Characterization of acute graft-versus-host disease following reduced-intensity stem-cell transplantation from an HLA-identical related donor

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To investigate clinical features of acute graft-versus-host disease (GVHD) following reduced intensity stem-cell transplantation (RIST), we retrospectively investigated medical records of 65 patients with hematologic malignancies who underwent RIST from a matched related donor. Preparative regimen comprised fludarabine 30 mg/m^2 (n = 53) or cladribine 0.11 mg/kg (n = 12) for 6 days plus busulfan 4 mg/kg for 2 days. Twelve patients received rabbit antithymocyte globulin 2.5 mg/kg/day for 2–4 consecutive days. Grade II to IV acute GVHD was diagnosed in 36 patients (55%). Its median onset was day 58 (range, 17–109), while it was bimodal, peaking day 15–29 (early-onset GVHD, n = 18) and day 75–89 days (late-onset GVHD, n = 18). Variables that were more common in early-onset GVHD than late-onset GVHD included skin rash (89% vs. 61%) and noninfectious fevers (33% vs. 11%). Desaturation, pulmonary infiltrates and hyperbilirubinemia (>2.0 mg/dL) were more common in late-onset GVHD (6% vs. 22%, 0% vs. 17%, and 6% vs. 33%, respectively). All of the patients with early-onset GVHD given corticosteroid responded to it, while 5 of the 18 patients with late-onset GVHD failed to respond it. Patients with either early-onset or late-onset GVHD tended to have better progression-free survival (PFS) than those without it; however, there was no significant difference in PFS between patients with early-onset GVHD and those with late-onset GVHD. This study suggests that several etiologies might have contributed to the development of acute GVHD following RIST. Am. J. Hematol. 83:630–634, 2008. © 2008 Wiley-Liss, Inc.

Introduction

Reduced-intensity stem cell transplantation (RIST) is a promising treatment for patients with hematologic malignancies who would not be candidates for myeloablative allogeneic stem cell transplantation (allo-SCT) because of their advanced age or comorbidity [1–3]. Acute GVHD is a significant cause of morbidity and mortality in RIST as well as myeloablative allo-SCT [4–6]; however, the characteristics of acute GVHD following RIST have not been fully investigated. Acute GVHD following RIST is likely to differ from that after myeloablative allo-SCT, since the immunological backgrounds are different between them. First, RIST has minimized the effects of conditioning regimens on tissue damage, thereby reducing inflammatory cytokine release [7,8]. Second, the intensity and duration of immunosuppressants used after RIST are different from those used after conventional allo-SCT [2,9–11]. Third, more antigen-presenting cells survive the RIST conditioning than do myeloablative conditioning, which may activate more donor T-cells to initiate graft-versus-host responses [12,13]. Last, the transient mixed chimerism following RIST may induce mutual tolerance, which may attenuate both GVHD and host-versus-graft (HVG) reactions [14,15]. Given few reports on acute GVHD after RIST [4,5,16–18], its overall characteristics need to be further investigated.

We retrospectively analyzed 65 patients with hematologic malignancies who underwent RIST from a matched sibling to investigate clinical features of acute GVHD after RIST.

Results

Engraftment

All of the 65 patients achieved engraftment with a median of day 11 (range, 5–16). No patients died of TRM within 28 days of RIST, while two patients died within 100 days of RIST due to acute GVHD and hemophagocytic syndrome. Within 100 days of transplant, underlying diseases progressed in two patients.

Clinical features of acute GVHD

Thirty-six patients developed Grade II–IV acute GVHD. Its median onset was day 58 (range, 17–109), while it was bimodal, peaking day 15–29 and day 75–89 days after transplantation (see Fig. 1). The number of patients who developed Grade II–IV acute GVHD before day 60 (early-onset GVHD) and those who developed it after day 61 (late-onset GVHD) were 18 each. The cumulative incidence of Grade II–IV acute GVHD was 26% at day 60 and 48% at day 120.

Clinical features of early-onset and late-onset GVHD were shown in Table I. Ten of 18 patients with early-onset acute GVHD had lymphoma. This contrasted with the finding that three of 18 patients with late-onset GVHD and two of 29 patients without acute GVHD had lymphoma. Variables which were more common in early-onset GVHD than late-onset GVHD included skin rash (89% vs. 61%), and

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Conflict of interest: Nothing to report.

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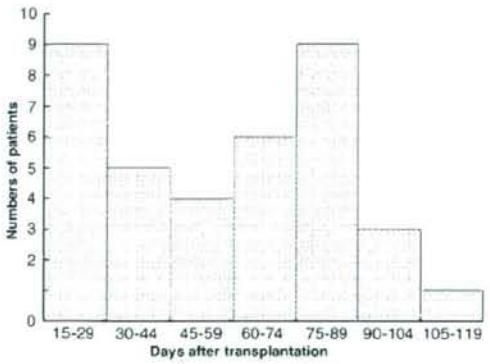

Figure 1. Onset of acute GVHD following RIST. Thirty-six patients developed Grade II–IV acute GVHD. Its median onset was day 58 (range, 17–109), while it was bimodal, peaking days 15–29 and days 75–89 after transplantation.

noninfectious fevers (33% vs. 11%), while desaturation, pulmonary infiltrates and hyperbilirubinemia (>2.0 mg/dL) were more common in late-onset GVHD than in early-onset GVHD (6% vs. 22%, 0% vs. 17%, and 6% vs. 33%, respectively). All of the patients who developed early GVHD had already achieved complete donor-type chimerism at day 60, whereas 42% of the patients who developed late Grade II–IV acute GVHD had achieved complete donor-type chimerism at day 60 ($P = 0.011$). Other variables, including use of ATG or methotrexate, were not significantly associated with the type of acute GVHD (Table I). Chimerism changes before and after GVHD development were shown in Table I. Four patients remained in mixed chimerism after development of GVHD.

Fifteen of the 18 patients with early-onset GVHD and all of the 23 patients with late-onset GVHD were given corticosteroid. Responses to corticosteroid were shown in Table I. All of the patients with early-onset GVHD given corticosteroid responded to it, while 5 of the 18 patients with late-onset GVHD failed to respond it.

Chronic GVHD

Of the evaluable 58 patients who survived without relapse longer than 100 days after RIST, 35 patients developed chronic GVHD. Clinical features of chronic GVHD were shown according to the types of acute GVHD (Table I). Twelve of 16 evaluable patients with early-onset acute GVHD and 14 of 17 evaluable patients with late-onset acute GVHD developed chronic GVHD in their clinical courses, while nine of 25 patients without prior history of acute GVHD developed chronic GVHD.

PFS and its prognostic factors

PFS was shown according to types of acute GVHD (see Fig. 2). Patients with either early-onset or late-onset GVHD tended to have better PFS than those without it; however, the differences were not statistically significant.

Results of univariate and multivariate analysis on prognostic factors of PFS were shown in Table II. Risk of underlying diseases and dose of transfused CD34-positive cells (X10E6/kg) were significant prognostic factors (relative risk (RR); 4.26, 95% confidence interval (CI); 1.32–14.1, *P*-value; 0.010, and RR; 0.64, 95% CI; 0.36–0.95, *P*-value 0.030, respectively). History of early-onset or late-onset GVHD was not a significant prognostic factor.

Discussion

The present study showed that median onset of acute GVHD following RIST was day 58 (range, 17–109), while it was bimodal, peaking day 15–029 and day 75–89 days after transplantation (see Fig. 1). These findings suggest that several etiologies might have contributed to the development of acute GVHD following RIST. In myeloablative allo-SCT, acute GVHD develops almost exclusively before day 60; however, the cumulative incidence of Grade II–IV acute GVHD was 26.2% at day 60 and 48% at day 120 in this study. We should recognize that ~30% of the patients who survive longer than 60 days without acute GVHD develop it after day 60.

The present study showed that RIST recipients frequently develop early GVHD, which is characterized by fever and rash. Steroid responsiveness was good; no patient with early GVHD developed late GVHD. Several researchers reported that engraftment syndrome, which is characterized by noninfectious fever, rash, and transient liver dysfunction, is commonly seen in RIST recipients [19,20]. The clinical features of early GVHD shown in this study are similar to those of engraftment syndrome. However, the median interval from engraftment to the development of early GVHD was 10 days, and early GVHD cannot be explained solely by immune reaction accompanied with engraftment. Little is known about immune reactions early after RIST. The Seattle group has reported that patients who developed GVHD within 50 days of nonmyeloablative transplantation were at high risk of TRM [21]. Although they did not describe different clinical features by the onset of GVHD, their results contrast with ours. The disparity may be associated with the distinct types of transplantation and different durations until donor-type chimerism achievement. All patients treated with our fludarabine or cladribine/busulfan-based regimen developed severe neutropenia [22]. The kinetics of engraftment and immune reactions early after transplantation might be different between RIST and nonmyeloablative transplantation. Further investigation is required to investigate the clinical features of early acute GVHD following RIST.

The clinical courses of late GVHD were different from those of early GVHD. Late GVHD often involved the gastrointestinal tract and the liver. The responsiveness to corticosteroid was poor compared with early GVHD. Three patients died of acute/chronic GVHD and five of noninfectious respiratory complications. Although the onset of late GVHD after RIST was late compared with GVHD after CST, its clinical courses were comparable with those of GVHD after CST. Pathogenesis of late-onset acute GVHD remains to be unknown, and further investigation is required. Since GVHD has high morbidity and mortality, its prophylaxis is crucial to improve the outcomes, and intense immunosuppressive therapy might be required for the treatment of late-onset acute GVHD compared with early-onset one.

Whether complete donor T-cell chimerism is needed before alloimmunity such as GVHD or the graft-versus-tumor (GVT) effect can occur is a major matter of controversy. While Childs et al., found that complete donor T-cell chimerism preceded the occurrence of GVHD and disease responses in RIST for metastatic renal cell carcinoma [23], other researchers demonstrated that both GVHD and GVL effects can occur in patients with mixed chimerism [24,25]. Since we have demonstrated that some patients remained mixed chimera even after GVHD development, complete chimerism is not essential in the development of GVHD following RIST. However, the majority of patients achieved complete chimerism after developing GVHD. The present study showed that underlying diseases, especially malignant lymphoma is associated with early-onset acute GVHD. Patients with malignant lymphoma might have received

TABLE I. Clinical Features of Early-Onset and Late-Onset Grade II–IV Acute GVHD

Types of acute GVHD			Early-onset (Day 15–60)	Late-onset (Day 61–)	No GVHD
Number of patients			18	18	29
Patients characteristics	Age	Year, median (range)	51.5 (30–61)	53.5 (37–64)	54 (25–67)
	Primary diseases	Acute leukemia	1	6	10
		Myelodysplastic syndrome	5	5	8
		Chronic myeloid leukemia	1	4	9
		Malignant lymphoma	10	3	2
		Others	1	0	0
	Sex mismacthes between recipient-donor pairs	Match	6	12	8
		Mismatch (donor: male/female)	7/5	2/4	6/15
	CD34-positive cells infused (10E6/kg)		3.4 (2.1–4.8)	2.6 (1.4–6.6)	3.3 (1.5–5.7)
	CD3-positive cells infused (10E7/kg)		3.0 (1.1–5.5)	3.7 (1.7–7.2)	3.0 (1.2–8.6)
	Use of methotrexate		1	4	5
	Use of antithymocyte globulin		3	2	8
	Engraftment	Day (range)	11 (6–12)	11 (5–14)	12 (7–16)
	Chimerism (complete/mixed)	Day 30	9/4	5/13	11/11
		Day 60	13/0	7/10	17/8
		Day 90	12/0	15/2	18/4
		Day 120	8/0	13/2	15/9
Acute GVHD	Grade	0–I/II/III–IV	0/11/6/1	2/11/7/0	NA
	Onset	Day (range)	29.5 (17–59)	82.5 (63–109)	NA
	Distribution of organ	Skin/liver/gut	16/1/10	11/6/8	NA
	Allogenic immune reaction	Noninfectionus fever	6	2	NA
		>5% weight gain	3	3	NA
		Desaturation[a]	1	4	NA
		Pulmonary infiltration	0	3	NA
	Changes of chimerism	NA to complete	4	0	NA
		NA to mixed	2	0	NA
		Mixed to complete	2	8	NA
		Mixed to mixed	0	2	NA
		Complete to complete	5	7	NA
	Primary therapy	Corticosteroid/supportive	15/3	18/0	NA
	Responses	CR/PR/NC/PD	11/4/0/0	9/4/4/1	NA
Chronic GVHD	Number of evaluable patients		16	17	25
	Number of patients with chronic GVHD		12	14	9
	Types of chronic GVHD	Progressive/quiescent/de novo	3/9/0	7/7/0	0/0/9
Causes of death		Total	4	5	9
		Disease progression	0	1	5
		Acute GVHD	1	1	0
		Chronic GVHD	1[b]	3[c]	0
		Others	2[d]	0	4[e]

NA, not applicable; TRM, transplant-related mortality; GVHD, graft-versus-host disease; CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

[a] Oxygen saturation <92%.

[b] The patient died of chronic GVHD complicated with invasive aspergillosis.

[c] These patients died of respiratory failure due to bronchiolitis obliterans ($n = 2$), and pneumonica caused by *P. aeruginosa* ($n = 1$).

[d] These included secondary cancer and interstitial pneumonitis.

[e] These included posttranplant lymphoproliferative disorder, liver failure, hemophagocytic syndrome, and multiple organ failure.

multiple courses of chemotherapy prior to RIST, and might have achieved complete chimerism earlier than patients with other types of hematologic malignancies. The present study showed a strong association between achievement of complete chimerism and the time of GVHD development. The observation is consistent with the previous report [25]. Patients with early GVHD achieved complete chimerism early, while complete chimerism achievement was late in those with late GVHD and those without GVHD. There are two possible explanations. First, early GVHD itself possibly attacks donor hematopoietic cells to lead to complete chimerism. Canine and murine models have shown that a state of mutual graft/host tolerance and absence of GVHD can be created by the introduction of stable mixed chimerism [26,27], while it is still an open question whether mixed chimerism per se protects the recipients against GVHD. Further investigations on the association between chimerism and GVHD are awaited.

Whether the GVT effect of early GVHD and that of late GVHD are different is a clinically important question. In the present study, PFS of the patients with either type of GVHD was longer than that of patients without GVHD (Table II). PFS after early-onset GVHD and that after late-onset GVHD were comparable. These findings suggest the possibility that the both types of GVHD have GVT effects and that the intensity of GVL effects is comparable. Whether the mechanisms of GVL effects by the two types of GVHD needs further investigations.

Although the present study provided novel information on acute GVHD after RIST, there are some problems to be discussed. First, it is a small-sized, retrospective study; unrecognized biases might have affected the results. Especially, enrolled patients had heterogeneous backgrounds with respect to the conditioning regimen and GVHD-prophylaxis. Greater proportion of patients with late-onset GVHD had received methotrexate as part of their prophylaxis. We

have attempted to address this heterogeneity in their multivariate analysis; however, given the overall small sample size, the power of respective adjustments remains questionable. Large-scale prospective evaluations are required to clarify the clinical features of acute GVHD following RIST. Second, little information is available concerning chronic GVHD [28], while the present study showed that either early-or late-onset acute GVHD is highly predictable for chronic GVHD. Manifestations typical of acute GVHD occasionally develop after day 100 in RIST recipients, and it is questionable whether current day 100 cut-off criteria is appropriate for separation of acute from chronic GVHD following RIST [29]. Patients with late-onset acute GVHD might show concurrent manifestation of chronic GVHD, while we could not collect enough information on this issue in this retrospective study, requiring further investigation. Third, optimal strategy should be established in the management of acute GVHD following RIST. It might be individualized according to the types of acute GVHD.

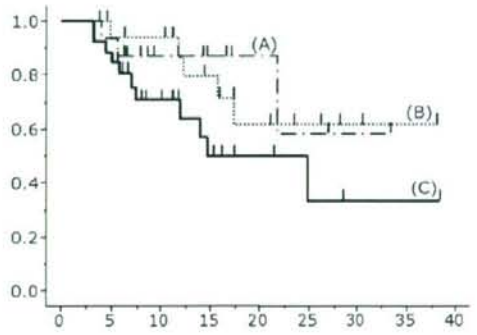

Figure 2. Progression-free survival according to the types of acute GVHD. (A) Patients with early-onset GVHD (n = 17). (B) Patients with late-onset GVHD (n = 18). (C) Patients without acute GVHD (n = 24). Patients who were surviving without relapse at day 100 after RIST were included in the analysis of prognostic factors of PFS. Patients with either early-onset or late-onset GVHD tended to have better PFS than those without GVHD; however, the differences were not statistically significant on either univariate or multivariate analyses.

Patients and Methods

Data collection and transplantation procedures

We reviewed the medical records of 65 consecutive patients with a median age of 53 years (range, 25–67) who underwent RIST from an HLA-identical sibling donor for the treatment of hematologic malignancies between January 2001 and October 2003 at the National Cancer Center Hospital. Patients with benign diseases were excluded since their management of acute GVHD is different from that in those with malignancies. Those who had undergone RIST twice were evaluated only for the first RIST. All the patients and donors gave their written informed consent in accordance with the requirements of the Institutional Review Boards of the National Cancer Center Hospital.

Transplantation procedures

All the patients received purine analogue-based preparative regimens. Fludarabine 30 mg/m^2 on days −8 to −3 (n = 53) or cladribine 0.11 mg/kg on days −8 to −3 (n = 12) plus busulfan 4 mg/kg on days −6 and −5 were administered [30]. Twelve patients received rabbit antithymocyte globulin (ATG, Thymoglobulin; IMTIX-SANGSTAT, Lyon, France) 2.5 mg/kg/day for 2–4 consecutive days. Cyclosporine 3 mg/kg continuous infusion was initiated for GVHD prophylaxis from day 1, and this was switched to oral administration when patients tolerated oral intake. The doses were adjusted with its whole blood levels [31]. Nine patients received additional short-term methotrexate (10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6). All the patients received granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood stem cells from an HLA-identical related donor. Supportive cares and management of infectious complications were reported previously [30,32]. Chimerism was assessed using polymerase chain reaction for variable numbers of tandem repeats with donor cells detected at a sensitivity of 10% [33]. CD3-positive cell chimerism was assessed at days 30, 60, 90, and 120.

Definition

Engraftment was defined as absolute neutrophil counts >0.5 × 10^9/L for two consecutive days. Diagnosis of acute GVHD was made by clinical judgment together with biopsy of the skin and gastrointestinal tract. Acute GVHD were graded according to the consensus criteria [34,35]. Grade II–IV acute GVHD was treated with 0.5–2.0 mg/kg/day of methylprednisolone in addition to cyclosporine. Response to corticosteroid

TABLE II. Univariate and Multivariate Analysis on Progression-Free Survival

Factors		Relative risk	95% Confidence interval	P-value
Univariate analysis				
Age		1.03	0.97–1.11	0.34
Sex	Female vs. male	0.81	0.30–2.17	0.68
Risk of primary diseases	High vs. low[a]	2.45	0.87–6.90	0.09
Use of antithymocyte globulin	Yes vs. no	2.1	0.81–5.45	0.13
Use of methotrexate	Yes vs. no	1.56	0.36–6.83	0.55
Dose of CD34-positive cells	per 1.0 × 10 E 6/kg	0.65	0.43–0.99	0.045[b]
Grade II–IV acute GVHD	None	1		0.34
	Early-onset	0.52	0.18–1.80	
	Late-onset	0.56	0.11–1.96	
Multivariate analysis				
Risk of primary diseases	High vs. low[a]	4.26	1.32–14.1	0.010[b]
Use of antithymocyte globulin	Yes vs. no	1.13	0.39–3.26	0.82
Dose of CD34-positive cells	per 1.0 × 10 E 6/kg	0.64	0.36–0.95	0.030[b]
Grade I–IV acute GVHD	None	1		0.12
	Early onset	0.43	0.22–2.0	
	Late onset	0.31	0.11–3.1	

GVHD, graft-versus-host disease.

[a] Low-risk patients were defined as those with acute leukemia in first or second remission, chronic myelogenous leukemia in chronic phase, and myelodysplastic syndrome refractory anemia. The others were classified into the high-risk group.

[b] Statistically significant.

was evaluated according to the report by Martin et al. [36]. Complete chimerism was defined as 90% or more donor-type. Transplant-related mortality (TRM) was defined as all causes of deaths without disease progression at any time after transplant.

Objectives and statistical analysis

The objective of this study was to investigate clinical features of acute GVHD following RIST. We investigated the onset and maximal grades of acute GVHD, maximal stages of skin, liver and gut, responses to corticosteroid, and progression-free survival (PFS) after RIST. Cumulative incidence of acute GVHD was calculated using Gray's method [37], treating death and/or disease progression without it as a competing risk. PFS was determined using the Kaplan-Meier method. Final follow-up was conducted in October 2003, with a median follow-up of surviving patients being 14.6 months (range, 3.8–46.8). Surviving patients were censored on the last day of follow-up.

A univariate analysis using the chi-square test and Mann-Whitney test were performed to compare clinical features of acute GVHD. A multivariate Cox proportional hazards model was used to identify independent and significant prognostic factors for PFS. The analyzed variables included age, sex, status of primary diseases at transplantation, doses of transfused CD34-positive cells, use of ATG and methotrexate as GVHD prophylaxis, and types of GVHD. Occurrence of the acute GVHD was included into the models as a time-dependent covariate. A significance level of 5% was set as the limit for inclusion in the model. Prognostic factors that were significant at $P < 0.05$ in the stepwise proportional model analysis were considered to be important in influencing PFS.

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ORIGINAL ARTICLE

Functional analysis of cytomegalovirus-specific T lymphocytes compared to tetramer assay in patients undergoing hematopoietic stem cell transplantation

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In order to evaluate whether we could predict reactivation of CMV by monitoring the number of CMV-specific cytotoxic T-lymphocytes (CTL), tetramer analysis was performed in 37 patients who underwent hematopoietic stem cell transplantation (HSCT). The results disclosed that the mean number of CMV-specific CTL at day 30 did not differ among patients who developed CMV antigenemia (22/μl) and those who did not (12/μl). Serial tetramer analysis showed that 21% of the patients had >10/μl CMV-specific CTL at the first detection of CMV antigenemia and 67% of the patients had more than 10/μl CMV-specific CTL at the onset of CMV disease. Intracellular staining upon stimulation by CMV lysates and peptide in patients with CMV colitis revealed that both IFN-γ producing CD4+ and CD8+ lymphocytes were suppressed at the onset of CMV colitis (1.6 and 8/μl), which increased with recovery of the disease (19 and 47/μl). These data suggest that it is difficult to predict CMV reactivation solely by the number of CMV-specific CTL. We suggest that additional functional analysis by intracellular cytokine assay may be useful for immunomonitoring against CMV.

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Introduction

Reactivation of CMV is one of the major complications in patients undergoing hematopoietic stem cell transplantation (HSCT) and is significantly related to morbidity and mortality despite the recent development of potent antiviral medications.[1,2] The decision to administer antiviral therapy is currently based on the clinical risk and the detection of viremia by various methods including PCR for CMV-derived DNA or CMV antigenemia assay. However, treatment with antiviral drugs such as ganciclovir and foscarnet increases the risk for secondary graft failure and other infectious complications due to myelotoxicity. To optimize the therapy with minimum drug exposure, it is important to monitor the recovery of CMV-specific immunity accurately. For this purpose, tetramer-based monitoring of CMV-specific cytotoxic T-cells (CTL) has been widely performed in patients with an HLA-A02 or HLA-B07 serotype.[3–11] Some of the results have demonstrated that the reconstitution of CMV-specific CTL as evaluated by quantitative tetramer to levels >10–20/μl is adequate for protection against CMV infection.[5–7] However, some patients with CMV-specific CTL above this level still experience CMV reactivation.[9] It has also been reported that the cellular response to CMV in immunosuppressed patients reflects functional impairment,[10] and CMV reactivation following HSCT has been shown to be associated with the presence of dysfunctional CMV-specific T-cells.[11] Therefore, by itself, the quantification of CMV-specific CTL seems to be insufficient and a simultaneous qualitative analysis of CMV-specific lymphocytes is needed. Furthermore, it is essential that we should develop a universal monitoring method, which is not limited to HLA to cover larger populations, since an epitope that is potent enough for immunomonitoring is not obtained in some HLA types such as HLA-A24.[12] In this study, simultaneous functional analysis of CMV-specific lymphocytes by intracellular cytokine assay upon stimulation with CMV lysate and antigen peptide were performed with tetramer-based CTL quantification in patients who underwent HSCT to identify an optimal monitoring system.

Materials and methods

Study patients

CMV seropositive patients with an HLA-A*0201 or HLA-A*0206 genotype who had undergone allogeneic non-T-cell

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depleted-HSCT between February 2002 and May 2005 were included in this study. Patients were eligible with the availability for 160 days of follow-up. The study was approved by the Ethics Committee and a written informed consent was given by all patients. Peripheral blood samples were obtained at days 30 ± 7 and 60 ± 7 after transplantation. When patients agreed to additional sampling, additional samples were obtained every 2–3 weeks. The median age of studied patients was 52 (21–68). The genotype for HLA-A02 in 37 eligible patients was HLA-A*0201 in 20 patients, HLA-A*0206 in 16 patients and both the HLA-A*0201 and HLA-A*0206 genotypes in one patient. Nine patients received BMT from an unrelated donor, two received BMT from a related donor and the remaining 26 received peripheral blood HSCT from a related donor. With regard to the conditioning regimen, 11 patients received a conventional regimen that included 120 mg/kg CY plus 16 mg/kg BU or 120 mg/kg CY plus 12 Gy of TBI, whereas 26 received a reduced-intensity regimen with 0.66 mg/kg cladribine (2-chlorodeoxyadenosine) plus 8 mg/kg BU or 180 mg/m^2 fludarabine plus 8 mg/kg BU. For patients who received a graft from an unrelated donor or DNA-mismatched donor, 4 Gy of TBI or 5 mg/kg of rabbit antithymocyte globulin (ATG) were added to reduced-intensity conditioning.

Diagnostic tests for CMV infection and CMV disease

CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. All patients and 31 donors (84%) were seropositive for CMV. CMV antigenemia was monitored weekly after engraftment to day 60, and at longer intervals thereafter, by using the immunocytochemical detection of pp65 antigen in leukocytes. Test results were considered to be positive when more than one cell per 50 000 leukocytes was positively stained. CMV disease was diagnosed clinically, with confirmation by biopsy of the involved organ. Pre-emptive antiviral therapy was given with an antigenemia of more than 10 positive cells per 50 000 leukocytes, which we defined as high antigenemia. The initial therapy was ganciclovir 5 mg/kg once per day, which was adjusted according to the follow-up CMV antigenemia value.

Peptide and CMV antigen

A >80% pure HLA-A02-binding peptide NLVPMVATV (AA 495–503, referred to as NLV peptide) from the CMV pp65 phosphoprotein was obtained using high-performance liquid chromatography (Qiagen, Tokyo, Japan).

Tetramer staining

Tetramer staining was performed as recently described.[13] Briefly, 5 µl CD8-FITC, CD4-PC5, CD19-PC5, CD13-PC5 and 2 µl PE-conjugated tetrameric HLA-A*0201 NLV peptide complex (CMV-tetramer), purchased from Beckman Coulter Inc. (Fullerton, CA, USA), were added to 100 µl heparinized blood and incubated for 30 min. After RBC were lysed and washed twice, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). More than 20 000 cells in the lymphocyte gate were acquired and analyzed using Cellquest software. The CD4−, CD19−, CD13− and CD8+ CMV-tetramer-positive fraction of the lymphocyte gate was defined as CMV-specific CTL.

Intracellular cytokine assay

Intracellular cytokine staining was performed as recently described[14] with the following modifications. Peripheral whole blood (1 ml) was stimulated for 6 h at 37 °C with 10 µg/ml NLV peptide or 1 µg/ml CMV lysate (Advanced Biotechnologies, Colombia, MD, USA), in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson, 1 µg/ml each). Breferdin A (Sigma, St Louis, MO, USA; 10 µg/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 µg/ml staphylococcal enterotoxin B or phosphate-buffered saline. Samples were lysed, permeabilized and stained with 2.5 µl CD69-FITC, 20 µl IFN-γ-PE, 0.6 µl CD3-APC and 10 µl CD8− or CD4− PerCP. More than 10 000 cells in the lymphocyte gate were acquired and analyzed using an FACS Calibur. The cells were gated on the CD3+ fraction of the lymphocyte gate and the proportion of IFN-γ and CD8 or CD4 was analyzed. CD69 was used as a marker for activated T-cells.

Statistical analysis

The difference between groups was compared with the Wilcoxon–Mann–Whitney *U*-test and the probabilities of $P<0.05$ were defined as statistically significant.

Results

Tetramer staining

CMV antigenemia was observed in 27 patients (73%) between day 23 and day 56 (median, day 34) after transplantation; 13 (35%) of them had a peak antigenemia level of >10/50 000 leukocytes (high antigenemia) which required ganciclovir therapy and four (11%) subsequently developed CMV disease. The median number of leukocytes and lymphocytes were 3500 (1300–17 200)/µl and 576 (228–3333)/µl at day 30 and 3900 (1400–9700)/µl and 1018 (192–6790)/µl at day 60, respectively. The median percentages of CD4+ and CD8+/lymphocytes were 35% (7–64%) and 38% (20–83%) at day 30 and 25% (6–37%) and 52% (27–83%) at day 60, respectively.

The tetramer analysis showed that the mean and median number of CMV-specific CTL at day 30 was, respectively, 11 and 1.9/µl for patients without CMV antigenemia, 23 and 7.8/µl for those with antigenemia, 33 and 15/µl for those with peak antigenemia <10/50 000, 12 and 3.7/µl for those with high antigenemia, and 21 and 2.4/µl for those who developed CMV disease. There was no significant correlation between the number of CMV-specific CTL and the incidence or severity of CMV antigenemia ($P>0.05$) (Figure 1).

To further evaluate the accurate number of CMV-specific CTL at the onset of CMV antigenemia, serial analysis of CMV-specific CTL was performed weekly in 14 patients (Figures 2 and 3). Patient's characteristics are shown in Table 1. CMV antigenemia was observed in 12 patients, and five of them (UPN1-5) developed high

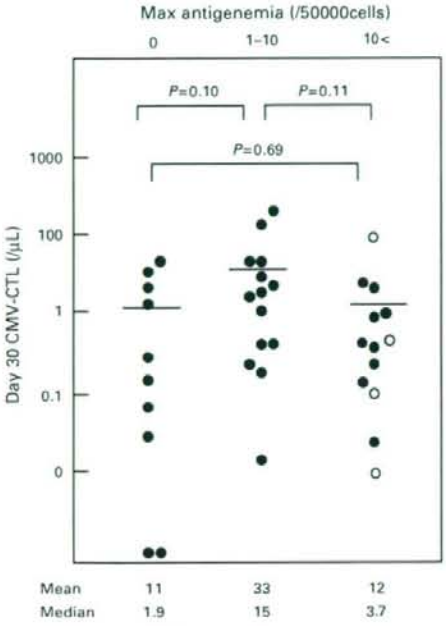

Figure 1 The number of CMV-specific CTL as evaluated by tetramer assay on day 30 post transplantation. The number of CMV-specific CTL did not differ between patients who did not develop CMV antigenemia, who had antigenemia below 10/50000, who had antigenemia of >10/50000. The outlined circle ○ indicates patients who developed CMV colitis.

antigenemia, including three (UPN1-3) with CMV colitis. The mean and median number of CMV-specific CTL at the first detection of CMV antigenemia was 21/μl and 4.7 (0–100)/μl in the 12 patients, and three (UPN2, 13, 14) showed >10/μl. For those who did not require antiviral therapy (UPN6-14), the number of CMV-specific CTL was widely ranged. While UPN6-8 showed <10/μl throughout the observation time, the maximum CTL count was >200/μl for UPN12-14. The number of CMV-specific CTL for UPN1 and UPN2 who developed CMV colitis showed >10/μl, which was 14 and 80/μl when diarrhea occurred, and 88 and 63/μl, respectively at the time of colon biopsy which proved CMV colitis.

It has been demonstrated that in patients coexpressing HLA-A02 and HLA-B07, CMV-specific cellular immune responses restricted by HLA-B07 dominate those restricted by HLA-A02, possibly because CD8+ T cells specific for dominant epitopes are able to suppress immune responses to less favored epitopes.[3] The allele frequency of HLA-B07 is low (5.2%) among Japanese[15] and only one patient coexpressed HLA-B07 in this study. We did not exclude this patient (UPN14) from the analysis because the number of HLA-A02-restricted CMV-specific CTL in this patient was 9.5/μl on day 30 and the maximum value reached 243/μl on day 128 suggesting that the coexpression of HLA-B07 seems not to have affected the immunoresponse of HLA-A2 in this patient.

Intracellular cytokine assay

Upon stimulation with CMV lysate, intracellular IFN-γ staining among five patients (UPN1–5) who developed high

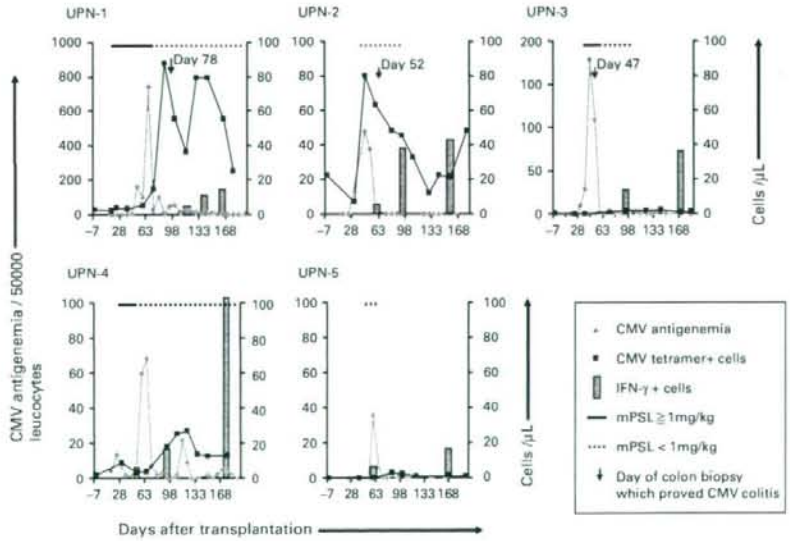

Figure 2 Serial analysis of patients who had high antigenemia of >10/50000. ■ indicates CMV-specific CTL as evaluated by tetramer assay, ◆ indicates CMV antigenemia, gray bar indicates the number of IFN-γ+cells/μl peripheral blood when stimulated with CMV lysate, the solid line indicates methylprednisolone administration of 1 mg/kg/day or more, the dashed line indicates corticosteroid administration less than 1 mg/kg/day and ↓ indicates the day of colon biopsy which CMV disease was diagnosed. UPN1, 2, 3 developed CMV disease. Intracellular IFN-γ was undetectable on day 60 and day 90 for UPN1 and on day 60 for UPN3.

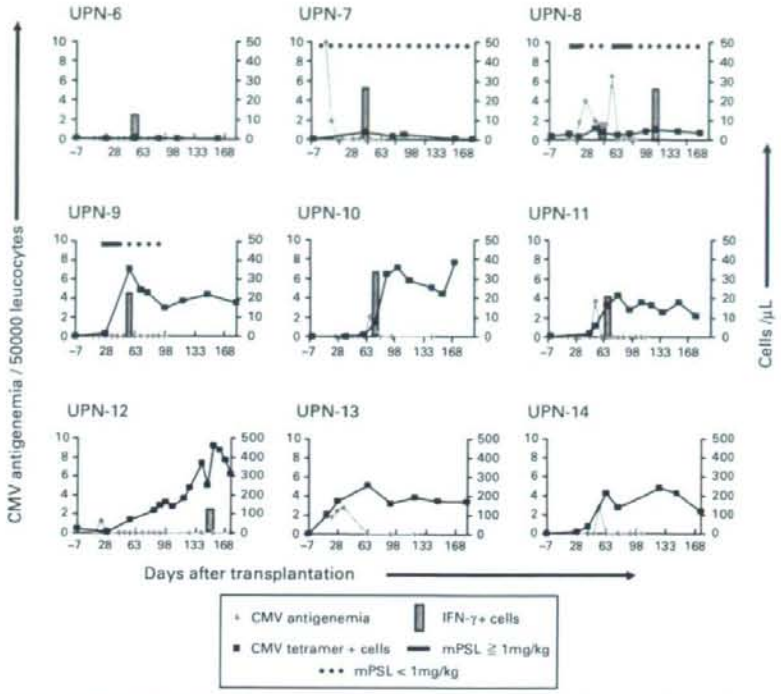

Figure 3 Serial analysis in patients with CMV antigenemia of <10/50000 or patients without CMV antigenemia. The legends are the same as Figure 2. Intracellular cytokine was not assessed for UPN13 and UPN14.

Table 1 Patients' characteristics

ID	*Age*	*HLA-A locus*	*Primary disease*	*Conditioning regimen*	*GVHD prophylaxis*	*Stem cell source*	*CMV serology*		*Max CMV-Ag*	*CMV disease*
							Recipient	*Donor*		
UPN-01	63	0201, 0206	CML (AP)	CdA/BU	CSP→TAC	PB	+	+	740	+
UPN-02	57	0201	NHL (DLBCL)	CdA/BU	CSP	PB	+	+	48	+
UPN-03	49	0201	NHL (low grade)	CdA/BU	CSP→TAC	PB	+	+	178	+
UPN-04	54	0206	MCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	68	–
UPN-05	59	0206	AML	CdA/BU/TBI	CSP+sMTX	UBM	+	+	35	–
UPN-06	66	0206	MDS (RA)	Flu/BU	CSP+sMTX	PB	+	–	0	–
UPN-07	61	0201	NHL (low grade)	Flu/BU/ATG	CSP	UBM	+	+	10	–
UPN-08	62	0201	AML	CdA/BU	TAC	PB	+	+	6.5	–
UPN-09	43	0201	MDS (RA)	BU/CY	CSP+sMTX	UBM	+	–	0	–
UPN-10	41	0206	AML	BU/CY	CSP+sMTX	RBM	+	+	2.1	–
UPN-11	54	0201	NHL (low grade)	Flu/BU	CSP+sMTX	PB	+	+	3.7	–
UPN-12	32	0206	RCC	CdA/BU	CSP	PB	+	+	2.8	–
UPN-13	42	0206	PCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	2.8	–
UPN-14	43	0206	RCC	CdA/BU/ ATG	CSP	PB	+	+	1.3	–

Abbreviations: ATG = antithymocyte globulin; CdA = cladribine; CML (AP) = CML (accelerated phase); CSP = cyclosporine; DLBCL = diffuse large B-cell lymphoma; Flu = fludarabine; MCL = mantle cell lymphoma; MDS (RA) = myelodysplastic syndrome (refractory anemia); NHL = non-Hodgkin lymphoma; PB = peripheral blood; PCL = plasma cell leukemia; RBM = related bone marrow; RCC = renal cell carcinoma; sMTX = short term methotrexate; TAC = tacrolimus; UBM = unrelated bone marrow.

antigenemia and required antiviral therapy showed that the mean number of IFN-γ-producing cells was 3.6 (0–6.7)/μl at day 60, which subsequently increased to 72 (15–250)/μl at day 160. As for three patients with CMV colitis (UPN1–3), only one patient (UPN2) had detectable level of IFN-γ-producing cells (4.8/μl) at the time of disease

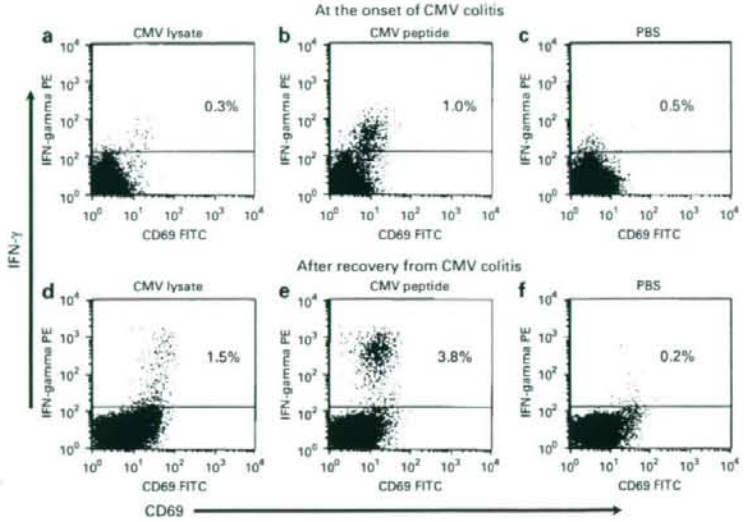

Figure 4 Intracellular cytokine assay in a patient with CMV colitis (UPN2). The samples were taken at the onset of CMV colitis (**a–c**) and after recovery from CMV colitis (**d–f**). The numbers of IFN-γ-producing cells on lysate stimulation (**a, d**) and peptide stimulation (**b, e**) both increased after recovery from CMV colitis. (**c**) and (**f**) are negative controls.

onset and were undetectable for the other two patients, which remained negative until day 90 for UPN1. The mean number of IFN-γ+ cells subsequently increased to 19 (5–38)/μl after recovery from CMV disease (Figures 2, 4a and d). Among the patients who did not require antiviral therapy, the IFN-γ-producing cells were all >10/μl at day 60.

When stimulated with CMV peptide, IFN-γ-producing cells numbered 8 (0–16)/μl at the time of disease onset with a subsequent increase to 47 (15–95)/μl after recovery from CMV disease (Figures 4b and e).

Regarding the phenotype of IFN-γ-producing cells, median of 81% (76–100) were CD4+ and <20% were CD8+ upon stimulation by CMV lysate. The staining of IFN-γ was brighter in CD4+ than in CD8+ cells and CD69 was positive for both CD4+ and CD8+ fraction. IFN-γ-producing cells were CD69 low positive and median of 42% (25–68) were CD8+, while the rest were CD8−/CD4− phenotype upon CMV peptide stimulation.

Discussion

Our results showed that it is difficult to predict CMV infection by the number of CMV-specific CTL alone as this did not correlate with the incidence and severity of CMV infection. While UPN1 and UPN2 developed CMV colitis after the recovery of sufficient number of CTL, UPN6, UPN7 and UPN8 did not require antiviral therapy despite low CMV-specific CTL. These results showed that CMV disease could occur after HSCT even in patients with >10/μl CMV-specific CTL as evaluated by tetramer assay, which has been considered to be sufficient to protect against CMV infection.[5,7]

CMV-specific CTL emerged immediately following the detection of antigenemia in most patients, suggesting that CMV infection can be a trigger for the recovery of CMV-specific immunity. However, UPN9 had recovery of CMV-specific CTL at day 60 even though his CMV antigenemia and CMV DNA as evaluated by PCR were negative throughout the course.

On the other hand, intracellular analysis revealed that IFN-γ production in both CD4+ and CD8+ T lymphocytes was depressed in patients with high antigenemia or CMV disease and this had subsequently recovered at disease resolution. Functional analysis methods for CMV-specific immune response by flow cytometry have been established,[16] and it was reported that patients who developed CMV disease after SCT had no detectable IFN-γ production by CD3+/4+ T-cells upon CMV AD-169 antigen stimulation.[17] It has also been demonstrated that levels of IFN-γ-producing CD4+ cells less than one cell/μl and CD8+ less than three cells/μl upon stimulation by CMV-infected autologous dendritic cells are not protective against recurrent infection.[18] As assessed by IFN-γ ELISPOT assay, the threshold level for protection against CMV reactivation was estimated as over one cell/μl peripheral blood upon CMV pp65 peptide stimulation.[19] The number of IFN-γ-producing cells upon CMV lysate stimulation were above ten cells/μl among patients whose antigenemia was <10/50 000 cells in our study, which may be sufficient for protection against CMV reactivation. It is difficult to determine the exact threshold level for protection against CMV since IFN-γ production differs among various stimulating agents. Also the magnitude of response is higher in the cytokine flow cytometry assay while the cytokine flow cytometry assay was less likely than the ELISPOT assay to detect low-level responses.[20]

Several studies on HIV-infected patients have shown the availability of analyzing the phenotype and other cytokine production of virus-specific T-cells such as IL-2, TNF-α.[21–23] It has been demonstrated that virus-specific T-cells, which produce both IFN-γ and IL-2 are important in virus-specific immunity, and that IFN-γ/IL-2 secreting CD8+ T-cells were CD45RA−/CCR7− phenotype and correlated with that of proliferating T-cells, whereas single IFN-γ-secreting cells were either CD45RA−/CCR7− or CD45RA+/CCR7−.[22] Another study has shown that immunorestored patients had increased levels of circulating CMV-specific CD8+ T-cells with 'early' (CD27+/CD28+/CD45RA+, CD27+/CD28+/CD45RA−) and 'intermediate' (CD27−/CD28+/CD45RA−) phenotype.[23] Only IFN-γ production was assessed in our study, however higher-order flow cytometry might have added more discriminatory value. Foster *et al*.[24] demonstrated that CMV-specific CD4+ T-helper cells show the same reconstitution kinetics as CD8+ CTL. Thus, functional analysis of lymphocytes upon lysate stimulation that can be used to assess both CD4+ and CD8+ cells is a useful tool for monitoring T cell immunity against CMV in patients after HSCT. This method is more widely applicable than peptide stimulation or tetramer assay, since it is not restricted to HLA or a single epitope. However, peptide stimulation and tetramer assay may still be a major procedure in the analysis of CD8+ T-cells, since tetramers are widely applied to adoptive immunotherapy of CMV[25] and the dominant population of IFN-γ-producing cells upon lysate stimulation was CD4+. Previous study has demonstrated that flow cytometry following stimulation of PBMC with pp65 and immediate early (IE)-1 peptide pools consisted of 15-aa peptides was highly sensitive and specific in predicting the presence of recognized epitope in the respective proteins.[26] Furthermore, it has been shown that IE-1-specific responses were more important in protective immunity than pp65-specific responses in heart and lung transplant recipients.[27] The stimulation with comprehensive peptide pools might have better assessed both functional CD4+ and CD8+ T-cell responses. Further study is needed to identify whether IE-1 is more important than pp65 in allogeneic HSCT patients, and the significance of IE-1 in Japanese population with low allele frequency of HLA-A1 (1.8%), -B7 (5.2%) or -B8 (<1%),[15] which is known to present IE-1 epitopes.

It is likely that the patients who did not have CMV reactivation despite low CMV-specific CTL had sufficient T-cell immune-recovery against CMV since the number of intracellular IFN-γ positive cells upon CMV lysate stimulation was as high as that in patients who had recovered from CMV reactivation. As for CD8+ T cells in these patients, CTL against other CMV-epitopes besides NLV might have helped to protect against CMV. It is reported that the recovery of CMV specific T-cells is earlier in patients who received reduced-intensity conditioning compared to conventional regimen and this was delayed by the use of ATG.[19,28] Additionally, the graft source and CD3+ T-cell dose significantly influence the recovery of CMV-specific immunity.[28] The difference of immune recovery according to the conditioning regimen and graft source was not demonstrated in this study, probably due to heterogeneous patients and small sample size. Functional depression of the lymphocytes due to corticosteroid for GVHD seems to be the major cause of CMV infection as documented in all patients with high antigenemia. Moreover, 75% of the patients with CMV disease were receiving more than 1 mg/kg/day of methylprednisolone (mPSL), while among those who did not require antiviral therapy, only 13% had received 1 mg/kg/day or more mPSL. The influence of corticosteroid on the number of CMV-specific CTL is controversial. Some studies have reported that a significant reduction of CMV-specific CTL occurred with corticosteroid therapy.[6,8] Others have shown that the frequency and the absolute number of CMV-specific CD8+ T cells were similar in patients receiving corticosteroids and those who didn't, while the CMV-specific CD8+ T cells showed decreased cytokine production.[10,11] Our result was consistent with the latter observation that while the number of CMV-specific CTL does not decrease significantly with corticosteroid therapy, IFN-γ production of CMV-specific CTL is severely suppressed. Therefore, concomitant assessment of T-cell function is essential in patients after HSCT, especially in those who are receiving corticosteroid therapy.

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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.

KEY WORDS

C-reactive protein • Allogeneic transplantation • Acute graft-versus-host disease • Nonrelapse mortality

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.

Table 1. *Patients' Characteristics*

	N (%)/ Median		
Variable	Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	*P* Value
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.

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) 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 0.5×10^9/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

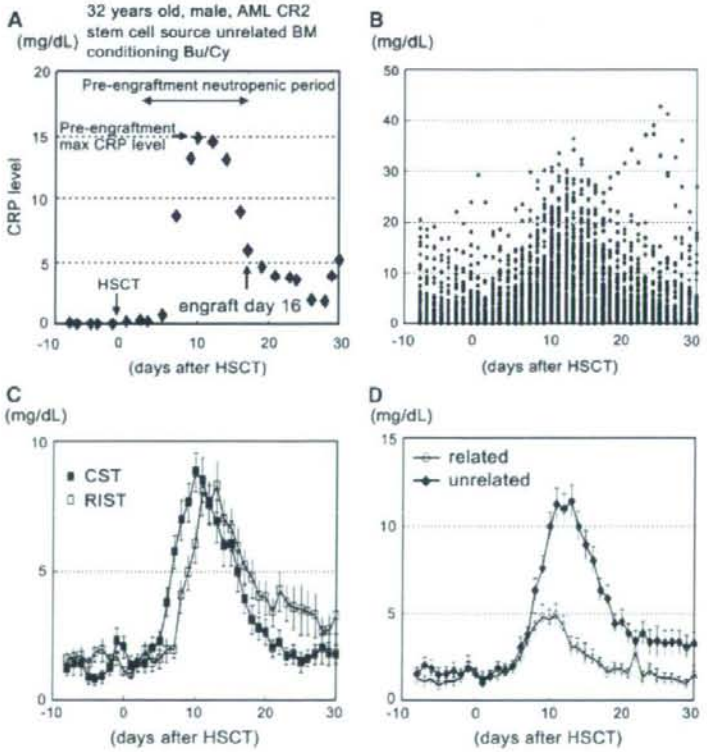

Figure 1. An example of how we measured CRP in a representative patient (A). Dot plot of the CRP level. All patients (B), CST versus RIST (C) and related versus unrelated (D).

statistics were used. Student *t*, chi-square, Fisher's exact test, and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. To analyze the pretransplant risk factors for a high CRP level, logistic analysis was used. OS was estimated using Kaplan-Meier curves. The cumulative incidence of aGVHD and NRM was estimated based on a Cox regression 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 assessed 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 logistic analysis and Cox proportional hazard modeling. In Japan, only BM and CB are allowed for unrelated transplantation, and most transplantations with a related 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 software (Chicago, IL). This analysis was approved by the institutional review board.

RESULTS

Infections

The median duration of follow-up in surviving patients 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 infections 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 incidence of central venous catheter infection was, respectively, 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 relation 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 logistic regression analysis, which showed that male, unrelated donor, stem cell source with BM or CB transplantation (versus PBSCT), HLA-mismatched donor, and immunosuppression with MTX were associated with high CRP values during neutropenia (Table 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), respectively, with a significant difference ($P = .017$) (Table 2). Notably, within the RIST group, the median CRP level was significantly lower in related than in unrelated transplantation (1.6 mg/dL [0.1-9.7] veruss 16.2 mg/dL [0.5-42.7]; $P < .001$). However, the logistic analysis failed to disclose any overall significant difference 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 $P = .04$, respectively). A Cox proportional hazard model showed that a high CRP level and CMV serostatus were associated 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 mismatch, conditioning, GVHD prophylaxis, stem cell source.

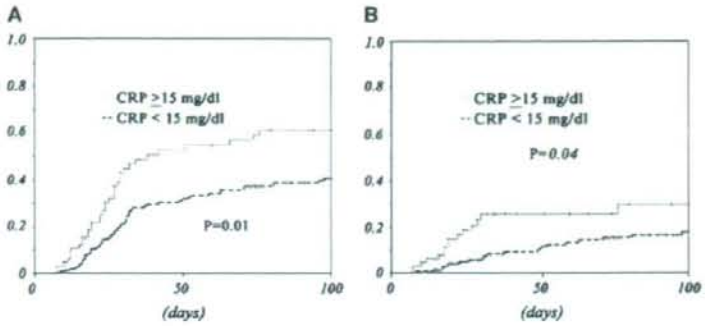

Figure 2. Cumulative incidence of grade II-IV aGVHD (A) and grade III-IV aGVHD (B) stratified according to the maximal CRP level during neutropenia.

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

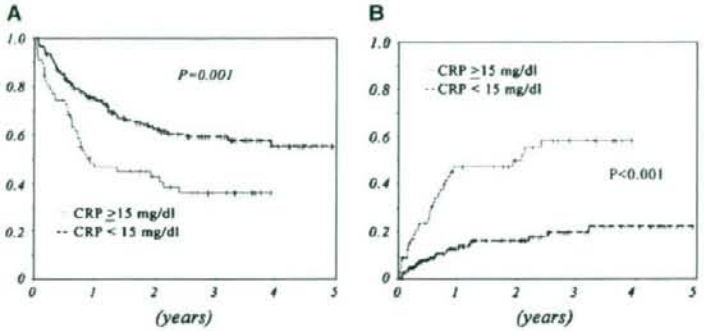

Figure 3. OS stratified according to the maximal CRP level during neutropenia (A). Cumulative incidence of TRM stratified according to the maximal CRP level during neutropenia (B).

worldwide, especially in Japan, to distinguish bacterial infections from other causes of fever [15-19]. Based on this practice, we reviewed the value of the CRP level after HSCT, and our data suggest that it might be useful to monitor the CRP value as a net surrogate marker for produced cytokines, and for predicting the subsequent development of aGVHD and NRM.

Our patients had various interacting backgrounds, and it is still difficult to predict whether a patient with a high CRP level is destined to suffer from GVHD or major infectious complications. Infectious diseases were previously reported to be a primary cause of elevated CRP [8,20], which might, in turn, affect the severity of aGVHD. In this study, we made every effort, including intense culture studies, to exclude infection as a primary cause of increased CRP, and showed that there were significantly more documented infections in the high-CRP group than in the low-CRP group. Current practice for the prevention of infection mostly focuses on the effective control of Gram-negative bacteria, considering the potent immediate pathologic effect of the organisms. However, if the hypothesis that decreasing the net production of cytokines is important for the prevention of subsequent GVHD is correct, more effort should be paid to broadly cover other types of organisms or even clinically less significant infection, that is, stomatitis, at least during the early period of neutropenia, particularly in patients carrying risk factors for high CRP, which included unrelated donor, HLA mismatch, BM, and CB transplantation in this study. The addition of other markers, such as procalcitonin, may be useful for identifying the risk of major infectious complications [24].

Table 5. *Causes of Death Stratified According to CRP Value during Neutropenia*

Causes of death	Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	P Value
Total	57 (36%)	39 (58%)	.002
Relapse/progressive disease	34 (22%)	8 (12%)	.09
acute GVHD (total)	6 (4%)	5 (7%)	.25
acute GVHD	5 (3%)	3 (5%)	.63
acute GVHD + infection	1 (1%)	2 (3%)	.16
chronic GVHD (total)	7 (4%)	11 (16%)	.003
chronic GVHD	3 (2%)	7 (10%)	.005
chronic GVHD + infection	4 (3%)	4 (6%)	.21
Infection*	0 (0%)	5 (7%)	.002
MOF†	0 (0%)	4 (6%)	.008
Respiratory failure‡	3 (2%)	4 (6%)	.11
Others	Stroke 2 VOD 2 Secondary cancer 1 Unknown 2	VOD 1 Myocardial infarction 1	

CRP indicates C-reactive protein; GVHD, graft-versus-host disease; TBI, total-body irradiation; MOF, multiple organ failure; VOD, veno-occlusive disease.

*Excluding infection during GVHD or GVHD treatment.

†Excluding MOF due to GVHD, infection.

‡Excluding respiratory failure because of GVHD, infection, and MOF.

Tissue damage caused by the conditioning regimen, complicated infections, and allogeneic immune reactions are the primary factors that are associated with the initial elevation of CRP early in the course of allogeneic HSCT. Consequently, it can be speculated that a reduced-intensity conditioning regimen results in decreased cytokine release and a resultant lower CRP value, which may lead to less chance of developing GVHD. Although the RIST regimens we used were relatively dose-intense, in this retrospective review we still found that CRP levels tended to be decreased after RIST compared to conventional myeloablative transplantation, particularly in a related compared to an unrelated transplantation setting. Because augmentation of allogeneic immune and inflammation reactions may induce a higher CRP value, we speculate that the benefit of RIST is diminished when a strong allogeneic reaction is induced, as in cases of unrelated transplantation.

To further evaluate the relationship between a higher CRP value during neutropenia and common risk factors associated with transplantation, we performed a multivariate analysis and showed that unrelated donor, HLA mismatch, and male sex were associated with higher CRP values. Additionally, from the finding in the multivariate analysis that unrelated donor and HLA mismatch were independently associated with high CRP, we surmised that the degree of genetic disparity might be associated with higher CRP during neutropenia. Based on a consideration of these findings together, we think that a higher CRP value may reflect the degree of tissue damage because of the transplant regimen and the subsequent magnitude of allogeneic immune reactions. Nevertheless, our analysis was hampered, because in Japan only BM and CB are allowed for unrelated transplantations, and most transplantations with a related donor use PBSC as a stem cell source. In these settings, a theoretically longer neutropenic period after unrelated BM or CB transplantation might be associated with a higher risk of infection, which could lead to higher CRP, as shown in this study.

In this study, the primary causes of death in the low-CRP group were mainly relapse and progression, whereas in the high-CRP group this was NRM. Notably, the observation that the relapse rate was higher in the low-CRP group than in the high-CRP group, as previously suggested by Min et al. [23], may further support our hypothesis that serum CRP values represent overall inflammation and cytokine production, which paves the way to GVHD and related graft-versus-leukemia (GVL) effects. A possible reason for this finding is that a low CRP level resulted in a lower incidence of GVHD and a resultant decrease in the GVL effect, or the high-CRP group developed earlier and more-frequent death from NRM compared to the low-CRP group, which left fewer patients for evaluation of the later occurrence of relapse.

In conclusion, our results suggest that the CRP value in the neutropenic period before engraftment in patients undergoing allogeneic HSCT may be a net surrogate marker of early inflammation that leads to the development of aGVHD/cGVHD and subsequent NRM, as has been proposed in mouse models. The intensity of the conditioning regimen, infectious diseases, and degree of allogeneic immune response attributed to HLA compatibility and the stem cell source may be the major factors that predict higher CRP values. Based on the results of this retrospective study, future clinical studies to evaluate the feasibility of earlier intervention and adjustment of the procedure for preventing GVHD and NRM based on monitoring of the early CRP value are warranted.

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