厚生労働科学研究費補助金

がん臨床研究事業

＜研究課題名＞

固形がんに対する同種細胞免疫療法を用いた標準的治療法の確立に関する研究
～転移固形腫瘍を対象としたミニ移植の安全性と有効性の検討～

平成 14 年度～16 年度　総合研究報告書

主任研究者　　高上　洋一

（所属機関　国立がんセンター中央病院）

平成 17（2005）年 4 月

厚生労働科学研究費補助金

がん臨床研究事業

＜研究課題名＞

固形がんに対する同種細胞免疫療法を用いた標準的治療法の確立に関する研究
～転移固形腫瘍を対象としたミニ移植の安全性と有効性の検討～

平成 14 年度～16 年度　総合研究報告書

主任研究者　　高上　洋一

（所属機関　国立がんセンター中央病院）

平成 17（2005）年 4 月

【 目　次 】

厚生労働科学研究費補助金　がん臨床研究事業
総合研究報告書

『 固形がんに対する同種細胞免疫療法を用いた標準的治療法の確立に関する研究 ～転移固形腫瘍を対象としたミニ移植の安全性と有効性の検討～ 』

主任研究者　　高上 洋一　国立がんセンター中央病院／薬物療法部 部長

研究要旨　転移性の腎癌患者において、同種抗腫瘍免疫効果(graft-versus-tumor: GVT 効果)を引き出すことを主旨とした骨髄非破壊的移植(ミニ移植)の安全性と有効性を、厳正な医師主導臨床試験を行って評価する。これに必要な治療効果判定基準、病理並びに放射線中央診断システムなどを含む固形腫瘍に対する移植領域に特化した標準手順書(SOP)を作成したうえで患者登録を開始した。現時点で、多施設共同新 GCP 試験には 6 名を、また第 I 相臨床試験には計 47 例を登録して試験を継続中である。腎細胞癌、大腸癌の一部などにおいては明らかな腫瘍縮小効果を観察した。班員施設でミニ移植を施行された腎癌患者について検討したところ、移植後 180 日以内の早期に死亡したのは診断時から転移巣を有する急速進行例や移植時全身状態の悪い患者であった。一方、これらを欠く患者では、長期の GVT 効果が得られることも判明した。付随研究の結果、GVT 効果には特異的免疫と非特異的免疫系の双方が関与する可能性が初めて示され、現在、標的抗原と効果担当細胞の解明を進めている。

分担研究者名（所属機関名・職名）
平家勇司（国立がんセンター研究所・主任研究官）
國頭英夫（国立がんセンター中央病院・医長）
高橋俊雄（東京都立駒込病院・名誉院長）
豊嶋崇徳（九州大学病院･助教授）
冨田善彦（山形大学医学部・教授）
公文裕巳（岡山大学大学院・教授）
並木幹夫（金沢大学大学院・教授）
三木恒治（京都府立医科大学・教授）
藤岡知昭（岩手医科大学・教授）
山上裕機（和歌山県立医科大学・教授）
岡島英二郎（国立がんセンター中央病院・医員）

A. 研究目的

難治性の腎癌や膵癌などの固形腫瘍に対する有効な治療法を開発することは急務である。本研究では、インターフェロン療法も含めた従来の治療法をもってしては治癒が期待できない転移性の腎癌患者を対象として、強力な同種抗腫瘍免疫効果を引き出すことを主旨とした骨髄非破壊的移植、いわゆるミニ移植の安全性と有効性を厳正な多施設共同第 I/II 相臨床治験を行って確認することを目指した。これによる波及効果として、我が国における移植領域の臨床試験体制の基盤整備も図った。

最近に至って、同種造血幹細胞移植のもつ抗腫瘍効果は、前治療として行う大量抗癌剤療法そのものよりも、むしろその後に患者体内で生着して増殖するドナー由来のリンパ球などを介して発生する GVT 効果と呼ばれる免疫学的効果の果たす役割が大きいことが明らかとなった。ただ通常の同種移植を行った場合には重度の副作用が多発するため、固形腫瘍患者に対しては施行されることはなかった。このため、前処置関連毒性を少なくすることで治療の安全性を著しく高めたミニ移植が開発された。本

研究では、難治性固形腫瘍患者に対して、この同種細胞免疫療法の特徴を強調したミニ移植を用いた新たな治療法を開発する。質の高いエビデンスを得るために厳正な臨床試験を行った。

B．研究方法

本研究の対象となったのは、他に有効な治療法がない転移性の腎癌(第I/II相臨床試験、予定登録症例30例)、あるいは膵癌や悪性黒色腫などの固形腫瘍患者(国立がんセンター中央病院における第I相臨床試験)である。説明同意を書面で得た後に、HLAの一致した血縁ドナーに顆粒球コロニー刺激因子(G-CSF)を皮下注射して末梢血幹細胞を動員した後に、アフェレーシスを行って幹細胞を採取して凍結保存する。患者には、ドナーの造血幹細胞とリンパ球の生着を促進する目的で、当院で開発した骨髄非破壊的前処置としてプリン誘導体であるフルダラビン(30 mg/kg/day x 6 日)とブスルファン (4 mg/kg x 4 日)、並びに抗胸腺リンパ球抗体(ATG) 2.5 mg/kg/日を2日間投与する。前処置療法終了後に保存した細胞を解凍して輸注する。移植前後の合併症の程度、生着の速さ、感染症の頻度、急性及び慢性 GVHD の頻度と程度、あるいは腫瘍縮小効果、長期生存と無病生存率などについて評価した。腎癌に対する第I/II相試験は新GCPに基づいて実施するために、データ管理は特定非営利活動法人である日本臨床研究支援ユニット（代表、大橋靖雄）に委託した。その他の癌に対する第I相試験については、国立がんセンター中央病院に専任のデータマネージャーを配して設置したデータセンターで厳正に執り行った。また、治療の均てん化を促進するために、地域主要がん診療施設からの研修者を積極的に受け入れた。

＜倫理面への配慮＞

本研究を実施するにあたっては、ヘルシンキ宣言や米国ベルモントレポート等の国際的倫理原則に従った。つまり、本研究に対する倫理審査委員会の承認が得られた施設からのみ患者登録を行う。対象患者については、いずれも患者本人に説明同意文書の内容を極力分かり易い言葉で説明し、説明同意文書2部を作製して本人に渡したうえで文書による同意を得た。

C．研究結果

多施設共同新GCP試験には6名を、また国立がんセンター中央病院における他に有効な治療を持たない転移性固形腫瘍患者を対象とした第I相臨床試験には計 47 例を登録して試験を継続中である。腎細胞癌、大腸癌の一部などにおいては明らかな腫瘍縮小効果を観察した。班員施設でミニ移植を施行された腎癌患者について検討したところ、移植後180日以内の早期に死亡したのは診断時から転移巣を有する急速進行例や移植時全身状態の悪い患者であった。一方、これらを欠く患者では、長期のGVT 効果が得られることが判明した。付随研究の結果、GVT 効果には特異的免疫と非特異的免疫系の双方が関与する可能性が初めて示され、現在、標的抗原と効果担当細胞の解明を進めている。本臨床研究を遂行する過程で、参加各施設においても臨床試験を推進するための人的基盤作りを積極的に行った。具体的には、若手医師を対象として試験計画書、症例報告書や各種の標準手順書の具体的な作成方法に関する教育会合を開いた。同時に、主要登録施設である国立がんセンター中央病院に研修医あるいは見学者として全国各施設の若手医師やコメディカルを受け入れ、合併症を含めた治療法、患者管理方法や試験管理法を具体的に教示することで治療の均てん化に大きく寄与した。全国各施設からの移植治療に関する長期の任意研修医数は、平成14年から現在に至る期間で21名に及ぶ。

D．考察

本研究では厳正なデータ管理を行うため、本治療法の安全性と有用性を短期間に高い信頼性で検証し、海外を先導する研究成果を示すことが可能となる。これによって、現在は有効な治療法のない多くの患者に対する根治的治療法を開発することは意義深い。同時に、該当領域における我が国の臨床試験基盤を構築し、患者や社会に対する情報公開に努め、治療の均てん化も推進したことで厚生労働行政にも大きく寄与したと考える。付随基礎研究の成果は、今後の特異的免疫療法の開発にも応用可能である。

E．結論

本研究の結果、難治性固形腫瘍に対する同種免疫反応を利用した治療が有効である可能性が示唆された。また、我が国における移植領域の臨床試験体制の基盤整備や均てん化の推進にも寄与した。

F．健康危機情報

現在までのところ、試験の中止に関わるような重大な有害事象の発生は認めていない。医薬品の適応拡大を目的とした医師主導臨床治験を計画・実施するうえでは、安全性情報の取扱いが最も重要な課題となる。このような質の高い臨床試験で得られる安全性情報は、将来的に国内の共有情報としてデータベース化することにより、規制当局への報告の際の重要なデータソースになると共に、我々自身に重要な意味を持って還元される貴重なデータとなり得る。また、これは、抗癌剤を含めた医薬品、医療機器、及び生物由来製品などの臨床開発、適正使用に大きく寄与すると考えられ、海外との情報交換も可能な形式での蓄積が必要不可欠であるため、本研究の重要課題の一つとして取り組む。

G．研究発表

一覧を別添する

H．知的財産権の出願・登録状況

1．特許取得

該当なし

2．実用新案登録

該当なし

3.その他

該当なし

研究成果の刊行に関する一覧

1. Shimokawa T, Ohashi H, Takaue Y, Kawano Y, Abe T, Kuroda Y: Successful umbilical cord blood transplantation in an infant with ALL who failed initial autologous peripheral blood stem cell transplantation. Med Pediatr Oncol, 38:60-61, 2002.
2. Watanabe T, Takaue Y, Kawano Y, Koike K, Kikuta A, Imaizumi M, Watanabe A, Eguchi H, Ohta S, Horikoshi Y, Iwai A, Makimoto A, Kuroda Y: HLA-identical sibling peripheral blood stem cell transplantation in children and adolescents. Biol Blood Marrow Transplant, 8:26-31, 2002.
3. Nakai K, Kanda Y, Mineishi S, Saito T, Ohnishi M, Niiya H, Chizuka A, Takeuchi T, Matsubara H, Kami M, Makimoto A, Tanosaki R, Kunitoh H, Tobinai K, Takaue Y: Suspected delayed immune recovery against cytomegalovirus after allogeneic blood stem cell transplantation with a reduced-intensity regimen. Bone Marrow Transplant, 29:237-241, 2002.
4. Saito T, Kanda Y, Kami M, Shoji N, Kato K, Shoji N, Kanai S, Ohnishi T, Kawano Y, Nakai K, Ogasawara T, Matsubara H, Makimoto A, Tanosaki R, Tobinai K, Wakasugi H, Takaue Y, Mineishi S: Therapeutic potential of a reduced-intensity preparative regimen for allogeneic transplantation with cladribine, busulfan and anti-thymocyte globulin against advanced/refractory acute leukemia/lymphoma. Clin Cancer Res, 8:1014-1020, 2002.
5. Ohnishi M, Kanda Y, Takeuchi T, Kim SW, Hori A, Niiya H, Chizuka A, Nakai K, Saito T, Makimoto A, Tanosaki R, Watanabe T, Kobayashi Y, Tobinai K, Takaue Y, Mineishi S: Limited efficacy of lamivudine against hepatitis B virus infection in allogeneic hematopoietic stem cell transplant recipients. Transplantation, 73:812-815, 2002.
6. Kanda Y, Mineishi S, Saito T, Saito A, Ohnishi M, Niiya H, Chizuka A, Nakai K, Takeuchi T, Matsubara H, Makimoto A, Tanosaki R, Kunitoh H, Tobinai K, Takaue Y: Response-oriented pre-emptive therapy against cytomegalovirus disease with low-dose ganciclovir: a prospective evaluation. Transplantation, 73:568-572, 2002.
7. Akiyama Y, Maruyama K, Mochizuki T, Sasaki K, Takaue Y, Yamaguchi K: Identification of HLA-A24-restricted CTL epitope encoded by the matrix protein pp65 of human cytomegalovirus. Immunol Lett, 83:21-30, 2002.
8. Kami M, Hori A, Takaue Y, Mutou Y: The gastrointestinal tract is a common target of invasive aspergillosis in patients receiving cytotoxic chemotherapy for hematological malignancy. Clin Infect Dis, 35:105-106, 2002.
9. Nagatoshi Y, Kawano Y, Watanabe T, Abe T, Okamoto Y, Kuroda Y, Takaue Y, Okamura J: Hematopoietic and immune recovery after allogeneic peripheral blood stem cell transplantation and bone marrow transplantation in a pediatric population. Pediatric Transplant, 6:319-326, 2002.
10. Kanda Y, Tanosaki R, Nakai K, Saito T, Ohnishi M, Niiya H, Chizuka A, Yakushijin K, Urahama N, Ueda K, Iijima K, Ando T, Matsubara H, Kami K, Makimoto A, Kobayashi Y, Tobinai K, Mineishi S, Takaue Y: Impact of stem cell source and conditioning regimen on erythrocyte recovery kinetics after allogeneic haematopoietic stem cell transplantation from an ABO-incompatible donor. Br J Haematol, 118:128-131, 2002.
11. Hamaki T, Katori H, Kami K, Yamato T, Yamakado H, Itoh T, Kusumi E, Igarashi M, Ueyama J, Kanda Y, Miyakoshi S, Mineishi S, Morinaga S, Mukai M, Hayashi M, Takaue Y, Hara S, Mutou Y: Successful allogeneic blood stem cell transplantation for aplastic anemia in a patient with renal insufficiency requiring dialysis. Bone Marrow Transplant, 30:195-198, 2002.
12. Lee JJ, Takei M, Hori S, Inoue Y, Harada Y, Tanosaki R, Kanda Y, Kami M, Makimoto A, Mineishi S, Kawai H, Shimosaka A, Heike Y, Ikarashi Y, Wakasugi H, Takaue Y, Hwang TJ, Kim HJ, Kakizoe T: The role of PGE2 in the differentiation of dendritic cells: how do dendritic cells influence T cell polarization and chemokine receptor expression ? Stem Cells, 20:448-459, 2002.

13. Nakai K, Mineishi S, Kami M, Kanda Y, Tanosaki R, Takaue Y: Chimerism induction and delayed onset of cytomegalovirus (CMV) infection after allogeneic reduced-intensity stem cell transplantation (RIST). Blood, 100:2674-2675, 2002.
14. Hamaki T, Kami M, Momomura S, Mineishi S, Kusumi E, Kanda Y, Ueyama J, Miyakoshi S, Morinaga S, Takaue Y, Mutou Y: Sustained molecular remission in a patient with CML in blastic crisis receiving dose-reduced hematopoietic stem-cell transplantation followed by early withdrawal of cyclosporine and prophylactic use of interferon-alpha. Am J Hematol, 71:196-199, 2002.
15. Nakai K, Kanda Y, Mineishi S, Hori A, Chizuka A, Niiya H, Tanimoto T, Ohnishi M, Kami M, Makimoto A, Tanosaki R, Matsuno Y, Yamazaki N, Tobinai K, Takaue Y: Primary cutaneous aspergillosis caused by *Aspergillus ustus* following reduced-intensity stem cell transplantation. Ann Hematol, 81:593-596, 2002.
16. Takaue Y: Mini-transplantation strategy for solid tumors. Int J Hematol, 76 Suppl 2:13-14, 2002.
17. Kami M, Kim SW, Takaue Y: Varicella vaccine in recipients of hematopoietic-cell transplants. N Engl J Med, 347:1624-1625, 2002.
18. Kami M, Machida U, Okuzumi K, Matsumura T, Mori S, Hori A, Kashima T, Kanda Y, Takaue Y, Sakamaki H, Hirai H, Yoneyama A, Mutou Y: Effect of fluconazole prophylaxis on fungal blood cultures: an autopsy-based study involving 720 patients with hematological malignancy. Br J Haematol, 117:40-46, 2002.
19. Nakai K, Mineishi S, Kami M, Kanda Y, Tanosaki R, Takaue Y: The feasibility of reduced-intensity allogeneic hematopoietic stem cell transplantation from a related donor with HLA one-antigen with or without one-allele mismatch. Haematologica, 88:115-117, 2003.
20. Watanabe T, Suzuya H, Onishi T, Kanai S, Kaneko M, Watanabe H, Nakagawa R, Kawano Y, Takaue Y, Kuroda Y, Talmadge JE: Effect of granulocyte colony-stimulating factor on bone metabolism during peripheral blood stem cell mobilization. Int J Hematol, 77:75-81, 2003.
21. Matsubara H, Takayama J, Makimoto A, Higa T, Kawamoto H, Ohira M, Yokoyama R, Beppu Y, Takaue Y: Possible benefits of high-dose chemotherapy as intensive consolidation in patients with high-risk rhabdomyosarcoma who achieve complete remission with conventional chemotherapy. Pediatr Hematol Oncol, 20: 201-210, 2003.
22. Kim SW, Kami M, Urahama N, Yamamoto R, Hori A, Imataki O, Kanda Y, Tanosaki R, Mineishi S, Takaue Y, Honda O: Feasibility of Acute Physiology and Chronic Health Evaluation (APACHE) II and III score-based screening in patients receiving allogeneic hematopoietic stem cell transplantation. Transplantation, 75:566-570, 2003.
23. Kami M, Hamaki T, Miyakoshi S, Murashige N, Kanda Y, Tanosaki R, Takaue Y, Taniguchi S, Hirai S, Ozawa K, Kasai M: Allogeneic hematopoietic stem-cell transplantation for the treatment of adult T-cell leukemia/lymphoma. Br J Haematol, 120:304-309, 2003.
24. Matsubara H, Makimoto A, Higa T, Kawamoto H, Kanda Y, Kami M, Tanosaki R, Mineishi S, Ohira M, Takaue Y: Successful treatment of meningoencephalitis caused by methicillin-resistant *Staphylococcus aureus* with intrathecal vancomycin in an allogeneic peripheral blood stem cell transplant recipient. Bone Marrow Transplant, 31:65-67, 2003.
25. Kato K, Ikarashi Y, Sugahara T, Yasumoto A, Sancho D, Yoshida M, Takaue Y, Kobayashi Y, Sanchez-Madrid F, Wakasugi H: U5A2-13, an antigen originally found on mouse NK-like T cells, is an early inducible cell surface antigen during lymphoid activation. Cell Immunol, 221:27-36, 2003.
26. Asada-Mikami R, Heike Y, Harada Y, Kanai S, Ikarashi Y, Kato K, Shirakawa K, Takaue T, Abe T, Wakasugi H: Increased expansion of Vα24+ T cells derived from G-CSF-mobilized peripheral blood stem cells as compared to peripheral blood mononuclear cells following α-galactosylceramide stimulation. Cancer Sci, 94:383-388, 2003.

27. Mineishi S, Kanda Y, Saito T, Nakai K, Makimoto A, Masahiro Kami M, Tanosaki R, Wakasugi H, Tobinai K, Takaue Y: Impact of GVHD in reduced-intensity stem cell transplantation for patients with hematological malignancies. Br J Hematol, 121:296-303, 2003.
28. Tanimoto T, Kusumi E, Hamaki T, Ueyama J, Miyakoshi S, Morinaga S, Kami M, Kanda Y, Ando T, Yoshihara S, Kim SW, Nakai K, Tobinai K, Tanosaki R, Mineishi S, Takaue Y, Muto Y: High complete response rate after allogeneic hematopoietic stem cell transplantation with reduced-intensity conditioning regimens in advanced malignant lymphoma. Bone Marrow Transplant, 32:131-137, 2003.
29. Murashige N, Kami M, Takaue Y: ZAP-70 in chronic lymphocytic leukemia. N Engl J Med, 349:506-507, 2003.
30. Kojima R, Kami M, Kim SW, Murashige N, Kishi Y, Hori A, Imataki O, Hamaki T, Sakiyama M, Arima F, Heike Y, Tanosaki R, Mineishi S, Takaue Y: Induction of graft-versus-autoimmune disease (GVA) effect against refractory psoriasis by complete donor-type chimerism and graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant, 32:439-442, 2003.
31. Saito T, Kanda Y, Kato K, Nakai K, SW Kim, Arima F, Kami M, Tanosaki R, Tobinai K, Wakasugi H, Heike Y, Mineishi S, Takaue Y: Immune reconstitution following reduced-intensity transplantation with cladribine, busulfan, and anti-thymocyte globulin: serial comparison with conventional myeloablative transplantation. Bone Marrow Transplant, 32:601-608, 2003.
32. Urahama N, Tanosaki R, Kami M, Iijima K, Chizuka A, Kim SW, Hori A, Kojima R, Imataki O, Makimito A, Mineishi S, Takaue Y: TRALI after the infusion of marrow cells in a patient with acute lymphoblastic leukemia. Transfusion, 43:1553-1557, 2003.
33. Yamamoto R, Kusumi E, Kami M, Yuji K, Hamaki T, Saito A, Murashige N, Hori A, Kim SW, Makimoto A, Ueyama J, Tanosaki R, Miyakoshi S, Mori S, Morinaga S, Heike Y, Taniguchi S, Masuo S, Takaue Y, Muto Y: Late hemorrhagic cystitis after reduced-intensity hematopoietic stem cell transplantation. Bone Marrow Transplant, 32:1089-1095, 2003.
34. Kami M, Takaue Y: Development of reduced-intensity hematopoietic stem cell transplantation (RIST) in the National Cancer Center Hospital, Japan. Therapeutic Apheresis Dialysis, 7:318-328, 2003.
35. Hamaki T, Kami M, Igarashi M, Kusumi E, Arase Y, Ishibashi T, Shimamura K, Miyakoshi S, Morinaga S, Takaue Y, Hayashi M, Mutou Y: Non-myeloablative hematopoietic stem-cell transplantation for the treatment of adult T-cell lymphoma in a patient with advanced hepatic impairment. Leuk Lymphoma, 44:703-708, 2003.
36. Murashige N, Kami M, Takaue Y: Reviparin after leg injury requiring immobilization. N Engl J Med, 348:1061-1063, 2003.
37. Imataki O, Kim SW, Kojima R, Hori A, Hamaki T, Sakiyama M, Murashige N, Satoh M, Kami M, Takaue Y: Life-threatening hypothyroidism associated with administration of cyclosporine in a patient treated with reduced-intensity hematopoietic stem cell transplantation for metastatic renal cell carcinoma. Transplantation, 75:898-907, 2003.
38. Nakai K, Mineishi S, Kami M, Saito T, Kamijo K, Hori A, Yamamoto R, Imataki O, Hamaki T, Yoshihara S, Ohnishi M, Kim SW, Ando T, Matsubara H, Kanda Y, Makimoto A, Tanosaki R, Kanai S, Heike Y, Tobinai K, Ohnishi T, Kawano Y, Wakasugi H, Takaue Y: Anti-thymocyte globulin affects the occurrence of acute and chronic graft-versus-host disease after a reduced-intensity conditioning regimen by modulating mixed chimerism induction. Transplantation, 75:2135-2143, 2003.
39. Kim SW, Kami M, Kobayashi K, Takaue Y, Honda O: Selective decontamination of digestive tract in intensive care. Lancet, 362:2118-2120, 2003.

40. Kusumi E, Miyakoshi S, Murashige N, Hamaki T, Kim SW, Yuji K, Kami M, Ueyama J, Morinaga S, Takaue Y, Muto Y: Successful reduced-intensity stem cell transplantation (RIST) with mismatched cord blood in a 70-year-old patient with severe aplastic anemia. Bone Marrow Transplant, 32:1111-1112, 2003.
41. Murashige N, Kami M, Takaue Y: G-CSF priming in acute myelogenous leukemia. N Engl J Med, 349:2071-2072, 2003.
42. Zheng Y, Watanabe N, Nagamura-Inoue T, Igura K, Nagayama H4, Tojo A, Tanosaki R, Takaue Y, Okamoto S, Takahashi TA: Ex vivo manipulation of umbilical cord blood-derived hematopoietic stem/progenitor cells with recombinant human stem cell factor can up-regulate levels of homing-essential molecules to increase their transmigratory potential. Exp Hematol, 31:1237-1246, 2003.
43. Hori S, Heike Y, Takei M, Maruyama M, Inoue Y, Lee JJ, Kim HJ, Harada Y, Kawai H, Shimosaka A, Kami M, Tanosaki MD, Wakasugi H, Saito S, Takaue Y, Kakizoe T: Freeze-thawing procedures have no influence on the phenotypic and functional development of dendritic cells generated from peripheral blood CD14+ monocytes. J Immunother, 27:27-35, 2004.
44. Hori A, Kami M, Kim SW, Murashige N, Sakiyama M, Kojima R, Hamaki T, Makimoto A, Miyakoshi S, Masuo S, Taniguchi S, Kunitoh H, Takaue Y: Urgent need for a validated tumor response evaluation system for use in immunotherapy. Bone Marrow Transplant, 33:255-256, 2004.
45. Kawano Y, Chan LL, Watanabe T, Abe T, Okamoto Y, Makimoto A, Nakagawa R, Watanabe H, Suzuya H, Takaue Y: Cryopreservation of mobilized blood stem cells at a higher cell concentration without the use of a programmed freezer. Ann Hematology, 83:50-54, 2004.
46. Yamasaki S, Ohno Y, Taniguchi S, Yoshida T, Hayashi S, Ogawa H, Shimazaki C, Takahashi S, Kasai M, Wake A, Nishimura M, Tokunaga K, Gondo H, Takaue Y, Harada M, Mineishi S: Allogeneic peripheral blood stem cell transplantation from 2- or 3-loci-mismathced related donors in adult Japanese patients with high-risk hematologic malignancies. Bone Marrow Transplant, 33:279-289, 2004.
47. Kamitsuji Y, Mori S, Kami M, Yamada H, Shirakawa K, Kishi Y, Murashige N, Kim SW, Heike Y, Takaue Y: Fatal deep vein thrombosis after allogeneic reduced-intensity hematopoietic stem-cell transplantation for the treatment of metastatic gastric cancer. Ann Hematol, 83:533-535, 2004.
48. Harashima N, Kurihara K, Utunomiya A, Tanosaki R, Hanabuchi S, Masuda M, Ohashi T, Fukui F, Hasegawa A, Masuda T, Takaue Y, Okamura J, Kannagi M: Graft-versus-human T-cell leukemia virus type-I response in adult T-cell leukemia patients following non-myeloablative hematopoietic stem cell transplantation. Cancer Res, 64:391-399, 2004.
49. Hori A, Kami M, Kim SW, Chizuka A, Kojima R, Imataki O, Sakiyama M, Hamaki T, Onishi Y, Usubuchi N, Kishi Y, Murashige N, Tajima K, Miyakoshi S, Heike Y, Masuo S, Taniguchi S, Takaue Y: Development of early neutropenic fever, with or without bacterial infection, is still a significant complication after reduced-intensity stem cell transplantation. Biol Blood Marrow Transplant, 10:65-72, 2004.
50. Mori T, Mori S, Kanda Y, Yakushiji K, Mineishi S, Takaue Y, Gondo H, Harada M, Sakamaki H, Yajima T, Iwao Y, Hibi T, Okamoto S: Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant, 33:431-434, 2004.
51. Kusumi E, Kami M, Yuji K, Hamaki T, Murashige N, Kim SW, Heike Y, Ueyama J, Miyakoshi S, Tanosaki R, Morinaga S, Mineishi S, Muto Y, Takaue Y: Feasibility of reduced-intensity hematopoietic stem cell transplantation from an HLA-matched unrelated donor. Bone Marrow Transplant, 33:697-702, 2004.
52. Hamaki T, Kami M, Kim SW, Onishi Y, Kishi Y, Murashige N, Hori A, Kojima R, Sakiyama M, Imataki O, Heike Y, Tanosaki R, Masuo S, Miyakoshi S, Taniguchi S, Tobinai K, Takaue Y:

Reduced-intensity stem cell transplantation from an HLA-identical sibling donor in patients with myeloid malignancies. Bone Marrow Transplant, 33:891-900, 2004.

53. Lee JJ, Nam CE, Nam JH, Lee HC, Chung IJ, Park MS, Choi BH, Song WH, Lee IK, Park KS, Kook H, Hwang TJ, Takei M, Takaue Y, Kim HJ: Generation of cytotoxic donor CD8+ T cells against relapsing leukemic cells following allogeneic transplantation by stimulation with leukemic cell- or leukemic lysate pulsed donor cell-derived dendritic cells. Leuk Res, 28:517-524, 2004.
54. Shimizu A, Sasaki H, Aoyagi K, Yoshida M, Kato K, Heike Y, Ikarashi Y, Shirakawa K, Takaue Y, Miyajima A, Terada M, Nagai H, Wakasugi H: The mouse natural killer T cell-associated antigen recognized by U5A2-13 monoclonal antibody is intercellular adhesion molecule-1. Immunol Lett 92:227-235, 2004.
55. Imataki O, Kim SW, Kami M, Gotoh M, Komaba S, Kasai M, S Hashino S, Naito K, Masuda M, Anan K, Teshima H, Togitani K, Inoue T, Nishimura M, Adachi Y, Fukuhara T, Yamashita T, Uike N, Kobayashi Y, Hamaguchi M, Higuchi M, Kawakami K, Takaue Y: A nationwide survey of deep fungal infections and fungal prophylaxis after hematopoietic stem cell transplantation in Japan. Bone Marrow Transplant, 33:1173-1179, 2004.
56. Tanimoto TE, Yamaguchi T, Tanaka Y, Saito A, Tajima K, Karasuno T, Kasai M, Kishi K, Mori T, Maseki N, Morishima S, Miyakoshi S, Kasai M, Ohno Y, Kim SW, Numata A, Kami M, Takaue Y, Mori S, Harada M: Comparative analysis of clinical outcomes after allogeneic bone marrow transplantation versus peripheral blood stem cell transplantation from a related donor in Japanese patients. Br J Haematol, 125:480-493, 2004.
57. Hori A, Kami M, Kim SW, Makimoto A, Tanosaki R, Takaue Y: Balance between acute graft-versus-host disease and graft-versus-tumor effect after reduced-intensity transplantation for metastatic renal cell carcinoma. Hematol J, 5:276-278, 2004.
58. Miyakoshi S, Yuji K, Kami M, Kusumi E, Kishi Y, Kobayashi K, Murashige N, Hamaki T, Kim SW, Ueyama J, Mori S, Morinaga S, Muto Y, Masuo S, Kanemaru M, Hayashi T, Takaue Y, Taniguchi S: Successful engraftment after reduced-intensity umbilical cord blood transplantation for adult patients with advanced hematological diseases. Clin Cancer Res, 10:3586-3592, 2004.
59. Sekiguchi N, Nishimoto J, Tanosaki R, Kubota N, Yokota Y, Kobayashi Y, Watanabe T, Kami M, Takaue Y, Matsuno Y, Tobinai K: EBV-positive Burkitt lymphoma as a late-onset posttransplantion lymphoproliferative disorder after allogeneic stem cell transplantation. Int J Hematol, 79:387-389, 2004.
60. Nakagawa T, Kami M, Hori A, Kim SW, Murashige N, Hamaki T, Kishi Y, Fujimoto H, Matsuoka N, Okajima E, Komiyama M, Tobisu K, Wakayama T, Uike N, Tajima K, Makimoto A, Mori S, Tanosaki R, Takaue Y, Kakizoe T: Allogeneic hematopoietic stem cell transplantation with a reduced-intensity conditioning regimen for the treatment of metastatic renal cell carcinoma: single institution experience with a minimum 1-year follow-up. Exp Hematol, 32:599-606, 2004.
61. Kishi Y, Miyakoshi S, Kami M, Ikeda M, Katayama Y, Murashige N, Kusumi E, Yuji K, Kobayashi K, Kato D, Hamaki T, Matsumura T, Kim SW, Morinaga S, Mori S, Kanemaru N, Hayashi T, Takaue Y, Taniguchi S: Early central nervous system complications after reduced-intensity stem cell transplantation. Biol Blood Marrow Transplant, 10:561-568, 2004.
62. Kojima R, Kami M, Nannya Y, Kusumi E, Sakai M, Tanaka Y, Kanda Y, Mori S, Chiba S, Miyakoshi S, Tajima K, Hirai H, Taniguchi S, Sakamaki H, Takaue Y: Incidence of invasive aspergillosis after allogeneic hematopoietic stem cell transplantation with a reduced-intensity regimen compared with transplantation with a conventional regimen. Biol Blood Marrow Transplant, 10:645-652, 2004.
63. Sakiyama M, Kami M, Hori A, Imataki O, Hamaki T, Murashige N, Kobayashi K, Kishi Y, Kojima R, Kim SW, Kusumi E, Yuji K, Miyakoshi S, Mori S, Tanosaki R, Taniguchi S, Takaue Y: Regimen-related toxicity following reduced-intensity stem-cell transplantation: comparison between

Seattle criteria and National Cancer Center Common Toxicity Criteria (NCI-CTC) version 2.0. Bone Marrow Transplant, 34:787-794, 2004.

64. Kami M, Makimoto A, Heike Y, Takaue Y: Reduced-intensity hematopoietic stem cell transplantation (RIST) for solid malignancies. Jap J Clin Oncol, 34:707-716, 2004.
65. Hori A, Kanda Y, Goyama S, Onishi Y, Komeno Y, Mitani K, Kishi Y, Ogawa S, Imataki O, Chiba S, Kojima R, Hamaki T, Sakiyama M, Kami M, Makimoto A, Tanosaki R, Takaue Y, Hirai H: A prospective trial to evaluate the safety and efficacy of pravastatin for the treatment of refractory chronic graft-versus-host disease. Transplantation, 79:372-374, 2004.
66. Miyakoshi S, Kami M, Kishi Y, Murashige N, Yuji K, Kusumi E, Matsumura T, Onishi Y, Kobayashi K, Kim SW, Hamaki T, Takaue Y, Taniguchi S: Fatal hepatic failure associated with graft rejection following reduced-intensity stem-cell transplantation for chronic idiopathic myelofibrosis (CIMF). Leuk Lymphoma, 45:2513-2516, 2004.
67. Kojima R, Kami M, Hori A, Murashige N, Ohnishi M, Kim SW, Hamaki T, Kishi Y, Tsutsumi Y, Masauzi N, Heike Y, Mori S, Kobayashi K, Masuo S, Tanosaki R, Takaue Y: Reduced-intensity allogeneic hematopoietic stem-cell transplantation as an immunotherapy for metastatic colorectal cancer. Transplantation, 78:1740-1746, 2004.
68. Kishi Y, Kami M, Murashige N, Tanaka Y, Fujisaki G, Kusumoto S, Mori S, Takaue Y, Tanosaki R: Hyperacute GVHD and emergence of peripheral CD3+CD56+ T-cells and activated natural killer cells are useful markers for early diagnosis of post-transplant hemophagocytic syndrome. Bone Marrow Transplant, 35:415-417, 2005.
69. Hori A, Kanda Y, Goyama S, Onishi Y, Komeno Y, Mitani K, Kishi Y, Ogawa S, Imataki O, Chiba S, Kojima R, Hamaki T, Sakiyama M, Kami M, Makimoto A, Tanosaki R, Takaue Y, Hirai H; Japan Hematology and Oncology Clinical Study Group. A prospective trial to evaluate the safety and efficacy of pravastatin for the treatment of refractory chronic graft-versus-host disease. Transplantation., 79:372-374, 2005.
70. Chizuka A, Kami M, Kanda Y, Murashige N, Kishi Y, Hamaki T, Kim SW, Hori A, Kojima R, Mori SI, Tanosaki R, Gomi H, Takaue Y: Value of surveillance blood culture for early diagnosis of occult bacteremia in patients on corticosteroid therapy following allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant, 35:577-582, 2005.
71. Akiyama Y, Tanosaki R, Inoue N, Shimada M, Hotate Y, Yamamoto A, Yamazaki N, Kawashima I, Nukaya I, Takesako K, Maruyama K, Takaue Y and Yamaguchi K: Clinical response in Japanese metastatic melanoma patients treated with peptide cocktail-pulsed dendritic cells. J Transl Med, 3:4, 2005.
72. Kobayashi K, Kami M, Ikeda M, Kishi Y, Murashige N, Tanosaki R, Mori S, Takaue Y: Fulminant septicemia caused by Bacillus cereus following reduced-intensity umbilical cord blood transplantation. Haematologica, 90:ECR06, 2005.

Bone Marrow Transplantation (2004) 33, 255–256

www.nature.com/bmt

Correspondence

Urgent need for a validated tumor response evaluation system for use in immunotherapy

Bone Marrow Transplantation (2004) 33, 255–256.
doi:10.1038/sj.bmt.1704371
Published online 15 December 2003

Hentschke *et al* recently reported a detailed case series on reduced-intensity stem-cell transplantation (RIST) for the treatment of renal cell and colon cancers.[1] While they provided important information on the feasibility of RIST and its possible antitumor effect, we would like to comment on their study design, especially focusing on the feasibility of response evaluation criteria. Although the Response Evaluation Criteria in Solid Tumors (RECIST) system has been used as a gold standard to evaluate the response of solid tumors to treatment,[2] mainly in the field of cancer chemotherapy, it has not been fully validated in the area of allogeneic transplantation for solid tumors, where the immune-mediated destruction of tumor cells is the principle mechanism of tumor destruction (graft-versus-tumor effect, GVT). Compared to hematological malignancies, solid tumors are generally more resistant to the cytotoxic agents used in conditioning regimens administered before transplantation. Consequently, we considered that there may be some important differences in evaluating the response of solid tumors between RIST and conventional chemotherapy.

First, the feasibility of directly applying RECIST, including the optimal timing of response evaluation, should be critically validated before its extensive application in transplantation. Currently available reports on RIST for solid tumors commonly note that tumor regression occurs several months after transplantation.[3] Some responses and GVHD effects, in general, occur during the late period of RIST. Thus, most tumors continue their natural growth until the manifestation of effective alloimmunity to restrain tumor growth. If the original RECIST criteria[2] are applied to patients undergoing RIST for solid tumors, most of the GVT effects would be evaluated as progressive disease (PD), which would preclude subsequent evaluation (Figure 1a). Therefore, RECIST may underestimate the efficacy of RIST. Furthermore, while there is no concept of spontaneous regression in the field of chemotherapy, this is quite common in RIST.

Second, the proper time to measure the tumor size as a baseline for evaluating a subsequent tumor response has not been clearly defined. In contrast to the results with chemotherapy, the tumor often temporarily increases in size following RIST. Some metastases initially progress slowly, while others progress rapidly. Accordingly, when the size at transplantation is used as a baseline, as in chemotherapy, a therapeutic effect following the initial progression could be overlooked or underestimated (Figure 1c). On the other hand, evaluating regression from the largest size after transplant certainly overestimates the effect of treatment (Figure 1b), and gives an unacceptable bias.

Third, the tumor size after RIST often fluctuates in response to a *de novo* GVT effect, post transplant immunotherapy including donor lymphocyte infusion, and adjustment of immunosuppressive agents (Figure 2). In this situation, it is clear that any evaluation of the response duration, such as progression-free survival and the overall response duration, is essentially impossible using the current RECIST criteria.

These limitations in tumor response evaluation are also expected to be present in other areas including tumor vaccination and dendritic cell therapy strategy. Improved overall survival will ultimately be evaluated in phase III trials. To reach this point, a global standard evaluation system that enables the effective screening of a therapeutic effect in an earlier phase II study will need to be established. We hope that this letter will inspire a productive discussion.

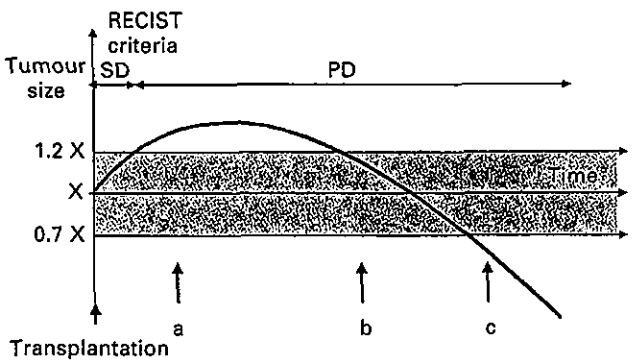

Figure 1 Course of tumor size after transplantation. Primary solid tumors are progressive, despite chemoradiotherapy prior to transplantation. (a) Most tumors continue their natural growth until the development of a GVT effect, which usually occurs several months after transplantation. (b) If the tumor has increased in size compared to that at the time of transplant, regression from the largest size may overestimate the treatment effect. (c) If the tumor size at transplant is defined as a baseline, some treatment effects, observed in patients whose lesions show initial progression followed by regression with the development of GVHD, will be underestimated.

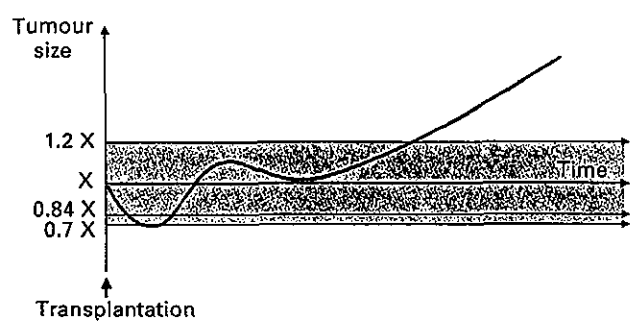

Figure 2 Fluctuation of tumor size after donor lymphocyte infusion or adjustment of immunosuppressive agents. It is difficult to handle patients in whom the tumor size fluctuates in response to post transplant immunotherapy, such as donor lymphocyte infusion and adjustment of immunosuppressive agents. Neither an appropriate timing of response evaluation nor an appropriate time to measure a baseline tumor size has been established in these cases.

A Hori[1]
M Kami[1]
S-W Kim[1]
N Murashige[1]
M Sakiyama[1]
R Kojima[1]
T Hamaki[1]
A Makimoto[1]
S Miyakoshi[2]
S Masuo[3]
S Taniguchi[2]
H Kunitoh[4]
Y Takaue[1]

[1]*Hematopoietic Stem Cell Transplant Unit, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku, Tokyo 100-0045, Japan;* [2]*Department of Hematology, Toranomon Hospital, Tokyo, Japan;* [3]*Department of Hematology and Rheumatology, JR Tokyo General Hospital, Tokyo, Japan; and* [4]*Department of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan*

References

1 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; **31**: 253–261.
2 Therasse P, Arbuck SG, Eisenhauer EA *et al.* New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205–216.
3 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; **343**: 750–758.

Ann Hematol (2004) 83:50–54
DOI 10.1007/s00277-003-0817-8

ORIGINAL ARTICLE

Yoshifumi Kawano · Chan Lee Lee ·
Tsutomu Watanabe · Takanori Abe · Hiroko Suzuya ·
Yasuhiro Okamoto · Atsushi Makimoto ·
Ryuji Nakagawa · Hiroyoshi Watanabe ·
Yoichi Takaue

Cryopreservation of mobilized blood stem cells at a higher cell concentration without the use of a programmed freezer

Received: 19 August 2003 / Accepted: 18 October 2003 / Published online: 13 November 2003

Abstract Cryopreservation of peripheral blood stem cells (PBSC) mobilized by chemotherapy combined with or without granulocyte colony-stimulating factor (G-CSF) is an essential part of procedure for anti-cancer strategies. We evaluated whether a higher cell concentration (2×10^8/ml) without the use of a programmed freezer was acceptable for the storage of mobilized PBSC in an autologous setting. Mobilized PBSC were enriched to mononuclear cells (MNC) by Percoll separation and then frozen at cell concentrations of $2–5\times10^7$/ml (group I, n=20) or 2×10^8/ml (group II, n=44) without the use of a programmed freezer using 5% DMSO, 6% hydroxy ethyl starch, and 4% autologous serum or human albumin. CD34+ cells purified by ISOLEX300 were frozen at 2×10^7/ml (group III, n=22) using the same method. The median recovery rates of CD34+ cells and CFU-GM were, respectively, n.d. (not determined) and 88% in group I, 103 and 64% in group II, and 98 and 53% in group III. There was a statistical significance between the recovery rate of CFU-GM in group III and that in group I (p=0.02). The median percentage of cell viability after thawing in each group was 89, 87, and 75%, respectively. The median numbers of days after PBSCT to achieve a WBC of $>1.0\times10^9$/l, an absolute neutrophil count of $>0.5\times10^9$/l, and a platelet count of $>50\times10^9$/l were, respectively, 11, 11 and 15 in group I; 12, 12 and 16 in group II; and 12, 12 and 27 in group III. These results suggest that enriched MNC from mobilized PBSC could be frozen at a higher cell concentration (2×10^8/ml) without the use of a programmed freezer, leading to reduction of the toxicities associated with infusion of thawed cells and of costly space required for cell storage.

Keywords Mobilized cells · Cryopreservation · Autologous · Cell concentration

Y. Kawano (✉)
Department of Pediatrics,
Graduate School of Medical and Dental Sciences,
Kagoshima University,
8-35-1 Sakuragaoka, 890-8520 Kagoshima, Japan
e-mail: ykawano@m3.kufm.kagoshima-u.ac.jp
Fax: +81-99-2657196

Y. Kawano · T. Watanabe · T. Abe · H. Suzuya · Y. Okamoto · A. Makimoto · R. Nakagawa · H. Watanabe · Y. Takaue
Department of Pediatrics,
University of Tokushima,
Tokushima, Japan

C. L. Lee
Department of Pediatrics,
University of Malaya,
Kuala Lumpur, Malaysia

Y. Takaue
Stem Cell Transplant Unit,
National Cancer Center Hospital,
Tokyo

Introduction

Peripheral blood stem cell transplantation (PBSCT) has replaced autologous marrow transplantation in the treatment of various types of cancers [1].Improvements in mobilization methods with recombinant cytokines enable us to collect larger numbers of PBSC. Harvested peripheral blood stem cells (PBSC) are also expected to be a source of targeting cells for various types of future cell therapies.

Cryopreservation of PBSC is essential in an autologous setting. It has been previously reported that cell concentration (2×10^7/ml) and rate control (−1°C/min) are critical in cell freezing procedure [2]. In case of PBSCT, the traditionally recommended concentration of 2×10^7/ml would result in large product volumes with correspondingly increased amounts of cryoprotectant, which could be toxic for the recipients [3, 4, 5]. On the other hand, rate-controlled freezing with a programmed freezer is expensive as a routine clinical procedure. To make this procedure simpler and more economical, a non-rate-controlled freezing method has been reported by several

investigators [6, 7, 8].It has been shown that rate-controlled cryopreservation with a programmed freezer at a higher cell concentration does not impair the post-thaw recovery of hematopoietic stem/progenitor cells [9].

However, the effect of cryopreservation at a higher cell concentration without the use of a programmed freezer on autologous PBSCT has been tested very little [10]. In this study, we compared the recovery of CFU-GM, CD34+ cells and engraftment kinetics of cryopreserved products, which were frozen without the use of a programmed freezer at low and higher cell concentrations.

Methods and materials

Subjects

Patients who underwent PBSC collection and autologous PBSCT from January 1992 to December 1999 in the Department of Pediatrics, University of Tokushima, were enrolled into this study after obtaining consent. The study subjects consisted of patients with acute lymphoblastic leukemia (ALL, n=26), acute nonlymphocytic leukemia (ANLL, n=11), non-Hodgkin's lymphoma (NHL, n=3), and various solid tumors (n=46). The solid tumors included neuroblastoma (n=13), brain tumor (10), Wilms tumor (6), ovarian cancer (5), breast cancer (4), retinoblastoma (2), rhabdomyosarcoma (2), testicular tumor (2), peripheral neuroectodermal tumor (1), and juvenile rheumatoid arthritis (1). Of 86 patients, 42 were male and 44 were female. Their ages ranged from 1 to 56 years, with a median age of 10 years.

Mobilization and collection of PBSC

PBSC were mobilized by intensive chemotherapy with or without recombinant human granulocyte colony-stimulating factor (G-CSF) as previously reported [11].They were collected in the recovery phase of chemotherapy with a Baxter CS3000 plus continuous-flow blood cell separator (Baxter Healthcare, Deerfield, IL, USA) [12].Collection was performed on the day the patient achieved a WBC of $>3\times10^9$/l and a platelet count of $>100\times10^9$/l in the recovery phase after chemotherapy. In some patients, PBSC were mobilized by G-CSF alone [13].In these cases, patients received 10 μg/kg of G-CSF once a day by subcutaneous injection for 5 days.Apheresis was initiated from days 4 to 6 after G-CSF injection, and 200–300 ml/kg (max. 10 liters) were processed per session.

Cryopreservation and thawing procedures

Apheresis-collected cells were separated using discontinuous gradients of 40 and 60% Percoll and centrifugation [14].Cells were resuspended in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% autologous serum, as previously reported. The freezing method reported by Makino et al. was introduced with minor modifications from the beginning of this study [7].Briefly, Percoll-separated cells were resuspended in DMEM with 10% autologous serum or 8% human albumin (Albumin Yoshitomi, Yoshitomi Pharmaceutical, Osaka) and mixed slowly with an equal volume of freezing solution containing 12% HES and 10% DMSO to give final concentrations of 5% DMSO and 6% HES. Prior to May 1995, PBSC were cryopreserved at concentrations of $2–5\times10^7$/ml (Group I, n=20). Purified CD34+ cells were frozen at 2×10^7/ml using the same method (Group III, n=22). Subsequently, a concentration of 2×10^8/ml was adopted (Group II, n=44). In all methods, cells were transferred to 5-ml polypropylene cryo-tubes (MS4605 W, Sumitomo Bakelite, Osaka), placed directly into a −80°C electric freezer, and then transferred to −135°C on the following day. The cells were stored in the same freezer until use.

Cells were thawed rapidly in a water bath maintained at 37°C. We divided stored cells into two portions that were infused over 2 days, when the volume of cells suspension was >300 ml [15]. For recovery analysis, an aliquot of cell suspension was quickly transferred to a 50-ml tube and diluted with thawing medium consisting of 10% fetal bovine serum (FBS, Filtron, Brooklyn, Australia) and 50 IU/ml of deoxyribonuclease (Sigma DN-25, Aldrich Japan, Tokyo) in DMEM by the stepwise addition of this medium at room temperature with gentle agitation. The cells were then collected by centrifugation, washed three times with the thawing medium, and resuspended in DMEM supplemented with 10% FBS for further experiments. Trypan blue staining method was used to measure cell viability.

CD34+ cell purification

G-CSF-mobilized PBSC collected by apheresis were enriched for CD34+ cells using an ISOLEX-300 (Baxter Healthcare, Deerfield, IL, USA) according to the manufacturer's suggestions. Briefly, excess platelets were removed by centrifugation for 20 min at 200×G at room temperature. Cells were incubated in phosphate-buffered saline (PBS, Nissui, Tokyo) containing 0.5% human-globulin (Gammagard, Baxter Japan, Tokyo) for 15 min to block Fc-receptors. One vial of anti-CD34 monoclonal antibody (9C5, 2 mg) was added to the cell suspension that contained $<5\times10^{10}$ cells. After 30 min of incubation at room temperature with gentle rotation (4/min), cells were washed three times with PBS containing 1% human serum albumin (Albumin-Yoshitomi, Yoshitomi Pharmaceutical, Osaka). Sensitized cells were incubated with sheep anti-mouse IgG_1-coated paramagnetic microspheres (Dynabeads, 10 ml; Dynal, Oslo). Cells rosetted with beads were captured on permanent magnets, and released by chymopapain or peptide capture included in the kit. These cells were frozen by the same method as described above.

Flow cytometry

CD34+ cells were assayed by Otsuka Assay Institute (Tokyo). Sample cells were shipped by air-cargo and assayed within 24 h. Cells that expressed the surface CD34 antigen were identified by flow cytometry analysis. Briefly, 100 μl of cell suspension were added to a test tube (Falcon 2052, Becton Dickinson, Lincoln Park, NJ, USA) containing isotype control (phycoerythrin-mouse IgG_1) and phycoerythrin-conjugated CD34 monoclonal antibody (Anti-HPCA2 antibody, Becton Dickinson) at a concentration of 1 μg antibody/10^6cells. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson). After function was verified, samples were drawn into the flow cytometer using FSC and SSC, as gating parameters, along with debris subtraction techniques to determine the characteristics of the cells. A total of 20,000 events were counted to identify the mononuclear cell fraction. The flow cytometric data were analyzed using a gated analysis via a set of SSC-FL parameters for CD34+ cells to calculate the percentage of positive cells. When a sample was substantially contaminated with RBC, it was lysed with a solution consisting of 0.826% (w/v) NH_4CL, 0.1% $KHCO_3$, and 0.004% EDTA-4Na.

Hematopoietic progenitor assay

Colony-forming cells were incubated in methylcellulose cultures supplemented with 20% FBS, 450 μg/ml of human transferrin (Sigma T-1147), 2 U/ml of recombinant human erythropoietin (Kirin Brewery, Tokyo), 1% deionized delipidated BSA (Calbiochem 12657, Hoechst Japan, Tokyo), and a combination of recombinant human G-CSF (filgrastim, Kirin), interleukin-3 (Kirin), and stem cell factor (Kirin). These stimulating factors were used at a final concentration of 20 ng/ml, which was the

previously determined optimal concentration in our laboratory. Triplicate or quadruplicate cultures were plated in volumes of 0.4 ml in 24-well tissue culture plates (Corning 258201, New York, NY) that were then placed in an ESPEC N_2-O_2-CO_2 BNP-110 incubator (Tabai ESPEC, Osaka, Japan), which maintained a humid atmosphere of 5% carbon dioxide, 5% oxygen, and 90% nitrogen at 37°C. Plates were incubated for 13–15 days and three types of colonies, including colony-forming unit for granulocyte-macrophage (CFU-GM), were counted using an inverted microscope. The mean number of colonies in four wells was calculated.

Transplant procedures

The transplant procedures in our institute have been previously described in detail [16]. Briefly, frozen cells were thawed rapidly in a water bath maintained at 37°C. The patients were given 5 mg/kg of hydrocortisone and/or antihistamines to prevent allergic reactions before infusion. The recovery speed after autografting was evaluated in terms of the number of days to achieve a WBC of $>1\times10^9$/l, an absolute neutrophil count (ANC) of $>0.5\times10^9$/l, and a platelet count of $>50\times10^9$/l. G-CSF was given to the patients only in group III after autografting.

Statistics

The Mann Whitney U-test was used to analyze the significance of differences. Data were analyzed using StatView (Version 4.5; Abacus Concepts, Berkeley, CA, USA) for a Macintosh computer.

Results

Frozen cells

After Percoll separation (groups II and I) or the purification procedure (group III), the median (range) numbers of frozen cells per kilogram of recipient body weight were determined, as shown in Table 1. MNC in group III (median, 2.6×10^6/kg) was significantly lower than those in groups II (5.9×10^8/kg) and I (13×10^8/kg) ($p<0.001$ each). The number of CD34+ cells in group II (5.7×10^6/kg) was also significantly higher than that in group III (2.2×10^6/kg) ($p=0.002$). The number of CFU-GM in group II (16×10^5/kg) was significantly higher than those in groups III (4.2×10^5/kg) and I (6.1×10^5/kg) ($p<0.001$ each). However, there was no difference in the number of CFU-GM between groups III and I.

Table 1 Numbers of frozen cells

	Group	MNC ($\times10^8$/kg)	CD34+ cell ($\times10^6$/kg)	CFU-GM ($\times10^5$/kg)
I	(2–5×10⁷/ml) n=20	13 (3.6–67.2)	n.d.	6.1 (1.5–22.0)
II	(2×10⁸/ml) n=44	5.9 (0.8–35.5)	5.7 (0.13–83.2)	16 (0.4–73.2)
III	(2×10⁷/ml) n=22	2.6×10⁶/kg (1.3–19)	2.2 (0.85–15.6)	4.2 (0.75–37.4)

Each value indicates a median value (range). The number of CD34+ cells in group II was significantly higher than that in group III ($p=0.0024$). The number of CFU-GM in group II was significantly higher than in groups I and III ($p=0.0009$ and 0.0003, respectively). *n.d.* not determined

Recovery rate and cell viability

Cell recovery rates after cryopreservation/thawing are shown in Table 2. These numbers ranged widely. Recovery rates of MNC were statistically identical among the three groups, but with different p values (II and I; 0.770, III and I; 0.100, III and I; 0.144). The viability of MNC after thawing in group III [75% (66–89)] was significantly lower than those in groups II and I ($p<0.001$, each). There was no difference in cell viability between groups II and I ($p=0.332$). The recovery rate of CFU-GM in group III [53% (13–202%)] was lower than that in group I ($p=0.023$). However, there were no significant differences between groups II and I ($p=0.091$), or groups III and II ($p=0.271$).

Engraftment kinetics

The median volume of cell suspension infused was 180 ml (range, 85 –700 ml) in group I, 60 ml (23–145 ml) in group II, and 8 ml (3.5–15 ml) in group III. The number of infused CFU-GM in group I [median, 5.6 (range, 1.5–20) $\times10^5$/kg] was statistically identical to that in group II [9.1 (0.3–90) $\times10^5$/kg] ($p=0.086$). The number of infused CD34+ cells in group III [2.1 (0.43 – 7.0) $\times10^6$/kg] was significantly lower than that in group II [5.3 (0.12–75) $\times10^6$/kg] ($p=0.001$), and the number of CFU-GM in group III [2.2 (0.3–12) $\times10^5$/kg] was significantly lower than those in groups II [9.1 (0.3–90) $\times10^5$/kg] and I [5.6 (1.5–20) $\times10^5$/kg] ($p<0.001$ each).

Table 2 Cell recovery rates and viabilities after thawing

	Group	MNC	Viability	CD34+ cell	CFU-GM
I	(2–5×10⁷/ml) n=20	67 (26–298)	89 (84–95)	n.d.	88 (33–373)
II	(2×10⁸/ml) n=44	72 (23–335)	87 (83–91)	103 (27–429)	64 (19–508)
III	(2×10⁷/ml) n=22	96 (23–204)	75 (66–89)	98 (33–196)	53* (13–202)

Each value indicates the percentage of a median value (ranges). *n.d.* not determined
*CFU-GM recovery in group III was significantly lower than that in group I with a p value of 0.023

Table 3 Engraftment speed after autografting

	Group	WBC ($>1\times10^9$/l)	ANC ($>5\times10^9$/l)	Platelets ($>50\times10^9$/l)
I	(2×10^7/ml) n=20	11 (8–29)	11 (8–22)	15 (10–88)
II	(2×10^8/ml) n=44	11 (8–18)	11 (8–19)	16 (10–37)
III	(2×10^7/ml) n=22	12 (9–20)	12 (9–19)	27* (11–60)

The data indicate median days (ranges) to achieve each criterion after autografting.
* Platelet recovery speed was significantly slower in group III than in group II and I with p values of 0.002 and 0.022, respectively

The engraftment rates determined by the number of days to achieve a WBC of $>1\times10^9$/l and ANC of 0.5×10^9/l after autografting were identical among the three groups. However, the platelet engraftment rate determined by the number of days to achieve a platelet count of $>50\times10^9$/l in group III was significantly delayed (p=0.022 and 0.002 versus groups II and I, respectively; Table 3).

Discussion

The rapid hematopoietic recovery after myeloablative therapy has prompted the use of PBSC in preference to BM cells [17]. Apheresis after mobilizing chemotherapy with or without G-CSF enables the collection of a large numbers of PBSC in comparison with marrow aspiration under general anesthesia. However, the collection and cryopreservation of PBSC is associated with intense labor and requires ample space for storage. Optimal conditions for PBSC cryopreservation have not yet been defined. In particular, an increase in the volume of cell suspension which will be frozen results in a concomitant increase in the volume of cryoprotectant, such as DMSO, which may become toxic at cell infusion [3, 5]. Under these circumstances, we applied a gradient centrifugation method with double-layered Percoll to deplete red cells, granulocytes, and platelets for clinical use in pediatric patients [18].

Rowley et al. reported that stem cell survival, as reflected in the post-thaw recovery of MNC, CFU-GM and CD34+ cells, was unaffected even when nucleated cells were frozen at a concentration of $3.7\pm1.9\times10^8$/ml [9].In addition, cryopreservation at different cell concentrations did not predict the time to engraftment or duration of aplasia [9]. Another study showed a reduced recovery of CFU-GM when a higher cell concentration of PBSC was compared with a lower cell concentration of bone marrow at the time of freezing. However, this did not translate into delayed hematopoietic recovery in clinical transplantation [19].Based upon these studies, we have initiated to cryopreserve PBSC at higher cell concentrations without the use of a programmed freezer. Benefit and efficacy of the procedure was evaluated by comparing hematopoietic recovery after autologous PBSCT.

In this study, we did not observe a significant difference in the freeze/thaw recovery rates of CD34+ cells or CFU-GM between groups II and I. However, recovery rate of CFU-GM and the number of reinfused cells in group III were significantly smaller than in the other two groups. The speeds of engraftment were not significantly different between groups II and I, although patients in group III showed a slower recovery of platelets. The neutrophil recovery might be enhanced by the administration of G-CSF in group III. On the other hand, there is another possibility that purification procedures with ISOLEX300 selectively affect on platelet-lineage progenitor cells or make them vulnerable to freeze/thaw procedure. Further investigations will be required to solve this problem.

Balint et al. reported that presence of 10% DMSO is an essential part of the cryopreservation procedure of very primitive murine stem cells [20]. On the other hand, DMSO is the primary factor related to toxicities at graft infusion and reduction of amount of DMSO by reducing the total volume of grafts should merit patients, particularly pediatric population. Thus, there is no suspicion for the superiority of using 5% DMSO when recovery rates of frozen cells and engraftment kinetics are identical.

In conclusion, the present results indicate that MNC in apheresis products be cryopreserved at 2×10^8/ml without the use of a programmed freezer, without jeopardizing their engraftment potential. Definition of the upper limit for the cryopreservation cell concentration will require further studies.

Acknowledgements The authors are grateful to Ms. Yasuda for her excellent technical assistance and to the nursing staff of the pediatric ward at the University Hospital of Tokushima.

References

1. Goldman JM, Schmitz N, Niethammer D, Gratwohl A (1998) Allogeneic and autologous transplantation for hematological disease, solid tumors and immune disorders: current practice in Europe in 1998. Bone Marrow Transplant 21:1–7
2. Goldman JM, Th'ng KH, Park DS, Spiers AS, Lowenthal RM, Ruutu T (1979) Collection, cryopreservation and subsequent viability of haemopoietic stem cells intended for treatment of chronic granulocytic leukemia in blast-cell transformation. Br J Haematol 40:185–195
3. Stroncek DF, Fautsh SK, Lasky LC, Hurd DD, Ramsay NK, McCullough J (1991) Adverse reactions in patients transfused with cryopreserved marrow. Transfusion 31:521–526
4. Okamoto Y, Takaue Y, Saito S, Shimizu T, Suzue T, Abe T, Sato J, Hirao A, Watanabe T, Kawano Y, Kuroda Y (1993) Toxicities associated with cryopreserved and thawed peripheral blood stem cell autografts in children with cancer. Transfusion 33:578–581
5. Al Alessandrino P, Bernasconi P, Caldera D, Colombo A, Bonfichi M, Malcovati L, Klersy C, Martinelli G, MaiocchiM, Pagnucco G, Varettoni M, Perotti C, Bernasconi C (1999) Adverse events occurring during bone marrow or peripheral blood progenitor cell infusion: analysis of 126 cases. Bone Marrow Transplant 23:533–537
6. Rowley SD (1992) Hematopoietic stem cell cryopreservation: a review of current techniques. J Hematother 1:233–250

7. Makino S, Harada M, Akashi K, Taniguchi S, Shibuya T, Inaba S, Niho Y (1991) A simplified method for cryopreservation of peripheral blood stem cells at −80°C without rate-controlled freezing. Bone Marrow Transplant 8:239–244
8. Takaue Y, Abe T, Kawano Y, Suzue T, Saito S, Hirao A, Sato J, Makimoto A, Kawahito M, Watanabe T, Shimokawa T, Kuroda Y (1994) Comparative analysis of engraftment after peripheral blood stem cell autografts cryopreserved by controlled vs uncontrolled-rate method. Bone Marrow Transplant 13:801–804
9. Rowley SD, Bensinger WI, Gooley TA, Buckner CD (1994) Effect of cell concentration on bone marrow and peripheral blood stem cell cryopreservation. Blood 83:2731–2736
10. Cabezudo E, Dalmases C, Ruz M, Sanchez JA, Torrico C, Sola C, Querol S, Garcia J (2000) Leukapheresis components may be cryopreserved at high cell concentrations without additional loss of HPC function. Transfusion 40:1223–1227
11. Takaue Y, Kawano Y, Kuroda Y (1995) Application of recombinant granulocyte colony-stimulating factor in peripheral blood stem-cell transplantation: a pediatric experience. In: Levitt D, Mertelsmann R (eds) Hematopoietic stem cells. Marcel Dekker, New York, pp 611–630
12. Makimoto A, Kawano Y, Abe T, Okamoto Y, Sato J, Nakagawa R, Watanabe H, Watanabe T, Kuroda Y, Sweet L, Takaue Y (1999) Comparative evaluation of procedures with a Baxter CS-3000 cell separator for collecting peripheral blood cells from children. J Hematother 8:305–310
13. Kawano Y, Takaue Y, Watanabe T, Abe T, Okamoto Y, Iwai A, Watanabe A, Ito E, Makimoto A, Nakagawa R, Watanabe H, Sato J, Suenaga K, Suzuya H, Ohnishi T, Kanamaru S, Kaneko S, Kuroda Y (1999) Efficacy of the mobilization of peripheral blood stem cells by granulocyte colony-stimulating factor in pediatric donors. Cancer Res 59:3321–3324
14. Takaue Y, Kawano Y, Watanabe A, Eguchi H, Abe T, Makimoto A, Okamoto Y, Kuroda Y (1996) Transplantation with purified or unmanipulated mobilized blood stem cells in children. In: Ikehara S, Takaku F, Good RA (eds) Bone marrow transplantation: basic and clinical studies. Springer, Berlin, Heidelberg, New York, pp 246–249
15. Kawano Y, Takaue Y, Watanabe T, Saito S, Abe T, Hirao A, Sato J, Ninomiya T, Suzue T, Koyama T, Shimokawa T, Yokobayashi A, Asano S, Masaoka T, Takaku F, Kuroda Y (1993) Effects of progenitor cell dose and preleukapheresis use of human recombinant granulocyte-macrophage colony-stimulating factor on the recovery of hematopoiesis after blood stem cell autografting in children. Exp Hematol 21:103–108
16. Takaue Y, Kawano Y, Abe T, Okamoto Y, Suzue T, Shimizu T, Saito S, Sato J, Makimoto A, Nakagawa R, Watanabe T, Ito M, Kuroda Y (1995) Collection and transplantation of peripheral blood stem cells in very small children weighing 20 kg or less. Blood 86:372–380
17. To LB, Roberts MM, Haylock DN, Dyson PG, Branford AL, Thorp D, Ho JQ, Dart GW, Horvath N, Davy ML, Olweny LM, Juttner CA (1992) Comparison of haematological recovery times and supportive care requirements of autologous recovery phase peripheral blood stem cell transplants, autologous bone marrow transplants and allogeneic bone marrow transplants. Bone Marrow Transplant 9:277–284
18. Takaue Y, Watanabe T, Kawano Y, Koyama T, Huq M, Suzue T, Abe T, Sato J, Shimokawa T, Kosaka M, Shimizu M, Ogura T, Ninomiya T, Kuroda Y (1989) Isolation and storage of peripheral blood hematopoietic stem cells for autotransplantation into children with cancer. Blood 74:1245–1251
19. Keung YK, Cobos E, Morgan D, Park M, Dixon S, Wu K, Park CH (1996) High cellular concentration of peripheral blood progenitor cells during cryopreservation adversely affects CFU-GM but not hematopoietic recovery. J Hematother 5:73–77
20. Balint B, Ivanovic Z, Petakov M, Taseski J, Jovcic G, Stojanovic N, Milenkovic P (1999) The cryopreservation protocol optimal for progenitor recovery is not optimal for preservation of marrow repopulating ability. Bone Marrow Transplant 23:613–619

[CANCER RESEARCH 64, 391-399, January 1, 2004]

Graft-*versus*-Tax Response in Adult T-Cell Leukemia Patients after Hematopoietic Stem Cell Transplantation

Nanae Harashima,[1] Kiyoshi Kurihara,[1] Atae Utsunomiya,[2] Ryuji Tanosaki,[3] Shino Hanabuchi,[1] Masato Masuda,[4] Takashi Ohashi,[1] Fumiyo Fukui,[1] Atsuhiko Hasegawa,[1] Takao Masuda,[1] Yoichi Takaue,[3] Jun Okamura,[5] and Mari Kannagi[1]

[1]Department of Immunotherapeutics, Tokyo Medical and Dental University, Medical Research Division, Tokyo; [2]Department of Hematology, Imamura Bun-in Hospital, Kagoshima; [3]National Cancer Center Hospital, Tokyo; [4]Second Department of Internal Medicine, University of the Ryukyus, Okinawa; and [5]National Kyushu Cancer Center, Fukuoka, Japan

ABSTRACT

Adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) is characterized by poor prognosis after chemotherapy. Recent clinical trials have indicated, however, that allogeneic but not autologous hematopoietic stem cell transplantation (HSCT) for ATL can yield better clinical outcomes. In the present study, we investigated cellular immune responses of ATL patients who obtained complete remission after nonmyeloablative allogeneic peripheral blood HSCT from HLA-identical sibling donors. In the culture of peripheral blood mononuclear cells (PBMCs) from a post-HSCT but *not pre-HSCT* ATL patient, $CD8^+$ CTLs proliferated vigorously in response to stimulation with autologous HTLV-I-infected T cells that had been established before HSCT *in vitro*. These CTLs contained a large number of monospecific CTL population directed to a HLA-A2-restricted HTLV-I Tax 11-19 epitope. The frequency of Tax 11-19-specific CD8+ CTLs in this patient markedly increased also *in vivo* after HSCT, as determined by staining with HLA-A2/Tax 11-19 tetramers. Similar clonal expansion of HTLV-I Tax-specific CTLs exclusively directed to a HLA-A24-restricted Tax 301-309 epitope was observed in the PBMCs from another ATL patient after HSCT from a HTLV-I-negative donor. Among four post-HSCT ATL patients tested, HTLV-I-specific CTLs were induced in the PBMC culture from three patients but not from the remaining one who had later recurrence of ATL. These observations suggested that reconstituted immunity against antigen presentation in ATL patients after HSCT resulted *in strong and selective* graft-*versus*-HTLV-I response, which might contribute to graft-*versus*-leukemia effects.

INTRODUCTION

Adult T-cell leukemia (ATL) is a T-cell malignancy that develops in ~5% of human T-cell leukemia virus type I (HTLV-I)-infected individuals and is characterized by mostly $CD4^+$ and $CD25^+$ mature T-lymphocyte phenotypes, onset at middle age or later, immune suppression, and poor prognosis (1–3). Clinical use of combination chemotherapy for ATL brought the 4-year overall survival rate up to 8 to 12%, which is still lower than those of other types of leukemia (4, 5). Recently, hematopoietic stem cell transplantation (HSCT) has been applied to a limited number of ATL patients. Initial studies of autologous HSCT revealed frequent recurrence of ATL (6). However, more recent studies have revealed that allogeneic HSCT could produce better results, although there was also a risk of graft-*versus*-host-disease (GVHD; Ref. 7). This strongly suggests that the cellular immune responses of donor against recipient, *i.e.*, graft-*versus*-leukemia (GVL) effects, contribute to eradicating ATL cells, as observed in other types of leukemia.

It has been demonstrated that allogeneic HSCT from HLA-identical siblings can cause GVHD to some degree, and the minor histocompatibility antigen (mHA) in the recipient has been referred to as the target antigen of GVHD (8). Several mHA, including the male-specific H-Y transplantation antigen (9), HA-1 antigen (10), CD31 molecule (11, 12), and human platelet antigens (12, 13), have been suggested to be involved in GVHD. It is known that the probability of recurrence of leukemia after allogeneic HSCT increases when the graft has been depleted of T cells or the donor is a genetically identical twin, indicating that GVL effects are important in preventing the recurrence of leukemia (14). Therefore, an augmentation of the donor T-cell response specific for mHA expressed in the recipient's hematopoietic cells but not in the nonhematopoietic cells has been proposed as one strategy for inducing GVL effects without causing GVHD (15). Tumor antigens such as bcr/abl fusion protein and WT-1, which are specific for or overexpressed in tumor cells, are also candidates for the target antigens of GVL effects (16, 17).

Host cellular immune responses against HTLV-I, especially outgrowth of cytotoxic T cells, are frequently found in peripheral blood mononuclear cell (PBMC) culture of asymptomatic HTLV-I carriers and HTLV-I-associated myelopathy/Tropical spastic paraparesis patients but infrequently in ATL patients (18, 19). Of the HTLV-I antigens such as *env*, *gag*, *pol*, and *pX* gene products, it has been shown that HTLV-I Tax is a dominant target antigen of HTLV-I-specific CTL (20, 21). Tax is also known to play a critical role in HTLV-I leukemogenesis by accelerating cell growth and inhibiting apoptosis (22, 23). These findings suggest that Tax-specific CTL may play a role in immune surveillance for HTLV-I leukemogenesis.

In a recently established animal model for HTLV-I-infected T-cell tumors, we demonstrated an antitumor effect of Tax-specific CTL *in vivo* (24, 25). In this model, otherwise fatal T-cell lymphomas in nude rats inoculated with syngeneic HTLV-I-infected cells could be eradicated by transferring fresh T cells from syngeneic immunocompetent rats vaccinated with either Tax-encoded DNA or peptides corresponding to a CTL epitope (26, 27). However, it is unclear whether such observations in experimental models apply to humans because HTLV-I expression is extremely low in human ATL cells in the periphery (28–30).

In the present study, we investigated the cellular immune responses of ATL patients after HSCT against spontaneously HTLV-I-infected T cells derived from the same patient before HSCT. These HTLV-I-infected cells were thought to possess antigens originating from the recipients, including targets for GVL effects. We found that in response to the recipient-origin cells, the PBMCs from post-HSCT patients exhibited vigorous HTLV-I-specific CTL responses that were directed to a limited number of Tax epitopes. Furthermore, such oligoclonal expansion of HTLV-I-specific CTL in post-HSCT PBMCs was observed also *in vivo*. These observations indicated that

Received 5/22/03; revised 9/18/03; accepted 10/28/03.

Grant support: Ministry of Health, Welfare, and Labor of Japan, the Ministry of Education, Science, Culture and Sports of Japan, and Japan Science and Technology Corporation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Mari Kannagi, Department of Immunotherapeutics, Tokyo Medical and Dental University, Medical Research Division, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81-3-5803-5798; Fax: 81-3-5803-0235; E-mail: kann.impt@tmd.ac.jp.

a strong graft-*versus*-HTLV-I response occurred in ATL patients after HSCT.

MATERIALS AND METHODS

Recipient/Donor Pairs and Blood Samples. Four acute type ATL patients, #37 (case 1), R07 (case 2), R11 (case 3), and #97 (case 4) and their corresponding HLA-identical sibling donors, #36, D07, D11, and #98, respectively, donated peripheral blood samples under written informed consent. The patients were participants in the clinical trial protocol for allogeneic HSCT for ATL with a reduced-intensity conditioning regimen that was supported by the Ministry of Health, Welfare, and Labor of Japan. After cyclophosphamide, doxorubicin, vincristine, prednisolone therapy, patient #37 at the beginning of recurrence, patients R07 and #97 in partial remission, and patient R11 in complete remission received conditioning treatment consisting of fludarabine (30 mg/m^2 i.v. days −8 to −3), busulfan (4 mg/kg p.o. days −6 and −5), and ATG (2.5 mg/kg days −2 and −1) before the infusion of granulocyte-colony stimulating factor-mobilized peripheral blood stem cells from the donors. Prophylaxis for GVHD was cyclosporine A alone starting from day −1. Although patients #37, R07, and #97 obtained complete remission within 2 months after HSCT, R11 had recurrence of ATL lymphoma in the neck 6 months after HSCT. Donor #36 was a HTLV-I carrier, but the other donors were not. The HLA and other clinical characteristics of the patients and donors are summarized in Table 1.

Cell Lines. PBMCs from the donors and recipients isolated on a Ficoll-Hypaque PLUS (Amersham Biosciences, Piscataway, NJ) gradient were partially stored in liquid nitrogen until use and partially used to obtain HTLV-I-infected IL-2-dependent T-cell (ILT) lines and EBV-transformed lymphoblastoid B-cell lines LCL. ILT-#37, ILT-R07, ILT-R11, and ILT-#97were spontaneously HTLV-I-infected T-cell lines originating from pre-HSCT recipients #37, R07, R11, and #97, respectively. To establish these ILT lines, PBMCs were stimulated with 1 μg/ml phytohemagglutinin (PHA)-P (Sigma, St. Louis, MO) after depletion of CD8$^+$ cells using a Dynabeads M450-CD8 (Dynal, Oslo, Norway) and then maintained in RPMI 1640 (Invitrogen-Life Technologies, Inc., Grand Island, NY) containing 10% FCS (Sigma), 10 units/ml recombinant human interleukin (IL)-2 (Shionogi, Osaka, Japan), or 10 ng/ml recombinant human IL-15 (Sigma) at 37°C with 5% CO_2 for over 2 months. An EBV-transformed B-cell line, LCL-#36, was established by maintaining positively separated CD19$^+$ PBMCs from donor #36 in RPMI 1640 with 10% FCS after infection with an EBV-containing culture supernatant of the B95-8 cell line (31). TCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9; Ref. 32), ILT-As-2 (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), ILT-Myj-3 (HLA-A2/A24, B54/B60, Cw1/Cw3, and DR4/DR5; Ref. 21), ILT-Nkz-2 (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and ILT-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are HTLV-I-infected T-cell lines, and LCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9), LCL-As (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), TOK (HLA-A24/-, B52/-, and DR2/-; Ref. 33), LCL-Nkz (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and LCL-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are EBV-transformed B-cell lines. An erythroblastoid cell line, K562 (34), was also used.

Flow Cytometry for Phenotyping and HTLV-I Expression. Cell surface phenotypes were determined using directly FITC-conjugated murine antihuman monoclonal antibodies (mAbs) followed by analysis on a FACSCalibur (Becton Dickinson, San Jose, CA), and data were analyzed using CellQuest software (Becton Dickinson). The mAbs used were anti-CD4 (clone: RPA-T4; BD PharMingen), anti-CD8 (clone: RPA-T8; BD PharMingen), anti-CD19 (clone: HIB19; BD PharMingen), and for isotype controls, antimouse IgG1. For detection of intracellular HTLV-I proteins, cells were stained with anti-Tax mAb (Lt-4; Ref. 35) and anti-Gag mAb (GIN-7; Ref. 36) after cell membrane permeabilization. These mAbs were kindly provided by Dr. Yuetsu Tanaka (University of the Ryukyus, Okinawa, Japan).

Induction of HTLV-I-Specific CTL. One million whole PBMCs from post-HSCT patient #37 were stimulated with 1 μg/ml PHA-P and then mixed with the same number of ILT-#37 cells, derived from pre-HSCT patient #37, and pretreated with 1% formaldehyde/PBS. These T cells were maintained in AIM-V medium (Invitrogen-Life Technologies, Inc.) supplemented with 100 units/ml penicillin, 0.5 mg/ml streptomycin, 10% heat-inactivated FCS, and 100 units/ml recombinant human IL-2 with periodic stimulation with formaldehyde-fixed respective ILT cells at 10–14-day intervals. PBMCs from donor #36 and pre-HSCT patient #37 were similarly stimulated with PHA and subsequently with formaldehyde-fixed ILT-#37 in cultures for CTL induction. CTL induced from CD8$^+$ cell-enriched PBMCs of donor #36 were also used in some experiments. In the other ATL cases tested (patients R07, R11, and #97), PHA-stimulated CD8$^+$ cell-enriched PBMCs from each post-HSCT

Table 1 *Summary of clinical status and T-cell immune response of the participants in hematopoietic stem cell transplantation (HSCT)*

Patient's ID	HSCT case	Donor/ recipient	Age (yrs)	Sex	Status	HTLV-I[a] infection	HLA	*In vitro* immune analysis of PBMC[b]			HTLV-I proviral DNA (copies/1000 PBMC)[d]		Clinical outcome after HSCT
								Sampling date (days after HSCT)	Stimulated with	Induction of CTL[c]	Pre-HSCT	Post-HSCT	
#36	Case 1	Donor	57	M	Healthy	+	A2/−, B46/−, Cw1/−, DR8/−	0	ILT-#37	+	Undetectable	N.T.	
#37	Case 1	Recipient	63	M	Acute ATL	+	A2/−, B46/−, Cw1/−, DR8/−	+183	ILT-#37	+	1150.3	Undetectable	Complete remission for more than 24 months
D07	Case 2	Donor	48	F	Healthy	−	A24/A32, B35/B60, DR4/−	N.T.	N.T.	N.T.	N.T.	N.T.	
R07	Case 2	Recipient	51	M	Acute ATL	+	A24/A32, B35/B60, DR4/−	+255	ILT-R07	+	26.7	Undetectable	Complete remission for more than 23 months
D11	Case 3	Donor	52	F	Healthy	−	A2/A26, B35/B61, DR4/6	N.T.	N.T.	N.T.	N.T.	N.T.	
R11	Case 3	Recipient	54	M	Acute ATL	+	A2/A26, B35/B61, DR4/6	+153	ILT-R11	−	440.8	Undetectable	Relapse of lymphoma at 6 months after HSCT
#98	Case 4	Donor	61	M	Healthy	−	A2/A26, B51/, DR4/5	N.T.	N.T.	N.T.	N.T.	N.T.	
#97	Case 4	Recipient	66	F	Acute ATL	+	A2/A26, B51/, DR4/5	+104	ILT-#97	+	3297.2	Undetectable	Died of GVHD at 9 months after HSCT

[a] HTLV-I, human T-cell leukemia virus type I; ATL, adult T-cell leukemia; PBMC, peripheral blood mononuclear cell; N.T., not tested; GVHD, graft-*versus*-host disease; ILT, IL-2-dependent T-cell line.

[b] PBMCs isolated from patients #37, R07, R11, and #97 at the indicated days after HSCT were repeatedly stimulated in culture with formalin-fixed autologous ILT cells established before HSCT, as described in the "Materials and Methods."

[c] Culture in which CTL specific for autologous ILT cells grew is indicated as (+) and that without CTL induction is indicated as (−).

[d] HTLV-I proviruses in the peripheral blood were measured just before and after HSCT at similar dates when *in vitro* immune responses were analysed. A level less than 0.5 copies/1000 cells was undetectable.