レジストリーデータの統計解析・活用のためのデータ整備

研究分担者　熱田 由子　名古屋大学大学院医学系研究科
造血細胞移植情報管理・生物統計学寄附講座 講師

A. 研究目的

本研究班では、非介入の臨床研究として造血細胞移植登録一元化データベースを用いた後方視的解析が重要な役割を果たす。造血細胞移植登録一元化データベースとは、日本造血細胞移植学会、日本小児血液学会、骨髄移植推進財団（骨髄バンク）、日本さい帯血バンクネットワークが協力して造血細胞移植登録の一元化・電子化を2006年より行っている造血細胞移植登録一元管理プログラム(TRUMP)データベースをﾌﾞす。2005年までに上記各組織が別々に紙調査票で収集した移植情報は2011年までにTRUMPに統合された。このデータベースは、データ収集を目的としたデータ構造であり、解析を目的としたデータ構造への変換には多くの手間を要する。本研究班で実施される後方視的解析の効率と質を向上することを目的とした。また、本研究班では、前向き臨床試験が重要な位置づけを担っているが、ここにおけるデータ管理でのTRUMPデータの利用に関しても検討した。

B. 方法

解析を目的としたデータ構造への変換のために解析に用いる基本項目を定めそのデータ構造を作成した。これに基づいた変数作成を実施した。血縁者間造血幹細胞移植のHLAデータは、入力不備データの確認および修正を昨年度に引き続き実施し、HLA座の入力不備の修正、非血縁者間移植においては各臍帯血バンクなどから提供されたHLA情報の反映、およびHLA一致度判定プログラムを作成した。

TRUMPは、日本造血細胞移植学会への年次報告に用いるプログラムであり、その場合は日本造血細胞移植学会データセンターでのみ解読が可能な暗号化を行ったデータセットが提出される。TRUMPの機能として、施設内での利用のために、汎用形式でのデータの出力が可能であり、1例での書き出しも出来る。臨床試験を実施における困難な点として、参加施設の報告書記入などの負担が挙げられるが、TRUMP dataを併用することにより負担を軽減する方法の検討を実施した。さらに、効率的な臨床試験実施体制に関しても検討を実施した。

C. 結果

解析データセット構造および､HLAを含み他変数の入力不備の修正も行った上での変数作成スクリプトを、今年度データセット用に変更し、日本造血細胞移植学会ホームページで公開した。本スクリプトでは、血縁者間造血幹細胞移植のHLAデータは、HLA座の入力不備の修正、非血縁者間移植においては各臍帯血バンクなどから提供されたHLA情報の反映、およびHLA一致度判定まで一度に実施するスクリプトである。

D　考察

造血細胞移植登録一元化データベースは、他の観察研究データベースと同様、継続的な新規症例の登録および既登録症例の生存・疾患状況・晩期合併症情報の更新が必要であり、常に変化し続けているデータベースである。さらに、調査項目も研究の重要あるいは定義の変化などに応じて変更し続けて行く必要がある。こういったliving databaseにおける質の管理および質の高い研究が行えるための統計解析におけるサポートは一度行えば事足りるものではなく、継続的に集中して取り組まなければならない。同時に、施設負担を減らし合理的に研究を行えるよう、臨床試験においてTRUMPの利用を増やせる工夫が今後必要である。

E. 結論

学会データベースを用いて本研究班で検討したい後方視的研究を実施するためのデータベース基盤整備を実施した。

バイオ人工細胞・臓器の開発による糖尿病その他の疾患の治療

バイオ人工細胞・臓器の開発による糖尿病その他の疾患の治療に関する研究

主任研究員　宮川周士　大阪大学大学院医学系研究科　小児成育外科　准教授

分担研究者　長島比呂志　明治大学農学部生命科学科　教授
岡部　勝　大阪大学遺伝情報実験センター　教授

研究協力者　大阪大学大学院医学系研究科　小児成育外科
上野豪久（助教）王　丹丹（院生）河村拓史（院生）前田　晃（研究員）
明治大学農学部生命科学科
渡邊 將人（研究員） 梅山一大（研究員） 松成ひとみ（研究員）、
中野和明（大学院生）
大阪大学遺伝情報実験センター
伊川正人（准教授）蓮輪英毅（助教）

A. 研究目的

目的は、医療用バイオ人工細胞・臓器の開発である。主眼をバイオ人工膵島とし、その細胞供給用の遺伝子改変ブタの作出をめざす。現有する糖転移酵素 GnT-III と補体制御因子 DAF(CD55)を遺伝子導入、かつ異種抗原α-Gal を knockout(KO)したブタに、他の補体制御因子、抗凝固因子、細胞性免疫制御分子やレトロウイルス制御用遺伝子を導入し、臨床応用可能な膵島細胞の供給源となるブタを作出する。将来的に糖尿病患者へのこのバイオ人工膵島を足がかりに、劇症肝炎治療へバイオ人工肝臓、透析患者にバイオ人工腎臓の供給を目指す。

現況としては、移植用臓器の開発は、我々が異種移植の超急性拒絶反応が宿主の補体と移植片の補体制御因子の種差に起因する反応である事を見いだし報告した、それに伴い1990年頃より世界的にベンチャー産業と結び付きヒトの遺伝子を導入（transgenic TG）、あるいはブタの遺伝子をつぶした(knockout KO)遺伝子改変ブタの開発競争が始まった。ハーバード大学はCD47ブタを開発。Mayo ClinicではGal-KO+MCP(CD46)-TGブタを作製。ピッツバーク大では既にGal-KO/hCD46/TFPI/CTLA4-Igブタを作成。欧州”XENOME”プロジェクトはGal-KO/CD55/CD59/CD39/HTブタを報告している。ハノーバー大でも、Gal-KO、DAF、TM、HO-1、ブタ内在性レトロウイルス(PERV)のKD、CTLA4-IgとA20のブタも作成。豪のメルボルン大では、Gal-KOをベースにCD55/CD59/CTLA4-Igを導入。韓国では、ソウル大が４年後の臨床実施に向けて、Gal-KO、HO-1、sTNFR-Igブタ、又 M-gen社が、DAF、HLA-Eブタを報告。台北大学でもDAFブタとHO-1ブタを開発している。一方、WHOはホームページに、35件の臨床報告を掲載している。特に注目すべきは、４年前よりニュージーランドでは免疫隔離膜下の膵島移植の臨床が始まっている（LCT社)。ロシアでもアルゼンチンでもこれに続いている。これに対し、我々は、H8年度よりトランジェニックブタ（DAF+糖転移酵素GnT-III）の作成に取りかかり、この線維芽細胞を使ってα-Gal抗原のKOに成功し2006年末ホモが産まれた。我々は既に何種類かの特許を所有し、この分野での極めて独自の異種移植用ブタを開発する事を目指している。

前年度には遺伝子導入ブタが得られなかったので、新たに遺伝子コンストラクトの構築と精製を行い、新たに遺伝子改変ブタを作成することを目的とした。さらに、既存のα-GalKOブタをベースに、より体細胞クローニングに適した細胞の樹立も目的とする。

B. 方法

1. 遺伝子の選択。現在世界で遺伝子改変ブタ作製に関係する分子は、

* 補体制御因子-----C1-INH, MCP(CD46), DAF(CD55), CD59
* 糖転移酵素----GnT-III, α-1,2FT, Endo-β-galC
* 凝固系（抗凝固因子）---TFPI、Thrombomodulin(TM)、CD39
* NK 細胞制御--HLA-G, HLA-E,
* Macrophage 制御--CD47
* T 細胞制御--CTLA4-Ig, FasL, TRAIL, CIITA

＊ 保存-----Hemoxygenase-1,
＊ PERV の KD
＊ Hanganutziu-Deicher (H-D) 抗原の遺伝子(CMAH) の KD 、等である。
今回の project では、下線を引いた分子を発現したブタの作出を目指す。

2. 遺伝子構築。
一般的に現在使われている promoter は、CMV や RSV のウイルス promoter、Chick β actin(pCAGGS)、human EF-1α、humanmouse H2k、あるいは rat insulin II or pig Insulin promoter である。加えて、導入 gene 本来の promoter である。膵島での遺伝子発現は、一般的に imsulin promoter が確実と思われるが、他の臓器での発現が望めない欠点が有る。一方、pCAGGS はユビキタスに発現するが、一部の報告では膵島での発現が弱いとされている。今回は CTDM を insuline promoter で、HLA-E を pCAGGS で発現させることにした。 諸外国のブタ作出方法はヒトの遺伝子=cDNA や genome を 1 つ 1 つ導入し、高発現の系統を樹立し、交配により重ね合わせる方法である。また、最近は IRES に換え 2A システムを用い、2-3 の分子を繋いで一度に発現させる方法も一部で始められている。
我々は(1). 高発現を得るのに、cDNA の codon を改変しブタで至適なものとする方法を取る。Codon 変換に関しては、各分子のアミノ酸配列を崩さず、ブタで最も頻度の高い t-RNA に合わせた DNA 配列に組み替える方法である。これまでに DAF を codon 変換し in vitro, in vivo (マウス) での強発現を確認している。
(2) . 各分子の機能ドメインを、同分子、別の分子間で繋いだ多重合分子 (hybrid) を作製し、この人工 cDNA をブタに遺伝子導入する方法をとる。
＊ CTDM: Thrombomoduline 部分に関しては, 直接抗凝固機能に関与する EGF4-6 と EGF3 の一部を選んだが、Thrombin の結合には EGF3 の部分のさらに 12 個のアミノ酸の必要と判断し、これを加えた。＜NCTDM＞
＊ HLA-Ev(147)(147 番目の S を C に変更する事により高発現が見込まれる)に 2A で human β2m を繋ぎ合わせた。＜HLA-Ev＞
＊ PERV の KD も試みる。既に H-1 promoter に pol 部分の siRNA を組み込んだが、又、U6 promoter も用意し、他の部分の siRNA を組み込んだ。

3. In vitro での確認
ブタの血管内皮細胞 (PEC) 及び繊維芽細胞で検定する。導入方法は, lipid 法 (リポフェクトアミン、等)、あるいは電気ショック法を用いた。発現の確認には FACS を用いた。

4. Transgenic マウス作り
昨年作った 2 種類のコンストラクト＜CTDM＞＜HLA-E*＞を、通常のマイクロインジェクションにより BDF1xBDF 1 にそれぞれ 150 個の胚を移植した。生後 8 週令のマウスの各臓器での発現を RT-PCR で検討した。

5. Transgenic ブタ作り
2 種類のコンストラクト<NCTDM>と<HLA-Ev>を、Intracytoplasmic sperm injection-mediated gene transfer (ICSI) 法によりブタ体外成熟卵へ注入した。精子との共培養に用いる DNA 濃度を 1.25ng/μl および 2.5ng/μl とし、顕微授精胚の正常分割率、胚盤胞形成率への影響を両区で比較した。得られた胚盤胞 (発生培養 7 日目) の PCR 解析により、遺伝子導入の有無 (一過性導入も含む) を調べた。以上の実験により、導入に用いる DNA の適性濃度を決定し、顕微授精胚のレシピエントブタへ移植を行った。
さらに既存の α-Gal KO ブタを野生型ブタと交配するなどして、近交化や発生に影響のある変異の進んだ系統への新たな血液の導入を図った。

C. 結果
1. 新規に作製した遺伝子構築。

* NCTDM < 補体制御 + α >
C1-INH - Thrombomodulin - DAF - MCP <NCTDM> ---pCAGGS/CTDM 及び pCPI(pig insulin promoter + CMV enhancer)/NCTDM----昨年の CTDM を改良した
* NK 細胞制御 pCAGGS/HLA-Ev(147)-2A-hβ2m <N-HLA-E>-- pCPI/N-HLA-E
* PERV の制御 PERV の KD------U6/siRNA-PERV(pol)

2. 細胞での発現確認
これらの遺伝子をブタの血管内皮細胞に導入し、FACS で発現を確認した。

3. マウスでの発現
pCPI/CTDMA および pCX/HLA-E をマウスに TG し、それぞれ 4 匹、2 匹の line を得た。内、2 匹, 1 匹の発現を生後 8 週令で RT-PCR で解析した（膵臓での発現を 1 とした)。
*CTDM#1 : 脳 (5.41)、心 (0.19)、肺 (1.17)、胸腺 (0.30)、肝 (0.06)、腎 (0.13)、腸 (0.39)、脾(1.61)、膵 (1) <n=2>
*CTDM#2 : 心 (2.45)、肺 (7.29)、胸腺 (9.25)、肝 (0.03)、腎 (0.71)、脾(14.25)、膵 (1)
*HLA-E : 脳 (2.75)、心 (70.71)、肺 (15.48)、胸腺 (0.17)、肝 (0.36)、腎 (1.28)、腸 (0.30)、脾(2.81)、筋 (12.86)、膵 (1)。であった。<n=3>
両方の promoter で膵臓での発現を確かめたが、pCPI の方が相対的に高発現と考えられた。

4. ブタでの発現
NCTDM および HLA-Ev 遺伝子両者ともに、1 25ng/μl 区では 2.5ng/μl 区に比してより高い胚盤胞形成率が得られる傾向であった (NCTDM : 47.8% [11/23] vs 24.0% [6/25], HLA-Ev : 34.8% [8/23] vs 22.7% [5/22])。一方、胚盤胞の遺伝子導入効率については、両区に差は見られなかった (75.0-100%)。
胚移植試験には、発生率が高い傾向であった 1 25ng/μl の DNA 濃度を採用した。NCTDM 遺伝子および HLA-Ev 遺伝子を導入した顕微授精胚、それぞれ 69 個および 79 個を 2 頭のレシピエントブタに移植した（さらに 2 頭に移植予定)。
α-Gal KO ブタ（雌）と野生型ブタとの交配によって得られた heterozygous KO 産仔を、他の α-Gal KO ブタ精子により受胎させて胎仔を回収し、新たに 9 ラインの α-Gal KO ブタ細胞を樹立した。

D. 考察
顕微授精法の応用により、HLA-E*および CTDM 遺伝子を導入したブタの作出は、十分可能であると考えられたが、今回 CTDM 遺伝子構築を再建し、再精製し、発生阻害性を検討した。
既存の α-Gal KO 細胞は、KO ブタの作成過程における近交化の影響やエピジェネティック変異の影響を受け、そのことが作出された体細胞クローン個体の正常性に影響していたと考えられる。既存の α-Gal KO ブタに新たな血液を導入したことで、発生異常を生じない新たな核ドナー細胞が樹立された可能性は高い。新たに作成・精製した遺伝子コンストラクトには顕著な発生阻害性が見られなかったので、遺伝子改変個体が得られる可能性は高く、表現形が確認されれば、新たに樹立した細胞への遺伝子導入に移ることができる。
また、個々の hybrid 遺伝子のブタ個体での発現を確認した後、これらを繋ぎ合わせた比較的長い構築を作製し、Gal-KO ブタからの fibroblast に in vitro で遺伝子導入し、高発現の line からの核移植により、遺伝子改変ブタを作製する方法を考えている。

E. 結論
プロジェクトの 2 年目であるが、昨年は流産のため、ブタ個体での導入遺伝子の発現に関する評価はできていない。マウスでの評価に戻すとともに、遺伝子に多少の変化を加えた。現時点で, 2 つの遺伝子の構築が終わり、それぞれ ICSI 法でブタに遺伝子導入し、ブタでの発現を検討中であるが、遺伝子導入した個体はまだ得られていない。表現形の解析には分娩を待たなければならない。

遺伝子構築及びマウス個体での発現に関する研究

分担研究者　岡部　勝　大阪大学遺伝情報実験センター教授
研究協力者　大阪大学遺伝情報実験センター
伊川正人（准教授）、蓮輪英毅（助教）

A. 研究目的

我々のグループは、既に糖転移酵素GnT-IIIとCD55のトランスジェニック（TG）ブタを作出している。今回は23年度に既出の遺伝子構築でのTGブタの重なる流産の原因を考えるため、マウスでの発現を検討した。

B. 方法

1.昨年度作出の遺伝子構築。

＊ CTDM＜補体制御 + 抗凝固因子＞

pCPI(pig insulin promoter + CMV enhancer)/CTDM C1-INH - Thrombomodulin - DAF - MCP <CTDM>

＊ NK細胞制御 pCAGGS/HLA-Ev(147)-IRES-hβ2m ＜ HLA-E* ＞

2.Transgenic マウス作り

昨年作出した2種類のコンストラクト＜CTDM＞＜HLA-E*＞を、通常のマイクロインジェクションによりBDF1xBDF1にそれぞれ150個の胚を移植した。生後８週令のマウスの各臓器での発現をRT-PCRで検討した。

C. 結果

８週令マウスでの発現

pCPI/CTDMAおよびpCX/HLA-Eをマウスに TG し、最終的にそれぞれ4匹、2匹の lineを得た。内、2匹,1匹の発現をRT-PCRで解析した（膵臓での発現を1とした）。

*CTDM#1．脳（5.41）、心（0 19）、肺（1.17）、胸腺（0.30）、肝（0.06）、腎（0.13）、腸（0.39）、脾(1.61)、膵（1）＜n＝2＞

*CTDM#2・心（2.45）、肺（7 29）、胸腺（9.25）、肝（0.03）、腎（0.71）、脾(14.25)、膵（1）

*HLA-E．脳（2.75）、心（70.71）、肺（15.48）、胸腺（0.17）、肝（0.36）、腎（1.28）、腸（0.30）、脾(2.81)、筋（12.86）、膵（1）。であった。＜n＝3＞

両方のpromoterで膵臓での発現を確かめたが、pCPIの方が相対的に高発現と考えられた。

D. 考察

HLA-E*およびCTDM遺伝子を導入したブタの作出は、流産したためin vivoでの発現に問題が有ると考えられたが、CTDM、HLA-E遺伝子コンストラクト自体は，in vivo（マウス）発現に問題は無いようであった。

E. 結論

両方のコンストラクトとも、in vivoで胎仔を得ることが可能であると考えられた。

遺伝子改変ブタの作出に関する研究

分担研究者　長嶋比呂志　明治大学農学部生命科学科教授
研究協力者　明治大学農学部生命科学科
渡邊 將人、梅山一大、松成ひとみ（以上研究員）、中野和明（大学院生）

A. 研究目的

前年度には遺伝子導入ブタが得られなかったので、新たに遺伝子コンストラクトの構築と精製を行い、超急性拒絶だけでなく遅延性拒絶にも対応し得る遺伝子改変ブタを作成することを目的とした。さらに、既存のα-Gal knockout(KO)ブタをベースに、より体細胞クローニングに適した細胞の樹立も目的とした。

B. 方法

2 種の遺伝子コンストラクト Pig insulin promoter+C1-INH+Thrombomodulin+DAF+MCPcyt(-) <NCTDM>とpCX/HLA-Ev(147)+2AP+human beta-2 microtubulin <HLA-Ev>を、Intracytoplasmic sperm injection-mediated gene transfer 法によりブタ体外成熟卵へ注入した。精子との共培養に用いる DNA 濃度を 1.25ng/μl および 2.5ng/μl とし、顕微授精胚の正常分割率、胚盤胞形成率への影響を両区で比較した。得られた胚盤胞（発生培養 7 日目）の PCR 解析により、遺伝子導入の有無（一過性導入も含む）を調べた。以上の実験により、導入に用いる DNA の適性濃度を決定し、顕微授精胚のレシピエントブタへ移植を行った。

さらに既存のα-Gal KO ブタを野生型ブタと交配するなどして、近交化や発生に影響のある変異の進んだ系統への新たな血液の導入を図った。

C. 結果

NCTDM および HLA-Ev 遺伝子両者ともに、1.25ng/μl 区では 2.5ng/μl 区に比してより高い胚盤胞形成率が得られる傾向であった（NCTDM：47.8% [11/23] vs 24.0% [6/25], HLA-Ev ・ 34.8% [8/23] vs 22.7% [5/22])。一方、胚盤胞の遺伝子導入効率については、両区に差は見られなかった（75.0-100%)。

胚移植試験には、発生率が高い傾向であった 1.25ng/μl の DNA 濃度を採用した。NCTDM 遺伝子および HLA-Ev 遺伝子を導入した顕微授精胚、それぞれ 69 個および 79 個を 2 頭のレシピエントブタに移植した（さらに 2 頭に移植予定)。

α-Gal KO ブタ（雌）と野生型ブタとの交配によって得られた heterozygous KO 産仔を、他のα-Gal KO ブタ精子により受胎させて胎仔を回収し、新たに 9 ラインのα-Gal KO ブタ細胞を樹立した。

D. 考察

既存のα-Gal KO 細胞は、KO ブタの作成過程における近交化の影響やエピジェネティック変異の影響を受け、そのことが作出された体細胞クローン個体の正常性に影響していたと考えられる。既存のα-Gal KO ブタに新たな血液を導入したことで、発生異常を生じない新たな核ドナー細胞が樹立された可能性は高い。新たに作成・精製した遺伝子コンストラクトには顕著な発生阻害性が見られなかったので、遺伝子改変個体が得られる可能性は高く、表現形が確認されれば、新たに樹立した細胞への遺伝子導入に移ることができる。

E. 結論

遺伝子導入した個体はまだ得られていないので、表現形の解析には分娩を待たなければならない。

遺伝子構築に関する研究

主任研究員　宮川周士　大阪大学大学院医学系研究科　小児成育外科　准教授

研究協力者　　上野豪久（助教）王　丹丹（院生）河村拓史（院生）前田　晃（研究員）

A. 研究目的

目的は、医療用バイオ人工細胞・臓器の開発である。主眼をバイオ人工膵島とし、その細胞供給用の遺伝子改変ブタの作出をめざす。

B. 方法

1. 遺伝子の選択。遺伝子改変ブタ作製に関係する分子は、補体制御因子 C1-INH, MCP(CD46), DAF(CD55)、凝固系（抗凝固因子）Thrombomodulin(TM)、NK 細胞制御 HLA-E、内在性ブタレトロウイルス（PERV）の KD である。

2. 遺伝子構築。(1)我々は高発現を得るのに、cDNA の codon を改変しブタで至適なものとする方法を取っている。また、(2)各分子の機能ドメインを、繋いだ多重合分子（hybrid）を作製し、この人工 cDNA をブタに遺伝子導入する方法をとる。

* <NCTDM> 昨年作成した CTDM（insuline promoter）の Thrombomodulin 部分を改変した。Thrombomoduline 部分に関しては，直接抗凝固機能に関与する EGF4-6 と EGF3 の一部を選んだが、Thrombin の結合には EGF3 の部分のさらに 12 個のアミノ酸の必要と判断し、これを加えた。Enhancer には CMV の enhancer を使用した。
* <HLA-Ev> HLA-Ev(147)(147 番目の S を C に変更する事により高発現が見込まれる)に 2A システムを使って human β2m を繋ぎ合わせ、pCAGGS にいれた。
* 新しい U6 promoter を使って、PERV の KD 用のベクターを作出した。

3. In vitro での確認。上記 TG 用遺伝子をブタの血管内皮細胞（PEC）で検定した。

C. 結果

1. 新規に作製した遺伝子構築。

* NCTDM < 補体制御 + α >
C1-INH - Thrombomodulin - DAF - MCP <NCTDM> ---pCAGGS/CTDM 及び pCPI(pig insulin promoter + CMV enhancer)/NCTDM----昨年の CTDM を改良した
* NK 細胞制御　pCAGGS/HLA-Ev(147)-2A-hβ2m <N-HLA-E>-- pCPI/N-HLA-E
* PERV の制御　　PERV の KD------U6/siRNA-PERV(pol)

2. 細胞での発現確認

これらの遺伝子をブタの血管内皮細胞に導入し、FACS で発現を確認した。

D. 考察

遺伝子構築に関しては，in vitro での発現を確認するのは勿論であるが，動物個体（in vivo）及び目的臓器（膵臓）での発現が重要と考えられる。今回マウスでの発現は、岡部チームにより確認できた。　最終的には、個々の hybrid 遺伝子の in vivo での発現を確認した後、これらを繋ぎ合わせた比較的長い構築を作製し、Gal-KO ブタからの fibroblast に in vitro で遺伝子導入し、高発現の line からの核移植により、遺伝子改変ブタを作製する方法を考えている。

E. 結論

プロジェクトの一年目は ICSI 法でブタに遺伝子導入したが流産した。その為、マウスで TG を作成し in vivo での発現を確認した。現時点で、二つの遺伝子の再構築が終わり、ICSI 法でブタに遺伝子導入し発現を検討中である。

VII.　研究成果の刊行物・印刷

Best Practice & Research Clinical Haematology 24 (2011) 477–483

Contents lists available at ScienceDirect

Best Practice & Research Clinical Haematology

journal homepage: www.elsevier.com/locate/beha

17

A novel BMT technique for treatment of various currently intractable diseases

Susumu Ikehara, Professor *

Department of Stem Cell Disorders, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka 570 8506, Japan

Keywords:
bone marrow transplantation (BMT)
stem cell disorder (SCD)
hemopoietic stem cell (HSC)
mesenchymal stem cell (MSC)
aspiration method (AM)
perfusion method (PM)
intra-bone marrow (IBM)
autoimmune disease
osteoporosis
emphysema

A recently-developed BMT method combines a "Perfusion Method" (PM) for collecting bone marrow cells (BMCs) with the Intra-Bone Marrow (IBM) injection of BMCs (IBM-BMT). As distinct from the conventional aspiration method (AM), the PM allows rapid (within 1 h) collection of BMCs without T cell contamination (T cells < 10%). Therefore, no GvHD occurs. Moreover, the burden on donors, such as back pain, bleeding and infection, can be reduced.

Full chimerism can be achieved even with only mild conditioning regimens if IBM-BMT is carried out, since IBM-BMT replaces not only the recipient's hemopoietic stem cells (HSCs) but also mesenchymal stem cells (MSCs) with donor-derived HSCs and MSCs.

Using this method, we show that most currently intractable diseases are HSC or MSC disorders, and that this novel strategy (PM + IBM–BMT) can be used to treat various otherwise intractable diseases (including autoimmune diseases and age-associated diseases).

We believe that the development of this technique will herald a revolution in the field of BMT, regeneration medicine and also organ transplantation.

Introduction

In 1985, we found that allogeneic bone marrow transplantation (BMT) (but not autologous BMT) could be used to prevent and treat autoimmune diseases in autoimmune-prone mice [1,2]. In addition, we succeeded in inducing autoimmune diseases in normal mice by the transplantation of T cell-

* Tel.: +81 6 6993 9625; Fax: +81 6 6993 9627.
E-mail address: ikehara@takii.kmu.ac.jp.

1521-6926/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.beha.2011.04.003

depleted bone marrow cells (BMCs) or partially purified hemopoietic stem cells (HSCs) from autoimmune-prone mice to normal mice [3,4].

Based on these findings, we have proposed that autoimmune diseases originate from defects in HSCs [3–7], and have also found that abnormal HSCs of autoimmune-prone mice are more resilient than normal HSCs [4,8,9]; abnormal HSCs can proliferate even in the allogeneic microenvironments, whilst normal HSCs can proliferate in collaboration with major histocompatibility complex (MHC)-compatible stromal cells (mesenchymal stem cells: MSCs), but not MHC-incompatible MSCs [4,8,9].

From these findings, we realized that, in the case of BMT across MHC barriers, we would have to transplant both donor-derived HSCs and MSCs to ensure that the donor-derived normal HSCs grow and survive in the allogeneic environments.

Recently, we have discovered that the injection of whole BMCs directly into the bone cavity (intra-bone marrow-BMT: IBM-BMT) provides distinct advantages, since IBM-BMT can efficiently recruit not only donor-derived HSCs but also MSCs. We here review our data regarding IBM-BMT plus the perfusion method (PM) (capable of efficiently collecting MSCs).

Advantages of novel BMT

As shown in Fig. 1, conventional BMT is carried out as follows: Bone marrow needles are inserted into the iliac bones more than 100 times, and the BMCs are collected by the aspiration method (AM). Therefore, contamination with peripheral blood (particularly T cells) is inevitable. When thus-collected cells are intravenously injected (IV-BMT), most cells become trapped in the lung and only a few cells migrate into the bone marrow (Fig. 1).

To apply our new BMT methods to humans, we established, using cynomolgus monkeys, a "PM", which minimizes the contamination of BMCs with T cells. As shown in Fig. 2, two needles are inserted into a long bone such as the humerus, femur, or tibia. The end of the extension tube is connected to a needle. The other end is placed in a syringe containing 0.5 ml heparin. The other needle is connected to a syringe containing 30 ml of saline, and the saline is then pushed gently from the syringe into the medullary cavity to flush out the bone marrow (BM). The saline containing the BM fluid is then collected.

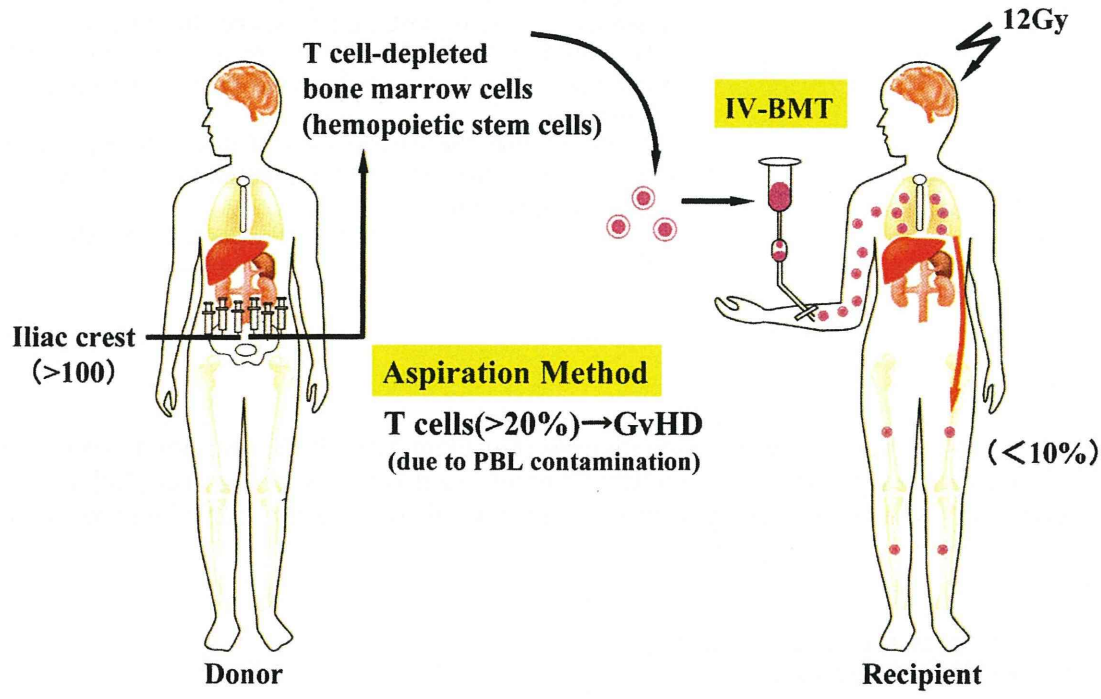

Fig. 1. Conventional BMT for allogeneic BMT. Conventional BMT is carried out using an aspiration method (AM), followed by the intravenous injection of BMCs (IV-BMT).

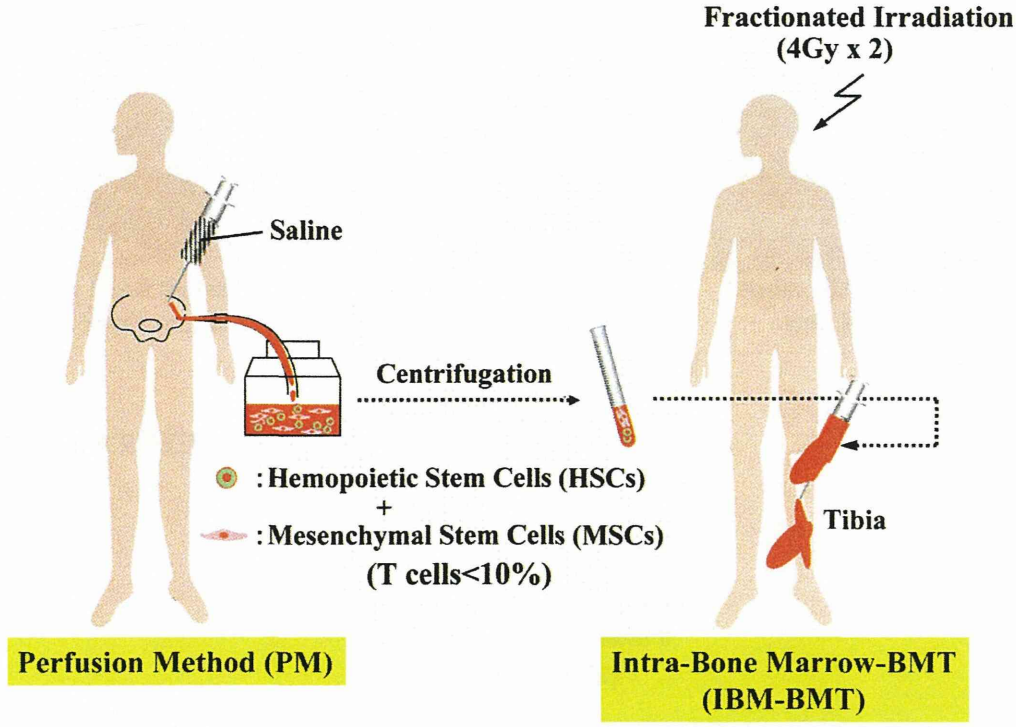

Fig. 2. New BMT method for allogeneic BMT. The new BMT method is carried out using a perfusion method (PM), followed by IBM–BMT.

There is significantly less contamination with T cells when using the PM (<10%) than with the conventional AM (>20%) [10,11]. Therefore, T cell-depletion is unnecessary with the PM, and whole BMCs can be used. However, in the case of the conventional AM, T cell-depletion is necessary, and the loss of some important cells such as MSCs during the process of T cell-depletion is inevitable. Furthermore, the number and progenitor activities of the cells harvested using the PM are greater than when using the conventional AM [10,11].

We have also found that the PM is applicable to the iliac bones as well as the long bones not only in monkeys but also in humans.

We are now starting a Phase I Study for the clinical application of PM + IBM–BMT.

IBM–BMT for organ transplantation

Since we have previously found that the combination of organ allografts and conventional IV-BMT from the same donors prevents the rejection of organ allografts [12], we attempted to apply IBM–BMT to organ allografts. IBM–BMT was the most effective strategy, since the radiation dose could be reduced to 4.0Gy × 2 in skin allografts [12,13]. In addition, we found that IBM–BMT is applicable to allografts of other organs and tissues in rats, such as pancreas islets [14] legs [15], lungs [16], and heart [17].

IBM–BMT for regeneration therapy

As it was apparent that donor stromal cells could be effectively recruited by "IBM–BMT", we next attempted to treat osteoporosis in SAMP6 mice; the SAMP6 mouse (a substrain of senescence-accelerated mice) spontaneously develops osteoporosis early in life and is therefore a useful model for examining the mechanisms underlying osteoporosis. After IBM–BMT, the hematolymphoid system was completely reconstituted with donor-type cells. Thus-treated SAMP6 mice (8 months after IBM-BMT) showed marked increases in trabecular bone even at 20 months of age (Fig. 3), and the bone mineral density (BMD) remained similar to that of normal B6 mice. Bone marrow stromal cells in "IBM-

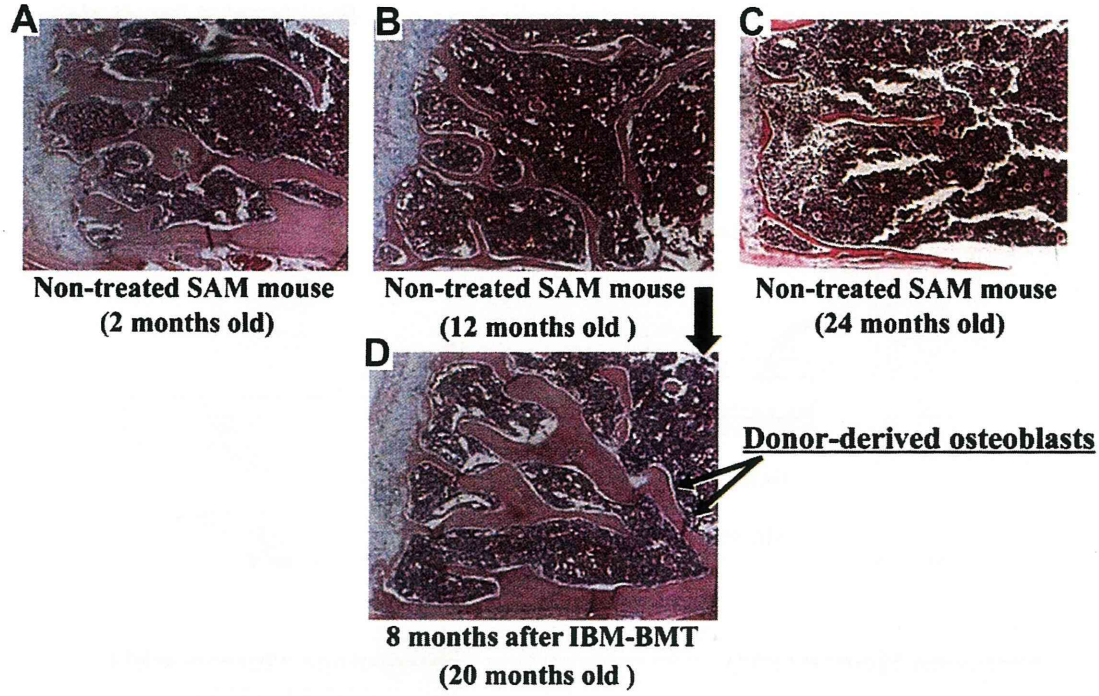

Fig. 3. Treatment of osteoporosis in SAMP6 mice by IBM–BMT from normal B6 mice.

BMT"-treated SAMP6 mice were replaced by donor stromal cells [18,19]. Thus, we succeeded in curing osteoporosis in SAMP6 mice by IBM–BMT, which can recruit both donor-derived HSCs and MSCs.

Since IBM–BMT appeared to be a powerful strategy in regeneration therapy, we next used tight-skin (Tsk) mice (an animal model for emphysema) to examine whether emphysema could be cured by IBM–BMT.

IBM-BMT was carried out from C3H mice into Tsk mice (8–10 weeks old) that had already shown emphysema. Eight months after the transplantation, the lungs of all the Tsk mice treated with IBM-BMT [C3H→Tsk] showed structures similar to those of normal mice, whereas the [Tsk→Tsk] mice showed emphysema, as seen in age-matched Tsk mice. Next, we attempted to transfer emphysema from Tsk mice to C3H mice by IBM–BMT. Six months after IBM-BMT, the [Tsk→C3H] mice showed emphysema [20]. These results strongly suggested that emphysema in Tsk mice originates from defects in the stem cells (probably MSCs and/or HSCs) in the bone marrow [20].

IBM–BMT + donor lymphocyte infusion (DLI) for treatment of malignant tumors

It is well known that the graft-versus-leukemia reaction (GvLR) can cure patients of a variety of hematological malignancies [21,22]. Recently, it has been reported that graft-versus-tumor (GvT) effects can induce partial (complete in some) remission of metastatic solid tumors such as breast cancer [23–25] and renal cell carcinoma [26–30]. Based on these findings, donor lymphocyte infusion (DLI) has recently been used for the treatment of malignant solid tumors even in humans. However, it is very difficult to completely eradicate the tumors, since extensive DLI induces graft-versus-host disease (GvHD). We therefore attempted to establish a new method for the treatment of malignant tumors, this method consisting of intra-bone marrow-IBM–BMT plus DLI, since we have recently found that IBM-BMT can allow a reduction in radiation doses as a conditioning regimen and prevent GvHD [31,32]. Using the Meth-A cell line (BALB/c-derived fibrosarcoma), we found that IBM-BMT plus the injection of $CD4^+$ T-cell-depleted (but not $CD8^+$ T-cell-depleted) spleen cells (as DLI) can prevent GvHD while suppressing tumor growth [33] (Fig. 4). In addition, we have found that IBM-BMT plus extensive DLI (3

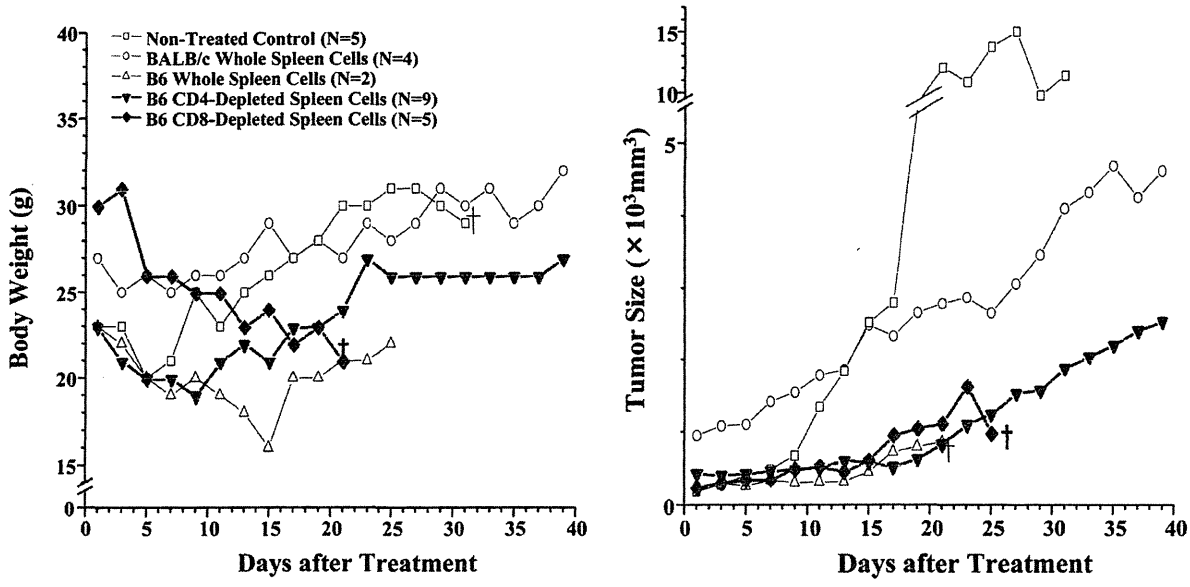

Fig. 4. Prevention of GvHD and suppression of tumor growth by IBM–BMT + DLI ($CD4^-$).

times every 2 weeks) leads to the complete rejection of the tumor, although the success rate (3/50) is not high so far [33].

In addition, we have examined whether this strategy (IBM-BMT plus DLI) is applicable to other tumors in other animals. We have obtained similar results in another system (colon cancer: ACL-15 in rats) [34]. We are now establishing more efficient strategies to eradicate malignant tumors.

IBM–BMT + thymus transplantation (TT) for modulation of age-associated diseases

We have recently proposed that age-associated diseases (AADs), such as osteoporosis and emphysema, are mesenchymal stem cell disorders.

Based on our findings, we attempted to prevent the progression of Alzheimer's disease using senescence-accelerated mice by IBM-BMT, and succeeded in preventing the development of Alzheimer's disease [35].

In addition, we succeeded in curing type 2 diabetes mellitus in db/db mice by IBM-BMT with TT [36].

These findings suggest that TT plays a crucial role in the prevention and treatment of AADs, since the recovery of T cell functions would be delayed after IBM–BMT alone (but not after IBM–BMT + TT).

Future directions

As described here, the new BMT method (PM + IBM–BMT) can be used to treat various otherwise intractable diseases, including i) autoimmune diseases, ii) AADs (osteoporosis, emphysema, etc.), iii) diseases curable by organ transplantation and iv) malignant tumors (including solid tumors) [33]. The PM can efficiently be used to collect whole BMCs (including HSCs and MSCs) without them being contaminated with T cells, and no GVHD therefore develops. IBM-BMT can efficiently transfer donor whole BMCs (both HSCs and MSCs) into recipients, and this method can therefore be used to quickly replace not only HSCs but also MSCs with donor-derived cells.

From the findings to date, it is conceivable that all the body's cells originate in the bone marrow, and that all diseases might therefore originate from defects in the bone marrow. One paper already suggests that gastric cancer originates from bone marrow-derived cells [37].

We believe that the development of our BMT method heralds a revolution in the field of transplantation (BMT and organ transplantation) and regeneration therapy.

Conflict of interest

No conflicts of interest to declare.

Acknowledgments

I thank Mr. Hilary Eastwick-Field and Ms. K. Ando for their help in the preparation of the manuscript. These studies were mainly supported by the 21st Century Center of Excellence (COE) program of the Ministry of Education, Culture, Sports, Science and Technology. Supported also by a grant from Haiteku Research Center of the Ministry of Education, Health and Labour Sciences Research Grants, a grant from the Science Frontier program of the Ministry of Education, Culture, Sports, Science and Technology, and a grant from the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Company, Ltd.), a grant from Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd., and a grant from Japan Immunoresearch Laboratories Co., Ltd. (JIMRO).

References

*[1] Ikehara S, Good RA, Nakamura T, et al. Rationale for bone marrow transplantation in the treatment of autoimmune diseases. Proc Natl Acad Sci USA 1985 Apr;82(8):2483–7.
[2] Ikehara S, Ohtsuki H, Good RA, et al. Prevention of type I diabetes in nonobese diabetic mice by allogeneic bone marrow transplantation. Proc Natl Acad Sci USA 1985 Nov;82(22):7743–7.
*[3] Ikehara S, Kawamura M, Takao F, et al. Organ-specific and systemic autoimmune diseases originate from defects in hematopoietic stem cells. Proc Natl Acad Sci USA 1990 Nov;87(21):8341–4.
[4] Kawamura M, Hisha H, Li Y, et al. Distinct qualitative differences between normal and abnormal hemopoietic stem cells in vivo and in vitro. Stem Cells 1997 Jan;15(1):56–62.
[5] Ikehara S. Autoimmune diseases as "stem cell disorders". Acta Histochem Cytochem 1997;30(1):3–12.
[6] Ikehara S. Autoimmune diseases as stem cell disorders: normal stem cell transplant for their treatment. Int J Mol Med 1998 Jan;1(1):5–16.
[7] Ikehara S. State-of-the-art review. A new concept of stem cell disorders and their new therapy. J Hematother Stem Cell Res 2003 Dec;12(6):643–53.
*[8] Hashimoto F, Sugiura K, Inoue K, et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. Blood 1997 Jan;89(1):49–54.
[9] Sugiura K, Hisha H, Ishikawa J, et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. Stem Cells 2001 Jan;19(1):46–58.
*[10] Inaba M, Adachi Y, Hisha H, et al. Extensive studies on perfusion metgod plus itra-bone marrow-bone marrow transplantation using cynomolgus monkeys. Stem Cells 2007 Aug;25(8):2098–103.
[11] Kushida T, Inaba M, Ikebukuro K, et al. Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys of various ages by perfusion or aspiration methods: a preclinical study for human BMT. Stem Cells 2002 Mar; 20(2):155–62.
[12] Nakamura T, Good RA, Inoue S, et al. Successful liver allografts in mice by combination with allogeneic bone marrow transplantation. Proc Natl Acad Sci USA 1986 Jan;83(12):4529–32.
*[13] Ikehara S. A novel method of bone marrow transplantation (BMT) for intractable autoimmune diseases. J Autoimmun 2008 May;30(3):108–15.
[14] Ikebukuro K, Adachi Y, Suzuki Y, et al. Synergistic effects of induction in transplantation of allogeneic pancreatic islets. Bone Marrow Transplant 2006 Nov;38(10):657–64.
[15] Esumi T, Inaba M, Ichioka N, et al. Successful allogeneic leg transplantation in rats by combination of intra-bone marrow (IBM) injection of donor bone marrow cells. Transplantation 2003 Dec;76(11):1543–8.
[16] Kaneda H, Adachi Y, Saito Y, et al. Long-term observation after simultaneous lung and intra-bone marrow-bone marrow transplantation. J Heart Lung Transplant 2005 Sept;24(9):1415–23.
[17] Guo K, Inaba M, Li M, et al. Long-term donor-specific tolerance in rat cardiac allografts by intra-bone marrow injection of donor bone marrow cells. Transplantation 2008 Jan;85(5):93–101.
[18] Ichioka N, Inaba M, Kushida T, et al. Prevention of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells. Stem Cells 2002 Nov;20(6):542–51.
*[19] Takada K, Inaba M, Ichioka N, et al. Treatment of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells. Stem Cells 2006 Feb;24(2):399–405.
*[20] Adachi Y, Oyaizu H, Taketani S, et al. Treatment and transfer of emphysema by a new bone marrow transplantation method from normal mice to Tsk mice and vice versa. Stem Cells 2006 Sep;24(9):2071–7.
[21] Thomas ED, Blume KG. Historical markers in the development of allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant 1999 Dec;5(6):341–6.
[22] Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host diseases in human recipients of allogeneic-marrow grafts. N Engl J Med 1979 May;300(19):1068–73.
[23] Ben-Yosef R, Or R, Nagker A, et al. Graft-versus-tumor and graft-versus-leukaemia effect in patient with concurrent breast cancer and acute myelocytic leukaemia. Lancet 1996 Nov. 29;348(9036):1242–3.
[24] Eibi B, Schwaigofer H, Nachbaur D, et al. Evidence for a graft-versus-tumor effect in a patient treated with marrow ablative chemotherapy and allogeneic bone marrow transplantation for breast cancer. Blood 1996 Aug;88(4):1501–8.

[25] Ueno NT, Rondon G, Mirza NQ, et al. Allogeneic peripheral-blood progenitor-cell transplantation for poor-risk patients with metastatic breast cancer. J Clin Oncol 1998 Mar;16(3):986–93.
[26] Childs RW, Clave E, Tisdale J, et al. Successful treatment of metastatic renal cell carcinoma with a nonmyeloablative allogeneic peripheral-blood progenitor-cell transplant: evidence for a graft-versus-tumor effect. J Clin Oncol 1999 Jul; 17(7):2044–9.
[27] Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. N Engl J Med 2000 Sept. 14;343(11):750–8.
[28] Appelbaum FR, Sandmaier B. Sensitivity of renal cell cancer to nonmyeloablative allogeneic hematopoietic cell transplantation: unusual or unusually important? J Clin Oncol 2002 Apr. 15;20(8):1965–7.
[29] Bregni M, Dodero A, Peccatori J, et al. Nonmyeloablative conditioning followed by hematopoietic cell allografting and donor lymphocyte infusions for patients with metastatic renal and breast cancer. Blood 2002 Jun. 1;99(11):4234–6.
[30] Hentschke P, Barkholt L, Uzunel M, et al. Low-intensity conditioning and hematopoietic stem cell transplantation in patients with renal and colon carcinoma. Bone Marrow Transplant 2003 Feb;31(4):253–61.
*[31] Kushida T, Inaba M, Hisha H, et al. Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 2001 May;97(10):3292–9.
[32] Rini BI, Zimmerman T, Stadler WM, et al. Allogeneic stem-cell transplantation of renal cell cancer after nonmyeloablative chemotherapy: feasibility, engraftment, and clinical results. J Clin Oncol 2002 Apr. 15;20(8):2017–24.
*[33] Suzuki Y, Adachi Y, Minamino K, et al. A new strategy for treatment of malignant tumor: intra-bone marrow-bone marrow transplantation plus $CD4^-$ donor lymphocyte infusion. Stem Cells 2005 Mar;23(3):365–70.
[34] Koike Y, Adachi Y, Suzuki Y, et al. Allogeneic intra-bone marrow-bone marrow transplantation plus donor lymphocyte infusion suppresses growth of colon cancer cells implanted in skin and liver of rats. Stem Cells 2007 Feb;25(2):385–91.
[35] Li M, Inaba M, Guo K, et al. Amelioration of cognitive ability in senescence-accelerated mouse prone 8 (SAMP8) by intra-bone marrow-bone marrow transplantation. Neurosci Lett 2009 Nov. 6;465(1):36–40.
*[36] Li M, Abraham NG, Vanella L, et al. Successful modulation of type 2 diabetes in db/db mice with intra-bone marrow-bone marrow transplantation plus concurrent thymic transplantation. J Autoimmun; 2010 Sep 28 [Epub ahead of print].
[37] Houghton J, Stoicov C, Nomura S, et al. Gastric cancer originating from bone marrow-derived cells. Science 2004 nov. 26; 306(5701):1568–71.

STEM CELLS AND DEVELOPMENT
Volume 21, Number 9, 2012

DOI: 10.1089/scd.2011.0358

Effects of Intrabone Marrow–Bone Marrow Transplantation Plus Adult Thymus Transplantation on Survival of Mice Bearing Leukemia

Yuming Zhang,[1,*] Naoki Hosaka,[2,*] Yunze Cui,[3,4] Ming Shi,[3] Ming Li,[3] Qing Li,[3] and Susumu Ikehara[3]

We recently found that allogeneic intrabone marrow–bone marrow transplantation (IBM–BMT) plus adult thymus transplantation (ATT) from the same donor is effective in mice bearing solid tumors. In the current study, we examined the effects of this strategy on the survival of mice with leukemia. One week after intravenous injection of 1×10^6 leukemic cells (EL-4, H-2^b) into 8-week-old B6 (H-2^b) mice, the mice were 8 Gy irradiated and transplanted with 1×10^7 bone marrow cells (BMCs) from 8-week-old BALB/c mice (H-2^d) by IBM–BMT with or without donor lymphocyte infusion (DLI) or ATT. All the mice without treatment died within 70 days after injection of EL-4. About 40% of those treated with IBM–BMT alone died within 100 days due to tumor relapse. In contrast, those treated with IBM–BMT + DLI or ATT showed the longest survival rate without relapse of leukemia. In addition, the former showed less graft versus host disease (GVHD) than the latter. The mice treated with IBM–BMT + ATT also showed an intermediate percentage of effector memory (EM) and central memory (CM) cells between those treated with BMT alone and those treated with IBM–BMT + DLI. The numbers and functions of T cells increased in those treated with IBM–BMT + ATT with interleukin-2 and interferon-γ production. These results suggest that IBM–BMT + ATT is effective in the treatment of leukemia with strong graft versus leukemia without increased risk of GVHD.

Introduction

ALLOGENEIC BONE MARROW transplantation (allo-BMT) has been used for the radical treatment of leukemia. However, allo-BMT has some side effects. Graft versus host disease (GVHD) occurs if anti-host reaction in donor T cells is too strong, whereas relapse occurs if it is too weak [1]. In addition, a failure of bone marrow cell (BMC) engraftment in the early phase of transplantation may induce immunodeficiency, which, in turn, leads to severe infection [2]. Although donor lymphocyte infusion (DLI) is sometimes used to enhance engraftment and/or graft versus leukemia (GVL) activity [3], this is associated with an increased risk of GVHD [4]. Therefore, new cellular-based methods are required.

We recently developed a new BMT method, intrabone marrow–bone marrow transplantation (IBM–BMT), in which BMCs are directly injected into the bone marrow cavity [5]. IBM–BMT results in a reduced incidence of GVHD and greater engraftment of donor cells, including mesenchymal stem cells (MSCs) than the conventional intravenous method [6,7].

We have also developed a BMT method that is combined with thymus transplantation (TT), which includes the transplantation of adult thymus transplantation (ATT), newborn thymus, and fetal thymus. The combination of BMT + TT is effective in restoring donor-derived T cell function even in aged, chimeric-resistant, supralethally irradiated, and low-dose irradiated mice, mice with metabolic diseases, and also in mice injected with a small number of BMCs [8–12]. Further, we demonstrated that IBM–BMT + TT is effective for graft versus tumor (GVT) and long-term survival with a low risk of GVHD [13,14].

In the current study, we examined the BMT + ATT method in mice with leukemia. We also performed BMT alone and BMT + DLI in these mice and compared the survival rate, degree of GVHD, and T-cell functions.

Materials and Methods

Mice

Female 6- to 8-week-old C57BL/6 (B6) (H-2^b) and BALB/c (H-2^d) mice were obtained from Shimizu Laboratory Supplies (Shizuoka, Japan) and maintained until use in our animal facilities under specific pathogen-free conditions. All

[1]Department of Pediatrics, Nanfang Hospital, Guangzhou, China.
[2]Department of Pathology, Kori Hospital-Kansai Medical University, Neyagawa, Osaka, Japan.
[3]Department of Stem Cell Disorders, Kansai Medical University, Moriguchi, Osaka, Japan.
[4]Japan Immunoresearch Laboratories Co., Ltd. (JIMRO), Takasaki City, Gunma, Japan.
*These two authors contributed equally to this work.

protocols for these animal experiments were performed in accordance with the Guidelines for Animal Experimentation, Kansai Medical University, and received approval from the Committee on Animal Experiments. EL-4 cells (H-2^b) were derived from thymoma in B6 mice. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum with antibiotics. These cells were intravenously transferred to the recipients (B6 mice).

IBM–BMT and ATT

Recipient B6 mice with tumors were irradiated (8 Gy) by using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International, Ottawa, ON, Canada) 7 days after transfer of the EL-4 cells. The next day, BMCs were flushed from the shafts of donor femora and tibiae, and single-cell suspensions were prepared. Next, 1×10^7 BMCs were directly injected into the bone marrow cavity of the recipient's tibia, as previously described for the IBM–BMT method [7]. Briefly, the knee was flexed to 90°, and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the bone cavity. Simultaneously, one quarter of the AT was grafted under the renal capsule of the left kidney in some mice.

Histology

Histological studies were performed in the liver, intestine (for evaluation of GVHD), and engrafted tumors from the recipients 4 weeks after the BMT. The tissues were fixed in 10% formaldehyde and embedded in paraffin. Serial tissue sections (4 μm thick) were prepared and stained by using hematoxylin and eosin. The degree of GVHD was evaluated by using a semiquantitative scoring system for abnormalities known to be associated with GVHD, as previously described [13].

Analysis of surface marker antigens and intracellular FoxP3 and cytokines by flow cytometry

Surface markers on lymphocytes from the spleen were analyzed by 3-color fluorescence staining by using a FACScan system (Becton Dickinson, Franklin Lakes, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-H-$2K^b$ (Pharmingen, San Diego, CA) mAbs and phycoerythrin (PE)-conjugated anti-H-$2K^d$ mAbs were used to determine chimerism. FITC, PE, or biotin-conjugated CD4, CD8, B220, CD44, or CD62L (Pharmingen) were used to analyze spleen cell subsets. Avidin-Cy5 (Dako, Kyoto, Japan) was used as the third color in the avidin/biotin system. Intracytoplasmic FoxP3 staining was performed by using an eBioscience FITC-anti mouse/rat FoxP3 staining kit in accordance with the manufacturer's instructions (eBioscience, San Diego, CA). Intracellular cytokines [interleukin (IL)-2, IL-4, IL-10, IL-17, interferon (IFN)-γ, and tumor necrosis factor] were detected by using an Intracellular Cytokine Staining Kit in accordance with the manufacturer's instructions (Becton Dickinson).

Mitogen responses

To analyze lymphocyte function, mitogen responses were examined in chimeric mice 2 months after transplantation. For mitogen response, a total of 2×10^5 splenocytes collected from chimeric mice and untreated B6 and BALB/c mice as responders were plated in 96-well flat-bottomed plates (Corning Glass Works, Corning, NY) containing 200 μL of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2 μL of glutamine (Wako Pure Chemicals, Tokyo, Japan), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal calf serum. For mitogen responses, responder cells were incubated with 2.5 μg/mL of Concanavalin A (ConA) (Calbiochem, San Diego, CA) or 25 μg/mL of lipopolysaccharide (LPS) (Difco Laboratories, Franklin Lakes, NJ) for 48 or 72 h. During the last 18 h of the culture period, 20 mL of 0.5 μCi ^{3}H-thymidine (^{3}H-TdR; New England Nuclear, Cambridge, MA) was introduced. Incorporation of ^{3}H-TdR was measured by using Microbeta TriLux (PerkinElmer, Wellesley, MA). The stimulation index was calculated as the average of ^{3}H-TdR incorporation in triplicate samples of responding cells with mitogen/^{3}H-TdR incorporation of responding cells in medium alone.

Statistical analyses and nonparametric analyses (Mann–Whitney *U*-test and log rank-test) were performed by using StatView software (Abacus Concepts, Berkley, CA). In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

Survival rate and body weight

First, we examined the effects of BMT alone, BMT+ATT, or BMT+DLI on the survival rate of mice transplanted with EL-4 (Fig. 1A). All the untreated mice transplanted with EL-4 died within 70 days due to tumor growth (Fig. 2B). Those treated with BMT alone showed a survival rate of about 60% 6 months after BMT. The remaining 40% of the mice died due to tumor growth. Interestingly, those treated with BMT+ATT or BMT+DLI showed the longest survival rate. Next, we investigated the weight of these mice. The mice not treated with EL-4 showed a gradual increase in weight (Fig. 1B), which was due to growth of the tumor (Fig. 2B). Those treated with IBM–BMT alone and IBM–BMT+ATT surviving for a long time showed a stable weight, and those treated with IBM–BMT+DLI showed a gradual weight loss.

Chimerism and histology

All mice treated with BMT showed donor-derived chimerism (H-$2K^d$), whereas untreated controls showed host-derived chimerism (H-$2K^b$) (Fig. 2A). The untreated mice showed massive infiltration of tumor cells throughout the whole body, including the liver, lung, mesenterium, muscle, and bone (Fig. 2B). All mice that died treated with BMT alone showed such tumor growth (as just mentioned). In contrast, most of those treated with BMT+ATT or BMT+DLI showed little tumor growth and long-term survival. The engrafted thymus showed a normal structure with cortical and medullar areas under the renal capsule (Fig. 2C). Normal T-cell differentiation was also observed in the thymus. In addition, those treated with BMT+ATT and BMT+DLI showed mild and moderate infiltrations of lymphocytes in the liver and small intestine, and the latter also showed some fibrosis with tissue destruction (Fig. 2D). Since the chimerism was of the donor type, this suggested the occurrence of

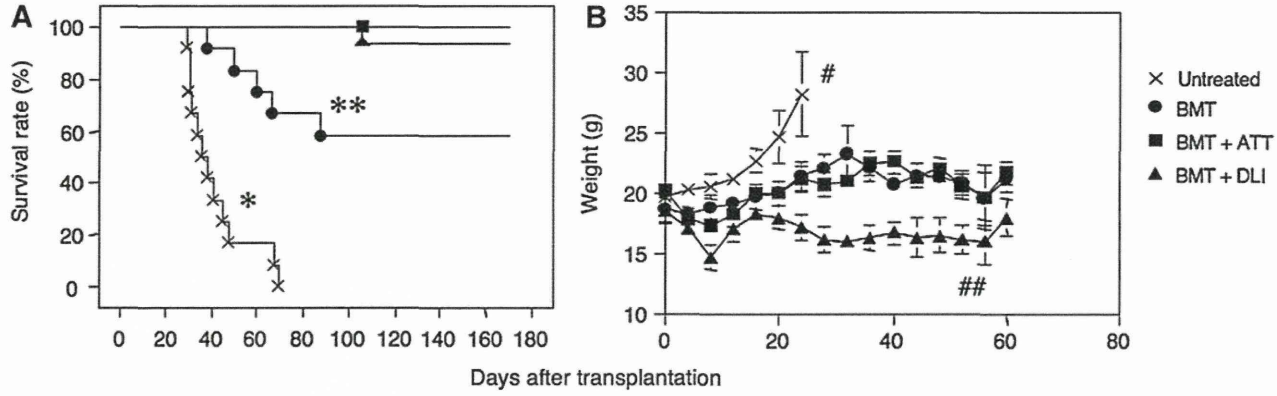

FIG. 1. Survival rate and body weight in mice with leukemia treated with BMT + TT. Survival rate **(A)** and weight **(B)** of mice with advanced tumors are shown. $^{*}P < 0.0001$ compared with those treated with BMT alone, BMT + ATT, or BMT + DLI. $^{**}P < 0.05$ compared with those treated with BMT + ATT or BMT + DLI. $^{\#}P < 0.05$ compared with those treated with BMT alone, BMT + ATT, or BMT + DLI. $^{\#\#}P < 0.05$ compared with those treated with BMT + ATT or BMT + DLI. Data are shown as means ± SE. Untreated controls ($n = 12$), those treated with BMT alone ($n = 12$), BMT + ATT ($n = 12$), or BMT + DLI ($n = 15$). ATT, adult thymus transplantation; BMT, bone marrow transplantation; DLI, donor lymphocyte infusion; TT, thymus transplantation.

GVHD. The GVHD scores are summarized in Fig. 2E. Those treated with BMT alone showed little GVHD, whereas those treated with BMT + ATT showed mild GVHD, and those treated with BMT + DLI showed moderate GVHD.

Lymphocyte subsets

We next analyzed the lymphocyte subsets in the spleen 4 weeks after transplantation. The number of $CD4^+$ T cells was significantly greater in the mice treated with BMT + ATT compared with those treated with BMT alone and those with BMT + DLI, in which the levels were comparable to those in normal BALB/c mice (Fig. 3). This was followed by those treated with BMT alone, followed by those treated with BMT + DLI, which showed the lowest levels. The percentage of $FoxP3^+$ regulatory T cells, which suppress immune responses, including GVH reactions [15,16], among $CD4^+$ T cells was the highest in the mice treated with BMT alone, the percentage being comparable to that in BALB/c mice. This was followed by those treated with BMT + ATT, whereas those treated with BMT + DLI showed the lowest percentage. The results for $CD8^+$ T cells were similar to those for $CD4^+$ T cells, although all values for mice treated with BMT were lower than those of BALB/c mice. The number of $B220^+$ T cells was lowest in those treated with BMT + DLI.

Effector memory, central memory, and naïve T cell subsets

T cells can be functionally divided into $CD62L^-CD44^-$ naïve, $CD62L^+CD44^+$ central memory (CM), and $CD62L^-CD44^+$ effector memory (EM) cells from prestimulation to terminal differentiation [17,18]. Therefore, we examined the proportions of these cells in both $CD4^+$ and $CD8^+$ subsets of T cells in the spleen. The percentage of EM among $CD4^+$ T cells was the highest in the mice treated with BMT + DLI followed by those treated with BMT + ATT (Fig. 4A). The lowest percentage of EM among $CD4^+$ T cells was seen in those treated with BMT alone, being comparable to that in BALB/c mice. Conversely, the percentage of CM was the highest in the mice treated with BMT alone and BALB/c mice, followed by those treated with BMT + ATT, and the lowest percentage of CM was seen in those treated with BMT + DLI. The percentage of naïve T cells was similar to that of CM cells, although the highest percentage was seen only in the BALB mice, and there were no significant differences between those treated with BMT + ATT and those treated with BMT + DLI. The results for $CD8^+$ T cells were similar to those for $CD4^+$ T cells (Fig. 4B).

Mitogen responses and cytokine production

Finally, we examined lymphocyte functions by monitoring mitogen responses (Con A for T cells and LPS for B cells) and cytokine production. The mice treated with BMT + ATT showed significantly increased Con A response compared with those treated with BMT alone, and the level was comparable to that in BALB/c mice (Fig. 5A). Those treated with BMT + DLI showed the lowest response. In contrast, the LPS response was almost the same in those treated with BMT alone, BMT + ATT, and BALB/c mice, and the lowest response was seen in BMT + DLI. With regard to cytokine production, those treated with BMT + ATT showed significant increases in IL-2 production compared with those treated with BMT alone and BMT + DLI, the level being comparable to that in BALB/c mice (Fig. 5B). In contrast, those treated with BMT + DLI showed significantly higher levels of IFN-γ production than those treated with BMT + ATT and BMT alone, although they did not reach the level of BALB/c mice. The production of IFN-γ was also elevated in those treated with BMT + TT, and was higher than in those treated with BMT alone.

Discussion

The current study was performed to examine the effects of BMT + ATT on leukemia in mice. The mice treated with BMT + ATT showed a longer survival than those treated with BMT alone, and milder GVHD than those treated with BMT + DLI. Leukemia showed little growth in BMT + ATT mice comparable to those treated with BMT + DLI. Those treated with BMT + ATT showed higher numbers of both

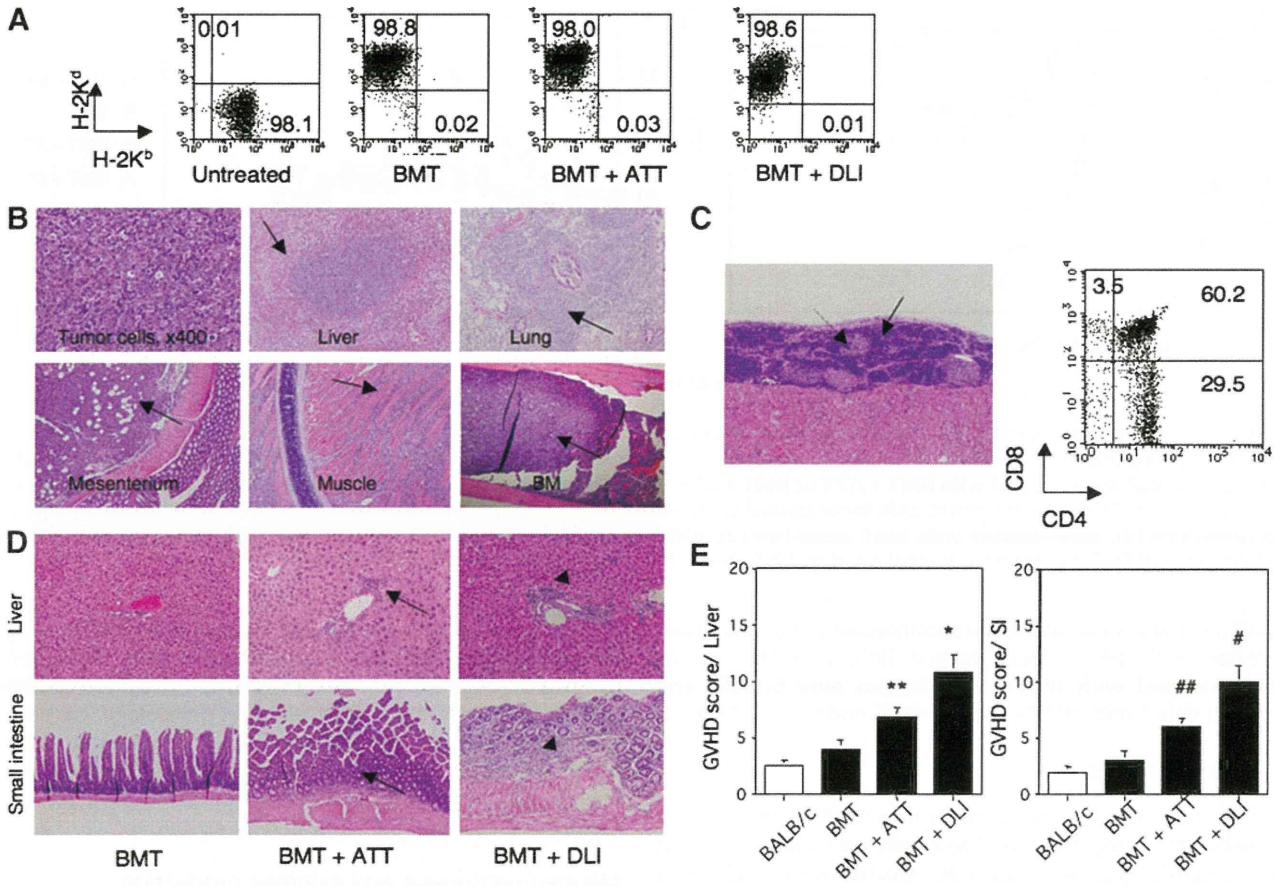

FIG. 2. Chimerism, histology, transplanted thymus, and GVHD in mice with leukemia treated with BMT + TT. Chimerism in experimental mice **(A)**, histology in the liver, lung, mesenterium, muscle, and BM from mice with leukemia **(B)**, histology in transplanted thymus and the FACS profile in **(C)**, histology for GVHD in chimeric mice **(D)** and the GVHD scores in the mice **(E)** are shown. Chimerism was analyzed in the spleens from mice transplanted with leukemia and those treated with BMT alone, BMT + ATT, or BMT + DLI **(A)**. The numbers in the profiles show the percentage. Tumor cells (*upper, left*) infiltrated the liver, lung, mesenterium, muscle, and BM in the mice transplanted with leukemia (*arrows*) **(B)**. Histological findings (*left*) and FACS profile (*right*) of thymocytes in the transplanted thymus from the mice treated with BMT and ATT **(C)**. *Plain arrow*, cortex; *dotted arrow*, medulla. The numbers in the profiles show the percentage. Representative data from 4 experiments are shown. The histology of GVHD is shown in the liver (*upper*) and small intestine (*lower*) **(D)**. Some lymphocytes infiltrated the liver and small intestine in BMT + ATT (*arrows*) and destroyed the tissue in BMT + DLI (*dotted arrow*). GVHD scores are shown in the liver (*left*) and small intestine (*right*) **(E)**. The GVHD score was calculated as described in Materials and Methods. $^{*}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone and BMT + ATT. $^{**}P < 0.05$ compared with normal BALB/c mice and the mice treated with BMT alone. $^{\#}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone and BMT + ATT. $^{\#\#}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone. The mice transplanted with leukemia cells (EL-4) were analyzed 5 weeks after transplantation and those treated with BMT alone, BMT + ATT, or BMT + DLI were analyzed 8 weeks after treatment. Data are shown as means ± SE. Normal BALB/c ($n = 4$), BMT ($n = 4$), BMT + ATT ($n = 4$), BMT + DLI ($n = 4$). GVHD, graft versus host disease.

$CD4^+$ and $CD8^+$ T cell subsets than those treated with BMT alone or with BMT + DLI. Interestingly, the percentages of $FoxP3^+$ regulatory T cells, CM, and EM T cells in those treated with BMT + ATT were intermediate between those treated with BMT alone and those treated with BMT + DLI. T-cell functions with production levels of some cytokines were also elevated in those treated with BMT + ATT. These findings suggest that the BMT + ATT method is more effective in the treatment of leukemia than previous methods.

First, we examined the survival rates in association with GVH and GVL effects. All mice with the development of leukemia died early, whereas those without leukemia showed long-term survival, with or without GVHD, thus indicating that the presence of leukemia is the factor with the greatest influence on mortality. However, we did not examine the further long-term effects of GVHD, and chronic GVHD may also lead to death in the long term [19]. Therefore, these observations suggest that BMT + ATT is superior to BMT alone and BMT + DLI.

We next investigated the mechanism of these effects. The numbers of both T-cell subsets significantly increased in mice treated with BMT + ATT compared with those treated with BMT alone and BMT + DLI. The low numbers of T-cell subsets as well as B cells in those treated with BMT + DLI may have resulted in GVHD [20]. Interestingly, those treated with BMT + ATT showed a lower percentage of regulatory T cells