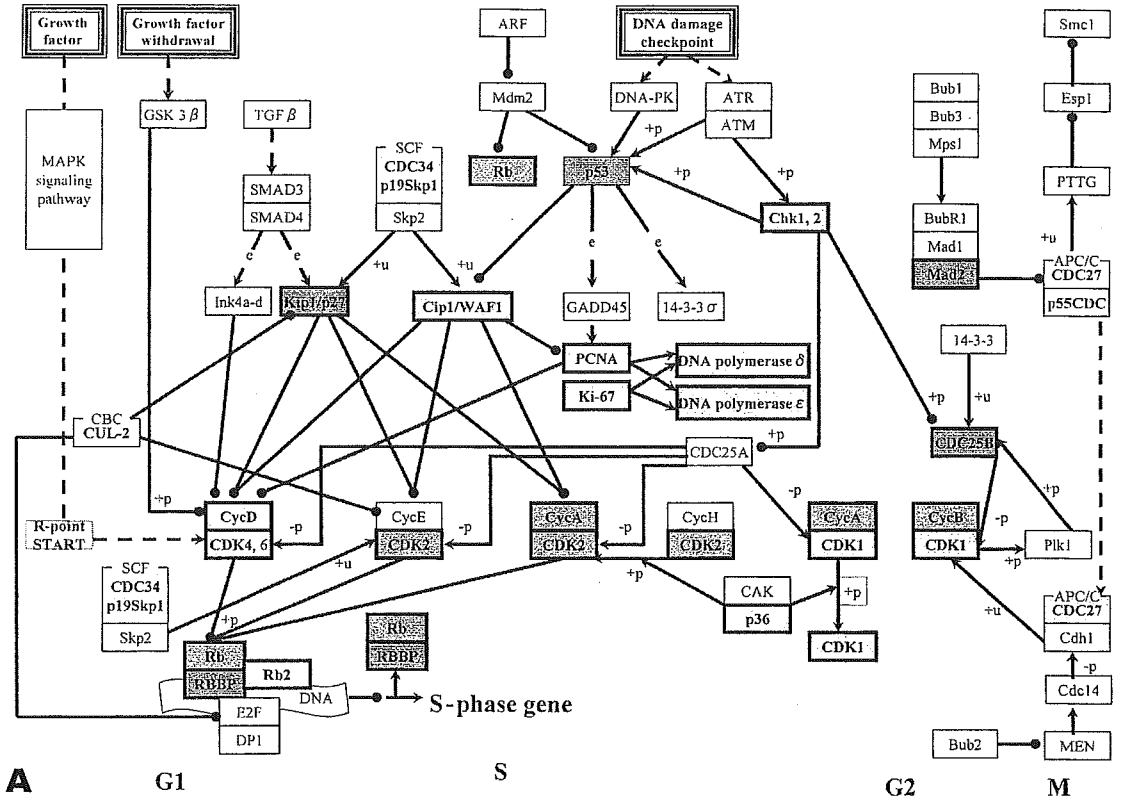
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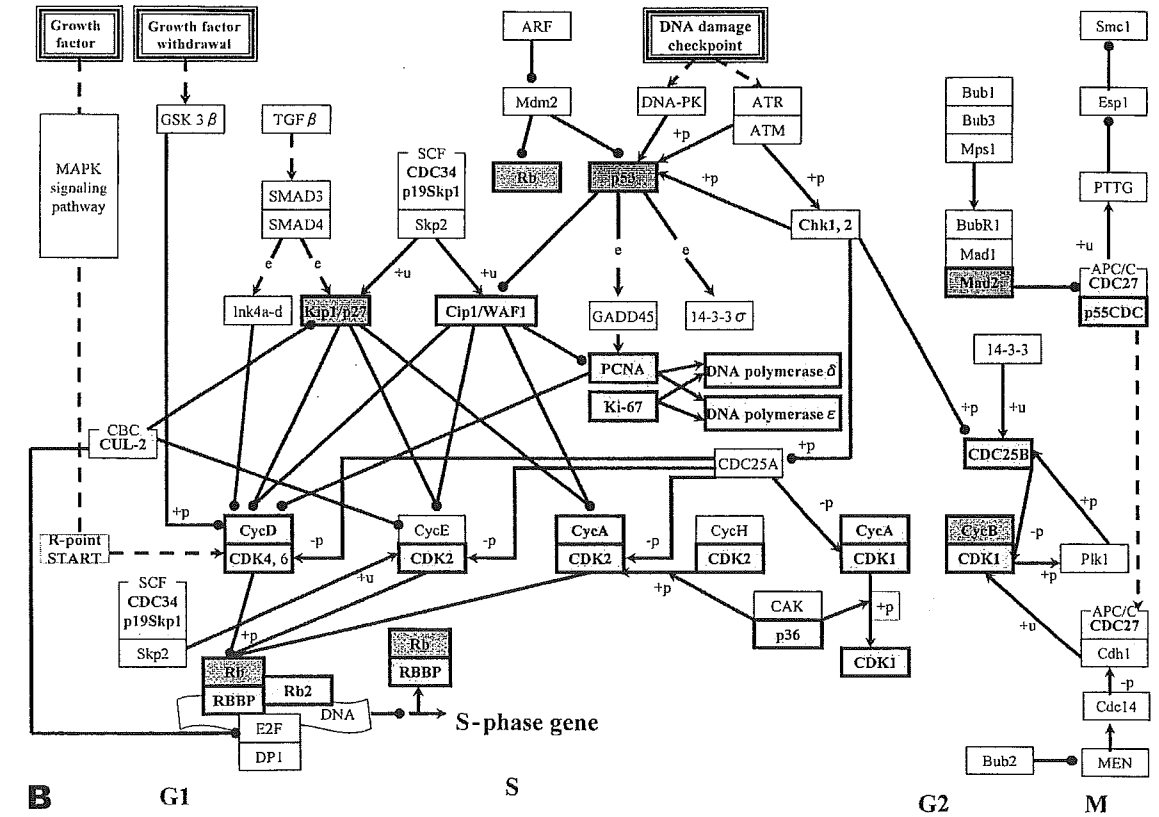
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CAK
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DNA
E2F
DP1
S-phase gene
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Bub3
Mps1
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Mad1
Mad2
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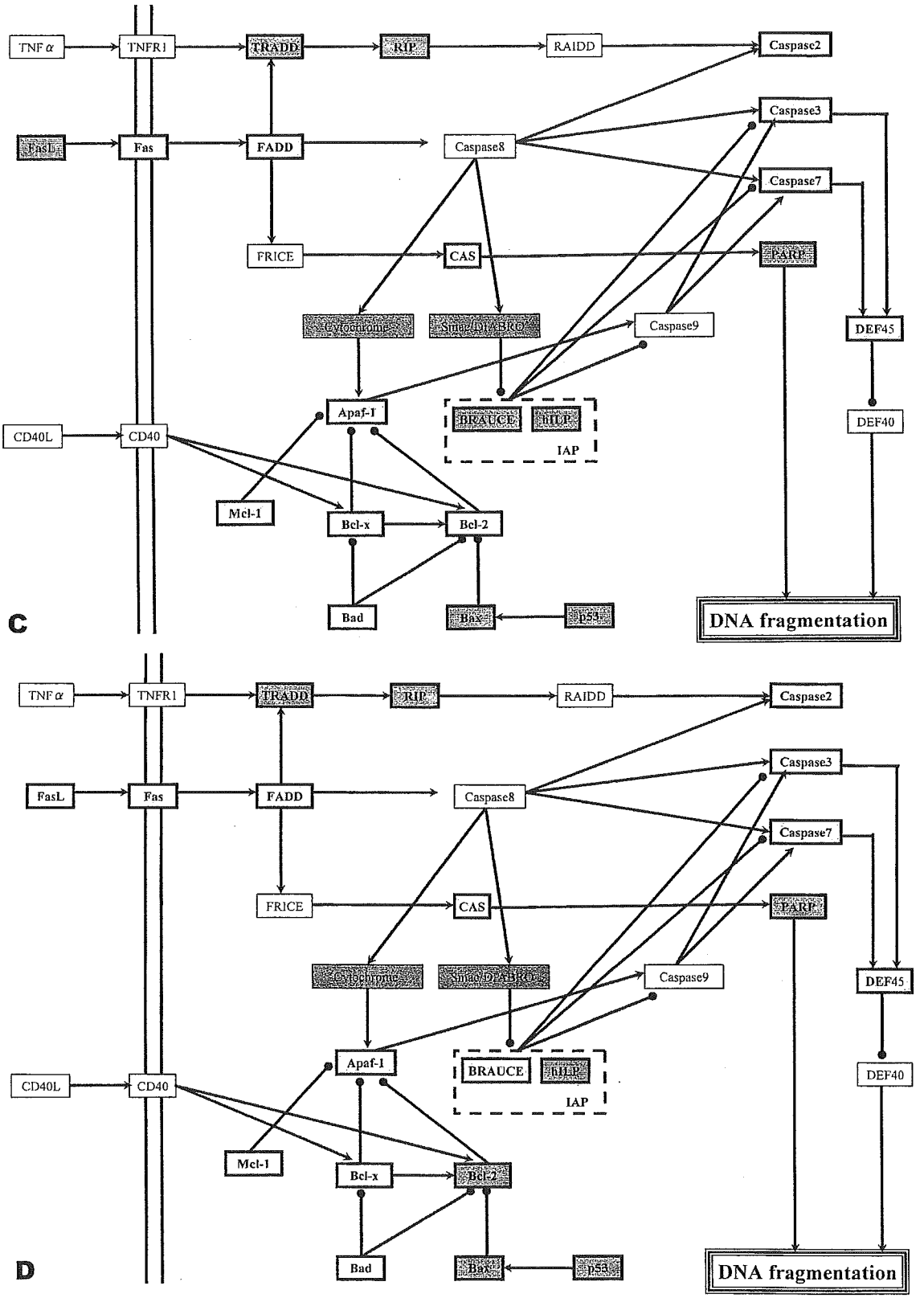

Figure 5. *Pathways of cell cycle and apoptosis induced by Ad-RGD-SART-1. (A): cell cycle pathway of A549, (B): cell cycle pathway of MCF7, (C): apoptosis pathway of A549, (D): apoptosis pathway of MCF7. Molecules marked with color (yellow, green and blue) were analyzed in the study. Yellow indicates "enhancement", green indicates "same level" and blue indicates "suppression".*

dendritic cells. Although these therapies induce general anti-tumor effects that yield some therapeutic benefit against metastatic sites, the potency is insufficient to control large tumors in advanced stages.

In this study, we used an RGD-fiber-modified adenovirus, Ad-RGD, to achieve high transduction efficiency. Previous reports have suggested that RGD-fiber-modified adenoviruses show higher transduction efficiency in freshly isolated human cells and various cell lines when compared with normal fiber adenoviruses. We confirmed that the transduction and expression levels of the RGD-fiber-modified adenoviruses were higher in the MCF-7 and A547 cell lines in preliminary experiments (unpublished data). The transduction and expression of SART-1 was evaluated by RT-PCR and a higher or equivalent expression of SART-1 mRNA was detected. Strong cell growth inhibition caused by cell cycle inhibition was observed after SART-1 gene transduction, which led to apoptosis (Figures 2-5). These data suggest that RGD-fiber-modified adenovirus vectors overcome the problem of low gene transduction efficiency.

SART-1 was identified as one of the tumor antigens recognized by established T cells. Our data also suggest that SART-1 gene transduction induced cell cycle inhibition, resulting in apoptosis (Figures 2-5). Precise analysis of cell cycle- and apoptosis-related molecules suggested the mechanism behind the SART-1 inhibition of the cell cycle and apparent induction of apoptosis (Figures 4 and 5). Our data showed that Kip1/p27, Mad2 and p53, which are cell cycle-related proteins, decreased in both cell lines (Figures 5A and C). Expression of p53 and Bax, which are apoptosis-related proteins, also decreased after SART-1 transduction in both cell lines (Figures 5B and D).

Previous reports suggest that enhancement of p53 and Adp27 (Kip1) expression inhibit cell proliferation. Cell cycle analysis has demonstrated that accumulation of cells in the G0/G1-phase at 24-120 h after transduction of the p53 or p27/kip1 genes is associated with an increase in early apoptosis (24-26). However, in our study, such molecules were decreased after SART-1 transduction, even though cell cycle arrest and apoptosis were induced by SART-1 gene transduction. Moreover, other apoptosis- and cell cycle-related proteins also exhibited inverse reactions or were not influenced by SART-1 gene transduction. Those results suggest that SART-1 induced cell cycle arrest and apoptosis *via* different pathways.

Based on the present results, two applications of SART-1 in cancer gene therapy are possible; SART-1 may be used as a cancer antigen, as reported previously (27, 28), or SART-1 can induce cell cycle arrest leading to apoptosis. The combination of these two concepts *via* transduction of a single gene is a novel approach for local and systemic therapy, and further studies are currently underway.

References

1 van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A and Boon T: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science *254*: 1643-1647, 1991.

2 Gaugler B, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethe B, Brasseur F and Boon T: Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. J Exp Med *179*: 921-930, 1994.

3 Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannelli JR, Appella E and Rosenberg SA: Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J Exp Med *180*: 347-352, 1994.

4 Shichijo S, Nakao M, Imai Y, Takasu H, Kawamoto M, Niiya F, Yang D, Toh Y, Yamana H and Itoh K: A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. J Exp Med *187*: 277-288, 1998.

5 Matsumoto H, Shichijo S, Kawano K, Nishida T, Sakamoto M and Itoh K: Expression of the SART-1 antigens in uterine cancers. Jpn J Cancer Res *89*: 1292-1295, 1998.

6 Ishida H, Komiya S, Inoue Y, Yutani S, Inoue A and Itoh K: Expression of the SART1 tumor-rejection antigen in human osteosarcomas. Int J Oncol *17*: 29-32, 2000.

7 Sasatomi T, Yamana H, Shichijo S, Tanaka S, Okamura T, Ogata Y, Itoh K and Shirouzu K: Expression of the SART1 tumor-rejection antigens in colorectal cancers. Dis Colon Rectum *43*: 1754-1758, 2000.

8 Shintaku I, Kawagoe N, Yutani S, Hoshi S, Orikasa S, Yoshizumi O and Itoh K: Expression of the SART1 tumor rejection antigen in renal cell carcinoma. Urol Res *28*: 178-184, 2000.

9 Yutani S, Shichijo S, Inoue Y, Kawagoe N, Okuda K, Kurohiji T, Tanaka M, Sata M and Itoh K: Expression of the SART1 tumor-rejection antigen in hepatocellular carcinomas. Oncol Rep *8*: 369-372, 2001.

10 Tanaka S, Tsuda N, Kawano K, Sakamoto M, Nishida T, Hashimoto T, Shichijo S, Kamura T and Itoh K: Expression of tumor-rejection antigens in gynecologic cancers. Jpn J Cancer Res *91*: 1177-1184, 2000.

11 Gohara R, Nakao M, Ogata Y, Isomoto H, Oizumi K and Itoh K: Histocompatibility leukocyte antigen-A2402-restricted cytotoxic T lymphocytes recognizing adenocarcinoma in tumor-infiltrating lymphocytes of patients with colon cancer. Jpn J Cancer Res *88*: 198-204, 1997.

12 Nakao M, Yamana H, Imai Y, Toh Y, Toh U, Kimura A, Yanoma S, Kakegawa T and Itoh K: HLA A2601-restricted CTLs recognize a peptide antigen expressed on squamous cell carcinoma. Cancer Res *55*: 4248-4252, 1995.

13 Kikuchi M, Nakao M, Inoue Y, Matsunaga K, Shichijo S, Yamana H and Itoh K: Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. Int J Cancer *81*: 459-466, 1999.

14 Kawamoto M, Shichijo S, Imai Y, Imaizumi T, Koga T, Yanaga H and Itoh K: Expression of the SART-1 tumor rejection antigen in breast cancer. Int J Cancer *80*: 64-67, 1999.

15 Imaizumi T, Kuramoto T, Matsunaga K, Shichijo S, Yutani S, Shigemori M, Oizumi K and Itoh K: Expression of the tumor-rejection antigen SART1 in brain tumors. Int J Cancer *83*: 760-764, 1999.

16 Matsunaga K, Nakao M, Masuoka K, Inoue Y, Gouhara R, Imaizumi T, Nishizaka S and Itoh K: Cytokines required for induction of histocompatibility leukocyte antigen-class I-restricted and tumor-specific cytotoxic T lymphocytes by a SART1-derived peptide. Jpn J Cancer Res *90*: 1007-1015, 1999.

17 Inoue Y, Nakao M, Matsunaga K, Kikuchi M, Gomi S, Toh U, Takamori S, Yamana H and Itoh K: Induction of human leukocyte antigen-A26-restricted and tumor-specific cytotoxic T lymphocytes by a single peptide of the SART1 antigen in patients with cancer with different A26 subtypes. J Immunother *23*: 296-303, 2000.

18 Imanishi T, Akazawa T, Kimura A, Tokunaga K and Gojobori T: Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. *In:* HLA(Tsuji K, Aizawa M and Sasazuki T, eds). Oxford, Oxford Scientific Publ, pp. 1065-1220, 1991.

19 Kim SH, Kim S and Robbins PD: Retroviral vectors. Adv Virus Res *55*: 545-563, 2000.

20 Mittereder N, Yei S, Bachurski C, Cuppoletti J, Whitsett JA, Tolstoshev P and Trapnell BC: Evaluation of the efficacy and safety of *in vitro*, adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA. Hum Gene Ther *5*: 717-729, 1994.

21 Haddada H, Cordier L and Perricaudet M: Gene therapy using adenovirus vectors. Curr Top Microbiol Immunol *199(Pt 3)*: 297-306, 1995.

22 Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G, Belousova N and Curiel DT: An adenovirus vector with genetically modified fibers demonstrates expanded tropism *via* utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. J Virol *72*: 9706-9713, 1998.

23 Hosokawa M, Hama S, Mandai K, Okuda K, Takashima S, Tajiri H, Eguchi K and Heike Y: Preparation of purified, sterilized, and stable adenovirus vectors using albumin. J Virol Methods *103*: 191-199, 2002.

24 Katner AL, Hoang QB, Gootam P, Jaruga E, Ma Q, Gnarra J and Rayford W: Induction of cell cycle arrest and apoptosis in human prostate carcinoma cells by a recombinant adenovirus expressing p27(Kip1). Prostate *53*: 77-87, 2002.

25 Ko LJ and Prives C: p53: puzzle and paradigm. Genes Dev *10*: 1054-1072, 1996.

26 Slee EA, O'Connor DJ and Lu X: To die or not to die: how does p53 decide? Oncogene *23*: 2809-2818, 2004.

27 Shimizu K, Yamaguchi Y, Ohta K, Miyahara E and Toge T: Analysis of T cell receptors reactive with squamous cell carcinoma antigen SART-1 presented by the HLA-A24 molecule. Oncol Rep *9*: 599-605, 2002.

28 Suzuki N, Maeda Y, Tanaka S, Hida N, Mine T, Yamamoto K, Oka M and Itoh K: Detection of peptide-specific cytotoxic T-lymphocyte precursors used for specific immunotherapy of pancreatic cancer. Int J Cancer *98*: 45-50, 2002.

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Reduced-Intensity Unrelated Cord Blood Transplantation for Patients with Advanced Malignant Lymphoma

Koichiro Yuji,[1] Shigesaburo Miyakoshi,[1] Daisuke Kato,[1] Yuji Miura,[1] Tomohiro Myojo,[1] Naoko Murashige,[2] Yukiko Kishi,[2] Kazuhiro Kobayashi,[3] Eiji Kusumi,[1] Hiroto Narimatsu,[1] Tamae Hamaki,[2] Tomoko Matsumura,[1] Masahiro Kami,[2] Takahiro Fukuda,[2] Shigeru Masuo,[3] Kazuhiro Masuoka,[1] Atsushi Wake,[1] Junichi Ueyama,[1] Akiko Yoneyama,[1] Ko Miyamoto,[4] Haruhisa Nagoshi,[4] Michio Matsuzaki,[1] Shinichi Morinaga,[1] Yoshitomo Muto,[1] Yoichi Takeue,[2] Shuichi Taniguchi,[1] for the Tokyo SCT Consortium

[1]Department of Hematology, Toranomon Hospital, Tokyo, Japan; [2]Hematopoietic Stem Cell Transplant Unit, the National Cancer Center Hospital, Tokyo, Japan; [3]Department of Hematology and Rheumatology, JR Tokyo General Hospital, Tokyo, Japan; [4]Division of Hematology and Oncology, St. Marianna University School of Medicine, Yokohama-City Seibu Hospital, Kanagawa, Japan

Correspondence and reprint requests: Shuichi Taniguchi, MD, Department of Hematology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku Tokyo, 105-8470 Japan (e-mail: taniguchi-s@toranomon.gr.jp).

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ABSTRACT

We report the results of reduced-intensity unrelated cord blood transplantation (RI-UCBT) in patients with advanced malignant lymphoma. Twenty patients (median age, 46.5 years; range, 27-66 years) underwent RI-UCBT with a preparative regimen consisting of fludarabine 125 mg/m^2, melphalan 80 mg/m^2, and 4 Gy of total body irradiation. The median infused total cell dose was 2.75 × 10^7/kg (range, 2.3-3.4 × 10^7/kg). Graft-versus-host disease (GVHD) prophylaxis was composed of cyclosporine or tacrolimus alone. Fifteen patients achieved primary neutrophil engraftment after a median of 20 days. Eight patients developed grade II to IV acute GVHD, and 2 developed chronic GVHD. Of the 16 patients with evaluable disease, 10 achieved a complete response. Primary disease recurred in 1 patient, and transplant-related mortality within 100 days occurred in 8 of 20 patients. The estimated 1-year probability of progression-free survival was 50%. These data suggest that RI-UCBT is a feasible option for patients with refractory lymphoma who lack an HLA-matched donor.

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KEY WORDS

Non-Hodgkin lymphoma • Reduced-intensity stem cell transplantation • Cord blood transplantation

INTRODUCTION

Allogeneic stem cell transplantation is a curative treatment for advanced or chemorefractory malignant lymphoma [1,2]. The therapeutic benefits are attributable to myeloablative radiochemotherapy and the graft-versus-malignancy effect [3], whereas severe regimen-related toxicity limits the efficacy of allogeneic hematopoietic stem cell transplantation to young patients without comorbidities. Reduced-intensity stem-cell transplantation (RIST) with a nonmyeloablative preparative regimen has been developed to decrease regimen-related toxicity while preserving an adequate antitumor effect. RIST may be a curative treatment for heavily pretreated elderly patients with malignant lymphoma [1,4-9]. Umbilical cord blood from unrelated donors has been used as an alternative stem cell source [10-13], and there have been a few reported cases of cord blood transplantation for refractory lymphoma [8,14-18]. However, the results in adult lymphoma patients of treatment with reduced-intensity unrelated cord blood transplantation (RI-UCBT) remain unclear. We analyzed the outcome of RI-UCBT in patients with relapsed or refractory lymphoma.

Table 1. *Patient Characteristics*

Patient No.	Age (y)/Sex	Histology	Stage	Previous Regimens/Previous Auto-HSCT	Previous Radiotherapy	Remission Status at RI-UCBT	Cord Blood Cell Dose (× 107/kg)	HLA Disparities	GVHD Prophylaxis
1	27/M	ALCL	IV	6/No	No	CR2	2.39	4/6	FK
2	32/F	DLBCL	IV	1/No	No	CR1	2.71	4/6	CSP
3	33/M	DLBCL	IV	3/No	Yes	PD	2.78	4/6	CSP
4	40/M	DLBCL	III	6/No	Yes	PD	2.27	4/6	FK
5	40/M	PTCL	IV	1/No	No	PR	3.4	4/6	CSP
6	41/F	DLBCL	IV	3/No	No	PD	3.2	4/6	FK
7	45/M	DLBCL	III	4/Yes (once)	Yes	PD	2.56	4/6	CSP
8	46/F	FL	IV	1/No	No	CR2	2.83	4/6	CSP
9	47/M	Nasal NK/T	II	1/yes (once)	Yes	PD	3	4/6	CSP
10	48/M	DLBCL	IV	2/No	No	PD	3.28	4/6	FK
11	48/F	FL	IV	5/No	No	PD	2.71	4/6	CSP
12	52/F	DLBCL	IV	3/No	No	PD	2.81	4/6	FK
13	55/M	DLBCL	III	4/No	Yes	PD	2.56	4/6	CSP
14	57/M	DLBCL	III	1/No	No	PD	2.9	4/6	CSP
15	57/F	DLBCL	IV	4/Yes (once)	Yes	PD	2.59	6/6	CSP
16	59/M	DLBCL	IV	3/Yes (once)	Yes	PD	2.26	4/6	FK
17	66/F	IVL	IV	2/No	No	PD	2.69	4/6	CSP
18	28/F	NS	IV	5/Yes (twice)	No	CR3	3.28	4/6	CSP
19	32/F	NS	IV	3/No	Yes	PD	2.83	4/6	FK
20	47/M	NS	III	7/Yes (once)	Yes	PD	2.34	4/6	CSP

ALCL indicates anaplastic large-cell lymphoma; auto, autologous; CR, complete remission; CSP, cyclosporine; DLBCL, diffuse large B-cell lymphoma; FK, tacrolimus; FL, follicular lymphoma; HSCT, hematopoietic stem cell transplantation; IVL, intravascular large B-cell lymphoma; Nasal NK/T, extranodal natural killer/T-cell lymphoma, nasal type; NS, nodular sclerosis classic Hodgkin lymphoma; PD, progression of disease; PR, partial remission; SCT, stem cell transplantation; PTCL, peripheral T-cell lymphoma, unspecified.

METHODS

Between September 2002 and April 2004, 20 adult patients with refractory lymphoma were treated with RI-UCBT at Toranomon Hospital, Japan. Biopsy-confirmed histologic diagnosis was based on the synonyms in the World Health Organization classification [19]. Of the 17 non-Hodgkin lymphoma patients, 2 had indolent lymphoma, and the other 15 had aggressive lymphoma (Table 1). Eligible patients had disease refractory to primary chemotherapy, had relapsed after first-line conventional chemotherapy or autologous transplantation, were considered inappropriate for conventional allogeneic hematopoietic stem cell transplantation because of the lack of an HLA-identical sibling or a suitable unrelated donor, were >50 years old, and/or had organ dysfunction. Those with chemosensitive diseases included all patients who had shown a response to the last therapy before transplantation (partial remission [PR] and complete remission [CR]); all other patients were classified as having chemoresistant diseases growing through multiple chemotherapy regimens (disease progression [PD]). All of the patients provided written informed consent in accordance with the requirements of the institutional review board. Data analysis was performed on December 1, 2004. The preparative regimen was composed of fludarabine 25 mg/m^2 daily for 5 days, melphalan 80 mg/m^2 daily for 1 day, and 4 Gy of total body irradiation in 2 fractions for 1 day. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporin A 3 mg/kg alone (n = 13) or tacrolimus 0.03 mg/kg alone (n = 7) from day −1 until the patients tolerated oral administration. The dose was tapered off from day 100 until 150. Patients were treated in laminar airflow rooms. All patients received prophylaxis with sulfamethoxazole or sulfadoxine against *Pneumocystis carinii* infection. Acyclovir and fluconazole prophylaxis were routinely used. Red blood cell and platelet transfusions were given to maintain hemoglobin levels >8 g/dL and platelet counts >10 × 10^9/L. Blood products were irradiated. Neutropenic patients received broad-spectrum intravenous antibiotics for the management of febrile neutropenia. Filgrastim 5 μg/kg/d was administered subcutaneously from day +1 until the neutrophil count was at least 1000/μL for 3 consecutive days. The cord blood unit was selected according to the number of nucleated cells per recipient's weight and HLA compatibility (HLA-A and -B by serology and HLA-DRB1 by high-resolution DNA typing). The chimerism status after RI-UCBT was determined by fluorescence in situ hybridization with a Y chromosome probe for sex-mismatched RI-UCBT or by polymerase chain reaction DNA typing of HLA antigens for HLA-mismatched RI-UCBT. Graft failure was defined as peripheral cytopenia and marrow hypoplasia occurring later than day 30, without detection of donor markers by cytogenetic or molecular techniques. The probability of overall and progression-free survival was estimated by the Kaplan-Meier method. Responses to transplantation were defined according to

Table 2. *Outcomes*

Patient No.	Neutrophils >5 × 10^8/L, d	Platelets >20 × 10^9/L, d	Acute GVHD Grade (Organ Involvement and Stage)	Chronic GVHD (Organ Involvement and Type)	Status post RI-UCBT (d)	Cause of Death
1	20	34	0	None	CR, 242+	
2	20	35	0	NE	Dead, 50, RL	Disease Progression
3	NE	NE	NE	NE	Dead, 15, NE	Pulmonary bleeding
4	17	36	III (skin 2, liver 0, gut 2)	None	PR, 292+	
5	16	40	0	Extensive (skin, lung, *de novo*)	CR, 352+	
6	NE	NE	NE	NE	Dead, 10, NE	Sepsis
7	14	36	III (skin 2, liver 0, gut 0)	None	CR, 471+	
8	23	72	III (skin 2, liver 0, gut 0)	None	CR, 482+	
9	NE	NE	NE	NE	Dead, 18, NE	Sepsis
10	23	39	0	None	CR, 306+	
11	33	NE	IV (skin 3, liver 3, gut 3)	NE	Dead, 74, CR	Sepsis
12	22	NE	0	NE	Dead, 40, CR	CMV pneumonia
13	GF	GF	NE	NE	Dead, 44, NE	Sepsis
14	NE	NE	NE	NE	Dead, 22, NE	Sepsis
15	12	26	0	None	CR, 392+	
16	18	29	II (skin 3, liver 0, gut 0)	None	CR, 213+	
17	14	57	III (skin 3, liver 0, gut 3)	NE	Dead, 73, CR	GVHD
18	13	43	I (skin 1, liver 0, gut 0)	None	CR, 406+	
19	25	48	III (skin 1, liver 0, gut 2)	Extensive (skin, gut, quiessent)	CR, 317+	
20	24	40	III (skin 0, liver 0, gut 2)	None	Dead, 113, CR	Pneumonia

CR indicates complete remission; GF, graft failure; GVHD, graft-versus-host disease; NE, not evaluable; PR, partial remission; RL, relapse.

the recommendations of the international working group [20].

RESULTS

The characteristics of the 20 patients and cord blood units are shown in Table 1. Nineteen (95%) of the 20 patients had advanced-stage disease at diagnosis (stage III/IV) and an International Prognostic Index score ≥2. Fifteen (75%) patients had refractory disease. Six patients (30%) had already experienced a treatment failure with front-line autologous transplantation. The median age of the patients was 46.5 years (range, 27-66 years), the median weight was 56 kg (range, 44-75 kg), and the median number of cryopreserved nucleated cells was 2.75 × 10^7/kg (range, 2.27-3.40 × 10^7/kg). Fifteen (75%) patients who underwent transplantation had a sustained engraftment as defined by neutrophil counts >0.5 × 10^9/L and an untransfused platelet count >20 × 10^9/L for at least 3 consecutive days (Table 2). Primary graft failure occurred in 1 patient, and autologous recovery was not observed. The median time to recovery to an absolute neutrophil count of 0.5 × 10^9/L was 20 days (range, 12-33 days), and the median time to achieve platelets >20 × 10^9/L was 39 days (range, 26-72 days). All patients with neutrophil engraftment showed full donor chimerism. Grade II to IV acute GVHD occurred in 8 of 15 evaluable patients, and chronic GVHD occurred in 2 of 11 evaluable patients. As for disease response, both patients with indolent lymphoma, 8 of 15 patients with aggressive lymphoma, and all 3 patients with Hodgkin lymphoma achieved CR after transplantation. A total of 16 patients had measurable disease at transplantation, and disease was reassessed at regular intervals after transplantation. The maximal response was CR in 10 patients and PR in 1. Among the CR patients, the median time to CR was 88 days (range, 32-220 days). Three of 4 patients in CR at RI-UCBT remain in sustained remission, and 1 has died of PD. One patient in PR at RI-UCBT achieved CR. Five of the 15 patients in PD at RI-UCBT achieved CR, 1 achieved PR, and 9 died of nonrelapse causes (sepsis/pneumonia, n = 7; acute GVHD, n = 1; pulmonary bleeding, n = 1). The cumulative incidence of nonrelapse mortality at 100 days was 41% (95% confidence interval, 19%-63%). At a median follow-up of 334.5 days (range, 213-482 days), 10 of the 20 patients were alive: 9 in CR and 1 in PR. The estimated 1-year overall and progression-free survival rates were both 50% (95% confidence interval, 28%-72%; Figure 1). Total nucleated cell dose HLA disparities, disease status, and preceding therapies were not associated with differences in survival (data not shown).

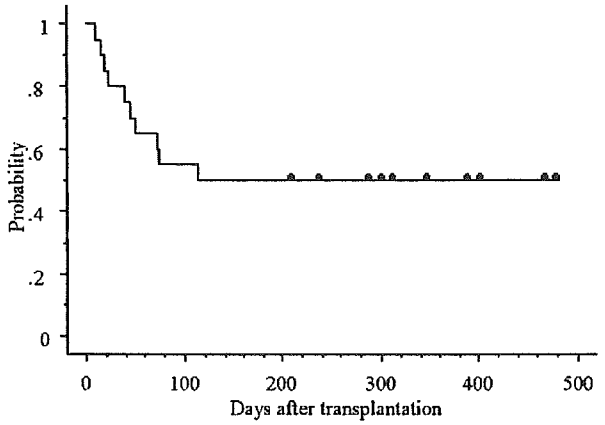

Figure 1. Overall survival (OS) and progression-free survival (PFS) after transplantation. The estimated 1-year OS and PFS were both 50.0% (95% CI, 28.1%-71.9%).

DISCUSSION

The results of our trial of RI-UCBT in patients with lymphoma whose disease recurred after a previous treatment are encouraging. The rapid availability of a unit of cord blood may be a particular advantage for lymphoma patients who require urgent transplantation, and cord blood can be an acceptable alternative stem cell source. Our patients were heavily pretreated and included chemoresistant and aggressive histologic types of disease whose outcome after RIST is reported to be very poor, with median survival rates from 19% to 32% at 1 year [21]. Despite our low-intensity conditioning regimen, a high rate of clinical remission was observed. This suggests that RI-UCBT does exert a strong allogeneic graft-versus-lymphoma effect. The median of 100 days between RI-UCBT and the appearance of a maximal response might be consistent with the time required for the activation and expansion of antitumor cytotoxic T cells.

The transplant-related mortality of 41% at day 100 in this study was higher than that reported for RIST in other studies [11,13,21-23]. Patient age, prior repeated therapies, disease status [24], and prolonged immunosuppression [25] would have played a role in the high transplant-related mortality after RI-UCBT. The proportion of deaths related to infection has recently been reported to be higher soon after cord blood transplantation than after bone marrow transplantation [12]. Additional strategies to promote engraftment and prevent early infectious complications should be developed, and this treatment strategy should be investigated among patients with less advanced diseases.

In conclusion, although our patient cohort was small and the observation period was limited, our result suggests that RI-UCBT is feasible for patients with refractory lymphoma who lack a suitable donor and require urgent treatment. RI-UCBT is associated with high transplant-related mortality, and this provides a rationale for a clinical study, which should be modified to focus on minimizing toxicities, controlling infectious complications, and enhancing any graft-versus-leukemia effect.

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REFERENCES

1. Chopra R, Goldstone AH, Pearce R, et al. Autologous versus allogeneic bone marrow transplantation for non-Hodgkin's lymphoma: a case-controlled analysis of the European Bone Marrow Transplant Group Registry data. *J Clin Oncol.* 1992; 10:1690-1695.
2. Verdonck LF, Dekker AW, Lokhorst HM, Petersen EJ, Nieuwenhuis HK. Allogeneic versus autologous bone marrow transplantation for refractory and recurrent low-grade non-Hodgkin's lymphoma. *Blood.* 1997;90:4201-4205.
3. Khouri IF, Keating M, Korbling M, et al. Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol.* 1998;16:2817-2824.
4. Goldstone AH, Kottaridis PD. The allogeneic effect in non-Hodgkin's lymphoma. *Leuk Lymphoma.* 2003;44(suppl 3):S91-S97.
5. Branson K, Chopra R, Kottaridis PD, et al. Role of nonmyeloablative allogeneic stem-cell transplantation after failure of autologous transplantation in patients with lymphoproliferative malignancies. *J Clin Oncol.* 2002;20:4022-4031.
6. Tanimoto TE, Kusumi E, Hamaki T, et al. High complete response rate after allogeneic hematopoietic stem cell transplantation with reduced-intensity conditioning regimens in advanced malignant lymphoma. *Bone Marrow Transplant.* 2003;32: 131-137.
7. Khouri IF, Saliba RM, Giralt SA, et al. Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. *Blood.* 2001; 98:3595-3599.
8. Herbert KE, Spencer A, Grigg A, Ryan G, McCormack C, Prince HM. Graft-versus-lymphoma effect in refractory cutaneous T-cell lymphoma after reduced-intensity HLA-matched sibling allogeneic stem cell transplantation. *Bone Marrow Transplant.* 2004;34:521-525.
9. Dhedin N, Giraudier S, Gaulard P, et al. Allogeneic bone marrow transplantation in aggressive non-Hodgkin's lymphoma (excluding Burkitt and lymphoblastic lymphoma): a se-

ries of 73 patients from the SFGM database. Societ Francaise de Greffe de Moelle. *Br J Haematol.* 1999;107:154-161.
10. Laughlin MJ, Barker J, Bambach B, et al. Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors. *N Engl J Med.* 2001;344:1815-1822.
11. Miyakoshi S, Yuji K, Kami M, et al. Successful engraftment after reduced-intensity umbilical cord blood transplantation for adult patients with advanced hematological diseases. *Clin Cancer Res.* 2004;10:3586-3592.
12. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med.* 2004;351:2265-2275.
13. Barker JN, Weisdorf DJ, DeFor TE, Blazar BR, Miller JS, Wagner JE. Rapid and complete donor chimerism in adult recipients of unrelated donor umbilical cord blood transplantation after reduced-intensity conditioning. *Blood.* 2003;102:1915-1919.
14. Rizzieri DA, Long GD, Vredenburgh JJ, et al. Successful allogeneic engraftment of mismatched unrelated cord blood following a nonmyeloablative preparative regimen. *Blood.* 2001;98:3486-3488.
15. Weinthal JA, Goldman SC, Lenarsky C. Successful treatment of relapsed Burkitt's lymphoma using unrelated cord blood transplantation as consolidation therapy. *Bone Marrow Transplant.* 2000;25:1311-1313.
16. Ooi J, Iseki T, Ito K, et al. Successful unrelated cord blood transplantation for relapse after autologous transplantation in non-Hodgkin's lymphoma. *Leuk Lymphoma.* 2002;43:653-655.
17. Yoshimasu T, Manabe A, Tanaka R, et al. Successful treatment of relapsed blastic natural killer cell lymphoma with unrelated cord blood transplantation. *Bone Marrow Transplant.* 2002;30:41-44.
18. Chao NJ, Koh LP, Long GD, et al. Adult recipients of umbilical cord blood transplants after nonmyeloablative preparative regimens. *Biol Blood Marrow Transplant.* 2004;10:569-575.
19. *Pathlogy and Genetics. Tumors of Hematopoietic and Lymphoid Tissues.* Geneva: World Health Organization; 2001.
20. Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. *J Clin Oncol.* 1999;17:1244.
21. Robinson SP, Goldstone AH, Mackinnon S, et al. Chemoresistant or aggressive lymphoma predicts for a poor outcome following reduced-intensity allogeneic progenitor cell transplantation: an analysis from the Lymphoma Working Party of the European Group for Blood and Bone Marrow Transplantation. *Blood.* 2002;100:4310-4316.
22. Toze CL, Barnett MJ, Connors JM, et al. Long-term disease-free survival of patients with advanced follicular lymphoma after allogeneic bone marrow transplantation. *Br J Haematol.* 2004;127:311-321.
23. Escalon MP, Champlin RE, Saliba RM, et al. Nonmyeloablative allogeneic hematopoietic transplantation: a promising salvage therapy for patients with non-Hodgkin's lymphoma whose disease has failed a prior autologous transplantation. *J Clin Oncol.* 2004;22:2419-2423.
24. Long GD, Laughlin M, Madan B, et al. Unrelated umbilical cord blood transplantation in adult patients. *Biol Blood Marrow Transplant.* 2003;9:772-780.
25. Klein AK, Patel DD, Gooding ME, et al. T-cell recovery in adults and children following umbilical cord blood transplantation. *Biol Blood Marrow Transplant.* 2001;7:454-466.

Erratum

In the March issue of *BBMT* the article by Drs. Lamb and Lopez (T cells: a new frontier for immunotherapy? *Biol Blood Marrow Transplant.* 2005;11:161-168) included an editing error. The phrase "guanosine monophosphate" was inadvertently inserted in place of "good manufacturing product". The correct passage is as follows: "Techniques are currently being developed for good manufacturing product–compatible clinical scale *ex vivo* expansion of T cells. The first clinical trials are expected within the next 6 to 12 months."

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ORIGINAL PAPER

Factors associated with granulocyte colony-stimulating factor-induced peripheral blood stem cell yield in healthy donors

H. Suzuya,[1] T. Watanabe,[1] R. Nakagawa,[1] H. Watanabe,[1] Y. Okamoto,[1] T. Onishi,[1] T. Abe,[1] Y. Kawano,[2] S. Kagami[1] & Y. Takaue[3]

[1]*Department of Pediatrics, University of Tokushima Graduate School of Medical Science, Tokushima, Japan*
[2]*Department of Pediatrics, University of Kagoshima Graduate School of Medical Science, Kagoshima, Japan*
[3]*Department of Internal Medicine, National Cancer Center Hospital, Tokyo, Japan*

Vox Sanguinis

Background and Objectives Poor collection results are a clinical problem in granulocyte-colony stimulating factor (G-CSF)-induced peripheral blood stem cell (PBSC) collection in healthy donors. It would be beneficial to be able to predict the PBSC yield from allogeneic donors before mobilization or harvesting.

Materials and Methods We examined the relationship between certain donor characteristics and the effectiveness of G-CSF-induced PBSC collection in 59 healthy family donors aged 3–63 years old (median 16 years). G-CSF was administered subcutaneously at 10 μg/kg for mobilization, daily for 5 days, and PBSC harvest using a continuous blood cell separator was started on day 5 of G-CSF treatment. Total cell yields were calculated as the number per unit of processed blood (l) per unit weight of the donor (kg).

Results In a univariate analysis, the donor's age, body mass index (BMI), white blood cell (WBC) count before mobilization, and platelet count before and during mobilization were significantly correlated with the yield of mononuclear cells (MNC), $CD34^+$ cells and granulocyte–macrophage colony-forming units (GM-CFU). Younger age ($P < 0{\cdot}001$), a low BMI ($P = 0{\cdot}002$), a high WBC count before mobilization ($P = 0{\cdot}004$), a high platelet count before ($P = 0{\cdot}012$) and during ($P < 0{\cdot}05$) mobilization, and a low speed of withdrawal ($P = 0{\cdot}019$) were associated with a higher $CD34^+$ cell yield. No significant correlation was found for gender, the type of G-CSF, the serum level of G-CSF, the type of cell separator, or the type of blood access. A multivariate forward and backward stepwise selection regression analysis showed that the factors associated with $CD34^+$ cell yield were age, platelet count before and during mobilization, and circulating $CD34^+$ cell concentration on day 2 of G-CSF treatment.

Conclusion In this small preliminary study, we found that donor age is the most important factor in predicting G-CSF-induced PBSC yields. Old age and low platelet counts before mobilization might be useful indicators for identifying poor mobilizers. Further validation of these findings in a larger number of donors are needed to establish whether these findings apply to other populations.

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Correspondence: Tsutomu Watanabe, MD, Department of Pediatrics, University of Tokushima Graduate School of Medical Science, Kuramoto-cho 2-18-15, Tokushima 770-8503, Japan
E-mail: twatanab@clin.med.tokushima-u.ac.jp

Introduction

Myeloablative chemotherapy and radiotherapy followed by allogeneic stem cell transplantation has been used as a curative treatment for haematological malignancies and life-threatening haematological disease. Allogeneic peripheral

blood stem cells (PBSC) have been increasingly used as the preferred source of haematopoietic stem cells because of improved neutrophil and platelet engraftment, avoidance of general anaesthesia for collection and potentially better graft-vs.-leukaemia effects [1]. Granulocyte colony-stimulating factor (G-CSF) has been exclusively used in normal donors to mobilize circulating stem/progenitor cells because of its predictability and safety [2]. Automated continuous blood cell separators are exclusively used for PBSC collection.

There is significant interindividual variability in donors with regard to the ability to collect PBSC, and there are donors who do not achieve the minimum target of $CD34^+$ cells recommended for allogeneic PBSC transplantation [3]. If we can predict poor collection before the use of G-CSF or early during G-CSF treatment, at least before apheresis, we may be able to avoid unnecessary G-CSF use and unwanted apheresis procedures. Furthermore, if a donor is the only donor available, we might perform another cycle of apheresis, or change to bone marrow (BM) harvest as a stem cell source or use some novel strategy, particularly for donors who are predicted to have poor stem cell mobilization with G-CSF.

In the autologous setting, the factors associated with poor-mobilizing cancer patients have been studied in detail [4–7]. The previous chemotherapy history affects mobilization [8]. In the allogeneic setting, there is little information available regarding the factors that predict harvested stem cell yields. Most previous studies that analysed the factors associated with PBSC yields were performed exclusively in adult donors [9,10]. Our study included paediatric donors who donated PBSC for use by their ill siblings. To date, there are no large studies that have included children in the study population. We also studied the factors associated with the apheresis procedures, which have been analysed only to minimally in previous studies.

Table 1 Donors' characteristics

Characteristics (n = 59)	
Age (years)	
Median (range)	16 (3–63)
No. of donors younger than 18 years of age	30
Sex (male/female)	34/25
Body weight (kg)	
Median (range)	54 (12–85)
BMI	
Median (range)	20·7 (13·0–32·8)
Mobilization	
Type of G-CSF (no. of donors)	
Filgrastim	29
Lenograstim	22
Nartograstim	8
Serum level of G-CSF (pg/ml)	
Median (range)	2014 (201–8450)
Mean ± SEM	2204 ± 362
Apheresis procedures	
Type of machine (no. of donors)	
COBE spectra	16
CS3000	43
Inlet lines (no. of donors)	
Radial artery	28
Antecubital vein	31
Blood drawing rate (ml/min)	
Median (range)	45 (15–70)
No. of aphereses	
Median (range)	3 (1–6)
Total processed volume (l)	
Median (range)	24 (7·2–50)

BMI, body mass index; G-CSF, granulocyte colony-stimulating factor; SEM, standard error of the mean.
Serum levels of G-CSF were measured on day 2.

Donors and methods

Donors

The donor characteristics are summarized in Table 1. All donors, except for children under 10 years old, agreed to PBSC donation after they had been informed about the procedures for both BM harvest and PBSC collection. The parents of donors younger than 10 years old were informed and gave their written consent to PBSC donation. The clinical protocols for PBSC harvest were approved by the institutional review board at Tokushima University Hospital.

Mobilization and collection of PBSC

Human recombinant G-CSF (filgrastim from Kirin Brewery Co., Tokyo, Japan, lenograstim from Chugai Pharmaceutical Co., Tokyo, Japan, or nartograstim from Kyowa-Hakko Kogyo Co., Tokyo, Japan), at a dose of 10 μg/kg, was injected subcutaneously into donors once a day, for 5 days, at 08:00 h to mobilize PBSC. Blood samples were drawn every day for further evaluations, just before injection of human recombinant G-CSF. Details of the harvesting procedure have been described previously [11]. Briefly, on day 5, PBSC harvest was started by apheresis using a Baxter CS3000 plus continuous-flow blood cell separator (Baxter Healthcare Corp., Deerfield, IL) or a COBE spectra (Gamblo Co., Lakewood, CO). Apheresis was initiated at 10:00 h on both days, and 150–300 ml/kg (maxumum 10 l) was processed per session. In children younger than 10 years old, blood was usually drawn from a radial artery using a 20–24 gauge catheter and given back to an antecubital vein or to veins in hands using an 18–24 gauge catheter. For donors weighing less than 20 kg, the extracorporeal circuit was preprimed with autologous blood that had been collected during the previous 3 weeks and stored,

depending on the machine and separating chambers. In adults, we used two antecubital veins for inlet and return lines to collect PBSC. When the blood withdrawal rate was poor, we used a radial artery, even in adult donors. Calcium gluconate was continuously infused at a dose of 1 ml per 200 ml blood processed during apheresis. The target doses were set at > 2×10^6/kg $CD34^+$ cells, according to our previous findings [12]. At least two aphereses were performed on day 5 in most donors. In some donors, we attempted to harvest more than three aphereses to obtain more than the target doses on days 6 and 7.

The numbers of mononuclear cells (MNC), $CD34^+$ cells and granulocyte–macrophage colony-forming units (GM-CFU) collected were counted. Total cell yields were calculated as the number per unit of processed blood (l) per unit weight of the donor (kg).

Cell counts, fluorescence-activated cell sorter assay for $CD34^+$ cells, and colony assay for GM-CFU

MNCs were determined using an automated counter (Beckman Coulter, Miami, FL). MNCs were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 fluorescein (Becton Dickinson, San Jose, CA) and with phycoerythrin (PE)-conjugated anti-CD34 phycoerythrin (Becton Dickinson) for 30 min at room temperature in the dark. Following incubation, red blood cells were lysed with fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson), and then washed twice with phosphate-buffered saline (PBS) containing 0·1% sodium azide and 10% bovine serum albumin (BSA), to remove unbound antibody. A total of 20 000 live events were counted in the fluorescence activated cell sorter (FACScan; Becton Dickinson). $CD34^+$ cells were selected based on their forward- and 90%-scatter properties and dim CD45 expression.

For the GM-CFU assay, MNC were cultured at a density of $0{\cdot}5–1{\cdot}0 \times 10^5$/ml in Iscove's modified Dulbecco's medium containing 0·8% methylcellulose, 20% fetal bovine serum (FBS), 450 μg/ml human transferrin and 1% deionized BSA. Recombinant human cytokines were added to cultures at various prescreened concentrations [200 ng/ml for stem cell factor (SCF), 200 ng/ml for interleukin (IL)-3, 2 U/ml for erythropoietin and 200 ng/ml for G-CSF]. Quadruplicate cultures were plated in a volume of 0·4 ml in 24-well tissue culture plates. The plates were incubated at 37 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% nitrogen for 14 days. Colonies, which were defined as clusters containing at least 40 cells, were scored after 14 days of culture using an inverted microscope, and the average value of triplicate data was used for calculations.

Measurement of serum G-CSF levels

We measured the daily serum concentration of G-CSF. We took samples 24 h after G-CSF administration and before the next G-CSF administration (i.e. as a trough level). An enzyme-linked immunosorbent assay (ELISA) kit for human G-CSF (Biotrek ELISA System; Amersham Life Science, Bucks., UK) was purchased. The manufacturer guaranteed the specificity of the assay by demonstrating that it failed to detect other known cytokines. The serum G-CSF level was measured by strict adherence to the manufacturer's instructions. In this assay system, the limit of sensitivity for the assay kit was 1·1 pg/ml for G-CSF.

Statistics

In a univariate analysis, the Spearman rank correlation test was used to analyse the effects of age, body mass index (BMI), the blood levels of G-CSF, blood withdrawal rate, white blood cell (WBC) count, platelet count, haemoglobin, MNC, the number of circulating $CD34^+$ cells and GM-CFU on cell yields. The Kruskal–Wallis test was used to analyse the effects of the type of G-CSF on cell yields. The Mann–Whitney *U*-test was used to analyse the influence of the type of cell separator, the type of inlet line, and gender on cell yields. A *P*-value of 0·05 was adopted as significant for all analyses. A multivariate forward and backward stepwise selection regression analysis was used to determine correlation coefficients and to derive a formula for predicting the cell yield. Statistical analysis was performed using StatView Version 5·0 for Macintosh (Abacus Concepts, Inc., Berkeley, CA).

Results

Procedure tolerability

All donors tolerated G-CSF administration. Three donors needed a reduced dose of G-CSF because of high leucocyte counts (> $50\,000 \times 10^6$/l) during mobilization. All donors tolerated the apheresis procedure. However, mild hypocalcemia was observed, which was resolved by increasing the dose of calcium gluconate infusion and did not require us to stop apheresis. Thrombocytopenia of < 100×10^9/l was seen in 16 donors after the completion of apheresis. Three donors showed a platelet count of less than 50×10^9/l after the completion of aphereses. However, there was no bleeding diasthesis.

Circulating progenitor cell kinetics and serum G-CSF levels during mobilization

The median WBC count before the initiation of G-CSF was 5800×10^6/l (range $2200–12\,000 \times 10^6$/l). The WBC count increased rapidly after G-CSF treatment, peaking on day 5 at $38\,450 \times 10^6$/l (range $21\,000–57\,300 \times 10^6$/l) (Fig. 1a). The $CD34^+$ cell number gradually increased from day 1 to day 3, and markedly increased on days 4 and 5 following the

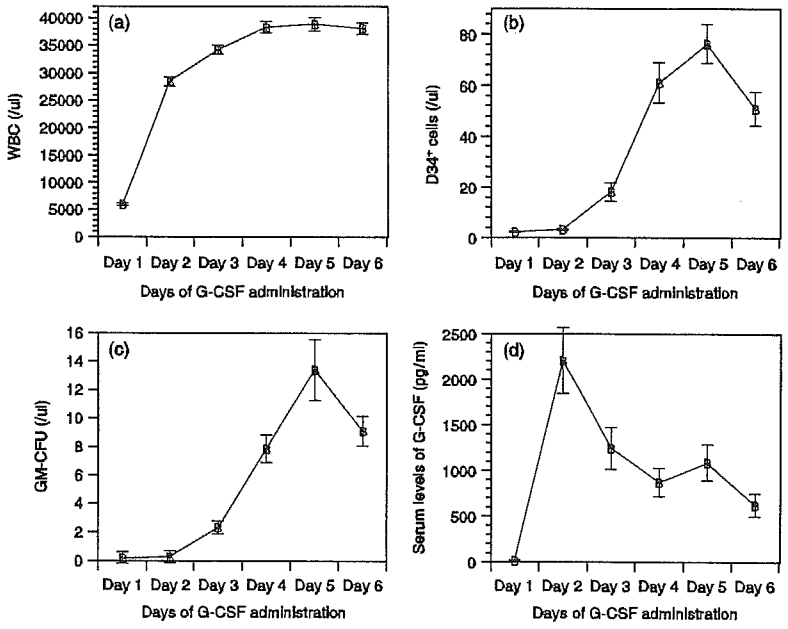

Fig. 1 Circulating progenitor cell kinetics and serum levels of granulocyte colony-stimulating factor (G-CSF) during G-CSF mobilization. (a) White blood cell; (b) $CD34^+$ cell; (c) granulocyte-macrophage colony-forming unit (GM-CFU); (d) serum levels of G-CSF. Results represent the mean value ± standard error of the mean (SEM).

initiation of G-CSF treatment (Fig. 1b). The GM-CFU number showed a gradual increase between days 1 and 3, and a marked increas on days 4 and 5 following the initiation of G-CSF treatment (Fig. 1c). The kinetics of $CD34^+$ cells and GM-CFU were well synchronized with the peaks on day 5. The average serum G-CSF level before G-CSF administration was 10·9 ± 8·1 (mean ± standard error of the mean) pg/ml; it showed a marked increase to 2204·8 ± 361·5 pg/ml on day 2, and then decreased from day 3 to day 6 (Fig. 1). We analysed the correlation between the serum G-CSF level on day 2 and PBSC yields.

Factors affecting G-CSF-induced PBSC yield

The cell yields were calculated as the number per unit of processed blood (l) per unit weight of the donor (kg). The blood withdrawal speed was calculated from the actual blood withdrawal speed by dividing by the donor's weight.

In a univariate analysis, age, BMI, blood withdrawal rate during collection, WBC count before mobilization, and platelet count before and during mobilization, were significantly correlated with MNC, $CD34^+$ cell and GM-CFU yields (Table 2). Younger age was associated with a higher MNC yield ($P <$ 0·001) (Fig. 2a), $CD34^+$ cell yield ($P <$ 0·001) (Fig. 2b) and GM-CFU yield ($P <$0·001) (Fig. 2c). A lower BMI was associated with a higher MNC yield ($P <$ 0·001), $CD34^+$ cell yield (P = 0·002) and GM-CFU yield (P = 0·022). A lower blood withdrawal rate was associated with a higher MNC yield ($P <$ 0·001), $CD34^+$ cell yield (P = 0·019) and GM-CFU yield (P = 0·003). A higher WBC count before mobilization was associated with a higher MNC yield (P = 0·001), $CD34^+$ cell yield (P = 0·004) and GM-CFU yield (P = 0·041). High platelet counts before and during mobilization were associated with a higher MNC yield (P = 0·004–0·017), $CD34^+$ cell yield (P = 0·003–0·019) and GM-CFU yield (P = 0·006–0·030). High haemoglobin levels on days 1–4 were correlated with a higher MNC yield (P = 0·010–0·029). A higher $CD34^+$ cell count in preapheresis blood (i.e. on day 5) was associated with a higher $CD34^+$ cell yield (P = 0·013). A higher GM-CFU in preapheresis blood (i.e. on day 5) was associated with a higher GM-CFU yield (P = 0·005). Gender, the type of G-CSF, the serum level of G-CSF on day 2, the type of cell separator, the type of inlet line, and the blood withdrawal rate had no significant effect on cell yields.

A multivariate forward and backward stepwise selection regression analysis showed that the factors associated with the MNC yield were age, WBC count before mobilization (i.e. on day 1) and WBC count on day 4; the factors associated with $CD34^+$ cell yield were age, platelet count before mobilization and circulating $CD34^+$ cell concentration on day 2 of G-CSF treatment; and the factors associated with GM-CFU yield were the platelet count on days 4 and 5.

Five of the total of 59 donors were poor mobilizers, where a poor mobilizer was defined as a donor in whom circulating $CD34^+$ cells never reached more than 20 per μl during mobilization.

Discussion

In our study, the most important factor for predicting good PBSC collection was the donor's age. Younger age was associated with a higher progenitor cell yield. It is not clear why

Table 2 Univariate analysis of factors (other than haematological parameters) for cell yields

Variables	MNC × 10^8/kg/l				$CD34^+$ cells × 10^6/kg/l				GM-CFU × 10^5/kg/l			
	Mean ± SD	Median	S.C.	*P*-value	Mean ± SD	Median	S.C.	*P*-value	Mean ± SD	Median	S.C.	*P*-value
Age			−0·546	< 0·001			−0·549	< 0·001			−0·492	< 0·001
BMI			−0·561	< 0·001			−0·412	0·002			−0·304	0·022
Gender				0·566				0·974				0·936
Female (n = 25)	0·67 ± 0·53	0·50			0·51± 0·52	0·42			1·21 ± 1·70	0·63		
Male (n = 34)	0·60 ± 0·50	0·47			0·47± 0·38	0·34			1·02 ± 1·05	0·72		
Type of G-CSF				0·420				0·114				0·058
Filgrastim (n = 29)	0·53 ± 0·35	0·44			0·39 ± 0·33	0·29			0·72 ± 0·75	0·48		
Lenograstim (n = 22)	0·82 ± 0·70	0·53			0·65 ± 0·57	0·47			1·61 ± 1·90	0·83		
Nartograstim (n = 8)	0·48 ± 0·17	0·47			0·38 ± 0·34	0·34			1·10 ± 0·87	0·88		
Serum level of G-CSF			−0·174	0·399			0·039	0·853			0·073	0·725
Type of machine				0·359				0·458				0·511
CS 3000 (n = 43)	0·62 ± 0·54	0·45			0·49 ± 0·49	0·34			1·12 ± 1·49	0·62		
COBE (n = 16)	0·65 ± 0·42	0·55			0·46 ± 0·24	0·43			1·06 ± 0·79	0·86		
Inlet lines				0·759				0·144				0·016
Radial artery (n = 31)	0·84 ± 0·64	0·53			0·57 ± 0·49	0·43			1·55 ± 1·71	0·87		
Antecubital vein (n = 28)	0·43 ± 0·18	0·45			0·40 ± 0·36	0·37			0·70 ± 0·66	0·60		
Blood withdrawal rate			−0·486	< 0·001			−0·302	0·019			−0·387	0·003

BMI, body mass index ; GM-CFU, granulocyte–macrophage colony-forming unit; G-CSF, granulocyte colony-stimulating factor; MNC, mononuclear cells; SD, standard deviation; S.C., Spearman Correlation.

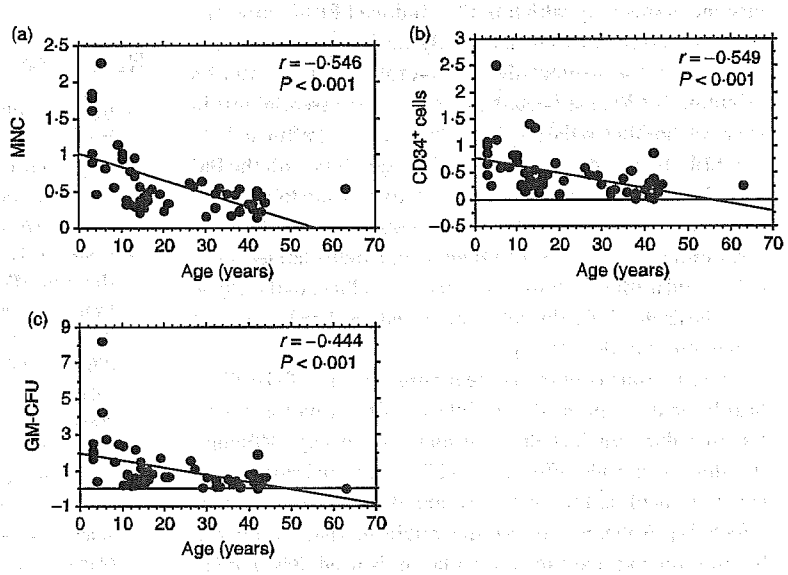

Fig. 2 Correlations between cell yields and the donor's age: (a) mononuclear cell (MNC) yield (× 10^8), (b) $CD34^+$ cell (× 10^6) yield, (c) granulocyte–macrophage colony-forming unit (GM-CFU) (× 10^5) yield. The cell yields were calculated as the number per unit of processed blood (l) per unit weight of the donor (kg).

age was found to be the most important predictor and, in fact, this is beyond the scope of the present study. Age-associated loss of BM haematopoietic function might play a role [13]. Further study is required to determine the mechanism of the age-associated loss of mobilization.

A higher $CD34^+$ cell count in preapheresis blood was associated with a higher $CD34^+$ cell yield, and a higher GM-CFU in preapheresis blood was associated with a higher GM-CFU yield. These findings suggest that the effectiveness of collection is related to mobilization, and are similar to results of previous studies [14]. However, these factors were determined after starting G-CSF administration and are thus inappropriate for practical use. We attempted to confirm the formula for predicting PBSC yield by using a multivariate forward and backward stepwise selection regression analysis. A multivariate analysis showed that the factors associated with the

$CD34^+$ cell yield were age, platelet count before mobilization and circulating $CD34^+$ cell concentration on day 2 of G-CSF treatment. However, the relatively low correlation coefficients limit the utility of these observations for any given patient.

Gender, the serum level of G-CSF and the type of G-CSF were not associated with cell yields. In this study, a fixed dose of G-CSF was used, and serum levels of G-CSF were within a wide range. However, we did not observe a correlation between the serum level of G-CSF and the effectiveness of mobilization. When the number of granulocytes increases, G-CSF is eliminated by cellular uptake via the G-CSF receptor and intracellular degradation, as well as by cleavage through neutrophil elastase [15,16]. Furthermore, serum G-CSF decreased with time in a log-linear manner, and the trough levels depend on metabolic clearance. Our data showed that the highest blood G-CSF level was observed on day 2, and it then decreased, which supports the above hypothesis. This indicates that the blood G-CSF level has limited predictive value in an individual donor. An analysis of gene polymorphism of the G-CSF receptor might be useful for predicting the response to G-CSF.

Interestingly, we found that the baseline donor platelet count and platelet count during mobilization correlated with the effectiveness of allogeneic PBSC mobilization. The precise mechanism by which G-CSF-induced PBSC enter the circulation is still unknown. Recently, the interaction between the chemokine stromal-derived factor-1 (SDF-1) and its receptor, CXCR4, has been shown to play an essential role in stem cell homing to the BM and subsequent engraftment [17–19]. SDF-1-mediated interaction of progenitors with the BM vascular niche allows the progenitors to relocate to a microenvironment that is suitable for megakaryocyte maturation and thrombopoiesis [20]. Our finding, of a high platelet count before and during mobilization, might be related to the above hypothesis that links thrombopoiesis and the development of progenitor motility [21,22].

There is some controversy regarding the use of G-CSF in healthy minors, especially in children who cannot assent to the procedure and lack decision-making capacity. Although the short-term side-effects of G-CSF in normal donors are generally benign, little is known about the long-term side-effects [2]. Apheresis procedures might be risky in minors because the extracorporeal circulation is used. BM harvest is also a harmful procedure for minors, although we have greater experience of BM harvest than PBSC collection in children. Children are frequently used in BM harvest, by their families and by doctors who extract their BM to try to save the life of a sibling. Although there are ethical considerations, PBSC transplantation may offer several advantages over BM transplantation for child donors and patients. In our study, parents were responsible for consenting to PBSC donation from minors younger than 10 years of age. Some donors had conditions for which general anaesthesia should be avoided, and most parents preferred PBSC collection over BM harvest. Parents are charged with the obligation to protect their children from harm, and most parents are capable of collecting and processing information necessary for making a decision to proceed with investigational medicine. The information provided to parents regarding the donor and the recipient must be detailed. In minors, PBSC donation is currently decided upon by well-informed parents and, if necessary, we may refer donors to an unbiased third party who is not concerned with the donation/treatment process. If this is not possible, PBSC collection from a minor should be avoided. This circumstance supports the need for a comprehensive study to evaluate the safety and efficacy of PBSC vs. BM collection in minors. In Japan, the Japanese Society of Blood Cell Transplantation established the donor follow-up programme to collect needed data on the side-effects of G-CSF on normal donors (including minors), in 1999, but the results are not yet available.

In conclusion, in this small preliminary study, we found that the donor's age is the most important factor in predicting G-CSF-induced PBSC yields. Old age and low platelet counts before mobilization might be useful for identifying poor mobilizers. Further validation of these findings in a larger number of donors are needed to establish whether these findings apply to other populations.

References

1 Bensinger WI, Martin PJ, Storer B, Clift R, Forman SJ, Negrin R, Kashyap A, Flowers ME, Lilleby K, Chauncey TR, Storb R, Appelbaum FR: Transplantation of bone marrow as compared with peripheral blood cells from HLA-identical relatives in patients with hematologic cancer. *N Engl J Med* 2001; 344:175–181

2 Anderlini P, Rizzo JD, Nugent ML, Schmitz N, Champlin RE, Horowitz MM: Peripheral blood stem cell donation: an analysis from the International Bone Marrow Transplant Registry (IBMTR) and European Group for Blood and Marrow Transplant (EBMT) database. *Bone Marrow Transplant* 2001; 27:689–692

3 Takeyama K, Ohto H: PBSC mobilization. *Transfus Apheresis Sci* 2004; 3:233–243

4 Pastore D, Specchia G, Mestice A, Liso A, Pannunzio A, Carluccio P, Buquicchio C, Greco G, Ciuffreda L, Pietrantuono G, Liso V: Good and poor $CD34^+$ cells mobilization in acute leukemia: analysis of factors affecting the yield of progenitor cells. *Bone Marrow Transplant* 2004; 33:1083–1087

5 Carral A, de la Rubia J, Martin G, Molla S, Martinez J, Sanz GF, Soler MA, Jarque I, Jimenez C, Sanz MA: Factors influencing the collection of peripheral blood stem cell in patients with acute myeloblastic leukemia and non-myeloid malignancies. *Leuk Res* 2003; 27:5–12

6 Nowrousian MR, Waschke S, Bojko P, Welt A, Schuett P, Ebeling P, Flasshove M, Moritz T, Schuette J, Seeber S: Impact of chemotherapy regimen and hematopoietic growth factor on mobilization and collection of peripheral blood stem cells in cancer patients. *Ann Oncol* 2003; 14:29–36

7 Delgado J, Fernandez-Jimenez MC, Martinez A, Sastre A,

Garcia-Miguel P, Hernandez-Navarro F, Arrieta R: Analysis of factors affecting PBPC collection in low-weight children with malignant disorders. *Cytotherapy* 2004; 6:43–49

8 Ford CD, Green W, Warenski S, Petersen FB: Effect of prior chemotherapy on hematopoietic stem cell mobilization. *Bone Marrow Transplant* 2004; 33:901–905

9 Brown RA, Adkins D, Goodnough LT, Haug JS, Todd G, Wehde M, Hendricks D, Ehlenbeck C, Laub L, DiPersio J: Factors that influence the collection and engraftment of allogeneic peripheral-blood stem cells in patients with hematologic malignancies. *J Clin Oncol* 1997; 15:3067–3074

10 Shimizu N, Asai T, Hashimoto S, Narita M, Kobayashi M, Ito M, Onoda M, Yokota A, Cho R, Nakaseko C, Nishimura M, Saito Y: Mobilization factors of peripheral blood stem cells in healthy donors. *Ther Apher* 2002; 6:413–418

11 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* 2002; 8:26–31

12 Kawano Y, Takaue Y, Watanabe T, Abe T, Okamoto Y, Iwai A, Iwai T, Watanabe A, Ito E, Makimoto A, Nakagawa R, Watanabe H, Sato J, Suenaga K, Suzuya H, Ohnishi T, Kanamaru S, Kaneko M, Kuroda Y: Efficacy of the mobilization of pediatric blood stem cells by granulocyte colony-stimulating factor in pediatric donors. *Cancer Res* 1999; 59:3321–3324

13 Carlo-Stella C, Di Nicola M, Milani R, Longoni P, Milanesi M, Bifulco C, Stucchi C, Guidetti A, Cleris L, Formelli F, Garotta G, Gianni AM: Age- and irradiation-associated loss of bone marrow hematopoietic function in mice is reversed by recombinant human growth hormone. *Exp Hematol* 2004; 32:171–178

14 Sohn SK, Kim JG, Chae YS, Kim DH, Lee NY, Suh JS, Lee KB: Large-volume leukapheresis using femoral venous access for harvesting peripheral blood stem cells with the Fenwall CS3000 Plus from normal healthy donors: predictors of $CD34^+$ cell yield and collection efficiency. *J Clin Apheresis* 2003; 18:10–15

15 El Ouriaghli F, Fujiwara H, Melenhorst JJ, Sconocchia G, Hensel N, Barrett J: Neutrophil elastase enzymatically antagonizes the in vitro action of G-CSF: implications for the regulation of granulopoiesis. *Blood* 2003; 101:1752–1758

16 Hunter MG, Druhan LJ, Massullo PR, Avalos B: Proteolytic cleavage of granulocyte colony-stimulating factor and its receptor by neutrophil elastase induced growth inhibition and decreased cell surface expression of the granulocyte colony-stimulating factor receptor. *Am J Hematol* 2003; 74:149–155

17 Papayannopoulou T: Current mechanistic scenario in hematopoietic stem/progenitor cell mobilization. *Blood* 2004; 103: 1580–1585

18 Fukuda S, Broxmeyer HE, Pelus LM: Flt3-ligand and the Flt3 receptor regulate hematopoietic cell migration by modulating the SDF-1{alpha} (CXCL 12)/CXCR4 axis. *Blood* 2004; 23:3117–3126

19 Pelus LM, Bian H, Fukuda S, Wong D, Merzouk A, Salari H: The CXCR4 agonist peptide, CTCE-0021, rapidly mobilizes polymorphonuclear neutrophils and hematopoietic progenitor cells into peripheral blood and synergizes with granulocyte colony-stimulating factor. *Exp Hematol* 2005; 33:295–307

20 Perez LE, Alpdogan O, Shieh JH, Wong D, Merzouk A, Salari H, O'Reilly RJ, van den Brink MR, Moore MA: Increased plasma levels of stromal-derived factor-1 (SDF-1/CXCL12) enhance human thrombopoiesis and mobilize human colony-forming cells (CFC) in NOD/SCJD mice. *Exp Hematol* 2004; 32:300–307

21 Avecilla ST, Hattori K, Heissig B, Tejada R, Liao F, Shido K, Jin DK, Dias S, Zhang F, Hartman TE, Hackett NR, Crystal RG, Witte L, Hicklin DJ, Bohlen P, Eaton D, Lyden D, de Sauvage F, Rafii S: Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med* 2004; 10:64–71

22 Nomura S, Inami N, Kanazawa S, Iwasaka T, Fukuhara S: Elevation of platelet activation markers and chemokines during peripheral blood stem cell harvest with G-CSF. *Stem Cells* 2004; 22:696–703

bjh research paper

Evaluation of cytomegalovirus-specific T-cell reconstitution in patients after various allogeneic haematopoietic stem cell transplantation using interferon-γ-enzyme-linked immunospot and human leucocyte antigen tetramer assays with an immunodominant T-cell epitope

Mutsuko Ohnishi,[1,2,3] Toshiharu Sakurai,[1] Yuji Heike,[2] Rie Yamazaki,[1] Yoshinobu Kanda,[4] Yoichi Takaue,[2] Hideaki Mizoguchi[3] and Yutaka Kawakami[1]

[1]Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, [2]Hematopoietic Stem Cell Transplantation Unit, National Cancer Center Hospital, [3]Departments of Hematology, Tokyo Women's Medical University, and [4]Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Tokyo, Japan

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Correspondence: Yutaka Kawakami MD PhD, Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjyukuku, Tokyo, 160-8582, Japan.
E-mail: yutakawa@sc.itc.keio.ac.jp

Summary

Cytomegalovirus (CMV) infection is a major complication for patients who received allogeneic haematopoietic stem cell transplantation (HSCT). Accurate monitoring of CMV-specific T-cell reconstitution is required for appropriate decision on treatment, such as anti-viral drugs, which have adverse effects. Although human leucocyte antigen (HLA) tetramer and interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assays have been used to measure CMV-specific T cells, detailed comparison of these assays and kinetics of anti-CMV T-cell reconstitution between reduced-intensity transplantation (RIST) and conventional HSCT has not yet been performed. In this study, we performed prospective comparative monitoring of CMV-specific T cells using HLA tetramer and IFN-γ-ELISPOT assays with a single immunodominant CMV_{495} peptide in 28 HLA-A*0201 and 9 HLA-A*0206 patients after various allogeneic HSCTs. The IFN-γ-ELISPOT assay was more sensitive for evaluation of functional T cells than the HLA tetramer assay, and CMV-specific T cells were reconstituted earlier in patients who received RIST without anti-thymocyte globulin (ATG) than those receiving RIST with ATG or conventional HSCT. The threshold level for protection from CMV reactivation was estimated as over 1×10^6 cells/l peripheral blood with the IFN-γ-ELISPOT assay. These results demonstrate that the IFN-γ-ELISPOT assay with CMV_{495} provides more accurate evaluation on CMV immunity in HLA-A*0201 and -A*0206 patients, and may be useful for determining timing of various treatments.

Keywords: cytomegalovirus, haematopoietic stem cell transplantation, interferon-γ-enzyme-linked immunospot, human leucocyte antigen tetramer, reduced-intensity transplantation.

Reactivation of latent cytomegalovirus (CMV) leads to an increased risk of life-threatening complications in immunocompromised hosts, including those receiving haemopoietic stem cell transplantation (HSCT) (Reusser *et al*, 1991; Li *et al*, 1994; Boeckh *et al*, 2003). CMV-specific T cells play an important role in the control of CMV reactivation (Reusser *et al*, 1991; Li *et al*, 1994; Walter *et al*, 1995; Einsele *et al*, 2002; Boeckh *et al*, 2003; Peggs *et al*, 2003). Although CMV antigenaemia (Nichols *et al*, 2001; Kanda *et al*, 2002) or polymerase chain reaction (PCR)-guided (Einsele *et al*, 1995) preemptive ganciclovir therapy have been shown to be effective for the prevention of CMV disease, ganciclovir administration results in an increased risk of cytopenia or late onset of CMV disease. Thus, accurate monitoring of CMV-specific T-cell recovery is important for ganciclovir administration timing (Cwynarski *et al*, 2001; Gratama *et al*, 2001; Ozdemir *et al*, 2002; Maris *et al*, 2003; Mohty *et al*, 2004).

The *in vitro* induction of CMV-specific T cells by stimulating peripheral blood mononuclear cells (PBMC) with viral infected cells has previously been performed (Reusser *et al*,

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1991; Li *et al*, 1994; Walter *et al*, 1995). Although this method provides whole anti-viral T-cell responses restricted by the patients human leucocyte antigens (HLAs), it is labour intensive and lacks quantitative analysis. The structural, major, late CMV matrix proteins, pp65 (Solache *et al*, 1999; Kuzushima *et al*, 2001) and IE (Hebart *et al*, 2003), were identified as immunodominant CMV antigens for cytotoxic T lymphocytes (CTL), and their T-cell epitopes have been identified. Quantitative evaluation of CMV-specific T cells using the HLA tetramer (Cwynarski *et al*, 2001; Gratama *et al*, 2001; Ozdemir *et al*, 2002; Mohty *et al*, 2004) and interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assays (Mohty *et al*, 2004) has been recently introduced. However, evaluation of the immunodominant nature of various CMV T-cell epitopes and direct comparison of these two assays with a single immunodominant peptide has not yet been performed in patients who have undergone HSCT.

Reduced-intensity transplantation (RIST) is increasingly applied for patients who are not eligible for conventional myeloablative HSCT (Mohty *et al*, 2000; Kanda *et al*, 2001; Chakrabarti *et al*, 2002; Junghanss *et al*, 2002; Nakai *et al*, 2002). The RIST regimens rely on immunosuppressive agents for prevention of graft rejection and acute graft-*versus*-host disease (GVHD), and sometimes use potent immunosuppressive drugs, such as antithymocyte globulin (ATG) (Mohty *et al*, 2000, 2004; Kanda *et al*, 2001, Nakai *et al*, 2002) or Campath-1H (Chakrabarti *et al*, 2002), which delay immune reconstitution after HSCT. Thus, it is important to investigate reconstitution of CMV-specific T cells among these different HSCT protocols.

In this study, we first determined the immunodominancy of the previously identified HLA-A*0201-restricted CMV peptides (Solache *et al*, 1999) among patients with various HLA-A2 subtypes. CMV-specific T cells were prospectively monitored with the immunodominant $pp65_{495-503}$, using both HLA tetramer and IFN-γ-ELISPOT assays in patients who received RIST with or without ATG or conventional HSCT. The IFN-γ-ELISPOT assay was found to be more sensitive for monitoring functional CMV-specific T cells in aiding the timing of ganciclovir administration and withdrawal, and that RIST without ATG showed an advantage for the earlier recovery of CMV immunity than other HSCT protocols.

Materials and methods

Patients and HSCT protocols

This research was approved by the Committee on Ethical and Clinical Investigation of the National Cancer Centre Hospital (NCCH). Informed consent was obtained from recipients and donors before study entry according to the Declaration of Helsinki. Thirty-seven patients (28 HLA-A*0201 and 9 HLA-A*0206) who underwent allogeneic peripheral blood stem cell transplantation (PBSCT) from HLA-matched siblings at NCCH between April 2001 and August 2003, were evaluated in this study (Table I). The HLA type of the donors and recipients was determined by PCR using sequence-specific primers. The preparative regimens were classified into three groups; (i) conventional regimen using 12 Gy of total body irradiation and 120 mg/kg of cyclophosphamide ($n = 5$) or the combination of 16 mg/kg oral busulphan and 120 mg/kg of cyclophosphamide ($n = 4$), (ii) RIST (8 mg/kg busulphan and 180 mg/m^2 fludarabine) with ($n = 11$) or (iii) without ($n = 17$) 5 mg/kg of rabbit ATG. Heparin-treated blood samples were taken at 1-week intervals after engraftment when

Table I. Characteristics of patients and transplant protocols.

Patient characteristics	No. of patients
Total, *n*	37
Median age, years (range)	44·8 (9–68)
Sex (male/female)	26/11
Disease	
Acute myeloid leukaemia	9
Acute lymphoblastic leukaemia	2
Myelodysplastic syndrome	5
Chronic myeloid leukaemia	5
Non-Hodgikin lymphoma	6
Multiple myeloma	1
Severe anaplastic anaemia	2
Solid tumour	7
Conditioning regimen	
Conventional	9
RIST ATG (+)	11
RIST ATG (−)	17
Source of stem cells	
Peripheral blood stem cells	37
CMV serologic status donor/recipient	
Positive/positive	32
Positive/negative	3
Negative/negative	2
HLA status	
HLA-A*0201	28
HLA-A*0206	9
Acute GVHD	
0-I	18
II	6
III-IV	13
Steroid use (mg/kg)	
0	20
1	11
2–10	6
Donor type	
Matched family	30
Mismatched family	7
Methods of GVHD prophylaxis	
CSP alone	18
CSP and MTX	19
Follow up day, median (range)	145·4 (42–325)

GVHD, graft-*versus*-host disease; CSP, cyclophosphamide; MTX, methotrexate; RIST, reduced-intensity transplantation; HLA, human leucocyte antigen.

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patients were hospitalised. HLA tetramer, ELISPOT, and antigenaemia assays were performed using the blood samples taken on the same day.

Evaluation of CMV infection

Pre-transplant sera from patients and donors were tested for CMV-specific immunoglobulin G (IgG) antibody (Ab) using an enzyme-linked immunosorbent assay (ELISA). CMV antigenaemia was evaluated using the previously described method (Gondo *et al*, 1994). Briefly, $1{\cdot}5 \times 10^5$ peripheral blood leucocytes were fixed on slides and incubated with anti-CMV pp65 monoclonal antibody (mAb) C7 conjugated with horse radish peroxidase (Teijin, Tokyo, Japan), stained using the direct immunoperoxidase method, and counted under a light microscope. The results are presented as numbers of positive cells per 50 000 leucocytes.

Induction of CMV-specific T cells with the synthesised CMV peptides

The CMV $pp65_{495-503}$ (NLVPMVATV), $pp65_{14-22}$ (VLGPISGHV), $pp65_{120-128}$ (MLNIPSINV) and melanoma antigen $gp100_{280-288}$ (YLEPGPVTA) were synthesised with a multiple peptide synthesiser (ACT 396; Advanced ChemTech, Louisville, KY, USA) using a standard solid-phase method with Fmoc chemistry, purified using reverse-phase high performance liquid chromatography (HPLC), and confirmed by mass spectral analysis. The purity of the peptides was over 90%. Two million PBMC were stimulated with 4 μg/ml of the CMV peptides in a well of 24-well plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% human pooled AB serum (HS), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C, and 100 IU/ml of recombinant human interleukin-2 (IL-2) (Shionogi, Osaka, Japan) was added on day 2. On days 7 and 14, the recovered cells were re-stimulated with irradiated peptide-pulsed autologous PBMC at a responder to stimulator ratio of 4:1, and IL-2 was added on day 9 and 16.

Production of HLA tetramers and analysis of specific T cells

Human leucocyte antigen-A*0201 tetramers were produced using previously described methods (Altman *et al*, 1996). Briefly, recombinant HLA-A*0201 heavy chains with the C terminal modification containing a substrate sequence for biotinylating enzyme BirA, and β_2-microglobulins were produced in *Escherichia coli* transformed with the relevant plasmids. Monomeric HLA-peptide complexes were generated by mixing the HLA heavy chains, β_2-microglobulins and antigenic peptides, then biotinylated by using recombinant BirA enzymes (Avidity, Denver, CO, USA) in the presence of biotin, and purified using gel filtration and anion exchange chromatography. HLA tetramers were then produced by mixing the biotynylated HLA-A2/peptide complexes with streptavidin-phycoerythrin (PE) (BD PharMingen, San Diego, CA, USA) at a molar ration of 10:1.

The CMV-specific CD8+ T cells in peripheral blood were evaluated with the HLA tetramers as follows: PBMC (10^6) were incubated with 2 μg/mL of PE-labelled HLA tetramers in 96-well plates for 30 min at 37°C, washed once with 200 μL of phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS), stained with fluorescein isothiocyanate (FITC)-labelled anti-CD8 mAb (T8-FITC; Beckman Coulter, Miami, FL, USA) for 1 h at 4°C, washed three times, and stained with 1 μg/ml 7-aminoactinomycin D (7-AAD) (Beckman Coulter) for 20 min at room temperature in 200 μL 5% FCS PBS. Stained cells were immediately analysed on a FACSCalibur using CELLQUEST software (Becton-Dickinson, Mountain View, CA, USA).

The frequency of CMV-specific T cells measured with the HLA tetramer in 17 CMV seropositive healthy individuals expressing either HLA-A*0201 or HLA-A*0206 was 0·76 ± 0·78% (mean ± 2SD) (range; 0·09–2·88%) of peripheral blood CD8+ T cells. Background staining obtained with the melanoma antigen $gp100_{280-288}$/HLA-A2 tetramer was 0·02%. The lower limit of detection by the CMV$pp65_{495}$ HLA tetramer was 0·02%, the lowest detection level of HLA tetramer positive cells by flow cytometry. The absolute number of the HLA tetramer positive T cells was calculated as: [lymphocyte count/peripheral blood (L)] × (numbers of HLA tetramer positive cells/total numbers of lymphocytes analysed by flow cytometry).

Measurement of IFN-γ producing CMV-specific T cells using ELISA or ELISPOT assay

The B-cell/T-cell hybrid cell line 174 CEM.T2 (T2) and COS-7 transfected with a plasmid expressing HLA-A*0206, were maintained in RPMI 1640 containing 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Fifty thousands T cells were cultured with either 1×10^5 HLA-A*0201 T2 cells or HLA-A*0206 transfected COS-7 cells pulsed with 5 μg/ml peptides for 16 h at 37°C in 2% human serum RPMI 1640 medium. IFN-γ in the culture supernatants was measured using IFN-γ-ELISA as previously described (Kawakami *et al*, 1994). The IFN-γ-ELISPOT assay was performed as previously described (Kuwana *et al*, 2003). Briefly, PBMC (2·5 or 1×10^5 per well) were stimulated with 5 μg/ml of the peptides, and incubated at 37°C for 16 h in 10% human serum IMDM medium, transferred to the multiscreen nitrocellulose 96-well immunoplate (Millipore, Bedford, MA, USA) coated with 15 μg/ml of anti IFN-γ-mAb (1-D1K; Mabtech, Stockholm, Sweden), and incubated at 37°C for 16 h. After 10 washes, it was mixed with 1 μg/ml of anti-IFN-γ biotinylated mAb (7-B6-1; Mabtech, Stockholm, Sweden) for 2 h at room temperature, washed 10 times with PBS, incubated with streptavidin-bound alkaline phosphatase (diluted 1 in 1000 in filtered) for 1 h at room temperature. After six washes with

PBS, IFN-γ spots were visualised by incubation with nitro blue tetrazolium/5-bromo-4-chloro-indolyl phosphate. Numbers of spot-forming cells (SFC) were counted using a computer-based evaluation system (KS-ELISPOT; Carl Zeiss, Hallbergmoos, Germany).

The frequency of CMV-specific T cells with the IFN-γ-ELISPOT assay was 84·7 ± 106·8 (range; 1–298·3) SFC/$2{\cdot}5 \times 10^5$ PBMC (mean ± 2SD) in 17 CMV seropositive healthy individuals expressing either HLA-A*0201 or HLA-A*0206. Background counts obtained with the $gp100_{280}$ was 5·3 SFC/$2{\cdot}5 \times 10^5$ PBMC, the lower limit of detection in this study. The absolute number of CMV-specific T-cell activity was calculated by the following formula; [lymphocyte and monocyte counts/peripheral blood (L)] × (numbers of IFN-γ positive spots/total number of added PBMC).

Statistical analysis

Spearman rank test was used to evaluate the correlation among the frequencies of IFN-γ producing cells, HLA tetramer-positive cells, and CMV antigenaemia. Paired and unpaired *t*-test were used for comparisons of the IFN-γ-ELISPOT and HLA tetramer assays as well as three different HSCT conditioning regimens. Kaplan–Meier analysis was applied for analysis of the relationship between the conditioning regimens and the day when CMV-specific T cells reached the threshold level after transplantation. Log-rank test was used to measure significant difference of cumulative rates of patients who reached the threshold level among patients with various HSCTs. All tests were two-tailed and were considered significant when $P < 0{\cdot}05$.

Results

*The CMV $pp65_{495-503}$ peptide is an immunodominant in both HLA-A*0201 and HLA-A*0206 individuals*

To evaluate the immunodominant nature of the previously identified CMV pp65 peptides restricted by HLA-A*0201 (Solache *et al*, 1999), including $pp65_{495-503}$ (NLVPMVATV), $pp65_{14-22}$ (VLGPISGHV), and $pp65_{120-128}$ (MLNIPSINV) in the Japanese population, whose popular HLA-A2 subtypes are HLA-A*0201 (10·6%), HLA-A*0206 (8·4%) and HLA-A*0207 (4·0%) (Tokunaga *et al*, 1997), we performed *in vitro* induction of CMV-specific T cells by stimulation with these three peptides from PBMC of 8 HLA-A*0201, 6 HLA-A*0206 and 3 HLA-A*0207, CMV-seropositive healthy donors. After 2–3 *in vitro* stimulations, CMV peptide-specific T cells were detected with IFN-γ release assay and HLA-A*0201 tetramer assay. CMV_{495}-specific T cells were induced from seven of eight HLA-A*0201 positive and all six HLA-A*0206 positive donors, while they were not induced or only detected in one case with other peptides or HLA-A*0207 donors (data not shown). It was noted that the CMV_{495}/HLA-A*0201 tetramer was able to specifically visualise not only HLA-A*0201 restricted, but also HLA-A*0206 restricted, CMV_{495}-specific T cells, indicating its use for immuno-monitoring HLA-A*0206 patients (data not shown). These results demonstrated that CMV_{495} is an immunodominant CMV epitope for both HLA-A*0201 and -A*0206 individuals. Therefore, we decided to use CMV_{495} to monitor CMV-specific T cells in patients after allogeneic HSCT, as it is important to use immunodominant epitopes to estimate total CMV immunity from the results with a single T-cell epitope.

The IFN-γ-ELISPOT assay is more sensitive to detect functional CMV-specific T cells than the HLA tetramer assay

Using IFN-γ-ELISPOT and HLA tetramer assays with the CMV_{495} peptide, we performed immuno-monitoring of CMV immunity in the patients who received various HSCTs. A significant correlation was observed between the frequencies obtained with the HLA tetramer and IFN-γ-ELISPOT assays ($P < 0{\cdot}001$). Absolute numbers of CMV-specific T cells determined with the HLA tetramer was higher than those obtained with the IFN-γ-ELISPOT assay probably because of detection of functional T cells with the ELISPOT assay among all CMV_{495}-specific T cells detected with the HLA tetramer.

The day that CMV-specific T cells were first detected after various HSCTs were evaluated in the HLA-A*0201 or -A*0206 positive, CMV seropositive patients. The patients who received steroid therapy before the first detectable time point of CMV-specific T cells after HSCTs were excluded from this analysis. The mean ± 2SD of first detected day was 55 ± 31 (range; 14–113 d) with the tetramer and day 37 ± 31 (13–113 d) with the ELISPOT, respectively (Fig 1, $P < 0{\cdot}001$), indicating that the IFN-γ-ELISPOT assay was more sensitive for detection of CMV-specific T cells than the HLA tetramer assay.

Early reconstitution of CMV-specific T cells in patients who received RIST without ATG

Recovery of CMV-specific T cells was then compared among the HLA-A*0201 or -A*0206 positive, CMV seropositive patients who received various allogeneic HSCTs using the HLA tetramer and IFN-γ-ELISPOT assays. With the tetramer or ELISPOT, CMV-specific CD8+ T cells were first detected on day 36 ± 16 (mean ± 2SD) (14–65 d), or day 22 ± 6 (14–29 d) after HSCT in the patients who received RIST without ATG, on day 62 ± 32 (23–104 d), or day 35 ± 4 (23–56 d) in the patients who received RIST with ATG, or on day 79 ± 39 (14–113 d), or day 73 ± 19 (13–113 d) in the patients who received conventional HSCT, respectively (Fig 2A, B). With the ELISPOT, the reconstitution of CMV-specific T cells was profoundly delayed in the patients who received conventional HSCT than those who received RIST with ($P = 0{\cdot}03$) or without ATG regimen ($P = 0{\cdot}001$). In the patients with RIST, ATG administration significantly delayed recovery of CMV-specific T cells ($P = 0{\cdot}007$).

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