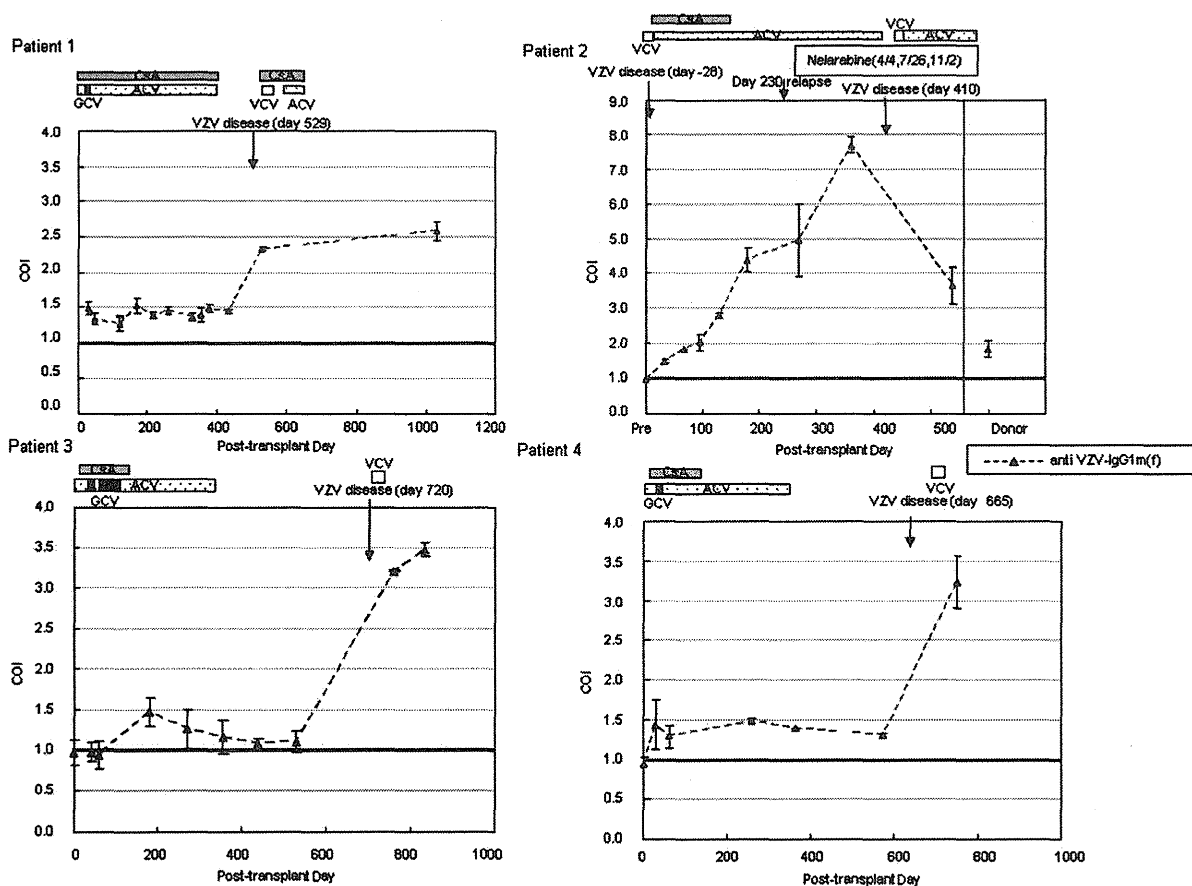

Figure 4. Clinical courses of the 4 IgG1m(f) homozygous-null patients (Patients 1 to 4) who underwent hematopoietic stem cell transplantation (HSCT) from IgG1m(f)-positive donors and developed varicella-zoster virus (VZV) disease after HSCT. Broken lines show VZV IgG1m(f) levels. CsA indicates cyclosporine; PSL, prednisolone; ACV, acyclovir; VCV, valacyclovir; GCV, ganciclovir.

12 months after HSCT was significantly lower than that of measles-IgG. In addition, 3 patients were weakly positive for donor-derived VZV IgG1m(f) at 6 to 9 months after HSCT, including 1 patient who underwent CBT (Patients 1, 3, and 4 in Table 2). This result may indicate that a small amount of VZV IgG was produced from donor-derived plasma cells that were continuously stimulated by latent virus antigen. Since all 3 of these patients subsequently developed VZV disease, the detection of donor-derived VZV IgG titer under low-dose ACV may reflect the amount of latent viral load and the possibility of future VZV reactivation. Although low-dose ACV prophylaxis effectively suppressed VZV reactivation in our study, the high incidence of VZV disease after ACV discontinuation still remains to be solved. IgG allotype analysis might be useful in estimating the risk for zoster after the discontinuation of low-dose ACV administration. Risk-adjusted decision-making of low-dose ACV discontinuation could be useful in reducing the long-term incidence of VZV reactivation. However, this study is too small and heterogeneous to make a definite conclusion because of the difficulty in collecting informative donor-recipient pairs, and thus, this hypothesis should be further evaluated in a larger study.

Some previous reports [7,22] hypothesized that the use of low-dose ACV could permit a subclinical reactivation of VZV, which may result in the reconstitution of sufficient VZV-specific T cell immunity. The findings in the current study also support the notion that subclinical VZV reactivation may exist and induce imperfect VZV-specific B cell immunity. However, without symptomatic VZV reactivation, donor-derived anti-VZV IgG did not reach titers comparable to those measured in healthy virus carriers.

Notably, donor-derived VZV IgG elevated vigorously in the early phase after HSCT in a patient who developed zoster just before allogeneic HSCT. Processed antigen presented on chemo-radiation–resistant recipient antigen-presenting cells might stimulate transferred donor B cells. Vaccination of the recipients before allogeneic HSCT may accelerate the reconstitution of donor-derived VZV IgG.

In conclusion, the production of anti-VZV IgG by recipient plasma cells persists long after RIC transplantation. In patients without symptomatic VZV reactivation, donor-derived anti-VZV IgG did not reach titers comparable to those measured in healthy virus carriers.

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CASE REPORT

Detection of donor-derived CMV-specific T cells in cerebrospinal fluid in a case of CMV meningoencephalitis after cord blood stem cell transplantation

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Abstract Cytomegalovirus (CMV) meningoencephalitis is a rather rare complication after allogeneic stem cell transplantation. We describe here the case of a 59-year-old man with acute myeloid leukemia who developed CMV meningoencephalitis after cord blood transplantation. The patient presented with a sudden onset of neurological symptoms, such as convulsion, on day 37. The analysis of cerebrospinal fluid (CSF) sample revealed an increase in the number of cells, which were of donor (cord blood) origin, consisting mainly of T cells. No bacteria were detected in the CSF sample. Real-time PCR analysis revealed that the CSF sample was positive for CMV, but was negative for HHV-6, adenovirus, or BK virus. The patient was diagnosed with CMV meningoencephalitis and received cidofovir. His neurological symptoms were gradually improved and completely disappeared by day 60. CMV-specific dextramer-positive $CD8^+$ T cells were detected in the peripheral blood and CSF samples, with the frequency being much higher in the CSF. To our knowledge, this is the first report on the appearance of CMV-specific T cells in CSF samples from a patient with CMV meningoencephalitis. Cord blood-derived CMV-specific T cells may develop early after transplantation, enter the intrathecal compartment, and likely contribute to the regulation of CMV-meningoencephalitis.

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Keywords Cytomegalovirus · Viral encephalitis · Cord blood transplantation · Dextramer

Introduction

Cytomegarovirus (CMV) meningoencephalitis (CMV-ME) is a rather rare complication, occurring in 6 % of patients with viral encephalitis after allogeneic stem cell transplantation (SCT) [1]. Recently, we had a patient with acute myeloid leukemia (AML) who developed CMV-ME after cord blood stem cell transplantation (CBT), and could, for the first time, confirm the presence of donor-derived CMV-specific CD8 T cells in the cerebrospinal fluid (CSF), using the dextramer staining procedure.

Case report

A 59-year-old Japanese man with AML (M6) evolving from myelodysplastic syndrome received a chemotherapy consisting of aclarubicin and cytarabine, and achieved complete remission morphologically, which was, however, considered as returning to refractory anemia because of the existence of deletion chromosome 20 in 17 of 20 cells in the karyotype analysis of bone marrow (BM) samples. The patient therefore was referred to our hospital for allogeneic SCT. Since there was no suitable donor in related and unrelated donor pools, we decided to perform CBT using reduced-intensity conditioning regimen, which consisted of fludarabine (FLU) 30 mg/m^2/day (day −6 to −2, total 150 mg), cyclophosphamide (CY) 50 mg/kg/day on day −6, and total body irradiation (TBI) 3 Gy on day −7 [2]. Graft-versus-host disease (GVHD) prophylaxis consisted of continuous infusion of cyclosporine (CsA) (target blood

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concentration was 300–400 ng/ml), and mycophenolate mofetil (MMF) 30 mg/kg, both of which started from day −3. Cord blood (CB) graft was female and contained 6.66×10^7 nucleated cells/kg and 4.77×10^5 $CD34^+$ cells/kg. The HLA profiles of the patient and CB unit were as follows: patient HLA A*24:02 *02:01, B*35:01 *56:01, DRB1*04:10 *09:01, and CB HLA A*24:02 *02:01, B*35:01 *40:01, DRB1*04:03 *09:01, which means mutual HLA 1 antigen mismatch in B locus. The clinical course of the patient is shown in Fig. 1. Hematopoietic engraftment was rapidly achieved, with absolute neutrophil count $>0.5 \times 10^9$/l on day 15 and platelet count $>50 \times 10^9$/l on day 29. On day 17, complete donor chimerism was confirmed in both $CD3^+$ and myeloid fractions of peripheral blood (PB) using informative short tandem repeat-PCR technique. The patient started having spiking fever >39 °C on day 7, which was considered as pre-engraftment immune reaction (PIR) [3]. Prednisolone was started at a dose of 5 mg/day on day 9 and increased to 10 mg/day on day 12. On day 12, the patient developed acute cutaneous GVHD (stage 1), which subsided a few days later without the need for any additional treatment. Foscarnet (FCN) 80 mg/kg/day was also started on day 8 as the prophylaxis of CMV or human herpes virus 6 (HHV6) infection. The fever subsided by day 20, but reappeared on day 32. CMV pp65 antigenemia, which was monitored weekly, continued to be negative from day −4 to day 87. Reactivation status for CMV, HHV6, adenovirus (ADV), and BK virus (BKV) was also monitored weekly using real-time PCR analysis of plasma samples. No viral reactivation was observed, except for a positive result for CMV (7.7×10^7 copy/μg DNA) on day 42.

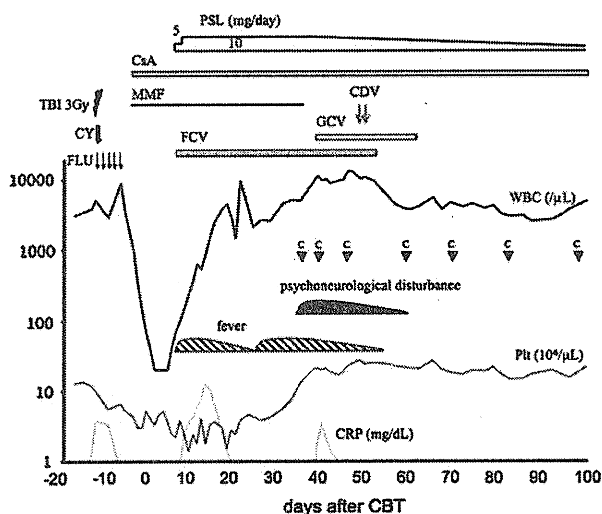

Fig. 1 Clinical course. *Bold*, *thin*, and *dotted lines* denote white blood cells (WBC) (/μL), platelets (10^4/μL), and CRP (mg/dL), respectively. *Flu* fludarabine, *CsA* cyclosporine, *MMF* mycophenolate mofetil, *PSL* prednisolone, *CY* cyclophosphamide, *TBI* total body irradiation, *FCN* foscarnet, *GCV* ganciclovir, *CDV* cidofovir, *C* CSF sample analysis

On the night of day 37, the patient's behavior suddenly became abnormal, such as cutting the infusion line with a pair of scissors or urinating on the bed board. Next morning, the patient was barely able to make even a simple conversation and his consciousness level was decreased, with the occurrence of general convulsion for a few minutes. An analysis of CSF sample on day 38 revealed that the cell number was 3464/3 μl (normal range 0–15/3 μl) consisting of polymorphonuclear leukocytes (PMN) 83 % and mononuclear cells (NMC) 17 %. The biochemical data of the CSF sample were: protein 261 mg/dl (normal range 40–75 mg/dl), Cl 116 mmol/l (normal range 120–130 mmol/l), and LDH 95 U/l (normal range 8–50 U/l). These results suggested that the patient had bacterial meningitis, but no bacterium was cultured in the CSF sample. MRI of the brain on day 39 showed no abnormal findings. Meropenem 3 g/day and ganciclovir (GCV) 3 mg/kg/day were started, with the administration of an increased dose of immunoglobulin. Real-time PCR data revealed that the CSF sample on day 38 was positive for CMV and negative for HHV6, while the PB sample was negative for the 2 viruses. There was no sign of CMV disease in other organs [4]. Follow-up data of the CSF are shown in Table 1. The CSF cell components turned to an MNC-dominant status after day 42. Although the reason why PMN was dominant in the CSF sample on day 38 is unknown, we speculate that PMN might have reflected a hyperacute inflammatory response in the central nervous system (CNS). Cidofovir 1 mg/kg/day was administered on days 50 and 52, and discontinued due to the elevation of serum creatinine level. His psychological and neurological symptoms gradually improved and completely disappeared on day 60. Follow-up brain MRI showed also normal results. He was discharged without any sequelae on day 104.

We performed the immunological characterization of CSF cells. The CSF cells on day 61 were of 100 % donor (CB) origin on chimerism analysis using STR-PCR. MNCs in the CSF mainly consisted of T cells: $CD3^+$ $CD4^+$ T cells 48.2 % and $CD3^+$ $CD8^+$ T cells 23.7 %, NK cells ($NKp46^+$ cells) 21.4 %, and B cells 0.8 %. The patient and CB shared HLA A*24:02 and A*02:01. We tested the presence of CMV-specific CD8 T cells in the PB and CSF samples on day 70 using CMV-specific HLA A*24:02-restricted and HLA A*02:01-restricted dextramers (Immudex, Copenhagen, Denmark). Dextramer staining was performed according to the manufacturer's protocol. Cells were stained with phycoerythrin-Cy7-conjugated anti-CD8, phycoerythrin-Cy5-conjugated anti-CD3 (Beckman Coulter Inc., Fullerton, CA, USA), and phycoerythrin- conjugated dextrameric-HLA A*02:01-restricted NLVPMVATV peptide complex or fluorescein isothiocyanate-conjugated

Table 1 Laboratory and viral PCR data of the CSF

Post CBT day	38	42	49	61	70	84	98
Cell number (/3 μl)	3464	1136	1336	861	376	277	217
PMN/MNC (%)	83/17	2/98	4/96	0/100	1/99	0/100	1/99
Protein (mg/dl)	261	268	289	118	107	100	96
CMV in CP CSF (copy/μg DNA)	2.8×10^6	7.5×10^5	2.0×10^4	1.6×10^2	NT	–	–
CMV in whole CSF (copy/ml)	2.8×10^6	4.9×10^7	9.1×10^5	6.7×10^2	NT	–	–
HHV6 in CP CSF (copying DNA)	–	–	–	–	NT	–	–
HHV6 in whole CSF (copy/ml)	–	–	–	–	NT	–	–
ADV in whole CSF (copy/ml)	–	–	–	–	NT	–	–
BKV in whole CSF (copy/ml)	–	–	–	–	NT	–	–

The amount of DNA of viruses, including CMV, HHV6, ADV, and BKV, in the whole or centrifuged pellet (CP) samples of the CSF was measured using real-time PCR. There was no PCR data on day 70 because most of the CSF sample was used for the flow cytometry and dextramer assay

PMN polymorphonuclear cells, *MNC* mononuclear cells, *CMV* cytomegalovirus, *HHV6* human herpes virus 6, *ADV* adenovirus, *BKV* BK virus, *NT* not tested

dextrameric-HLA A*24:02-restricted QYDPVAALF peptide complex (Immudex, Copenhagen, Denmark). After lysing red blood cells and washing twice with bovine serum albumin containing phosphate-buffered saline, cells were examined on a flow cytometer (Cytomics FC 500, Beckman Coulter, Inc., USA). More than 100,000 cells were acquired in the lymphocyte gate and analyzed using CXP software. The percentage of CMV-specific dextramer-positive cells in the $CD3^+$ $CD8^+$ fraction is shown in Table 2. The dextramer-negative control value in the CSF was a little high; however, these data suggest that the percentage of CMV-specific T cells is higher in the CSF than in the PB at least for A*02:01 dextramer. CMV-specific CD8 T cells seemed to be dominantly HLA A*02:01-restricted, but direct comparison was limited due to the difference in the efficacy of the two dextramers. Of note, CSF cell numbers were maintained still at high levels even after CMV DNA became undetectable (Table 1).

Table 2 CMV-specific T cells (%) in the $CD3^+$ $CD8^+$ fraction

	A*02:01 dextramer	A*24:02 dextramer	Dextramer (−)
PB	0.01	0.26	0.01
CSF	1.19	0.47	0.19

Discussion

Cytomegalovirus disease of the CNS is a rare complication after allogeneic SCT in patients. Reddy et al. [4] recently summarized 11 cases of CMV disease of the CNS after SCT. According to their report, all cases developed CMV CNS disease at late onset (occurring 166 or more days after transplantation), were ganciclovir resistant, and ten of them expired despite antiviral combination therapy. Drug resistance was pointed out to be a key factor in the occurrence of CMV CNS disease [5]. In our case, the CMV disease was also suggested to be relatively FCN resistant, since CMV-ME developed during prophylactic FCN administration. In accordance with the previous report [4], there was no evidence of CMV disease in organs other than CNS. The patient did not even show CMV antigenemia or positive PCR test for CMV DNA using the plasma samples except for one (PCR data on day 42). The occurrence of CMV CNS lesion in an isolated form may reflect a relatively low penetration of FCN, as described by Reddy et al. CMV disease of the CNS is reported to develop at late onset because drug resistant virus appears after a relatively long period of drug therapy. On the other hand, CMV-ME in our case that developed in a form as related to PIR in the engraftment period is similar to post-transplant HHV-6 encephalitis, which was reported to develop in association with the production of inflammatory cytokines such as interleukin-6 [6]. Furthermore, in our case, the absence of abnormal findings of MRI of the brain may have resulted in complete recovery of this serious complication.

There have been no reports showing the presence of CMV-specific CTLs in the CFS of patients with CMV-ME. Regarding the detection of virus-specific CTLs in the CSF, JC virus-specific CTLs in patients with progressive multifocal leukoencephalopathy [7], and HIV-specific $CD8^+$ T cells in antiretroviral therapy-naïve HIV-positive subjects [8], have been reported. These studies suggest that the presence of virus-specific CTLs in the CSF has a beneficial effect in controlling these viral CNS diseases. Likewise, the presence of CMV-specific CTLs in the CSF in our case may have exerted some beneficial effects, although ganciclovir and/or cidofovir are considered to have contributed to controlling CMV-ME. In the present report, we first showed the existence of CMV-specific T cells in CSF samples of the patient with CMV-ME. In addition, we

underlined that CMV-specific T cells were of donor origin (CB derived), and that the frequency of CMV-specific T cells was higher in CSF than in PB. In macaques, activated T cells were reported to preferentially enter the intrathecal compartment and increase in frequency early after acute simian immunodeficiency virus infection [9]. Furthermore, rodent data suggest that the expression of viral antigens in the brain may upregulate endothelial cell major histocompatibility complex class I expression, contributing to $CD8^{+}$ T cell migration into the brain [10]. Taken together with these findings, we consider in our case that CB-derived CMV-specific T cells may develop early in transplantation and enter the intrathecal compartment.

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Conflict of interest The authors declare no competing financial interests.

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ORIGINAL ARTICLE

Frequency of CD4$^+$FOXP3$^+$ regulatory T-cells at early stages after HLA-mismatched allogeneic hematopoietic SCT predicts the incidence of acute GVHD

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Acute GVHD (aGVHD) is a major obstacle to allogeneic hematopoietic SCT (alloHSCT). Although it is thought that aGVHD is initiated in secondary lymphoid organs at a very early stage of alloHSCT, whether CD4$^+$FOXP3$^+$ regulatory T-cells (Tregs) have an impact on aGVHD development during this period remains unclear. Here, we measured Tregs in peripheral blood as early as possible after HLA-mismatched alloHSCT, and assessed the incidence of aGVHD. Flow cytometric analyses revealed that at the second week after HSCT, patients with aGVHD had significantly ($P = 0.018$) lower Treg:CD4$^+$T-cell ratios than those without aGVHD. As these differences were seen before the development of aGVHD, these ratios can predict the incidence of aGVHD. The cumulative incidence of aGVHD in patients with ratios of $<9\%$ was significantly higher than that in patients with ratios of $\geqslant 9\%$ ($P = 0.0082$, log-rank test). Additionally, the specific ratio of Tregs:CD4$^+$T-cells was the most significant value among all other possible lymphocyte-associated ratios and absolute cell counts. These findings suggest that the ratio of Tregs:CD4$^+$T-cells at the second week post HLA-mismatched alloHSCT might be a potent predictor of aGVHD in these patients. The practical efficacy of this finding should be verified in further interventional studies.

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Keywords: regulatory T-cells; GVHD; allogeneic SCT; HLA mismatch

INTRODUCTION

Although allogeneic hematopoietic SCT (alloHSCT) has the potential to cure many hematological disorders, GVHD continues to be a major obstacle associated with morbidity and mortality. Naturally occurring regulatory T-cells (Tregs) initially found in CD4$^+$CD25^{high}T-cell fractions[1,2] suppress autoreactive[1] and alloreactive[3–5] immunoreactions. Other researchers have investigated the relationship between the frequency of CD3$^+$CD4$^+$CD25^{high}Tregs in peripheral blood and the incidence of GVHD after alloHSCT, but results have been inconsistent, possibly due to differences in the definition of CD25^{high}.[6–8] The intracellular protein derived from the *FOXP3* gene has since been detected using flow cytometry, and is recognized as both a master regulatory gene and a unique marker for these Tregs.[9,10] This procedure enables the specific measurement of Tregs, and distinguishes them from activated conventional CD4$^+$CD25$^+$ T-cells. Subsequent studies have applied this procedure and suggested the role of Tregs in attenuating GVHD, mostly after HLA-matched alloHSCT.[11–14]

Here we examined Treg frequencies in the peripheral blood of patients who received alloHSCT from an HLA-mismatched related donor without T-cell depletion. As donor T-cells rapidly recover under our HSCT clinical protocol,[15,16] we analyzed the frequencies of Tregs and other lymphocyte populations as early as possible following HSCT, and examined the relationship between Treg frequency and the subsequent incidence of acute GVHD (aGVHD).

PATIENTS AND METHODS

Patients and samples

Forty-seven patients who underwent alloHSCT from partially HLA-mismatched related donors without T-cell depletion were evaluated. All patients received treatment at the Hyogo College of Medicine Hospital (Nishinomiya City, Japan) between July 2007 and August 2010 in accordance with the protocols approved by the institutional review board. Of these 47 patients, 45 received HLA-haploidentical HSCT. Patient characteristics are summarized in Table 1. After the provision of written informed consent, peripheral blood samples were obtained weekly on a fixed day of the week from the first to the eighth week after transplantation. Data acquired between day 1 and 7 were accordingly defined as data of the first week, those between day 8 and 14 as data of the second week, and so on.

Transplant procedure

Thirty and seventeen patients were preconditioned with a nonmyeloablative and myeloablative regimen, respectively, as reported previously.[15,16] In brief, the nonmyeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, for 6 days), BU (3.2 mg/kg/day, for 2 days, i.v.), and either anti-T-lymphocyte globulin (Fresenius Biotech GmbH, Munich, Germany) or anti-thymocyte globulin (Genzyme, Cambridge, MA, USA) (8 mg/kg or 2–4 mg/kg of the total dose, respectively). The myeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, 4 days), cytosine arabinoside (2 g/m^2/day, 4 times over 2 days), CY (60 mg/kg/day, for 2 days) and TBI (8 Gy delivered in 4 fractions). The GVHD prophylaxis regimen for nonmyeloablative HSCT consisted of tacrolimus (0.02 mg/kg/day) and methylprednisolone (1 mg/kg/day), and that for myeloablative HSCT consisted of tacrolimus (0.03 mg/kg/day), MTX

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Table 1. Patient characteristics

	No GVHD	GVHD	P-value
Number	25	22	
Median age	37	34	0.49
Sex			*0.33*
Male	9	11	
Female	16	11	
Diagnosis			*0.91*
ALL	6	8	
AML	6	6	
Non-Hodgkin lymphoma	7	4	
Myelodysplastic syndrome	3	2	
Hodgkin lymphoma	1	1	
CLL	1	1	
CML	1	0	
Conditioning intensity			*0.98*
Nonmyeloablative	16	14	
Myeloablative	9	8	
Source of stem cells			*0.16*
PBSC	12	15	
BM	13	7	
GVHD grade			*NA*
I		11	
II		7	
III		4	
IV		0	

Abbreviation: NA = not applicable.

(10 mg/m^2 on day 1 and 7 mg/m^2 on day 3), methylprednisolone (2 mg/kg/day) and mycophenolate mofetil (15 mg/kg). After transplantation, degrees of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood were assessed by quantitative PCR for STR markers, as previously reported.[17] Assessment of aGVHD was based on clinical symptoms in accordance with commonly accepted criteria.[18,19] Unless patient condition precluded them, skin, liver and gastrointestinal tract biopsies were performed to support the diagnoses. Gastric biopsy was essentially required for the diagnosis of gut GVHD without manifest diarrhea (stage 1).

Flow cytometric analysis of Treg

Peripheral blood samples were collected using EDTA anticoagulant, and PBMCs were isolated by density-gradient centrifugation for analysis without cryopreservation. Flow cytometric analysis was performed using a Coulter cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) with CXP software (Beckman Coulter), using the following Abs: FITC-conjugated anti-CD3, phycoerythrin-Texas Red energy-coupled dye-conjugated anti-CD25, and phycoerythrin-Cy5-conjugated anti-CD4 (Beckman Coulter). For FOXP3 intracellular staining, the phycoerythrin-conjugated anti-FOXP3 Staining Set (eBioscience, San Diego, CA, USA) was used according to the manufacturer's instructions. FOXP3 staining was performed independently after staining with other Abs.

Statistical analysis

Differences in characteristics between patient groups were assessed by the Mann – Whitney *U*-test for continuous variables and the χ^2 test for categorical values. Median Treg frequencies were compared using the Mann – Whitney *U*-test. Treg frequencies were adjusted for differences between patients with and without aGVHD by multiple regression with logistic analysis. The sensitivity and specificity of Tregs in predicting aGVHD were assessed by receiver operating characteristic curve analysis. Cumulative incidences of aGVHD were plotted according to the Kaplan – Meier method and compared using the log-rank test.

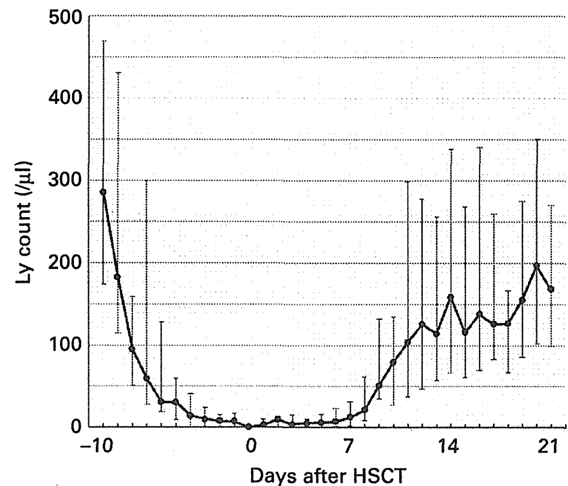

Figure 1. Recovery of lymphocyte (Ly) counts after HLA-mismatched HSCT. Median lymphocyte counts in peripheral blood after HLA-mismatched HSCT are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively.

RESULTS

Patients

Of the 47 patients, 22 presented with aGVHD *vs* 25 who did not (Table 1). The onset of aGVHD occurred at a median of 38 days after transplantation (range: 14–102). None of the characteristics examined had any significant impact on aGVHD incidence. As described previously, the degree of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood achieves the complete donor type within 2 weeks after HLA-mismatched HSCT in our hospital.[15–17] Here, assessment once per week confirmed that on average complete donor-type chimerism in T-cells was achieved on day 10 (median, range: 5–23). Lymphocytes recovered during the second week (Figure 1) were therefore considered to consist almost entirely of donor-originated lymphocytes.

Flow cytometric analysis of Tregs

Representative results of a patient 4 weeks after HSCT are shown in Figures 2a–d. FOXP3$^+$ Tregs were analyzed using a flow cytometric plot gated by CD3$^+$CD4$^+$ fractions (Figure 2a). Although CD25 staining alone showed a large overlap between CD25$^+$ and CD25$^-$ cells (Figure 2c), FOXP3 staining was able to separate FOXP3$^+$ cells as an isolated population (Figure 2d). As demonstrated previously, CD25 staining alone is frequently incapable of revealing an unequivocal boundary that discriminates Tregs from CD4$^+$CD25$^+$-activated conventional T-cells in almost all cases.[20] As FOXP3 staining has apparent objectivity and, moreover, FOXP3 is the key molecule for this type of Treg,[9,10] we defined Tregs simply as CD4$^+$FOXP3$^+$T-cells, regardless of CD25 expression.

Treg:CD4$^+$T-cell ratios at the second week were significantly lower in patients with aGVHD

As lymphocyte numbers were markedly low during the first week, as shown in Figure 1, flow cytometric analysis was unable to detect any Tregs. By the second week after HSCT, in contrast, lymphocyte numbers increased to levels that made analysis possible in almost all cases (41 of 47 patients). Representative results of patients without and with aGVHD are shown in Figures 2e and f, respectively. Of the remaining six patients with slower lymphocyte recovery, two could be assessed at the third week,

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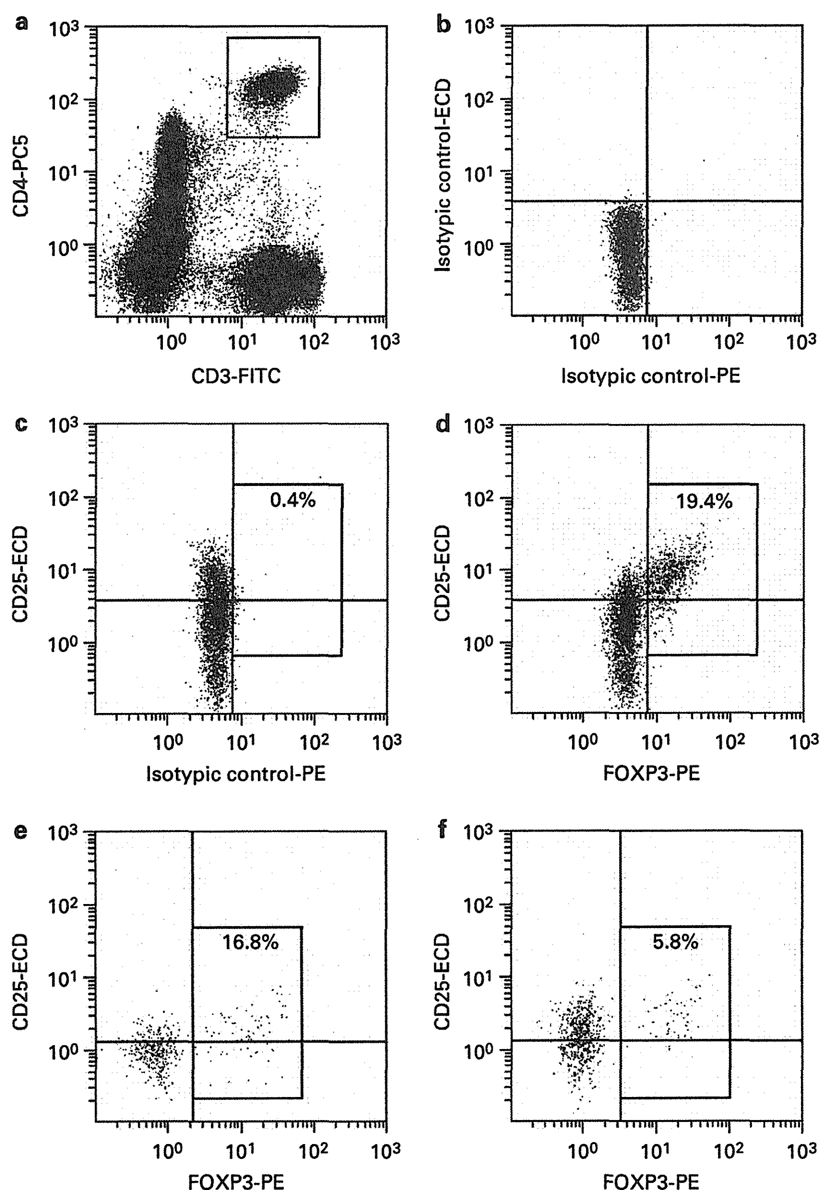

Figure 2. Flow cytometric analysis of Tregs. Representative results of four-color flow cytometric analyses performed for Tregs. Peripheral mononuclear blood cells were stained with CD3-FITC, CD4-PC5, CD25-energy-coupled dye (ECD) and FOXP3-phycoerythrin (PE). All dot plots were gated into lymphocyte populations according to forward- and side-scatter properties, and the gate of $CD3^+CD4^+$ fractions shown on plot **a** was used for the other dot plots with CD25/FOXP3 axes. The percentage of $FOXP3^+$ cells was calculated by subtracting the background percentage of the gate found in plot **c** from the gate shown on plot **d**. Plots **a**–**d** are representative results of a patient 4 weeks after HSCT. Plot **e** is a representative result of a patient without aGVHD in the second week and plot **f** is one of a patient with aGVHD in the second week.

one at the fifth week, one at the sixth week and two at the seventh week.

Figure 3 shows Treg:$CD4^+$T-cell ratios after HSCT ($n=41$), which are the most meaningful values as described in the following paragraph. On average, Tregs were collected on day 12 (median, range: 8–14) during the second week. Patients with aGVHD had significantly lower ratios in the second week after HSCT than those without aGVHD (median (range), 5.23 (0.32–44.8) *vs* 15.5 (0.00–37.1); $P=0.018$). Similar tendencies were seen during the following weeks, but the differences were not statistically significant. Multivariate analysis using logistic regression, which incorporated patient characteristics and transplantation settings, showed that Treg:$CD4^+$T-cell ratio was a unique independent and significant factor related to the incidence of aGVHD (Table 2).

Treg:$CD4^+$T-cell ratio is the most significant value among all other ratios and absolute counts

We also examined the significance of all other ratios between two major lymphocyte populations during the second week. As summarized in Table 3A, although $CD4^+$T-cell:whole T-cell ratio (median (range), 0.32 (0.09–0.78) in the aGVHD (+) group *vs* 0.16 (0.02–0.79) in the aGVHD (−) group; $P=0.026$) and

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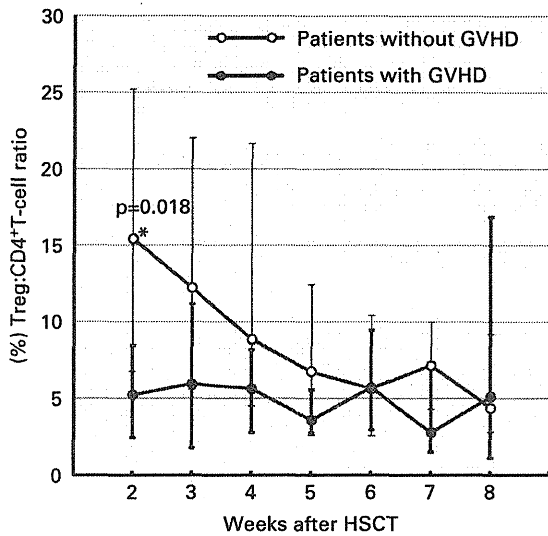

Figure 3. Frequency of Tregs in peripheral blood after HLA-mismatched HSCT. The frequencies of Tregs were assessed by flow cytometry weekly until the eighth week after HSCT. The median Treg:CD4$^+$T-cell ratios of patients with or without aGVHD are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively. Patients with aGVHD had significantly ($P = 0.018$) lower median ratios at the second week after HSCT than those without aGVHD.

Table 2. Multivariate analysis

Parameter	*P-value*
Age	0.643
Sex	0.295
Diagnosis	0.774
Conditioning intensity	0.732
Source of stem cells	0.397
Treg:CD4$^+$T-cell ratio	0.032*

Abbreviation: Tregs = regulatory T-cells. *Indicates statistical significance ($P < 0.05$).

CD8$^+$T-cell:CD4$^+$T-cell ratio (median (range), 1.98 (0.43–9.79) in the aGVHD (+) group *vs* 4.13 (0.38–32.3) in the aGVHD (−) group; $P = 0.043$) were significant, the Treg:CD4$^+$T-cell ratio ($P = 0.018$) had statistically the most significant value. Additionally, neither absolute numbers of whole lymphocytes nor the respective lymphocyte fraction (including Tregs) significantly correlated with the incidence of aGVHD (Table 3B).

Treg:CD4$^+$T-cell ratio at the second week predicts the incidence of aGVHD

As aGVHD occurred at a median of 38 days after HSCT (range, 14–102), while the significant decreases in Treg:CD4$^+$T-cell ratio were observed during the second week, this ratio can serve to predict the incidence of aGVHD. A receiver operating characteristic curve was generated by plotting the true positive rate of aGVHD against the false-positive rate for different cutoff-ratio values (Figure 4). The area under the curve was 0.73, indicating that the Treg:CD4$^+$T-cell ratio at the second week is a good predictor of aGVHD. Further analysis revealed that a cutoff-ratio value of 9% yielded the most accurate predictions of future aGVHD incidence (Figures 4, 69.6% sensitivity and 77.8% specificity). Treg:CD4$^+$T-cell ratios of $<9\%$ predicted a significantly higher incidence of aGVHD than ratios of $\geqslant 9\%$ (Figure 5, $P = 0.0082$, log-rank test).

Table 3. *P*-values of each ratio (A) and absolute number (B)

A

denominator \ numerator	Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
Ly		0.636	0.093	0.674	0.203	0.478
T			0.026*	0.237	0.318	0.478
CD4$^+$T				0.043*	0.774	0.018*
CD8$^+$T					0.213	0.713
B						0.139
Treg						

B

Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
0.674	0.636	0.083	0.979	0.213	0.875

*Indicates statistical significance ($P < 0.05$).

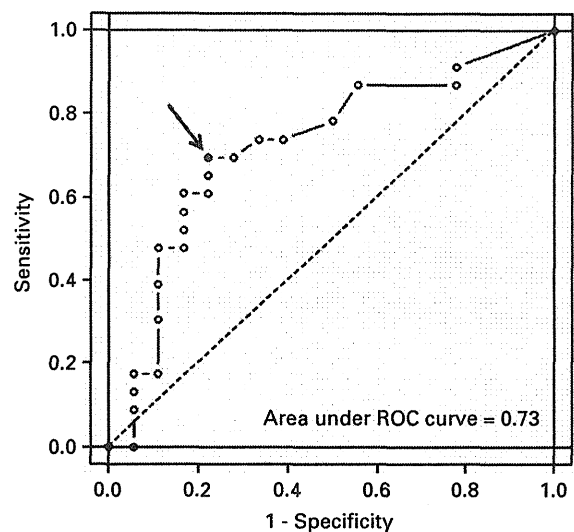

Figure 4. Receiver operating characteristic (ROC) analysis. ROC curve for the ratios of Tregs:CD4$^+$T-cells in identifying patients with aGVHD. The dashed diagonal line represents non-discrimination. Arrow, cutoff ratio at which the sensitivity and specificity resulted in a maximal Youden's index (cutoff ratio, 9%; sensitivity, 69.6%; specificity, 77.8%).

DISCUSSION

In this study, we found that ratios of Treg:CD4$^+$T-cells during the second week after HLA-mismatched HSCT without T-cell depletion accurately predicted the incidence of future aGVHD. Other investigators have used flow cytometry with intracellular staining of FOXP3 to demonstrate a relationship between aGVHD and Treg frequency in peripheral blood. Rezvani *et al.*[11] demonstrated a significant decrease in Treg frequencies at days 30 and 45 when comparing patients with and without aGVHD at the time of Treg sampling, as did Ratajczak *et al.*[14] at a mean of 3 months. Furthermore, Magenau *et al.*[13] assessed Tregs at the onset of aGVHD and demonstrated significant decreases in Treg frequencies in aGVHD patients, with comparison done using samples at GVHD onset and from patients without GVHD, such that the two groups were balanced for the time of acquisition. While their study sampled Tregs more than 4 weeks post transplantation, our study reports significant differences at less than half this time. Although further investigation is needed to determine whether early-stage Treg measurements are possible in other alloHSCT settings, our method under the condition of HLA-mismatched HSCT with rapid hematopoietic reconstitution[15–17]

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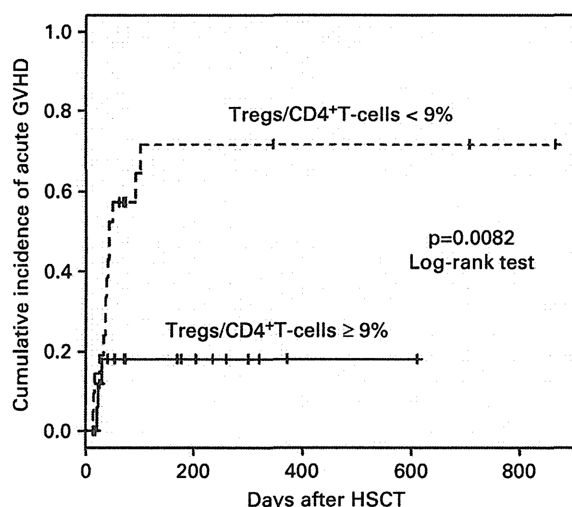

Figure 5. Cumulative incidence of aGVHD. Patients with Treg:CD4$^+$ T-cell ratios <9% had a significant higher incidence of aGVHD than those with ratios ⩾9% ($P = 0.0082$, log-rank test). Measurements were taken in the second week after HSCT.

produced the earliest reported differences in Treg frequency, a finding with practical applications that allows for the prediction of future incidence of aGVHD.

It is a commonly accepted theory that GVHD is initiated in the priming phase, in which donor T-cells activate and proliferate in response to host APCs in secondary lymphoid organs,[21] where Tregs presumably function effectively by suppressing APC function.[22] TNFα is also well known as a central cytokine that peaks immediately after HSCT and stimulates APCs to prime T-cells during this phase.[21,23] Choi *et al.*[24] and Willems *et al.*[25] have both demonstrated that levels of TNFα receptor 1 (a surrogate marker of TNFα) at day 7 correlate with subsequent development of GVHD after myeloablative and nonmyeloablative alloHSCT, respectively. Although a number of studies using animal models have contributed to theories underlying GVHD pathogenesis,[26–30] their finding that this priming phase is limited to a very short duration immediately after HSCT in humans is particularly valuable. The most important point in our study is that conducting investigations at the earliest possible time point after HSCT enabled us to obtain our findings from a very narrow time window. Although it remains unclear whether lower Treg frequencies in peripheral blood reflect lower frequencies in secondary lymphoid organs, integrating our findings and previous studies in which the decline in Treg frequency in peripheral blood was seen during the initial phase of GVHD,[31] it is reasonable to assume that this is indeed the case, and thus that it causes the development of GVHD.

We also found that while absolute numbers of each lymphocyte population did not predict the occurrence of aGVHD, the Treg:CD4$^+$T-cell ratio was the most significant predictor among all other ratios (Table 3). As Tregs can work in cooperation with other cells, including APCs and other T-cells,[22] it is considered rational that ratio rather than absolute number is the relevant factor for predicting aGVHD. However, the reason why the specific ratio of Tregs:CD4$^+$T-cells is the most significant predictor remains uncertain. Although CD4$^+$T-cells recognize MHC class II molecules and have been shown to induce GVHD in a class II-mismatched (class I-matched) murine HSCT model,[32] the importance of CD4$^+$T-cells in GVHD pathogenesis has been demonstrated in fully MHC-mismatched (both class I and class II) murine models[33–35] and even in an HLA class I-mismatched HSCT.[36] Beilhack *et al.*[33] visualized initial proliferation of CD4$^+$T-cells followed by CD8$^+$T-cells in secondary lymphoid organs, and Ewing *et al.*[34] and Yu *et al.*[35] have demonstrated that the activity of CD4$^+$T-cells in the early phase contributes to subsequent development of aGVHD by CD8$^+$T-cells. Accordingly, CD4$^+$T-cells would likely have a leading role during the priming phase of aGVHD, and only then would activation and proliferation of CD8$^+$T-cells proceed. Our observation that both higher CD4$^+$ T-cell:whole T-cell and lower CD8$^+$T-cell:CD4$^+$T-cell ratios in the second week exhibit a significant relationship with aGVHD development does not conflict with these findings, as they both indicate a greater abundance of CD4$^+$T-cells than CD8$^+$T-cells. Furthermore, CD4$^+$T-cells have a particularly direct relationship with Tregs, with CD4$^+$T-cells being the principal targets that Tregs suppress in APC-dependent[37] and -independent[38] manners. Considering this, the high significance of the Treg:CD4$^+$T-cell ratio is reasonable.

Whereas 22 patients developed aGVHD in this study, half of those had grade 1 aGVHD. As previously described,[15] once aGVHD appears in these HLA-mismatched HSCT cases, it inevitably and rapidly progresses to more severe disease, resulting in fatal outcome. All the 11 patients with grade I aGVHD had stage 1 or 2 skin disease at onset. We were therefore obliged to treat them at the earliest time possible, usually within 24 h, with a combination of topical treatment and dose escalation of internal corticosteroid as initial treatment. Additionally, in cases where a skin biopsy was performed, we were unable to delay treatment while waiting for the results, although they would have been helpful for subsequent validation of treatment. Consequently, the disease remained at grade 1 in half of the patients, whereas progression could not be prevented in the other half. It is notable that Treg:CD4$^+$T-cell ratios are able to predict even mild cases of aGVHD, as even grade 1 aGVHD poses a high risk of causing more serious conditions, and should be avoided if possible. In contrast to previous studies,[13,14] we did not observe a significant inverse relationship between aGVHD grade and Treg:CD4$^+$T-cell ratio (data not shown). We attribute this to the early intervention and/or unequal distribution of patients for each grade.

We have demonstrated that patients who developed aGVHD had significantly lower Treg:CD4$^+$T-cell ratios at the second week after HLA-mismatched HSCT, well in advance of clinical aGVHD symptoms. The measurement of Tregs during the second week therefore provides a means to predict the development of aGVHD. Our results suggest that Tregs have a vital role in regulating aGVHD progression, and support the efficacy of early infusions of donor Tregs to prevent GVHD in HLA-haploidentical HSCT.[39] Further studies are needed to confirm whether interventions lead to improved outcomes for patients who show a high risk of aGVHD during the second week post HSCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We appreciate the conscientious technical assistance of Ms Aya Yano and Ms Kimiko Yamamoto in the flow cytometric and donor – recipient chimerism analyses.

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Irreversible neurological defects in the lower extremities after haploidentical stem cell transplantation: Possible association with nelarabine

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Severe peripheral neuropathy and myelopathy are rare complications after stem cell transplantation (SCT). In our institution, seven patients of precursor T lymphoblastic leukemia/lymphoma without the central nervous involvement who had been treated by nelarabine to control their diseases received SCT from HLA-haploidentical familial donor (HLA-haploidentical SCT) with the conditioning regimen including high-dose cytarabine (HDAC). Three of evaluable six patients developed irreversible paresthesia and muscle weakness in both lower extremities after neutrophil engraftment. The results of nerve conduction studies and short latency somatosensory evoked potentials suggested axonal neuropathy of both lower extremities in all three patients and myelopathy in two patients. Negative findings of PET-CT, and analyses of repeated cerebrospinal fluid samples and the bone marrow also indicated that tumor involvement was improbable. In all three patients, the symptoms worsened or persisted despite administration of corticosteroid and intravenous immunoglobulin. The high frequency of the neurological symptoms in our patients previously treated by nelarabine strongly suggested the association of the nelarabine use. Furthermore, the HLA-haploidentical SCT setting and the use of a potentially neurotoxic agent, HDAC might augment the neurotoxicity of nelarabine. It may be desirable that HLA-haploidentical SCT candidates avoid receiving nelarabine. Am. J. Hematol. 88:853–857, 2013. © 2013 Wiley Periodicals, Inc.

Introduction

Severe peripheral neuropathy and myelopathy are rare complications after stem cell transplantation (SCT). SCT of unmanipulated bone marrow (BM) and peripheral blood stem cell (PBSC) from HLA-haploidentical familial donors (HLA-haploidentical SCT) using only pharmacological GVHD prophylaxis has been investigated in our institution [1,2]. In both myeloablative conditioning (MAC) and reduced intensity conditioning (RIC), the rate of Grade II or higher acute GVHD was kept at low level (36.7% in MAC and 20.0% in RIC, respectively) by the early therapeutic intervention for GVH reaction or Grade I GVHD, and that of extended type chronic GVHD was also low (29.2% in MAC and 25.0% in RIC, respectively). Except for the relatively high rate of viral hemorrhagic cystitis (36.7% in MAC and 42.3% in RIC, respectively), other complications including neurotoxicity were at the acceptable levels, indicating that the HLA-haploidentical SCT was feasible procedure. Seven recipients of HLA-haploidentical SCT in our institution had received nelarabine therapy to control their precursor T lymphoblastic leukemia/lymphoma (T-ALL/LBL). Among evaluable six patients, three developed irreversible neurological defects in both lower extremities early after SCT (day +18 to +24) as we describe here. We discuss the etiologies of their neurological symptoms and examine their association with the use of nelarabine.

Patients and Methods

Seven patients with T-ALL/LBL (3 T-ALL, 4 T-LBL) who had been treated by nelarabine to control their diseases received HLA-haploidentical SCT between November 2008 and July 2012. One of them, who had already received SCTs twice from HLA-identical donors, developed pulmonary hemorrhage requiring intubation and multiple organ failure on day +7, and also resulting the death on day +35, and was considered not to be evaluable. Therefore, the remaining six patients were analyzed.

The characteristics of the six patients are shown in Table I. Disease status at the HLA-haploidentical SCT was primary refractory disease in three patients, refractory relapse in two, and second CR in one. In all five nonCR patients, BM involvement was positive. Prior to the SCT, cerebrospinal fluid (CSF) involvement was not detected in any patients. Various chemotherapies as shown in Table I were performed and the intra-thecal (IT) chemotherapies containing methotrexate (MTX) and cytarabine (Ara-C) were administered only at the prophylaxis doses. All six patients received at least one course (1–3 courses) of nelarabine therapy (1.5 g/m^2 on days 1, 3, and 5) to control their diseases. The median time period from the last administration of nelarabine to the SCT was 1.3 months (range: 0.5–8 months). Prior to the HLA-haploidentical SCT, two patients had received another allogeneic SCT: PBSCT using RIC regimen consisting of fludarabine and melphalan 140 mg/m^2 from HLA-identical sister in patient 1, and BMT using MAC regimen consisting of etoposide (ETP), cyclophosphamide, and total body irradiation (TBI) 12 Gy from HLA-1-antigen mismatched father in patient 5. As conditioning for HLA-haploidentical SCT, three patients (patients 2, 3, and 6) received MAC regimen consisting of high-dose cytarabine (HDAC) 2 g/m^2 for 4 days, fludarabine 30 mg/m^2 for 4 days, cyclophosphamide 50 mg/kg for 2 days, and TBI 8 Gy (2 Gy for 4 days) as described previously [1]. The remaining three patients (patients 1, 4, and 5) received RIC regimen consisting of fludarabine 30 mg/m^2 for 6 days and melphalan 70 mg/m^2 for 2 days with or without TBI 3 Gy (Table II). One patient (patient 1) received carmustine (BCNU) 60 mg/m^2 for 3 days before the RIC regimen. Rabbit antithymocyte globulin (ATG) was administered to four patients: Fresenius (F) 2 mg/kg for 4 days to patient 4, Thymoglobulin (T) 1 mg/kg for 4 days to patient 1 and 0.5 mg/kg for 2 days to patients 5 and 6. Unmanipulated BM and PBSC obtained from HLA-haploidentical familial donor were

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TABLE I. Disease Status and Therapies Prior to the Haploidentical SCT

Patient	1	2	3	4	5	6
Age at SCT	53	31	47	54	33	19
Sex	Male	Male	Male	Male	Male	Male
Disease	T-LBL	T-LBL	T-ALL	T-ALL	T-LBL	T-LBL
Disease status at SCT	2nd refractory relapse	Primary refractory	Primary refractory	1st refractory relapse	2nd CR	Primary refractory
Disease site at SCT	Mediastinum, para-aorta, ribs, iliac crest, BM	Mediastinum, BM	BM	BM	(Mediastinum)	Neck, mediastinum, BM
CSF involvement	Negative	Negative	Negative	Negative	Negative	Negative
Disease status (months before SCT) Therapies prior to SCT (Number of courses)	*Onset (31M)* CHOP (8) RT 30.6 Gy →*CR* *1st relapse (21M)* Hyper CVAD (1) →*CR* HDAC+ETP (1) allo-PBSCT *2nd relapse (5M)* Hyper CVAD (1) HDAC+ETP (1) nelarabine (2)	*Onset (6M)* hyper CVAD (1) ALL 202 (1) ID-MTX (1) HDAC+ETP (1) nelarabine (3)	*Onset (4M)* hyper CVAD (1) Ida+Ara-C (1) nelarabine (1) ALL 202 (1)	*Onset (22M)* Ad-VP (1) Hyper CVAD (4)→*CR* *1st relapse (4M)* nelarabine (3)	*Onset (14M)* Hyper CVAD / HD-MA (3) nelarabine (1) allo-BMT →*CR* *1st relapse (3M)* ALL 202 (1) RT 24Gy →*CR*	*Onset (8M)* CHOP (1) Hyper CVAD (2) CHOP (1) nelarabine (1)
Months from the last nelarabine to SCT	0.5	1	2.5	1.5	8	1

CHOP, cyclophosphamide, doxorubicin, and prednisone; RT, radiation therapy; Hyper CVAD, fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone; HDAC, high-dose cytarabine; ETP, etopside; allo-PBSCT, allogeneic peripheral blood stem cell transplantation; ALL 202, cyclophosphamide, daunorubicin, vincristine, and L-asparaginase; ID-MTX, intermediate dose methotrexate; Ida, idarubicine; Ara-C, cytarabine; Ad-VP, Doxorubicin, vincristine, and prednisone; HD-MA, high-dose methotrexate and cytarabine; allo-BMT, allogeneic bone marrow transplantation.

TABLE II. Summary of the Haploidentical SCT

Patient	1	2	3	4	5	6
Donor	Son	Brother	Daughter	Daughter	Sister	Mother
HLA disparity in GVH direction	2 Ag	3 Ag	3 Ag	2 Ag	3 Ag	3 Ag
Stem cell source	PBSC	BM	BM	PBSC	PBSC	PBSC
Conditioning regimen	RIC/BCNU	MAC	MAC	RIC	RIC	MAC
TBI	–	8 Gy	8 Gy	–	3 Gy	8 Gy
ATG	4 mg/kg (T)	–	–	8 mg/kg (F)	2 mg/kg (T)	2 mg/kg (T)
GVHD prophylaxis	TAC, MMF, mPSL 1 mg/kg	TAC, MTX, MMF, mPSL 2 mg/kg	TAC, MTX, MMF, mPSL 2 mg/kg	TAC, MMF, mPSL 1 mg/kg	TAC, mPSL 1 mg/kg	TAC, mPSL 1 mg/kg
Acute GVHD	None	None	None	Grade 2	None	None
Chronic GVHD	None	None	Extended	None	None	None
Confirmation of CR	Day +24	Day +48	Day +25	Day +23	Day +48	Not achieved
Relapse	BM (day +105)	Pericardium (day +75)	None	BM (day +91)	Mediastinum, para- aorta (day +211)	–
CSF involvement	Negative	Negative	Negative	Negative	Negative	Positive (day +65)
Neurological symptoms of lower extremities	Complete paraplesia, complete numbness	Inability of walk, paresthesia	Tendency to fall, paresthenia	None	None	(Facial, oculomotor nerve pulsey)
Onset of the symptoms	Day +24	Day +18	Day +24	–	–	(Day +60)
Outcome	Dead (day +154)	Dead (day +176)	Dead (day +381)	Dead (day +221)	Dead (day +231)	Alive

BCNU, carmustine; TBI, total body irradiation; ATG, antithymocyte globulin; TAC, taclorimus; MMF, mycophenolate mofetil.

transplanted into two and four patients, respectively. HLA disparity in GVH direction was 2 antigens in two patients and 3 antigens in four patients, respectively. As graft-versus-host disease (GVHD) prophylaxis, all six patients received tacrolimus (TAC) and methylprednisolone (mPSL) at the dosage of 1 mg/kg for those receiving ATG (patients 1, 4–6) and 2 mg/kg for those not receiving ATG (patients 2 and 3). Mycophenolate mofetil (MMF) 15 mg/kg was administered to patients 1–4. MTX 5 mg on days 1 and 3 was added to patients not receiving ATG (patients 2 and 3). The doses of mPSL and MMF were planned to be tapered in the absence of acute GVHD after neutrophil engraftment.

Results

All six patients achieved rapid neutrophil engraftment (neutrophil count >500/μl) and full donor chimerism. As shown in Table II, acute GVHD occurred in one patient (Grade 2 in patient 4). Extended type chronic GVHD

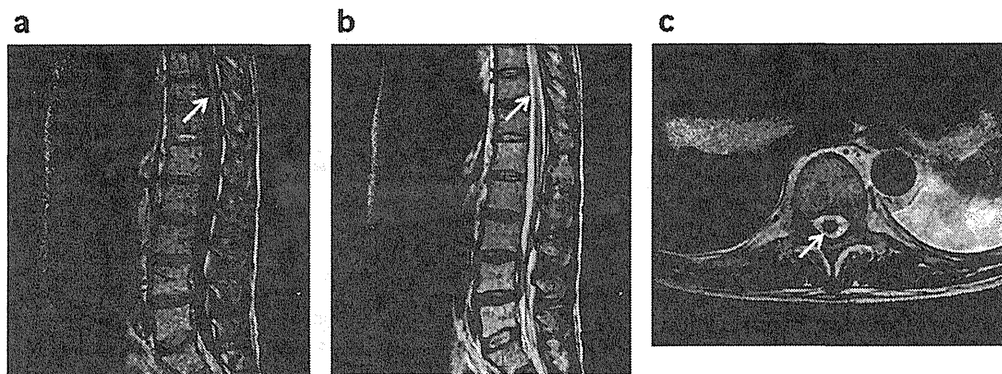

Figure 1. MRI images of patient 1. (a) Sagittal FLAIR, (b) sagittal T2, and (c) axial T2 weighted magnetic resonance images showing increased signal intensity in the spinal cord at the levels under the lower thoracic vertebral bodies (arrow). The axial image shows the spinal cord at the level of the T 11 vertebral body.

occurred in one patient (patient 3). Five patients (patients 1–5) achieved CR. Among them, four patients developed relapse, but CSF relapse was not detected in any of them. In one patient who did not achieve CR (patient 6), leukemic cells became detectable in CSF on day +65. As for neurological symptoms prior to the SCT, somnolence occurred after nelarabine therapy and persisted for one week in patient 1. In the remaining five patients, any neurological symptoms were not obvious. After the SCT, three patients developed irreversible neurological defects in their lower extremities as described below. In patient 6, facial and oculomotor nerve palsy occurred on day +60, which was considered to be caused by the CNS relapse, but neuropathy in his lower extremities was not observed.

Patient 1

A 53-year-old man with T-LBL underwent an HLA-haploidentical SCT in June 2009 for the treatment of his second refractory relapse, which was detected as multiple uptakes in the mediastinal and paraaortic lymph nodes, ribs, and iliac crest using ^{18}F-fluoro-2-deoxyglucose positron emission tomography-computed tomography (PET-CT) and lymphoma cells in the BM. Neutrophil engraftment was achieved on day +11 while his platelet count remained <20,000 /μl. Since no acute GVHD occurred, MMF was stopped on day +18 and the corticosteroid dose was rapidly tapered to 5 mg of prednisolone (PSL) on day +24. A BM aspiration on day +22 did not reveal any lymphoma cells, while a PET-CT on day +24 revealed no abnormal uptake, indicating the achievement of CR. On day +24, he complained of paresthesia, weakness in his legs, and inability to stand. He also complained of difficulty in urination that required urethral catheterization. The deep tendon reflexes (DTRs) of his lower extremities were absent and his plantar responses were both extensor. By day +40, the symptoms had worsened to complete numbness under the Th 5 dermatome and complete paraplegia. CSF analysis on day +27 showed neither pleocytosis nor lymphoma involvement but a mild increase in protein content (68 mg/dl). Polymerase-chain-reaction (PCR) tests for human herpes virus type 6 (HHV 6) and cytomegalovirus (CMV) were negative. Magnetic resonance imaging (MRI) of the supine on day +28 showed abnormal hyper-intense signals on both T2- and fluid attenuated inversion recovery (FLAIR)-weighted images of the spinal cord at levels under the lower thoracic vertebral bodies (Fig. 1). A nerve conduction study (NCS) on day +31 showed that the amplitude of compound muscle action potentials (CMAPs) of the right tibial nerve was reduced and the number of F-waves was also decreased. However, an NCS of the right median nerve showed no significant abnormal findings. No evoked potentials were found in the short latency somatosensory evoked potentials (SSEP) of the lower limbs. Moreover, we could not find evoked potentials except at the Erb points in the SSEP of the upper limbs. From these findings, we considered that the patient suffered from both axonal peripheral neuropathy in the lower extremities and myelopathy.

Despite the negative findings of the PET-CT and CSF analyses, two IT administrations of Ara-C and 30 Gy of irradiation to the area from Th 10 to L3 were performed because of the possibility of intraspinal lymphoma involvement. However, by day +60, the paresis had extended to both upper extremities. From day +67, intravenous immunoglobulin (IVIG) 0.4 g/kg was administered for 5 days, although the symptoms did not improve. CSF analysis on day +84 showed only a mild increase in protein content (62 mg/dl) without pleocytosis and lymphoma cell involvement, similar to that observed on day +27, while a test for the anti-GM1 ganglioside antibody was negative. Despite mPSL pulse therapy, his symptoms did not improve. A relapse in the BM was confirmed on day +105 and the patient died on day +154 because of progression of lymphoma.

Patient 2

A 31-year-old man with T-LBL underwent an HLA-haploidentical SCT on September 2010 for the treatment of his primary refractory disease presenting as a large mediastinal mass, pericardial effusion, and BM involvement. Neutrophil engraftment was achieved on day +15, and the platelet count reached 20 × 10^3/μl without transfusions on day +71. He developed a cutaneous fungal infection on day +2, which was treated by liposomal amphotericin B and micafungin. He also developed BK virus-associated cystitis on day +13. Since there was no sign of acute GVHD, mPSL and MMF were tapered gradually from day +20. CR was confirmed by PET-CT on day +34 and a BM aspiration on day +48. On day +18, he complained of muscle weakness and loss of position sense of both lower extremities, and developed a tendency to fall. By day +23, he was unable to walk without a walking frame. During the continuation of rehabilitation, he complained a loss of thermal nociception and on day +67, he was unable to walk even with a walking aid. DTR of his lower extremities was absent and his plantar responses were both extensor. An MRI of the supine showed an abnormal hyper-intense T2 signal in the lower cervical spinal cord. CSF analysis showed neither pleocytosis nor any lymphoma cells but a mild increase in protein content (80 mg/dl). PCR tests for HHV 6 and CMV were negative. Analysis for oligoclonal bands, myelin basic protein, and bacterial culture of the CSF were all negative. The amplitude of CMAPs was decreased slightly in his lower extremities in a

NCS. The results of a SSEP indicated that conduction of both peripheral nerves and spinal cord was disturbed. These findings suggested peripheral neuropathy in the lower extremities and myelopathy. IVIG 0.4 g/kg was administered for 5 days, although his symptoms did not improve. A PET-CT on day +75 revealed pericardial and pleural tumors with abnormal uptakes, indicating a relapse of his lymphoma. On day +175, he died because of progression of lymphoma.

Patient 3

A 45-year-old man with T-ALL underwent an HLA-haploidentical SCT in May 2009 for the treatment of his primary refractory disease. Neutrophil engraftment was achieved on day +11, although platelet engraftment (untransfused platelet counts of >20,000/μl) was not achieved. CR was confirmed by a BM aspiration on day +25. On day +24, when the doses of mPSL and MMF for GVHD prophylaxis had been tapered to 1.4 and 10 mg/kg, respectively, he complained of generalized paresthesia and muscle weakness in his lower extremities, and developed a tendency to fall. CSF analysis on day +31 showed neither pleocytosis nor any leukemic cells, but a mild increase in protein content (59 mg/dl). NCS revealed that the amplitude of CMAPs of the posterior tibial nerve, peroneal nerve, and sural nerve was reduced, indicating axonal peripheral neuropathy in the lower extremities. The patient did not develop acute GVHD but developed extensive chronic GVHD involving the skin, oral mucosa, lung, and eyes, which was controlled by increase of PSL dosage. Since thrombocytopenia that had persisted after the SCT worsened, he received IVIG on day +318 considering the possibility of immune thrombocytopenia. However, no significant improvement was observed in the thrombocytopenia or neurological symptoms. He continued to be in CR, although his clinical course was complicated by chronic GVHD and thrombocytopenia, possibly caused by thrombotic microangiopathy. He died of a gastrointestinal hemorrhage on day +381.

Discussion

Among evaluable six recipients undergoing HLA-haploidentical SCT who had received nelarabine before the SCT, three patients developed paresthesia and muscle weakness of both lower extremities after engraftment of neutrophils, on days +18, +24, +24, respectively. In patient 1, the symptoms worsened to involve complete numbness under the level of Th 5, complete paraplegia, and an inability to urinate by day +40. This patient's paresis also extended to both upper extremities by day +60. In patient 2, the thermal and position senses of his lower extremities were lost and he was unable to walk by day +67. In patient 3, a tendency to fall was noticed. The results of NCS and SSEP suggested axonal peripheral neuropathy of the lower extremities in all three patients and myelopathy in patients 1 and 2.

On the basis of the following findings, we considered that these neurological symptoms were not due to tumor involvement. First, the CRs of their diseases were confirmed by PET-CT and BM analysis when their neurological symptoms occurred. Second, repeated CSF analysis did not reveal any tumor cell involvement in any of the patients. Third, in patient 1, the neurological symptoms worsened despite IT chemotherapy and RT. In patient 3, he continued to be in CR, whereas his neurological symptoms did not improve.

Openshaw classified the etiologies of peripheral neuropathy after allogeneic SCT into three categories [3]. The first category is critical illness polyneuropathy (CIPN). It is thought to be caused by inadequate perfusion of peripheral nerves, which may occur in association with septic shock after SCT, and is most often recognized as a predominantly motor axonal neuropathy with a moderate to severe neurological deficit that interferes with respiratory weaning. The second category is immunologically mediated neuropathy, a demyelinating neuropathy similar to the Guillan–Barré Syndrome (GBS). The third category is toxic neuropathy caused by various chemotherapeutic agents. Possible etiologies that were reported for myelopathy developing after SCT include radiation myelitis caused by a combination of TBI for conditioning and local radiation for consolidation after SCT, HHV6-associated encephalitis/myelitis, and immune-mediated myelitis, which is responsive to corticosteroid, cyclophosphamide, or IVIG [4–7].

In our patients, CIPN could be excluded as clinical episodes causing hypotension were lacking. Viral encephalitis/myelitis could also be excluded as CSF findings did not reveal pleocytosis, and PCR tests for HHV6, CMV were negative.

The pathogenesis of GBS remains incompletely understood, but an aberrant immune response against peripheral nerve component has been postulated. GBS is a rare complication following SCT, which may occur in association with acute and chronic GVHD as well as in the absence of GVHD [8]. Wen et al. described four patients with GBS after allogeneic SCT [9]. In all four patients, GBS developed 4–12 months (mean 8.2 months) after SCT. CSF analysis was unremarkable except for increased protein. NCS showed conduction blocks consistent with demyelinating neuropathy. Three patients improved with plasmapheresis, whereas one patient died soon after treatment with IVIG and plasmapheresis. On the other hand, Rodriguez et al. reported three pediatric patients with T-cell leukemia/lymphoma who developed GBS early (days +2, +3, and +6) after allogeneic SCT [10]. Prior to SCT, all three patients were heavily treated with chemotherapy including HDAC and one patient received nelarabine therapy. As conditioning for SCT, all three patients received myeloablative regimen containing HDAC. CSF analysis was unremarkable except for increased protein. The neurological symptoms worsened despite IVIG therapy in two patients and stabilized in one patient. The close temporal relationship between the onset of their neurological symptoms and drug administration suggested a neurotoxic effect precipitated by the conditioning regimen as the etiology of their neurological symptoms. In our all three patients, the onset of the neurological symptoms was relatively early and IVIG therapy was not effective. In these points, our patients resemble the latter case series presented by Rodriguez et al. While some cases with GBS after SCT showed NCS findings consistent with axonal neuropathy, most cases showed those consistent with demyelinating neuropathy [11,12]. In our three patients, NCS findings were consistent with axonal neuropathy, which is frequently observed in toxic neuropathy caused by various chemotherapeutic agents [13]. The early onset and NCS findings consistent with axonal neuropathy in our patients suggested the neurological symptoms to be attributable to toxic effect of chemotherapeutic agents.

The two structurally similar agents administered to our patients, that is HDAC and nelarabine, have been reported to cause neurotoxicity including peripheral neuropathy [14–19]. HDAC induces CNS toxicity in which cerebellar toxicity is the most common at a frequency of 8–26%. This agent causes peripheral neuropathy in only 1% of patients [14]. The reported onset of neuropathy following the start of HDAC treatment ranges from 6 hr to 20 days. The clinical presentations are variable and range from sensory to rapidly progressive life-threatening motor neuropathy requires ventilator support. Destruction of the myelin sheath and axonal degeneration has both been reported as neuropathologic features of HDAC-induced peripheral neuropathy [15]. It has also been postulated that the peripheral neuropathy may be due to either direct drug toxicity or autoimmune responses.

Nelarabine is a soluble pro-drug of 9-β-D-arabinofuranosylguanine (ara-G), a deoxyguanosine derivative with a T-cell specific action. Kurtzberg et al. reported a phase I trial of nelarabine for hematologic malignancies in which various doses (5–70 mg/kg) of this agent were administered daily for 5 consecutive days [16]. Neurologic events attributable to nelarabine were observed in 67 (72%) of the 93 patients enrolled in the study. Transient somnolence, malaise, and fatigue began 6 to 8 days after the initiation of drug therapy and have been reported most frequently. Among 69 patients who had been treated at the dose levels higher than 30 mg/kg, 11 patients developed neurotoxicity in the form of hypoesthesias, paresthesias, or peripheral neuropathies. These symptoms were graded as severe in three of the 11 patients and remained unresolved in four patients at the time of final data analysis. Gőkbuget et al. reported a single-arm phase II study conducted in adults with relapsed/refractory T-ALL/LBL [17]. In that study, nelarabine was administered at a dosage of 1.5 g/m^2 daily on days 1, 3, and 5, which is now accepted as the standard dose for adult patients. Toxicity was evaluated in 201 cycles administered in 126 patients. Neurologic toxicities of any degree were observed after 26 cycles (13%) in 20 patients (16%), with the majority of events being transient and reversible. Grade III–IV neurotoxicities were observed after four cycles (4%) in nine patients (7%). In one patient, treatment had to be stopped because of a Guillan-Barré-like syndrome with tetraparesis, hallucinations, and reduced vigilance, which developed at day 3 during the first cycle. These symptoms improved slowly after withdrawal. Berg et al. reported a phase II study in which a total 151 of children and young adults received nelarabine for 5 consecutive days at daily doses of either 0.4, 0.6, 0.9, or 1.2 g/m^2 [18]. Thirty-one episodes of ≥Grade 3 neurologic adverse events were observed in 27 patients (18% of patients). Of these, 13 episodes were peripheral neurologic adverse events consisting of peripheral neuropathy manifested by weakness, numbness, and/or paresthesias, predominantly in the lower extremities. In at least two cases, the peripheral neuropathy appeared to have improved gradually. In the other cases, the patients died of progressive disease before resolution of the neuropathy. Papayannidis et al. reported the case of a 30-year-old male with ALL at the second relapse who developed severe neurologic symptoms resembling those in our cases after receiving two courses of nelarabine therapy at the standard dose of 1.5 g/m^2 on days 1, 3, and 5 [19]. On the third day of the second course, severe paresthesia in both legs occurred and rapidly extended to the anterior abdominal wall and to the gluteus skin. He also developed severe difficulty in standing caused by a significant defect in maintaining equilibrium and a strong-force defect, especially involving the legs, in addition to alterations in urinary and bowel functions. While a CSF showed no leukemic or infective involvement, an MRI showed an abnormal hyperintense T2 signal in the mid-thoracic spinal cord at T5, which resembles the MRI findings observed in our patients 1 and 2. The ineffectiveness of corticosteroid therapy suggested cumulative drug toxicity as the etiology of his symptoms, mainly attributable to the administration of nelarabine.

Among evaluable six recipients of HLA-haploidentical SCT in the present study who had been treated by nelarabine for disease control, three patients developed severe neurological symptoms as mentioned earlier. This high frequency of neurological symptoms strongly suggested that there was an association between nelarabine and these symptoms. Additionally, the short time period (0.5–2.5 months) between the last administration of nelarabine and HLA-haploidentical SCT in them might argument the neurotoxicity of this agent. The potentially neurotoxic agent, HDAC, which was used for conditioning in all six patients, was also considered to have augmented the neurotoxicity of nelarabine.

In the above cited phase II study of nelarabine reported by Gőkbuget et al., a total of 36 of 45 patients who achieved a CR with this agent subsequently received a SCT in continuous CR [17]. Any severe neurological complications were not observed and transplantation-related mortality was as low as 11% (GVHD in one patient, infection in three patients). In contrast to our patients, the conditioning regimens used in this earlier study did not contain HDAC (TBI 12 Gy and cyclophosphamide in 21 patients, other TBI-based regimens in four, busulfan-based regimens in five, melphalan and fludarabine in five, and treosulfan in one). Furthermore, almost all patients received HLA-identical SCT except one who received HLA-haploidentical and another autologous SCT. Therefore, it is possible that the HLA-haploidentical SCT setting in addition to HDAC use may also have contributed to the development of neurological defects in our patients.

Considering our experience, it may be desirable that patients who have previously received nelarabine avoid intensive conditioning regimens containing HDAC and that HLA-haploidentical SCT candidates avoid receiving nelarabine.

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CASE REPORT

Post-transplant gastric antral vascular ectasia after intra-venous busulfan regimen

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Abstract Gastric antral vascular ectasia (GAVE) is an angiodysplastic disorder that causes gastric bleeding. GAVE can develop as a complication of hematopoietic stem cell transplantation (HSCT-GAVE), and it has been suggested that it may be associated with oral administration of busulfan. We report two cases of HSCT-GAVE after a conditioning regimen containing intra-venous busulfan (ivBu), not oral busulfan. The first case, a 42-year-old woman with blastic plasmacytoid dendritic cell neoplasm, underwent second allogeneic HSCT with conditioning regimen consisting of cyclophosphamide (120 mg/kg) and ivBu (12.8 mg/kg). HSCT-GAVE developed on day 84 post-transplant, and argon plasma coagulation (APC) was performed successfully. The second case, a 60-year-old woman with acute myelogenous leukemia, underwent allogeneic HSCT with the conditioning regimen consisting of ivBu (12.8 mg/kg) and fludarabine (150 mg/kg). She developed melena and was diagnosed with GAVE by endoscopy on day 145 post-transplant. Although complete hemostasis was not achieved despite four administrations of APCs, the melena spontaneously terminated on day 235 post-transplant. To our knowledge, this is the first report describing HSCT-GAVE after ivBU-based HSCT. Although there is no established therapy for HSCT-GAVE, APC may be an option for HSCT-GAVE.

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Introduction

Gastric antral vascular ectasia (GAVE) is reported to account for up to 4 % of all non-variceal upper-gastrointestinal bleeding [1]. GAVE is localized mainly at the gastric antrum and characterized by a typical endoscopic pattern showing red spots which are either organized in stripes extending radially from the pylorus ("watermelon stomach"), or distributed randomly ("honeycomb stomach") [2].

GAVE, pathogenesis of which is still obscure, is often associated with systemic illnesses, such as liver cirrhosis, autoimmune connective-tissue disorders, and chronic renal failure. It has been known that GAVE sometimes develops as a complication of post-hematopoietic stem cell transplantation (HSCT-GAVE). The incidence of HSCT-GAVE is reported to be 0.13–2.2 % [3, 4]. Interestingly, all HSCT-GAVE described in these literatures occurred after oral busulfan-containing regimen and thus, relationship between oral busulfan and development of GAVE has been postulated. Recently, intra-venous formula of busulfan (ivBu) began to be in use. There are, however, no reports describing HSCT-GAVE after ivBu-containing conditioning regimen. We report two cases in which HSCT-GAVE developed after the ivBu regimen-based hematopoietic stem cell transplantation.

Case 1

Case 1 was a 41-year-old woman with blastic plasmacytoid dendritic cell neoplasm. The clinical stage was defined as

IVB according to the modified Ann Arbor staging system. The lesions extended to the nasal cavity, lymph nodes of neck and supraclavicular fossa, but not to the stomach. After the first complete remission was achieved by combination chemotherapy, autologous peripheral blood stem cell transplantation (aPBSCT) was performed. The tumor relapsed in the nasal cavity 18 months after aPBSCT.

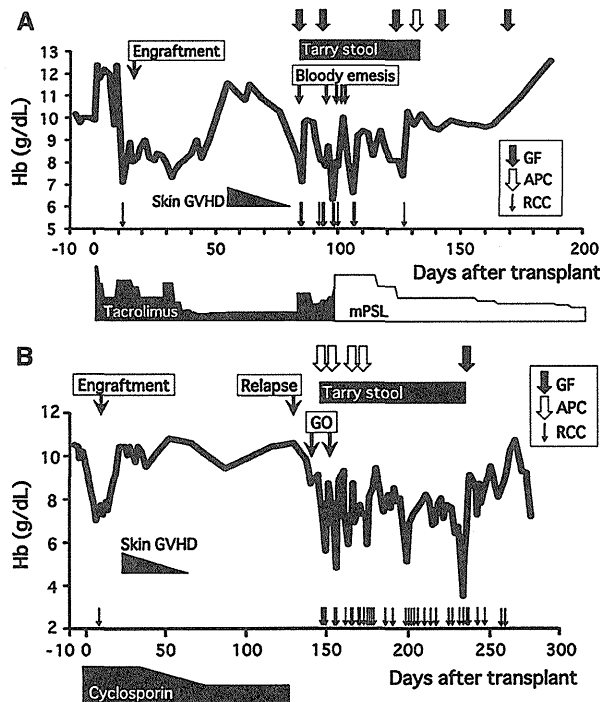

Fig. 1 Clinical course of the cases. Clinical courses of the case 1 (**a**) and case 2 (**b**) were shown. The *line* shows a level of hemoglobin. *GF* gastric endoscopy, *APC* argon plasma coagulation, *RCC* transfusion of red cell concentrate, *Hb* hemoglobin level, *mPSL* methylprednisolone, *GVHD* graft-versus-host disease, *GO* gemtuzumab ozogamicin

After the second complete remission was achieved by salvage chemotherapy, she received allogeneic bone marrow transplantation (BMT) from an unrelated HLA-matched donor. Conditioning regimen consisted of 120 mg/kg of cyclophosphamide and 12.8 mg/kg of ivBu. IvBu was administered at 0.8 mg/kg q.i.d. for 4 days through the central-venous catheter. Tacrolimus and short-term methotrexate were used as acute graft-versus-host disease (GVHD) prophylaxis. Neutrophil engraftment was achieved on day 15 post-transplant. On day 55, grade 1 GVHD was seen only in the skin, which alleviated with topical steroids (Fig. 1).

Hematemesis appeared on day 84 and the hemoglobin level was decreased from 11.0 to 7.8 g/dl. Platelet count was $6.3 \times 10^4/\text{mm}^2$. Upper-gastrointestinal endoscopy revealed multiple blood oozing points at the antrum without ulcers. Pathological examination of the gastric biopsy did not provide a definitive diagnosis. There were no signs that suggested TMA, CMV gastritis and relapse of lymphoma (Fig. 2b). There was no elevation of red cell fragmentation that made to think about TMA. Since the bleeding was thought to be attributed to upper-gastrointestinal acute GVHD, we increased dose of tacrolimus and used methylprednisolone. But tarry stool persisted. Computed tomography showed no sign of portal hypertension. Gastrointestinal endoscopy on day 124 post-transplant revealed randomly distributed red spots in the antrum, which was considered as "honeycomb stomach" indicating GAVE (Fig. 2a). Argon plasma coagulation (APC) therapy performed on day 131 succeeded in stopping tarry stool; the patient experienced no active bleeding after the therapy.

Case 2

Case 2 was a 59-year-old woman with acute myeloid leukemia (AML) with maturation. After she obtained a first

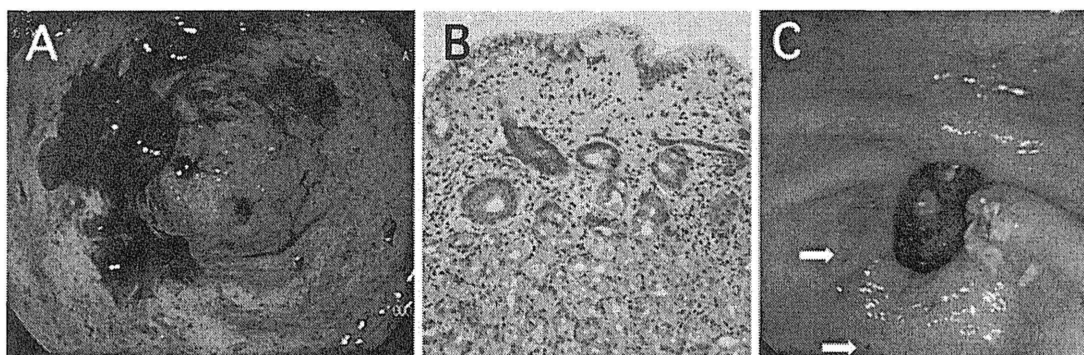

Fig. 2 Images of gastric endoscopy and hematoxylin-eosin staining of gastric specimen. An endoscopic image of case 1 on day 124 post-transplant. There are randomly distributed red spots in the antrum, which formed "honeycomb stomach" (**a**). Hematoxylin-eosin staining of gastric specimen on day 84 showed no specific findings in case 1 (**b**). An endoscopic image of case 2 on day 150 post-transplant. Easily bleeding red spots caused by capillary dilatation in the antrum (*arrows*) were seen (**c**)

complete remission by two cycles of remission-induction chemotherapy, allogeneic BMT from her HLA-matched sibling was performed. The conditioning regimen consisted of myeloablative dose of ivBu (12.8 mg/kg) and fludarabine (150 mg/m^2). Tacrolimus and mycophenolate mofetil were used as acute GVHD prophylaxis. Neutrophil engraftment was achieved on day 15 and GVHD requiring treatment was not observed. The AML relapsed on day 129 and gemtuzumab ozogamicin (GO) was administered on day 139. Tarry stool appeared on day 145, although platelet count was maintained above 2.0×10^4/mm^2. We diagnosed GAVE based on an endoscopic observation of an easily bleeding red spots in the antrum (Fig. 2c). There were no signs suggesting TMA such as elevated value of lactase dehydrogenase, unexplainable renal disorder and central nervous system damage, CMV gastritis, and GVHD. Although APC was performed four times between days 150–167, tarry stool continued. Endoscopy revealed disappearance of GAVE lesions, although small ulcers were present at the spots where APC therapy had been performed. We chose only observation after the continuous bleeding after APC, given the risk of invasive treatments. A conservative management brought improvement of tarry stool, which eventually disappeared after day 235. Despite second gemtuzumab ozogamicin and donor lymphocyte infusion, AML was not controlled. She died due to progression of AML on day 332.

Discussion

We reported two cases of HSCT-GAVE that developed after ivBu-containing conditioning regimen-based HSCT. We experienced another case in which GAVE was highly suspected 131 days after allogeneic BMT with ivBu (12.8 mg/kg) as conditioning. Diagnosis of GAVE was made from the endoscopic findings, although gastric GVHD, TMA, and CMV gastritis could not be fully excluded. APC was effective in obtaining hemostasis in the third case.

GAVE developed on day 84 post-transplant in the case 1 and APC effectively stopped bleeding. In case 2, the GAVE developed on day 145 post-transplant. Although APC cured GAVE lesions, tarry stool continued long time because of ulceration induced by APC. There was no pathological finding of gastric GVHD in case 1. In case 2, GO might have influenced the clinical course, although there are no literatures describing cases developing GAVE after administration of GO.

Twenty-nine cases of HSCT-GAVE have been reported in the literature [3–7]. In these cases, HSCT-GAVE developed at a median time of day 69 post-transplant. All the 29 patients received conditioning regimens containing orally administered busulfan (poBu). Ohashi et al. [4] reported that five of 134 patients who received busulfan-containing regimen developed HSCT-GAVE. In contrast, HSCT-GAVE was not observed among 96 patients who received non-busulfan regimen. The authors concluded that the incidence of HSCT-GAVE was increased significantly after busulfan-containing regimen. In our center, poBu was given to 42 cases, and ivBu was to 31 cases as a conditioning regimen from 1990 to 2012. Three cases developed GAVE, and all cases had been administered with IVBu.

Busulfan was administered via the oral route in all the previously reported cases of HSCT-GAVE. This fact raised a possibility that poBu causes mucosal damage at the gastric antrum through the direct contact to the mucosal membrane or the high local drug concentration at the antrum, because a large amount of drug stagnates there [3]. Meanwhile, our two cases received busulfan intravenously showing HSCT-GAVE could occur after ivBu. This implicates the presence of other mechanisms in addition to the “local” theory. One possible cause of HSCT-GAVE is that increased portal-vein pressure caused by sinusoidal obstruction under the influence of busulfan induces ectasia of antral vein, although this scenario does not apply to our cases directly because they did not show any signs of sinusoidal obstruction. Our cases did not have coexisting diseases which were suspected to associate with the pathogenesis of GAVE, such as gastric disease, liver cirrhosis, chronic renal failure, aortic stenosis, and autoimmune diseases.

Although there is no established therapy for HSCT-GAVE, endoscopic therapy has been used for many cases. Among these, effectiveness of yttrium aluminum garnet (YAG) laser was reported; more in detail, it was effective in eight among nine YAG-treated patients [3, 5].

Effectiveness of APC for HSCT-GAVE has not been demonstrated, but has been reported in GAVE-related bleeding in 20 patients with liver cirrhosis after a median of 3 sessions (range 1–10) [2]. Taken together, APC might be another option for HSCT-GAVE.

Conflict of interest There is no ethical problem or conflict of interest with regard to this manuscript.

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