Hematopoietic recovery

A total of 113 patients achieved primary engraftment with a median time to reach a neutrophil count of 0.5 × 10^9/L or higher and a platelet count of 2.0 × 10^9/L or higher of 14 d (range, 10–40 d) and 22 d (range, 8–105 d), respectively. The median times to reach these neutrophil and platelet counts were earlier in the RIC group than the MAC group (neutrophil: 14 vs. 19 d, $P < 0.001$; platelet: 21 vs. 29 d, $P = 0.005$), as shown in Table 2. None of the patients experienced primary graft failure. All but two patients, who died before day 30 after allo-HCT without evidence of engraftment, were assessed for hematopoietic recovery, and 6 (5%) experienced secondary graft failure.

Graft-versus-host disease

The 113 patients who achieved engraftment was evaluated for aGVHD. The incidence of grade II–IV aGVHD was 42% and that of grade III–IV aGVHD was 14%, as shown in Table 2. There was no significant difference between the RIC and MAC groups in the incidence of aGVHD. Among the 107 patients who survived more than 100 d after allo-HCT, 10 (9%) developed limited cGVHD and 48 (45%) developed extensive cGVHD. There was no significant difference between the RIC and MAC groups with regard to the incidence of cGVHD.

Non-relapse mortality

The 4-yr incidence of NRM was 29% in the MAC group and 33% in the RIC group ($P = 0.89$) (Fig. 1A). In a univariate analysis, covariates associated with a higher incidence of NRM were recipient sex [female, hazard ratio (HR) 2.9, 95% CI 1.1–7.5, $P = 0.03$], IPSS risk at diagnosis (Int-2/High, HR 2.2, 95% CI 1.1–4.7, $P = 0.04$), the FAB stage at peak (RAEB/CMMoL, HR 2.8, 95% CI 1.0–7.7, $P = 0.05$), cytogenetic risk at diagnosis (poor, HR 2.0, 95% CI 1.1–4.0, $P = 0.03$), BM blasts at HCT (20% or higher, HR 4.1, 95% CI 1.7–10.2, $P = 0.002$), and the presence of aGVHD (grade III–IV, HR 4.4, 95% CI 2.2–9.0, $P < 0.001$), as shown in Table S1. In a multivariate analysis (Table 3), the covariates associated with a higher incidence of NRM were the presence of aGVHD (grade III–IV, HR 6.9, 95% CI 2.7–17.4, $P < 0.001$) and BM blasts at HCT (20% or higher, HR 3.6, 95% CI 1.3–9.9, $P = 0.01$). cGVHD in this model was not an independent factor for NRM when substituted for grade III–IV aGVHD (data not shown).

Relapse

The 4-yr incidence of relapse was 26% in the MAC group and 25% in the RIC group ($P = 0.97$) (Fig. 1B). In a univariate analysis, the only covariate associated with a higher relapse rate was prior chemotherapy (HR 2.5, 95% CI 1.1–5.8, $P = 0.04$), as shown in Table S1. In a multivariate analysis (Table 3), covariates associated with a higher relapse rate were prior chemotherapy (HR 4.3, 95% CI 1.2–15.9, $P = 0.03$), BM blasts at HCT (5–19%, HR 4.3, 95% CI 1.5–12.8, $P = 0.008$) and the absence of cGVHD (HR 12.7, 95% CI 3.1–52.6, $P < 0.001$). Grade II–IV or III–IV aGVHD in this model was not an independent factor for relapse when substituted for cGVHD (data not shown).

Table 2 Transplantation outcome

No. of patients	All $N = 115$	MAC $N = 34$	RIC $N = 81$
Graft failure (%)			
Primary	0 (0)	0 (0)	0 (0)
Secondary	6 (5)	1 (3)	5 (6)
Engraftment			
Neutrophils ≧ 0.5 × 10^9/L Median days (range)	14 (10–40)	19 (10–40)	14 (10–27)
Platelets ≧ 20 × 10^9/L Median days (range)	22 (8–105)	29 (13–90)	21 (8–105)
Acute GVHD (%)			
II–IV	48 (42)	12 (35)	36 (44)
III–IV	16 (14)	4 (11)	12 (15)
Onset, median days (range)	30 (5–98)	34 (9–66)	31 (9–68)
Chronic GVHD (%)			
Limited	10 (10)	4 (14)	6 (8)
Extensive	48 (47)	11 (39)	37 (50)
Onset, median days (range)	138 (100–1090)	124 (100–245)	134 (100–1090)

MAC, myeloablative conditioning; RIC, reduced intensity conditioning; GVHD, graft-versus-host disease.

Overall survival

In the overall population, the 4-yr OS was 44%. Although patients in the RIC group were older and had a worse cytogenetic risk, no difference in OS was seen between the two groups (47% in the MAC group vs. 42% in the RIC group, $P = 0.84$) (Fig. 1C). Fifty two patients (45%) were alive and 63 (55%) had died. Disease relapse or progression (40%) was the most common cause of death, followed by non-relapse causes complicated by organ failure (23%), infection (19%), GVHD (6%), and others (12%) (Table 4). In a univariate analysis, covariates associated with a worse OS were older age (60 yrs or older, HR 1.7, 95% CI 1.0–2.9, $P = 0.04$), the FAB stage at diagnosis (RAEB/CMMoL, HR 1.8, 95% CI 1.0–3.2, $P = 0.04$), IPSS risk at diagnosis (Int-2/High, HR 2.4, 95% CI 1.3–4.4, $P < 0.001$), the FAB stage at peak (RAEB/CMMoL, HR 2.3, 95% CI 1.0–5.2, $P = 0.04$ RAEB-T/AML-MLD, HR 2.6, 95% CI 1.2–5.7,

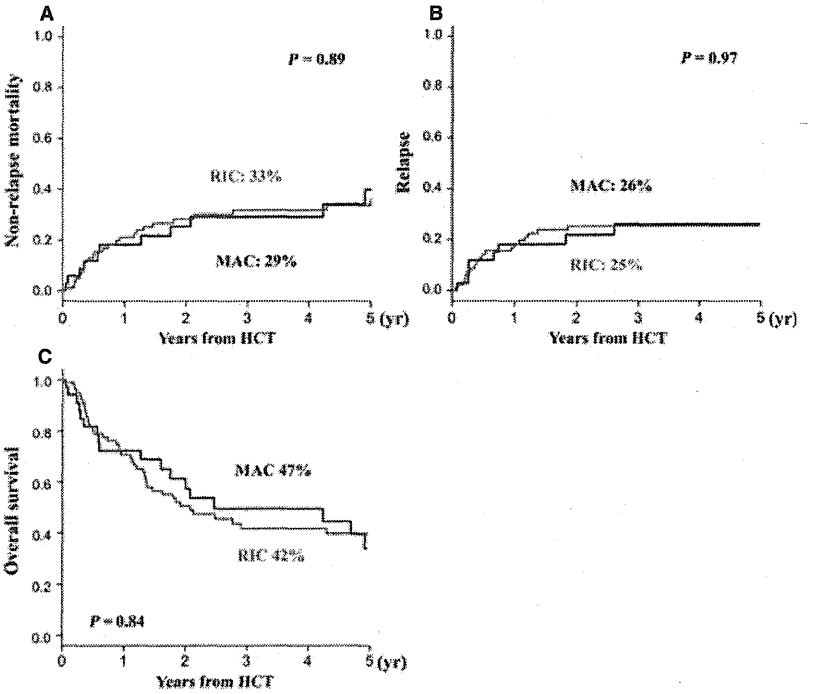

Figure 1 Outcomes stratified according to the intensity of the conditioning regimens. non-relapse mortality (A), Relapse (B) and overall survival (C) of patients with myelodysplastic syndrome receiving allo-hematopoietic cell transplantation after myeloablative conditioning or reduced-intensity conditioning regimens.

$P = 0.01$), IPSS risk at peak (Int-2/High, HR 2.3, 95% CI 1.1–5.0, $P = 0.02$), cytogenetic risk at diagnosis (poor, HR 2.2, 95% CI 1.3–3.7, $P < 0.001$), BM blasts at HCT (20% or higher, HR 3.4, 95% CI 1.6–7.2, $P < 0.001$), and the presence of aGVHD (Grade III–IV, HR 2.8, 95% CI 1.5–5.4, $P = 0.001$), as shown in Table S1. In a multivariate analysis (Table 3), covariates associated with a worse OS were the FAB stage at peak (RAEB-T/AML-MLD, HR 3.3, 95% CI 1.2–8.6, $P = 0.02$), cytogenetic risk at diagnosis (poor, HR 2.1, 95% CI 1.1–6.9, $P = 0.01$), BM blasts at HCT (20% or higher, HR 3.0, 95% CI 1.3–6.9, $P = 0.01$) and the absence of cGVHD (HR 2.0, 95% CI 1.1–4.0, $P = 0.04$). The presence of grade III–IV aGVHD was significantly associated with a worse OS (HR 5.4, 95% CI 2.5–11.4, $P < 0.001$) when this was substituted for cGVHD in this model.

In semi-landmark analyses for the entire population, the OS of patients with cGVHD tended to be better than that of patients without cGVHD ($P = 0.11$) (Fig. 2A). When the analysis was limited to the RIC group, the OS of patients with cGVHD was significantly better than that of patients without cGVHD ($P = 0.005$) (Fig. 2B). We also found that, in patients with poor cytogenetic risk, the OS of patients with cGVHD was significantly better than that of patients without cGVHD ($P = 0.003$) (Fig. 2C), whereas in patients with good/intermediate cytogenetic risk, there was no significant difference in OS between the two groups ($P = 0.76$) (Fig. 2D). In patients with BM blasts 5% or higher at HCT, the OS of patients with cGVHD was significantly better than that of patients without cGVHD ($P = 0.02$) (Fig. S1A), whereas in patients with BM blasts <5% at HCT, there was no significant difference in OS between the two groups ($P = 0.59$) (Fig. S1B).

Impact of extensive cGVHD in the RIC group

The median age in the RIC group was 57 (19–68) yrs. Among the 81 patients in the RIC group, 46 patients (58%) had cGVHD. The majority (86%) of patients with cGVHD developed extensive cGVHD. We also conducted a multivariate analysis limited to the patients pre-treated with RIC (Table S2) and found that the absence of extensive cGVHD was significantly associated with a worse OS (HR 2.4, 95% CI 1.2–5.5, $P = 0.001$) and a higher relapse rate (HR 13.1, 95% CI 4.0–43.9, $P < 0.001$). The presence of extensive cGVHD in this model was not an independent factor for NRM (HR 0.9, 95% CI 0.3–2.7, $P = 0.85$) when substituted for Grade III–IV aGVHD.

Discussion

We performed retrospective analyses of 115 patients with *de novo* MDS or AML-MLD who received their first allo-HCT at our center. By multivariate analyses, we found that the presence of cGVHD significantly reduced relapse and improved OS. To evaluate these results, we considered GVHD to be a time-dependent covariate and analyzed data from all patients to avoid bias from not considering patients

Table 3 Multivariate analysis for NRM, relapse, and OS

Variable	NRM		Relapse		OS	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age						
<60 yrs			1	0.72	1	0.33
≧60 yrs			1.2 (0.5–3.2)		1.4 (0.7–2.6)	
Prior chemotherapy						
No			1	0.03		
Yes			4.3 (1.2–15.9)			
Conditioning regimens						
MAC	1	0.33	1	0.77	1	0.63
RIC	0.7 (0.3–1.5)		0.9 (0.3–2.6)		1.2 (0.6–2.5)	
FAB stage at peak						
RA/RARS	1		1		1	
RAEB/CMMoL	1.2 (0.5–2.7)	0.68	0.6 (0.1–4.8)	0.57	1.9 (0.6–5.9)	0.28
RAEB-T/AML-MLD	2.3 (0.7–7.3)	0.14	0.7 (0.1–4.8)	0.73	3.3 (1.2–8.6)	0.02
Cytogenetic risk group						
Good/Intermediate	1	0.68	1	0.04	1	0.01
Poor	1.2 (0.5–2.7)		2.7 (1.1–6.9)		2.1 (1.1–6.9)	
BM blasts at HCT						
≦4%	1		1		1	
5–19%	1.2 (0.5–2.9)	0.75	4.3 (1.5–12.8)	0.008	1.6 (0.7–3.4)	0.28
≧20%	3.6 (1.3–9.9)	0.01	4.6 (0.9–23.4)	0.07	3.0 (1.3–6.9)	0.01
GVHD						
Grade III–IV aGVHD						
No	1	<0.001				
Yes	6.9 (2.7–17.4)					
cGVHD						
Yes			1	<0.001	1	0.04
No			12.7 (3.1–52.6)		2.0 (1.1–4.0)	

NRM, non-relapse mortality; OS, overall survival; HCT, allogeneic hematopoietic cell transplantation; HR, hazard ratio; CI, confidence interval; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; CMMoL, chronic myelomonocytic leukemia; RAEB-T, refractory anemia with excess blasts in transformation; AML-MLD, acute myeloid leukemia with multilineage dysplasia; BM, bone marrow; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease.

Covariates examined for NRM; Period of HCT, Patient sex, Conditioning regimens, FAB stage at peak, Cytogenetic risk group, BM blast at HCT, The presence of Grade III–IV aGVHD. Covariates examined for Relapse rate; Period of HCT, Age, Patient sex, Prior chemotherapy, Conditioning regimens, FAB stage at peak, Cytogenetic risk group, BM blast at HCT, The presence of cGVHD. Covariates examined for OS; Period of HCT, Conditioning regimens, FAB stage at peak, Cytogenetic risk group, BM blast at HCT, The presence of cGVHD.

who died or relapsed too early to develop acute or chronic GVHD. Some studies that used the same statistical method reported that cGVHD had beneficial effects on relapse in patients receiving allo-HCT after MAC (14, 15). In addition, others showed that the presence of cGVHD was an independent factor in reducing relapse and improving progression-free survival (PFS) in the setting of non-MAC regimens (12) or RIC regimens (16). Similar to our study, Valcárcel *et al.* (16) demonstrated that the development of cGVHD was the strongest factor in reducing relapse and improving survival in patients with high-risk MDS and AML receiving allo-HCT after RIC.

There has been no previous study on the effect of cGVHD on OS according to the conditioning regimen and disease status at allo-HCT. To clarify these questions, we used semi-landmark analyses to evaluate the effect of cGVHD on OS in various subgroups. In the current study, the presence of cGVHD predominantly improved OS in the setting of RIC, but did not affect OS in the MAC group (data not shown). In addition, the presence of cGVHD was significantly associated with the improvement in OS in high-risk patients with BM blasts of 5% or higher at allo-HCT or poor cytogenetic risk, whereas it did not affect OS in low-risk patients. These findings suggest that the benefit of the GVL effect appeared to be more prominent in patients with high-risk MDS who did not receive intensive preparative regimens.

Our findings may suggest that extensive cGVHD is beneficial for patients pre-treated with RIC because of elderly age or less-fit conditions. Valcárcel *et al.* reported that cGVHD was significantly associated with reducing relapse and improving OS without increasing NRM in high-risk AML and MDS patients pre-treated with RIC. In their study,

Table 4 Cause of death

No. of patients	All $N = 115$	MAC $N = 34$	RIC $N = 81$
Cause of death			
All Causes (% of all patients)	63 (55)	18 (53)	45 (56)
Progression (% of all death)	25 (40)	7 (39)	18 (40)
Organ failure (%)	14 (23)	5 (28)	9 (20)
Multiple organ failure	3	1	2
Veno-occlusive disease	3	1	0
Renal failure	1	0	1
Cardiac failure	1	1	0
Diffuse alveolar hemorrhage	7	2	5
Infection (%)	12 (19)	3 (17)	9 (20)
Bacterium	7	2	5
Fungus	3	0	3
Virus	2	1	1
Bleeding (%)	2 (3)	0 (0)	2 (4)
Secondary cancer (%)	4 (6)	0 (0)	4 (10)
GVHD (%)	4 (6)	2 (11)	2 (4)
Unknown (%)	2 (3)	1 (5)	1 (2)

MAC, myeloablative conditioning; RIC, reduced intensity conditioning; GVHD, graft-versus-host disease.

the cumulative incidence of cGVHD was 53% and extensive cGVHD accounted for the majority (94%) of that (16). Baron *et al.* (12) showed a comparable incidence of extensive cGVHD and reported the same results in AML and MDS patients with extensive cGVHD pre-treated with non-MAC regimens.

It is difficult to induce cGVHD 'moderately' on purpose, and the induction of cGVHD may lead to an increased risk of NRM. When we wish for the presence of cGVHD without a devastating outcome, there are two possible choices. First, G-CSF-mobilized peripheral blood mononuclear cells (G-PBMC) may be a preferable stem cell source when compared with BM. Some studies have shown that the use of G-PBMC as a stem cell source increased the frequency of cGVHD with comparable survival as compared with BM (17–19). Second, GVHD prophylaxis without ATG may be another beneficial option, as ATG has been shown to significantly decrease the incidence of cGVHD (20–22).

As the major causes of treatment failure were disease relapse and progression, treatment strategies before or after allo-HCT to reduce the risk of relapse remain a significant consideration for patients with high-risk MDS. The use of some additional treatment might be effective, especially for patients with high-risk MDS without cGVHD. Azacitidine is a DNA hypomethylating agent to show a significantly prolonged OS compared with conventional care regimens in patients with intermediate-2 and high-risk MDS (23, 24). The use of low-dose azacitidine as pre-emptive and maintenance treatment may prolong survival in patients with higher-risk MDS or AML after allo-HCT (25–27). Azacitidine also appears to induce leukemic cell differentiation and increase the expression of human leukemic antigen DR-1 (HLA-DR) and several tumor-associated antigens that could potentially enhance the GVL effect (28–30). We were not

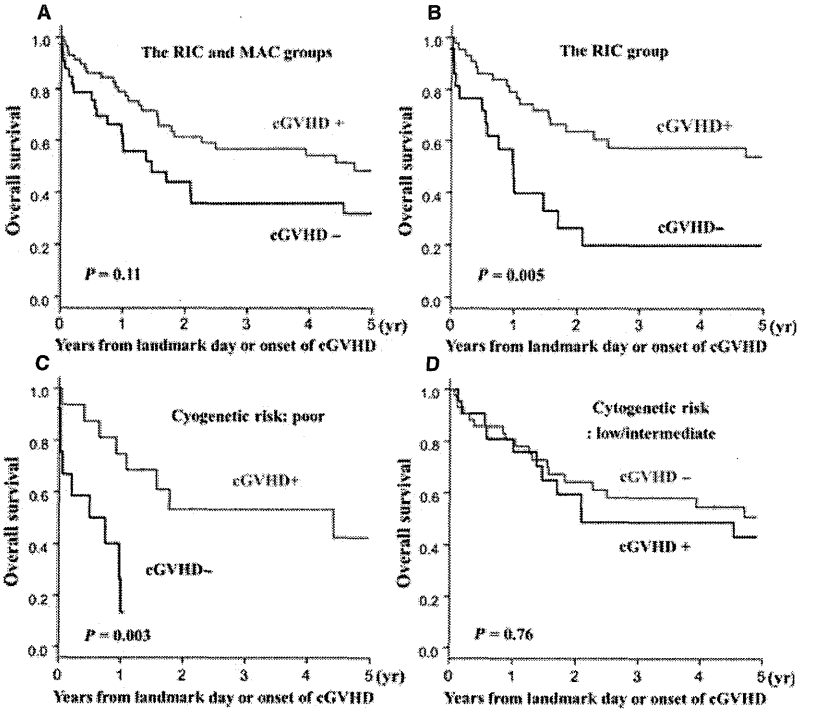

Figure 2 Semilandmark plots illustrating the impact of chronic graft-versus-host disease (GVHD) on overall survival (OS) of patients with myelodysplastic syndrome receiving allo-hematopoietic cell transplantation. OS curves of patients with or without chronic GVHD are shown for the entire population (A), the reduced-intensity conditioning group (B), patients with poor cytogenetic risk (C), and patients with low/intermediate cytogenetic risk (D).

able to assess the effect of Azacitidine before or after allo-HCT in patients with MDS, because patients who received Azacitidine were not included in our study. These issues need to be addressed in a prospective study.

We also analyzed the impact of aGVHD on outcomes after allo-HCT. The presence of grade II–IV aGVHD did not significantly influence the outcome. On the other hand, the presence of grade III–IV aGVHD was significantly associated with a worse OS and a higher incidence of NRM. Several studies have analyzed the effect of aGVHD on the prognosis after allo-HCT, but only a few have shown that aGVHD has a positive impact (12, 15, 16, 31). Kanda *et al.* (31) reported that grade I aGVHD had a beneficial effect on PFS in high-risk patients. However, we were not able to evaluate the effect of grade I aGVHD because of the small number of patients.

In the present study, OS, relapse and NRM did not differ significantly between the MAC and RIC groups, although the RIC group had significantly higher proportions of elderly patients and those with poor cytogenetic risk. Several previous studies have analyzed MDS and AML patients who received allo-HCT after MAC or RIC regimens (2, 6, 32, 33). In some studies, OS and PFS tended to be similar between the MAC and RIC groups, with a decreased incidence of NRM offset by an increased incidence of relapse in the RIC group. In other studies, there were no differences in relapse or NRM between the MAC and RIC groups, with a comparable OS (34, 35), and our results were consistent with the latter results.

The other major covariates that influenced OS in the present study were poor cytogenetic risk at diagnosis and the disease status at allo-HCT. Poor cytogenetic risk was also a significant factor for the increased risk of relapse, which was consistent with previous reports (32, 33, 36, 37). Although some studies have reported that a low pre-transplant tumor burden was essential for the success of allo-HCT in patients with MDS (35, 38, 39), it remains to be determined whether induction chemotherapy should be given to reduce the tumor burden before allo-HCT. Previous studies have shown that chemotherapy prior to allo-HCT did not improve OS because of the possibility of an increased incidence of NRM (38–40). In the present study, prior chemotherapy was significantly associated with an increased risk of relapse, but did not affect OS or NRM. This result may be explained by the fact that patients who need chemotherapy prior to HCT are probably those with high-risk disease.

Our study has several limitations, and thus the results must be interpreted with caution. These limitations include the retrospective nature of the study including the fact that therapeutic strategies were chosen at the discretion of physicians, the small number of patients analyzed, the heterogeneity of the groups of patients, and a short follow-up period. Nevertheless, the present data from more than 100 patients treated in a single center allowed us to identify factors that were associated with the prognosis in patients with MDS after allo-HCT.

In summary, the presence of cGVHD significantly reduced the risk of relapse and improved OS without increasing the incidence of NRM in patients with MDS. We also found that the presence of cGVHD significantly improved OS in high-risk patients or the RIC group, which suggests that the GVL effect may be beneficial in high-risk patients who do not receive intensive preparative regimens. For elderly or unfit patients with MDS, allo-HCT with RIC regimens was a potentially curative therapeutic option comparable with MAC regimens. As the major causes of treatment failure were disease relapse and progression, the treatment strategies to reduce the risk of relapse before and after allo-HCT are still a significant consideration for patients with high-risk MDS.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

N.H. designed the study, prepared the data file, performed the analysis, interpreted data, and wrote the manuscript; S.K was primarily responsible for the study design, data analysis, and interpretation of the data; K.O., T.K., Y.K., A.S., Y.I., R.U. and T.T. provided the patients' data; S-W.K., Y.T., and Y.H. interpreted data and reviewed the manuscript; K.T. supported the statistical analysis; T.F. provided the patients' data, interpreted data, and helped to write the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Semilandmark plots illustrating impact of chronic GVHD on OS of patients with MDS receiving allo-HCT.

Table S1. Univariate analysis for NRM, relapse, and OS.

Table S2. Multivariate analysis for NRM, relapse and OS in the RIC group (patients pretreated with RIC).

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ORIGINAL ARTICLE

Reduced-intensity conditioning regimen with low-dose ATG-F for unrelated bone marrow transplant is associated with lower non-relapse mortality than a regimen with low-dose TBI: a single-center retrospective analysis of 103 cases

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Abstract Although anti-T lymphocyte globulin-Fresenius (ATG-F) is commonly used as prophylaxis for graft-versus-host disease (GVHD), the appropriate dosage of ATG-F in the setting of a reduced-intensity conditioning (RIC) regimen has not been determined. In the present study, we retrospectively analyzed the clinical outcomes of 103 patients after unrelated bone marrow transplant (uBMT) with RIC regimens. RIC regimens consisted of purine analogue plus busulfan with low-dose TBI or ATG-F (5–10 mg/kg in total). Median age was 57 years (range 20–68). The incidence of grade II–IV acute GVHD and chronic GVHD with ATG-F was significantly lower than that with TBI 2 Gy (15 vs. 61 %, $P < 0.05$; 33 vs. 57 %, $P < 0.05$). The incidence of 2-year NRM with ATG-F was significantly lower than that with TBI 2 Gy (6 vs. 28 %, $P < 0.05$). There was no statistically significant difference in the cumulative incidence of 2-year relapse between the ATG-F and TBI 2 Gy groups (37 vs. 20 %, $P = 0.13$). In conclusion, the addition of low-dose ATG-F to GVHD prophylaxis in patients who received uBMT resulted in decreased incidence of acute and chronic GVHD, which led to a significantly reduced risk of NRM without compromising overall survival. The beneficial effect of low-dose ATG-F should be assessed in a prospective clinical trial.

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Keywords ATG · Unrelated bone marrow transplant · GVHD

Introduction

After Slavin and co-workers [1–3] introduced a reduced-intensity conditioning (RIC) regimen using fludarabine (Flu)/busulfan (Bu), similar regimens have been widely used worldwide. Bornhäuser et al. [2] reported that a RIC regimen using Flu/Bu/Anti-T lymphocyte globulin (ATG) was associated with a high risk of graft failure (GF). The incidence of GF was higher in patients who received bone marrow (BM, 31 %) as compared to unmanipulated peripheral blood stem cells (PBSC, 10 %), although this difference was not statistically significant [2]. In contrast, Nagler et al. [3] reported that a RIC regimen using Flu/Bu/ATG followed by an unrelated BMT (uBMT) was not associated with GF. In general, PBSC is preferred in the setting of RIC, due to the risk of GF [4]. However, in Japan, only BM was able to be harvested from an unrelated volunteer donor. Therefore, we incorporated TBI 4 Gy in addition to purine analogue plus Bu to ensure engraftment [5]. With this conditioning regimen, while all patients ($n = 17$) achieved engraftment, 5 had grade IV acute graft-versus-host disease (GVHD) and the incidence of non-relapse mortality (NRM) was unacceptably high (1-year NRM 46 %). Therefore, we thereafter reduced the dose of TBI from 4 to 2 Gy.

Finke et al. [6, 7] reported that ATG-F as GVHD prophylaxis reduced the incidences of acute and chronic GVHD without comprising survival in patients with an unrelated HSCT following a myeloablative conditioning regimen. Therefore, we also incorporated low-dose ATG-F instead of TBI to decrease GVHD-related deaths. Since

Asian populations have a lower risk of GVHD than Caucasian populations, we used low-dose ATG-F (5 or 10 mg/kg in total) [8–10]. Here, we retrospectively analyzed the clinical outcomes in patients who received uBMT with a RIC regimen in our institute. We focused on a comparison of currently used regimens, and particularly those that contain TBI 2 Gy and ATG-F.

Patients and methods

Study design

This was a single-center retrospective study that compared 3 RIC regimens (TBI 4 Gy-containing, $n = 30$; TBI 2 Gy-containing, $n = 40$; ATG-F-containing, $n = 33$) in patients who received uBMT from December 2001 to May 2009. In Japan in the era considered in this study, only BMT could be performed from an unrelated volunteer donor. This study was approved by the Institutional Review Board of National Cancer Center, Tokyo, Japan.

Clinical outcomes

Endpoints included neutrophil recovery, overall survival (OS), progression-free survival (PFS), NRM, acute GVHD, chronic GVHD and discontinuation of immunosuppressive drugs. Neutrophil recovery was defined as an absolute neutrophil count (ANC) of $\geq 0.5 \times 10^9$/L for 3 consecutive days. Incidences of grade II–IV or III–IV acute GVHD were based on standard criteria [11]. Chronic GVHD was defined according to Seattle's group criteria [12]. When typical chronic GVHD occurred before 100 days after uBMT, it was also defined as chronic GVHD in this study. Primary GF was defined in accordance with a previous report as an ANC that did not exceed 500/mm^3 or the absence of donor T cells (<5 %) before relapse, disease progression, second HSCT, or death [13]. Secondary GF was defined as a decrease in ANC of <100/mm^3 at 3 determinations or the absence of donor T cells (<5 %) after the initial engraftment without recovery before relapse, disease progression, second HSCT, or death.

Statistical analysis

The probabilities of OS and PFS were calculated by the Kaplan–Meier method. Cox proportional-hazards regression model was used to analyze OS and PFS. The cumulative incidences of engraftment, NRM, GVHD and discontinuation of immunosuppressive drugs were evaluated using a model by Fine and Grey for the univariate and multivariate analyses of cumulative incidence. In the competing risk models for engraftment, GVHD and discontinuation of immunosuppressive drugs, relapse and death before these events were defined as competing risks. In the competing risk models for NRM, relapse was defined as a competing risk. In the competing risk models for GF, relapse and NRM were defined as competing risks. Factors that were associated with a two-sided P value <0.10 in the univariate analysis were included in a multivariate analysis. We used a backward-stepwise selection algorithm and retained only the statistically significant variables in the final model. A two-sided P value <0.05 was considered statistically significant. The variables evaluated in these analyses were as follows: sex, patient's age at the time of uBMT (age $\geq$ 55 years vs. age < 55), disease risk (standard risk vs. high risk), conditioning regimen (TBI 4 Gy vs. TBI 2 Gy vs. ATG-F), and HLA disparity assessed by allele typing of HLA A, B, C and DRB1. Standard risk was defined as the first complete remission of acute leukemia or the first chronic phase of chronic myeloid leukemia. High risk was defined as other hematological malignancies. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) [14]. More precisely, it is a modified version of R commander (version 1.6-3) that was designed to add statistical functions that are frequently used in biostatistics.

Results

Patients' characteristics

The characteristics of the 103 patients are shown in Table 1. The median age was 57 years (range 20–68). Thirty-six (35 %) and 15 (15 %) patients received bone marrow from a donor with an HLA antigen and allele mismatch, respectively. All patients received a RIC regimen as defined by previous reports [15, 16]. The conditioning regimen included Flu ($n = 87$, 180 mg/kg) or cladribine ($n = 16$, 0.66 mg/kg) + Bu (8 mg/kg oral or 6.4 mg/kg i.v.). Targeting of Bu was not performed. The total dose of ATG-F was 10 mg/kg ($n = 13$) or 5 mg/kg ($n = 20$). As GVHD prophylaxis, tacrolimus (TAC) was mainly used in the TBI 2 Gy group and ATG-F group (90 and 90 %, respectively) and cyclosporine (CSP) was mainly used in the TBI 4 Gy group (93 %).

Posttransplant complications

Surviving patients were followed up for a median of 1,494 days after uBMT (range 524–3,466 days). The median follow-up of surviving patients in the TBI 4 Gy group was significantly longer than those in the other

Table 1 Patient characteristics

	TBI 4 Gy *N* (%)	TBI 2 Gy *N* (%)	ATG-F *N* (%)	*P* value
No. of patients	30	40	33	
Age, median (range), year	57 (27–67)	56 (20–68)	57 (24–66)	
Sex (Male/Female)	17/13	28/12	17/16	0.25
Diagnosis				
AML	15 (50)	17 (43)	15 (50)	0.02
MDS	6 (20)	3 (8)	3 (10)	
Lymphoma	5 (17)	20 (50)	14 (47)	
Others[a]	4 (13)	0 (0)	1 (3)	
Disease risk				
Standard	5 (17)	7 (18)	6 (18)	1.00
High	25 (83)	33 (83)	27 (82)	
HLA mismatch				
None	17 (57)	22 (55)	13 (39)	0.30
Mismatch	13 (43)	18 (45)	20 (61)	
GVHD prophylaxis				
CSP-based	28 (93)	4 (10)	5 (15)	<0.05
TAC-based	2 (7)	36 (90)	28 (85)	
Conditioning				
Fludarabine	19 (63)	35 (88)	33 (100)	<0.05
Cladribine	11 (37)	5 (13)	0 (0)	
Time period				
2001–2003	22 (73)	0 (0)	0 (0)	<0.05
2004–2006	8 (27)	16 (40)	11 (33)	
2007–2009	0 (0)	24 (60)	22 (66)	

Cladribine group included more patients with CSP as GVHD prophylaxis (10 patients, 63 %) and more patients transplanted from an HLA matched donor (14 patients, 88 %) as compared to fludarabine group. In patients who received ATG-F, 20 and 13 patients received 5 mg/kg and 10 mg/kg ATG-F, respectively. Whereas all patients with 5 mg/kg received TAC as GVHD prophylaxis, 8 patients (62 %) received TAC in patients who received 10 mg/kg

AML acute myeloid leukemia, *MDS* myelodysplastic syndrome, *CSP* cyclosporin, *TAC* tacrolimus

[a] Others included multiple myeloma, myeloproliferative disorder and acute lymphoblastic leukemia

groups (TBI 4 Gy 2,782 days, TBI 2 Gy 1,164 days, ATG-F 1,473 days), which reflects the change in our clinical practice, but there was no significant difference in the follow-up period between the TBI 2 Gy group and ATG-F group. Neutrophil engraftment was observed in 102 patients (median 18 days, range 11–32 days, Fig. 1a). Engraftment was achieved in 100, 93 and 97 % of the patients at 30 days after uBMT in the TBI 4 Gy, TBI 2 Gy and ATG-F groups, and there was no significant difference among the 3 groups. Primary GF occurred in one patient who received a conditioning regimen with ATG-F. Secondary GF occurred in 7 patients (TBI 4 Gy $n = 1$, ATG-F $n = 6$). Five patients who had GF after uBMT underwent salvage HSCT (cord blood transplant $n = 4$, haploidentical transplant $n = 1$), and 4 patients were successfully rescued. The other two patients had an autologous recovery and one patient had progressive disease before a planned salvage HSCT. The proportion of patients with GF with ATG-F was significantly higher than those in the other 2 groups (3, 0, 21 % in TBI 4 Gy, TBI 2 Gy and ATG-F, respectively; $P = 0.002$). The cumulative incidences of GF including both primary and secondary GF were 3.3, 0, 21.2 % in the TBI 4 Gy, TBI 2 Gy and ATG-F groups, respectively (Fig. 1b). In multivariate analysis, the use of ATG-F was associated with an increased risk of GF as compared to the use of low-dose TBI including both 2 and 4 Gy (HR 16.5, 95 % CI 2.1–130.9, $P = 0.008$).

The cumulative incidences of grade II-IV acute GVHD were 50, 61 and 15 % in the TBI 4 Gy, TBI 2 Gy and ATG-F groups, respectively (Fig. 1c). The use of ATG-F was associated with a significantly lower incidence of grade II–IV acute GVHD as compared to TBI 2 Gy ($P < 0.001$). Multivariate analysis showed that the ATG-F group was associated with a decreased risk of grade II–IV acute GVHD as compared to TBI 2 Gy (hazard ratio [HR] 0.17, 95 % CI 0.06–0.44, $P < 0.001$). There was no statistically significant difference between TBI 4 Gy and ATG-F groups. The cumulative incidences of grade III–IV acute GVHD were 30, 8 and 0 % with TBI 4 Gy, TBI 2 Gy and ATG-F, respectively (Fig. 1d).

The cumulative incidences of chronic GVHD were 50, 57 and 33 % with TBI 4 Gy, TBI 2 Gy and ATG-F, respectively (Fig. 1e). The ATG-F group had a significantly lower incidence of chronic GVHD as compared to the TBI 2 Gy group ($P = 0.038$). There was no statistically significant difference between TBI 4 Gy and ATG-F groups. Multivariate analysis showed that the use of ATG-F was associated with a decreased risk of chronic GVHD as compared to TBI 2 Gy (HR 0.45, 95 % CI 0.23–0.88, $P = 0.019$). The cumulative incidences of extensive chronic GVHD were 30, 45 and 21 % with TBI 4 Gy, TBI 2 Gy and ATG-F, respectively (Fig. 1f). There was no statistically significant difference between TBI 4 Gy and ATG-F groups. The ATG-F group had a significantly lower incidence of chronic GVHD than the TBI 2 Gy group ($P = 0.022$). Multivariate analysis showed that the use of ATG-F was associated with a lower incidence of extensive chronic GVHD as compared to TBI 2 Gy (HR 0.38, 95 % CI 0.17–0.89, $P = 0.025$). There was no statistically significant difference between TBI 4 Gy and ATG-F groups. In the ATG-F group, there was no statistically significant difference between 5 and 10 mg/kg ATG-F in terms of posttransplant complications, including acute and chronic GVHD (data not shown).

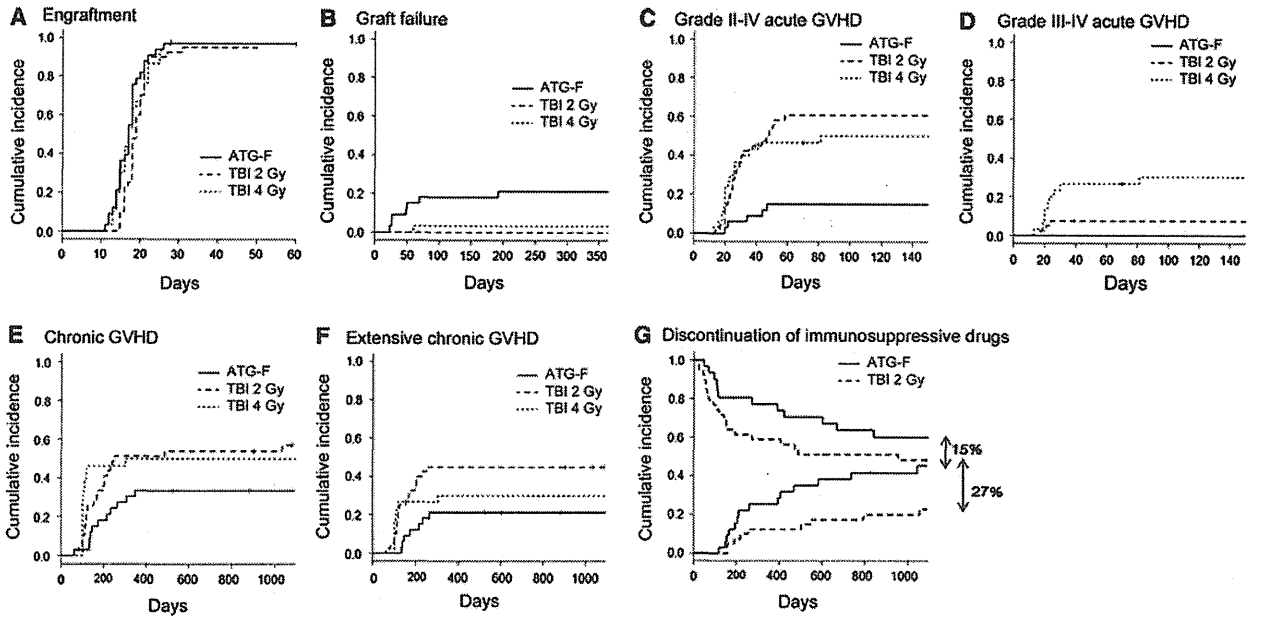

Fig. 1 **a** Engraftment, **b** graft failure, **c** grade II–IV acute GVHD, **d** grade III–IV acute GVHD, **e** chronic GVHD, **f** extensive chronic GVHD and **g** discontinuation of immunosuppressive drugs (*2 lower curves*). The competing risks of relapse and death are shown in the *2 upper curves*. *Bidirectional arrows* show the proportion of surviving patients who continued to receive immunosuppressive drugs at 3 years after uBMT (27 vs. 15 % with TBI 2 Gy and ATG-F, respectively; $P = 0.09$)

The cumulative incidences of the discontinuation of immunosuppressive drugs are shown in Fig. 1g. We excluded the TBI 4 Gy group from this analysis because the incidence of competing events (NRM and relapse) was high. At 3 years after uBMT, immunosuppressive drugs were discontinued in 46 % and 23 % of the patients with ATG-F and TBI 2 Gy, respectively. The probability that surviving patients would continue immunosuppressive drugs in the TBI 2 Gy group tended to be higher than that in the ATG-F group (27 vs. 15 %, $P = 0.09$).

There were no reported cases of PTLD in this case series.

Survival

The probabilities of 2-year OS were 40, 60 and 69 % with TBI 4 Gy, TBI 2 Gy and ATG-F, respectively (Fig. 2a). There was no statistically significant difference among three groups. One year after uBMT, the probability of OS with ATG-F was significantly better than that with TBI 2 Gy and TBI 4 Gy (ATG-F 91 % vs. TBI 2 Gy 68 %, $P = 0.01$; vs. TBI 4 Gy 47 %, $P < 0.01$). A multivariate analysis for OS showed that older age (age ≥55) was associated with an inferior outcome (HR 2.1, 95 % CI 1.1–4.2, $P = 0.03$). The probabilities of 2-year PFS were 40, 53 and 57 % with TBI 4 Gy, TBI 2 Gy and ATG-F, respectively (Fig. 2b). There was no statistically significant difference among three groups. The cumulative incidences of relapse at 2 years after uBMT were 10, 20 and 37 % with TBI 4 Gy, TBI 2 Gy and ATG-F, respectively (Fig. 2c). There was no statistically significant difference among three groups. No covariate including the use of ATG-F was associated with an increased risk for relapse. The cumulative incidences of 2-year NRM were 50, 28 and 6 % with TBI 4 Gy, TBI 2 Gy and ATG-F, respectively (Fig. 2d). A multivariate analysis showed that the ATG-F group had a lower NRM as compared to both TBI 2 Gy and TBI 4 Gy groups, respectively (HR 0.24, 95 % CI 0.07–0.80, $P = 0.020$; HR 0.12, 95 % CI 0.04–0.38, $P < 0.01$). In comparison between TBI 2 Gy and TBI 4 Gy, there was a trend toward an increased risk of NRM with TBI 4 Gy as compared to TBI 2 Gy ($P = 0.074$). In the ATG-F group, there was no statistically significant difference in the clinical outcomes between 5 and 10 mg/kg ATG-F, including OS, PFS and NRM (data not shown). In terms of the type of purine analogue, there was no statistically significant difference in the clinical outcomes between Flu and cladribine, including OS, PFS and NRM (data not shown).

Discussion

We assessed the impact of low-dose ATG-F and low-dose TBI on the clinical outcomes in patients who received an uBMT with a purine analogue plus busulfan-based RIC

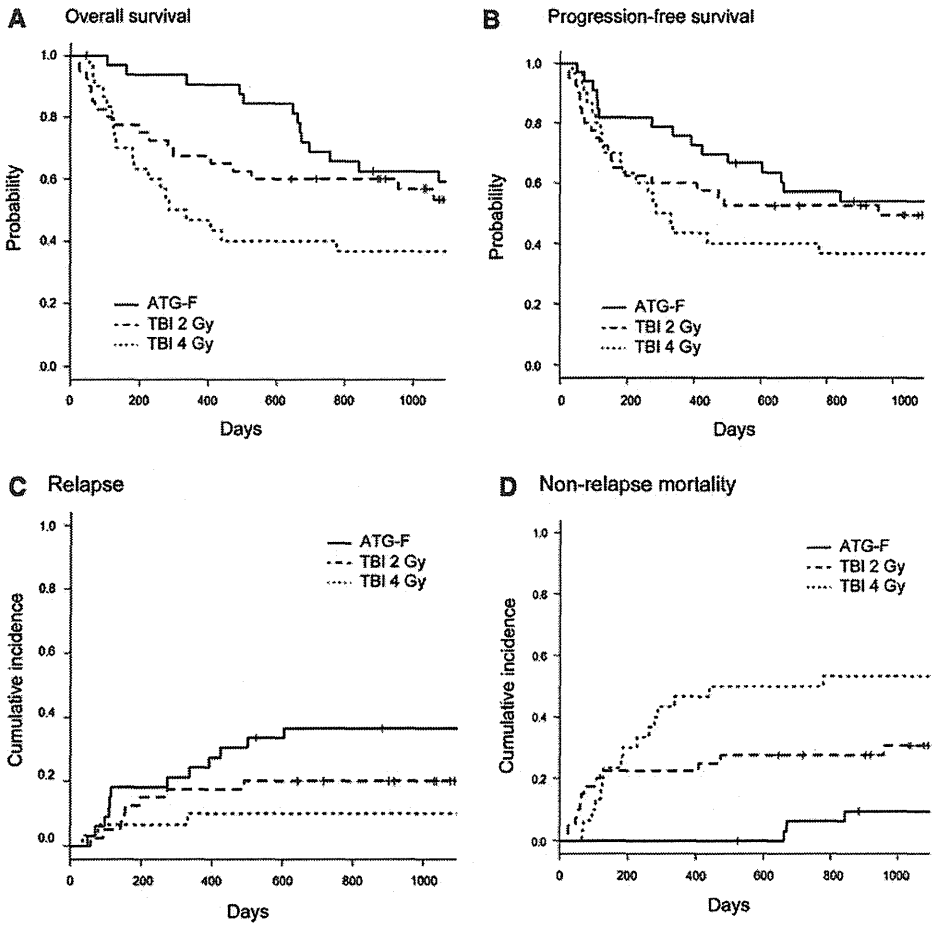

Fig. 2 **a** Overall survival, **b** progression-free survival, **c** relapse and **d** non-relapse mortality

regimen. The incidence of acute and chronic GVHD in patients with ATG-F was low, considering that all patients received BMT from an unrelated donor and about half of the patients received BMT from an unrelated donor with HLA mismatch. The promisingly low incidence of NRM in the ATG-F group is also an important finding in this study, considering the patients' characteristics such as old age and HLA mismatch. Although this study was not a prospective randomized-controlled trial, the incidence of NRM in the ATG-F group was significantly lower than that in the TBI 4 Gy and TBI 2 Gy groups.

In Western countries, the total dose of ATG-F for GVHD prophylaxis is usually 30–60 mg/kg [6]. In Asian countries, a smaller dose of ATG is commonly used, since the incidence of GVHD itself in Asian patients has been shown to be lower than that in Caucasian patients [8, 9]. Kim et al. [17] reported that the use of low-dose ATG (Thymoglobulin, 2.5 mg/kg) was associated with a low incidence of acute GVHD in patients who received an HLA-mismatched unrelated HSCT. In our study, we used ATG-F at a dose of 5 or 10 mg/kg. Use of the lower dose of ATG (5 mg/kg) did not increase the incidence of GVHD and was associated with similar clinical outcomes as compared to the higher dose (10 mg/kg), albeit the size of the study was limited. Soiffer et al. [18] reported that the adverse impact of ATG, which increased the incidence of infectious diseases and relapse, outweighed the lowered risk of GVHD in patients who received a RIC regimen. Therefore, the optimal dosage of ATG-F could differ depending on the intensity of the conditioning regimen. As shown in this study, the regimen with low-dose ATG-F significantly reduced the incidence of GVHD as compared to the TBI-containing regimen without compromising OS, possibly because low-dose ATG-F did not intensively suppress the recovery of lymphocytes that recognize infectious organisms or hematological malignancies. To confirm our finding, prospective studies are needed to assess the impact of low-dose ATG-F with a uniform conditioning regimen and GVHD prophylaxis.

Finke et al. [6] conducted a large randomized-control trial which demonstrated that the incidence of acute GVHD was reduced with ATG-F as GVHD prophylaxis without compromising OS in patients who underwent unrelated HSCT following a myeloablative conditioning regimen.

Furthermore, long-term follow-up revealed that the use of ATG-F significantly reduced the incidence of chronic GVHD [7]. Another randomized trial using ATG in unrelated HSCT also showed that chronic GVHD, especially chronic lung dysfunction, was reduced in patients who received ATG [19]. Consistent with their results, the incidences of acute and chronic GVHD with low-dose ATG-F were significantly lower than those with low-dose TBI in our study. The probability of OS at 1 year after uBMT was significantly better with low-dose ATG-F than with low-dose TBI, which reflects the decrease in GVHD-related deaths in the early phase. In addition to the decreased risk of death in the earlier time period after uBMT, more surviving patients with ATG-F discontinued immunosuppressive drugs as compared to those with TBI 2 Gy. The reduction and better control of chronic GVHD should be associated with an improved quality of life. Such beneficial effects are important in long-term survivors after uBMT.

The major concern with ATG-F in combination with RIC in this study was the high incidence of GF ($n = 7$, 21 %). Even though 4 patients were rescued by salvage HSCT and 2 patients had autologous recovery, GF is a lethal complication after allogeneic HSCT. Therefore, it would be better to avoid a RIC regimen with ATG-F in patients with a high risk of GF, for example untreated MDS with a history of transfusion [20]. One option is to use PBSC instead of BM, since BM is a well-known risk factor for GF [21, 22]. Although another option to improve the rate of engraftment in such cases could be the combination of TBI 2 Gy and ATG-F, we have not yet tested this regimen.

Another concern with ATG-F is PTLD. The incidence of PTLD with in vivo T cell depletion using ATG varies. Soiffer et al. [18] reported that the incidence of PTLD was 2 % in patients who received a RIC regimen with ATG. In our study, there were no cases of PTLD. Although the size of our study was small, the risk of PTLD might be tolerable because T cell depletion was not intense.

The limitations of this study should be clarified. This was a retrospective study that assessed the impact of ATG-F or TBI on the clinical outcomes in patients who received a RIC regimen. At first, TBI 4 Gy group included patients who received uBMT during an earlier time period as compared to the other 2 groups (Table 1). Poor clinical outcome in TBI 4 Gy group was similar to our previous report ($n = 17$, NRM 46 % at 1 year) [4]. The high incidence of NRM in the TBI 4 Gy group in the current study ($n = 30$, NRM 50 % at 2 years) might be partly explained by the increased regimen-related toxicity. Considering the high incidence of severe acute GVHD in the TBI 4 Gy group, GVHD prophylaxis using CSP might be insufficient in uBMT with a RIC regimen. The improvement of supportive care might also affect the incidence of NRM in recent years in the TBI 2 Gy and ATG-F groups as reported [23, 24]. Second, the decision on whether a patient received ATG-F or TBI 2 Gy was based on the preference of each transplant physician and patient, which could lead to a significant selection bias. Furthermore, the characteristics of the patients were heterogeneous. Especially, the underlying disease varied significantly. The benefit of low-dose ATG-F should be re-evaluated.

In conclusion, the use of low-dose ATG-F in combination with a purine analogue plus Bu-based RIC regimen was associated with a promisingly low incidence of acute and chronic GVHD without compromising OS. The NRM rate in the ATG-F group was significantly lower than that in the TBI 4 Gy and TBI 2 Gy groups. The role of low-dose ATG-F as prophylaxis for GVHD should be further assessed with a uniform conditioning regimen in a prospective clinical trial.

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Conflict of interest There is no conflict of interest to declare.

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ORIGINAL ARTICLE

Identification of molecular markers for pre-engraftment immune reactions after cord blood transplantation by SELDI-TOF MS

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Cord blood transplantation (CBT) is frequently associated with pre-engraftment immune reaction (PIR), which is characterized by high-grade fever that peaks around day 9 of transplantation. PIR mimics hyperacute GVHD or engraftment syndrome; however, it is considered to be of different etiology as it occurs before engraftment. Proteomic patterns have been studied in the fields of transplantation, but no specific marker has been identified. As there are no data to confirm the mechanism of PIR, we used a surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF MS) system to identify a specific marker for PIR. The protein expression profile of serum samples from CBT patients was analyzed with a SELDI-TOF MS system. A protein peak that commonly predominated in PIR was purified by an anion exchange column, isolated by SDS-PAGE, and identified by in-gel trypsin digestion, and mass fingerprinting. A 8.6-kDa protein and 11-kDa protein that increased by 10- to 100-fold in the serum of patients during PIR was identified as anaphylatoxin C4a and serum amyloid A. SELDI-TOF MS system in combination with other proteomic methods could serve as a potential diagnostic tool in discovering biomarkers for PIR after CBT.
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Introduction

High-grade fever before engraftment without any other obvious signs of infection, which mimics hyperacute GVHD or engraftment syndrome, is frequently observed in patients who undergo cord blood transplantation (CBT).[1,2] In previous reports, when patients with no evidence of infection or adverse effects of medication exhibited skin eruption, diarrhea, jaundice or body weight gain greater than 10% of baseline, these conditions were defined as 'immune reactions.' These reactions were classified as 'pre-engraftment immune reaction (PIR)' if they developed 6 or more days before engraftment, whereas those within 5 days of engraftment were defined as 'engraftment syndrome' (1). The reported incidence of PIR has ranged from 78–83% (1–2). This PIR peaks at around day 9 of CBT, and is often accompanied by high-grade fever. Although PIR responds well to corticosteroid therapy, the prolonged use of steroid often causes an increased incidence of infectious complications, leading to significant treatment-related mortality, particularly in the elderly. GVHD prophylaxis with tacrolimus, compared with CsA, is less likely to be associated with PIR[3,4] and the addition of MTX may further reduce the risk.[5,6] It has been speculated that cytokines induced by the initial immune/inflammation reaction are the primary cause of PIR, but no data are available to confirm this supposition. To clarify this question, we evaluated the protein expression profile of serum in CBT recipients using a surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF MS) system and found potential markers for PIR.

Materials and methods

Study patients and samples

Patients who received treatment for hematological malignancies at the National Cancer Center Hospital or Toranomon Hospital between February 2002 and May 2005 were included in this study. The study was approved by the Ethics Committee, and written informed consent was given by all patients. A total of 78 peripheral blood samples taken from 57 patients, including 34 samples taken from 13 patients who had undergone allogeneic CBT, were eligible for the analysis. Samples from CBT patients were taken on three different occasions, that is, (1) afebrile period before PIR onset: the median of day 3 (1–6) post transplant; (2) onset of fever: the median of day 8 (6–13); and (3) after resolution of fever: the median of day 26.5 (15–60). To analyze the protein profile that was specific to PIR, samples taken from patients with documented infection

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Table 1 Characteristics of 13 patients

Age	
Median 52 years	
Range (26–70 years)	
Sex	
Male	6
Female	7
Primary disease	
AML	5
ALL	2
Adult T-cell leukemia	2
Diffuse large B-cell lymphoma	2
Peripheral T-cell lymphoma-unspecified	1
Myelofibrosis	1
Conditioning regimen	
Flu 180 mg/m^2, BU 8 mg/kg, TBI 4 Gy	6
Flu 180 mg/m^2, BU 8 mg/kg, TBI 8 Gy	1
Flu 125 mg/m^2, Mel 80 mg/m^2, TBI 4 Gy	5
Flu 125 mg/m^2, Mel 80 mg/m^2, TBI 2 Gy	1
GVHD Prophylaxis	
CsA	6
CsA + short-term MTX	4
Tacrolimus	3
No. of HLA mismatch	
1 locus	3
2 loci	9
4 loci	1
Day of engraftment (n = 11)	
Median 18 days	
Range 13–29 days	
Day of PIR onset	
Median 8 days	
Range 6–13 days	
Day between PIR and resolution	
Median 15.5 days	
Range 7–49 days	
Treatment for PIR	
None	3
Empiric antibiotics	4
Corticosteroids and empiric antibiotics	6

Abbreviations: Flu = fludarabine; Mel = melphalan; PIR = pre-engraftment immune reaction.

or those who were suffering from engraftment syndrome were excluded from the analysis. All 13 CBT patients received reduced-intensity conditioning, and graft rejection occurred in 2 patients (16%). As for the treatment and its outcome for PIR, six patients responded well to corticosteroid and seven patients improved without any treatment or empiric antibiotics alone. One of the patients who developed graft failure received corticosteroids for the treatment of PIR. The mean neutrophil count at PIR was 15 (0–100)/μl. The patients' characteristics are shown in Table 1.

SELDI-TOF MS analysis

The relative protein expression levels were determined as previously described with the following modifications using a SELDI TOF-MS system (Bio-Rad Laboratories, Hercules, CA, USA).[7,8] The protein was processed using a Biomek 2000 Laboratory Work Station (Beckman Coulter, Fullerton, CA, USA). Samples were analyzed in duplicate and 28 spectra were obtained from five serum fractions with four kinds of chips (IMAC30, CM10, H50, Q10), four different binding buffers, two kinds of energy absorption molecules and two focus mass ranges.

Serum fractionation

The serum samples were centrifuged at 20 000 × *g* and the supernatant was vigorously mixed with denaturation buffer U9 (9 M urea: 2% CHAPS: 50 mM Tris-HCl, pH9) for 20 min. Serum samples were fractionated into four fractions by the following methods. Briefly, the strong anion exchange resin BioSepra Q Ceramic HyperD F (Pall, NY, USA) was equilibrated with 50 mM Tris-HCl, pH 9, in advance, and 180 μl per well was loaded onto a filter plate. The loaded resin was equilibrated three times with 200 μl of U1 buffer (U9 buffer diluted 1:10 with 50 mM Tris-HCl). Denatured serum was added to the resin, the sample well was washed with 50 μl of U1 buffer, and the sample was incubated for 30 min at 4 °C. The non-binding fraction was collected, and protein was eluted by a phased pH gradient at pH 5.8, pH 4 and below pH 4.

Protein binding

IMAC30 (immobilized metal affinity capture), CM10 (cation exchange), H50 (reverse-phase) and Q10 (anion exchange) ProteinChip arrays were used for the analysis. To immobilize copper ion on the IMAC30 surface, each spot was incubated with 50 μl of 100 mM copper sulfate for 10 min at room temperature. Excess copper was removed by washing twice with distilled water and incubated with 50 μl of 100 mM sodium acetate (pH 4) for 5 min at room temperature. Each spot was rinsed twice with distilled water before the analysis step.

The following buffers were used for binding and dilution of the samples: 100 mM sodium acetate (pH 4) or 50 mM HEPES (pH 7) for CM10, 100 mM sodium phosphate (pH 7) + 0.5 M NaCl for IMAC30, 50 mM HEPES (pH 7) for H50, and 50 mM Tris-HCl (pH 8) for Q10. The following procedure was commonly used for all chip analyses: (1) Each spot was equilibrated twice with 150 μl of binding buffer on a shaker for 5 min, and excess buffer was removed. (2) The fractionated and unfractionated samples were diluted 10-fold with binding buffer. The diluted samples were loaded onto a chip, and incubated on a shaker for 30 min at room temperature. (3) The chip was washed three times on a shaker for 5 min with 150 μl per spot of buffer. (4) The chip was rinsed twice with 200 μl of distilled water and dried. (5) Each spot was treated with two kinds of energy absorption molecules: 50% saturated sinapinic acid and α-cyano-4-hydroxycinnamic acid.

Protein detection

Captured proteins were detected using a ProteinChip SELDI system (PCS4000 Enterprise, Bio-Rad Laboratories). The maximum detection range was 100 000 with a focus mass range of 3000–10 000 for low MW, and 200 000 with a focus mass range of 10 000–30 000 for high MW. Quantitative analysis of proteins was performed using

ProteinChip Software version 3.2 and ProteinChip Data Manager Software (Bio-Rad Laboratories).

Protein purification and identification

The serum samples were denatured with urea and fractionated by an anion exchange column (ProteinChip Q Spin Columns, Bio-Rad Laboratories) to remove albumin by binding it to the column. The fraction that passed through the anion exchange column at pH 9 was collected. The sample was diluted threefold with 50 mM Tris-HCl (pH 8) and loaded onto an anion exchange column to bind the objective peak protein. The protein was eluted in a phased manner with 50–300 mM NaCl. After demineralization and concentration, the proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. In-gel digestion by Trypsin was performed on the objective band. The protein was determined by mass fingerprinting of the digested peaks against the ProFound database (Rockefeller University

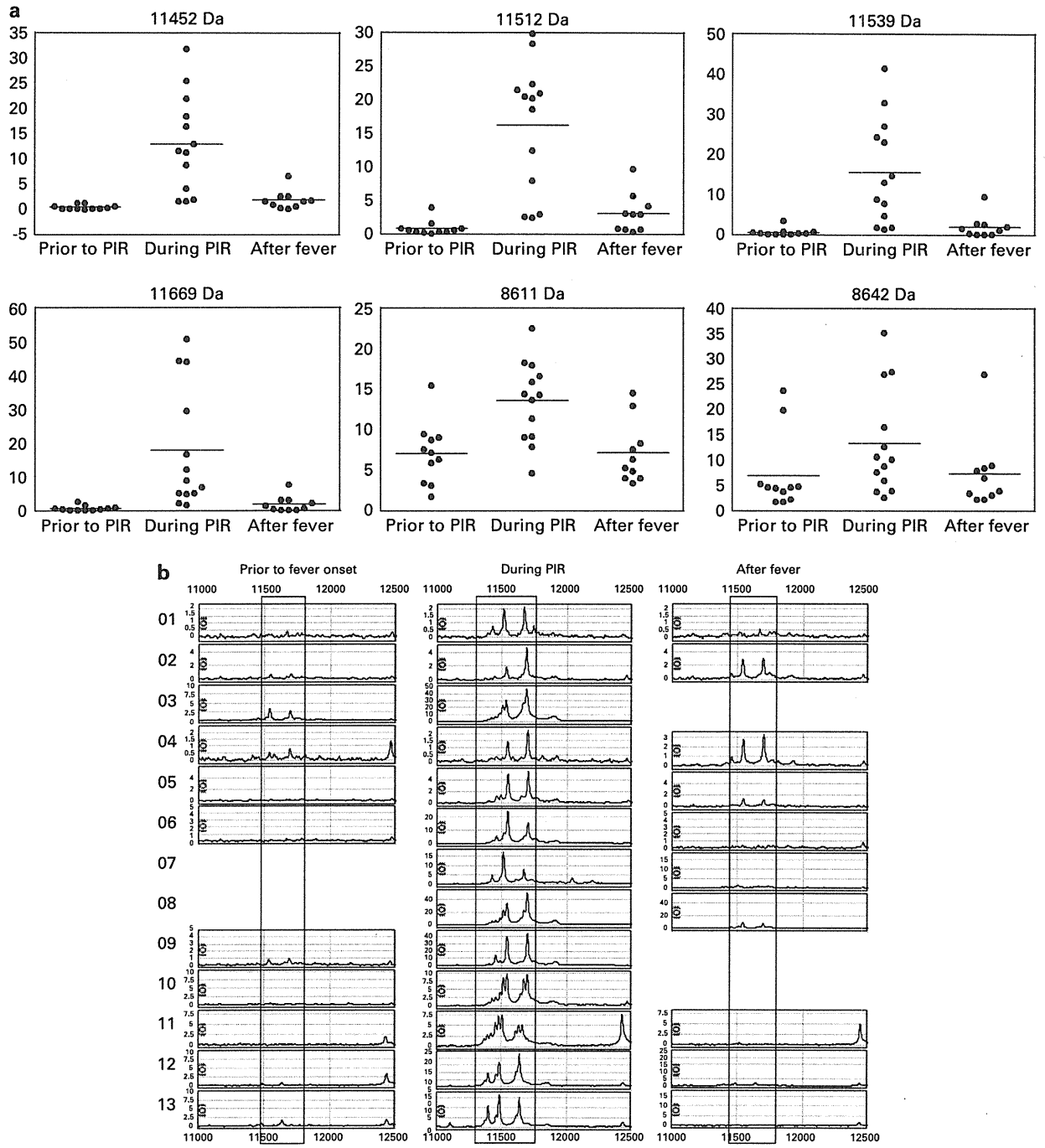

Figure 1 (**a**) Peak intensity levels of six protein peaks that commonly increased at the time of PIR. (**b**) Typical response pattern of the 11-kDa protein peak in 13 patients, in a trace view.

edition), and the amino-acid sequence was determined using the PCI-QSTAR MS/MS search engine.

Statistical analysis

Data were analyzed using ProteinChip Data Manager Software. After baseline correction, MW calibration was performed using eight standard protein molecules followed by a total ion current normalization process. To identify distinct and significant peaks, we used a signal-to-noise cutoff of 2 ($s/n > 2$), which selects peaks with a signal level that is significantly above the calculated background noise.

For the statistical analysis, the Kruskal–Wallis H-test was used to compare differences among three groups. The differences between the two groups were compared with the Wilcoxon–Mann–Whitney *U*-test. Probabilities of $P < 0.05$ were defined as statistically significant.

Results

Protein profiles

A total of 3005 protein peaks for which $s/n > 2$ were detected. Of these, 743 showed a significant difference between the febrile and afebrile periods. After we further excluded noise peaks, 469 peaks still showed a significant difference, and after excluding variations between individuals, 19 candidate peaks that were commonly elevated at PIR in more than 11 patients (84.6%) were selected. Reproducibility was tested, and six protein peaks that commonly increased at the time of PIR, with molecular masses of 8611, 8642, 11452, 11512, 11539 and 11669 Da, were identified (Figure 1). The assay conditions under which the proteins were identified are shown in Table 2.

Purification and determination of target proteins

Protein peaks were fractionated by an anion exchange column, and the elution fraction at pH 9 was used for purification and identification because the albumin that overlaps the candidate peak was removed from this fraction. The protein was eluted from the column with 100–150 mM NaCl. SDS-PAGE after demineralization and concentration of the protein showed an 11-kDa band (Figure 2a). In-gel digestion was performed on the cutout band, and mass fingerprinting was performed for eight peptides with mass values of 1455, 1463, 1550, 1611, 1670, 1706, 1941 and 2097 (Figure 2b). Six of these values were consistent with serum amyloid A (SAA), which consists of

Table 2 Assay conditions by which marker proteins were detected

MW	*Fraction*	*Chip*	*Binding buffer*	*EAM*	*Focus mass range*
8611	pH 9	CM10	100 mM Na Acetate (pH 4)	SPA	3000–10 000
8642	pH 9	CM10	50 mM HEPES (pH 7)	SPA	3000–10 000
11452	Unfractionated	IMAC30	100 mM Na Phosphate (pH 7) +0.5 M NaCl	SPA	10 000–30 000
11512	Unfractionated	IMAC30	100 mM Na Phosphate (pH 7) +0.5 M NaCl	SPA	10 000–30 000
	pH 9	CM10	50 mM HEPES (pH 7)	SPA	10 000–30 000
11539	Unfractionated	Q10	50 mM Tris-HCl (pH 8)	SPA	10 000–30 000
11669	Unfractionated	IMAC30	100 mM Na Phosphate (pH 7) +0.5 M NaCl	SPA	10 000–30 000
	pH 9	CM10	50 mM HEPES (pH 7)	SPA	10 000–30 000
	Unfractionated	Q10	50 mM Tris-HCl (pH 8)	SPA	10 000–30 000

Abbreviations: CM10 = cation exchange; EAM = energy absorption molecule; IMAC30 = immobilized metal affinity capture; SPA = 50% saturated sinapinic acid; Q10 = anion exchange.

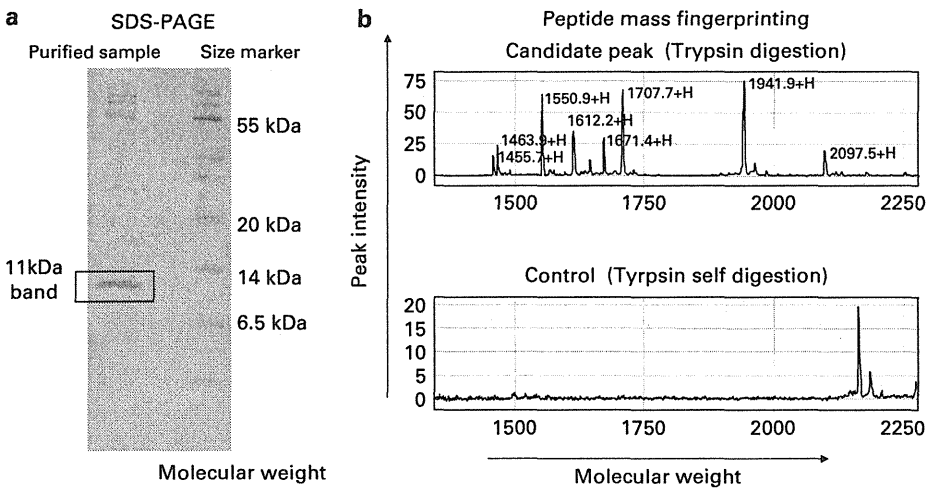

Figure 2 Representative data of SDS-PAGE and peptide mass fingerprinting from the sample taken during PIR. (**a**) SDS-PAGE showing the 11-kDa band. Coomassie Brilliant Blue (CBB) staining. (**b**) Peptide mass fingerprinting of the marker protein.

104 amino acids and has a MW of 11 622, or its isoforms, in which serine and/or arginine is deleted from the N-terminal portion (Figure 3). The amino-acid sequences of all six peptide masses were consistent with SAA by MS/MS analysis.

The SAA level was measured by ELISA in the same sample that was assessed by SELDI-TOF MS. The mean SAA level measured by ELISA before fever onset was 14 (3–51) μg/ml, and this increased to 883 (40–2470) μg/ml at the time of PIR and decreased to 45 (8–126) μg/ml after resolution of the fever (Figure 4a). The data obtained by ELISA agreed with the SELDI-TOF MS peak intensity value (Figure 4b). Although the 8.6 kDa peak was not determined in this experiment, it was most likely to be anaphylatoxin C4a based on its MW (8650) and isoelectric point (9.45).

Serum amyloid A value in different conditions

Seven of the 13 patients with PIR developed acute GVHD, and 2 patients had graft failure. The patients who developed graft failure showed high levels of SAA at PIR (2040 and 2390 μg/ml). The mean and median values of SAA at PIR in seven patients who developed acute GVHD were 677 μg/ml and 451 (60–2470) μg/ml, respectively, which were not significantly different from the values in the four patients without acute GVHD (432 and 506 (40–675) μg/ml) ($P=0.93$).

The SAA value was assessed in 24 non-transplant febrile patients: (a) 12 samples from patients with documented infection, including sepsis, (b) 6 samples from patients with tumor fever and (c) 6 samples from patients with drug-induced fever. The mean and median values and statistical significance when compared with PIR were (a) 477 μg/ml and 347 (31–1240) μg/ml ($P=0.63$), (b) 432 μg/ml and 248 (127–1080) μg/ml ($P=0.75$) and (c) 49 μg/ml and 42 (31–73) μg/ml ($P=0.0013$), respectively.

The SAA values during acute GVHD in other transplantation settings were assessed in 20 patients: (d) 10 samples from related allo-PBSCT recipients including 5 febrile patients and (e) 10 samples from unrelated BMT recipients including 4 febrile patients. The mean and median values and statistical significance when compared with PIR were (d) 293 and 238 (19–645) μg/ml ($P=0.20$) and (e) 366 and 344 (31–724) μg/ml ($P=0.31$), respectively (Figure 4a). The level of SAA elevation was not as high as that in PIR, but the sample size was too small to show specificity.

Discussion

Proteomic analysis has been widely used to assess the allogeneic response, including GVHD in hematopoietic SCT.[8–11] The two most important methods that are used to investigate biomarkers, for example, detection of early GVHD, are SELDI-TOF MS and capillary zone electrophoresis mass spectrometry (CE-MS). Although the resolution and sensitivity of SELDI-TOF MS are not as high as those of CE-MS, it has the benefits of relatively low cost and ease of use.[9] It has been reported that proteomic pattern analysis by SELDI-TOF MS can be used to accurately distinguish GVHD samples from post transplant non-GVHD samples and pretransplant samples with 100% specificity and 100% sensitivity.[8] Furthermore, with the CE-MS system, 16 polypeptide patterns excreted in the urine could be used to discriminate patients with GVHD from patients without complications, with 82% specificity and 100% sensitivity. In addition, 13 sepsis-specific polypeptides

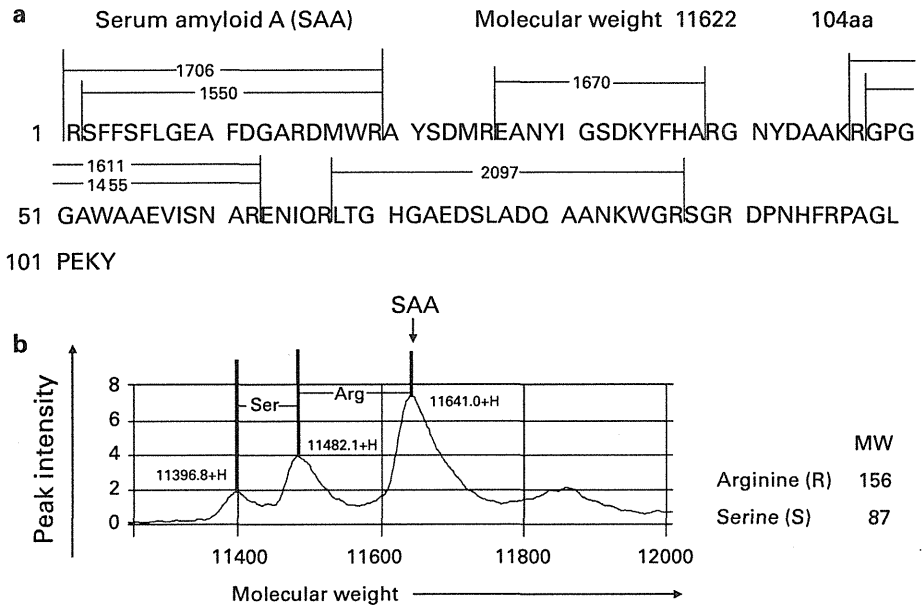

Figure 3 (a) Amino-acid sequences of the target protein. Six peptide sequences that matched an MS/MS database search were identical to amino acids of SAA. (b) The analyzed peak was determined to be SAA and its isoform produced by the deletion of serine and/or arginine from the N terminus.

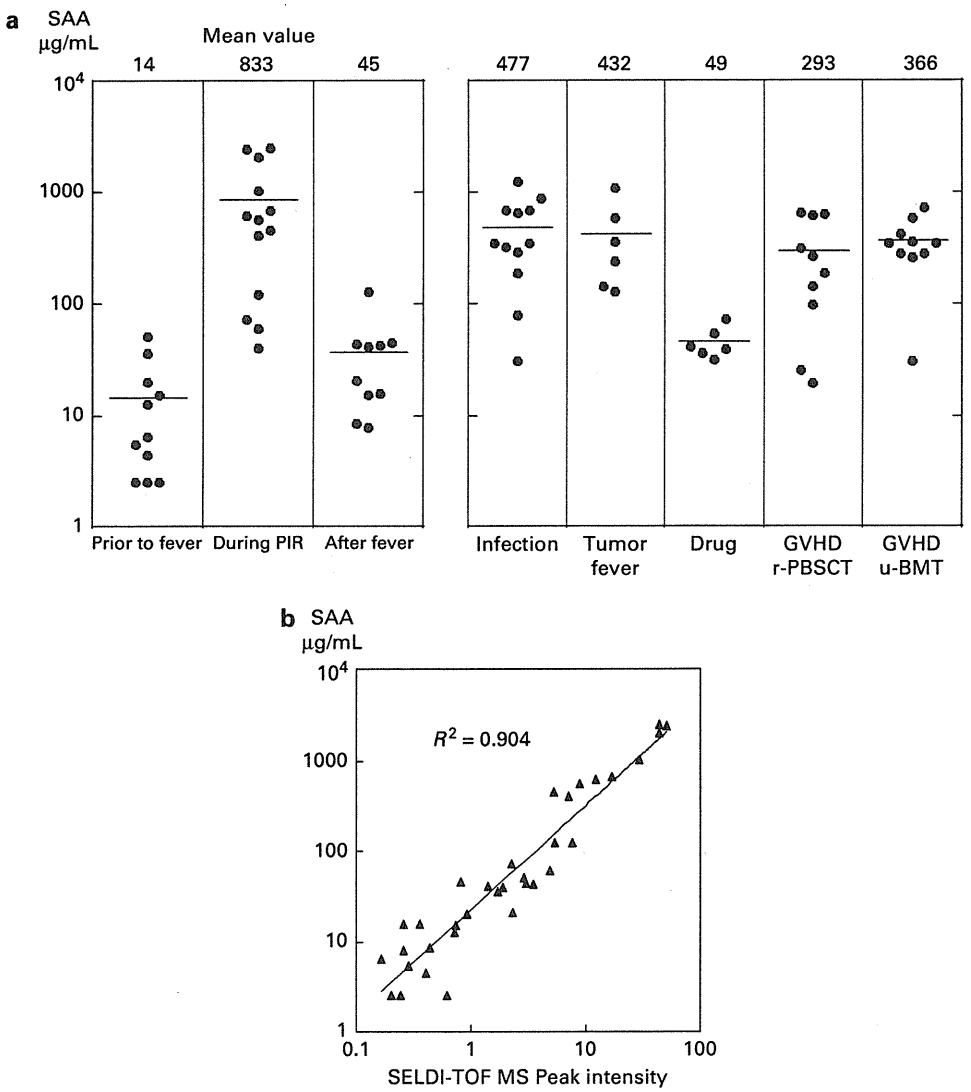

Figure 4 SAA level measured by ELISA. (**a**) SAA level in different conditions: Before fever, during PIR and after fever resolution in 13 CBT recipients. Documented infection including sepsis, tumor fever, drug-induced fever, GVHD in related allo-PBSCT (r-PBSCT) and GVHD in unrelated BMT (u-BMT). (**b**) The data obtained by ELISA correlated well with the SELDI-TOF MS peak intensity value ($n = 34$).

could be used to distinguish sepsis from GVHD, with a specificity of 97% and a sensitivity of 100%.[10] The diagnosis of acute GVHD, even before a clinical diagnosis, is possible with the use of a GVHD-specific model consisting of 31 polypeptides.[11]

Proteomic analysis has also been applied to the analysis of an allograft response in organ transplantation in animal models.[12,13] In a mouse skin transplant model, several protein biomarker candidates were detected by ProteinChip technology based on their molecular mass, which could be used to clearly differentiate between rejection and non-rejection groups, before a clinical manifestation.[12] In a rat small bowel transplantation model, two migration inhibitory factor-related proteins and lysozyme that increased during allograft rejection were identified by a SELDI-TOF MS system.[13] Thus, we believe that ProteinChip technology should be a useful tool for identifying specific markers related to PIR.

Previous studies have shown that combinations of several biomarkers are more sensitive and accurate than the use of a single marker in the diagnosis of an allogeneic response.[11] However, most biomarkers are not well characterized and can only be detected by the ProteinChip system. As the ProteinChip system is not routinely available in clinical practice, we thought it would be necessary to identify a marker that could be monitored easily. In this study, SAA was identified as a candidate marker for PIR. Furthermore, this study showed the feasibility of quantitative analysis by the ProteinChip system, although the ProteinChip system has previously been considered to be a tool for semiquantitative analysis.

Serum biomarkers associated with leukemia[14] and cancer[15–21] have also been identified by the SELDI