Table 1 Patients characteristics

	Group 1 (N = 21)	*Group 2 (N = 23)*	*Group 3 (N = 12)*	*Total G-CSF alone (N = 56)*	*Chemotherapy plus G-CSF (N = 18)*
Age: (Median (range))	7 (1–21)	8 (1–21)	6 (2–13)	7 (1–21)	7 (1–21)
Sex (M/F)	14/7	18/5	10/2	42/14	13/5
Primary tumor	Germ cell tumor 6 Medulloblastoma 3 Pilocytic astrocytoma 3 PNET/Ewing's 3 Rhabdomyosarcoma 2 Pontine glioma 1 Adrenal cancer 1 Retinoblastoma 1 Ependymoma 1	Medulloblastoma 8 Neuroblastoma 4 Germ cell tumor 4 Wilms' Tumor 2 Osteosarcoma 2 NHL 1 MRK 1 Hepatoblastoma 1	Medulloblastoma 5 Neuroblastoma 3 Germ cell tumor 2 Pilocytic astrocytoma 1 Wilms' tumor 1	Medulloblastoma 16 Germ cell tumor 13 Neuroblastoma 5 Pilocytic astrocytoma 4 Wilms' tumor 3 Osteosarcoma 3 PNET/Ewing's 3 Rhabdomyosarcoma 2 Others 10	Medulloblastoma 7 Neuroblastoma 3 NHL 3 Rhabdomyosarcoma 3 PNET/Ewing's 1 Osteosarcoma 1
Metastasis to bone marrow	1	1	2	4	2
Prior chemotherapy					
Platinum-based	14	11	0	25	4
Alkylator-Based	5	3	0	8	1
Platinum/Alkylator-based	2	9	12	23	6
No. of cycles	3 (2–3)	10 (4–12)	8 (3–15)	6 (2–15)	5 (3–10)
No. of high-dose therapies	0	0	2 (1–3)	2 (1–3)	0
Local irradiation	4	1	0	5	1
Extensive irradiation	0	5	5	10	5
Time from onset to PBSC harvest (months)	4 (1–13)	7 (4–25)	19 (9–48)	6 (1–30)	5 (3–15)
Last chemotherapy to G-CSF (days)	19 (0–35)	19 (11–41)	29 (15–85)	24 (0–85)	18 (11–26)
Types of G-CSF					
Filgrastim	9	11	6	26	10
Lenograstim	10	10	6	26	8
Nartograstim	2	2	0	4	0

Abbreviations: G-CSF = granulocyte colony-stimulating factor; MRK = malignant rhabdoid tumor; NHL = non-Hodgkin's Lymphoma; PBSC = peripheral blood stem cell; PNET = primitive neuroectodermal tumor.

Colony-forming units-granulocyte/macrophage assay
Colony-forming units-granulocyte/macrophage were determined using MethoCult GF H4434V (VERITAS Co., Tokyo, Japan) according to the manufacturer's guidelines. Briefly, MNCs were incubated in methylcellulose medium supplemented with recombinant human erythropoietin, recombinant human stem cell factor, recombinant human GM-CSF, recombinant human G-CSF and recombinant human IL-3. Cells were placed in 35-mm culture dishes in triplicate and incubated in an ESPEC N_2–O_2–CO_2, a,m,o-110 incubator (Tabai ESPEC Co., Osaka, Japan), which maintained a humid atmosphere of 5% carbon dioxide, 5% oxygen and 90% nitrogen. After 14 days of incubation, the number of CFU-GM was counted under an inverted microscope. The number of circulating CFU-GM was calculated with the following formula:

$$\text{CFU} - \text{GM/l} = \frac{\text{WBC } (\times 10^6/\text{l}) \times \text{MNC } (\%/100) \times \text{no. of colonies per well}}{\text{no. of scattered MNC per well}}$$

High-dose chemotherapy and autologous peripheral blood stem cell transplantation
The high-dose regimen depended on the type of cancer. Mostly, regimens consisted of 200 mg/kg cyclophosphamide and 1600 mg/m^2 carboplatinum for pilocytic astrocytoma and medulloblastoma, 750 mg/m^2 ThioTEPA, 450 mg/m^2 MCNU and 1500 mg/m^2 cyclophosphamide for medulloblastoma, 1600 mg/m^2 carboplatinum and 1500 mg/m^2 etoposide for germ cell tumor, 1600 mg/m^2 etoposide, 750 mg/m^2 ThioTEPA and 180 mg/m^2 melphalan for rhabdomyosarcoma and peripheral neuroectodermal tumor, 750 mg/m^2 ThioTEPA and 180 mg/m^2 melphalan for osteosarcoma, 1600 mg/m^2 carboplatinum, 1500 mg/m^2 etoposide and 180 mg/m^2 melphalan for neuroblastoma and 450 mg/m^2 MCNU, 16 g/m^2 cytosine arabinoside (Ara-C), 1600 mg/m^2 etoposide and 100 mg/kg cyclophosphamide for non-Hodgkin's lymphoma. Post transplant G-CSF use was limited to patients who showed poor PBSC collection, such as Group 3 patients or for patients, who were complicated with severe infection.

Statistical analysis
Statistical analysis was performed using SPSS statistical software (SPSS Co., Tokyo, Japan). Groups were compared using the non-paired *t*-test. The relationships between different hematological parameters and PBSC yields were analyzed by the Spearman rank correlation test. Pearson's sample correlation coefficient and the corresponding *P*-value were calculated for the null hypothesis of no correlation.

Results

Circulating cell kinetics during mobilization with granulocyte colony-stimulating factor alone (Figure 1)
The changes in WBC, platelet, circulating CD34$^+$ cells and CFU-GM in patients mobilized with G-CSF alone are shown in Figure 1. The median WBC before the initiation of G-CSF was 3.35 × 10^9/l (range: 1.20–7.90). The median hemoglobin level and platelet count before the initiation of G-CSF were 9.6 g/dl (7.4–13.5) and 249 × 10^9/l (70–647), respectively. WBC increased rapidly after G-CSF treatment, with a peak of 29.67 × 10^9/l (range: 9.30–62.00) on day 6. The platelet count decreased gradually during G-CSF treatment, but this decrease was not significant. After apheresis on day 5, the platelet count significantly decreased to 144 × 10^9/l (44–410). The kinetics of circula-

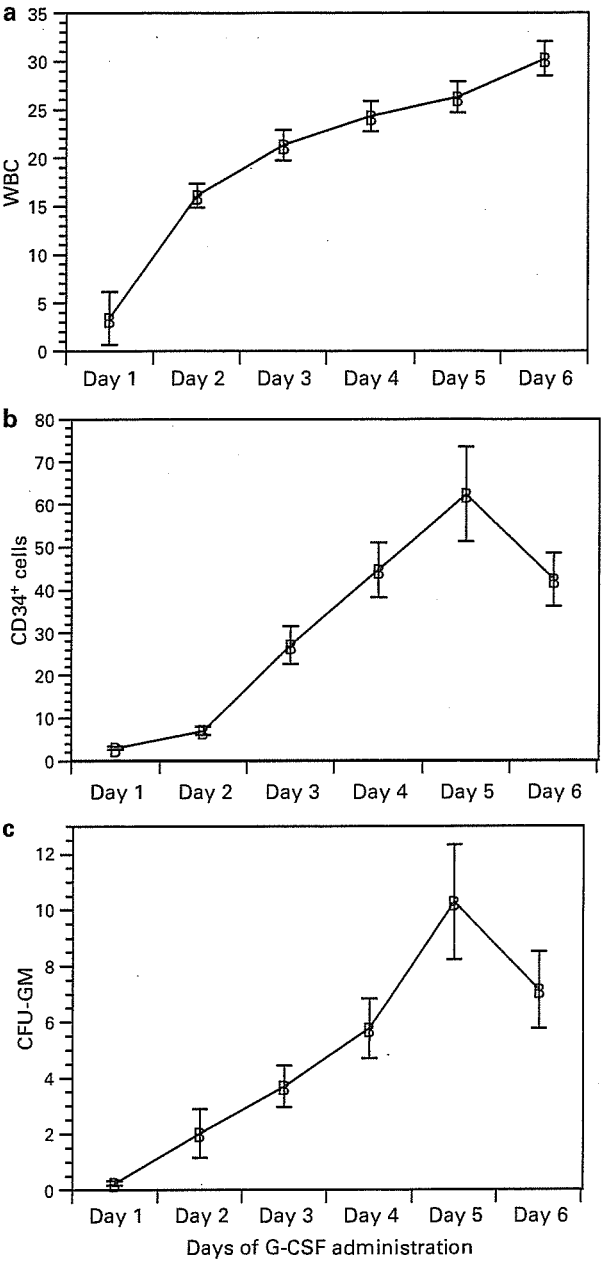

Figure 1 Circulating cell kinetics during granulocyte colony-stimulating factor mobilization. (a) WBC ($\times 10^9$/l), (b) circulating CD34+ cells ($\times 10^6$/l), (c) circulating colony-forming units-granulocyte/macrophage ($\times 10^6$/l).

tory $CD34^+$ cells and CFU-GM during mobilization were synchronized, and both of the peak values occurred on days 4 through 6 of G-CSF treatment. The numbers of $CD34^+$ cells and CFU-GM gradually increased between days 1 and 3, and markedly increased on days 4 and 6 following the initiation of G-CSF treatment. The peak $CD34^+$ cell level was observed on day 5 in 37 of 56 patients (66%), on day 4 in 10 patients (18%) and on day 6 in nine patients (16%). The peak CFU-GM level was observed on day 5 in 40 of 56 patients (71%), on day 4 in seven patients (13%) and on day 6 in nine patients (16%). The median numbers of circulating $CD34^+$ cells and CFU-GM on day 5 were 62.4×10^6/l (4.13–192.60) and 11.16×10^6/l (0.056–48.98), respectively.

Mobilization effects and peripheral blood stem cell apheresis yields

The results of mobilization and apheresis are shown in Table 2. Peripheral blood stem cell yields were calculated as number per unit of processed blood (l) per unit weight of the patient (kg). The peak values of circulating CD34+ cells and CFU-GM in Group 3 were significantly lower than those in Groups 1 ($P=0.002$) and 2 ($P=0.049$). Compared to patients mobilized by chemotherapy plus G-CSF, the $CD34^+$ cell yields were significantly lower in all of the patients mobilized with G-CSF alone ($P=0.045$). Compared to patients mobilized by chemotherapy plus G-CSF, the $CD34^+$ cell yields were significantly lower in Group 3 patients ($P=0.014$). Compared to patients mobilized by chemotherapy plus G-CSF, the CFU-GM yields were significantly lower in Group 2 ($P=0.033$). The $CD34^+$ cell yield was significantly lower in Group 3 compared to Group 1 ($P=0.014$). There were no differences in $CD34^+$ cell yields between Groups 1 and 2, and the control group. The CFU-GM yield in Group 1 was significantly higher than that in Groups 2 and 3 ($P=0.033$ and 0.048, respectively).

We analyzed several factors that might be used to predict PBSC yields such as age and hematological parameters before and during G-CSF treatment in all of the patients who were mobilized with G-CSF alone. In all of these patients, the number of circulating $CD34^+$ cells on day 5 was significantly correlated with the $CD34^+$ yield (Figure 2). There was no significant correlation between any other factors and PBSC yields.

Toxicity of the mobilization procedure

No patient required a reduced dose of G-CSF. The adverse effects induced by G-CSF injection were tolerable in all groups. Although patients mobilized with G-CSF alone who were older than 10 years sometimes complained of slight fever, bone pain, lumbago or mild headache, this was sustained with or without medication. Clinical signs of severe infection were not evident in patients mobilized with G-CSF alone. However, among patients mobilized with chemotherapy plus G-CSF, almost all of the patients experienced neutropenia, and the nadir ANC was 0.1–0.4×10^9/l. There were episodes of fever during neutropenia

Table 2 Mobilization and collection of PBSC by apheresis

	Group 1 (N=21)	*Group 2 (N=23)*	*Group 3 (N=12)*	*All (N=56)*	*Chemotherapy plus G-CSF (N=18)*
$CD34^+$ cell($\times 10^6$)/l (peak)					NE
Mean	72.6	81.3	19.1	62.4	
Median (range)	82.9 (34.3–118.5)	46.3 (14.0–361.2)	16.5 (4.2–40.0)	34.3 (4.2–361.2)	
CFU-GM ($\times 10^6$)/l (peak)					NE
Mean	16.6	10.6	2.8	10.3	
Median (range)	9.3 (0.0–48.9)	7.8 (0.1–32.6)	0.9 (0.0–10.4)	3.3 (0.0–48.9)	
*Apheresis number**					
Mean	2.8	3.0	3.1	2.9	2.7
Median (range)	3 (2–4)	3 (2–4)	3 (2–4)	3 (2–4)	2 (1–5)
MNC/kg/l collected					
Mean	0.94	1.08	1.12	1.02	0.69
Median (range)	0.61 (0.18–2.10)	0.67 (0.12–6.26)	1.05 (0.28–2.59)	0.67(0.12–6.26)	0.64 (0.09–1.39)
$CD34^+$ cells/kg/l collected					
Mean	1.33	1.19	0.42*	1.06*	2.62
Median (range)	0.94 (0.07–3.82)	0.83 (0.08–7.78)	0.36 (0.08–1.39)	0.60 (0.07–7.78)	0.67 (0.01–13.88)
CFU-GM/kg/l collected					
Mean	2.01	1.09*	1.35	1.37*	3.71
Median (range)	1.54 (0.21–5.01)	0.77 (0.10–3.56)	0.68 (0.05–4.15)	0.82 (0.05–5.01)	0.70 (0.06–20.25)
Mobilization failure (no)	1	2	1	4	1

Abbreviations: CFU-GM = colony-forming units-granulocyte/macrophage; G-CSF = granulocyte colony-stimulating factor; PBSC = peripheral blood stem cells.

PBSC were collected to support double sequential high-dose chemotherapy in three patients in Group 1, 12 patients in Group 2, four patients in Group 3 and no patients in the control group.

Number was divided by the target number of planned high-dose chemotherapies.

*Significantly low compared to the control group.

in 13 of 18 patients (72%) mobilized with chemotherapy plus G-CSF, which required treatment with intravenous antibiotics and/or antifungal agents. Culture was positive for five patients who were mobilized with chemotherapy plus G-CSF.

Engraftment

The results of engraftment following autologous PBSCT are shown in Table 3. In Group 1, high-dose chemotherapy was cancelled because of progressive disease in one. In Group 2, high-dose chemotherapy was cancelled because of stable disease in one and poor PBSC collection in one. In Group 3, only one patient declined high-dose chemotherapy because of poor PBSC collection, and proceeded to allogeneic cord blood cell transplantation.

Three patients in Group 1, 12 in Group 2 and four in Group 3 received double sequential high-dose chemotherapy. None of the patients in the control group received double transplantation. There were no significant differences in WBC and ANC engraftment among all groups, although platelet recovery was delayed in patients mobilized with G-CSF alone, especially Group 3 patients. Graft failure was not observed in any of the transplanted patients.

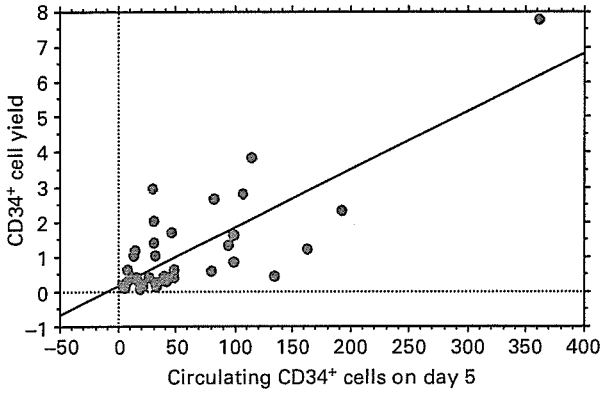

Figure 2 Correlation between circulating $CD34^+$ cells ($\times 10^6$/l) on day 5 of granulocyte colony-stimulating factor (G-CSF) administration and $CD34^+$ cell yields in patients mobilized with G-CSF alone ($N=38$). $CD34^+$ cell yields were calculated as number per unit of processed blood (l) per unit weight of the patient (kg).

Discussion

This study was a retrospective analysis of pediatric and adolescent patients with solid tumors treated with autologous PBSCT at our institution. Therefore, the disease and conditioning regimens are heterogeneous. In addition, the relatively small number of patients in some treatment groups and the retrospective analysis may make it difficult to draw any definitive conclusions. Nevertheless, our data suggest that G-CSF alone may be capable of mobilizing a sufficient number of PBSC for successful autografting after high-dose chemotherapy in pediatric and adolescent patients with solid tumors. The study of mobilization kinetics showed a similar pattern of PBSC mobilization with allogeneic donors, and the peaks of circulating $CD34^+$ cells and CFU-GM were observed on days 4 through 6 of G-CSF treatment. Thus, when PBSC are mobilized by G-CSF alone, we can easily determine the

Table 3 Engraftment results

	Group 1 (N=21)	*Group 2 (N=23)*	*Group 3 (N=12)*	*All (N=56)*	*Chemotherapy plus G-CSF (N=18)*
Total no. of transplants	24	35	16	75	18
No. of cases of double transplant	3	12	4	19	0
No. of transplanted cells					
MNC ($\times 10^8$/kg)					
Mean	4.79	3.33	4.35	4.01	5.36
Median (range)	4.03 (1.30–13.20)	2.59 (1.40–13.70)	5.58 (0.91–6.19)	3.20 (0.91–13.70)	4.60 (2.13–13.2)
$CD34^+$ cells ($\times 10^6$/kg)					
Mean	10.36	4.10*	2.43*	6.39	13.91
Median (range)	4.51 (1.20–48.20)	3.38 (0.36–9.65)	1.47 (0.41–5.90)	3.8 (0.36–48.2)	8.7 (4.46–74.5)
CFU-GM ($\times 10^5$/kg)					
Mean	11.02	4.04*	0.99*	5.54*	17.77
Median (range)	3.60 (0.30–14.00)	3.65 (0.10–12.80)	0.42 (0–3.74)	3.49 (0–14.00)	14.85 (0.55–90)
Engraftment days (median (range))					
WBC > 1.0×10^9/l	11 (8–26)	12 (7–21)	12 (9–28)	11 (8–28)	12 (9–16)
ANC > 0.5×10^9/l	11 (8–26)	13 (9–22)	12 (9–28)	12 (9–28)	12 (9–16)
Platelet > 20×10^9/l	12 (7–28)	15 (8–31)	22** (8–55)	15 (8–55)	10 (7–16)
Platelet > 50×10^9/l	16 (10–56)	27** (8–49)	27* (20–149)	20** (8–149)	14 (7–28)
Post transplant G-CSF use (no.)	6	16	14	36	3

Abbreviations: CFU-GM = colony-forming units-granulocyte/macrophage; G-CSF = granulocyte colony-stimulating factor.
*Significantly low compared to the control group.
**Significantly delayed compared to the control group.

timing of commencing PBSC harvest without monitoring the daily CD34+ cell concentration. In contrast, in mobilization with chemotherapy plus G-CSF, it might be difficult to determine the optimal timing of PBSC harvest following chemotherapy because of the wide range of optimal mobilization time points.[9] In mobilization with chemotherapy plus G-CSF, suggested predictive factors for initiating harvests have included the peripheral WBC count, circulating CD34+ cells, the peripheral absolute monocyte count and the platelet count.[10,13] The measurement of CD34+ cells in peripheral blood before PBSC harvest has begun to be used, but it is too labor intensive to determine this value every day around the time of PBSC harvest. Furthermore, when chemotherapy is used for PBSC mobilization, myelosuppression induced by chemotherapy is inevitable, and we might repeat unwanted chemotherapy simply to mobilize PBSC, and thus cause additional costs for patient care. Most patients who received chemotherapy for PBSC mobilization required treatment of neutropenic fever,[14] as in our study. Drawbacks for mobilization with G-CSF alone include a 1-week delay of scheduled chemotherapy and a lack of antitumor effects. However, if we started to harvest after two or three courses of chemotherapy instead of in the early phase of chemotherapy, this delay might not affect the outcome of the disease. Although the dose of G-CSF is higher in mobilization with G-CSF alone than in post-chemotherapy mobilization, the duration of G-CSF administration can be much longer in post-chemotherapy administration than in mobilization with G-CSF alone. The total dose of G-CSF in post-chemotherapy mobilization might be greater than in mobilization with G-CSF alone. Thus, mobilization with G-CSF alone might offer clinical and economic advantages, and might increase convenience and improve the QOL of patients.

It is often difficult to obtain the minimum requirement of CD34+ cells for safe engraftment after autologous PBSCT in heavily pre-treated patients. In adult lymphoma patients, predictors of poor mobilization were more than two prior treatment regimens and the WBC count on the first day of apheresis.[15,16] In another study, patients who failed to mobilize had received more than seven cycles, and the authors advocated that PBSC collection should be planned early in the course of chemotherapy.[17] In adult cancer patients, factors that predict a higher likelihood of poor mobilization include increasing numbers of cycles of prior chemotherapy, prior radiation therapy and the presence of overt marrow metastasis.[18–20] Autologous donors are assumed to have sustained an injury to the hematopoietic stem cell system that is responsible for the poor mobilization. Limited data are available with regard to PBSC collection with G-CSF alone in pediatric patients with cancer.[21] We showed that PBSC could be mobilized by G-CSF alone even in most of the heavily treated pediatric patients with solid tumors using the same dose as in healthy donors. Most of the patients received platinum-based or alkylator-based chemotherapy before PBSC harvest. We could not analyze the effects of specific chemotherapeutic agents on PBSC mobilization. We merely analyzed the effects of the degree of previous chemotherapy on PBSC mobilization. Obviously, when the patients were categorized according to prior treatment, the frequency of poor mobilization depended on the degree of previous treatment. In our study, we stopped collecting PBSC in four patients. A previous study showed that the frequency of poor mobilizers was 14% in patients mobilized with G-CSF alone,[14] which was similar to our experience. The dose-escalation effects of G-CSF alone or in combination with other cytokines in such patients should be investigated to improve the efficacy of PBSC mobilization.

Peripheral blood stem cell yields might be lower in the G-CSF alone group than in the chemotherapy plus G-CSF mobilization group. However, there is no difference in the number of aphereses between the G-CSF alone group and the chemotherapy plus G-CSF group. A threshold level of infused progenitors might exist for successful PBSCT. Most pediatric and adolescent patients with solid tumors who are candidates for high-dose chemotherapy with autologous PBSC support mobilize enough PBSC to ensure the collection of a PBSC product that is capable of rapid hematological recovery, when mobilization is attempted with G-CSF alone or in combination with chemotherapy. Mobilization with G-CSF alone is capable of collecting PBSC over this threshold level, and there were no graft failures, even in heavily pre-treated patients.

In conclusion, mobilization with G-CSF alone was effective even in pediatric and adolescent patients with solid tumors who received more than three cycles of chemotherapy or with extensive irradiation, although platelet recovery following autologous PBSCT was delayed. Mobilization with G-CSF alone in cancer patients might offer some advantages, such as ease of determining a collection schedule without a daily determination of CD34+ cells in the blood, and the avoidance of neutropenic fever and additional transfusion.

Acknowledgements

We are grateful to Ms A Itho for her technical assistance.

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Myeloablative allogeneic hematopoietic stem cell transplantation for non-Hodgkin lymphoma: a nationwide survey in Japan

Sung-Won Kim, Tetsuya E. Tanimoto, Noriyuki Hirabayashi, Seiichi Goto, Masahiro Kami, Satoshi Yoshioka, Toshiki Uchida, Kenji Kishi, Yuji Tanaka, Akio Kohno, Masaharu Kasai, Masakazu Higuchi, Masanobu Kasai, Shin-ichiro Mori, Takahiro Fukuda, Koji Izutsu, Hiroshi Sao, Takayuki Ishikawa, Tatsuo Ichinohe, Kengo Takeuchi, Kinuko Tajima, Ryuji Tanosaki, Mine Harada, Shuichi Taniguchi, Kensei Tobinai, Tomomitsu Hotta, and Yoichi Takaue

We retrospectively surveyed the data of 233 patients who underwent myeloablative allogeneic hematopoietic stem cell transplantation (allo-HSCT) for non-Hodgkin lymphoma (NHL). Donors were HLA-matched relatives in 154 patients (66%) or unrelated volunteers in 60 (26%). Ninety patients (39%) were in complete remission. One hundred ninety-three (83%) received a total body irradiation (TBI)–based regimen, and 40 (17%) received a non–TBI-based regimen. Acute graft-versus-host disease (GVHD) occurred in 155 (67%) of the 233 evaluable patients; grade II to IV in 90 (39%), and grade III to IV in 37 (16%). Treatment-related mortality (TRM) was observed in 98 patients (42%), and 68% of them were related to GVHD. In a multivariate analysis, chemoresistance, prior autograft, and chronic GVHD were identified as adverse prognostic factors for TRM. Relapse or progression of lymphoma was observed in 21%. The 2-year overall survival rates of the patients with indolent (n = 38), aggressive (n = 111), and lymphoblastic lymphoma (n = 84) were 57%, 42%, and 41%, respectively. In a multivariate analysis, chemoresistance, prior autograft, and prior radiotherapy were identified as adverse prognostic factors for overall survival. Although myeloablative allo-HSCT represents an effective therapeutic option for patients with NHL, more work is still needed to decrease TRM and relapse. (Blood. 2006;108:382-389)

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Introduction

Hematopoietic stem cell transplantation (HSCT) for patients with non-Hodgkin lymphoma (NHL) has been mainly focused on an autograft strategy. High-dose therapy with autologous HSCT (auto-HSCT) can increase remission rates and possibly prolong disease-free survival and overall survival (OS) in patients with chemotherapy-sensitive NHL at relapse.[1] This is also effective as first-line therapy for those with advanced aggressive lymphoma.[2] Nevertheless, relapse is a frequent cause of treatment failure after auto-HSCT.[1,3]

Allogeneic HSCT (allo-HSCT) has several advantages over auto-HSCT, because the former can avoid the reinfusion of malignant cells and can also be associated with a graft-versus-lymphoma (GVL) effect, which might reduce the risk of relapse. Most physicians believe that a small fraction of patients with end-stage aggressive lymphoma can still achieve prolonged lymphoma-free survival with the application of allo-HSCT. However, the high incidence of treatment-related mortality (TRM) (up to 55%) associated with allogeneic HSCT with a myeloablative regimen has prevented the wider application of this strategy.[4-8] Several reports on allo-HSCT for refractory or advanced lymphoma, as well as studies comparing auto- versus allo-HSCT for NHL, have been published over the past decade.[8-10] However, most of these studies were small and nonrandomized, and incorporated patients who had heterogeneous backgrounds. Thus, the role of allo-HSCT in the treatment of NHL remains controversial. Moreover, the outcome of allo-HSCT in each histologic subtype has not been fully determined. Previous studies have suggested that allo-HSCT improves the prognosis of patients with advanced follicular lymphoma (FL),[7,10,11] whereas few reports have been published on its benefit in aggressive lymphoma.[12,13] In particular, there has been very little information available on subtypes, including mantle-cell lymphoma[11,14]; peripheral T-cell lymphoma, unspecified (PTCL)[15]; natural killer (NK) cell lymphoma[16]; and anaplastic large cell lymphoma.

The application of reduced-intensity stem cell transplantation (RIST) or "nonmyeloablative" HSCT has been reported to decrease

From the Hematology and Hematopoietic Stem Cell Transplantation Division, National Cancer Center Hospital, Tokyo, Japan; the Department of Hematology, Tottori University Hospital, Tottori, Japan; the Department of Internal Medicine, Nagoya Daini Red Cross Hospital, Nagoya, Japan; the Department of Hematology/Oncology, Kyoto University Hospital, Kyoto, Japan; the Division of Hematology, Tokai University Hospital, Isehara, Japan; the Department of Hematology, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan; the Department of Hematology and Oncology, JA Aichi Showa Hospital, Konan, Japan; the Department of Internal Medicine, Sapporo Hokuyu Hospital, Sapporo, Japan; the Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan; the Department of Cell Therapy and Transplantation Medicine, University of Tokyo, Tokyo, Japan; the Department of Hematology, Meitetsu Hospital, Nagoya, Japan; the Department of Pathology, Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan; the First Department of Internal Medicine (Medicine and Biosystemic Science), Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan; and the Department of Hematology, Toranomon Hospital, Tokyo, Japan.

Submitted March 1, 2005; accepted December 22, 2005. Prepublished online as *Blood* First Edition Paper, March 7, 2006; DOI 10.1182/blood-2005-02-0596.

Supported in part by grants from the Ministry of Health, Labor and Welfare, Japan.

Presented in part as a poster presentation at the 44th annual meeting of the American Society of Hematology, Philadelphia, PA, December 2002.

Reprints: Yoichi Takaue, Medical Oncology, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan; e-mail: ytakaue@ncc.go.jp.

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TRM.[17-19] Additionally, the recent development of supportive treatments may have decreased the risk of TRM and facilitated the application of allo-HSCT to NHL.[20] Therefore, we conducted a retrospective nationwide survey on Japanese patients with NHL who had undergone conventional allo-HSCT to establish a benchmark of myeloablative allo-HSCT in the treatment of NHL.

Patients, materials, and methods

Data sources

This survey collected the data of 233 consecutive patients who received myeloablative allo-HSCT for NHL between 1990 and 2001 in 56 participating hospitals. Data were derived from questionnaires distributed to each hospital. Additional questionnaires were sent to confirm the follow-up data, including the occurrence of graft-versus-host disease (GVHD). The indications for allo-HSCT were left to the discretion of each institution. The patients included in this study received a conditioning regimen with an intensity that was equivalent to that of total body irradiation (TBI) plus cyclophosphamide or busulfan plus cyclophosphamide. Patients who had previously received monoclonal antibody therapy or T-cell–depleted transplantation, those younger than 14 years, and those who received RIST were not included. Additionally, those with adult T-cell leukemia/lymphoma were excluded because their clinical course differed from that of other types of lymphoma. The minimum data required for the inclusion of a patient in this study were age, sex, histologic diagnosis, prior treatment details, status at transplantation, donor information, conditioning regimen, date of transplantation, therapy-related complications, date of last follow-up, disease status at follow-up, date of disease progression/death, and cause of death. Approval was obtained from the institutional review board. Informed consent was provided according to the Declaration of Helsinki.

Definitions

The initial institutional histologic diagnosis was further reviewed by a pathologist (K. Takeuchi) using the WHO classification.[21] Briefly, NHL was divided into 3 clinical subtypes: indolent, aggressive, and lymphoblastic lymphoma. Indolent lymphoma included all grades of FL and extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma). Aggressive lymphoma included all lymphomas except for indolent and lymphoblastic lymphoma. Transformed indolent lymphoma and Burkitt lymphoma were classified as aggressive lymphoma. Furthermore, because most of the patients were evaluated before publication of the WHO classification, this analysis only included those who had tumors that formed lesions, such as T-cell lymphoblastic lymphoma (T-LBL), and all other patients who had features of leukemia were excluded. Those with chemosensitive disease included all patients who had shown a response to the last chemotherapy prior to transplantation (partial remission [PR], complete remission [CR] unconfirmed, and CR), whereas chemoresistant disease included those with primary refractory disease or refractory relapse prior to transplantation. Acute and chronic GVHD was graded according to the consensus criteria.[22,23] Patients who survived 100 days were evaluable for the assessment of chronic GVHD. OS was measured as the time from the day of transplantation until death from any cause, and progression-free survival (PFS) was the time from the day of transplantation until disease progression (PD)/relapse or death from any cause. Patients who died from transplantation-related causes were classified as TRM regardless of their disease status.

Statistical analysis

OS and PFS were calculated using the Kaplan-Meier method.[24] Surviving patients were censored on the last day of follow-up, in July 2002. The associations among patient-, disease-, and transplantation-related factors and OS were assessed by using univariate and multivariate Cox proportional hazards models. The associations between these factors and TRM were assessed by using univariate and multivariate logistic models. The variables analyzed included age, clinical subtype, histologic diagnosis, chemosensitivity, history of autograft or radiotherapy, years of transplantation, donor, source of stem cells, TBI-containing regimen, GVHD prophylaxis, and acute and chronic GVHD. Acute GVHD was treated as a time-dependent covariate in the Cox model. Stepwise variable selection at a significance level of .05 was used to identify covariates associated with outcomes. TRM and disease progression/relapse were calculated by using cumulative incidence. The statistical analysis was performed with the SAS 8.2 program package (SAS Institute, Cary, NC).

Results

Patients' characteristics

The patients' characteristics are listed in Table 1. All patients were younger than 60 years at the time of transplantation, with a median age of 31 years. Thirty-eight patients (16%) had indolent lymphoma, 111 (48%) had aggressive lymphoma (diffuse large B-cell, n = 44; PTCL, n = 22; extranodal NK/T-cell, n = 19; anaplastic large cell, n = 7; mantle cell, n = 5; Burkitt, n = 4; angioimmunoblastic T cell, n = 2; blastic NK cell, n = 2; hepatosplenic T-cell, n = 2; subcutaneous panniculitis like T cell, n = 2; mycosis fungoides with visceral dissemination, n = 2), and 84 (36%) had lymphoblastic lymphoma. Ninety patients (39%) were in CR, 38 (16%) were in PR, 42 (18%) were in primary refractory, and 63 (27%) had refractory relapse at the time of allo-HSCT. Ninety patients (39%) had received 4 or more chemotherapy regimens before allo-HSCT. Forty patients (17%) had received prior autograft, and 81 (35%) had received prior radiotherapy. One hundred fifty-four patients (66%) received a transplant from a human leukocyte antigen (HLA)–matched related donor, 19 (8%) from a 1-antigen–mismatched related donor, 43 (19%) from a matched unrelated donor, and 17 (7%) from a 1-antigen–mismatched unrelated donor. One hundred fifty-nine (68%) patients received bone marrow (60 from an unrelated donor) and 70 (30%) received granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood. One hundred ninety-three patients (83%) received TBI-based myeloablative regimens, including TBI 12 Gy plus cyclophosphamide (n = 60); a combination of TBI, cyclophosphamide, and etoposide (n = 47); or TBI, cyclophosphamide, and cytarabine (n = 40). Forty patients (17%) received a non–TBI-based myeloablative regimen, including a combination of busulfan and cyclophosphamide with or without other agents (n = 27); melphalan, thiotepa, and busulfan (n = 3); cytarabine, ranimustine, carboplatin, cyclophosphamide, and total lymphoid irradiation (n = 2); or cytarabine, etoposide, and busulfan (n = 2). The remaining 6 patients received individualized regimens. GVHD prophylaxis included a combination of cyclosporin and methotrexate in 204 (88%) or tacrolimus and methotrexate in 22 (9%). Two hundred twenty-six patients (97%) were treated with G-CSF, starting at days +1 to +6 after graft infusion until engraftment.

GVHD

Acute GVHD occurred in 155 (67%) of the 233 patients: grade I in 65 (28%), grade II to IV in 90 (39%), and grade III to IV in 37 (16%) patients. Of the 165 patients who survived the initial 100 days after allo-HSCT, chronic GVHD occurred in 79 (48%), with extensive type in 48 (29%). In allo-HSCT from related (n = 173) and unrelated (n = 60) donors, grade II to IV acute GVHD occurred, respectively, in 61 (35%) and 30 (50%), grade III to acute GVHD occurred in 25 (15%) and 12 (20%), chronic GVHD occurred in 54 (31%) and 25 (42%) patients, and chronic extensive

Table 1. Patient-, disease-, and transplantation-related characteristics

Variable	No. (%)*
Patient characteristics	
Younger than 40 y	158 (68)
40 y or older	75 (32)
Male sex	150 (64)
Disease characteristics at diagnosis	
Histology	
Indolent	38 (16)
Follicular	37 (16)
MALT	1 (0)
Aggressive	111 (48)
Diffuse large B cell	44 (19)
Peripheral T cell, unspecified	22 (9)
Extranodal NK/T cell, nasal type	19 (8)
Anaplastic large cell	7 (3)
Mantle cell	5 (2)
Others	14 (6)
Lymphoblastic	84 (36)
Precursor B cell	7 (3)
Precursor T cell	77 (33)
Stage I	9 (4)
Stage II	25 (11)
Stage III	30 (13)
Stage IV	150 (64)
No data	19 (8)
Disease characteristics at transplantation	
Response to chemotherapy†	
Sensitive	128 (55)
Complete remission‡	90 (39)
Partial remission	38 (16)
Resistant	104 (45)
Primary refractory disease	41 (18)
Refractory relapse	63 (27)
No. of prior chemotherapy regimens†	3 (0-11)
Fewer than 4 regimens	143 (61)
At least 4 regimens	90 (39)
Prior autograft	40 (17)
Prior radiotherapy	81 (35)
Transplantation characteristics	
Year of transplantation	
1990-1995	46 (20)
1996-2001	187 (80)
No. of patients receiving a transplant per hospital	
Fewer than 9 patients	146 (63)
At least 9 patients	87 (37)
Donor	
HLA-matched related	154 (66)
HLA-1 antigen-mismatched related	19 (8)
HLA-matched unrelated	43 (19)
HLA-1 antigen-mismatched unrelated	17 (7)
Donor-recipient sex match	
Male-male	80 (34)
Male-female	66 (28)
Female-male	33 (14)
Female-female	46 (20)
Donor-recipient CMV status§	
+/+	131 (57)
−/+	14 (6)
+/−	14 (6)
−/−	11 (5)
Source of stem cells	
Bone marrow	159 (68)
Peripheral blood cells	70 (30)
Bone marrow + peripheral blood cells	2 (1)
Cord blood	2 (1)
Conditioning regimen	
TBI-containing	193 (83)
Non-TBI	40 (17)
GVHD prophylaxis	
Cyclosporin + methotrexate	204 (88)
Tacrolimus + methotrexate	22 (9)
Others	7 (3)

The study included 233 patients. The median age was 31 years (range, 15-59 years). Age was a continuous variable.

MALT indicates extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; NK, natural killer; HLA, human leukocyte antigen; CMV, cytomegalovirus; TBI, total body irradiation; GVHD, graft-versus-host disease.

*Categoric variable.

†One patient with mediastinal B-LBL did not receive prior chemotherapy for an unknown reason but did receive prior radiotherapy.

‡Includes 2 patients in complete remission, unconfirmed.

§Sixty-three pairs were not evaluated for CMV status.

GVHD occurred in 33 (19%) and 16 (27%). In allo-HSCT from HLA-matched (n = 197) and mismatched (n = 36) donors, grade II to IV acute GVHD occurred, respectively, in 76 (39%) and 15 (42%), grade III to IV acute GVHD occurred in 30 (15%) and 7 (19%), chronic GVHD occurred in 65 (33%) and 14 (39%), and chronic extensive GVHD occurred in 41 (21%) and 7 (19%). The distribution pattern of the incidences of acute and chronic GVHD by background factors was analyzed by using a chi-square test. Although none of the factors correlated with acute GVHD, the incidence of chronic GVHD was higher in patients who had GVHD prophylaxis with tacrolimus plus methotrexate than in those with cyclosporin plus methotrexate ($P = .015$, chi-square test; $P = 0.023$, Fisher exact test).

Disease response

Of the 143 patients who had measurable disease at allo-HSCT, 89 (62%) achieved CR, 7 (5%) PR, 6 (4%) stable disease (SD), and 12 (8%) PD, whereas 29 (20%) were not evaluable because of early death. Of the 90 patients who were in CR at transplantation, 80 (89%) maintained CR, 4 (4%) showed PD, and 6 (7%) were not evaluable because of early death. Thirty-five patients died before the first response evaluation, with a median survival of 29 days (range, 0-72 days) after allo-HSCT. In the 27 patients with indolent lymphoma who had measurable disease at allo-HSCT, 22 (81%) achieved CR or PR. In the 72 patients with aggressive lymphoma who had measurable disease at allo-HSCT, 49 (68%) achieved CR or PR. In the 41 patients with lymphoblastic lymphoma who had measurable disease at allo-HSCT, 26 (63%) achieved CR.

TRM, disease relapse, and progression

Ninety-eight patients (42%) died of TRM, and its cumulative incidence is shown in Figure 1. Of the 98 patients who died of therapy-related complications, 60 (61%) died within day 100 of transplantation and 38 (39%) died thereafter. The major causes of TRM included GVHD (n = 11), infection (n = 29), interstitial pneumonitis (n = 16), venoocclusive disease of the liver (n = 11), thrombotic microangiopathy (n = 8), heart failure (n = 7), hemorrhage (n = 4), renal failure (n = 3), and others (n = 9), as shown in Table 2. The causes of infection-related mortality (n = 29) were bacterial (n = 13), fungal (n = 11), or viral (n = 5). Seventeen (59%) of 29 patients died of infections within 100 days of allo-HSCT, 7 (24%) from 101 days to 1 year and 5 (17%) thereafter. Fourteen patients died of TRM before engraftment. Of the 98 patients who died of TRM, 67 (68%) had GVHD, and 11 of

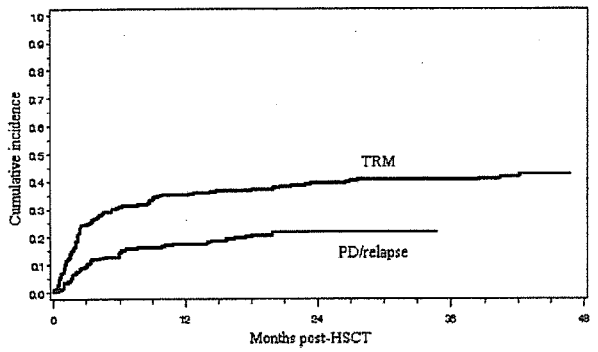

Figure 1. Cumulative incidences of treatment-related mortality (TRM) and disease relapse/progression (PD/relapse).

these died of GVHD (6 acute, 5 chronic) itself. The 14 factors shown in Table 3 were assessed with regard to their relation to TRM. A univariate analysis revealed that 6 factors, including older patient age, chemoresistant disease, prior autograft, prior radiotherapy, aggressive lymphoma other than PTCL, and chronic GVHD, were associated with a significantly increased risk of TRM. In a multivariate analysis using a logistic model, chemoresistant disease, prior autograft, and chronic GVHD remained significant.

The cumulative incidence of relapse and PD is shown in Figure 1. Relapse or progression of lymphoma after allo-HSCT was observed in 49 patients (21%; 5 indolent, 19 aggressive, 25 LBL), and 32 (14%; 3 indolent, 13 aggressive, and 16 LBL) died of PD. Of the 105 patients with chemoresistant disease before allo-HSCT, 61 (58%) died of treatment-related complications, 19 (18%) died of PD, and 25 (24%) are alive with a median follow-up of 20.9 months (range, 1.8-136.0 months). Of the 128 patients with chemosensitive disease before allo-HSCT, 37 (29%) died of treatment-related complications, 12 (9%) died of PD, and 79 (62%) are alive with a median follow-up of 35.2 months (range, 4.4-140.2 months). Eight (16%) of the 49 patients who showed PD died of treatment-related complications such as infection (n = 4), interstitial pneumonitis (n = 3), and GVHD (n = 1). Only 6 of the 70 patients who had passed 2 years after transplantation developed relapse thereafter.

Donor lymphocyte infusion

Donor lymphocyte infusions (DLIs) were given after the withdrawal of immunosuppressive therapy to those who relapsed or showed evidence of disease progression or persistent disease without any sign of GVHD. A total of 7 patients, including 5 with T-LBL, received DLI after allo-HSCT from an HLA-matched related donor (n = 6) or a -matched unrelated donor (n = 1). Two patients who received DLI from an HLA-matched related donor developed grade II acute GVHD, which subsequently extended to extensive chronic GVHD; one of them with T-LBL died without a response, whereas the other with T-cell lymphoma is still alive without disease progression 3.8 years after allo-HSCT. Five patients did not develop GVHD following DLI; 3 patients subsequently died of disease progression, but 2 patients with T-LBL are still alive without disease progression at 361 and 783 days after allo-HSCT.

OS and PFS

One hundred four (45%) of the 233 patients are currently alive with a median follow-up of 31 months (range, 1.8-138 months). The OS and PFS are, respectively, 45% and 40% at 2 years, and 39% and 36% at 5 years after allo-HSCT (Figure 2). Median OS and PFS are, respectively, 15.6 months (95% confidence interval, 9.6-27.6 months) and 9.6 months (6-18 months). The 2-year OS of those with indolent, aggressive, and lymphoblastic lymphoma was, respectively, 57%, 42%, and 41%. Patients with indolent lymphoma tended to have a better survival ($P = .131$, log rank test; $P = .064$, G. Wilcoxon test) (Figure 3). Kaplan-Meier estimates of OS of patients with 4 histologic subtypes of aggressive lymphoma, including diffuse large B-cell lymphoma (n = 44), PTCL (n = 22), extranodal NK/T-cell lymphoma, nasal type (n = 19), and others (n = 26), are shown in Figure 4.

The 14 clinical factors shown in Table 4 were assessed with regard to their relation to OS. A univariate analysis revealed that 5 factors, including chemoresistant disease, prior autograft, prior radiotherapy, aggressive lymphoma other than PTCL, and clinical subtype (aggressive versus indolent), were associated with a significantly worse OS. In a multivariate analysis using Cox proportional hazard models, chemoresistant disease, prior autograft, and prior radiotherapy were associated with a worse OS (Table 4). Acute GVHD, which was treated as a time-dependent variable, was not a significant factor for OS in both univariate and multivariate models. The relation between OS and response to chemotherapy is shown in Figure 5.

Discussion

This report describes the general outcome of patients with NHL who underwent modern allo-HSCT with a myeloablative regimen

Table 2. Causes of treatment-related mortality

Causes of TRM	Patients, no. (%)	No. of patients with GVHD	No. of patients without GVHD	Early death, no.*
GVHD	11 (11)			
Infection	29 (30)	15	8	6
Interstitial pneumonitis	16 (17)	15	0	1
Venoocclusive disease	11 (11)	5	4	2
Thrombotic microangiopathy	8 (8)	7	1	0
Heart failure	7 (7)	3	1	3
Hemorrhage	4 (4)	3	1	0
Renal failure	3 (3)	2	1	0
Others†	9 (9)	6	1	2
Total	98 (100)	56	17	14

GVHD indicates graft-versus-host disease.

*Early death was defined as treatment-related death before engraftment.

†Others (n = 9) were acute respiratory distress syndrome (n = 2), hepatic failure (n = 2), leukoencephalopathy (n = 1), secondary solid cancer (n = 1), suicide (n = 1), and unknown cause (n = 2).

Table 3. Univariate and multivariate analyses of treatment-related mortality

Variable	No.	Univariate analysis Hazard ratio (95% CI)	Univariate analysis P	Multivariate analysis Hazard ratio (95% CI)	Multivariate analysis P
Age at transplantation			.035		—
Younger than 40 y	158	1.00		—	
40 y or older	75	1.82 (1.04-3.17)		—	
Clinical subtype			.349		—
Indolent	38	1.00		—	
Lymphoblastic	84	1.47 (0.66-3.32)		—	
Clinical subtype			.103		—
Indolent	38	1.00		—	
Aggressive	111	1.91 (0.88-4.16)		—	
Aggressive lymphoma			.045		—
PTCL	22	1.00		—	
Non-PTCL	89	2.85 (1.02-7.94)		—	
Response to chemotherapy			< .001		< .001
Sensitive	128	1.00		1.00	
Resistant	105	3.41 (1.97-5.88)		2.95 (1.66-5.25)	
Prior autograft			< .001		< .001
No	193	1.00		1.00	
Yes	40	4.74 (2.23-10.07)		4.09 (1.85-9.04)	
Prior radiotherapy			.010		—
No	152	1.00		—	
Yes	81	2.05 (1.18-3.55)		—	
Years of transplantation			.225		—
1996-2001	187	1.00		—	
1990-1995	46	1.49 (0.78-2.86)		—	
Donor			.295		—
HLA-matched	197	1.00		—	
HLA-mismatched	36	1.46 (0.72-2.98)		—	
HLA-matched donor			.437		—
Related	154	1.00		—	
Unrelated	43	1.24 (0.72-2.15)		—	
Source of stem cells*			.544		—
BM	159	1.00		—	
PBSCs	70	1.09 (0.82-1.46)		—	
Conditioning regimen			.144		—
TBI-containing	193	1.00		—	
Others	40	1.67 (0.84-3.30)		—	
GVHD prophylaxis†			.169		—
Cyclosporin + methotrexate	204	1.00		—	
Tacrolimus + methotrexate	22	1.86 (0.77-4.51)		—	
Acute GVHD			.537		—
No	78	1.00		—	
Yes	155	1.19 (0.69-2.06)		—	
Chronic GVHD			< .001		.029
No	79	1.00		1.00	
Yes	154	2.76 (1.53-4.98)		2.02 (1.07-3.77)	

CI indicates confidence interval; PTCL, peripheral T-cell lymphoma; HLA, human leukocyte antigen; BM, bone marrow; GVHD, graft-versus-host disease; and —, not applicable.

*Those who received cord blood (n = 2) or BM + PBSC (n = 2) were excluded because of the small number of patients.

†Seven patients using other GVHD prophylaxis were excluded.

in Japan, focusing on the background problems of myeloablative therapy and the identification of risk factors for TRM and OS. We showed that long-term, lymphoma-free survival could be achieved in approximately 40% of patients. Patients with FL had a better prognosis, consistent with previous reports.[8,10] Even in patients with aggressive lymphoma or LBL, long-term survival of 35% was identified, consistent with previous reports.[8,9] However, there were no significant differences between clinical subtypes (eg, aggressive versus indolent or PTCL versus non-PTCL) in a multivariate analysis. Because rituximab became commercially available after 2001 in Japan, patients with B-cell NHL who received anti-CD20 antibody therapy were not included in this study. The clinical effect of the introduction of rituximab on outcome after allogeneic transplantation should be carefully evaluated in a future study.

Our study confirmed a high TRM rate (42%) after conventional allo-HSCT with a myeloablative regimen, consistent with previous reports.[4-8,25] One of the major causes of death was severe regimen-related toxicities, which included interstitial pneumonitis, venoocclusive disease, cardiac and renal toxicity, and organ hemorrhage. Although TBI-based regimens are frequently chosen because lymphoma cells are considered to be sensitive to irradiation, they have also been associated with long-term complications, including

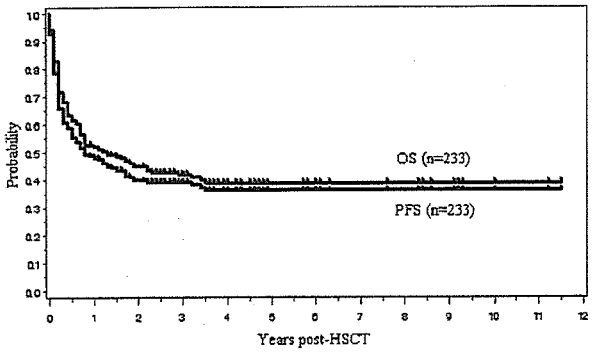

Figure 2. Overall survival (OS) and progression-free survival (PFS) for all 233 patients.

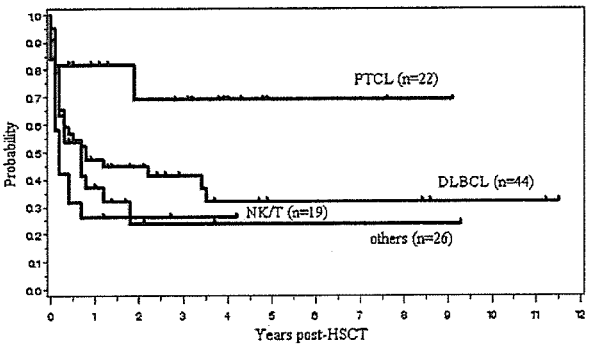

Figure 4. Overall survival for patients with 4 histologic subtypes of aggressive lymphoma. PTCL indicates peripheral T-cell lymphoma, unspecified; DLBCL, diffuse large B-cell lymphoma; NK/T, extranodal NK/T-cell lymphoma, nasal type.

interstitial pneumonitis.[26,27] Because most patients received TBI-based regimens as reported,[4,5,7] we failed to detect any significant differences in TRM between those who received or did not receive TBI.

Another major cause of death in our study was GVHD and/or infection. Of the 98 patients who died of treatment-related complications in our study, 29 (30%) died of infection. At least half of the patients (15 of 29) who died of infectious complications also had GVHD. In a prospective trial of allo-HSCT for patients with NHL, infection accounted for 63% of all TRM,[28] whereas other studies, including ours, have reported an incidence of 25% to 30%.[4,6] In practical transplantation procedures, complications are usually multifactorial, and it is always very difficult to define the exact cause of death, which may account for the wide variations in the incidence of infections among those who died of TRM (18%-63%) in previous reports.[4,5,28,29]

In this study, the incidence of chronic GVHD was high (48%), and chronic GVHD was a risk factor for TRM. The reason for the higher incidence of chronic GVHD in our study compared with the IBMTR report[9,30] was that the IBMTR study included data of patients who died within 100 days after allo-HSCT, whereas we excluded these patients. Unexpectedly, the incidence of chronic GVHD was higher in patients who had GVHD prophylaxis with tacrolimus plus methotrexate than in those with cyclosporin plus methotrexate. In Japan, there is a clear tendency to select tacrolimus rather than cyclosporine for GVHD prophylaxis in unrelated or HLA-mismatched transplantation.[31,32] In addition, PBSCT is not yet permitted for unrelated transplantation. Altogether, the higher incidence of GVHD observed in the tacrolimus group may simply reflect that patients with a higher risk of GVHD were selected to receive tacrolimus.

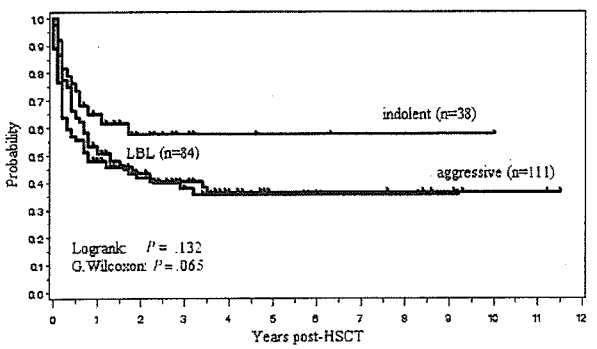

Figure 3. Overall survival stratified according to the clinical subtype. Indolent lymphoma included all grades of FL and extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue. Aggressive lymphoma included all lymphomas except for indolent and lymphoblastic lymphoma (LBL).

We found that the incidence of disease relapse/progression of NHL was low (21%). High TRM in the early phase of the transplantation course may mask later disease relapse/progression, and this made it difficult to estimate the relapse rate in this study. OS and PFS were not affected by the severity of acute GVHD. Our limited analysis failed to confirm a GVL effect after myeloablative allo-HSCT. Although the risk of relapse for patients with acute or chronic GVHD was not significantly different from that of patients without acute or chronic GVHD in previous studies with malignant lymphoma,[8,10,30] a study from the Japan Marrow Donor Program showed that the development of grade II to IV acute GVHD was associated with a lower incidence of disease progression after unrelated HSCT.[31] It has been reported that a low level of acute GVHD was associated with improved OS, and all levels of acute GVHD were associated with a decrease in the relapse rate for intermediate-grade NHL.[8] High levels of acute GVHD had a deleterious effect on OS but were associated with an improved relapse rate for LBL.[8] Thus, our study confirmed that greater effort is required to reduce GVHD-related complications after myeloablative allo-HSCT.

We confirmed that chemoresistance before allo-HSCT and prior autograft were significant risk factors for both OS and TRM. RIST or a less organ-toxic myeloablative allo-HSCT using a combination of fludarabine plus intravenous busulfan may be applied more safely in this population to reduce TRM.[19-21,33,34] However, further studies are needed to determine whether reduced-intensity conditioning could control activity of chemoresistant disease. In contrast to previous studies, we showed that prior radiotherapy was associated with a significantly worse OS, which may be related to the fact that 44 (54%) of the 81 patients who had a history of local radiotherapy had refractory disease at transplantation. Hence, it might be that prior radiotherapy was a marker of survival for more advanced and refractory disease.

In conclusion, we confirmed that myeloablative allo-HSCT is a curative therapeutic option in a subset of patients with NHL, but it carries a high risk of toxicities and TRM. Chemoresistant disease and a history of previous autograft are risk factors for both OS and TRM. Whether the introduction of a reduced-intensity transplantation procedure results in reduction of TRM should be evaluated, and more effective GVHD prophylaxis while maintaining a GVL effect should be developed.

Table 4. Univariate and multivariate analyses of overall survival

Variable	No.	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Age at transplant			.134	—	—
Younger than 40 y	158	1.00		—	—
40 y or older	75	1.32 (0.92-1.90)		—	—
Clinical subtype			.126	—	—
Indolent	38	1.00		—	—
Lymphoblastic	84	1.57 (0.88-2.80)		—	—
Clinical subtype			.045	—	—
Indolent	38	1.00		—	—
Aggressive	111	1.77 (1.01-3.11)		—	—
Aggressive lymphoma			.004	—	—
PTCL	22	1.00		—	—
Non-PTCL	89	3.45 (1.47-7.69)		—	—
Response to chemotherapy			< .001	—	—
Sensitive	128	1.00		—	—
Resistant	105	3.31 (2.30-4.76)		3.12 (2.16-4.51)	< .001
Prior autograft			< .001	—	—
No	193	1.00		—	—
Yes	40	2.59 (1.73-3.87)		2.18 (1.43-3.30)	< .001
Prior radiotherapy			< .001	—	—
No	152	1.00		—	—
Yes	81	1.99 (1.41-2.83)		1.47 (1.02-2.11)	.037
Years of transplantation			.932	—	—
1996-2001	187	1.00		—	—
1990-1995	46	1.02 (0.67-1.54)		—	—
Donor			.076	—	—
HLA-matched	197	1.00		—	—
HLA-mismatched	36	1.50 (0.96-2.33)		—	—
HLA-matched donor			.769	—	—
Related	154	1.00		—	—
Unrelated	43	0.93 (0.58-1.50)		—	—
Source of stem cells*			.095	—	—
BM	159	1.00		—	—
PBSCs	70	1.37 (0.95-2.00)		—	—
Conditioning regimen			.107	—	—
TBI-containing	193	1.00		—	—
Others	40	1.42 (0.93-2.17)		—	—
GVHD prophylaxis†			.227	—	—
Cyclosporin + methotrexate	204	1.00		—	—
Tacrolimus + methotrexate	22	1.40 (0.81-2.40)		—	—
Acute GVHD-time‡	—	1.25 (0.85-1.84)	.264	1.28 (0.87-1.90)	.213

CI indicates confidence interval; PTCL, peripheral T-cell lymphoma; HLA, human leukocyte antigen; BM, bone marrow; GVHD, graft-versus-host disease; and —, not applicable.

*Those who received cord blood (n = 2) or BM + PBSCs (n = 2) were excluded because of the small number of patients.

†Seven patients using other GVHD prophylaxis were excluded.

‡Acute GVHD was treated as time-dependent variable.

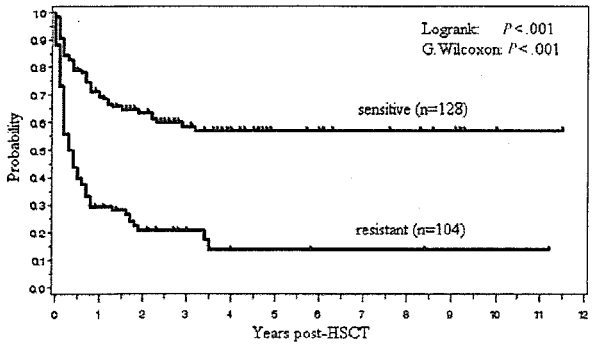

Figure 5. The relation between overall survival and response to chemotherapy.

Acknowledgments

We thank the many physicians in the participating teams who contributed to this survey.

Appendix

The following institutions contributed data to this study: Asahikawa Medical College Hospital, Hokkaido University Hospital, Sapporo Hokuyu Hospital, Akita University Hospital, Gunma Saiseikai Maebashi Hospital, Jichi Medical School Hospital, Suifu Hospital, Saitama Cancer Center Hospital, Jikei University Kashiwa Hospital, Chiba Aoba Municipal

Hospital, Tokyo Metropolitan Komagome Hospital, National Cancer Center Hospital, Keio University Hospital, Toranomon Hospital, Yokohama City University Hospital, Kanagawa Cancer Center, Tokai University Hospital, Kurobe City Hospital, Kanazawa University Hospital, Ishikawa Prefectural Central Hospital, Nagoya City University Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, JA Aichi Showa Hospital, Kyoto University Hospital, Kyoto Prefectural University of Medicine Hospital, Osaka University Hospital, Osaka City University Hospital, Kansai Medical University Hospital, Kinki University Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka City General Hospital, Rinku General Medical Center Izumisano Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Okayama University Hospital, Okayama Medical Center, Shimane Prefectural Central Hospital, Takamatsu Red Cross Hospital, Ehime Prefectural Central Hospital, University of Occupational and Environmental Health Hospital, Kitakyushu Municipal Medical Center, Kyushu Cancer Center, Kokura Memorial Hospital, Fukuoka University Hospital, Hamanomachi Hospital, Harasanshin Hospital, Saga Prefectural Hospital Koseikan, Sasebo Municipal General Hospital, Miyazaki Prefectural Miyazaki Hospital, Imamura Bun-in Hospital, and Ryukyu University Hospital.

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Int. J. Cancer: **119,** 1360–1367 (2006)
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Monitoring of WT1-specific cytotoxic T lymphocytes after allogeneic hematopoietic stem cell transplantation

Yuriko Morita[1,2], Yuji Heike[1], Mami Kawakami[1], Osamu Miura[2], Shin-ichi Nakatsuka[3], Michiko Ebisawa[4], Shin-ichiro Mori[1], Ryuji Tanosaki[1], Takahiro Fukuda[1], Sung-Won Kim[1], Kensei Tobinai[1] and Yoichi Takaue[1]*

[1]*Division of Hematology and Stem Cell Transplantation, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan*
[2]*Department of Hematology and Oncology, Graduate School of Medicine, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*
[3]*Department of Clinical Laboratory, Osaka Minami Medical Center, National Hospital Organization, Kawachi-nagano, Osaka, Japan*
[4]*SRL, Inc., Tokyo, Japan*

Donor-derived cytotoxic T lymphocytes (CTL) that respond to tumor antigens emerge after hematopoietic stem cell transplantation (HSCT), particularly in association with the status of immune recovery. To analyze the frequency of CTL against PR1, PRAME and WT1 after HSCT, a tetramer-based analysis was performed in 97 samples taken from 35 patients (9 AML, 11 MDS, 2 CML, 4 ALL, 7 lymphoma and 2 renal cell carcinoma [RCC]) with the HLA-A02 phenotype. Regarding PR1, only 1 sample showed the presence of tetramer-positive cells (0.04%/lymphocyte). Similarly, in PRAME, only 10 of 97 samples were sporadically positive with low titers. For WT1, positive results were detected in 39 of 97 samples and 7 (2 CML, 1 ALL, 2 lymphoma and 2 RCC) patients clearly showed positive results more than once. On the basis of these results, we performed serial analyses of WT1-specific CTL during the clinical course in 2 patients with RCC, who underwent HSCT with a reduced-intensity regimen, to examine the precise correlation between the kinetics of CTL, the occurrence of GVHD and the observed clinical response. A higher positive rate for WT1-specific CTL and a correlation with the clinical response suggest that WT1 may be a useful antigen for a wider monitoring application.
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Key words: WT1; cytotoxic T lymphocytes; allogeneic hematopoietic stem cell transplantation; renal cell carcinoma

Allogeneic hematopoietic stem cell transplantation (HSCT) is considered to be the most powerful and distinguished immunotherapy since the concept of graft-*versus*-leukemia/tumor (GVL/GVT) effect has been established.[1] However, the underlying mechanism of the anticancer effect is not yet clear. Although the generation of donor-derived cytotoxic T lymphocytes (CTLs) in response to tumor antigens is considered to be the primary reason,[2,3] the potential of any tumor-specific antigens to induce CTL should be critically evaluated in correlation with the clinical response. It is very likely that the immunogenic antigen, which plays the predominant role in the GVT effect, will be a potent candidate antigen for clinically realistic immunotherapy, including tumor vaccine, dendritic cell therapy and adoptive CTL infusion, to treat malignant disorders. WT1, PR1 and PRAME have been attractive targets for immunotherapy because of their expression in a wide variety of tumors, with a relative lack of expression in most normal tissues.[4–6]

One of the Wilms' tumor genes, WT1, encodes a zinc finger transcription factor,[7] and binds to the early growth response-1 DNA consensus sequence in growth factor gene promoters.[8] WT1 is expressed at a high level in most types of leukemia[9] and various types of solid tumors, including melanoma, renal cell carcinoma (RCC), and lung, breast, testicular and ovarian cancer.[10,11] Although WT1 is expressed at low amounts in the nuclei of some normal cells, it is limited to very few tissues, including splenic capsule and stroma, some gonadal cells and hematopoietic precursor cells.[12] It has been reported that MHC class I-restricted, WT1-specific CTLs were generated from human peripheral blood mononuclear cells (PBMC) by *in vitro* stimulation with WT1 peptide.[13–15] Additionally, an anticancer effect of WT1-specific CTL and the rejection of WT1-expressing tumor cells have been reported.[16–18]

The other candidates include PR1, which is derived from proteinase 3, a myeloid tissue-restricted serine protease present in azurophil granules in myeloid cells.[5] PR1 is overexpressed in some leukemia cells, and it has been reported that PR1-specific CTL selectively lyses chronic myelogenous leukemia (CML).[19] PRAME (preferentially expressed antigen in melanoma) is encoded by genes that are overexpressed in a wide variety of tumors, including melanoma (95% of patients), RCC (41%), lung cancer (50%), acute leukemia (30%) and multiple myeloma (52%).[20] PRAME is not expressed in normal tissue, except for testis, and very low levels are found in endometrium, ovaries and adrenals.[6,20–23]

In this study, we assessed the frequency of CTL against WT1, PR1 and PRAME by tetramer assay in peripheral blood taken from patients who underwent HSCT, to identify the best candidate antigen for clinically applicable immunotherapy.

Material and methods

Subjects

After we obtained written informed consent, peripheral blood samples were obtained from patients with an HLA-A02 serotype who had undergone allogeneic HSCT, with a conventional (CST) or reduced-intensity regimen (RIST), between August 2000 and May 2004 at National Cancer Center Hospital (Tokyo, Japan). Patients who received non-T-cell depleted-HSCT from a serologically full HLA-matched donor for hematological malignancy or RCC were eligible for this study.

Samples were taken at least 3 weeks after transplantation with confirmed hematological engraftment and more than 90% donor chimerism. Additional requirements for sampling included complete remission in leukemia patients and disease without bone marrow involvement in those with lymphoma or RCC.

Tumor antigen epitope peptides and cytomegalovirus peptide

The following >80% pure HLA-A*0201 binding peptides were obtained for the experiments, using high-performance liquid chromatography (Qiagen, Tokyo): WT1 peptide RMFPNAPYL (amino acids [AA] 126–134), PR1 peptide VLQELNVTV (AA 169–177) and PRAME peptide ALYVDSLFFL (AA 300–309); cytomegalovirus (CMV) pp65 peptide NLVPMVATV (AA 495–503) was used as a positive control.

Tetramer staining

Antibodies to CD4, CD8, CD19, CD13, CD45RA, CD45RO, CD27, CD57, CCR7, PE-conjugated CMV-tetramer and PR1-tetramer were purchased form Beckman Coulter (Fullerton, CA), and

***Correspondence to:** Department of Medical Oncology, National Cancer Center Hospital, 1.1. Tsukiji 5-Chome, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3/3248-1510. E-mail: ytakaue@ncc.go.jp
Received 30 October 2005; Accepted 1 February 2006
DOI 10.1002/ijc.21960
Published online 4 April 2006 in Wiley InterScience (www.interscience.wiley.com).

APC-conjugated CMV, WT1 and PRAME-tetramer were purchased from ProImmune (Oxford, UK). The antibodies and tetramer complexes were added to 200 μL heparinized whole blood or cell suspension, and incubated for 15 min at room temperature in the dark. For the quantification of antigen-specific CTL, whole blood samples were used, and the red blood cells were lysed with ammonium chloride-based lysing solution after antibody staining. After being washed twice with BSA-containing PBS, the cells were fixed and analyzed on a flow cytometer (FACS Calibur, Becton Dickinson). Analysis was performed using Cellquest software. For the quantification of antigen-specific-CTL, whole blood samples were used, and $CD4^-$, $CD19^-$, $CD13^-$, $CD8^+$ and $tetramer^+$ fraction of the lymphocyte gate were defined as antigen-specific CTL. Samples with more than 0.02% antigen-specific CTL per lymphocyte were defined as positive results. For immunophenotyping of the antigen-specific CTL, fresh PBMC, separated from heparinized blood by Ficoll-Hypaque (IBL, Japan) density gradient centrifugation, was used to acquire a higher number of lymphocytes (minimum of 10^5) per analysis. The cells were gated on $CD8^+$ and $tetramer^+$ fraction of the lymphocyte, and then the positive ratios for CCR7, CD45RA, CD45RO, CD27 and CD57 were analyzed.

Expansion of antigen-specific CTL

PBMC was diluted at 1×10^6 cells/mL in RPMI 1640 (Sigma) supplemented with 10% FBS, gentamicin and streptomycin (hereafter referred to as culture medium). PBMC (2×10^6 cells) were seeded in a 24-well plate, and the peptide was added to a final concentration of 5 μM on day 0. The peptide was diluted to 10 mg/mL in dimethyl sulfoxide (DMSO) prior to use, and the same amount of DMSO alone was used as a negative control. The culture was fed on days 4 and 7 by replacing half of the medium with a fresh culture medium containing 20 U/mL IL-2 and 10 μM peptide. Cells were cultured for 14 days.

Intracellular cytokine staining

The cultured cells were washed with culture medium, and 5×10^4 cells per test were suspended in 200 μL medium. Tetramer was added to the test samples, and incubated for 15 min at 37°C in the dark. For peptide-stimulated cells, specific peptides were added to a final concentration of 10 μM and incubated for 6 hr at 37°C. Breferdin A (Sigma; 10 μg/mL) was added during the last 4 hr of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μg/mL staphylococcal enterotoxin B or PBS. Samples were washed, permeabilized and stained with anti-IFN-γ and anti-CD8 antibodies, and analyzed using a FACS Calibur.

Genotyping of the HLA-A02 locus

The DNA genotype of the HLA-A02 locus was examined using a Micro SSP allele-specific HLA class 1 DNA typing tray (One Lambda, CA, USA), according to the manufacturer's protocol. In brief, DNA was extracted from the lymphocytes, and added to a PCR reaction buffer containing dNTP and Taq polymerase. The sample-reaction mixture was applied to a 96 PCR tray that had been preloaded with allele-specific primers for HLA-A02. After the PCR reaction, the samples were electrophoresed on a gel, and photographed by an UV transilluminator. HLA-genotype was determined by the patterns of the allele-specific PCR product.

Results

Tetramer assay

Samples were obtained from 35 patients (9 AML, 11 MDS, 2 CML, 4 ALL, 7 lymphoma and 2 RCC): 10 had received CST and 25 received RIST. The stem cell source was peripheral blood stem cells from a related donor in 24, related bone marrow in 2 and unrelated bone marrow in 9. The genotype for the HLA-A02 allele was analyzed in 27 patients: 13 had A*0201, 12 had A*0206, 1 had A*0207 and 1 had both A*0201 and A*0206 genotypes.

TABLE I – DETECTION OF ANTIGEN-SPECIFIC CTL BY TETRAMER ASSAY

Antigen	Disease	No. of patients	CTL-positive patients	No. of samples	Samples with 0.02% ≤ CTL
WT1	AML	9	0	16	1
	MDS	11	0	17	2
	CML	2	2	8	4
	ALL	4	1	7	3
	NHL	7	2	14	7
	RCC	2	2	35	22
	Total	*35*	*7 (20)*[2]	*97*	*39 (40)*
PRAME	AML	9	0	16	2
	MDS	11	0	17	2
	CML	2	0	8	0
	ALL	4	0	7	1
	NHL	7	0	14	0
	RCC	2	1	35	5
	Total	*35*	*1 (2.8)*	*97*	*10 (10)*
PR1	AML	9	0	16	1
	MDS	11	0	17	0
	CML	2	0	8	0
	ALL	4	0	7	0
	NHL	7	0	14	0
	RCC	2	0	8	0
	Total	*35*	*0 (0)*	*70*	*1 (1.4)*

[1]When the patients showed 0.02% ≤ CTL at least twice, they were considered to be positive.–[2]Values in parentheses indicate percentages.

All samples from CMV-seropositive patients were positive for CMV-specific-CTL (CMV-CTL) with high titers (mean 2.3%), and the same result was obtained using CMV-tetramer purchased from Beckman Coulter or ProImmune. The frequency of CMV-CTL did not differ between the A*0201 and A*0206 genotypes, but the patient with the A*0207 genotype was negative for CMV-CTL, since he was seronegative for CMV.

Regarding WT1-specific CTL (WT1-CTL), 39 of 97 samples had more than 0.02% CTL per lymphocyte. Seven patients (2 CML, 1 ALL, 2 lymphoma and 2 RCC) showed positive results at least twice, and we defined them as WT1-CTL positive patients. Among those with positive WT1-CTL between days 40 and 520 postHSCT, 1 ALL patient and 1 CML patient received CST, while the other 5 patients received RIST. All of the WT1-CTL-positive patients had experienced skin involvement by graft-*versus*-host disease (GVHD) of grade 1–3 prior to the detection of WT1-CTL, except for 1 ALL patient. The other target organs of GHVD were the liver in 1 patient, and gut in 1 patient. The HLA-A*02 genotype in WT1-CTL-positive patients was A*0206 in 5, A*0201 in 1 and both the A*0201 and A*0206 genotypes in 1.

Regarding PR1, all 70 samples were 0%, except for 3 samples that showed 0.01 and 0.04%. The sample with 0.04% PR1-specific-CTL was taken from a patient with AML at day 925 postSCT. However, another sample taken at day 966 from the same patient was negative (0%), suggesting that the initial result was a false-positive one. Similarly, in PRAME, 87 of 97 samples were negative and 10 samples from 7 different leukemia, lymphoma or RCC patients were sporadically positive with low titers (<0.05%), but positive results were not found at different occasions. Only 1 RCC patient showed a positive result more than once. However, the staining of PRAME-tetramer was dull compared to the prominent positive staining of WT1-tetramer, which suggests that the result may have been false-positive (Table I).

Expansion of antigen-specific CTL

The samples taken from 7 patients (4 AML, 2 CML and 1 RCC) were cultured with WT1, PR1, PRAME, CMV peptide or DMSO. The frequency of antigen-specific-CTL was analyzed by tetramer assay before and 14 days after culture. The CMV-CTL expanded in all 7 samples by 2- to 50-fold, whereas none of the PR1 or PRAME

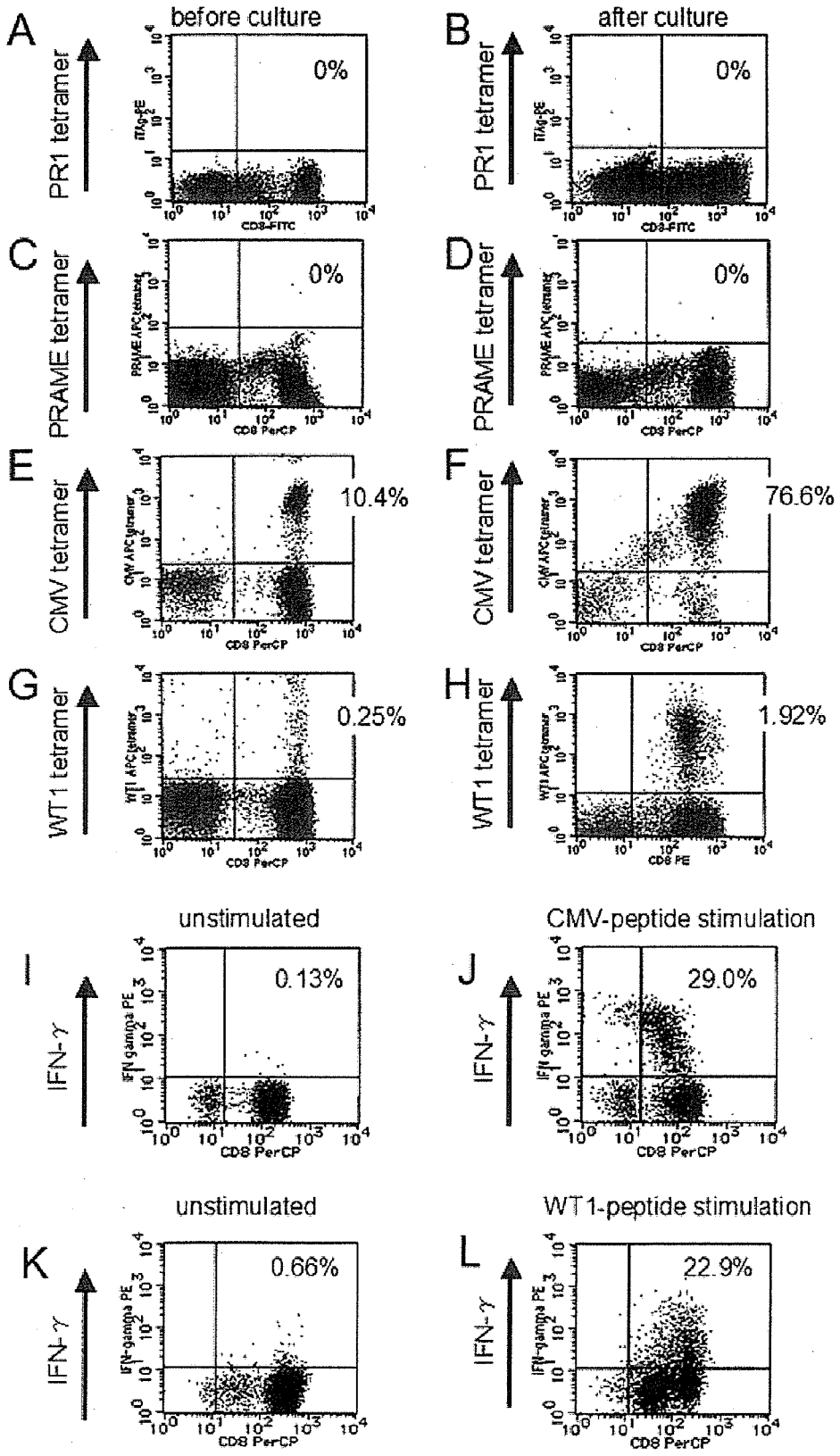

FIGURE 1 – Cell expansion of antigen-specific CTL and intracellular IFN-γ staining. PR1-CTL culture in CML patient (*a*, *b*) and PRAME-CTL culture in a RCC patient (*c*, *d*) remained in undetectable levels, even after expansion culture. CTL showed an expansion of CMV-CTL (*e*, *f*) and WT1-CTL (*g*, *h*). The intracellular IFN-γ staining showed that the CMV-CTL and WT1-CTL produced IFN-γ when stimulated with the peptide (*j*, *l*), while the unstimulated control did not (*i*, *k*). The cells are gated on a tetramer$^+$ fraction.

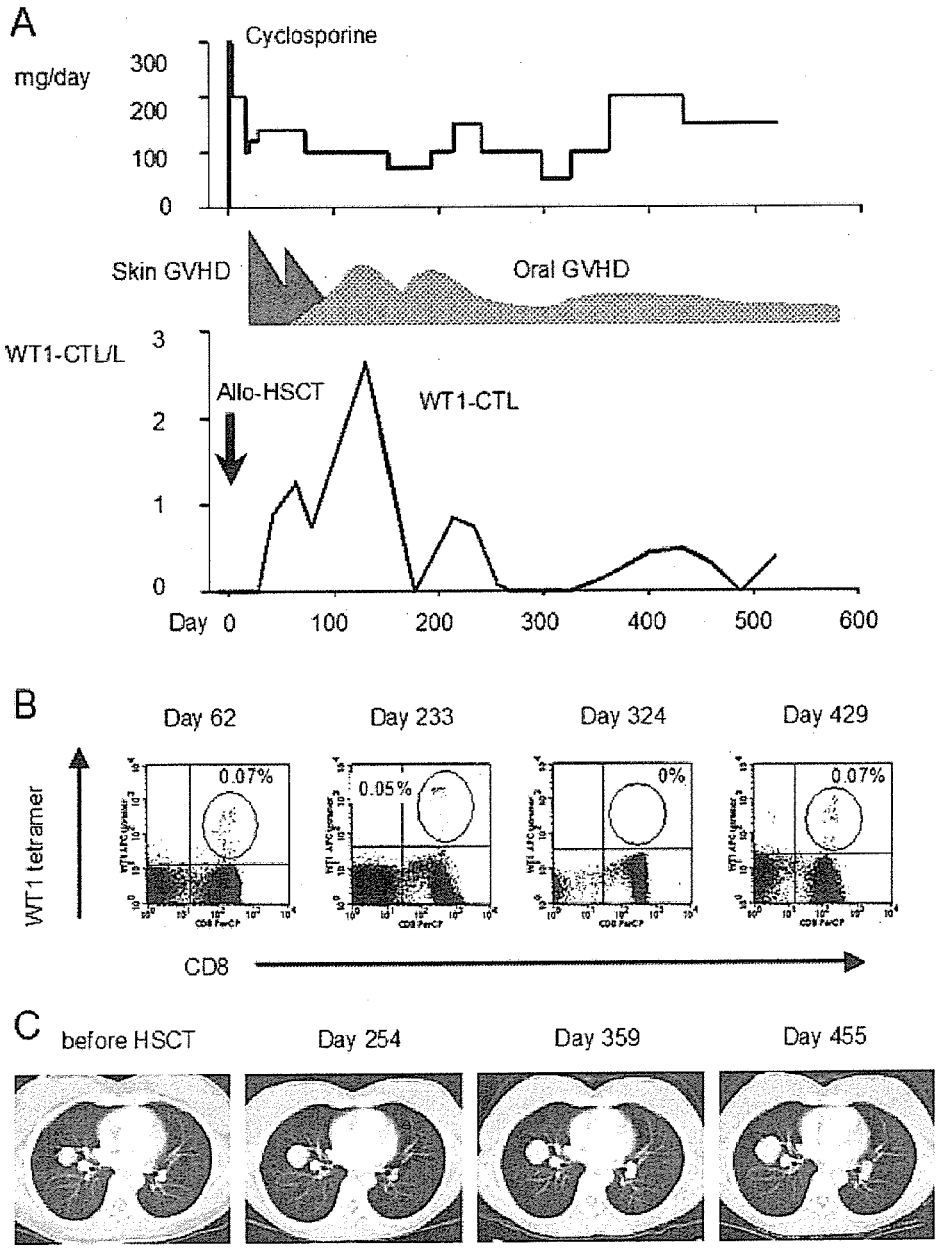

FIGURE 2 – Clinical course and tetramer analysis of Case 1 with RCC WT1-CTL was detected after the occurrence of skin GVHD, followed by a peak on day 128 when oral GVHD developed (*a*, *b*). Lung metastases slowly progressed while WT1-CTL disappeared (*c*).

peptide-containing cultures showed expansion of antigen-specific CTL, with all samples showing 0% for PR1-CTL and PRAME-CTL even after culture. The sample taken from a patient with RCC showed a meaningful expansion of WT1-CTL (Figs. 1*a*–1*h*).

Intracellular cytokine staining

The CMV-CTL and WT1-CTL, expanded by culture, were analyzed for intracellular IFN-γ. The cells were gated on tetramer-positive fraction of the lymphocyte gate, and the positive rate of CD8 and IFN-γ was analyzed. As for the CMV peptide cultured cells obtained from 7 patients, the mean rate of $CD8^+$/IFN-γ^+ in the CMV-$tetramer^+$ lymphocyte gate was 31.8% when stimulated with CMV peptide, whereas it was 1.72% when stimulated with PBS (negative control). A demonstrative result is shown in Figures 1*i* and 1*j*. For cells taken from a RCC patient and cultured with WT1 peptide, the rate of $CD8^+$/IFN-γ^+ in the WT1-$tetramer^+$ lymphocyte gate was 22.9% when stimulated with WT1 peptide, whereas it was 0.66% for negative control (Figs. 1*k* and 1*l*).

Serial analysis of WT1-CTL in patients with RCC

On the basis of Based on these results, we performed serial analyses of WT1-specific CTL during the clinical course of 2 patients with RCC who underwent RIST. The samples were obtained biweekly until day 200 and at longer intervals thereafter.

The first case is a 32-year-old female who had undergone resection of the primary disease, but had multiple lung metastases that were resistant to interferon therapy (Fig. 2). The histology of the primary disease was mixed RCC, which was positive for WT1 (Figs. 3*a* and 3*b*). She received RIST after conditioning with cladribine and busulfan, and cyclosporine (CSP) was administrated as GVHD prophylaxis. Engraftment was achieved on day 12, which was followed by skin GVHD that extended to the whole

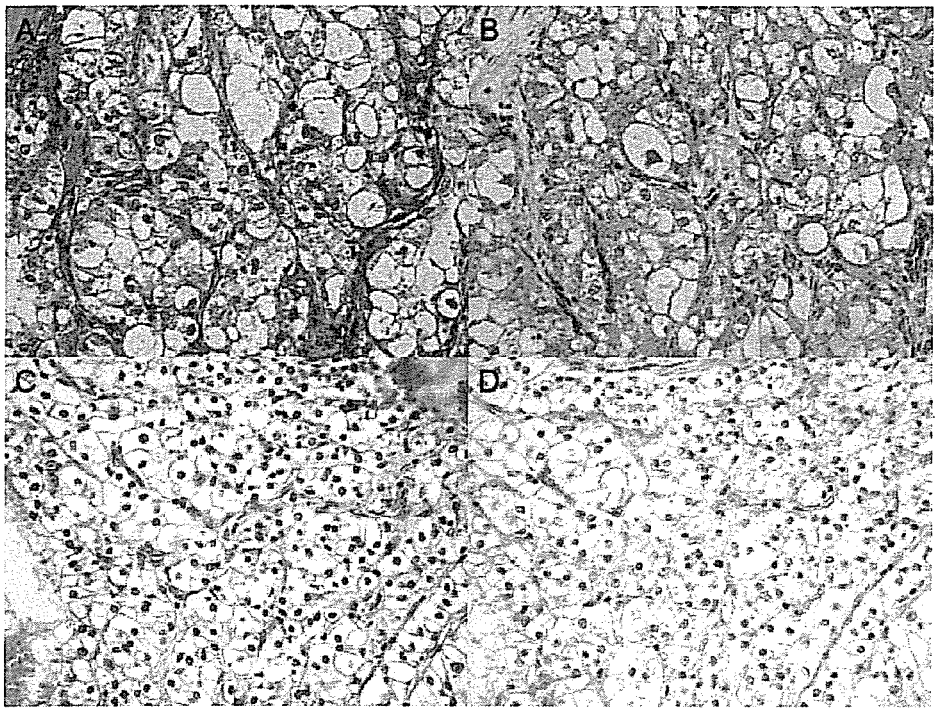

FIGURE 3 – Histology of resected RCC. The resected tumor sample in the first case with hematoxylin–eosin staining (*a*) confirmed a mixed cell carcinoma of the kidney. Immunostaining with WT1 (*b*) was positive for WT1. The second case with clear cell carcinoma (*c*) also showed positive staining for WT1 (*d*). Original magnification ×200 for (*a*, *b*) and ×100 for (*c*, *d*).

body. She was treated with topical corticosteroid after skin biopsy, which provided prompt resolution. WT1-CTL was detected at day 40 when the skin rash recurred, and the peak formation of WT1-CTL occurred on day 128 when oral chronic GVHD developed. The lung metastasis showed a stable disease until day 254, when the tumor started to grow with a slight improvement of oral GVHD, and WT1-CTL became undetectable on day 268. However, with a subsequent slight exaggeration of oral GVHD from day 359, a low titer of WT1-CTL once again became detectable from day 399. This patient is currently doing well at day 520 postHSCT, with a 24% increase in lung metastasis but with no new lesion.

The second case is a 43-year-old male patient who had the primary disease resected, but developed multiple lung metastases, which progressed despite interferon therapy. The histology of the primary disease was clear cell carcinoma that was positive for WT1 (Figs. 3*c* and 3*d*). The patient received RIST after conditioning with fludarabine busulfan, and anti-thymocyte globulin with CSP for GVHD prophylaxis (Fig. 4). He developed liver acute GVHD on day 83, after a rapid reduction in the dose of CSP. Liver GVHD was successfully treated by resuming CSP at a dose of 400 mg/body. He became positive for WT1-CTL on day 90; however, it disappeared along with the remission of liver GVHD. After CSP was tapered, skin GVHD occurred and WT1-CTL became detectable again. However, WT1-CTL disappeared from day 239 with the remission of skin GVHD, and the disease showed rapid progression. Donor lymphocyte infusion was performed on day 350 to induce a GVT effect, but WT1-CTL was not induced, and the patient died of respiratory failure because of disease progression on day 377 postHSCT.

Immunophenotype of WT1-CTL

The immunophenotype of the WT1-CTL in the RCC patients described earlier was analyzed (Fig. 5). The samples obtained at days 40, 77, 128, 149 and 233 posttransplantation from the first patient and at days 97, 146 and 196 in the second patient had adequate numbers of WT1-CTL for analysis. The phenotype did not differ significantly among samples taken from the same patient at different occasions. The WT1-CTL was effector phenotype in both patients, but different among the 2 patients as described later. In the first patient, WT1-CTL was mainly effector memory phenotype. Seventy percent of the WT1-CTL expressed $CD45RA^+/CD45R0^-$ phenotype, 53% were of $CD57^+/CD45R0^-$ phenotype and 22% were of $CD57^-/CD45R0^-$ phenotype. In the other classification, 38% were $CD27^-/CD45RA^+$ and 34% were $CD27^+/CD45RA^+$. In the second patient, 80% of the WT1-CTL had the $CD45RA^-/CD45R0^+$ phenotype and 57% expressed $CD57^+/CD45RO^+$. In the other classification, 66% were $CD45RA^-/CD27^-$ and 21% were $CD45RA^-/CD27^+$. In both patients, over 95% of the WT1-CTL were negative for CCR7.

Discussion

Our study showed that CTL with avidity for the WT1 antigen are present in the peripheral blood of patients who underwent allogeneic HSCT for malignant disease. A GVT effect is thought to be mediated by expanding donor T cells, and a relationship has been reported between GVHD and disease control.[24] However, an optimal immune-monitoring system for tumor antigen-specific CTL, which is thought to be the effector cell for the GVT effect, has not been well established. Few studies on WT1-CTL have been reported, and most have focused on patients with leukemia[25] or those who received peptide vaccination.[16,26] This is the first report of the kinetics of WT1-CTL in patients with RCC.

In the tetramer assay, we were able to reduce the background staining by sorting T cells with phenotypes, including CD4, CD13 and CD19, in addition to a lymphocyte gate using FSC and SSC. By this procedure, bright and distinct tetramer staining with fewer false-positive results was obtained, which made it possible to detect antigen-specific CTL present at very low levels. Tetramer assay using fresh peripheral blood is the best screening procedure, since it could be performed more easily and quickly than conventional procedures. Previous studies have used peptide stimulation and cytokine production analysis, such as ELISPOT assay or intracellular cytokine assay, to detect antigen-specific CTL.[25,27] However, they are only semiquantitative, as it is impossible to exclude nonspecific cytokine production. We have overcome these problems by simultaneously staining the cells with tetramer and intracellular cytokine, which visualized the IFN-γ production pattern of antigen-specific CTL.

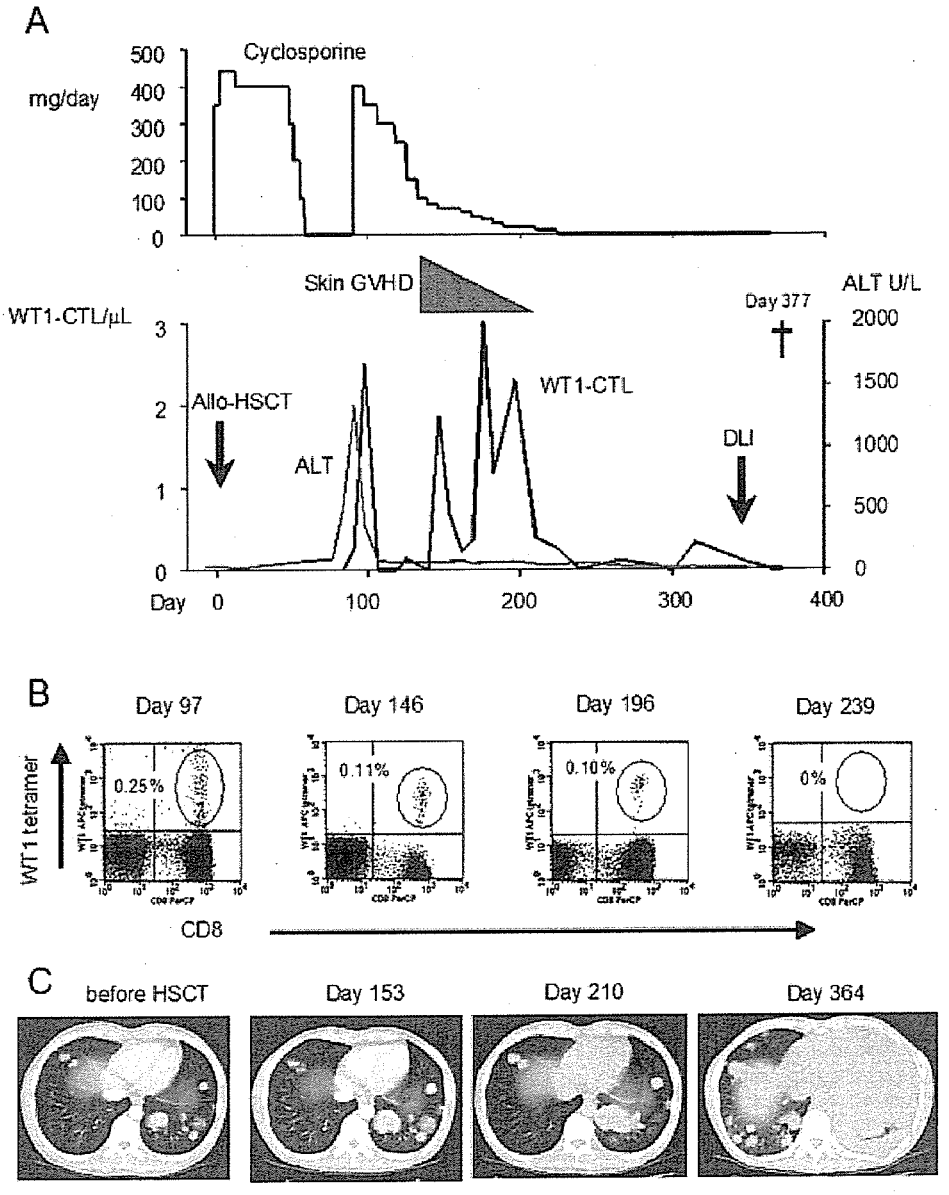

FIGURE 4 – Clinical course and tetramer kinetics in Case 2 with RCC. WT1-CTL emerged after the occurrence of liver and skin GVHD (*a*, *b*). The tumor rapidly grew after the disappearance of WT1-CTL (*c*).

The serial analysis of WT1 tetramer in 2 RCC patients clearly showed that WT1-CTL emerges after HSCT in relation with GVHD, and that they are associated with disease control/progression. It has been reported that the expansion of $CD8^+$ IFN-γ-producing T cells and the incidence of GVHD are associated with the clinical response to nonmyeloablative allogeneic HSCT for RCC.[2] Our results suggest that WT1-CTL can be included among these $CD8^+$ IFN-γ-producing T cells.

The immunostaining of WT1 showed a cytoplasmic pattern in both cases. Although WT1 is usually a nuclear protein, it is reported that some types of adenocarcinomas show cytoplasmic pattern.[4,28] Also, a recent study showed that WT1 shuttles between the nucleus and cytoplasm, and thereby 10–50% of total cellular WT1 can be detected in the cytoplasm.[29] From these evidences, we conclude that the RCC cells in the 2 patients expressed WT1, which was present as a tumor antigen.

The WT1-CTL was detected in a relatively short period after HSCT, when the patient obtained full donor chimerism, which may suggest that the precursor of WT1-CTL was already present in the donor graft. Since the WT1-CTL in the donor graft was under the level of detection of the tetramer assay and the WT1-CTL emerged soon after the occurrence of GVHD, it is quite likely that an immunological event associated with GVHD induced rapid expansion of the WT1-CTL. We can assume that GVHD drove the tumor-antigen to a peripheral circulation and stimulated WT1-CTL, together with a significant amount of cytokines, which were produced in the very early phase of HSCT and GVHD.

The immunophenotyping of antigen-specific CTL may be useful for predicting the function of CTL and disease prognosis.[30–34] In RCC patients, most of the WT1-CTL detected was $CCR7^-/CD57^+$, consistent with an effector memory phenotype. The first case showed a relatively high frequency of CD45RA+/$CD45RO^-$ phenotype with an equal ratio of $CD27^+$ and $CD27^-$, while most of the WT1-CTL in the second case had $CD45RA^-/CD45RO^+$ and $CD27^-$ phenotypes. From a previous report, a cytokine-producing memory T cell subset capable of rapidly inducing IFN-γ and TNF-α synthesis shows the $CD27^-$ phenotype, with varying degrees of CD45RA/CD45RO expression.[35] In another article, CTL with phe-