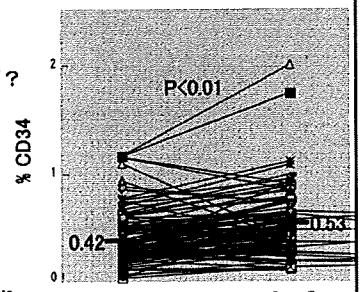
採取開始 day 4 vs day 5

Day4 開始
G-CSFリスク軽減
全拘束期間短縮

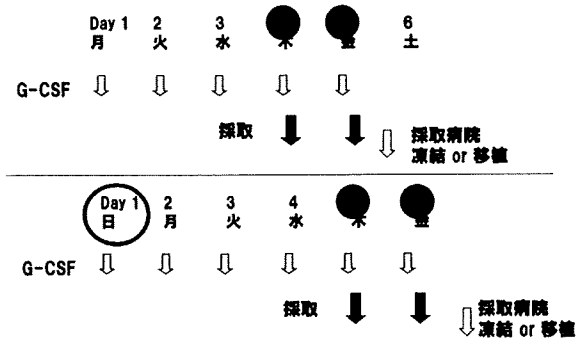
1回採取成功率低下？

2回以上apheresisを施行した患者での
CD34比較
(n=96)



九州大学病院 遺伝子・細胞療法部

Day 4 vs Day 5



Blood access

カテーテル挿入：ドナーのリスクの観点から望ましくない

カテーテル必要：　健常人ドナー：15/155（約10%）

九州大学病院 遺伝子・細胞療法部

カテーテル認めないと，10%の採取不可のリスク

Blood access

カテーテル挿入を認めない場合：

ドナーの意思決定の前，コーディネーター中に
血管（正中静脈）の評価が必要

採取病院の検診でとれそうにないと判断されたらどうする？

G-CSFを打った後で入院後，
十分なフローがとれないとどうする？
採取医が穿刺失敗したらどうする？

Blood access

中心静脈カテーテル挿入について

既存のガイドラインなど

名古屋大学医学部附属病院　中心静脈カテーテル挿入マニュアル
http://www.mms-net.com/~med.nagoya-u/anesth/cv/CVmanual2.pdf

日本麻酔科学会
安全な中心静脈カテーテル挿入・管理のための手引き2009
http://www.anesth.or.jp/dbps_data/_material_/localhost/a.pdf

Blood access

中心静脈カテーテル挿入について

中心静脈へのアクセスについて

1. PBドナーの適格性に、採取に適する末梢静脈がある　を創る
　　確認検査時に、適格性を判断

2. 末梢静脈から採取できると判断されていたが
　　実際に採取したら、血流量が確保できない
　　血管穿刺がうまくゆかず、末梢静脈が確保できない

3. 上記の場合は、大腿静脈にアクセスする

中心静脈へアクセスする可能性があることを
あらかじめドナーにインフォーム

考察1

- 採取を外来主体で行うか、入院主体で行うかは、採取施設、ドナー、ドナーと採取施設の地理的関係等により異なる
- 採取開始日は、day4　day5　いずれも許容される

考察

- 中心静脈へのアクセスについて
  1. 明らかに末梢からの採取が無理
     ドナー適格性について
  2. 末梢から採取したが無理で、移行
     血管が取れないので、
     G-CSFを投与した後に中止は実際的でない
- 中心静脈へアクセスする可能性があることをあらかじめドナーにインフォーム
- 中心静脈にアクセスした場合、後出血に対する対応
  1. 退院のスケジュール
  2. 穿刺部の処置

UR-PBSCTの将来

UR-PBSCTの臨床試験に関する私見

UR-PBSCTにおける臨床試験について

1. GVHD予防法を筆頭に、移植前処置法など適切な移植方法がわからない状態で、これらの移植方法を統一した多施設共同臨床試験の実施は難しい。
2. 経験の豊富なUR-BMTと経験の少ないUR-PBSCTを適切に比較するためには、UR-PBSCTについてもある程度の臨床経験と移植方法の至適化が必要である。

UR-PBSCTにおける臨床試験について

1. したがってUR-PBSCTの多施設共同臨床試験を行うのであれば、単群でGVHD予防法などの至適化を目指した臨床試験を計画すべきである。
2. しかし、様々な因子を調整しなければならない現状で、画一的な用量設定試験の実施は困難であり、おそらくLearning curveによる移植成績の改善を妨げる。
3. かといって、各施設の最良にゆだねた診療のretrospective studyには限界がある。(必要な情報を収集できない。)

UR-PBSCTにおける臨床試験について

1. そこで、移植前に前処置、GVHD予防法などの移植方法について、いくつかの選択肢を設定して担当医の裁量で選択できるようにして、その選択理由とともに登録する方式によって、より精度の高い解析ができるような臨床試験を実施する。

患者側の希望
□PBSCTのみ (理由:)
□BMT/PBSCTいずれでも可
ドナー側の希望
□PBSCTのみ (理由:)
□BMT/PBSCTいずれでも可

移植前処置
□CY-TBI
□BU-CY
□FLU-MEL □FLU-MEL-TBI (Gy)
□FLU-BU □FLU-BU-TBI (Gy)
□FLU-CY □FLU-CY-TBI (GY)
□その他 ( )

前処置選択理由
□施設の標準前処置
□高齢のため
□臓器障害(障害)のため

GVHD予防法 (予防として投与を予定しているものをすべて選択)
☐CSA ☐TAC ☐MTX
☐MMF ☐PSL

CSA、TACを用いる場合、その投与方法、開始用量、目標血中濃度
開始用量 _____mg/kg
☐持続静注 (目標血中濃度 ng/ml)
☐1日2回点滴静注 (目標血中濃度 ng/ml ☐トラフ・☐投与__時間後)
☐1日1回点滴静注 (目標血中濃度 ng/ml ☐トラフ・☐投与__時間後)

MTXを用いる場合、その投与日と投与量
☐15 mg/m² (day 1) + 10 mg/m² (day 3,6,11)
☐10 mg/m² (day 1) + 7 mg/m² (day 3,6,11)
☐15 mg/m² (day 1) + 10 mg/m² (day 3,6)
☐10 mg/m² (day 1) + 7 mg/m² (day 3,6)
☐その他 (__mg/m² (day ___) + (__mg/m² (day ___)

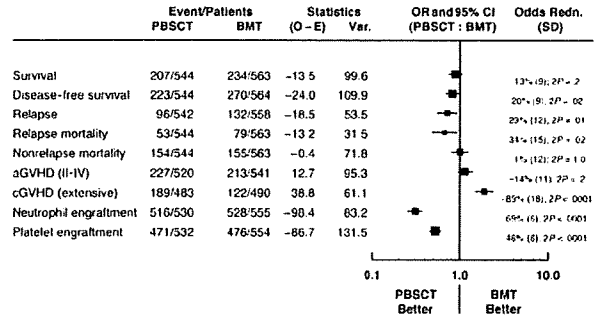
MMFを用いる場合、その投与日と投与量
☐ (_____mg/m² (day ___～___)

CSAの減量開始予定日(GVHDの発症がない場合)
移植後 _____日
CSAの投与終了予定日(GVHDの発症がない場合)
移植後 _____日

今後、明らかにしていきたいこと

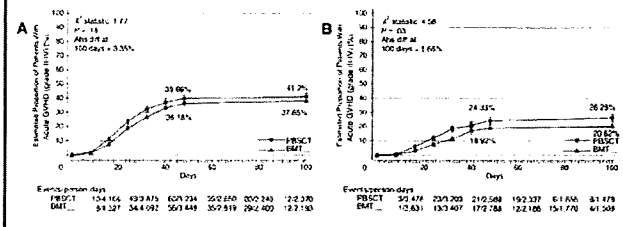
1. 前方視的情報収集
↓
2. UR-PBSCTの移植方法の至適化
↓
3. UR-BMTとの比較 (RCT?)
生存率のみならず、QOL、医療費についても。

血縁者間PBSCTvsBMT



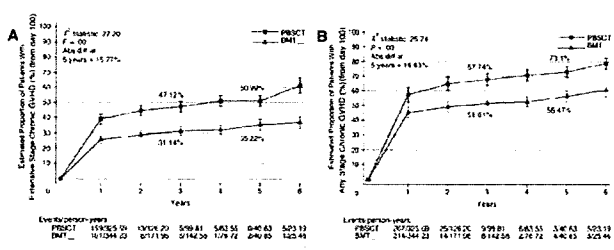
J Clin Oncol 23:5074-5087.

血縁者間PBSCTvsBMT



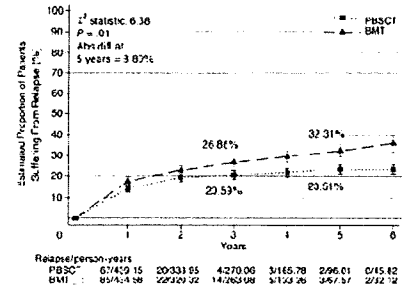
J Clin Oncol 23:5074-5087.

血縁者間PBSCTvsBMT



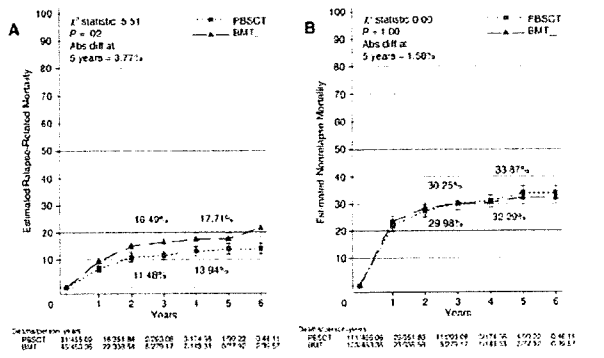
J Clin Oncol 23:5074-5087.

血縁者間PBSCTvsBMT



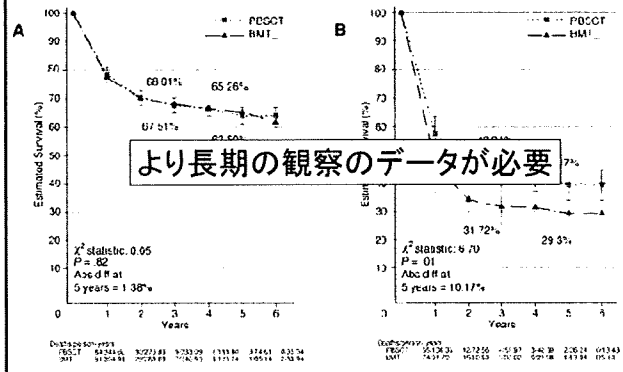
J Clin Oncol 23:5074-5087.

血縁者間PBSCTvsBMT



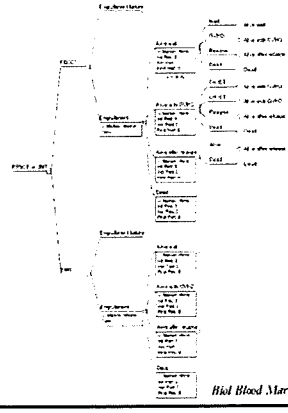
J Clin Oncol 23:5074-5087.

血縁者間PBSCTvsBMT



J Clin Oncol 23:5074-5087.

血縁者間PBSCTvsBMT 決断分析



Biol Blood Marrow Transplant 15: 1415-1421 (2009)

血縁者間PBSCTvsBMT 決断分析

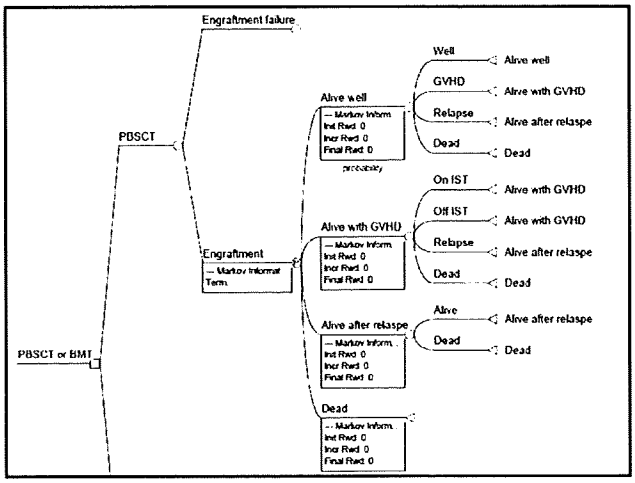
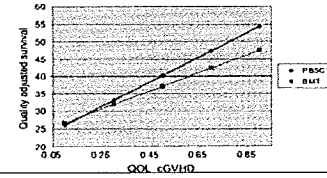


Table 1. Probability Estimates with Data Sources

Probability	Data Source	Estimate (PBSCT)	Adjusted for Month Cycle Length (PBSCT)	Estimate (BMT)	Adjusted for Month Cycle Length (BMT)	Range for Sensitivity Analyses
Engraftment failure	Meta-analysis	0.03	0.03	0.05	0.05	0.01-0.08
aGVHD	Meta-analysis	0.412	0.137	0.379	0.126	0.12-0.8
Death from aGVHD	Meta-analysis	RRI*early TRM	RRI*early TRM	RRI*early TRM	RRI*early TRM	RRI 1-5
Relapse, year 1	Meta-analysis	0.153	0.01275	0.156	0.013	0.03
Relapse, year 2	Meta-analysis	0.06	0.005	0.069	0.0058	0.03-0.12
Relapse, year 3	Meta-analysis	0.0143	0.0012	0.053	0.0044	0.005-0.08
Treatment success aGVHD	Literature	0.4	0.067	0.4	0.067	0.25-0.75
cGVHD through year 1	Meta-analysis	0.59	0.098	0.45	0.075	0.05-0.7
cGVHD beyond	Meta-analysis	0.09	0.0075	0.08	0.0067	0.05-0.15
cGVHD complications through year 1	Meta-analysis	0.4	0.067	0.25	0.042	0.1-0.5
cGVHD complications beyond	Meta-analysis	0.05	0.0042	0.04	0.0033	0.03-0.06
Transplant complications	Literature	0.125	0.01	0.125	0.01	0.05-0.2
Treatment success cGVHD	Literature	0.3	0.0083	0.3	0.0083	0-0.7
Taper IST	Stewart et al [33]	0.20	0.0056	0.4	0.011	0.05-0.5
Death from relapse, early	Meta-analysis	0.07	0.0058	0.1	0.0083	0.05-0.3
Death from relapse, late	Meta-analysis	0.045	0.00375	0.065	0.0054	0.04-0.08
Early TRM	Meta-analysis	0.125	0.01	0.125	0.01	0.05-0.2
Late TRM	Meta-analysis	0.02	0.0017	0.02	0.0017	0-0.1
Quality of life	Literature	estimates (see Methods)*		*estimates (see methods)		0-1.0
aGVHD complications	Meta-analysis	0.26	0.087	0.20	0.067	0.09-0.39
Death from cGVHD	Meta-analysis	RRI*late TRM	RRI*late TRM	RRI*late TRM	RRI*late TRM	RRI 1-5
Age, years	Base case	35		35		18-65
ASR mortality	Literature	*U.S. standard ASR mortality		*U.S. standard ASR mortality		

aGVHD indicates acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; TRM, treatment-related mortality; IST, immunosuppressive therapy; RRI, relative risk increase; ASR, age/sex/race.

血縁者間PBSCTvsBMT 決断分析



同様の手法でcost-effectivenessについても評価可能。

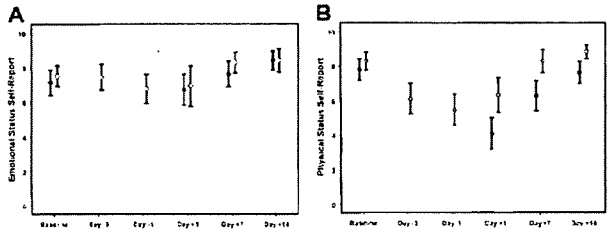
Table 2. Survival Outcomes for PBSCT versus BMT

	PBSCT	BMT
Overall life expectancy, months	61	54
QALE	56	49

PBSCT indicates peripheral blood stem cell transplantation; BMT, blood marrow transplantation; QALE, quality-adjusted life expectancy.

Biol Blood Marrow Transplant 15: 1415-1421 (2009)

ドナーのQOL (Seattle RCT)



(Blood. 2001;97:2541-2548)

ドナーのQOL (NMDP)

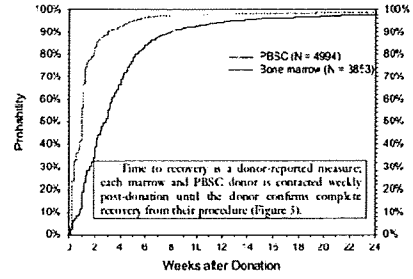


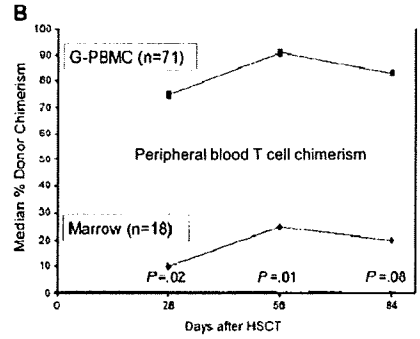
Figure 5. Kaplan-Meier plots of time to recovery from stem cell donation (first donations performed from November 2001 through March 2006).

Biology of Blood and Marrow Transplantation 14:29-36 (2008)

ドナープールの拡大

- 理想的にはBM、PBどちらでもよいと言ってくれるドナーの増加。
- しかし、「PBなら・・・」というドナーがドナープールの増大に貢献する可能性は否定できない。
- まずはPBを入り口にして、PB採取、BM採取の両者を知っていただくことで、そのようなドナーが増えることを期待する。

RICT,NST from UR-donor



(Blood. 2003;102:2021-2030)

平成20年度 全国調査報告書

9.3.3c. 非血縁者からの移植・骨髄 UR-BMT

年齢 Age	'91～'96	'97	'98	'99	'00	'01	'02	'03	'04	'05	'06	'07	合計 Total
0～4	67	27	31	14	16	20	31	26	26	26	39	26	385
5～9	90	31	41	38	43	55	46	36	46	37	38	26	527
10～14	168	35	28	42	38	47	41	39	42	43	37	39	527
15～19	155	57	54	72	72	65	65	48	45	44	43	45	716
20～24	139	57	92	65	71	55	53	40	32	35	45	42	706
25～29	165	46	92	62	70	50	62	56	42	57	49	53	744
30～34	140	36	50	64	59	82	69	61	62	67	59	51	770
35～39	95	32	43	59	67	59	68	57	75	71	74	71	791
40～44	88	34	40	50	75	81	60	50	68	66	81	67	757
45～49	40	28	37	45	70	76	71	85	59	95	81	91	778
50～54	9	7	8	17	54	59	74	72	78	87	87	103	655
55～59	0	0	0	6	9	19	27	50	71	91	110	127	510
60～64	0	0	0	1	0	4	3	16	30	59	46	61	220
65～69	0	0	0	0	0	1	2	5	8	14	12	16	58
70～74	0	0	0	0	0	1	0	0	0	1	2	0	1
75～79	0	0	0	0	0	0	0	0	0	0	0	0	0
80～84	0	0	0	0	0	0	0	0	0	0	0	0	0
85～89	0	0	0	0	0	0	0	0	0	0	0	0	0
90～95	0	0	0	0	0	0	0	0	0	0	0	0	0
不明	0	0	0	0	0	0	0	0	0	0	0	0	0
合計 Total	1,006	390	446	535	669	706	675	641	684	793	803	819	8,158

非血縁者間移植数増加の最大の要因

UR-PBSCTの導入によって

- ドナーQOLの改善
- 非血縁ミニ移植の拡大
- 細胞療法への応用(多数の幹細胞、T細胞)
- 緊急時の対応(貯血が不要)

厚生労働省科学研究事業
「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　耕一　班長

平成21年度　第2回　研究班会議（2010/1/31）

末梢血幹細胞非凍結での移植の経験

都立駒込病院　造血細胞移植チーム
坂巻壽、奥山美樹、比留間潔

背景・目的

同種造血幹細胞移植の幹細胞源として末梢血幹細胞（PBSC）の有用性が高まりつつあるが、十分なCD34陽性細胞の得られないドナーが少なからず存在することから、数日間にわたり採取、凍結保存して十分な細胞数を確保してから移植されることが多い。しかし、適切な移植細胞数に関しては必ずしも定まっているとは言えない。

当院では原則、採取末梢血は凍結保存せずに移植を行ってきた。採取末梢血を凍結保存せずに移植を行った症例を後方視的に解析したので報告する。

駒込病院における非凍結末梢血CD34+細胞を用いた同種移植

対象および方法

◆ 1996年6月～2009年12月、同種PBSCT（RISTを除く）72例。
患者年齢；中央値37才（17～60才）
患者性別；男/女＝47/25

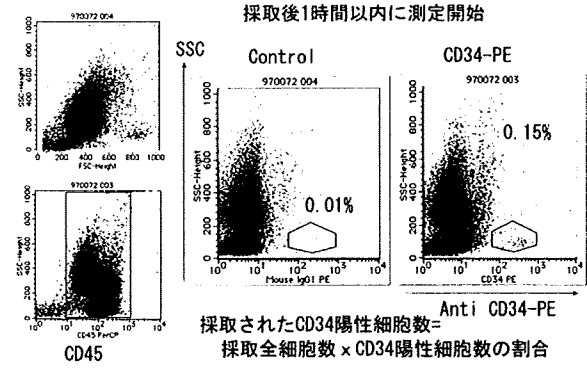
AML	31例	MDS	5
ALL	15	MM	4
NHL	8	SAA	2
CML	7		

◆ ドナーはHLA一致の血縁者で、G-CSF 10μg/kg/dayを皮下注し投与4～5日後にCobe Spectraで1～2日間PBSCを採取した。
処理量は150～200 ml/kg（ドナー体重）。

CD34+細胞数は2x10⁶/kgを目標、1x10⁶/kgを最少限度とすることを目安に採取した。

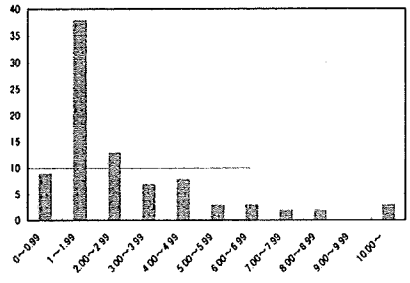
末梢血幹細胞中のCD34陽性細胞測定法

採取後1時間以内に測定開始



採取されたCD34陽性細胞数＝
採取全細胞数 x CD34陽性細胞数の割合

採取されたCD34陽性細胞数の度数分布

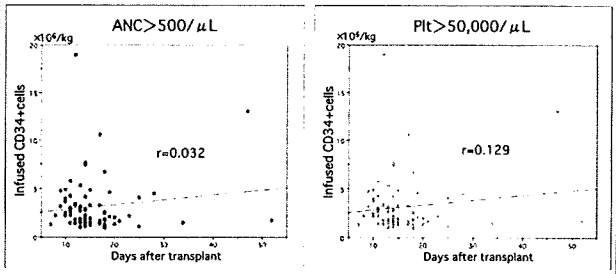


CD34+cells ×10⁶/Kg

初日採取細胞数の少なかったケースでの最終輸注細胞数

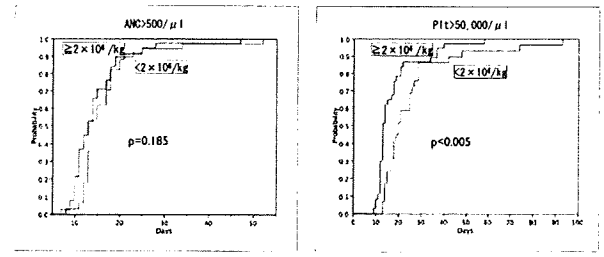
1日目採取CD34細胞数 (X10⁶/kg)	2日目採取CD34細胞数 (X10⁶/kg)	移植CD34陽性細胞数 (X10⁶/kg)
0.28	0.81	1.09
0.39	1.41	1.8
0.58	0.84	1.42
0.65	1.03	1.68
0.67	0.32	0.99
0.75	0.38	1.13
0.93		0.93
0.95	1.21	2.16
0.98	0.85	1.83

輸注CD34+細胞と造血回復

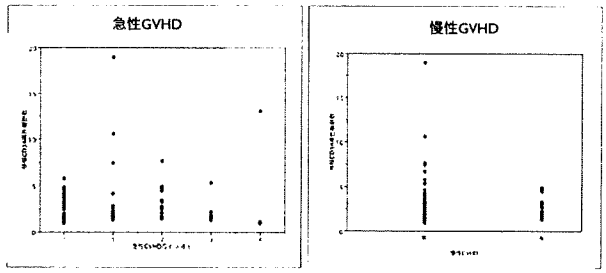


輸注CD34+細胞と造血回復

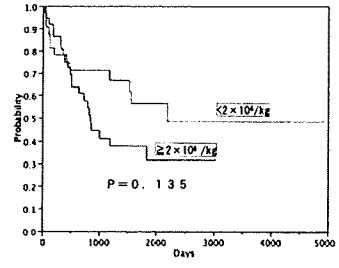
CD34+<2.0x10⁶とCD34+≧2.0x10⁶の比較



輸注CD34+細胞とGVHD



輸注CD34+細胞と生存率



まとめ

- 72回の移植の内、ドナーから2日にわたり採取したのは14例であった。
- 輸注されたCD34+細胞数は中央値2.16 X10⁶/Kg（0.93～18.92 X10⁶/Kg ）
- 全例で生着を認めた。
- CD34+細胞＞2.0 X10⁶/Kg輸注された症例では血小板の回復が有意に促進されたが、好中球数の回復には有意差を認めなかった。
- 急性・慢性GVHDとも輸注されたCD34+細胞数との相関は認めなかった

VII. 研究成果の刊行物・印刷

Long-Term Donor-Specific Tolerance in Rat Cardiac Allografts by Intrabone Marrow Injection of Donor Bone Marrow Cells

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Background. Donor-specific central tolerance in cardiac allograft can be induced by hematopoietic chimerism via conventional intravenous bone marrow transplantation (IV-BMT). However, there are problems with IV-BMT, such as the risk of graft failure and of the toxicity from conditioning regimens.

Methods. A new method for heart transplantation is presented. This method consists of administration of fludarabine phosphate (50 mg/kg) and fractionated low-dose irradiation (3.5 Gy×2 or 4.0 Gy×2), followed by intrabone marrow injection of whole bone marrow cells (IBM-BMT) plus heterotopic heart transplantation.

Results. Cardiac allografts with IBM-BMT were accepted and survived long-term (>10 months) showing neither acute rejection nor chronic rejection including cardiac allograft vasculopathy by such conditioning regimens. In contrast, cardiac allografts with conventional IV-BMT were rejected within 1 month after the treatment with irradiation of 3.5 Gy×2 or within 3 months after the treatment with irradiation of 4.0 Gy×2. Macrochimerism (>70%) was favorably established and stably maintained by IBM-BMT but not IV-BMT. Low levels of transient mixed chimerism (<7%) were induced by IV-BMT with fludarabine plus 4.0 Gy×2, but the chimerism was lost within 1 month after the treatment.

Conclusions. These findings indicate that IBM-BMT is a feasible strategy for the induction of persistent donor-specific tolerance, enables the use of reduced radiation doses as conditioning regimens, and obviates the need for immunosuppressants.

Keywords: Tolerance induction, Heart transplantation, Intrabone marrow injection, Bone marrow transplantation.

(*Transplantation* 2008;85: 93–101)

Despite recent advances in immunosuppressive agents, chronic rejection and side effects associated with the lifelong usage of nonspecific immunosuppressants remain a barrier to successful clinical solid organ transplantation (*1*). Mixed hematopoietic chimerism has proven its efficacy in the induction of persistent tolerance in rodents, large animals (including nonhuman primates) and recently a few renal patients by conventional intravenous (IV) bone marrow transplantation (BMT) (*2–4*). However, obstacles that hinder the clinical application of BMT as a feasible strategy for inducing tolerance in a clinical setting include the toxicity of conditioning regimens, the risk of graft failure, and the problem of graft-versus-host disease (GvHD) (*5*).

Recent studies in animal models have therefore aimed at minimizing conditioning and optimizing selective immunosuppression. Included in these approaches is the use of nonmyeloablative conditioning regimens, T-cell depletion, donor lymphocyte infusion, and blockade of stimulatory and costimulatory pathways (*6–9*). We have recently found that the injection of donor bone marrow cells (BMCs) directly into the bone marrow cavity (intrabone marrow BMT [IBM-BMT]) induces persistent donor-specific tolerance in mice even if the radiation doses are reduced to sublethal levels (*10*). IBM-BMT also enhances the rapid recovery or reconstitution of the hematolymphoid system (including bone marrow stromal cells) of donor origin, resulting in the complete amelioration of autoimmune diseases in MRL/lpr mice, in which conventional IV-BMT had been unsuccessful (*10*). It is of interest that the recipients treated with IBM-BMT have no clinical or histopathological signs of GvHD or graft failure (*10, 11*). In addition, we have more recently extended this new approach to the induction of tolerance in the transplantation of the leg (*12*), lung (*13*), and pancreatic islets (*14*) in rats. Therefore, the new BMT method (IBM-BMT) seems to have significant advantages in achieving successful allogeneic organ transplantation.

It has been reported that fludarabine eliminates normal and malignant mononuclear cells in animals and humans through the inhibition of DNA synthesis. It has also been shown to have cytotoxic effects on lymphoid cells (*15*), particularly T cells (*16*). Furthermore, the prior injection of flu-

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darabine has proven to facilitate the establishment of high levels of hematopoietic chimerism with low doses of irradiation, as shown in our previous leg (*12*) and pancreatic islet transplantation (*14*). In view of both its selective lympholytic activity (especially to eliminate donor-reactive T cells in the recipients) and relatively mild side-effect profile (*17*), we have used this immunosuppressive agent as a part of our nonmyeloablative conditioning regimens.

In the present study, we report that the combination of the injection of fludarabine, low-dose fractionated irradiation, and IBM-BMT provides a feasible clinical strategy for inducing permanent tolerance without using any immunosuppressants.

MATERIALS AND METHODS

Animals

Brown Norway (BN: $RT1^n$), Fischer 344 (F344: $RT1^l$), and PVG (PVG: $RT1^c$) rats were purchased from SLC (Shizuoka, Japan) and maintained until use in our animal facilities under specific pathogen-free conditions. BN rats at the age of 8–10 weeks were used as recipients, and F344 rats at the age of 8–10 weeks were used as donors for the transplantations of the heart, skin, and BMCs. PVG rats were used as third party stimulators in mixed leukocyte reactions (MLRs). All animal research was reviewed and approved by the Animal Experimentation Committee, Kansai Medical University.

Heterotopic Heart Transplantation

Heterotopic heart transplantation was performed, as described by Tomita Y (*18*). Briefly, after induction of anesthesia with intraperitoneal injection of sodium pentobarbital, vascularized heart transplantation was performed heterotopically into the right cervical portion of recipients using a microsurgical cuff technique. Donor hearts were procured and stored in a cold saline bath. The donor brachiocephalic artery and main pulmonary artery were anastomosed with the recipient right common carotid artery and right external jugular vein, respectively. Allograft survival was assessed by daily palpation. Graft rejection was defined as complete cessation of spontaneous ventricular contraction.

Experimental Protocol for BMT

BN rats were injected intravenously with 50 mg/kg of fludarabine phosphate, followed by fractionated whole body irradiation (3.5 Gy×2 to 5.0 Gy×2) by ^{137}Cs (Gammacell 40 Exactor; Nordion International Inc, Ontario, Canada) 1 day before the bone marrow and heart transplantation (day −1). As described previously (*11*), BMCs (3×10^7 or 10×10^7 cells/60 μL) obtained from the femurs and tibias of donor F344 rats were injected intravenously (IV-BMT) or directly into the bone marrow cavity (IBM-BMT) of the left tibia of the recipient BN rats on day 0, and the cardiac allografts from F344 rats were implanted simultaneously. In terms of the IBM-BMT technique, 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 tibia through the patellar tendon and then inserted into the bone cavity.

Analyses for Cell Surface Antigens

Spleen cells, peripheral blood mononuclear cells (PBMNCs), and BMCs from the recipient rats were stained with fluorescein isothiocyanate (FITC)-conjugated rat antiRT1^l monoclonal antibody (mAb; PharMingen, San Diego, CA) to identify the donor-derived cells, and purified mouse antiRT1^n mAb (Serotec Ltd., Oxford, United Kingdom) followed by phycoerythrin (PE)-conjugated goat antimouse immunoglobulin G (Serotec Ltd.) to identify the recipient-derived cells. Donor-derived cells bearing a lineage-specific phenotype were also analyzed by FITC-antiRT1^l mAb plus PE-conjugated mAb against CD45R (B220) (PharMingen), CD4, CD8, or CD11b (Caltag Laboratories, Burlingame, CA). The stained cells were analyzed by a FACScan (Becton Dickinson & Co., Mountain View, CA).

Mixed Leukocyte Reactions

MLRs were performed as described previously (*13*). Briefly, the responder spleen cells (2×10^5) were cultured with 2×10^5 irradiated (12 Gy) stimulator spleen cells from F344, BN, and PVG rats for 72 hr and pulsed with 0.5 μCi of [^{3}H]-thymidine for the last 16 hr of the culturing period.

Skin Transplantation

Full-thickness F344 donor or PVG third-party skin grafts of 2×3 cm were prepared and transplanted with 6–0 Prolene on the dorsolateral thorax of BN recipient rats. Grafts were secured with sutures and covered with sterile gauze and an elastic protective tape. The first inspection was carried out on the seventh day, followed by daily inspections for 3 weeks. The graft rejection was defined as the complete loss of viable epidermal graft tissue when more than 50% of the graft became raised, necrotic, or covered by eschar.

Histological Studies

Fresh heart necropsy tissue was fixed in 10% formalin for 48 hr and embedded in paraffin according to standard procedures. Three-micrometer-thick sections were stained with hematoxylin and eosin (H-E), Elastica-van Gieson's (EvG), and Masson's trichrome (MT). Antiα-smooth muscle actin (α-SMA) mAb (DAKO A/S, Denmark) was used for immunohistochemical staining. Furthermore, the frozen specimens were also prepared for fluorescent staining using FITC-conjugated rat antiRT1^l mAb (PharMingen) and purified OX-62 mAb, reactive to rat dendritic cells (DCs) and integrin α_E chain (PharMingen), followed by PE-conjugated goat antimouse immunoglobulin G (Serotec Ltd.) to identify the donor-derived DCs in the recipient thymus by a confocal laser scanning microscope (LSM-GB200; Olympus, Tokyo, Japan). To determine GvHD, the liver, intestine, and skin were also histologically examined after H-E staining.

Statistical Analysis

Statistical analysis of the survival rate of the cardiac allografts was performed using a log-rank test. A P value of <0.05 was considered statistically significant using Student's t test.

RESULTS

Establishment of Hematopoietic Chimerism after IBM-BMT

We first determined not only the number of donor BMCs (for injection) but also the conditioning regimens for

BMT. Recipient rats were given fractionated irradiation (≥4.5 Gy×2) with prior injection of fludarabine, and BMCs (1×10^8) from donor F344 rats were injected intravenously (IV-BMT). In these conditions (1×10^8 BMCs and ≥4.5 Gy×2), macrochimerism (>90%) was established in the recipient rats regardless of IBM-BMT or IV-BMT (Table 1). Therefore, we next carried out BMT with a lower dose of BMCs (3×10^7) and a lower irradiation dose (3.5 Gy×2) to make differences in chimersim clear. PBMNCs were collected from the recipient rats every 2 weeks from the second week until the 24th week after the treatment and stained with donor-specific antirat $RT1^l$ mAb and recipient-specific antirat $RT1^n$ mAb to examine the chimerism.

As shown in Figure 1A, all the recipient rats with fludarabine plus irradiation of either 3.5 Gy×2 or 4.0 Gy×2 showed macrochimerism (>70%) after IBM-BMT. It is noted that approximately 80% of PBMNCs were of donor origin even 180 days after IBM-BMT at the lower irradiation dose (3.5 Gy×2), although the PBMNCs of the recipients treated with fludarabine plus 3.5 Gy×2, followed by IV-BMT, were of host origin (Fig. 1A). This macrochimerism achieved by IBM-BMT was maintained stably even 10 months after transplantation (data not shown). Hematolymphoid cells bearing mature lineage markers were also shown to be of donor origin when the cells in the spleen, PBMNCs, and BM were stained with antibodies against CD45R, CD4, CD8, and CD11b plus donor $RT1^l$ (Table 1 and Supplemental Table 1, available for viewing online only). It is noted that the reconstitution of donor multilineage hematolymphoid cells was similarly observed in the bone marrow of injected and noninjected sites (Supplemental Table 2, available for viewing online only). In contrast, IV-BMT after fludarabine plus 3.5 Gy×2 failed to reconstitute the recipients with donor-derived hematolymphoid cells (Table 1). Only a low level of transient donor chimerism, which lasted only about 1 month, was achieved when the rats were conditioned with fludarabine plus 4.0 Gy×2. Thus, the donor macrochimerism was stably maintained by IBM-BMT, and the low levels of chimerism seen at 2 weeks after IV-BMT (fludarabine plus 4.0 Gy×2) were almost undetectable at 4 weeks. These findings clearly indicate that IBM-BMT facilitated the rapid recovery and reconstitution of donor hematolymphoid cells even with less myelotoxic conditioning regimens (fludarabine plus 3.5 Gy×2) in comparison with conventional IV-BMT.

Survival of Cardiac Allografts

After IBM-BMT, cardiac allografts survived for more than 10 months without any signs of rejection or GvHD in the recipients conditioned with either fludarabine plus 3.5 Gy×2 or fludarabine plus 4.0 Gy×2 (Fig. 1B). In contrast, the cardiac allografts with IV-BMT were rejected within 1 month after BMT in the recipients conditioned with fludarabine plus 3.5 Gy×2 (mean survival time: 22.8±5.4

TABLE 1. Analyses of cell surface antigens on donor-derived cells in the spleen of the recipient rats

Rat	N	BMT	Radiation (Gy)	BMCs ($\times10^7$)	Cell surface antigen (%) Donor-derived CD4[a]	Donor-derived CD8	Donor-derived CD45R	Donor-derived CD11b	Donor-derived cells in chimeric rats (%)
[F344→BN]	5	IV	3.5Gy×2	3	0	0	0	0	0
					9.65±0.60	10.34±0.60	68.34±0.67	10.49±1.23	
[F344→BN]	5	IBM	3.5Gy×2	3	6.45±0.56	15.38±1.21	44.87±1.34	11.54±1.85	73.26±8.54
					10.34±0.25	21.29±0.35	62.78±0.64	16.32±0.76	
[F344→BN]	10	IV	4.0Gy×2	3	0	0.02±0.01	0.07±0.02	0	0.12±0.04
					7.67±0.58	16.66±1.25	62.04±015	8.24±0.47	
[F344→BN]	10	IBM	4.0Gy×2	3	12.21±1.42	12.46±2.43	38.25±1.81	6.88±0.88	81.36±8.86
					14.38±2.05	16.04±0.37	54.45±0.62	11.19±0.54	
[F344→BN]	10	IV	4.5Gy×2	10	10.02±1.25	17.68±2.54	56.56±2.47	9.47±1.57	94.26±7.83
					11.32±0.57	19.54±1.25	66.14±0.61	9.83±1.56	
[F344→BN]	10	IBM	4.5Gy×2	10	11.35±2.01	9.98±1.48	67.81±1.56	12.76±2.04	98.34±2.16
					12.54±0.41	10.82±0.63	68.75±0.83	14.87±0.29	
[F344→BN]	10	IV	5.0Gy×2	10	15.22±2.05	19.84±1.34	52.14±2.06	10.01±1.07	96.27±1.80
					16.11±2.57	22.14±1.72	56.13±1.05	10.04±0.21	
[F344→BN]	10	IBM	5.0Gy×2	10	23.65±1.02	11.22±1.84	52.23±2.63	8.39±0.96	99.26±0.45
					24.21±1.12	11.04±0.56	53.74±0.32	8.73±0.57	
F344	5	–	–	–	–	–	–	–	–
					21.29±0.58	26.13±0.54	59.17±0.71	9.13±0.32	
BN	5	–	–	–	–	–	–	–	–
					26.13±0.60	17.63±0.41	49.29±0.16	10.81±0.27	

The cells from the spleen were prepared from the recipient rats 12 weeks after IBM-BMT or IV-BMT, and stained with donor-specific antirat $RT1^l$ mAb and mAb against mature lineage markers (CD4, CD8, CD45R, and CD11b) to examine the reconstitution of the hematolymphoid system. The cells were analyzed using a FACScan. The results are expressed as the mean±SD of more than five rats.

[a]Donor- and recipient-derived cells.

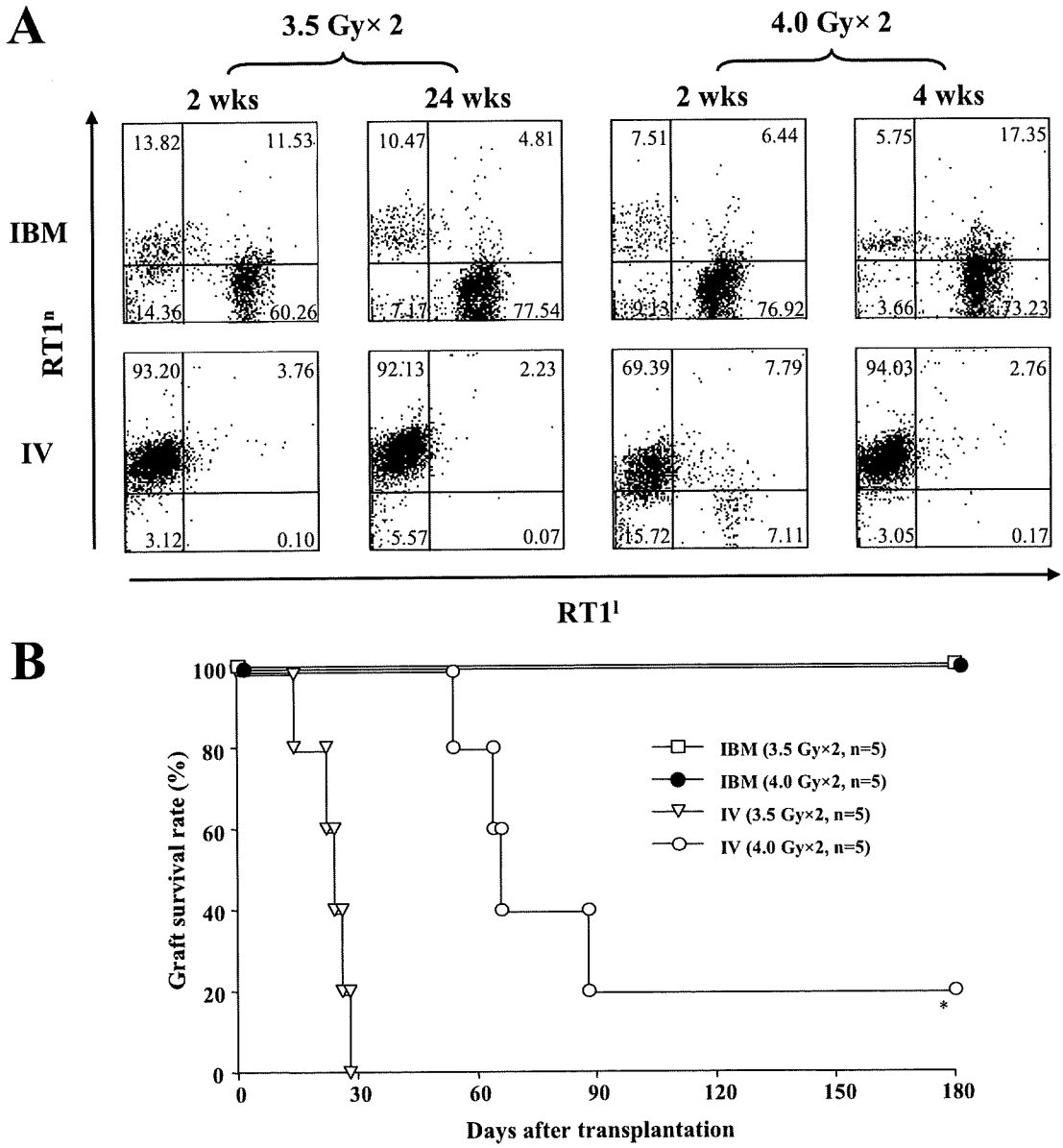

FIGURE 1. Analyses for donor-derived cells in the PBMNCs of recipients after IBM-BMT or IV-BMT. (A) PBMNCs were obtained from the recipients every 2 weeks from the 2nd to 24th week after IBM-BMT or IV-BMT plus heart transplantation, then stained with donor-specific antirat $RT1^l$ mAb (X-axis) and recipient-specific antirat $RT1^n$ mAb (Y-axis) to examine the donor cell engraftment. The stained cells were analyzed using a FACScan. FACS profiles represent representative data of five rats. Quadrants in the figures were set by the staining profile of the cells treated with isotype-matched Ig controls. Note that macrochimerism (>70%) was maintained stably after 180 days by IBM-BMT, but the transient chimerism induced by IV-BMT (fludarabine plus 4.0 Gy×2) was almost undetectable at 4 weeks. (B) Survival of cardiac allografts after IBM-BMT or IV-BMT. BN rats were injected intravenously with 50 mg/kg of fludarabine phosphate, followed by fractionated irradiation (3.5 Gy×2 or 4.0 Gy×2) 1 day before the bone marrow and heart transplantation (day −1). BMCs (3×10^7 or 10×10^7 cells/60 μL) from donor F344 rats were injected intravenously (IV-BMT) or directly into the bone marrow cavity (IBM-BMT) of the left tibia of the recipient BN rat on day 0. Vascularized heart transplantation was performed heterotopically into the right cervical portion of recipients using a microsurgical cuff technique. Allograft survival was assessed by daily palpation. Graft rejection was defined as complete cessation of spontaneous ventricular contraction.*The cardiac allograft of 1 of five rats treated with IV-BMT (4.0 Gy×2) was removed for immunohistochemical examination on day 180.

days, n=5) or within 3 months after BMT in those with fludarabine plus as much as 4.0 Gy×2 (mean survival time: 68±14.3 days, n=4) except in one instance.

Histology of Cardiac Allografts

All cardiac allografts were sectioned and stained with H-E, EvG, MT, and α-SMA mAb at the time of rejection or at

180 days after the transplantation. As already described, the cardiac allografts after IV-BMT were rejected within 1 month in the recipients conditioned with fludarabine plus 3.5 Gy×2. This was clearly confirmed by the histological findings of severe lymphocyte infiltration and extensive myocyte damage (Fig. 2D), showing evidence of acute rejection. Furthermore, despite the transient mixed chimerism induced by IV-BMT (4.0 Gy×2), severely thickened intima (cardiac allograft vasculopathy: CAV) was observed in the intramyocardial and epicardial arteries of the rejected allografts, and this was further confirmed by α-SMA staining and EvG staining (Fig. 2E). In contrast, in the allografts of the recipients conditioned with fludarabine plus 3.5 Gy×2 or 4.0 Gy×2 and treated with IBM-BMT, the arterial intima was maintained almost intact without any appearance of CAV (Fig. 2B, C).

MT staining was also performed to assess the cardiac remodeling of the allografts with respect to interstitial fibrosis during the chronic phase. While the allografts with IV-BMT (3.5 Gy×2, or 4.0 Gy×2) showed moderate to severe fibrosis in both the epicardium and intramyocardium (Fig. 2D, E and Fig. 3C, D), the prevalence of interstitial fibrosis in the allografts with IBM-BMT (3.5 Gy×2, or 4.0 Gy×2) was significantly lower than that in the allografts with IV-BMT (Fig. 2B, C and Fig. 3A, B). In addition, the coronary vessels in the allografts with IBM-BMT developed minimal myointimal thickening, compared with moderate to severe thickening with IV-BMT. Furthermore, in-

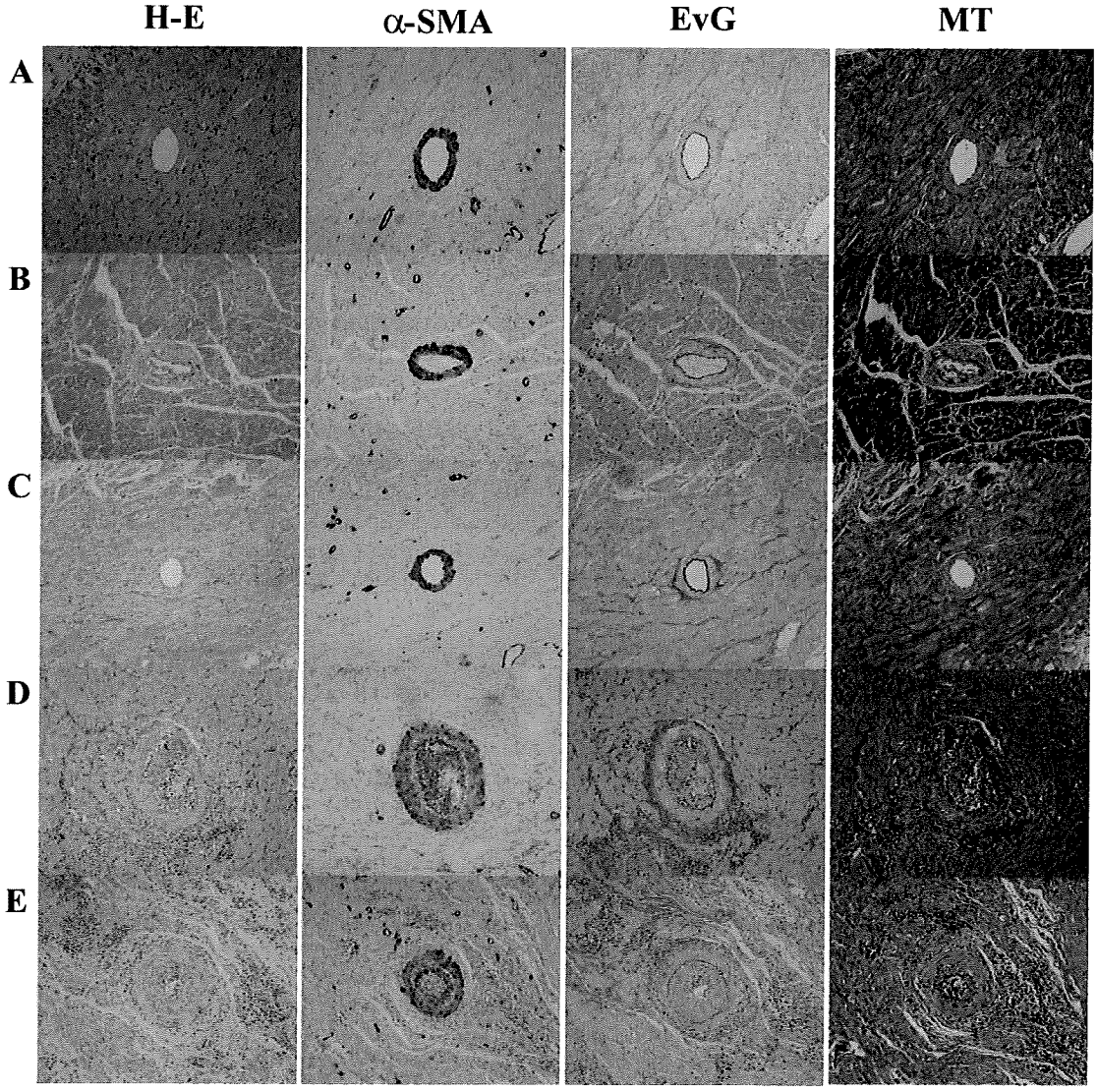

FIGURE 2. Histological findings in cardiac allografts after IBM-BMT or IV-BMT. The cardiac allografts were histologically examined at the time of rejection or 180 days after the treatment. H-E, α-SMA, EvG, or MT staining (×100) was performed to determine the rejection or the severity of CAV. (A) Intact isograft after syngeneic heart transplantation. (B) Allograft with minimal intimal thickening and sparse interstitial fibrosis at 180 days after IBM-BMT (fludarabine plus 3.5 Gy×2). (C) Allograft with well-preserved intact intima and mild interstitial fibrosis at 180 days after IBM-BMT (fludarabine plus 4.0 Gy×2). (D) Rejected allograft with severe lymphocytic infiltration and severe interstitial fibrosis at 28 days after IV-BMT (fludarabine plus 3.5 Gy×2). (E) Allograft with severe CAV and remarkable proliferation of elastic and collagenic fibers at 86 days after IV-BMT (fludarabine plus 4.0 Gy×2).

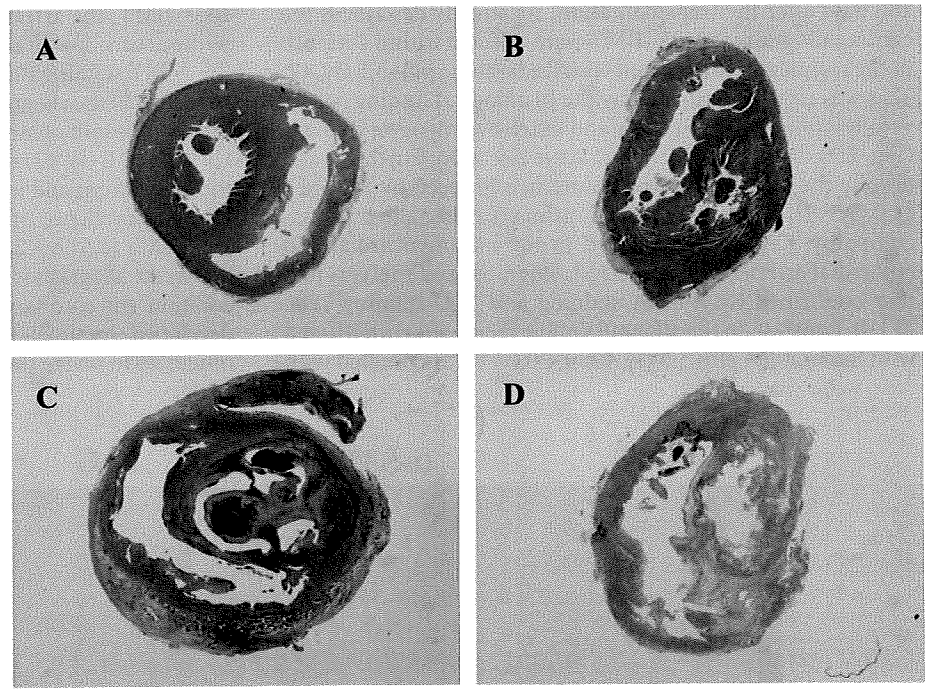

FIGURE 3. Macroscopic appearance in cardiac allografts after IBM-BMT or IV-BMT. The cardiac allografts from IBM-BMT (A, B) and IV-BMT groups (C, D) were stained with MT and coronal sections examined (×5) at the time of rejection. Allografts examined after IBM-BMT and irradiation with 3.5 Gy×2 (A) or 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

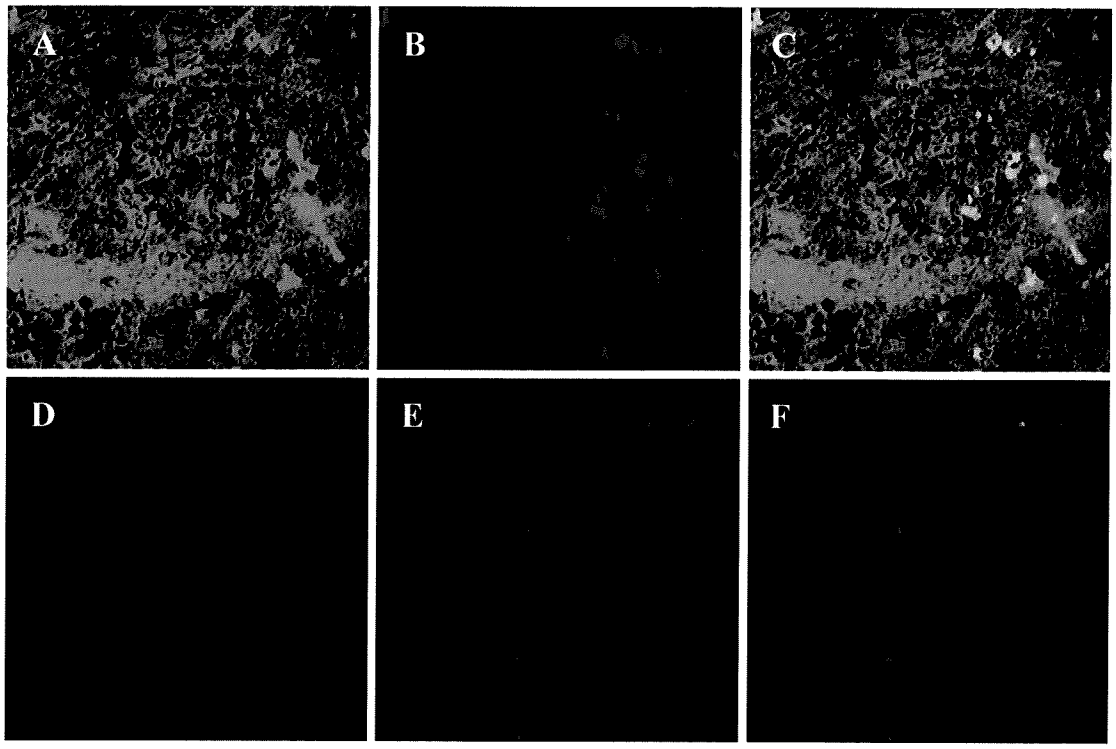

FIGURE 4. Presence of donor-derived DCs in the thymus of the rats treated with IBM-BMT or IV-BMT. Thymic sections were double-stained with FITC-antiRT1[1] mAb (A and D, cells colored green) and purified OX-62 mAb followed by PE-conjugated goat antimouse immunoglobulin G (B and E, cells colored red) for 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).

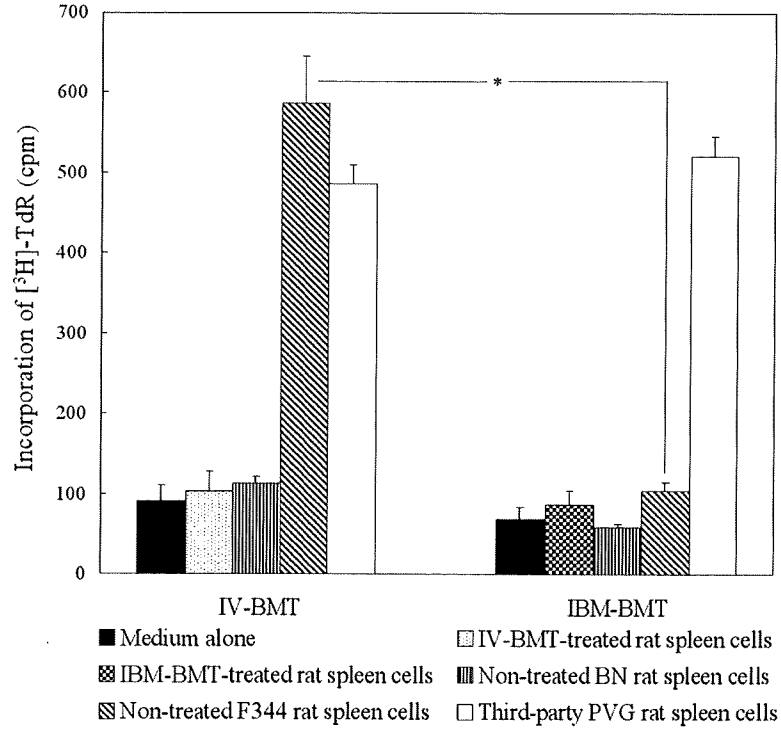

FIGURE 5. MLRs in recipient rats treated with heart transplantation plus IBM-BMT or IV-BMT. MLRs were performed 180 days after the treatment. The recipient rats treated with IBM-BMT or IV-BMT were assessed using untreated BN, F344 and PVG rats (the third party). Responder T cells (2×10^5) from the rat 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 (≥4.5 Gy×2). However, using lower doses of BMCs (3×10^7) under less myelotoxic conditioning regimens (≤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.

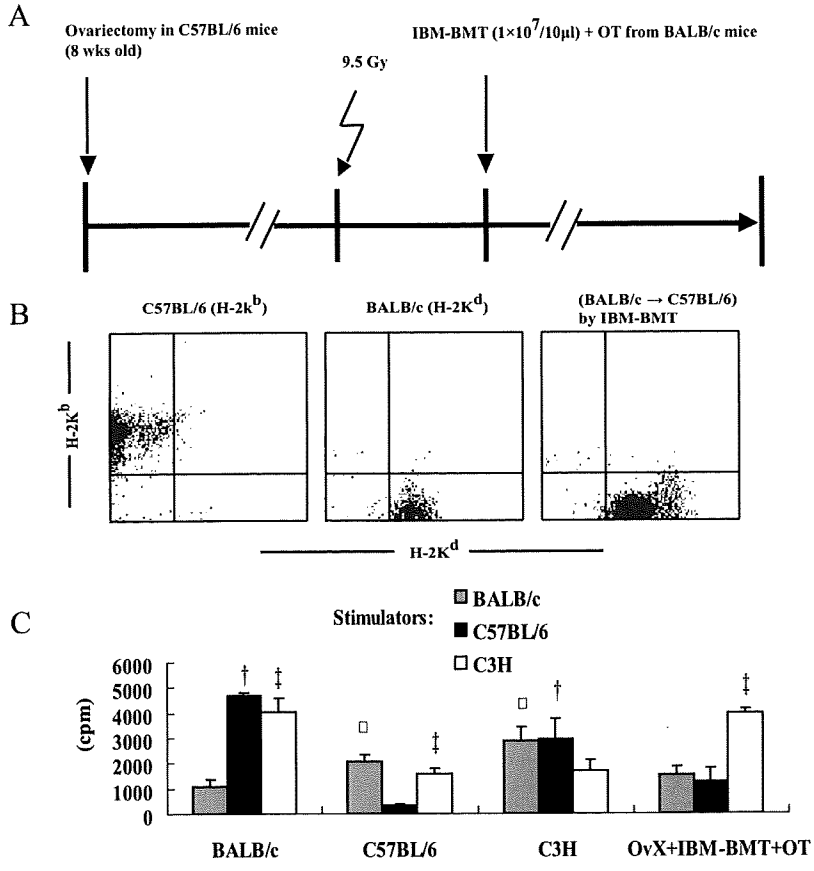

FIGURE 1. OvX mice treated with IBM-BMT. B6 mice at the age of 8 weeks were OvX. After 3 months, the mice were irradiated with a lethal dose (9.5 Gy), and BMCs from normal BALB/c mice were injected directly into the bone marrow cavity (IBM-BMT) of the left tibia (A). Three months after IBM-BMT and OT, 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. $^{\square}P<0.01$ vs. BALB/c; $^{\dagger}P<0.01$ vs. C57BL/6; $^{\ddagger}P<0.01$ vs. C3H.