[12%]). Prophylaxis for GVHD consisted of cyclosporin (CsA) alone (n = 55), Cyclosporin with short-term methotrexate (sMTX) (n = 38), tacrolimus alone (n = 13), or tacrolimus with sMTX (n = 11).

In 81 of the 117 patients, the source of stem cells was G-PBMC from a related donor, which contained a mean of 3.3×10^6 CD34$^+$ cells/kg (range: 1.5-7.0 $\times 10^6$ CD34$^+$ cells/kg) and 8.7×10^7 CD3$^+$ cells/kg (range: 6.4-86.1 $\times 10^7$ CD3$^+$ cells/kg). The other 36 patients received related (n = 4) or unrelated (n = 32) bone marrow, which contained a mean of 2.9×10^8 total nucleated cells (TNC)/kg (range: 0.97-6.53 $\times 10^8$ TNC/kg).

A total of 9 patients received donor lymphocyte infusion (DLI), mainly after day 90, and all of them received DLI for relapse of disease. There was no patient who received DLI for low donor T cell chimerism.

Informed consent was obtained according to the Declaration of Helsinki.

Definitions

Graft failure was defined as (1) failure of absolute neutrophil count (ANC) to surpass 500 /mm^3 at day 30 after HSCT or (2) decrease in ANC <100 /mm^3 at 3 determinations after the initial engraftment or (3) absence of donor T cells (<5%) before relapse, disease progression, second HSCT, or death. The diagnosis and clinical grading of acute and chronic GVHD (aGVHD, cGVHD) were performed according to established criteria [15-17]. Complete remission (CR) was defined as according to the International Workshop Criteria in AML [18] and lymphoma [19] patients. Low disease risk was defined as AML or ALL in first CR, MDS-refractory anemia, and CML in first chronic phase. All other diagnoses were classified as high risk.

Chimerism Analysis

We assessed donor-recipient chimerism by the polymerase chain reaction (PCR)-based amplification of a polymorphic short tandem repeat region. Chimerism was evaluated using peripheral blood samples on days 30, 60, and 90 after transplantation. Samples were separated using Ficoll-hypaque into mononuclear cells and a precipitate that included red blood cells and granulocytes. Mononuclear cells were further separated into CD3-positive and -negative fractions with immunomagnetic beads (CD3 Magnetic Particles-DM, BD Pharmingen, San Diego, CA). Granulocytes were collected by lysing red blood cells in the precipitate. Briefly, DNA was extracted from selected cells using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Multiplex PCR was performed using primer sets (AmpFISTR Identifiler Kit, Applied Biosystems, Foster City, CA). Five-color fluorescence detection was performed on an ABI 3100-Avant Genetic

Analyzer (Applied Biosystems). For each STR allele, the area under the curve for the corresponding signal was automatically processed using GeneScan 3.7 software (Applied Biosystems). The percentage of donor cells was calculated as (area signal donor)/(area signal donor + area signal recipient). The range of the error of chimerism was regarded as 5% at our laboratory (Heike et al., unpublished data).

Statistical Analysis

The chi-square test, Fisher's exact test, and Pearson correlation coefficients were used to evaluate the association of percent donor chimerism with various clinical factors such as patient age at the time of RIST (with 55 years as a cutoff), disease type (acute leukemia, MDS/myeloproliferative disease [MPD], lymphoma), disease risk (high, low), stem cell source (G-PBMC, bone marrow), serologic HLA matching (match, mismatch), and conditioning with TBI (yes, no) or ATG (yes, no).

Overall survival (OS) was defined as the time between stem cell infusion to death from any cause. Event-free survival (EFS) was defined as the time from stem cell infusion to graft failure, PD, or nonrelapse mortality (NRM), whichever occurred earlier. OS and EFS were estimated by the Kaplan-Meier method [20]. The log-rank test and the generalized Wilcoxon test were used to compare the probabilities of survival after HSCT over time across patient subgroups. Multiple Cox regression models were used for multivariate risk factor analysis for OS and EFS. Clinical factors evaluated in the OS and EFS analyses were donor T cell chimerism at day 30 (with 60% as a cutoff), patient age at the time of RIST, disease type, disease risk, stem cell source, HLA matching, and conditioning. Logistic regression models were used for multivariate risk factor analysis for low donor T cell chimerism (<60%) at day 30. Clinical factors evaluated for the risk of low donor T cell chimerism at day 30 were number of prior chemotherapy regimens (≥2, <2) and donor type in addition to the variables mentioned above. We considered 2-sided P-values of <.05 to be statistically significant. Statistical analyses were performed with SAS version 8.2 (SAS Inc., Cary, NC).

RESULTS

Kinetics of Chimerism

Whereas 43% of the patients retained mixed donor chimerism (<90% donor) in the T cell fraction, 92% achieved complete chimerism (≥90%) in the granulocyte fraction at day 30 after RIST (Figure 1). In the peripheral blood mononuclear cell (PBMC) fraction, 72% of the patients achieved complete chimerism
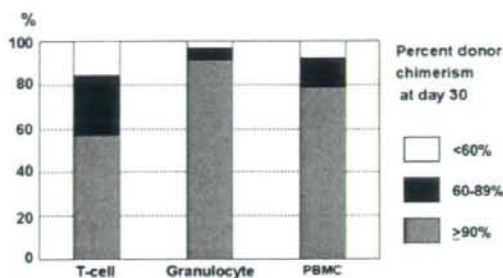
Figure 1. Distribution of chimerism status at day 30 after RIST.

($\geq 90\%$). The percent donor-chimerism was significantly higher in granulocyte than T cell fraction throughout the entire course, and the median (mean) values were, respectively, 100% (96%) versus 95% (83%), 100% (98%) versus 100 % (89%), and 100% (98%) versus 100% (91%) at days 30, 60, and 90, respectively after RIST (Figure 2).

In univariate and multivariate analyses (Table 2), having received <2 types of chemotherapy regimens before RIST was the only factor that was significantly associated with low donor T cell chimerism (<60%) at day 30 (hazard ratio [HR]: 6.1; 95% confidence interval [CI], 2.1-18.4; $P < .01$). Non-TBI regimens and related donor also tended to be associated with lower donor T cell chimerism.

Graft Composition and Donor Chimerism

By examining the impact of graft composition of G-PBMC on donor chimerism, we found that increases in TNC and CD3$^+$ T cells contents paralleled the increase in donor T cell chimerism at day 30 ($P < .03$ and $P < .05$, respectively). The same relationship was observed between CD34$^+$ cell contents and granulocyte chimerism ($P = .06$). In patients who received bone marrow, a higher number of TNC infused was associated with a higher level of donor T cell chimerism at day 30 ($P < .01$).
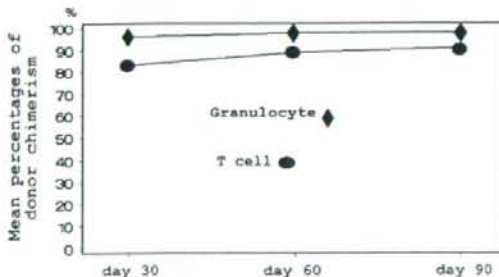

Figure 2. Kinetics of chimerism status after RIST (mean percentages of donor chimerism levels). Percent donor cell chimerism was significantly higher in granulocyte than T cell fraction throughout the entire course, and the mean values were, respectively, 96% versus 83%, 98% versus 89 %, and 98% versus 91% at days 30, 60, and 90 after RIST.

Table 2. Factors affecting low donor T cell chimerism (<60%) at day 30

Characteristics	Univariate analysis		Multivariate analysis	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Patient age, years				
<55	1			
≥55	2.04 (0.71 - 5.87)	0.19		
Disease type				
Lymphoma	1			
MDS/MPD	2.33 (0.69 - 7.87)	0.17		
Acute leukemia	0.86 (0.24 - 3.03)	0.81		
Disease risk				
Low	1			
High	1.51 (0.40 - 5.69)	0.54		
No. of prior chemotherapy regimens				
≥2	1		1	
<2	5.07 (1.73-14.83)	<0.01	6.08 (2.01-18.41)	<0.01
Stem cell source				
G-PBMC	1			
Bone marrow	0.84 (0.28 - 2.57)	0.77		
Donor				
Unrelated	1		1	
Related	3.48 (0.75-16.08)	0.11	4.21 (0.86-20.49)	0.08
HLA				
Match	1			
Mismatch	0.63 (0.17 - 2.34)	0.49		
TBI				
No	1		1	
Yes	0.17 (0.02 - 1.38)	0.10	0.13 (0.02-1.05)	0.06
ATG				
No	1			
Yes	1.08 (0.35 - 3.32)	0.89		

Association between Donor T Cell Chimerism at Day 30 and RIST Outcome

Graft failure

The median (mean) percentage of donor T cell chimerism at day 30 was 9% (18%) (0%-63%) in 5 patients who experienced graft failure, which was significantly lower than those in the other patients (97% [86%], 15%-100%, $P < .01$), as shown in Figure 3. Day 30 T cell chimerism below 60% was associated with a significantly increased risk of graft failure (Table 3). Among the 5 patients who experienced graft failure, 4 had achieved complete donor chimerism at day 30 when evaluated in the granulocyte fraction.

Whereas 4 of the 5 patients (80%) who experienced graft failure received HLA-mismatched grafts, 23 of the 112 patients (21%) who did not experience graft failure received HLA-mismatched grafts ($P = .01$). In a multivariate analysis, however, neither day 30 T cell chimerism below 60% nor HLA mismatch was associated with an increased risk of graft failure. Among 18 patients with <60% donor T cell chimerism at day 30, HLA mismatch was significantly associated with an increased risk of grafts failure (3 of 3 who received HLA-mismatched graft versus 1 of 15 who received HLA-matched grafts, $P = .005$). In contrast, HLA mismatch was not associated with an increased risk of graft failure in 99 patients with 60% or more donor T cell chimerism at day 30 (1 of 24
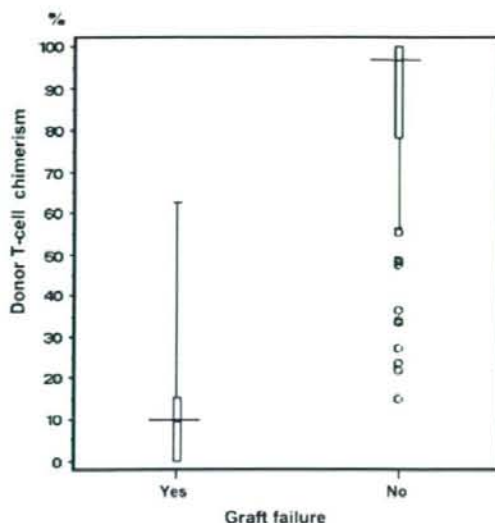
Figure 3. Donor T cell chimerism levels at day 30 in patients with or without subsequent graft failure. Five of the 117 patients (4%) who experienced graft failure had a significantly lower donor T cell chimerism level than the other engrafted patients (n = 112) (donor T cell chimerism, median 9% [range: 0%-63%] versus 97% [range: 15%-100%], respectively) (p < .01). Horizontal lines, median; boxes, 25-75 percentile; vertical lines, 10-90 percentile; circles, individual data outside the 10-90 percentile.

who received HLA-mismatched grafts versus 0 of 75 who received HLA-matched grafts, $P = .24$).

GVHD

Grade II-IV aGVHD occurred in 54 patients (46%), and cGVHD occurred in 63 patients (64%). No correlation was found between the kinetics of T

Table 3. Association between donor T-cell chimerism at day 30 and clinical outcome

| | | T-cell chimerism at day 30 | | |
Outcome	Total (n=117)	<60% (n=18)	≥60% (n=99)	P
Graft failure				
No	112 (96%)	14 (78%)	98 (99%)	<0.01
Yes	5 (4%)	4 (22%)	1 (1%)	
Acute GVHD				
0-I	64 (55%)	11 (61%)	53 (54%)	
II-IV	53 (45%)	7 (39%)	46 (46%)	0.55
Chronic GVHD*				
No	36 (36%)	7 (50%)	29 (34%)	0.25
Yes	63 (64%)	7 (50%)	56 (66%)	
NRM (at 1 year)	11.0%	11.1%	10.9%	0.26
PD (at 1 year)	27.3%	22.6%	28.1%	0.45
OS (at 1 year)	78.0%	65.7%	80.3%	0.02
EFS (at 1 year)	61.8%	55.6%	62.8%	0.02

GVHD indicates graft-versus-host disease; NRM, non-relapse mortality; PD, relapse or progressive disease; OS, overall survival; EFS, event-free survival;

*Proportion of patients with chronic GVHD was assessed among 99 evaluable patients.

cell chimerism and the occurrence of aGVHD or cGVHD, as shown in Table 3.

NRM and PD

Nineteen patients experienced NRM, with a 1-year probability of 11% (Table 3). No correlation was found between T cell chimerism at day 30 and the incidence of NRM.

PD was observed in 39 patients, with a 1-year probability of 27% (Table 3). No correlation was found between T cell chimerism at day 30 and the incidence of PD.

Cause of death

Among the 18 patients who had <60% donor T cell chimerism at day 30, 7 (39%) died of PD and 4 (22%) died of NRM, including bacteria sepsis (n = 2), pneumonitis (n = 1), and secondary carcinoma (n = 1). In contrast, among the remaining 99 patients who achieved 60% or more donor T cell chimerism, 21 (21%) died of PD and 15 (15%) died of NRM, including pneumonitis (n = 8), sepsis (n = 3), hemorrhage (n = 1), GVHD (n = 1), cerebral infarction (n = 1), and unknown cause (n = 1).

OS and EFS

Seventy patients (60%) are currently alive at a median follow-up of 1040 days after RIST (range: 153-2535). The 1-year probabilities of OS and EFS among all of the patients were 78% and 62%, respectively. As shown in Figure 4, OS was significantly better in patients who achieved 60% or more donor T cell chimerism at day 30 than in those who did not ($P = .02$). In a Cox proportional hazard model, low T cell donor chimerism (<60%) at day 30 was associated with poor OS (HR: 2.2; 95% CI, 1.1-4.5; $P = .02$) and EFS (HR: 2.0; 95% CI, 1.1-3.8; $P = .02$) adjusted for other significant prognostic factors (Table 4). In addition, high-risk disease and patient age (≥55 years) were associated with an increased risk of poor EFS (HR: 2.4; 95% CI, 1.2-5.0; $P = .02$, HR: 1.8; 95% CI, 1.1-3.0; $P = .03$, respectively) (Table 4).
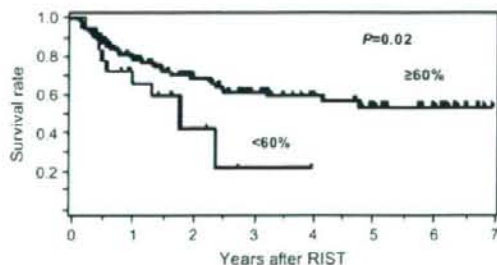

Figure 4. OS stratified according to donor T cell chimerism at day 30. OS was significantly better in patients who achieved 60% or more donor T cell chimerism at day 30 than in those who did not ($P = .02$).

Table 4. Multivariate analysis: factors associated with clinical outcome

Outcome	Variable	Harzard ratio	95% CI	P
OS				
	Donor T-cell chimerism at day 30			
	≥60%	1		
	<60%	2.25	1.13–4.47	0.02
EFS				
	Donor T-cell chimerism at day 30			
	≥60%	1		
	<60%	2.05	1.10–3.81	0.02
	Patients age, years			
	<55	1		
	≥55	1.80	1.07–3.04	0.03
	Disease risk			
	Low	1		
	High	2.44	1.19–5.01	0.02

Clinical factors evaluated in the OS and EFS analyses were donor T-cell chimerism at day 30 (with 60% as a cutoff), patient age at the time of RIST, disease type, disease risk, stem cell source, HLA matching and conditioning.

DISCUSSION

In this retrospective study of RIST with Bu, we showed that 43% of the patients retained mixed donor T cell chimerism (<90%), whereas 92% achieved complete chimerism in the granulocyte fraction, which was consistent with previously published observational studies in RIST [4,10,11,13,21]. Furthermore, we showed that low donor T cell chimerism of <60% at day 30 predicted poor OS and EFS, which suggests that the kinetics of T cell chimerism are important after Bu-containing RIST.

Consistent with other reports, we found that the induction of complete chimerism in T cell fraction after a Bu-containing regimen was rather slow, and granulocyte engraftment was earlier than T cell engraftment compared to patients who received RIC regimens containing a combination of Flu and Mel [10]. When the combination of Cy and Flu was used for RIST conditioning, full donor chimerism was achieved earlier in T cells than in myelogenous cells [1,22]. Interestingly, when alemtuzumab was used in a RIC regimen, 58% retained mixed donor chimerism at day 90 after RIC [13]. This may be because of the fact that alemtuzumab remained in the peripheral circulation long after RIST, which suppressed not only host but also donor lymphocytes. Based on these reports, we suspected that a Cy-containing regimen suppresses host granulocytes less intensely than a Bu-containing regimen, whereas a Mel-containing regimen suppresses host lymphocytes more intensely than a Bu-containing regimen.

The only significant variable associated with a lower level of donor T cell chimerism at day 30 was having received <2 regimens of chemotherapy pretransplant in our results. This result was consistent with previous reports [4,10]. When a patient is treated with RIST, such as our low-dose Bu-containing regimen, prior chemotherapy may facilitate the achievement of higher levels of donor T cell chimerism by decreasing the recipient immunocompetence.

In previous reports there has been some controversy regarding whether there are any differences in the levels of donor T cell chimerism after RIST with or without low-dose TBI [11,13]. In our study with Bu-containing regimens, regimens that included additional low-dose TBI tended to offer higher donor T cell chimerism in a multivariate analysis. However, there was no correlation between ATG-conditioning regimens and donor T cell chimerism at day 30, which was consistent with other regimens [13]. This might be because of the lower dose of ATG (Fresenius, 5-10 mg/kg) in our regimens compared to other studies that utilized the same ATG preparation (Fresenius, 40-90 mg/kg) [23,24]. Alternatively, this might be simply because of the small number of patients who received ATG in our study.

In previous reports, recipients of G-PBMC after RIST showed higher percentages of donor T cell chimerism than those who received bone marrow [4,25], which was not confirmed in our study. With regard to regimens that include Bu, no previous large-scale study has analyzed the correlation between the type of stem cell source and T cell engraftment. When low-dose Bu is contained in the RIC regimen, the stem cell source may no longer influence the level of T cell chimerism. Alternatively, this may be because of the fact that most of the bone marrow recipients in our study also received an additional 2-4 Gy TBI. There was a trend toward a decreased risk of low donor T cell chimerism in recipients of unrelated grafts, although the difference was not significant. We speculate that a lower probability of low donor T cell chimerism might be because of the addition of low dose TBI for patients who underwent unrelated HSCT.

Patients who received G-PBMC showed an increase in TNC and $CD3^+$ T cells that paralleled an increase in donor T cell chimerism at day 30 after RIST in our study. The same relationship was observed between $CD34^+$ cell contents and granulocyte chimerism. Baron et al. [26] reported that higher numbers of donor T cells and $CD34^+$ progenitor cells in the grafts were associated with higher levels of day 28 donor T cell chimerism. Similarly, Carvallo et al. [22] reported that higher levels of $CD34^+$ progenitor cells in the grafts were associated with higher levels of donor myeloid chimerism early after RIST.

In this study, donor T cell chimerism levels of below 60% early after RIST were significantly associated with an increased risk of graft failure. It has been reported that patients with <50% donor T cell chimerism early after nonmyeloablative HSCT were more likely to have graft failure than those with more than

50% donor T cell chimerism [4]. After Bu-containing RIC, Mattsson et al. [21] reported that 2 of the 8 patients who had >50% recipient T cells on day 28 had graft failure or rejection, whereas this was not seen in any of the 22 patients with <50% recipient T cells. Lower donor natural killer NK-cell chimerism after Bu-containing RIST was associated with an increased risk of graft failure [4,27]. Although significant associations of low donor T cell chimerism and HLA mismatch with graft failure disappeared in our multivariate model, our data suggested that HLA mismatch was an important predictor of graft failure only in patients with <60% donor T cell chimerism at day 30. The current study demonstrated that patients at high risk of graft failure could be identified by chimerism analysis at day 30 in T cell fractions, but not in granulocyte fractions, and that chimerism analysis at day 30 after Bu-containing RIST may allow early interventions aimed at reversing graft failure.

Our results suggest that low donor T cell chimerism of <60% at day 30 may predict a poor outcome, although levels of donor T cell chimerism were not associated with NRM PD. In our study, the levels of donor T cell chimerism were not associated with aGVHD or cGVHD, although some reports have stated that donor T cell chimerism was associated with the risk of GVHD [1,4,13,19,28]. It is still controversial whether or not achievement of complete donor T cell chimerism is needed to improve OS and reduce the relapse risk in patients who undergo RIST. Baron et al. [9] suggested that the assessment of donor chimerism levels helps to identify patients who are at higher risk of relapse after nonmyeloablative HSCT. High donor chimerism levels among immune competent cells including T cells and NK cells might be a surrogate for a high graft-versus-tumor effect, and a fractionated chimerism analysis may be useful for detecting and quantifying minimal residual disease after RIST. In a small case series of Bu-containing RIST, mixed donor chimerism was associated with an increased risk of relapse and a worse prognosis [12,29]. In contrast, among patients who underwent RIST that contained Flu, Bu, and alemtuzumab, those who showed mixed donor chimerism beyond day 100 were associated with an improved OS and a lower incidence of GVHD and NRM, without any effect on the relapse risk [13]. Further studies are needed to determine whether the achievement of complete chimerism after RIST is beneficial with less risk of PD and/or more risk of NRM.

In conclusion, within the limitations of a retrospective study, we found that the percentage of donor chimerism was significantly higher in granulocyte than T cell fraction throughout the entire course after Bu-containing RIST. Low donor T cell chimerism of <60% at day 30 may predict a poor outcome, and

a prospective study to examine the value of early intervention based on chimerism data is warranted.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Reduced-intensity unrelated donor bone marrow transplantation for hematologic malignancies

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Abstract To review a current experience of unrelated bone marrow transplantation (BMT) with reduced-intensity conditioning (RIC) regimens, we conducted a nationwide survey with 77 patients (age, 25–68 years). The backbone RIC regimen was a combination of fludarabine or cladribine, busulfan or melphalan and total body irradiation at 2–4 Gy. Five patients died early, but 71 (92%) achieved initial neutrophil recovery. Thereafter, 36 patients (47%) died of therapy-related complications, 23 (30%) of whom died within day 100. Grades II–IV acute graft-versus-host disease (GVHD) occurred in 34 of the 68 evaluable patients (50%). In a multivariate analysis, a regimen containing antithymocyte globulin (ATG) was significantly associated with a decreased risk of acute GVHD ($P = 0.041$). Thirty-three patients are currently alive with a median follow-up of 439 days (28–2002 days), with an OS of 50% at 1 year. In conclusion, unrelated BMT with RIC regimens can be a curative treatment in a subset of patients.

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1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a possible curative approach for patients with various hematologic malignancies. Recently, the application of reduced-intensity conditioning (RIC) regimens, mostly incorporating fludarabine as a backbone agent, has been explored for patients whose age or concomitant medical conditions contraindicate the use of conventional myeloablative regimens [1–3]. Since only 30–40% of patients have an appropriate family donor available [4], the establishment of an unrelated donor transplantation program with RIC regimens is urgently needed.

Graft rejection, regimen-related toxicities and graft-versus-host disease (GVHD) have been the major problems in unrelated HSCT with RIC [5–13]. In unrelated transplantation, engraftment is influenced by the source of stem cells and superior results have been observed with peripheral blood stem cells (PBSC) compared to bone marrow [9, 14]. Nevertheless, PBSC has not yet been approved as a graft source for unrelated transplantation in Japan [15]. The level of regimen-related toxicities directly depends on the intensity of the regimen, and the incidence of GVHD increases with unrelated donors compared to related donors. Although attempts have been made to overcome these problems, a suitable procedure for unrelated bone marrow transplantation (BMT) with RIC regimens has not yet been established. To accumulate further expertise, we conducted a nationwide survey of Japanese patients with hematologic malignancy who had undergone BMT from an HLA-matched or -mismatched unrelated donor with RIC regimens. Although the present data were obtained from a limited population of patients, these findings may show a current status of unrelated BMT with RIC.

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2 Patients and methods

2.1 Data sources

This survey collected the data of 77 consecutive patients in 17 participating hospitals who received unrelated BMT with RIC for hematologic malignancies between 2000 and 2004. Data were derived from questionnaires distributed to each hospital. Additional questionnaires were sent to confirm the follow-up data, including the occurrence of GVHD. The minimum data required for inclusion of a patient in this study were age, sex, histological diagnosis, status at transplant, donor information, conditioning regimen, date of transplant, donor chimerism status, therapy-related complications, date of last follow-up, disease status at follow-up, date of disease progression (PD)/death and cause of death.

This study was approved by institutional review board of each individual center. All patients provided written informed consent according to the Declaration of Helsinki. Unrelated donors provided consent through the Japan Marrow Donor Program as part of its standard procedures. The indications, conditioning regimens, management of GVHD and supportive care for BMT were left to the discretion of each institution. Patients who had previously received allogeneic HSCT and those younger than 20 years were not included. Patients younger than 50 years who had organ dysfunction and/or have previously received high-dose chemotherapy with autologous HSCT were also included.

2.2 Definitions

RIC regimens were defined as previously reported [6, 9, 10], and conditioning regimens that included either beyond 4 Gy of total body irradiation (TBI), 8 mg/kg of busulfan or 140 mg/m^2 of melphalan were excluded from the study. Alleles at the HLA-A, -B, and -DRB1 loci were identified by middle-resolution DNA typing as described previously [16]. Risk status at transplantation was categorized as either standard risk or high risk. Standard-risk diseases included acute leukemia in first complete remission, chronic myeloid leukemia in first chronic phase, and refractory anemia of myelodysplastic syndrome (MDS). Other diseases were categorized as high-risk disease. Graft failure was analyzed in patients who survived more than 28 days posttransplant according to the criteria reported by Petersdorf et al. [17]. Briefly, the definition included failure of the absolute neutrophil count (ANC) to surpass 500/mm^3 before relapse, death or second transplantation, as well as a decrease in the ANC to less than 100/mm^3 on at least three consecutive determinations with a finding of severe hypoplastic marrow. The degree of donor chimerism among peripheral blood T cells was assessed several times

between day 28 and day 100 after HSCT using fluorescence in situ hybridization (FISH) to detect X and Y chromosomes for recipients of grafts from sex-mismatched donors, and polymerase chain reaction-based analyses of polymorphic microsatellite regions for recipients of sex-matched or sex-mismatched transplants. Mixed chimerism was defined as the detection of 5–90% of donor cells in the peripheral blood. Acute and chronic GVHD were graded according to the consensus criteria [18, 19]. Patients who survived 100 days were evaluable for the assessment of chronic GVHD. Overall survival (OS) was measured as the time from the day of transplantation until death from any cause, and progression-free survival (PFS) was the time from the day of transplantation until PD/relapse or death from any cause. Patients who died from transplantation-related causes were classified as non-relapse mortality (NRM) regardless of their disease status.

2.3 Statistical analysis

The primary endpoint of this study was OS and chimerism. The secondary endpoints were PFS, NRM, PD, and the incidence of acute and chronic GVHD. Descriptive statistical analysis was performed to assess patient baseline information. Patients were divided into two groups: age 60 or above and less than 60. OS and PFS were calculated using the Kaplan–Meier method. The cumulative incidence of acute GVHD was calculated using the method described by Gooley et al. [20] to eliminate the effect of competing risks. The competing event for acute GVHD was defined as death without grades II–IV acute GVHD. For each endpoint, a Cox proportional hazard model was used for univariate and multivariate analyses. The factors included in the analysis were HLA disparity (mismatch vs. identical), recipient age (age 60 or above vs. less than 60), use of TBI (yes vs. no), use of ATG (yes vs. no), diagnosis of AML (yes vs. no), risk status (high vs. standard) and acute GVHD (II–IV vs. 0–I). Acute GVHD in the model was treated as a time-varying covariate. We defined statistical significance as a P value less than 0.05. All statistical analyses were performed using STATA version 8 (College Station, TX).

3 Results

3.1 Patients and diagnoses

The patients' characteristics are listed in Table 1. The median age of the patients was 54 years (range, 25–68 years) as a whole. Twenty-one patients (27%) had acute myelogenous leukemia (AML), 2 (3%) had acute lymphoblastic leukemia, 5 (7%) had chronic myeloid leukemia, 20 (26%) had MDS or myeloproliferative disease (refractory anemia,

$n = 8$; refractory anemia with excess blasts, $n = 9$; others, $n = 3$), 19 (25%) had non-Hodgkin lymphoma (follicular lymphoma, $n = 12$; diffuse large B-cell lymphoma, $n = 4$; mantle cell lymphoma, $n = 2$; peripheral T-cell lymphoma, unspecified, $n = 1$), 7 (9%) had adult T-cell leukemia/lymphoma, and 3 (4%) had multiple myeloma. Sixty-three patients (82%) had high-risk disease at the time of allogeneic BMT.

3.2 Conditioning regimens

Conditioning regimens are shown in Table 2. None received ex vivo T-cell depleted transplantation.

3.3 HSCT procedure and supportive care

Forty-seven patients (61%) were transplanted from a matched, 24 (31%) were from a 1 allele-mismatched, and 6 (8%) were from a 2 or 3 allele-mismatched unrelated donor. All patients received bone marrow as a source of stem cells. The prophylaxis of GVHD was either cyclosporine- or tacrolimus-based. Thirty-nine patients (51%) received cyclosporine with methotrexate, including five patients who received an ATG-containing preparative regimen. Nine patients (12%) received cyclosporine alone, including five patients who received ATG. Each patient received cyclosporine with mycophenolate mofetil and cyclosporine with prednisolone, respectively. Twenty-five patients (33%) received tacrolimus with methotrexate, including one patient who received ATG. Two patients (3%) received tacrolimus alone, including one who received ATG. Granulocyte colony-stimulating factor was administered intravenously from day +1 or +6 until neutrophil engraftment in all patients.

3.4 Engraftment and chimerism

Five patients died before the engraftment evaluation, with a median survival time of 15 days (range, 2–17 days). Seventy-one patients (92%) achieved initial neutrophil recovery, but three patients (two AMLs and one MDS) later experienced secondary graft failure; one each with AML and MDS after unrelated BMT from an HLA-1 allele-mismatched donor received a second transplantation when they failed to achieve subsequent complete donor-type chimerism, but both died of infectious complications. The other patient with AML after unrelated BMT from an HLA-6 allele-matched donor achieved initial complete chimerism, but later developed secondary graft failure upon the administration of ganciclovir for cytomegalovirus antigenemia. However, this patient achieved the spontaneous recovery of autologous marrow function and is currently surviving beyond 2,000 days.

Table 1 Patient characteristics

Variable	Younger than 60 years (n = 60)	60 years or older (n = 17)
Patient age (range, median)	25–59, 52	60–68, 63
Disease		
Acute myelogenous leukemia	16 (27%)	5 (29%)
Acute lymphoblastic leukemia	2 (3%)	0
Chronic myeloid leukemia	5 (8%)	0
Myelodysplastic syndrome or myeloproliferative disease	12 (20%)	8 (47%)
Malignant lymphoma	16 (27%)	3 (18%)
Adult T-cell leukemia/lymphoma	7 (12%)	0
Multiple myeloma	2 (3%)	1 (6%)
Risk status		
Standard	13 (22%)	1 (6%)
High	47 (78%)	16 (94%)
HLA disparity		
Matched	37 (62%)	10 (59%)
One-mismatched	19 (32%)	5 (29%)
Two or more mismatched	4 (7%)	2 (12%)
Donor–recipient sex match		
Male–male	20 (33%)	11 (65%)
Male–female	16 (27%)	2 (12%)
Female–male	9 (15%)	4 (24%)
Female–female	15 (25%)	0
GVHD prophylaxis		
Cyclosporine ± methotrexate	38 (63%)	10 (59%)
Tacrolimus ± methotrexate	21 (35%)	6 (35%)
Others	1 (2%)	1 (6%)
Median nucleated cell dose infused ($\times 10^8$/kg, range)	2.80 (0.39–5.52)[a]	2.92 (0.76–4.30)

HLA Human leukocyte antigen, *GVHD* graft-versus-host disease

[a] The data of two patients were excluded because infused nucleated cell dose was unknown

Chimerism was evaluated in 68 patients (88%), with short tandem repeats analysis (n = 52), variable number of tandem repeats analysis (n = 5) and FISH analysis in the case of sex mismatch (n = 11). Complete donor chimerism was confirmed in 58 (85%) within day 100. Mixed chimerism was confirmed in nine patients (13%), but two later reverted to recipient type. One patient failed to achieve donor-type chimerism due to disease relapse on day 20. The incidence of complete donor chimerism was similar in those younger and older than 60 years (85 and 86%), with a similar incidence of mixed chimerism (15 and 14%). No patients received donor lymphocyte infusion.

3.5 GVHD

Acute GVHD occurred in 41 of the 68 evaluable patients (60%), grades II–IV in 34 (50%) and grades III–IV in 14 patients (21%). Chronic GVHD occurred in 26 of the 42 evaluable patients (62%), with extensive type in 23 (55%). The incidence of grades II–IV acute GVHD was the same in patients younger and older than 60 years (50%). The incidence of grades III–IV acute GVHD (22 and 14%) and extensive chronic GVHD (56 and 50%) was similar. In unrelated BMT, from HLA-6 allele-matched (n = 40), HLA-1 allele-mismatched (n = 23), and HLA-2 or 3 allele-mismatched (n = 5) donors, grades II–IV acute GVHD occurred, respectively, in 18 (45%), 10 (43%) and 3 patients (60%), and chronic GVHD occurred in 15 (38%), 9 (39%) and 2 patients (40%). In univariate and multivariate analyses, an ATG-containing regimen was significantly associated with a decreased risk of the onset of grades II–IV acute GVHD (data not shown).

3.6 Survival

Thirty-three patients are currently alive with a median follow-up of 439 days (28–2,002 days), with an OS of 50% at 1 year and 46% at 2 years. The OS of patients younger than 60 years was 49% at 2 years (95% confidence interval [CI], 34–62%), and this could not be defined in older patients (95% CI, 15–45%). Patients younger than 60 years

Table 2 Conditioning regimens

Conditioning regimens	Younger than 60 years ($n = 60$)	60 years or older ($n = 17$)
TBI-containing		
Fludarabine 180 mg/m^2 (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, TBI 4 Gy	30 (50%)	6 (35%)
Fludarabine 125–180 mg/m^2, melphalan 80–140 mg/m^2, TBI 4 Gy	5 (8%)	3 (18%)
Fludarabine 180 mg/m^2 (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, TBI 2 Gy	2 (3%)	0 (0%)
Fludarabine 180 mg/m^2, TBI 4 Gy	0 (0%)	1 (6%)
ATG-containing		
Fludarabine 180 mg/m^2 (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, ATG	5 (8%)	4 (24%)
Fludarabine 180 mg/m^2, cyclophosphamide 60 mg/kg, ATG	1 (2%)	0 (0%)
Fludarabine 180 mg/m^2, ATG	1 (2%)	0 (0%)
TBI and ATG-containing		
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg, TBI 4 Gy, ATG	1 (2%)	1 (6%)
Non-TBI and non-ATG		
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg	6 (10%)	2 (12%)
Fludarabine 125–180 mg/m^2, melphalan 140 mg/m^2	5 (8%)	0 (0%)
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg, cyclophosphamide 60 mg/kg	2 (3%)	0 (0%)
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg, thiotepa 10 mg/kg	1 (2%)	0 (0%)
Fludarabine 180 mg/m^2, cyclophosphamide 60 mg/kg	1 (2%)	0 (0%)

TBI Total body irradiation, *ATG* antithymocyte globulin (ATG-Fresenius 10 mg/kg or thymoglobulin 5 mg/kg)

tended to show better survival than older patients ($P = 0.124$). The HLA disparity (match vs. mismatch), TBI vs. non-TBI, ATG vs. non-ATG-containing regimen, and disease category (AML vs. MDS or myeloproliferative disease vs. lymphoid malignancies) was not significantly associated with OS (data not shown). Patients with standard risk tended to show better survival than those with high risk ($P = 0.129$). In univariate and multivariate analyses, no variables were significantly associated with OS (data not shown).

3.7 NRM and PD

Thirty-six patients (47%) died of therapy-related complications, with a cumulative incidence of NRM at 1 year of 43% (95% CI, 31–56%). Of the patients who died of therapy-related complications, 23 (30%) died within day 100 of transplantation and 13 (17%) died thereafter. The NRM at 1 year in patients younger and older than 60 years was 38% (95% CI, 25–53%) and 61% (95% CI, 36–85%), respectively, as shown in Fig. 1. The causes of NRM were infection (23%), regimen-related toxicity (14%) and GVHD (9%). GVHD-related mortality was found in 26%. Infection was the major cause of death in patients younger than 60 years. Regimen-related toxicity, mainly pulmonary complications, was the major cause of treatment failure for patients older than 60 years. In univariate and multivariate analyses, no variables were significantly associated with
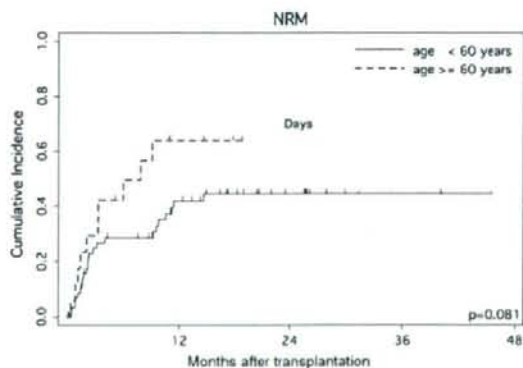

Fig. 1 Non-relapse mortality stratified according to patient age, younger or older than 60 years

NRM (data not shown). Relapse or progression of primary disease after unrelated BMT with RIC regimens was observed in 13 patients (17%; 10 patients younger than 60 years and 3 older than 60 years). There were no relapsed patients after transplantation in standard risk group. The incidence of death due to relapse or progression of primary disease was 14%. In univariate and multivariate analyses, no variables were significantly associated with PD although patients with grades II–IV acute GVHD showed a relatively lower incidence of PD (data not shown).

4 Discussion

This report reviews the current experience of unrelated BMT with RIC regimens in Japan, with particular focus on the risk factors for engraftment, GVHD, NRM, survival and PD. Although the engraftment rate has been reported to be lower when RIC unrelated transplantation was performed with bone marrow compared to peripheral blood cells [9, 10], we observed that sustained engraftment was achieved in 99% of evaluable patients, with complete donor chimerism confirmed in 85%. The incidence of graft failure was not different from that in RIC transplantation from related donors in Japan; 3.7% in recipients with an HLA-matched donor and 5.7% in those with a 1-locus-mismatched donor [21]. Complete donor chimerism in our study was comparable with that reported from the National Marrow Donor Program (85 vs. 84%) [22]. In our study, two-thirds of patients successfully received 2–4 Gy TBI-containing regimens, which were aimed at the enhancement of engraftment, as suggested in a previous report with patients with aplastic anemia [23], while 2 of the 12 patients who received an ATG-containing regimen had late graft failure, similar to a previous report which noted an incidence of 19% [5]. It has been reported that the Japanese population is more homogenous than others in terms of the distribution of HLA. Thus, it would be possible that the impact of minor HLA disparities on engraftment may become prominent after RIC transplantation.

Despite the observed satisfactory engraftment rate, we confirmed a high NRM rate (47%) after unrelated BMT with variable RIC regimens, due mostly to GVHD-related complications, including infections under steroid therapy, as previously designated by Wong et al. [10]. On the other hand, the incidence of death due to relapse or progression of primary disease was low (14%). Hence, successful prophylaxis and treatment of GVHD is particularly important in this procedure, and studies with ATG [5, 24] or alemtuzumab [25–27] have reported encouraging results. Although the number of patients was still small, in our study an ATG-containing regimen resulted in a decreased incidence of acute and chronic GVHD, despite the use of a lower dose (ATG-Fresenius 10 mg/kg or Thymoglobulin 5 mg/kg) than reported elsewhere. This study showed that age older than 60 years tended to be associated with a higher risk of NRM after unrelated HSCT with RIC regimens, though this relation was not statistically significant in a multivariate analysis. This finding, however, is limited by the small sample size. Additional use of ATG may reduce the incidence of GVHD-related NRM even in older patients but ATG should be carefully incorporated since about 20% of patients who received an ATG-containing regimen developed late graft failure in our study.

This study suggested that the onset of grades II–IV acute GVHD was associated with a lower incidence of PD, although this was not statistically significant in a multivariate analysis, possibly due to the small sample size. However, GVHD in turn resulted in a higher incidence of NRM, and a desirable graft-versus-leukemia or lymphoma effect would be offset, particularly in older patients [10, 28]. Hence, our observation echoes the warning that the intentional induction of GVHD should be avoided.

Compared to the long-term follow-up data after unrelated HSCT with RIC from the NMDP reported by Giralt et al. [22], our NRM at 1 year was worse (43 vs. 30%), but OS was likely to be better (50% at 1 year and 46% at 2 years vs. 44% at 1 year, 28% at 3 years and 23% at 5 years). In their report, disease stage, performance status, stem cell source, HLA matching, and timing of transplant were the most important prognostic factors for survival after RIC unrelated donor transplantation. This study suggested that high risk and HLA-mismatched patients were associated with worse OS, although this was not statistically significant in the multivariate analysis. Interpretation of these results, however, should be careful because of relatively short period of follow-up and the small sample size in our study. Although high risk patients was 82%, rate of relapse were unexpectedly low in our study. This might be due to earlier mortality, which precludes estimate of relapse rate. Alternately, more patients (60%) received more intense conditioning composed of 8 mg/kg of busulfan or 80–140 mg/m^2 of melphalan and 4 Gy TBI in our study.

In conclusion, we confirmed that unrelated BMT with RIC regimens can be a curative therapeutic option in a subset of patients with advanced hematologic malignancy, but at the expense of a high risk of severe complications and NRM. The incorporation of low-dose TBI may be advantageous for enhancing engraftment, and a suitable prophylaxis for GVHD still remains a primary target of clinical research. Based on the observed data, a prospective trial is currently underway to determine the value of a lower dose of ATG (ATG-Fresenius 5 mg/kg) to be added to the combination of fludarabine and busulfan.

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LETTER TO THE EDITOR

Small intestinal CMV disease detected by capsule endoscopy after allogeneic hematopoietic SCT

CMV disease is a serious complication after allogeneic hematopoietic SCT (Allo-HSCT) in addition to GVHD.[1] CMV disease can involve many organs and the gastro-intestinal tract is a common site.[2] There are several reports on small intestinal endoscopic findings of GVHD detected by capsule endoscopy,[3] [6] but only limited information has been published regarding endoscopic findings of CMV enteritis after Allo-HSCT using capsule endoscopy.[4] We report herein a case of CMV enteritis involving the small intestine after Allo-HSCT that was detected by capsule endoscopy.

A 58-year-old man with myelodysplastic syndrome underwent Allo-HSCT with HLA mismatched unrelated cord blood at the National Cancer Center Hospital in Tokyo, Japan. The conditioning regimen consisted of fludarabine ($125 \, mg/m^2$), melphalan ($80 \, mg/m^2$) and 4 Gy TBI. Tacrolimus was administered for GVHD prophylaxis.

The transplantation course was uneventful for 7 months, but the patient then started experiencing epigastric pain and watery diarrhea. Total colonoscopy revealed several erosions surrounding a single ulceration in the ascending colon (Figure 1a). Biopsy specimens obtained from the ulceration and erosions showed enlarged endothelial cells with nuclear inclusion bodies (Figure 2a) that were positive for CMV by immunohistochemical staining (Figure 2b). No definite histological feature to support GVHD was found. A simultaneous CMV antigenemia assay using the monoclonal antibody C7-HRP (Teijin, Tokyo, Japan) indicated two positive cells per 44 000 cells. A subsequent capsule endoscopy (PillCam SB, Given Imaging Inc., Israel) also revealed a single ulceration with satellite erosions in the jejunum, but we were unable to perform a biopsy because of the primary limitation of capsule endoscopy, that is, lack of any biopsy capability. The ulceration in the jejunum was very similar to the one found in the ascending colon (Figure 1b).

Antiviral therapy was started with ganciclovir (10 mg/kg/day) followed by foscarnet (60 mg/kg/day) for 6 weeks and the patient's symptoms resolved completely. A follow-up CMV antigenemia assay was negative and endoscopic findings by capsule endoscopy and total colonoscopy revealed healing scars without any active lesions.

Intestinal complications after Allo-HSCT predominantly affect the small intestine,[7] but macroscopic findings of small intestinal disorders following Allo-HSCT have not been fully investigated. Traditional small bowel examinations such as push enteroscopy are somewhat invasive in nature and most patients undergoing Allo-HSCT cannot tolerate such procedures due to the seriousness of their condition. Capsule endoscopy is now widely accepted for small intestinal investigation as being far less invasive. In the present case, this advanced technology enabled us to obtain clear endoscopic images of CMV enteritis in the small intestine after Allo-HSCT.

CMV antigenemia assay is one of the most widely used methods to detect CMV reactivation in a variety of clinical settings;[8] however, it is of little value in predicting and diagnosing gastrointestinal CMV disease.[9] The differential diagnosis of intestinal disorders following Allo-HSCT includes intestinal GVHD, thrombotic microangiopathy, treatment-related toxicities and *clostridium difficile*
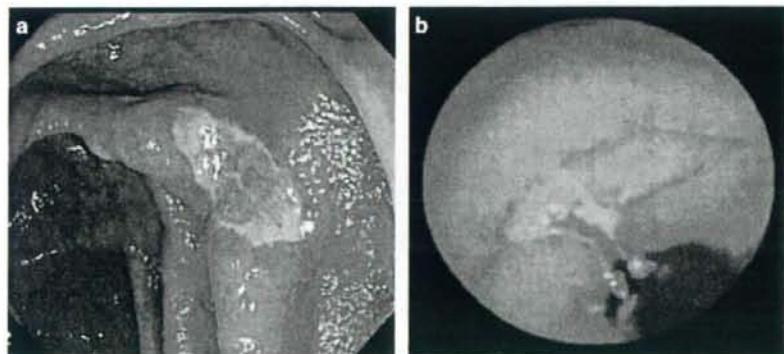

Figure 1 Total colonoscopy revealed a single ulceration (a) with several erosions in the ascending colon. Capsule endoscopy also revealed a single ulceration (b) with several erosions in the jejunum very similar to the one found in the ascending colon.
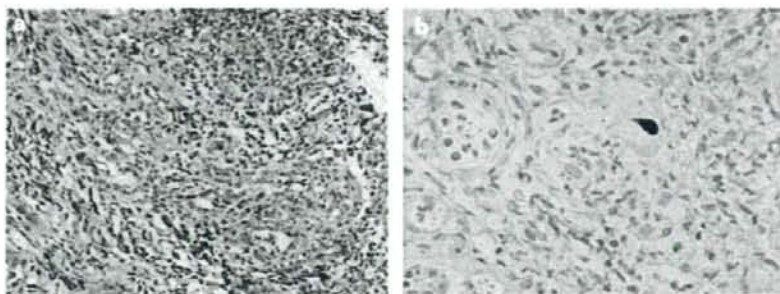
Figure 2 Biopsy specimens obtained from the ulceration and erosions showed cellular enlargement of endothelial cells with nuclear inclusion bodies (a) that were positive for CMV by immunohistochemical staining (b).

enterocolitis as well as CMV enterocolitis.[7] Actual diagnosis is usually based on pathological examinations of endoscopically obtained mucosal biopsy specimens, but this is not possible with capsule endoscopy due to its lack of biopsy capability.

In the present case, we were able to make a diagnosis of CMV enteritis in the small bowel without histological biopsy because of similar coincident findings in the ascending colon that were proven by pathological examination to be CMV colitis. This diagnosis was subsequently confirmed clinically by prompt resolution of intestinal symptoms, endoscopic findings and CMV antigenemia assay after antiviral treatment using ganciclovir and foscarnet.

Fortunately, CMV enteritis involved both the small intestine and colon in this case. CMV colitis, which was proven by biopsy, was a factor in diagnosing small intestinal CMV disease; however, CMV enteritis involving only the small intestine without colon involvement may not be rare after Allo-HSCT because the small intestine is a frequent and severe site for gastrointestinal complications following Allo-HSCT.[7] In such a situation, our picture (Figure 1a) showing ulceration detected by capsule endoscopy may be useful in diagnosing small intestinal CMV disease after Allo-HSCT.

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Preengraftment Serum C-Reactive Protein (CRP) Value May Predict Acute Graft-versus-Host Disease and Nonrelapse Mortality after Allogeneic Hematopoietic Stem Cell Transplantation

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ABSTRACT

In a mouse model, inflammatory cytokines play a primary role in the development of acute graft-versus-host disease (aGVHD). Here, we retrospectively evaluated whether the preengraftment C-reactive protein (CRP) value, which is used as a surrogate marker of inflammation, could predict posttransplant complications including GVHD. Two hundred twenty-four adult patients (median age, 47 years; range: 18-68 years) underwent conventional stem cell transplantation (CST, n = 105) or reduced-intensity stem cell transplantation (RIST, n = 119). Patients were categorized according to the maximum CRP value during neutropenia: the "low-CRP" group (CRP < 15 mg/dL, n = 157) and the "high-CRP" group (CRP ≥ 15 mg/dL, n = 67). The incidence of documented infections during neutropenia was higher in the high-CRP group (34% versus 17%, P = .004). When patients with proven infections were excluded, the CRP value was significantly lower after RIST than after CST (P = .017) or after related than after unrelated transplantation (P < .001). A multivariate analysis showed that male sex, unrelated donor, and HLA-mismatched donor were associated with high CRP values. The high-CRP group developed significantly more grade II-IV aGVHD (P = .01) and nonrelapse mortality (NRM) (P < .001), but less relapse (P = .02). The present findings suggest that the CRP value may reflect the net degree of tissue damage because of the conditioning regimen, infection, and allogeneic immune reactions, all of which lead to subsequent aGVHD and NRM.
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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is associated with high treatment-related mortality (TRM) because of acute graft-versus-host disease (aGVHD) and infections [1,2]. Inflammatory cytokines, for example, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 [3-11], are produced following conditioning and play a primary role in activating T cells, leading to GVHD and resultant target tissue destruction [12,13]. An acute-phase protein, C-reactive protein (CRP), is produced by hepatocytes downstream of IL-6 [14] and is widely used as a reliable

surrogate marker of infectious diseases [15-19]. This process is further stimulated by other cytokines including TNF-α [12,13]. After allogeneic HSCT, the elevation of CRP was observed with infectious complications, but not in uncomplicated aGVHD [8,20]. On the other hand, elevation of CRP has been shown to be associated with TRM [21-24]. Nevertheless, these previous studies adopted the sporadic measurement of CRP and mostly focused on patients undergoing conventional HSCT (CST) with a myeloablative regimen. It has been hypothesized that recently developed reduced-intensity HSCT (RIST) decreases regimen-related toxicities and, hence, may reduce inflammation

that augments the subsequent allogeneic immune reaction to induce GVHD and nonrelapse mortality (NRM).

In this study, the correlation between the preengraftment CRP value and subsequent clinical events was analyzed to test whether high CRP reflected the degree of tissue damage because of the conditioning regimen, infections, and allogeneic immune reactions and/or inflammation, all of which could contribute to subsequent aGVHD and NRM.

MATERIALS AND METHODS

Patient Characteristics

The data from a cohort of 224 consecutive adult patients with hematologic malignancies, who were treated between January 2002 and July 2006 at the National Cancer Center Hospital (NCCH, Tokyo, Japan), were reviewed retrospectively. Patients who developed graft failure or who had previous allogeneic transplantation were excluded. Their characteristics are listed in Table 1. The median age of the patients was 47 years (range: 18-68 years), and their diagnosis included acute myeloid leukemia (AML, $n = 94$), acute lymphoblastic leukemia (ALL, $n = 23$), non-Hodgkin lymphoma (NHL, $n = 62$), myelodysplastic syndrome (MDS, $n = 27$) and chronic myeloid leukemia (CML, $n = 12$). Standard risk included acute leukemia in first complete remission, chronic leukemia in the first chronic phase, MDS in refractory anemia, and NHL in complete remission, with the rest of the patients categorized as a high-risk group. Stem cell sources used for transplantation included bone marrow (BM, $n = 108$), peripheral blood stem cells (PBSC, $n = 98$) and cord blood cells (CB, $n = 18$). One-hundred five patients received a CST regimen including total-body irridiation (TBI)-based ($n = 50$) and non-TBI-based busulfan-containing regimens ($n = 55$), whereas 119 patients received a RIST regimen including fludarabine or cladribine plus busulfan or melphalan (Table 1). CMV serostatus was positive in 157 patients and negative in 67 patients. The median age of the patients was 49 years in the high-CRP group (range: 19-67) and 47 years in the low-CRP group (range: 18- 68). Written informed consent was obtained according to the Declaration of Helsinki.

Transplantation Procedures

GVHD prophylaxis included cyclosporine- ($n = 174$) and tacrolimus-based regimens ($n = 50$), with an additional short course of methotrexate (MTX) in 165 patients. Granulocyte colony-stimulating factor (G-CSF) was administered in all patients from day $+6$ of transplantation until engraftment was confirmed. Most patients received ciprofloxacin (200 mg orally 3 times daily) for bacterial prophylaxis until neutrophil engraftment. Fluconazole (100 mg once daily)

Table 1. *Patients' Characteristics*

Variable	N (%)/ Median		P Value
	Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	
Age (year)	47 (18-68)	49 (19-67)	.85
<40	53 (34)	26 (39)	
≥40	104 (66)	41 (61)	.47
Patient sex			
Male	84 (54)	48 (72)	
Female	73 (46)	19 (28)	.01
Donor sex			
Male	81 (52)	30 (45)	
Female	76 (48)	37 (55)	.35
CMV serostatus			
Positive	140 (89)	64 (96)	
Negative	17 (11)	3 (4)	.20
Disease risk			
Standard	35 (22)	17 (25)	
High	122 (78)	50 (75)	.62
Conditioning			
CST	72 (47)	33 (50)	
RIST	85 (53)	34 (50)	.64
GVHD prophylaxis			
Cyclosporin-based	122 (78)	52 (78)	
Tacrolimus-based	35 (22)	15 (22)	.99
Short term MTX (+)	107 (68)	58 (87)	.004
Relation to donor			
Related	94 (60)	13 (19)	
Unrelated	63 (40)	54 (81)	<.001
Stem cell source			
Bone marrow	63 (40)	45 (67)	
PBSC	87 (55)	11 (16)	
Cord blood	7 (5)	11 (16)	<.001

CRP indicates C-reactive protein; CMV, cytomegalovirus; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; GVHD, graft-versus-host disease; MTX, methotrexate; PBSC, peripheral blood stem cells; HLA, human leukocyte antigen.

was administered for fungal prophylaxis. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and varicella zoster virus until the cessation of immunosuppressive agents. Prophylaxis against *Pneumocystis jiroveci* infection was provided with trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day -3 of transplantation, and from day $+28$ until day $+180$ or the discontinuation of immunosuppressive agents. Patients with fever during the neutropenic period were treated with cefepime, and additional agents including vancomycin and aminoglycosides, and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the absolute neutrophil count exceeded $0.5 \times 10^9/L$. In our institute, the CRP level was serially measured as part of our routine checkup at least 3 times a week. Hence, all serially admitted patients were subjected to this analysis. Every patient had started CRP measurement

Table 2. *Comparison of Preengraftment CRP Value Stratified According to the Conditioning Regimen (CST versus RIST) and the Relation to Donor (Related versus Unrelated)*

Patients' Characteristics	CRP Value Median (Range)
All patients	8.9 (0.1-42.7)
CST	10.5 (0.3-31.3)*
Related	9.4 (0.6-30.0)†
Unrelated	10.6 (0.3-31.3)†
RIST	6.2 (0.1-42.7)*
Related	1.6 (0.1-9.7)‡
Unrelated	16.2 (0.5-42.7)‡

CST indicates conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation.

*$P = .017$.
†$P = .33$.
‡$P < .001$.

before the initiation of the conditioning regimen, and the median pretransplant CRP level was 0.3 mg/dL (range: 0.0-20.5 mg/dL). The median maximum CRP value during neutropenia was 8.9 mg/dL (0.1-42.7, Table 2).

The "maximum CRP level" was determined by measuring both the CRP level and the neutrophil count, as shown in the example in Figure 1A. The average number of levels assessed for each patient was 8 (range: 1-30). The median day of the maximum CRP level was day 10 of HSCT (range: 0-25), with 79% of patients developing this in later days (≥8 days). The patients were categorized according to the maximum CRP level after the threshold CRP level was determined following a preliminary analysis of the maximum CRP level after CST using an ROC curve analysis (data not shown). The "low-CRP" group (CRP <15 mg/dL) included 157 patients and the "high-CRP" group (CRP ≥15 mg/dL) included 67 patients.

Statistical Analyses

The primary endpoint of this study was the occurrence of grade II-IV and grade III-IV aGVHD, according to the Consensus Criteria [25]. The secondary endpoints were overall survival (OS) and nonrelapse mortality (NRM). Standard descriptive
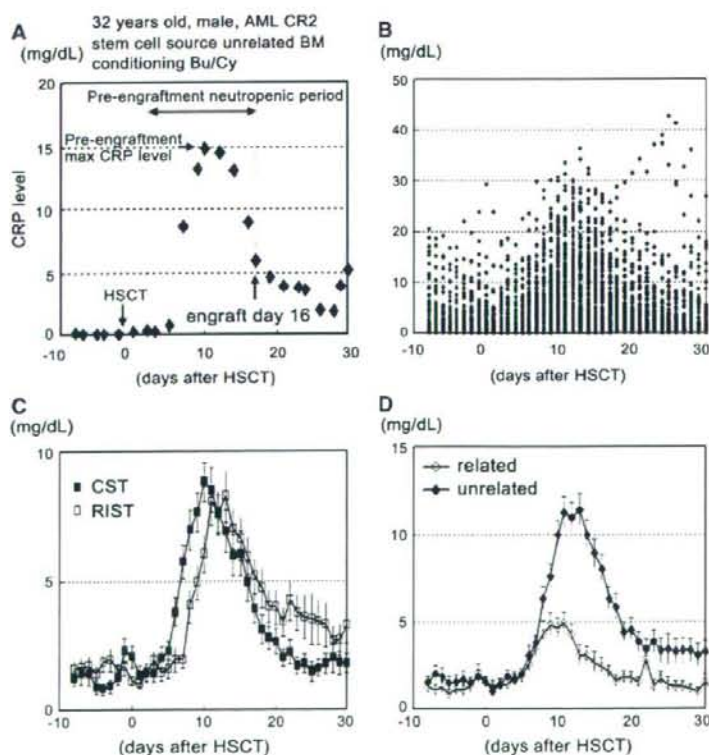

Figure 1. An example of how we measured CRP in a representative patient (A). Dot plot of the CRP level. All patients (B), CST versus RIST (C) and related versus unrelated (D).

statistics were used. Student *t*, chi-square, Fisher's ex-
act test, and Wilcoxon rank-sum tests were used to
compare clinical and patient characteristics. To ana-
lyze the pretransplant risk factors for a high CRP level,
logistic analysis was used. OS was estimated using Ka-
plan-Meier curves. The cumulative incidence of
aGVHD and NRM was estimated based on a Cox re-
gression model for cause-specific hazards by treating
progressive disease or relapse as a competing event.
Cox proportional hazard models were used for the
multivariate analysis of variables in aGVHD, NRM,
and OS after HSCT. Clinical factors that were as-
sessed for their association with aGVHD included
patient age, patient sex, donor sex, CMV serostatus,
conditioning regimen (CST versus RIST), donor
(human leukocyte antigen [HLA]-matched versus
HLA-mismatched, related versus unrelated), GVHD
prophylaxis (cyclosporine-based versus tacrolimus-
based, short-term MTX versus no MTX) and disease
risk (standard versus high risk). NRM and OS were
also assessed for their association with these factors.
Factors with $P < .10$ in the univariate analyses were
subjected to a multivariate analysis using a multiple lo-
gistic analysis and Cox proportional hazard modeling.
In Japan, only BM and CB are allowed for unrelated
transplantation, and most transplantations with a re-
lated donor use PBSC as a stem cell source. Therefore,
the stem cell source was not included as a factor in the
multivariate analysis. A level of $P < .05$ was defined as
statistically significant. All P values are 2-sided. All
analyses were made with SPSS ver 10.0 statistical soft-
ware (Chicago, IL). This analysis was approved by the
institutional review board.

RESULTS

Infections

The median duration of follow-up in surviving pa-
tients was 965 days (61 to 1432 days) in the high-CRP
group and 915 days (76 to 1803 days) in the low-CRP
group, and the incidence of total documented infec-
tions during neutropenia was, respectively, 23 cases
in the high-CRP group (34%) and 27 cases in the
low-CRP group (17%, $P = .004$). The incidence of
bacteremia was, respectively, 20 cases (30%) and 20
cases (13%, $P = .002$), and the incidence of pneumonia
was 7 cases (10%) and 4 cases (3%, $P = .01$). The in-
cidence of central venous catheter infection was, re-
spectively, 4 cases (6%) and 7 cases (4%, $P = .63$).

Serial changes in the CRP level are shown in
Figure 1B; in most cases, the CRP level was elevated
within 2 weeks of HSCT. Stratified data according
to conditioning regimen (CST versus RIST) or rela-
tion to donor (related versus unrelated) are shown in
Figure 1C and D, respectively.

To clarify the pretransplant risk factors for high
CRP values during neutropenia, we performed a logis-

tic regression analysis, which showed that male, unre-
lated donor, stem cell source with BM or CB
transplantation (versus PBSCT), HLA-mismatched
donor, and immunosuppression with MTX were asso-
ciated with high CRP values during neutropenia (Ta-
ble 1). Factors that showed significant associations ($P
< .1$) were subjected to a multiple logistic regression
analysis, and the results showed that unrelated donor,
HLA mismatch and male sex were associated with high
CRP ($P < .001$, $P = .005$, $P = .028$, respectively), as
shown in Table 3. The median CRP levels after CST
and RIST were 10.5 (0.3-31.3) and 6.2 (0.1-42.7), re-
spectively, with a significant difference ($P = .017$) (Ta-
ble 2). Notably, within the RIST group, the median
CRP level was significantly lower in related than in un-
related transplantation (1.6 mg/dL [0.1-9.7] veruss
16.2 mg/dL [0.5-42.7]: $P < .001$). However, the logis-
tic analysis failed to disclose any overall significant dif-
ference between CST and RIST.

Primary Outcomes

The cumulative incidences of aGVHD grade II-IV
and grade III-IV are shown, respectively, in Figure 2A
and B. Grade II-IV and grade III-IV aGVHD were
both more frequent in the high-CRP group than in
the low-CRP group ($P = .001$ and $P = .04$, respec-
tively). A Cox proportional hazard model showed
that a high CRP level and CMV serostatus were asso-
ciated with an increased risk of grade II-IV aGVHD
(Table 4). Similar results were obtained when we
included only the patients who received a myeloablative
conditioning regimen (grade II-IV aGVHD 25% in
the low-CRP group and 58% in the high-CRP group,
$P < .001$, grade III-IV aGVHD 7% in the low-CRP
group and 21% in the high-CRP group, $P = .047$).

Secondary Outcomes

OS and NRM are shown, respectively, in
Figure 3A and B. OS was significantly worse in the

Table 3. *Multiple Logistic Regression Analysis of Risk Factors for High
CRP during Neutropenia*
*Factors with $P < .10$ in a Multivariate Analysis Was Shown**

Outcomes and Variables	Multiple Logistic Regression Analysis		
	Odds	95% CI	P Value
Unrelated donor	4.6	2.2-9.6	<.001
HLA mismatch	2.6	1.3-5.0	.005
Patient sex (male)	2.1	1.1-4.2	.0028

CRP indicates C-reactive protein; CI, confidence interval; HLA,
human leukocyte antigen; CMV, cytomegalovirus.
*Factors included in univariate analysis: patient sex, donor sex, CMV
serostatus, use of short-term MTX, relation to donor, HLA mis-
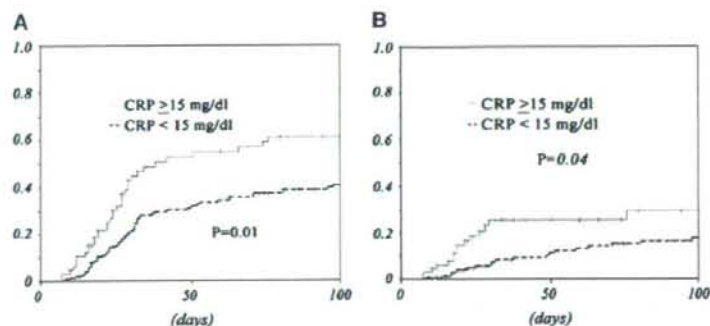match, conditioning, GVHD prophylaxis, stem cell source.

Figure 2. Cumulative incidence of grade II-IV aGVHD (A) and grade III-IV aGVHD (B) stratified according to the maximal CRP level during neutropenia.

high-CRP group than in the low-CRP group (1-year OS 47% versus 75%, P = .001). NRM was significantly higher in the high-CRP group than in the low-CRP group (1-year NRM 47% versus 13%, P < .001). Similar results were obtained when we included only patients who received a myeloablative conditioning regimen (1-year NRM 8% in the low-CRP group and 38% in the high-CRP group, P = .007). A Cox proportional hazard model showed that the risk factors for poor OS were high CRP (P = .002, hazard ratio [HR] 2.0, 95% confidence interval [CI] 1.3-3.1) and high-risk disease (P = .015, HR 2.2, 95% CI 1.2-4.0), whereas those for high NRM were high CRP (P < .001, HR 4.0, 95% CI 2.0-8.0) and high-risk disease (P = .029, HR 2.6, 95% CI 1.1-6.2), as shown in Table 4. When the threshold was set at 15 mg/dL, the sensitivity and specificity of the CRP level for prediction of grade II-IV aGVHD, NRM, or OS were 37% and 75%, 59% and 79%, and 40% and 78%, respectively. The relapse rate was significantly lower in the high-CRP group than in the low-CRP group (1-year relapse 21% versus 33%, P = .02).

Causes of death are summarized in Table 5. A total of 57 patients (36%) in the low-CRP group and 39 patients (58%) in the high-CRP group died (P = .002, OR 2.4 [1.4-4.4]). Six patients (4%) in the low- and 5 (7%) in the high-CRP group died because of aGVHD, for example, death because of infectious diseases associated with aGVHD and its treatment. Seven patients (4%) in the low- and 11 (16%) in the high-CRP group (P = .003, OR 4.2 [1.6-11.4]) died because of chronic GVHD (cGVHD), including death because of infectious diseases associated with cGVHD and its treatment. No patient (0%) in the low- and 5 (7%) in the high-CRP group (P = .002) died because of infectious diseases excluding infectious disease concomitant with GVHD. No patient in the low-CRP group and 4 (6%) in the high-CRP group (P = .008) died because of multiple-organ failure (MOF) excluding MOF because of GVHD and infectious disease.

DISCUSSION

The results of this retrospective study suggested that higher CRP values during the neutropenic period may reflect net inflammation secondary to tissue damage because of the conditioning regimen, infection, and subsequent allogeneic immune reactions, all of which lead to aGVHD/cGVHD and ultimate NRM. In a mouse model, the concept that the production of inflammatory cytokines plays an important role in the development of aGVHD, by affecting the afferent and effector phase [12,13], has been accepted. Cooke et al. [26] showed that LPS antagonism reduced aGVHD in a mouse model, as indicated by Ferrara et al. [4]. However, in human studies, the value of determining individual levels of cytokines to monitor aGVHD has not been fully explored, because this approach is very costly and requires sophisticated techniques, which impedes its universal applicability. On the other hand, CRP is already being widely used

Table 4. *Multiple Variate Analysis for aGVHD, NRM, and OS**

Outcomes and Variables	Hazard Ratio	95% CI	P value
Grade II-IV aGVHD			
High CRP	1.7	1.1-2.6	.02
CMV positivity	3.1	1.0-9.8	.5
Disease risk (high)	1.6	0.9-2.7	.10
NRM			
High CRP	4.0	2.0-8.0	<.001
Age (≥40 years old)	1.9	0.9-3.9	.07
Disease risk (high)	2.6	1.1-6.2	.03
OS			
High CRP	2.0	1.3-3.1	.002
Disease risk (high)	2.2	1.2-4.0	.02

CRP indicates C-reactive protein; CI, confidence interval; CMV, cytomegalovirus; GVHD, graft-versus -host disease; TBI, total body irradiation; NRM, nonrelapse mortality; OS, overall
*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source