified for 72 h in formic acid-EDTA. The tissues were embedded in paraffin, sectioned, and stained with Hematoxylin & Eosin (H&E) or prepared for immunocytochemical studies. After antigen unmasking at high temperature, the sections were stained with donor-specific biotin-conjugated anti-H-2^d mAb (BD Pharmingen) followed by streptavidin-FITC-conjugated mAb (CALTAG Laboratories, Invitrogen Corp., Carlsbad, CA) to identify the donor-derived cells. These specimens were also stained with PE-conjugated anti-c-kit (CD117) mAb (Miltenyi Biotec K.K. Tokyo, Japan) to examine the engraftment or maintenance of donor progenitor cells. Nuclei were stained with DAPI (Nacalai Tesque, Inc., Kyoto, Japan). In addition, the sections were stained with FITC-conjugated anti-CD34 mAb (eBioscience) and PE-conjugated anti-Ly-6A/E (Sca-1) mAb (BD Pharmingen) to detect the characterization of HSCs. The specimens stained with FITC-anti-H-2^d mAb and DAPI were imaged by differential interference contrast (DIC).

The stained samples were analyzed using an optical microscope (Nikon Eclipse E1000M, Digital Sight ACT-1 for L-1 software version 2.62; Nikon Co, Ltd, Tokyo, Japan) and a confocal laser microscope (LSM 510 META; Carl Zeiss IMT Corporation, Oberkochen, Germany). The cells expressing c-kit, CD34, and Sca-1 were analyzed by using an image-processing and analysis program (Scion Image, Scion Corporation) in 10 intermediate-power field (IPF) at 63× magnification, chosen randomly.

Statistical analyses

Statistical analyses were performed by Student's t-test, and statistical analysis of the survival rate of recipients was carried out using a log rank test.

RESULTS

Engraftment of donor-derived cells by IBM-BMT

Using a serial BMT method, we have previously shown that IBM-BMT can efficiently reconstitute the hematopoietic system with cells of donor origin, in contrast to the conventional IV-BMT, in the MHC-matched/CD45-disparate combination. This was the case when the ser-

TABLE 1. PERCENTAGES OF DONOR-DERIVED HEMOPOIETIC CELLS AFTER IBM-BMT

Cells (%)	BMT	Primary # recipient	Secondary # recipient	Tertiary recipient # Donor -derived	Tertiary recipient # Recipient -derived	Non-treated BALB mice (Control)
CD4	IBM	13.7 ± 2.4	9.7 ± 0.0	4.3 ± 0.3	1.6 ± 0.2	22.2 ± 1.3
	IV	12.7 ± 2.8	9.3 ± 0.4	1.6 ± 1.0	1.6 ± 0.9	
CD8	IBM	4.3 ± 0.5	3.5 ± 0.0	1.7 ± 0.2	0.3 ± 0.0	9.8 ± 1.4
	IV	3.6 ± 0.6	3.2 ± 0.4	0.9 ± 0.6	0.5 ± 0.3	
B220	IBM	56.8 ± 6.2	56.6 ± 6.7	40.2 ± 2.9*	0.2 ± 0.0	48.4 ± 1.7
	IV	58.0 ± 2.9	55.3 ± 3.6	17.7 ± 1.8*	0.7 ± 0.1	
CD11b	IBM	6.3 ± 2.0	6.6 ± 1.3	13.1 ± 2.8	3.5 ± 0.3*	4.1 ± 0.8
	IV	7.8 ± 3.2	4.9 ± 0.2	12.9 ± 1.7	25.5 ± 2.7*	
Gr-1	IBM	4.5 ± 0.8	3.3 ± 0.8	12.1 ± 2.6	0.3 ± 0.2*	10.1 ± 0.3
	IV	5.8 ± 2.5	2.8 ± 0.3	10.9 ± 1.4	25.0 ± 2.7*	

#The spleen cells were prepared from the primary, secondary, and tertiary recipients 2 mo after the transplantation, and the cells were then stained with FITC-conjugated anti H-2^d (donor type) or anti-H-2^k (recipient type) mAb and PE-conjugated mAbs against CD4, CD8, B220, CD11b, and Gr-1 to identify the donor-derived cells with mature lineage markers. The stained cells were analyzed by a FACScan. Numbers represent mean % ± SD of 6 mice. *represents statistical significance ($P < 0.01$).

ial BMT was performed in MHC-disparate combinations as follows: The percentages of the donor-derived hemopoietic cells in the spleen were examined about 2 months after the treatment with IBM-BMT in the primary, secondary, and tertiary recipients, and we compared these data with those from the recipients of IV-BMT. As shown in Table 1, the frequencies of cells with each lineage in both the $[BALB \rightarrow C3H]^{1\text{-}IBM}$ and the $[BALB \rightarrow C3H]^{2\text{-}IBM}$ were similar to those observed in the $[BALB \rightarrow C3H]^{1\text{-}IV}$ and the $[BALB \rightarrow C3H]^{2\text{-}IV}$: The cells in hematolymphoid lineages were completely of donor origin. In the $[BALB \rightarrow C3H]^{3\text{-}IBM}$, the cells in various lineages were mainly found to be of donor origin, whereas 30–50% of T cells and ~70% of macro-

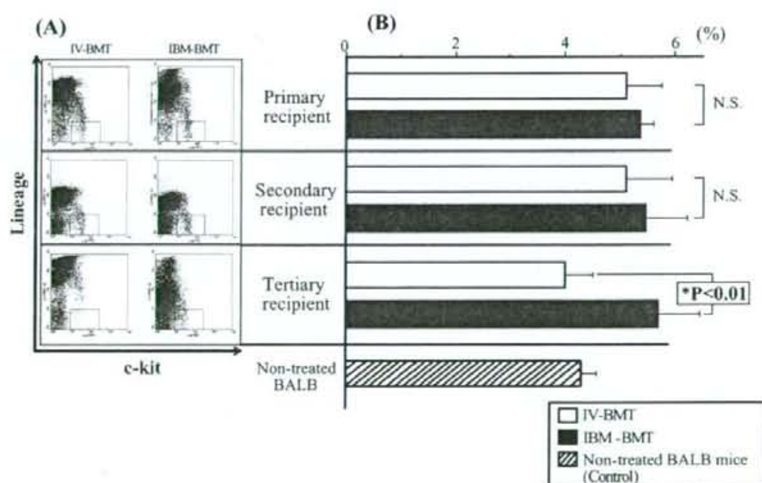

FIG. 2. Donor-derived progenitor cells after serial BMT. The donor-derived hematopoietic progenitor cells of ($Lin^-/c\text{-}kit^+/H\text{-}2^{d+}$ cells) in the bone marrow were determined by flow cytometry 2 months after BMT using the primary, secondary, and tertiary recipients. (**A**) Representative FACS profiles of $Lin^-/c\text{-}kit^+$ cells (gated of H-2^{d+} cells) after serial IBM-BMT or IV-BMT are shown. (**B**) The frequency of donor-derived hematopoietic progenitor cells after serial IBM-BMT (closed bars) was compared with that after serial IV-BMT (open bars). Nontreated BALB mice at 10 weeks of age (hatched bar) represent a control. Bars and vertical lines in the figure represent mean % and SD of donor-derived hematopoietic progenitor cells of 6 mice. (*) Statistical significance ($p < 0.01$).

phages (CD11b$^+$) and granulocytes (Gr-1$^+$)in the [BALB → C3H]$^{3\text{-IV}}$ were of recipient origin. It is noted that, in 25% of the [BALB → C3H]$^{3\text{-IV}}$, no donor-derived Lin$^+$ cells were detected. We have observed similar results when the peripheral blood cells were analyzed in the primary, secondary, and tertiary recipients. The results are shown in Supplemental Table 1, where the differences in chimerism in the tertiary recipients in the

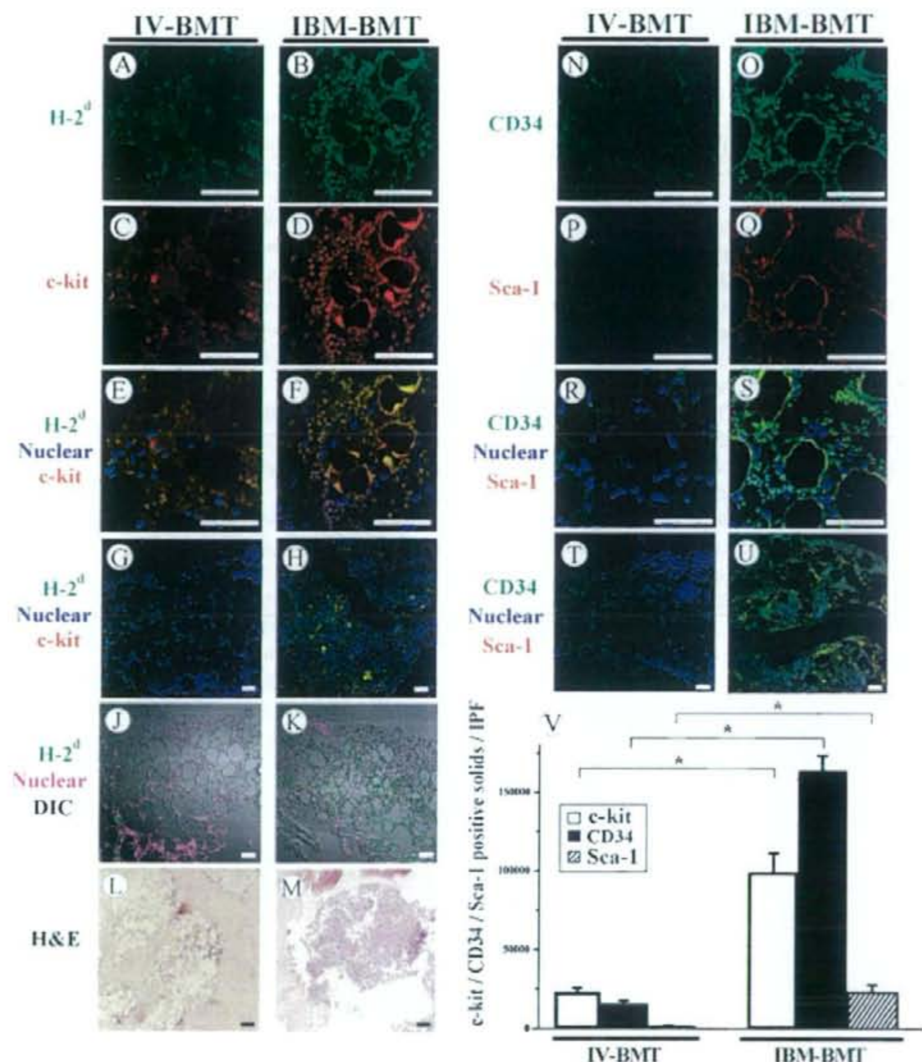

FIG. 3. Histological findings of the femur of the tertiary recipients. The right femora of the tertiary recipients were removed, fixed, and embedded in paraffin. They were then sectioned and stained with donor-specific biotin-conjugated anti-H-2^d mAb followed by streptavidin-FITC (green, **A–B,E–K**) and PE-conjugated anti-c-kit (CD117) mAb (red, **C,D,E–H**) to examine the engraftment of donor progenitor cells. Merged images are shown in **E–K**. The specimens from the right femora were also stained with CD34 (green, **N–O, R–U**) and Sca-1 (red, **P,Q,R–U**). Merged images are shown in **R–U**. Nuclei were stained with DAPI (blue, **E–K,R–U**). The specimens stained with FITC-anti-H-2^d mAb and DAPI were imaged by DIC (**J,K**), and the same sections were stained with H&E (**L,M**). Bars, 50 μm (**A–U**). c-kit$^+$ cells (open bars), CD34$^+$ cells (closed bars), or Sca-1$^+$ cells (hatched bars) were counted in 10 IPFs randomly chosen and are summarized in **V**. Columns and bars represent means ±SDs of 6 mice. (*) Statistical significance ($p < 0.01$).

[BALB → C3H]$^{3\text{-IBM}}$ were also mainly in B cells observed in the spleen, whereas 60% of monocytes (CD11b$^+$) in the [BALB → C3H]$^{3\text{-IV}}$ were of recipient origin. This was the case when BMCs were examined (data not shown).

Long-term maintenance of donor-derived progenitor cells by IBM-BMT

When progenitor cells with the immunophenotype of Lin$^-$/c-kit$^+$/H-2^{d+} cells were examined (Fig. 2), the frequency of these cells was slightly (but not statistically significantly) higher in the [BALB → C3H]$^{1\text{-IBM}}$ and [BALB → C3H]$^{2\text{-IBM}}$ than in the [BALB → C3H]$^{1\text{-IV}}$ and [BALB → C3H]$^{2\text{-IV}}$. However, in the [BALB → C3H]$^{3\text{-IBM}}$, the frequency of progenitor cells was significantly higher than in the [BALB → C3H]$^{3\text{-IV}}$. It is noted that the progenitor cells observed in the [BALB → C3H]$^{3\text{-IBM}}$ were mainly of donor origin, whereas those in the [BALB → C3H]$^{3\text{-IV}}$ were a mixture of donor and recipient origin. It should be noted that no donor-derived progenitor cells (Lin$^-$/c-kit$^+$/H-2^{d+} cells) were detected in 25% of the [BALB → C3H]$^{3\text{-IV}}$.

The efficient engraftment of progenitor cells was confirmed by the results shown in Supplemental Fig. 1, where donor-derived hematopoietic progenitor cells (Lin$^-$/c-kit$^+$/H-2^{d+} cells) significantly increased at the site of IBM-BMT (left tibia) at 2 days and 7 days when compared to those observed in the noninjected bone marrow (right tibia) and in both the tibiae of recipients treated with IV-BMT, whereas mature fully differentiated T cells immediately migrated into the periphery (data not shown).

The facilitated engraftment of progenitor cells was also confirmed by histological examination, as shown in Fig. 3, where donor-derived progenitors (H-2^{d+}/c-kit$^+$) were clearly and highly observed in both the right femur (note that BMCs were injected into the left tibia) (Fig. 3B,D,F,H) and the spleen (data not shown) of the [BALB → C3H]$^{3\text{-IBM}}$. These cells were rarely detected in the femora of [BALB → C3H]$^{3\text{-IV}}$ (Fig. 3A,C,E,G). In addition, the femora of the [BALB → C3H]$^{3\text{-IV}}$ showed hypoplastic bone marrow, as shown in Fig. 3 (J and K [DIC images] and L and M [H&E staining]).

When more primitive hematopoietic progenitors with the immunophenotype of CD34$^+$ or Sca-1$^+$ were examined histologically, the frequency of either CD34$^+$ or Sca-1$^+$ cells in the BM was clearly higher in the [BALB → C3H]$^{3\text{-IBM}}$ than in the [BALB → C3H]$^{3\text{-IV}}$, as shown in Fig. 3 (N,P,R,T and O,Q,S,U). The numbers of c-kit$^+$, CD34$^+$, or Sca-1$^+$ cells in 10 IPFs chosen randomly are summarized in Fig. 3V, where these hematopoietic progenitors were detected in significantly higher numbers in the [BALB → C3H]$^{3\text{-IBM}}$ than in the [BALB → C3H]$^{3\text{-IV}}$. These findings clearly indicate that IBM-BMT can facilitate not only the development of various lineage cells but also the effective generation and maintenance of progenitor cells.

Analyses of long-surviving recipients treated with IBM-BMT

The engraftments of HSCs and progenitor cells are crucially important to maintain hematopoiesis. The advantages of IBM-BMT to support the effective generation of donor-derived hematolymphoid cells and to maintain the progenitor cells were also shown in the results of long-surviving recipients treated with IBM-BMT. As shown

TABLE 2. DONOR-DERIVED HEMOPOIETIC CELLS IN LONG-SURVIVING RECIPIENTS AFTER IBM-BMT

Cells (%)	*BMT*	*Long-surviving recipients*	*Age-matched control (non-treated BALB mice)*	*12 weeks control (non-treated BALB mice)*
CD4	IBM	17.7 ± 0.3	18.2 ± 0.4	25.6 ± 0.2
	IV	17.6 ± 0.8		
CD8	IBM	5.1 ± 0.2	8.1 ± 0.1	12.0 ± 0.3
	IV	6.2 ± 0.4		
B220	IBM	45.8 ± 0.5	47.8 ± 0.5	37.7 ± 1.4
	IV	40.2 ± 2.0		
CD11b	IBM	4.4 ± 0.1	5.8 ± 0.9	2.9 ± 0.2
	IV	4.1 ± 0.0		
Gr-1	IBM	3.0 ± 0.0	8.9 ± 0.2	1.6 ± 0.0
	IV	2.5 ± 0.2		
Lin$^-$/H-2^{d+}/c-kit$^+$	IBM	5.1 ± 0.1*	5.0 ± 0.3	4.1 ± 0.2
	IV	4.0 ± 0.1*		

[a]The spleen cells were prepared from the primary recipients 1 year after the treatment with IBM-BMT or IV-BMT. The cells were then stained with the mAbs listed in the footnote for Table 1 to examine mature hematolymphoid cells and progenitor cells. Numbers represent mean % ± SD of 5 mice. *represents statistical significance ($P < 0.01$).

in Table 2, donor-derived cells with mature lineage markers remained at normal levels 1 year after the treatment in both the recipients treated with IBM-BMT and those treated with IV-BMT. The frequency of donor-derived progenitor cells was higher in the recipients of IBM-BMT than in those of IV-BMT, indicating that IBM-BMT can support the engraftment and maintenance of HSCs of donor origin. Histological findings in the right femora also revealed that the number of donor-derived progenitors ($H\text{-}2^{d+}$/c-kit$^+$) in the recipients of IBM-BMT (Fig. 4B,D,F,H) was clearly higher than in those of IV-BMT (Fig. 4A,C,E,G). This was the case when CD34$^+$ or Sca-1$^+$ cells were also examined (Fig. 4J,L,N,P and K,M,O,Q). The number of these primitive progenitors was also higher in the recipients treated with IBM-BMT than in those treated with IV-BMT in 10 IPFs chosen randomly (Fig. 4R). Therefore, in the BM of the recipients of BMCs by IBM-BMT, the progenitors or more primi-

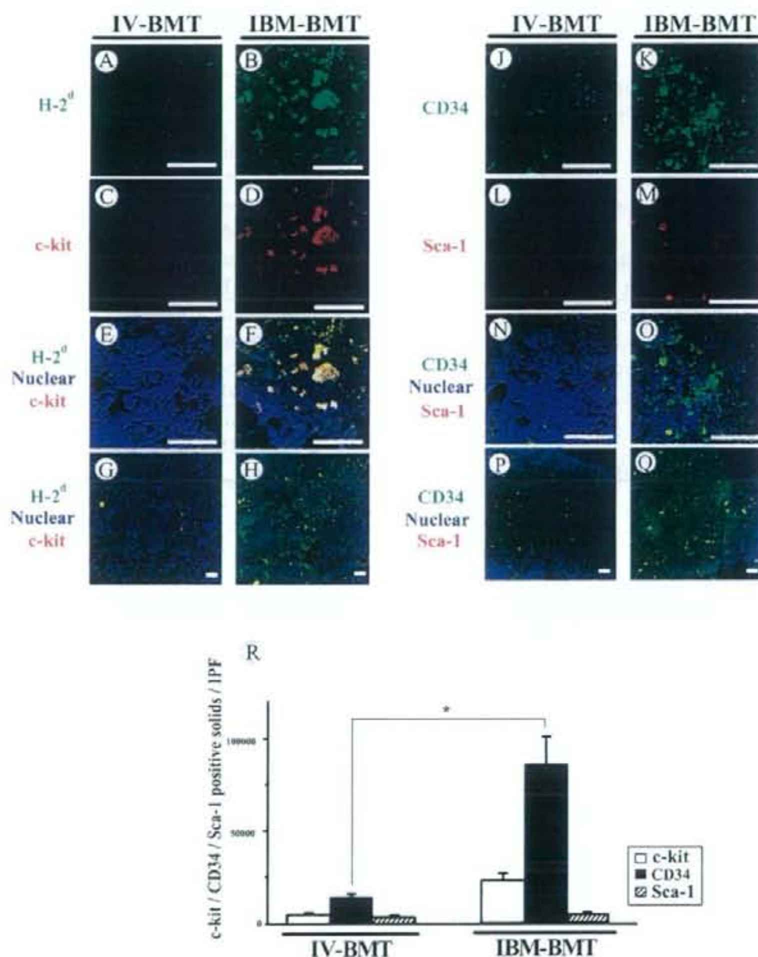

FIG. 4. Histological findings of the femur of the long-surviving recipients. The femora of the long-surviving recipients (>1 year after the primary BMT, but not serial BMT, were removed 1 year after the treatment and the specimens were stained with donor-specific biotin-conjugated anti-H-2^d mAb (green, **A,B,E–H**) and PE-conjugated anti-c-kit mAb (red, **C,D,E–H**). Merged images are shown in the figure (**E,G**, femur from the recipients treated with IV-BMT; **F,H**, femur from the recipients treated with IBM-BMT). The specimens were also stained with CD34 (green, **J,K,N±Q**) and Sca-1 (red, **L,M,N±Q**). Merged images are shown in **N±Q**. Nuclei were stained with DAPI (blue, **E±H,N±Q**). Bars, 50 μm (**A±Q**). c-kit$^+$ cells (open bars), CD34$^+$ cells (closed bars), or Sca-1$^+$ cells (hatched bars) were counted in 10 IPFs randomly chosen and are summarized in **R**. Columns and bars represent means ± SD of 6 mice. (*) Statistical significance ($p < 0.01$).

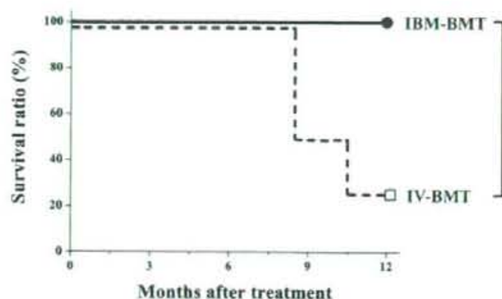

FIG. 5. Survival rates after BMT. Survival rates of 5 mice treated with IBM-BMT or IV-BMT were observed for 12 months after IV- or IBM-BMT, and were statistically analyzed by a log rank test. (*) Statistical significance ($p < 0.01$).

tive progenitors might well proliferate there for a long time to maintain these cells compared with BMCs transferred by IV-BMT.

Finally, the survival rate of the recipients treated with the primary IBM-BMT (without carrying out serial BMT) was significantly higher than that of the recipients treated with IV-BMT, indicating the efficacy of the long-term maintenance of progenitor cells or HSCs after IBM-BMT (Fig. 5).

DISCUSSION

In our previous work, we have shown that IBM-BMT can facilitate donor cell engraftment. This might be due to the efficient transplantation of both donor-derived pluripotent (P)-HSCs and stromal cells (including MSCs), because we have shown that MHC-matched stromal cells are required for the proliferation and differentiation of P-HSCs, and that the donor-derived stromal cells play a crucial role in successful allogeneic BMT [14,15]. However, in the case of conventional IV-BMT, most donor-derived BMCs (including MSCs) are trapped in the lung and liver, and only a few cells migrate into the BM [10]. Therefore, conventional IV-BMT had a transient effect on the treatment of autoimmune diseases because of the insufficient engraftment of donor-derived cells, resulting in the recurrence of the diseases due to the recovery of recipient-derived cells [16,17]. Furthermore, we have found that IBM-BMT is a highly sensitive method to detect severe combined immunodeficiency (SCID)-repopulating cell activity, including homing and/or seeding [18,19]. These findings clearly show that IBM-BMT is a powerful strategy to improve the efficiency of homing, seeding, and repopulating of P-HSCs. Indeed, we succeeded in preventing the recurrence of autoimmune diseases in chimeric-resistant MRL/lpr mice by IBM-BMT [1,2]. Furthermore, the facilitated engraftment of progenitor cells was clearly observed when serial analyses of donor-derived hematopoietic progenitor cells ($Lin^-/c\text{-}kit^+/H\text{-}2^{d+}$ cells) at the injected and non-injected (contralateral) sites were carried out over time to examine the localization of hematopoietic cells immediately after the injection into the bone marrow (IBM-BMT) (see Supplemental Fig. 1). This conformed with our previous results where donor-derived $Lin^-/c\text{-}kit^+$ cells had increased at the site of BMC injection [20]. Therefore, the hematopoietic progenitor cells actually persisted in the bone marrow microenvironment of the injected site (left tibia), thus suggesting that hematopoietic stem cells contained in the injected BMCs can efficiently localize in the physiological stem cell niche when BMCs are directly injected into the BM cavity (IBM-BMT).

However, it has been poorly understood how long the donor-derived BMCs containing HSCs or progenitors can be maintained in the recipients. Therefore, the long-term maintenance of donor-derived HSCs or progenitors should be compared between the recipients of IBM-BMT and IV-BMT. This is an important subject in clinical stem cell transplantation: establishing which strategy can rescue the recipients without the recurrence of disease and allow them to survive without graft failure. Indeed, we have recently shown that the hematopoietic system of donor-origin can be efficiently maintained over the long term by IBM-BMT using the serial BMT protocol in the MHC-matched combination [3]. The hematolymphoid system of the secondary recipients of BMCs from the primary recipients treated with IBM-BMT recovered earlier than the secondary recipients of BMCs from the primary recipients treated with IV-BMT [3].

The present study confirmed the effectiveness of IBM-BMT in the long-term maintenance of donor-derived hematopoiesis, even in the complete MHC-disparate mouse strain combinations ([BALB → C3H] and [BALB → B6]). The frequency of donor-derived cells in various lineages and progenitor cells was higher in the tertiary recipients of BMCs by IBM-BMT than in those by IV-BMT, as measured by flow cytometry and immunohistochemistry (Table 1; Figs. 2 and 3). Thus, the advantages of IBM-BMT seem to be in both lymphoid and myeloid engraftment. Furthermore, it is noted that neither donor-derived progenitor cells nor mature hematolymphoid cells were detected in ~25% of the tertiary recipients of BMCs by IV-BMT, indicating that progenitor cells can be efficiently maintained by IBM-BMT but not by IV-BMT (Table 1). The difference in the frequencies of donor-derived progenitor cells between the recipients treated with IBM-BMT and IV-BMT was more clearly confirmed in the tertiary recipients than in the primary and the secondary recipients. These findings indicate that IBM-BMT is suitable for long-term maintenance of the hematolymphoid system of donor origin. Indeed,

we have confirmed that IBM-BMT is far superior to IV-BMT in the assay of long-surviving recipient mice without carrying out serial BMT (Fig. 5). The recipients of BMCs by IV-BMT gradually died of infection, which was due to graft failure, resulting from fighting between donor and recipient cells, because the donor progenitor cells gradually lose their self-renewal capacity, but radio-resistant recipient P-HSCs gradually recover in collaboration with recipient stromal cells.

This was the case when the progenitor cells (c-kit$^+$) or more primitive progenitor cells (CD34$^+$/Sca-1$^+$) [21] were examined in the long-surviving recipient mice (Table 2 and Fig. 5); the frequency of donor-derived progenitor cells was significantly higher in the recipients of IBM-BMT than in those of IV-BMT, indicating that IBM-BMT can support the engraftment and maintenance of HSCs of donor origin.

Thus, self-renewal of P-HSCs is a key to their long-term repopulating ability (LTRA), and this can be supported by IBM-BMT, resulting in the long-term maintenance of donor-derived hematopoiesis. Previously, we have shown that donor-derived stromal cells (including MSCs) are essential for P-HSCs to proliferate and differentiate, and that MHC restriction exists between P-HSCs and MSCs [1,14,15]. IBM-BMT can efficiently recruit both donor-derived MSCs and P-HSCs in recipients, although IV-BMT can recruit only progenitor cells (not P-HSCs or MSCs) [18,22]. The efficient engraftment of both P-HSCs and MSCs by IBM-BMT can be applicable to the treatment of senile osteoporosis in the senescence-accelerated mouse, SAMP6, which spontaneously develops osteoporosis early in life partly due to the decreased production of interleukin-11 (IL-11), and the transfer of osteoporosis to the normal strain by IBM-BMT confirmed the advantages of this strategy [23]. Therefore, possibly due to the efficient engraftment of MSCs of donor origin, the frequencies of Sca-1$^+$ cells and c-kit$^+$ cells in the recipients of BMCs by IBM-BMT were significantly higher than those by IV-BMT (Figs. 3 and 4), and the frequency of CD34$^+$ cells was also higher in the recipients of BMCs by IBM-BMT. The advantageous effect of IBM-BMT in the reconstitution of Lin$^+$ cells was only observed in the tertiary recipients; however, this clearly indicates that P-HSCs could be efficiently maintained only by IBM-BMT, and again suggests the importance of MSCs, simultaneously recruited, to construct a hematopoietic microenvironment. Thus the difference observed in frequencies of Sca-1$^+$, c-kit$^+$, and CD34$^+$ cells might be associated with a significantly higher survival rate after IBM-BMT observed in long-surviving recipients.

The immunophenotype of P-HSCs is still controversial and complicated; it has been reported that Lin$^-$/Sca-1$^+$/c-kit$^+$/CD34$^-$ cells [24] and Mac-1$^-$/CD34$^\pm$ or Mac-1^{low}/CD34$^\pm$ cells [25] have the capacity of LTRA. We have previously shown that Lin$^-$/c-kit$^-$ cells have LTRA (>2 years) using the serial IV-BMT assay [26]. Further studies on the surface phenotype of real P-HSCs are now under investigation using IBM-BMT.

It is unlikely that the residual host immune system preferentially killed allogeneic BMCs in the case of IV-BMT, but not in IBM-BMT because the radiation dose used as a preconditioning for BMT was lethal to the C3H mice, and in the first BMT the donor cell engraftment of the recipients treated with IV-BMT was similar to that treated with IBM-BMT. Furthermore, we can rule out the possibility that changes in the features of the stromal cells that might support hematopoiesis are induced by injury as a result of the IBM-BMT, because mock IBM-BMT together with IV-BMT showed similar results to IV-BMT alone when the primary, secondary, and tertiary recipients were examined (data not shown). A possible explanation for the novel findings here is that the niche is constructed particularly efficiently when BMCs including P-HSCs are injected via IBM-BMT.

Although the practical relevance for the treatment of patients is not entirely clear, and Hagglund reported that there was no difference in hematopoietic recovery between conventional IV-BMT and IBM-BMT [27], the recent report by Ibatico et al. showed that IBM-BMT could shorten the time of engraftment when allogeneic cord blood cells were infused into 1–3 HLA-mismatched recipients [28]. Recently, we successfully carried out IBM-BMT with the perfusion method to a patient with intractable β-thalassemia major; the white blood cell count reached 8,600/μl on day 55, and 98% of the peripheral blood cells were of donor origin [29]. These findings indicate that IBM-BMT can be applicable to the clinical trials to overcome delayed engraftment and also available for the treatment of patients with various intractable diseases.

In conclusion, we have demonstrated that IBM-BMT facilitates the lodging of HSCs, thereby resulting in the long-term maintenance of HSCs and supporting the efficient development of multilineage hematopoiesis even in completely MHC-disparate combinations ([BALB $\rightarrow$ C3H] and [BALB $\rightarrow$ B6]). Therefore, our work opens a new avenue of BMT and stem cell biology.

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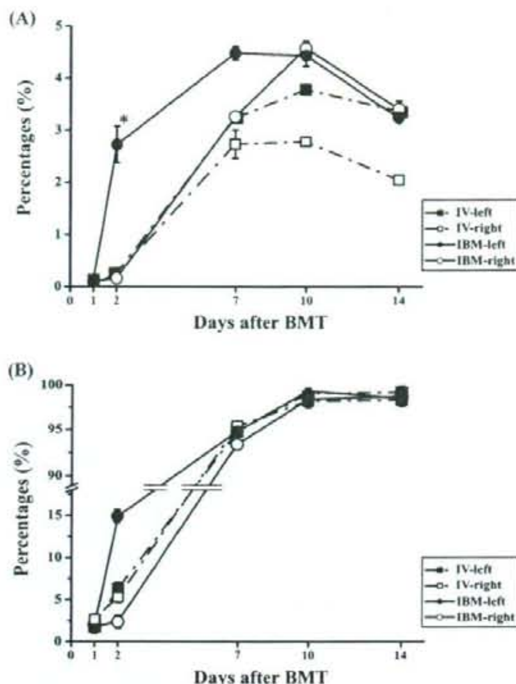

Supplemental FIG. 1. Analysis of donor-derived progenitor cells immediately after IBM-BMT. BMCs (2×10^7 cells) from BALB mice were injected into the left tibia. The donor-derived hemopoietic progenitor cells of (Lin$^-$/c-kit$^+$/H-2^{d+} cells) at the injected (closed circle, line) and noninjected (contralateral, open circle, line) sites were serially analyzed at 1, 2, 7, 10, and 14 days to examine the localization of the hematopoietic cells immediately after the injection into the bone marrow (IBM-BMT). The recipients treated with IV-BMT (left and right tibiae, open and closed square, dotted lines) served as controls. (**A**) Kinetics of donor-derived hematopoietic progenitor cells of (Lin$^-$/c-kit$^+$/H-2^{d+} cells). (**B**) Kinetics of donor-derived cells (H-2^{d+} cells). Symbols and bars represent means $\pm$ SDs of 4 mice. (*) Statistical significance ($p < 0.01$).

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Facilitation of hematopoietic recovery by bone grafts with intra-bone marrow–bone marrow transplantation

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Abstract

We have previously shown that T cells can acquire donor-type major histocompatibility complex (MHC) restriction and can interact with both donor-type antigen-presenting cells (APCs) and B cells, when adult donor bones are co-grafted with intravenous (IV) injection of bone marrow cells (BMCs) in order to supply donor bone marrow (BM) stromal cells. We have also found that the direct injection of donor BMCs into recipient BM (intra-bone marrow–bone marrow transplantation: IBM–BMT) produces more rapid reconstitution (including T-cell functions) and higher survival rates than IV injection (IV-BMT) even in chimerism-resistant combinations. In the present study, we show that the co-administration of bones from suckling (2–3 days old) donor mice is also effective in the IBM–BMT system. Even when a relatively low number of BMCs were injected into adult (more than 15 weeks old) mice, complete reconstitution was achieved in the mice that had received IBM–BMT + bone grafts, but not in the mice that had received IBM–BMT alone. Most BM and splenic adherent cells obtained from the recipients that had received IBM–BMT + bone grafts were reconstituted by donor-type cells. Both T-cell proliferation and plaque-forming cell assays indicated that the T cells of such mice showed donor-type MHC restriction. Moreover, the analyses of thymic sections using confocal microscopy revealed that donor BM stromal cells had migrated into the thymus. Thus, the co-administration of donor bones has great advantages for allogeneic BMT in adult mice.
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Keywords: Bone marrow transplantation; Bone marrow cells; Bone graft; Stromal cells

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Introduction

Allogeneic bone marrow transplantation (BMT) has been utilized for complete treatments for refractory diseases such as leukemia and aplastic anemia. It has

been reported, however, that humoral immunodeficiency lasts for a longer period in adult patients than in infant patients even after donor-type cells are reconstituted (Onoe et al., 1980). Similar observations are obtained in animal studies; primary humoral responses against T cell-dependent antigens cannot be completely reconstituted in fully allogeneic BMT (El-Badri and Good, 1994; Gerritsen et al., 1994). We have previously shown that such a deficiency can be overcome if donor bones are engrafted in conjunction with BMT, since donor BM stromal cells migrate into the thymus, where they are engaged in positive selection. T cells in such mice can acquire donor-type major histocompatibility complex (MHC) restriction and can interact with both donor-type antigen-presenting cells (APCs) and B cells (Li et al., 2000). In these experiments, adult (8–10 weeks old) mice were used as recipients, and bones obtained from adult (8–10 weeks old) donor mice were engrafted into the mice that had received 2×10^6 T cell-, macrophage- and stromal cell-depleted BMCs by the conventional intravenous (IV) route.

In several earlier experiments, we have demonstrated that the co-administration of adult donor bones facilitates the acceptance of donor BMCs even in chimeric-resistant combinations, such as [normal → MRL/lpr] (Ishida et al., 1994) and [DBA/2 → B6] (Hisha et al., 1995). Donor-type stromal cells were detected in the BM adherent cells obtained from the treated chimeric mice. This indicates that BM stromal cells contained in the engrafted bones migrate into the recipient BM cavity and provide a suitable environment for donor hemopoietic stem cells (HSCs). We have also found that an MHC restriction exists between HSCs and BM stromal cells (Hashimoto et al., 1997; Sugiura et al., 2001); we grafted bones obtained from various mouse strains to one recipient mouse and BMCs were then injected by the IV route (Hashimoto et al., 1997). Significant cell accumulation of the injected BMCs was observed in the engrafted bones having the same MHC phenotype as the BMCs, whereas only a few BMCs were detected in the engrafted bones having different MHC phenotypes from the BMCs (Hashimoto et al., 1997). In accordance with this concept, clinical approaches using co-administration of donor bone fragments have been performed in patients who received non-myeloablative BMT and the facilitating effect of the grafted donor BM stromal cells was observed (Cahill et al., 2004; Jones et al., 2004).

More recently, we have found that the direct injection of donor BMCs (intra-bone marrow injection: IBM) produces more rapid reconstitution (including T-cell functions) and higher survival rates than IV injection even in chimerism-resistant combinations [normal → MRL/lpr mice] (Kushida et al., 2001). Moreover, we have shown that senile osteoporosis in SAMP6 mice can be prevented and treated effectively by IBM–BMT using normal mouse BMCs (Ichioka et al., 2002; Takada et al., 2006). In the recipient mice, the proliferation of donor stromal cells was also observed at the site of injection (Kushida et al., 2001; Takada et al., 2006). These results indicate the possibility that the stromal cells contained in donor BMCs play an important role in IBM–BMT. Therefore, we next performed simultaneous injection of donor BMCs and a stromal cell line (PA6 cells) into recipient bone cavity in a normal mouse combination. This approach allowed for a higher reconstitution of donor-type cells in the mice that had received IBM–BMT + injection of PA6 cells than in those that had received IV-BMT + injection of PA6 cells or that had received IBM–BMT alone (Zhang et al., 2004). The IBM–BMT group showed the highest survival rate of the three groups up to 60 days after BMT. When allogeneic BM adherent cells were used instead of the PA6 cells, a similar facilitating effect was observed in the IBM–BMT group (Zhang et al., 2004). These results indicate that co-administration of stromal cells in IBM–BMT provides a great advantage for the acceptance of donor cells.

Based on these findings, we hypothesized that complete reconstitution would be achieved in adult (more than 15 weeks old) mice if BMCs were administered by the IBM route in conjunction with bone grafts from younger mice. Therefore, in the present study, we first compared the *in vitro* proliferation and hemopoiesis-supporting ability of BM stromal cells obtained from suckling (2–3 days old) and young adult (4–5 weeks old) mice. Then we examined whether bone grafts could induce a complete reconstitution, including T-cell functions, in adult (more than 15 weeks old) mice that had received donor BMCs by the IV or IBM routes. To investigate precisely the facilitating effect of bone grafts, a lower number (5×10^6 per mouse) of whole BMCs was injected into the recipient mice, because we have administered 3×10^7 whole BMCs into the recipient mice (without donor bone grafts) in most of our previous experiments (Kushida et al., 2001; Ichioka et al., 2002; Takada et al., 2006). Moreover, we examined whether donor BM stromal cells migrate from the grafted bones into recipient BM, spleen and thymus after IBM–BMT and participate in positive selection.

Materials and methods

Animals

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