Table 4 Univariate and multivariate analyses of risk factors for overall survival

	Univariate HR (95% CI)	*P* value	Multivariate HR (95% CI)	*P* value
Age (>60)	7.0 (2.0–24)	0.002	6.6 (1.8–24)	0.004
Sex (F:M)	1.7 (0.4–8.1)	0.48	–	–
HLA mismatch	0.6 (0.1–2.9)	0.56	–	
Disease status (high)	2.9 (0.9–9.4)	0.07	2.6 (0.8–8.6)	0.11
Acute GVHD (III–IV)	2.8 (0.8–11)	0.13	–	–
Intravenous busulfan	0.9 (0.3–3.3)	0.85	–	–

HR hazard ratio, *CI* confidence interval

associated with worse OS (HR 6.6, 95% CI 1.8–24, $P = 0.004$).

4 Discussion

Our simple procedure with T-cell replete, reduced-intensity conditioning without TBI, with the use of the classical combination of CsA and sMTX as GVHD prophylaxis, allowed stable engraftment and manageable GVHD, and led to very low NRM in u-RIST.

In reports of u-RIST with regimens that contained ATG [1–7] or alemtuzumab [5, 8–10], NRM rates were 10–39 and 15–30%, respectively. Although ATG or alemtuzumab-containing regimens were effective in preventing both chronic and acute GVHD [1–10] with the use of a standard dose of alemtuzumab, high relapse and infection rates due to delayed immune reconstitution are still a problem [1, 5–7, 9, 10, 22–24]. The optimal dose of ATG or alemtuzumab in u-RIST has been explored. Recent reports showed that reduced doses of ATG led to lower NRM in u-RIST [21, 29, 30].

We found five additional reports of u-RIST without use of TBI, ATG, or alemtuzumab [31–35]. NRM at 1 year was reported to be 19–32% [31–34]. In our study, the observed NRM of 3% at day 100 and 10% at 2 years is relatively low compared to that in previous reports of u-RIST. Important contributors to these favorable results might be that patients all had a history of prior chemotherapy, bone marrow alone was used as a stem cell source, and our study population was Japanese.

In RIST using ATG, alemtuzumab, or TBI, the reported incidence of acute GVHD was 19–47% [1–7], 16–36% [5, 8–10], and 3–77% [11–18], respectively. In RIST with TBI, the incidence of acute GVHD tended to be higher than in RIST with T-cell depletion. Taking differences in stem cell source, race, degree of HLA disparity, and GVHD prophylaxis into consideration, we cannot make a simple comparison among regimens. However, the observed incidence of 42% acute GVHD and of 60% chronic GVHD in our study was not higher than with RIST that involved the use of T-cell depletion. Japanese patients have a lower incidence of acute GVHD than Caucasian patients after hematopoietic stem cell transplantation from either HLA-matched siblings or unrelated donors. Although the incidence of acute GVHD was not particularly low in our study, the 77% efficacy rate of initial treatment for acute GVHD with high-dose steroids was relatively high [36], which might have contributed to the lower NRM. Furthermore, TBI was not required for conditioning to achieve rapid, complete T-cell chimerism and obtain sustained engraftment, probably because the study patients were limited to those with a history of prior chemotherapy.

A prospective, multi-institutional clinical trial of u-RIST in the Japanese population using a cladribine/Bu/TBI 4 Gy regimen showed a high NRM rate of 54% at 1 year, which largely derived from GVHD with co-existent infection [37]. This result suggests that the use of TBI 4 Gy may increase mortality associated with GVHD due to increased tissue damage, particularly in elderly patients.

Conversely, with the truly non-myeloablative conditioning developed by the Seattle Group, consisting of low-dose TBI 2 Gy with/without Flu and with CsA/MMF as post-grafting immune suppression, NRM rates were relatively low at 11–20% [11–14]. These results suggest that not only is TBI intensity important in itself, but that the intensity of conditioning combined with TBI, and post-grafting immunosuppressants are also important. However, a lower rate of sustained engraftment (10 of 18 patients, 56%) was reported when unrelated bone marrow was used [12]. Truly non-myeloablative conditioning is not likely to be sufficient to ensure sustained engraftment in unrelated bone marrow transplantation.

In our study, the achievement of complete donor T-cell chimerism by day 100 was significantly different between the oral and intravenous Bu groups. However, in the oral busulfan group, two of three cases of incomplete T-cell chimerism were caused by relapse rejection. It is therefore unknown how much the intensified busulfan dose contributed to increase lymphoablation of recipient T cells.

The current 37% cumulative incidence of relapse at 1 year appeared to be high. Predominant relapse and

progression occurred beyond 100 days after u-RIST (62% of cases relapsed or progressed). On the other hand, 38% of cases relapsed or progressed in the early phase after transplantation. For early relapse or progression of disease, a more intense conditioning regimen such as Flu/melphalan [38], Flu/Bu4 [39] or up-front allogeneic hematopoietic stem cell transplantation after cytoreductive induction therapy [40] may be effective. However, for late disease relapse/progression, a treatment strategy that enhances the graft-versus-leukemia/lymphoma effect would be needed, including the selective use of peripheral blood stem cells for chronic GVHD [41], or prophylactic DLI [42].

This study has the following limitations: (1) it was retrospective and performed at a single institution; (2) the population was small; (3) diseases were heterogeneous; and (4) HLA-mismatched donors were included. The characteristics of patients in previous reports of u-RIST were likewise heterogeneous. Ideally therefore, we should on an individual basis determine the magnitude of immunosuppression and the intensity of a conditioning regimen that is necessary and sufficient to obtain engraftment, and control GVHD and disease after taking into account numerous factors including patient age, intensity of prior chemotherapy, prior transfusion volume, residual host immune capacity, HLA compatibility, the stem cell source, ethnic background, and tumor burden.

Our regimen achieved lower NRM and better survival than those in previous reports, particularly in patients with a standard disease risk. However, our results cannot be applied to HLA-mismatched transplantation because of the small number of patients with HLA-mismatched transplantation in the present study. In addition, we cannot conclude with certainty that the low NRM was attributable to our regimen alone, based on comparison of the NRM rate in our regimen and that in other RISTs from the literature that included total-body irradiation or in vivo T-cell-depletion. To solve this problem would require a prospective study that addresses optimal conditioning for u-RIST in a cohort with as homogeneous a population as is possible.

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Conflict of interest None of the authors has a conflict of interest.

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Allogeneic stem cell transplantation as treatment for heavily treated, refractory acute graft-versus-host disease after HLA-mismatched stem cell transplantation

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Objective. **No effective treatment has been established for patients with steroid-refractory acute graft-versus-host disease (GVHD). Recently, we demonstrated in a murine tandem bone marrow transplantation model that life-threatening GVHD established by the first bone marrow transplantation was successfully treated by engraftment of a second donor graft after reduced-intensity conditioning. We named the effect by which allografts counteract GVHD "graft-versus-GVHD."**

Materials and Methods. **To investigate the efficacy of graft-versus-GVHD treatment clinically, 16 patients who developed, after human leukocyte antigen–mismatched stem cell transplantation, severe GVHD, refractory to three to five lines of GVHD-specific treatments, underwent 17 allogeneic stem cell transplantations using reduced-intensity conditioning regimens with grafts from a second donor.**

Results. **Among the 15 transplantations that could be evaluated, rescue donor grafts were engrafted in 11 cases and rejected in 4 cases. For patients who achieved rescue donor engraftment, the response rate was 90.9% (eight complete response, two partial response, and one stable disease). Six of the eight patients with complete response survived without GVHD symptoms, with a median follow-up of 2128 days. No new development of GVHD by the second graft was observed. No patients had recurrence of the original malignant disease. In contrast, no long-term survivors were observed in patients who rejected rescue donor grafts.**

Conclusions. **We propose here a novel graft-versus-GVHD treatment to treat refractory GVHD, and these results strongly suggest that GVHD can be successfully treated by eliminating the harmful lymphocytes responsible for GVHD by a second allogeneic stem cell transplantation.**

Graft-versus-host-disease (GVHD) is a major obstacle to successful allogeneic bone marrow transplantation (BMT), and greatly limits the applications and efficacy of allogeneic BMT. In particular, for steroid-refractory GVHD, no consensus treatment has been established [1,2], although a number of therapeutic approaches, including mesenchymal stem cells, pentostatin, infliximab, and a variety of monoclonal antibodies, have been reported [3–7].

We and others have attempted to treat patients with severe GVHD by second transplantation using autologous or syngeneic hematopoietic cells to ablate the lymphoid cells responsible for GVHD [8–10]. Although severe GVHD resolved or partially improved after these transplantations, relapse of the original tumor occurred in the majority of patients.

Therefore, we intended to use a second allogeneic donor as a graft source for rescue transplantation against GVHD. We recently demonstrated in a murine tandem BMT model where the three mouse strains shared one major histocompatibility complex haplotype and the other major histocompatibility complex haplotype was different, that

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life-threatening GVHD established by the first BMT using myeloablative conditioning was successfully treated by engraftment of a second donor graft using reduced-intensity conditioning treatment [11]. In allogeneic stem cell transplantation (SCT) for autoimmune diseases, donor lymphocytes are considered to have the capacity to eliminate all residual self-reactive host lymphocytes through a process known as graft-versus-autoimmunity effects [12], with analogy to graft-versus-leukemia (GVL) in leukemia. Thus, we named the effects by which second allografts counteract GVHD through permanent elimination or transient reduction of first donor harmful lymphocytes, "graft-versus-GVHD" [11].

In addition, clinically, we recently developed a novel unmanipulated human leukocyte antigen (HLA)–haploidentical nonmyeloablative SCT using a conditioning treatment consisting of fludarabine + busulfan + anti–T-lymphocyte globulin (ATG), and GVHD prophylaxis consisting of tacrolimus (FK506) + methylprednisolone (mPSL) (1 mg/kg), in which the incidence of acute GVHD was only 20% [13]. As some GVHDs occurred after donor lymphocyte infusion or rapid tapering of immunosuppressive agents for early relapse or severe viral infections, the actual incidence of GVHD was estimated to be 10%; therefore, we applied this HLA-haploidentical nonmyeloablative SCT to rescue transplantation for refractory GVHD.

In the present study, we investigated whether second allogeneic SCT could treat patients with severe, steroid-refractory GVHD.

Materials and methods

Patients

From February 2001 to December 2008, 320 patients underwent allogeneic SCT at Osaka University Hospital or at the Hospital of Hyogo College of Medicine. Among them, 16 consecutive adult patients who developed severe refractory GVHD after HLA-mismatched SCT underwent a second allogeneic SCT to treat GVHD. All of these patients were in remission at the time of rescue transplantation. The major objectives in this study were improved GVHD and survival at 6 months. GVHD was diagnosed from a biopsy of at least one involved organ. Patients with severe GVHD (≥grade II) who did not respond to mPSL (≥2 mg/kg) or who had recurrent GVHD at a dose of steroids ≥1 mg/kg mPSL were eligible for the study; however, patients who were finally enrolled received a median of four (range of two to five) lines of GVHD-specific treatments, including tumor necrosis factor blocker, ATG, and mycophenolate mofetil, by the time of the rescue transplantation (Table 1). In general, GVHD occurring after HLA-mismatched SCT progresses very rapidly, and quickly becomes irreversible; therefore, in the first SCT inducing GVHD, when the manifestations of GVHD worsened during 3 days of treatment, other immunosuppressive agents were added [14], sometimes in combination. Regarding the eligibility criteria for the rescue transplantation, patients who had HLA-identical or HLA 1–3 antigen-mismatched related donors were eligible. Patients were not eligible for rescue transplantation if they had severe renal, heart, or lung disease: serum creatinine level >1.5 times the normal upper limit, ejection fraction <50% on an echocardiogram, or oxygen saturation <93%, respectively. Patients were not eligible for rescue transplantation if they had severe liver disease that was considered to be caused by diseases other than GVHD; total bilirubin level >2.0 mg/dL, and aspartate aminotransferase >2.5 times the normal upper limit.

The characteristics of the patients and first transplantation inducing severe GVHD are shown in Table 1. Because one patient underwent allogeneic rescue SCT twice, 17 graft-versus-GVHD treatments were performed. Among the 16 patients, 14 had developed acute GVHD after allogeneic SCT, including 3 patients who had developed recurrent acute GVHD >100 days after transplantation and 2 after donor lymphocyte infusion. Institutional review board approval was obtained for the treatment protocol, and written informed consent was obtained from the patients and their families.

Four patients underwent the first transplantation (inducing severe GVHD) using a graft from an HLA 2–3 antigen-mismatched donor, and underwent the second (rescue) transplantation using a graft from an HLA-matched or 1 antigen-mismatched donor (Table 2). The donor in the first transplantation was selected for the following reasons. We recently reported that unmanipulated HLA-haploidentical SCT was useful for treating patients with hematologic malignant diseases in the advanced stage [13,15,16]. Thus, in our HLA-haploidentical SCT protocol, patients with a full-blown relapse can undergo allogeneic SCT using a graft from an HLA-haploidentical donor, even when an HLA-matched (or 1 antigen-mismatched) related donor is available. Such decisions were made at the recommendation of the physicians and with the concurrence of the patient and family members after considering the overall risks of recurrent malignancy, graft rejection, and severe GVHD with the two different types of donors.

Rescue transplantation procedure

Details of the rescue transplantation are shown in Table 2. Median interval between the previous allogeneic SCT and the rescue transplantation was 59 days (range, 32–481 days). All patients received a reduced-intensity conditioning treatment. The conditioning consisted of 30 mg/m^2 fludarabine intravenously for 3 consecutive days on days −6 to −4, ATG (Fresenius) 2 mg/kg/day for 4 days (day −4 to day −1) with or without total body irradiation 3 Gy on day 0. Eight patients could not receive total body irradiation because they had received total body or local irradiation as previous treatments. One patient (no. 10–2) who rejected the first rescue transplantation received thiotepa 10 mg/kg on day −2 and total body irradiation 4 Gy on day −1 in addition to fludarabine and ATG. In all cases, peripheral blood stem cells were used as the stem cell source.

GVHD prophylaxis was performed with FK506 and mPSL (1 mg/kg), as reported previously [13]. In brief, FK506 treatment was initiated the day before transplantation and given at a dose of 0.02 mg/kg/day as a continuous infusion. The target blood concentration of FK506 was set between 8 and 10 ng/mL until day 30, and was thereafter tapered in the absence of acute GVHD. Patients received intravenous FK506 therapy until they could reliably receive oral medications after transplantation. Intravenous administration of mPSL was started at a dose of 1 mg/kg/day from day −4. mPSL tapering was started in the third week and was performed relatively rapidly until day 30 using the serum soluble

Table 1. Patients' characteristics and first transplantation inducing severe GVHD

No	Sex/Age	Disease	Disease status	Conditioning regimen	Donor	HLA disparity	PS	grade	Stage			prior treatment for GVHD
									skin	gut	liver	
1	23/F	ALL	PR	full	Mother	2/2†	50	II	3	1	0	MTX, MMF, mPSL(2), Flu,
2	17/M	LBL	Re3	full	Cousin	2/3	10	III	3	3	1	Flu, ATG, MTX, MMF(inc)
3	33/M	ALL	PR	full	Sibling	3/3	20	III	3	4	0	MTX, MMF(inc), Flu, ATG,
4	37/M	MDS	RAEB	full	Offspring	3/3	20	III	3	4	0	Flu, MMF(inc), infliximab, ATG, pulse mPSL
5	25/M	CML	Re(autoBM)*	full	Sibling	2/2	70	II	3	0	0	PSL(inc). MMF
6	21/F	NHL	CR2(autoPB)	full	Mother	2/0	50	II	3	0	0	MMF, infliximab
7	19/M	HD	RR	full	Father	3/2	50	IV	4	0	0	MTX, ATG, infliximab
8	22/M	ALL	Re2	full	Sibling	3/2	10	III	0	3	3	infliximab, ATG, pulse mPSL, MTX, basiliximab
9	19/F	CML	BC	full	Sibling	2/2	70	II	3	0	0	infliximab, ATG, pulse mPSL, MTX, MMF(inc)
10-1	19/M	SNCL	IF	full	Sibling	2/2	50	III	3	4	0	MTX, infliximab, pulse mPSL, ATG
10-2	19/M	SNCL	IF	RIST	Mother	2/2	30	III	3	2	0	mPSL(inc), infliximab, MMF, ATG
11	41/F	LAHS	IF	full	Offspring	2/3	20	III	3	2	3	ATG, infliximab, MMF, pulse mPSL
12	21/F	AML	Re(alloBM)	RIST	Father	3/3	20	III	2	2	2	infliximab, pulse mPSL, MMF, ATG
13	49/M	CML	CP	RIST	Offspring	2/3	30	IV	4	2	0	ATG, MTX, infliximab, pulse mPSL
14	19/F	ALL	Re2	full	Sibling	3/1	40	III	2	3	1	pulse mPSL, MMF, etanercept, ATG
15	47/F	ALL	Re(alloPB)	RIST	UCB	4/2	40	III	3	3	0	etanercept, MMF, pulse mPSL
16	31/F	ALL	RR	full	Sibling	3/2	60	III	2	2	0	PSL(inc), pulse mPSL, MTX

AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's lymphoma; SNCL, small non-cleaved lymphoma; LAHS, lymphoma-associated hemophagocytic syndrome; CR2, second complete remission; PR, partial remission; Re, relapse; Re2 or Re3, second or third relapse; RR, resistant relapse; RAEB, refractory anemia with excess of blasts; CP, chronic phase; BC, blastic crisis; IF, induction failure; full, full regimen; RIST, reduced intensity of conditioning treatment; PS, Karnofsky performance status; MTX, methotrexate; MMF, mycophelate mofetil; mPSL(2), methylprednisolone 2 mg/kg; pulse mPSL, pulse therapy of methylprednisolone; Flu, fludarabine; ATG, anti-T-lymphocyte globulin; inc, increase in dose; autoBM, autologous bone marrow transplantation; autoPB, autologous peripheral blood stem cell transplantation; alloBM, allogeneic bone marrow transplantation; alloPB, allogeneic peripheral blood stem cell transplantation.

*Transplantation in parentheses indicates previous stem cell transplantation.

†Numbers before or after a slash indicate mismatched HLA antigens in GVH or HVG directions, respectively.

Table 2. Details of the rescue transplantation

No	Interval between 2 transplantations (days)	Donor relationship	Donor Sex/ Age	HLA disparity	Conditioning treatment	Cell dose CD34 cells $\times 10^6$/kg	Cell dose CD3 cells $\times 10^8$/kg	Engraftment of rescue graft	days	Hematological recovery Neu>0.5 $\times 10^9$/l (days)	Hematological recovery PLT>20 $\times 10^9$/l (days)	GvGVHD effect	survival (days)	Cause of death
1	94	Sibling	F/25	2/2	chemo	3.90	1.61	+	15	9	101	complete	+3304	-
2	40	Sibling	F/22	0/0	chemo	6.60	5.81	NE		8	-	NA	10	TMA
3	145	Mother	F/58	1/1	chemo	3.76	5.38	NE		-	-	NA	13	Renal failure
4	40	Offspring	M/12	3/3	chemo	16.50	4.19	+	20	10	-	partial	135	GVHD
5	481	Mother	F/55	2/2	chemo+TBI	3.90	2.50	+	34	not decreased	not decreased	complete	+2714	-
6	213	Sibling	F/23	1/0	chemo+TBI	5.20	6.71	+	29	10	36	complete	831	Cardiac failure
7	47	Mother	F/45	2/2	chemo	3.60	3.57	−		26	-	partial	76	Pneumonia
8	98	Mother	F/47	3/3	chemo+TBI	6.20	3.11	+	11	10	-	partial	23	Pneumonia
9	227	Mother	F/48	2/3	chemo+TBI	4.51	2.06	+	17	not decreased	not decreased	complete	+2170	-
10-1	59	Mother	F/51	2/2	chemo+TBI	2.80	2.12	−		not decreased	-	transient	+42	-
10-2	101	Mother	F/51	2/2	chemo+TBI*	2.30	2.27	+	14	9	32	complete	+2086	-
11	63	Sibling	M/37	0/0	chemo	7.10	1.71	−		not decreased	not decreased	partial	33	VOD
12	32	Mother	F/ 51	3/0	chemo+TBI	23.00	3.49	−		not decreased	-	partial	46	GVHD
13	36	Offspring	M/22	2/3	chemo+TBI	7.16	3.22	+	52	8	9	complete	+1637	-
14	59	Sibling	F/12	3/1	chemo+TBI	18.60	8.10	+	14	10	-	transient	72	TTP
15	49	Offspring	M/16	3/3	chemo	17.10	4.30	+	8	8	-	complete	163	Hepatic failure
16	39	Sibling	F/27	3/2	chemo	14.00	2.66	+	107	not decreased	16	complete	+490	-

chemo, chemotherapy consisting of fludarabine 30 mg/m2 and anti-T-lymphocyte globulin; TBI, total body irradiation 3Gy; NE, not evaluable; not decreased, neutrophils or platelet counts did not decrease below 0.5 x 109/l or 20 x 109/l, respectively; GvGVHD effect, graft-versus-GVHD effect; complete, complete response; partial, partial response; TMA, thrombotic microagiopathy; VOD, hepatic veno-occlusive disease; TTP, thrombotic thrombocytopenic purpura.

*Thiotepa 10 mg/kg and TBI 4 Gy were given in addition to fludarabine and ATG.

interleukin-2 receptor level [17,18], as an indicator, and was thereafter continued carefully.

Acute GVHD was graded according to standard criteria [19] and GVHD beyond 100 days after transplantation was diagnosed based on the proposed National Institutes of Health criteria [20]. Patient status before rescue transplantation was assessed by the Karnofsky performance rating. We defined the response to treatment as follows: complete response: loss of all symptoms of acute GVHD; partial response: improvement of at least one GVHD grade; stable disease: no change in GVHD grade; progressive disease: worsening of GVHD. Regarding the assessment of GVHD after the rescue transplantation, if the symptoms of patients were considered to have been caused mainly by a complication other than GVHD, their GVHD stages were downgraded by one stage, according to the recommendation in the 1994 consensus conference on acute GVHD grading [21]. A diagnosis based on autopsy directly reflected the assessment of response.

Each patient was isolated in a laminar air-flow room and standard decontamination procedures were followed. Oral antibiotics (ciprofloxacin, vancomycin, amphotericin B) were administered to sterilize the bowel. Patients with negative cytomegalovirus (CMV) IgG titers received blood products from CMV seronegative donors. Intravenous immunoglobulin was administered at a minimum dose of 100 mg/kg every 2 weeks until day 100. Cotrimoxazole was given for at least 1 year for prophylaxis of *Pneumocystis jirovecii* infections. Acyclovir was administered at a dose of 1000 mg/day for 5 weeks after transplantation to prevent herpes simplex infections.

Ganciclovir 7.5 mg/kg divided in three doses per day was administered from day −10 to day −3 as prophylaxis for CMV infection. Thrombotic microangiopathy was diagnosed according to Zeigler's criteria [22], and based on the recommendations reported by Nishida et al. [23].

Chimerism analysis

Chimerism between the donor and recipient was analyzed as described previously [13]. Chimerism analysis was continued twice a week after transplantation until donor engraftment or rejection. Blood samples were analyzed to determine the degree of donor/recipient chimerism in the T-cell or neutrophil-enriched cell fraction, using polymerase chain reaction amplification of informative microsatellite regions, which identified differences between the donor and recipient (based on polymorphisms found in pretransplantation donor/recipient samples) [24]. To remove monocytes, KAC-2 silica beads (Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan) were mixed with heparinized peripheral blood and incubated at 37°C for 1 hour. To enrich T cells, a negative selection system (RosetteSep; StemCell Technologies) was used [25]. To obtain a T-cell–enriched cell fraction, a cocktail containing anti-CD16, anti-CD19, anti-CD36, and anti-CD56 antibodies was added to the blood samples after they were treated with Silica beads. After Ficoll-Paque (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient centrifugation, $CD3^+$ cells were recovered from the Ficoll: plasma interface with a purity >95%. Neutrophils were recovered from the Ficoll:RBC interface with a purity >99%.

Statistical analysis

The protocol was designed as a phase II study with sufficient power to detect a response rate of ≥20% with a standard error of 10%. Comparison of patients who did or did not achieve rescue donor engraftment for the response for GVHD was evaluated using the χ^2 test. Survival data from patients achieving rescue donor engraftment or not were compared based on the results of log-rank tests. Results were considered significant at $p < 0.05$.

Data were "locked" for analysis on May 31, 2010.

Results

Engraftment of rescue donor grafts

To treat GVHD, patients received peripheral blood stem cells from a second allogeneic donor with a median of 6.40×10^6 (range, $2.30–23.00 \times 10^6$) $CD34^+$ cells/kg, including a median of 3.22×10^8 (range, $1.61–8.10 \times 10^8$) $CD3^+$ cells/kg, without T-cell depletion. As shown in Table 1, 16 patients received 17 rescue transplantations to treat GVHD. Because of a poor performance status at transplantation, two patients (nos. 2 and 3) died early (days 10 and 13, respectively) and could not be evaluated for the effects of rescue transplantation; therefore, data from 15 transplantations were analyzed.

Among the 15 transplantations that could be evaluated, rescue donor grafts engrafted in 11 cases, but not in 4 cases. T-cell engraftment preceded neutrophil engraftment (data not shown). In chimerism analysis, all patients showed 100% first donor chimerism in both T-cell and myeloid cell components before the rescue transplantation. It was difficult to obtain continuous chimerism data between first and second (rescue) donors within 1 week after transplantation because of lymphocytopenia. Changes of T-cell chimerism of patients, in whom the chimeric status could be consecutively measured, are shown in Figure 1. In the four patients rejecting a rescue graft, although transiently increasing up to 35% on day 4, rescue donor-derived T cells, thereafter decreased and became undetectable up to 2 weeks after transplantation. Regarding patients who achieved engraftment, donor T-cell chimerism rapidly or gradually increased after transplantation, and full T-cell chimerism of the rescue donor was achieved in a median of 15 days (range, 7–106 days).

Regarding neutrophil recovery, in 6 of the 15 patients, absolute neutrophil counts did not decrease to $<0.5 \times 10^9$/L, and in the remaining 9 patients, absolute neutrophil counts increased to $>0.5 \times 10^9$/L at a median of 10 days (range, 8–26 days). The platelet counts did not decrease to $<20 \times 10^9$/L in three patients (nos. 5, 10–2, and 12). Among the remaining 12 patients, platelet recovery occurred in 5 patients at a median of 32 days (range, 9–101 days), but not in the remaining 7 patients because of early death or subsequent transplantation.

Graft-versus-GVHD effects

Clinical effects of rescue transplantation are shown in Table 3. For successful graft-versus-GVHD treatment, engraftment of the rescue donor graft was mandatory in our murine model [11], in which immunosuppressive agents were not used. In the present clinical study, in which immunosuppressive agents

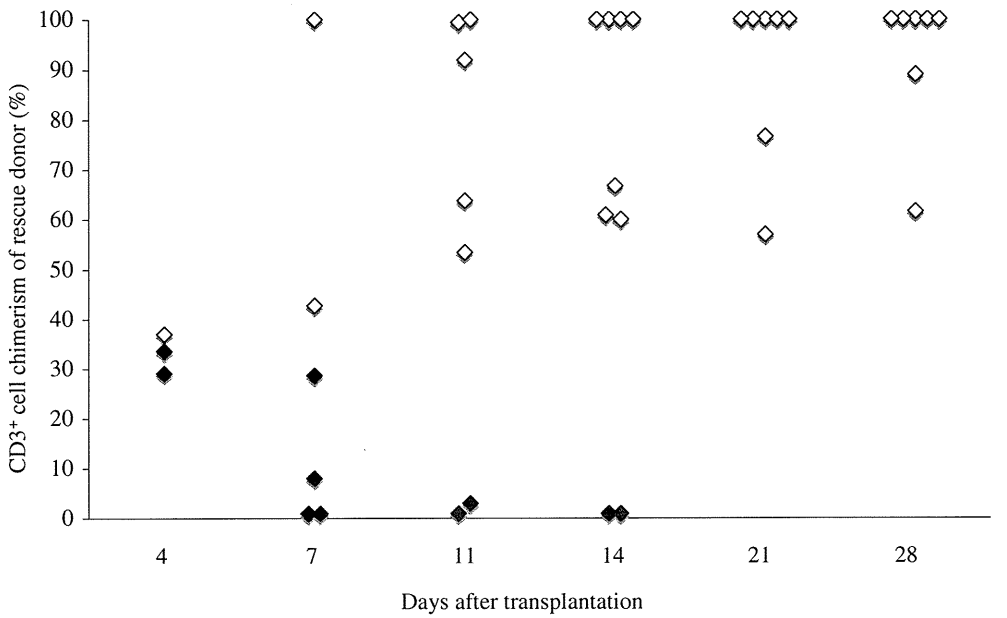

Figure 1. T-cell chimerism between first and second (rescue) donors in patients who did or did not achieve rescue donor engraftment. Open or closed diamonds denote patients who did or did not achieve rescue donor engraftment, respectively.

were naturally used in the transplantation, the response rate for patients achieving rescue donor engraftment or not was 90.9% (eight complete response, two partial response, and one stable disease) and 50% (one complete response, one partial response and one stable disease), respectively. Patients achieving rescue donor engraftment tended to show a higher response than patients not achieving engraftment ($p = 0.080$, χ^2 test). For the response of each organ, patients achieving rescue donor engraftment showed a significantly higher response with cutaneous GVHD than patients not achieving engraftment ($p = 0.016$), but there was no significant difference in response for intestinal and hepatic GVHDs between patients who did and did not achieve rescue donor engraftment. Regardless of achieving engraftment of the rescue donor graft, most GVHD symptoms began to improve during the conditioning treatment, and continued to improve by 1 week after transplantation. Thereafter, in patients who achieved rescue donor engraftment, the majority of GVHD symptoms continued to improve and disappeared within 40 days after transplantation, whereas in patients not achieving engraftment, some GVHD

Table 3. Change of the severity of GVHD

		stage			
No.	engraftment	skin	gut	liver†	grade
1	yes	3 → 0 (19)*	1 → 0 (0)	0 → 0	II → 0 (19)
4	yes	3 → 0 (6)	4 → 2 (12)	0 → 0	III → III
5	yes	3 → 0 (19)	0 → 0	0 → 0	II → 0 (19)
6	yes	3 → 0 (21)	0 → 0	0 → 0	II → 0 (21)
8	yes	0 → 0	3 → 1 (9)	3 → 0 (11)	III → II (11)
9	yes	3 → 0 (4)	0 → 0	0 → 0	II → 0 (4)
10-2	yes	3 → 0 (7)	2 → 0 (15)	0 → 0	III → 0 (15)
13	yes	4 → 0 (38)	2 → 0 (20)	0 → 0	IV → 0 (38)
14	yes	2 → 0 (−5)	3 → 0 (30)	1 → 1	III → II (30)
15	yes	3 → 0 (10)	3 → 0 (30)	0 → 0	III → 0 (30)
16	yes	2 → 0 (−6)	2 → 0 (5)	0 → 0	III → 0 (5)
7	no	4 → 1 (2)	0 → 0	0 → 0	IV → I (2)
10-1	no	3 → 1 (5)	4 → 1 (5) → 2 (19)	0 → 0	III → II (5) → III(19)
11	no	3 → 0 (−5)	2 → 0 (−5)	3 → 0†	III → 0†
12	no	2 → 0 (10)	2 → 0 (15)	2 → 3 (4) → 2(13)	III → III

*Numbers in parentheses denote the day after rescue transplantation when the stage or grade of GVHD was changed.

†Staging of hepatic GVHD was decided based on the serum bilirubin levels. Patient No.11 had an increased bilirubin level and died on day 33, but the main cause of death of the patient was diagnosed from autopsied samples with hepatic veno-occusive disease without no evidence of GVHD.

symptoms disappeared and others became stable or rebounded. Once a complete response was achieved, no rebound of GVHD occurred. In 8 patients who achieved rescue donor engraftment and who had a complete response, the median time for achieving a complete response was 19 days (range, 4–38 days) after transplantation. Among three patients not achieving a complete response despite rescue donor engraftment, one patient (no. 4) showed a complete response for cutaneous GVHD, but had continued diarrhea. The diarrhea was diagnosed to be mainly caused by thrombotic microangiopathy because of partial improvement of the symptom by tapering the immunosuppressants [23]. In another patient (no. 8), the serum bilirubin level was normalized after rescue transplantation and diarrhea had also improved (stage 3 → stage 1) by day 23 when the patient died of aspergillus pneumonia. The remaining patient (no. 14) showed a complete response of cutaneous and gut GVHDs, but serum bilirubin levels continued to increase. The aggravation of jaundice was diagnosed to be caused by thrombotic thrombocytopenic purpura based on the presence of severe hemolysis and renal failure. In four patients who rejected rescue donor grafts, one patient (no. 11) showed a complete response of cutaneous and intestinal GVHDs, but showed a progressive increase in serum bilirubin levels and died on day 33. The patient was diagnosed from autopsied liver samples with hepatic veno-occlusive disease with no evidence of GVHD. Patient no. 7 achieved a partial response (stage 4 → stage 1) of cutaneous GVHD but died of pneumonia on day 76. Patient no. 12 showed a complete response for cutaneous and intestinal GVHDs, but showed no response of hepatic GVHD, and died of aggravated GVHD on day 46. The remaining patient (no. 10–1) showed a partial response of cutaneous GVHD and also showed partial improvement of intestinal GVHD by day 5, when diarrhea rebounded and was progressively aggravated; therefore, he underwent a second rescue transplantation, after which he achieved rescue donor engraftment and ultimately had a complete response.

Regarding chronic GVHD, only 1 of the 10 patients who survived for >100 days developed limited-type chronic GVHD (skin lesion).

Adverse effects (Table 4)

CMV antigenemia occurred in 11 of 15 transplants (73.3%). The median peak number of CMV antigen-positive leukocytes was 15.4 per 50,000 white blood cells (15.4/50,000), with a range of 2.8/50,000 to 285.7/50,000. No CMV disease was observed.

Three patients developed bacterial infections: one (no. 7) had fatal pneumonia from *Enterococcus cloacae*, and one (no.16) had *Escherichia coli* sepsis, and one (no. 15) had sinusitis, all were successfully treated with administration of antibiotics. Two patients developed aspergillus pneumonia: one patient (no. 13) was successfully treated by antibiotics and another patient (no. 8) with a pulmonary aspergillus lesion before rescue transplantation died of aggravated pneumonia and brain fungal embolism. One patient (no. 14) developed fatal thrombotic thrombocytopenic purpura and one (no. 11) fatal hepatic veno-occlusive disease. One patient (no. 10–1) developed pancreatitis, which was improved by conventional treatment. Ten patients (62.5%) developed liver dysfunction with an increase to more than three times the normal upper limit of the transaminase level. The majority of cases of liver dysfunction were due to steroid- or drug-induced toxicities, and the transaminase level in these patients was normalized after tapering or discontinuation of the causative drugs. Other adverse events are shown in Table 4.

Relapse, cause of death, and overall survival

No patients had recurrence of the original disease. Two patients died early because of a poor performance status at rescue transplantation. Among them, 1 patient (no. 2) had severe GVHD accompanied by sepsis hyperbilirubinemia (10.2 mg/dL), and died of multiorgan failure on day 10. Another (no. 3) developed renal failure after the start of conditioning treatment. Despite receiving hemodialysis, he died of renal failure on day 13.

Overall survival at 6 months and 3 years was 44.6% (95% confidence interval [CI], 19.8–86.8%), and 37.2% (95% CI, 12.4–62.0%), respectively. Patients who achieved rescue donor engraftment showed a significantly improved survival rate compared with those who rejected grafts (log-rank test, $p = 0.013$) (Fig. 2). Six of the eight patients who achieved a complete response survived without any GVHD symptoms or relapse of the original diseases, with a median follow-up of 2128 days (range, 490–3304 days). Two of these patients needed no immunosuppressive agents and the others a small dose of steroids. Two of the patients who achieved a complete response died of cardiac failure on day 831 (no. 6) and of hepatic failure on day 163 (no. 15). Three patients who achieved rescue donor engraftment and who did not achieve a complete response died of multiorgan failure, including thrombotic microangiopathy on day 135 (no. 4), fungal pneumonia on day 23 (no. 8), and thrombotic thrombocytopenic purpura on day 72 (no. 14), as described previously. On the other hand, no long-term survivors were observed in patients who rejected rescue donor grafts. The causes of death for patients who rejected grafts were as described here. Performance status at rescue transplantation was important because no long-term survivors were observed among patients with ≤20% Karnofsky performance score.

Discussion

In the present study, we clearly showed that severe, steroid-refractory GVHD was successfully treated by allogeneic SCT using grafts from a second allogeneic donor. The response rate was 80.0% (90.9% for patients achieving engraftment and 50.0% for patients rejecting graft).

Table 4. Adverse events (%)

Infection	bacteria	bacteremia	1 (5.9)
		others	2 (11.8)
	fungus		2 (11.8)*
	virus	cytomegalovirus	0 (0)
		herpes zoster	2 (11.8)
	pneumocystis jiroveci		0 (0)
Hypoxemia			1 (5.9)
Hemorrhagic cystitis			2 (11.8)
Thrombotic thrombocytopenic purpura			1 (5.9)
Thrombotic microangiopathy			2 (11.8)
Venoocclusive disease			1 (5.9)
Pancreatitis			1 (5.9)
Liver dysfunction†			10 (58.8)
Hypertension			4 (23.5)
Aseptic necrosis			2 (11.8)
Cataract			2 (11.8)
Hyperglycemia‡			8 (47.1)
Nephrotoxicity§			1 (5.9)
Insufficiency of adrenal gland			1 (5.9)

*One patient had aspergillus pneumonia before transplantation.
†An increase to > 3 times the normal upper limit of transaminase.
‡Insulin dose of >30U/day was needed to control blood sugar.
§Nephrotoxicity that needed hemodialysis.

Although patients who were enrolled in the present study had a severe GVHD after HLA-mismatched SCT, which is known to be very difficult to control [26], the overall survival at 6 months and 3 years was 44.6% and 37.2%, respectively. Furthermore, the GVHDs were not only steroid-resistant, but also heavily treated: these patients were refractory to a median of four lines of GVHD-specific treatments (12 patients received tumor necrosis factor blockade, 12 ATG, 11 mycophenolate mofetil, and 9 a pulse therapy of mPSL). The rationale for graft-versus-GVHD treatment is that allogeneically harmful lymphocytes responsible for GVHD are all eliminated by retransplantation using a second allogeneic graft [11]. In the realization of the graft-versus-GVHD concept, there are two major barriers to be overcome: organ toxicity by conditioning treatment and new development of GVHD by a second allogeneic graft.

Regarding the organ toxicities of conditioning treatment, patients with severe GVHD are in a poor state of health due to GVHD-related organ damage, and therefore cannot

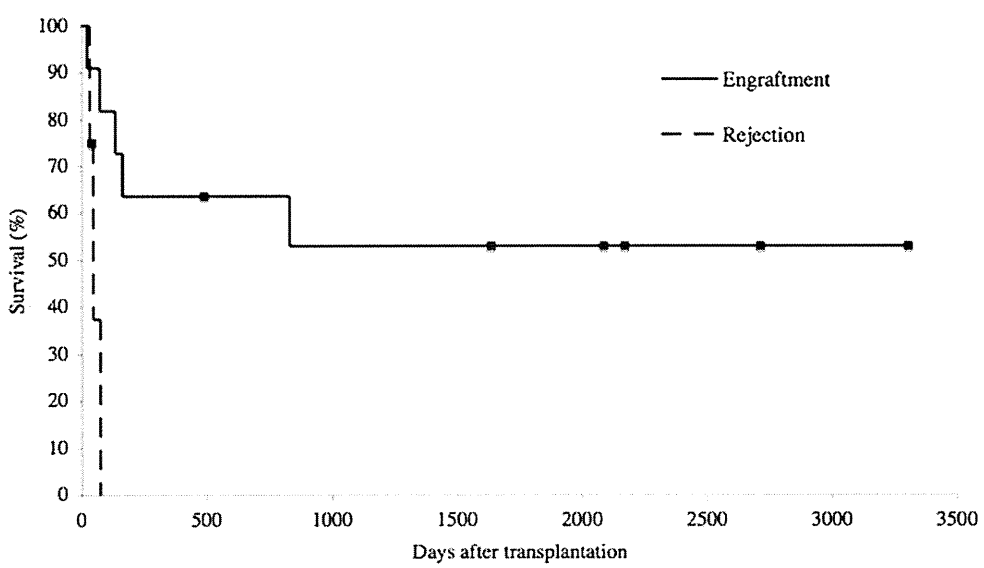

Figure 2. Overall survival of patients with refractory GVHD who did or did not achieve rescue donor engraftment. Patients achieving rescue donor engraftment showed a significantly improved survival rate compared with those rejecting grafts (p = 0.013). The survival rate of patients (n = 11) who achieved rescue donor engraftment was 63.6% (95% CI, 34.6–92.6%) at 6 months and 53.0% (95% CI, 22.0–84.0%) at 3 years, respectively.

Table 5. Relationship between first (GVHD-induced) and second (rescue) donors in graft-versus-GVHD treatment

No.	First donor	Second donor	Relationship of 2 donors (second to first donors)	HLA disparity in the direction of 1st to 2nd donors	GVH-target HLA antigens in 2nd donor*	Engraftment of rescue grafts
14	first son	second son	HLA-matched sibling	0	–	+
15	younger sister	youngest sister	HLA-matched sibling	0	–	+
17	brother	sister	HLA-matched sibling	0	–	+
1	mother	sister	Daughter and mother	2	–	+
9	sister	mother	Mother and daughter	3	–	+
10	brother	mother	Mother and son	3	–	+
16	UCB	son	Unrelated	2	–	+
5	first son	second son	HLA-haploidentical sibling	3	A24B48DR14	+
6	mother	brother	Son and mother	2	A26B59	+
7	mother	sister	Daughter and mother	2	B7DR1	+
11	brother	mother	Mother and son	2	B52DR15	+/–†
12	son	brother	Uncle and nephew	2	B54DR4	–
8	father	mother	Spouse	5	A11B35DR4	–
13	father	mother	Spouse	6	A33B44DR13	–

*HLA determinants of 2nd donors that could be major targets for GVH reaction in first (GVHD-induced) transplantation.
†The patient rejected the first rescue transplantation, but achieved engraftment of the second rescue transplantation.

usually tolerate an intensified conditioning treatment. However, as shown in our murine BMT model, recipients with severe GVHD were in a profoundly immunosuppressive state as a result of GVHD-related activation-induced cell death [27,28] and, therefore, with the help of unmanipulated (T-cell replete) grafts, could easily accept second allografts, even under minimal conditioning treatment, which was advantageous for recipients with serious organ damage.

Regarding GVHD induced by second allogeneic grafts, we demonstrated that second GVHD could be suppressed by conventional GVHD prophylaxis consisting of FK506 and a small dose of mPSL. This was fully expected because, in the unmanipulated HLA-haploidentical reduced-intensity SCT that we recently developed, using a conditioning treatment consisting of fludarabine + busulfan + ATG, and GVHD prophylaxis consisting of FK506 + mPSL 1 mg/kg, the actual incidence of GVHD was only 10% [13]. We consider that, in addition to in vivo T-cell purging by ATG, reduced-intensity conditioning, and a small dose of mPSL effectively suppressed inflammatory cytokine production in the transplantation period, which was shown to be closely involved in the pathophysiology of GVHD [29]. The molecular and cellular mechanisms of the high resistance to GVHD development have not been fully determined: however, in our murine studies, significantly reduced interferon-γ levels and a significantly increased percentage of $CD3^+CD4^+foxp3^+$ cells [30,31] were observed in day 7 spleens of second rescue BMT recipients compared with recipients of first BMT with severe GVHD. In addition, antigen-presenting cells (APCs) in the recipient spleen were found to have already been replaced by those of first-donor origin at the time of the second BMT. When APCs are replaced with first donor-derived cells from host cells, the first donor APCs need to cross-present host antigens to second donor T cells to induce GVHD; however, it was reported using major histocompatibility complex–matched, minor antigen-mismatched, murine BMT systems that this cross-presentation was insufficient to induce GVHD [32]. Although the present study includes mostly HLA-mismatched donor/recipient combinations, the limited ability of first donor-derived APCs to cross-present host antigens is considered to reduce the magnitude of the GVH reaction, at least compared with the first transplantation, in which host-type APCs directly present host antigens.

For successful graft-versus-GVHD treatment, engraftment of the rescue donor graft was mandatory in our murine model in which immunosuppressive agents were not used. In the present clinical study, even in patients who rejected the rescue graft, some GVHD symptoms improved within a week after the second transplantation because of conditioning treatment, including immunosuppressive agents and possibly because of the alloreactive response of second donor grafts to dampen first donor lymphocytes. Although these effects may have potential to completely control GVHD coupled with GVHD prophylaxis after second transplantation, as observed in patient no. 11, basically as long as the alloresponse from first donor-derived lymphocytes is maintained, GVHD symptoms continue or are aggravated, as shown in most patients who rejected second grafts. In fact, 8 of the 11 patients achieving rescue donor engraftment had a complete response, and 6 of the 8 patients survived without GVHD symptoms, with a median follow-up of 2128 days. These results strongly suggest, also in humans, that the engraftment of second donor grafts contributes to enduring control of GVHD and longer survival of patients with severe, refractory GVHD. Regarding HLA disparity between the first and second (rescue) donors, when rescue donors did not have HLA determinants that could be major targets for the GVH reaction in transplantation inducing GVHD, no rejection occurred (Table 5). As the extent

of HLA disparity in the direction of the first to second donor became greater, rejection tended to occur more frequently. When the 2 donors were HLA-matched siblings, 100% rescue donor chimerism was gradually achieved over 2 to 3 months.

Furthermore, as suggested in our murine model, the timing of rescue transplantation was another key factor for obtaining a positive graft-versus-GVHD effect. In particular, graft-versus-GVHD treatment in the late stage of GVHD is not effective. When organ damage due to GVHD proceeds fully, although the cell components involved in GVHD are all eliminated, recovery from severe organ damage is difficult, as shown by the lack of long-term survivors among recipients with a low PS score ≤20%. Thus, graft-versus-GVHD treatment may be started as one of the treatments for steroid-refractory GVHD before patients are heavily treated.

As patients did not show relapse of the original disease after successful graft-versus-GVHD treatment, and the majority of GVHD patients treated by autologous SCT had a relapse of the original disease [8–10], this strongly suggests GVL effects of second rescue allografts. In autologous transplant settings for GVHD, autografts can reintroduce malignant cells into the recipients in addition to the absence of GVL effects. Furthermore, in rescue transplantation of GVHD by allogeneic grafts, there is a possibility that malignant cells may have been eliminated by allogeneic NK cells as ATG was integrated into the conditioning treatment [33]. Thus, graft-versus-GVHD treatment has a unique feature in that it exerts GVL effects together with treating GVHD, which indicates the achievement of separating GVL from GVHD, a goal in allogeneic SCT.

We have proposed here the novel concept of graft-versus-GVHD and clinically showed that, using reduced-intensity conditioning and T-cell–replete grafts mostly from an HLA-mismatched donor, the second allogeneic SCT succeeded in eliminating harmful lymphocytes responsible for GVHD without the new development of GVHD. Thus, this graft-versus-GVHD strategy may be a promising treatment for refractory GVHD, although our results will have to be confirmed in a large-scale study.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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ORIGINAL ARTICLE

Drug interaction between voriconazole and tacrolimus and its association with the bioavailability of oral voriconazole in recipients of allogeneic hematopoietic stem cell transplantation

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Abstract In a previous study, we noted wide inter-individual variability in drug interactions between voriconazole and tacrolimus, but that analysis did not take into account the routes of administration. In the present study, we analyzed interactions between these two drugs when both agents were administered orally after allogeneic hematopoietic stem cell transplantation (HSCT); the effect of plasma voriconazole levels on the magnitude of the drug interaction was also examined. Twenty-five allogeneic HSCT recipients were evaluated. Trough concentrations of tacrolimus were measured prior to, and periodically for 7–10 days after, initiating voriconazole (400 mg/day) to determine the concentration/dose (C/D) ratio of tacrolimus. The median C/D ratio of tacrolimus increased significantly from 172.8 (range 28.6–1110.7) to 537.5 (range 127.8–1933.3) (ng/mL)/(mg/kg) ($P < 0.01$) following initiation of voriconazole; the median increase was 138.8 % (range −32.0 to 685.7 %). The plasma concentration of voriconazole did not correlate with the increase of the tacrolimus C/D ratio ($\rho = 0.16$, $P = 0.44$). These results indicate that oral voriconazole has a significant drug interaction with oral tacrolimus with a wide inter-individual variability, which cannot be explained by the bioavailability of voriconazole. Other possible mechanisms should be explored in future studies.

Keywords Voriconazole · Tacrolimus · Drug interaction · Hematopoietic stem cell transplantation

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Introduction

Recipients of hematopoietic stem cell transplantation (HSCT) are at high risk of developing invasive fungal disease (IFD), and incidence of invasive aspergillosis has been increasing [1–3]. At present, several anti-fungal agents active against *Aspergillus species* are widely used therapeutically or prophylactically. Voriconazole is recommended as the first-line agent for invasive aspergillosis since it yields a superior outcome as compared with amphotericin-B [4–7]. Voriconazole is metabolized by cytochrome P450 (CYP) enzymes, namely CYP 2C9, 2C19, and 3A4; it can also be an inhibitor of these enzymes [5, 8]. Therefore, its drug interaction with a variety of drugs metabolized by these enzymes has been well known. Calcineurin inhibitors such as tacrolimus and cyclosporine A (CsA), which are essentially used in allogeneic HSCT recipients, are recognized as agents with a clinically significant drug interaction [8]. A uniform dose reduction rate of calcineurin inhibitors upon initiating voriconazole was recommended by the manufacturer based on the results obtained from a limited number of renal transplant recipients and healthy subjects [9–11]. In our previous study, however, we systematically assessed the effects of voriconazole administration on the concentration of calcinuerin inhibitors in the recipients of allogeneic HSCT and disclosed a notably wide inter-individual variability in the magnitude of the drug interaction [12]. However, the limitations of the study were (1) the analysis did not take into account the route of administration (intravenous or oral) of voriconazole and calcineurin inhibitors, and (2) both treatment groups (intravenous and oral) included only a small number of patients. Therefore, the purpose of the present study was to further elucidate the drug interaction between voriconazole and tacrolimus in recipients of allogeneic HSCT by increasing the number of patients evaluated

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and selecting the cases given both voriconazole and tacrolimus orally. In addition, the plasma concentration of voriconazole was also measured to examine its effect on the inter-individual variability of the drug interaction.

Patients and method

Patient selection and drug administration

Recipients of allogeneic HSCT who had already been on a steady dose of oral tacrolimus, and were started on oral voriconazole for the treatment or prophylaxis of aspergillosis, were included in this study. By reviewing the medical chart and database, 25 patients were evaluable and retrospectively evaluated. HSCT was performed between April 2006 and October 2010. During this period, 91 patients underwent allogeneic HSCT, and 66 patients were not included since they did not fulfill the criteria described above. The patient characteristics are shown in Table 1. Tacrolimus was initially administered by continuous intravenous infusion at a dose of 0.03 mg/kg starting on day −1. When the patients were able to eat and did not develop gastrointestinal GVHD or other diseases, oral administration of tacrolimus was started in 2 doses given every 12 h. Voriconazole was administered orally under fasting conditions at a maintenance dose of 200 mg/body every 12 h (400 mg/body/day).

Table 1 Patient characteristics ($n = 25$)

Median age (range)	46 (19–62)
Gender	
Male/Female	9/16
Median body weight, kg (range)	53.0 (38.3–70.3)
Disease	
Acute leukemia	7
Multiple myeloma	6
Myelodysplastic syndrome	6
Non-Hodgkin lymphoma	3
Aplastic anemia	2
Myeloproliferative disease	1
Donor type and stem cell	
Unrelated, bone marrow	23
Mismatched related, bone marrow	1
Mismatched unrelated, cord blood	1
Conditioning	
Myeloablative	14
Reduced-intensity	11
Acute graft-versus-host disease	
Grades 0–I	14
Grade II	8
Grade III	3
Grade IV	0

Median post-transplant day of initiating voriconazole administration was 41 (range 31–113). Both tacrolimus and voriconazole were given simultaneously every day. The daily voriconazole dose per kg body weight was between 5.7 and 10.4 mg (median 7.6 mg). All patients had a stable renal and hepatic function, and did not have gastrointestinal symptoms such as nausea, vomiting, or diarrhea on initiating oral tacrolimus or voriconazole.

Determination of the concentration/dose (C/D) ratio of calcineurin inhibitors

Whole blood concentrations of tacrolimus were measured using a standard microparticle enzyme immunoassay. Blood concentrations of tacrolimus were measured just before and every 1–2 days after initiating voriconazole for 7–10 days. The concentration/dose [(ng/mL)/(mg/kg)] ratio of tacrolimus was used as a parameter reflecting both the drug dose and concentration simultaneously [12]. The C/D ratio of tacrolimus was calculated 7–10 days after initiating voriconazole, when the increased blood levels of tacrolimus had stabilized. The increase in the C/D ratio after initiating voriconazole was determined in comparison with that just before initiating the drug.

Measurement of the plasma concentration of voriconazole

The plasma concentrations of voriconazole were measured by high-performance liquid chromatography as described previously [13]. The day of applicable plasma concentration of voriconazole was determined on the same day that the C/D ratio was evaluated in each patient after initiating voriconazole.

Statistical analysis

The Wilcoxon signed rank test was used to compare the difference in the C/D ratio of tacrolimus before and after initiating voriconazole. The Spearman rank correlation coefficient was calculated to assess the correlation between the increase in the C/D ratio and the plasma level of voriconazole. P values less than 0.05 were accepted as statistically significant.

Results

Effect of voriconazole administration on the C/D ratio of calcineurin inhibitors

The blood concentrations of tacrolimus increased steadily after initiating voriconazole in all patients but one

(Table 2). The median C/D ratio of tacrolimus 7–10 days after initiating voriconazole was 537.5 (range 127.8–1933.3) (ng/mL)/(mg/kg), which was significantly higher than that before initiating voriconazole [172.8 (range 28.6–1110.7); $P < 0.01$, Table 2]. The median increase in the C/D ratio was 138.8 % (range −32.0 to 685.7 %). No significant adverse effects associated with increased level of tacrolimus were observed.

Association of the plasma voriconazole concentration with drug interaction

The median plasma concentration of voriconazole on the day of evaluation of the C/D ratio of tacrolimus was 1.85 μg/ml (range 0.44–7.28, Table 2). The concentration was below 1.0 μg/ml in 4 (16 %), below 2.0 μg/ml in 13 (52 %), and above 4.0 μg/ml in 3 patients (12 %, Table 2). There was no significant correlation between the plasma concentration of voriconazole and the increase in the C/D ratio of tacrolimus ($\rho = 0.16$, $P = 0.44$; Fig. 1). In addition, there was no significant correlation between the C/D ratio of tacrolimus before voriconazole administration and the plasma concentration of voriconazole ($\rho = 0.23$, $P = 0.28$).

Discussion

Although the drug interaction between voriconazole and tacrolimus is well recognized, data on this interaction in

Table 2 Concentrations of tacrolimus before and after voriconazole administration

Case	Concentrations and C/D ratio of tacrolimus				Increase of C/D ratio (%)	Concentration of voriconazole (μg/ml)
	Before voriconazole		After voriconazole			
	Trough level (ng/ml)	C/D ratio	Trough level (ng/ml)	C/D ratio		
1	6.6	133.6	26.9	544.5	307.6	2.14
2	14.2	208.3	13.9	611.6	193.6	1.46
3	5.6	28.6	8.8	224.4	685.7	1.67
4	12.5	325.0	14.6	759.2	133.6	2.20
5	14.0	541.3	12.7	368.3	−32.0	1.62
6	11.6	166.3	12.5	537.5	223.2	3.14
7	12.9	119.7	10.2	591.6	394.2	1.58
8	8.4	128.1	10.5	640.5	400.0	2.74
9	10.8	180.7	10.8	542.2	200.0	5.83
10	11.0	152.4	9.2	557.8	266.0	3.37
11	13.4	285.7	11.7	415.7	45.5	0.60
12	6.3	38.1	12.7	127.8	235.4	0.44
13	11.6	114.6	13.4	264.7	130.9	1.85
14	9.7	371.5	7.1	543.9	46.4	7.28
15	9.1	109.2	15.9	190.8	74.7	1.34
16	8.9	216.1	7.5	758.8	251.1	2.60
17	11.8	265.5	16.4	369.0	39.0	1.70
18	11.1	161.0	9.3	359.6	123.4	0.81
19	11.2	166.4	19.1	397.3	138.8	4.38
20	10.2	183.6	13.1	353.7	92.6	0.74
21	13.6	240.3	9.9	524.7	118.4	2.23
22	13.5	172.8	10.3	659.2	281.5	2.81
23	9.4	136.3	14.0	541.3	297.1	1.48
24	10.9	334.1	8.0	490.4	46.8	1.41
25	15.8	1110.7	16.5	1933.3	74.1	3.72
Median		172.8	–	537.5[a]	138.8 %	1.85

[a] Significantly higher than that before voriconazole administration

C/D concentration/dose (ng/mL)/(mg/kg)

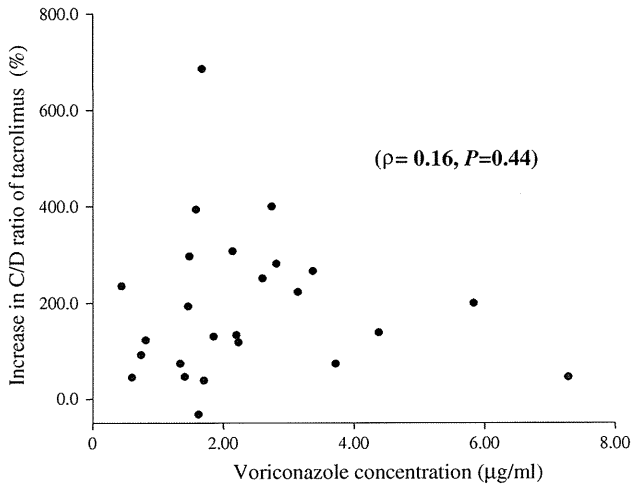

Fig. 1 Relation between the plasma voriconazole and the increase in the concentration/dose ratio of tacrolimus after initiating voriconazole administration. There was no significant correlation between the two variables ($\rho = 0.16$, $P = 0.44$)

HSCT recipients are quite limited. To the best of our knowledge, our previous report was the first to systematically evaluate the drug interaction in HSCT recipients. In that report, we showed a wide inter-individual variability in the magnitude of drug interaction between voriconazole and tacrolimus/CsA in the recipients of allogeneic HSCT in whom both agents were given intravenously or orally. Therefore, the aim of the present study was to further evaluate the drug interaction using a more homogeneous subject group: all patients received both agents orally, which we think is the most common setting in the management of allogeneic HSCT recipients. In the present study, we confirmed that the drug interaction between voriconazole and tacrolimus was significant when both agents were given orally. However, much as in the results of our previous study, there was a notable wide variability among the patients: the increase in the C/D ratio of tacrolimus after initiating voriconazole ranged between −32.0 and 685.7 % (mean 138.8 %). This wide inter-individual variability makes it difficult to determine a specific dose reduction rate for tacrolimus upon the initiation of voriconazole, despite the fact that the manufacturer does specify such a rate [11]. Therefore, we again strongly recommend close and periodic monitoring of the tacrolimus concentration, especially within 7 days after initiating voriconazole, which should be followed by the dose adjustment on an individual basis. This could minimize the dose-related toxicity and maximize the efficacy of calcineurin inhibitors even under this significant drug interaction.

The reasons for the notable inter-individual differences in the drug interaction between voriconazole and calcineurin inhibitors remain to be elucidated. One possible explanation is the variability of the pharmacokinetics of voriconazole. Several studies have shown that voriconazole shows variable pharmacokinetics among individuals not only when used orally but also when administered intravenously [13–18]. Based on these findings, although the optimal concentration has yet to be established, therapeutic drug monitoring of voriconazole is generally recommended to improve its efficacy and to prevent its toxicity. Consistent with the results of other studies, the present study has shown that the trough plasma concentration of voriconazole measured 7–10 days after initiation showed a wide variability among the patients, ranging between 0.44 and 7.28 μg/ml (median 1.85). This variability of voriconazole concentration did not exhibit a significant correlation with the increase in the C/D ratio of tacrolimus, in contrast to the results of our previous study evaluating the same effect in patients receiving itraconazole [19]. Thus, it is unlikely that the difference in the bioavailability of orally administered voriconazole plays a critical role in the variability of its drug interaction with tacrolimus. However, there are studies suggesting the intra-patient variability of voriconazole concentration measured at different times [18, 20], the effects of which should also be examined in a future study.

Another possibility is the difference in the activity of CYP among patients. Tacrolimus is extensively metabolized by a CYP 3A subgroup consisting of 3A4 and 3A5. In contrast, voriconazole is metabolized more actively by CYP 2C9 and 2C19, and less actively by 3A isoenzymes. In addition, voriconazole acts as an inhibitor of these CYP isoenzymes. The activities of voriconazole as a substrate and an inhibitor of these isoenzymes in combination are certainly considered the major sources of its intensive drug interaction. Genetic polymorphisms have been identified in these isoenzymes, and can cause significant differences in the kinetics of the drugs that the isoenzymes metabolize [8, 21–24]. Therefore, it is plausible that the lower the activity of CYP isoenzymes, the higher is the voriconazole concentration and the greater is the magnitude of interaction with tacrolimus. However, the results of the present study strongly suggest that such a relation is unlikely, based on the lack of correlation between the voriconazole levels and magnitude of interaction.

Since it is reportedly different from the other CYP enzymes, the role of CYP 3A4 could be a possible explanation for the wide variability of drug interaction between tacrolimus and voriconazole. In addition to the recently identified significant genetic polymorphisms in the metabolism of tacrolimus, the activity of CYP 3A4 is known to vary widely among the individuals not subject to its genetic polymorphisms [8, 21, 22]. Not only hepatic but also gastrointestinal tract CYP varies significantly in its activity among individuals. In addition, hepatic and

gastrointestinal tract CYP 3A4 activities are known to be regulated independently [25]. Since both agents were administered orally in the present study, we speculate that the magnitude of the interaction between voriconazole and tacrolimus locally in the small intestine might differ significantly depending on the level of gastrointestinal CYP 3A4 activity in each individual. Examination in the setting of both or either agent given intravenously would be of help in further elucidating the variability of the drug interaction.

In addition to CYP enzymes, transporters such as *P*-glycoprotein should generally be considered as possible candidates for affecting the drug interaction [26]. *P*-glycoprotein is a major transporter and plays critical roles in the carrier-mediated process of drug disposition of tacrolimus and azoles. Much as for the CYP enzymes, there has been increasing evidence of genetic heterogeneity in the transporters. However, unlike other azoles, voriconazole has been shown to be neither a substrate nor an inhibitor of *P*-glycoprotein [27, 28]. Therefore, *P*-glycoprotein is unlikely to affect the drug interaction between voriconazole and tacrolimus and the magnitude of the drug interaction.

We conclude that the magnitude of the drug interaction between voriconazole and tacrolimus is highly variable, and thus the dose adjustment of tacrolimus upon initiating voriconazole should be performed on an individual basis. The bioavailability of oral voriconazole could not explain the variability, and future studies examining the mechanism are required.

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