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ORIGINAL ARTICLE

Positive impact of chronic graft-versus-host disease on the outcome of patients with *de novo* myelodysplastic syndrome after allogeneic hematopoietic cell transplantation: a single-center analysis of 115 patients

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Abstract

To evaluate the impact of graft-versus-host disease (GVHD) and prognostic factors for patients with myelodysplastic syndrome (MDS) after allogeneic hematopoietic cell transplantation (allo-HCT), we retrospectively reviewed 115 patients with MDS or acute myeloid leukemia with multilineage dysplasia (AML-MLD) after allo-HCT at our center. Eighty one patients received reduced-intensity conditioning (RIC) regimens, whereas 34 received myeloablative conditioning regimens. Although the RIC group was significantly older and included more patients with poor cytogenetic risk, no difference in 4-yr overall survival (OS) was seen between the two groups. In a multivariate analysis, covariates associated with a worse OS were the French-American-British stage of refractory anemia excess blasts in transformation/AML-MLD at peak, poor cytogenetic risk, bone marrow blasts of 20% or higher at HCT and the absence of chronic GVHD (cGVHD). By using semi-landmark analyses, we found that the presence of cGVHD significantly improved OS in high-risk patients or the RIC group. However, there was no difference in OS between those with and without cGVHD among low-risk MDS patients. These findings suggest that the graft-versus-leukemia effect may be more beneficial in high-risk patients who do not receive intensive preparative regimens.

Key words myelodysplastic syndrome; allogeneic hematopoietic cell transplantation; graft-versus-host disease; graft-versus-leukemia effect

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Allogeneic hematopoietic cell transplantation (allo-HCT) has been assumed to be the only treatment modality with curative potential for patients with myelodysplastic syndrome (MDS). However, about 90% of MDS cases occur in elderly patients above the age of 60 yrs (1) and a substantial proportion of them are more likely to have a worse performance status and an increased comorbidity. As a result, myeloablative conditioning (MAC) regimens are less commonly used for patients with MDS because of an increased risk of non-relapse mortality (NRM). However, some studies have reported that the dose intensity of the conditioning regimen plays an important role in controlling the disease after allo-HCT for MDS or acute myeloid leukemia (AML) (2, 3). Reduced-intensity conditioning regimens (RIC) have been developed to decrease the risk of NRM with less-intensive conditioning for elderly or less-fit patients while preserving a graft-versus-leukemia (GVL) effect by an alloimmune reaction as an antitumor effect (4, 5). The European Group for Blood and Marrow Transplantation reported that, among patients with MDS who underwent allo-HCT from a sibling donor, the RIC group was associated with a lower incidence of NRM and a higher risk of relapse in comparison with the

MAC group, whereas overall survival (OS) was similar in both groups (6).

Although an alloimmune reaction by donor T-cells is important for disease control after allo-HCT, especially in the RIC setting, the significance of this effect has not been well documented in patients with MDS. Therefore, we retrospectively reviewed the medical records of 115 patients with *de novo* MDS or AML with multilineage dysplasia (AML-MLD) who underwent their first allo-HCT at our center, and evaluated the impact of graft-versus-host disease (GVHD) and prognostic factors for the outcome in patients with MDS after allo-HCT.

Patients and methods

Patients

This study included patients with *de novo* MDS or AML-MLD who underwent their first allo-HCT at our center between January 2000 and December 2009. The study protocol was reviewed and approved by the institutional ethics committee. Therapy-related MDS and cord blood transplant recipients were excluded. Therapy-related MDS was defined as disease arising in patients who were treated with irradiation, chemotherapy, or both for hematologic malignancies or other cancers. Disease stages were categorized according to the French-American-British (FAB) classification (7). AML-MLD was defined as AML with more than 30% bone marrow (BM) myeloblasts and morphological features of myelodysplasia, or a prior history of MDS. Patients with MDS were classified into two diagnostic groups (Low/Intermediate-1 and Intermediate-2/High) at diagnosis and at peak according to the International Prognostic Scoring System (IPSS) (8). Cytogenetic risk groups were determined according to IPSS using the cytogenetic information at diagnosis. Matching between the donor and recipient was determined according to donor–recipient HLA-A, HLA-B, and HLA-DR compatibility.

Myeloablative conditioning regimens included cyclophosphamide (Cy, 60 mg/kg for 2 d) plus busulfan (Bu, orally 4 mg/kg for 4 d or i.v. 3.2 mg/kg for 4 d) (Bu/Cy) or total body irradiation (TBI, 12 Gy) (TBI/Cy). RIC regimens included Bu (orally 4 mg/kg for 2 d or i.v. 3.2 mg/kg for 2 d) plus fludarabine (Flu, 30 mg/m^2 for 6 d) (Flu/Bu) or cladribine (2-CdA, 0.11 mg/kg for 6 d) (2-CdA/Bu). In a subset of patients who received RIC, low-dose TBI (2 or 4 Gy) and/or low-dose antithymocyte globulin (ATG) (total dose 5–10 mg/kg Fresenius or 2.5–5 mg/kg Thymoglobulin) were added. GVHD prophylaxis included either cyclosporine or tacrolimus alone or a combination of either of the calcineurin inhibitors and methotrexate. The decision regarding the intensity of the conditioning regimen and GVHD prophylaxis for each patient was made at the discretion of the attending physicians based on a review of the patient's age, disease status, comorbidities, performance status and HLA compatibility.

Neutrophil and platelet engraftment dates were defined as the first of three consecutive days with an absolute neutrophil count of 0.5×10^9/L or higher and an untransfused platelet count of 2.0×10^9/L or higher. Acute and chronic GVHD (cGVHD) were diagnosed and graded according to standard criteria (9). Response and relapse of the disease were defined according to standard hematologic criteria.

Statistical analysis

We used the Chi-square analysis and Fisher's exact test to compare categorical covariates and the Mann–Whitney *U* test to compare continuous covariates. OS was estimated by the Kaplan–Meier method, and differences between groups were evaluated by the log-rank test. Relapse and NRM were considered as competing risk events for each other. The probabilities of relapse and NRM were estimated by the cumulative incidence functions, and differences between groups were evaluated by the Gray test (10, 11). OS and the incidences of relapse and NRM were estimated as probabilities at 4 yrs from allo-HCT. To evaluate the effect of cGVHD on OS, we performed semi-landmark analyses (12). For patients with cGVHD, OS was estimated as the probability from the onset of cGVHD by the Kaplan–Meier method. A landmark comparison group consisted of survivors without cGVHD at day 138 (landmark day), which was the median time of the onset of cGVHD with OS for this group estimated as the probability from the landmark day. The Cox proportional hazards regression model was used for univariate and multivariate analyses, and a hazard ratio was calculated in conjunction with a 95% confidence interval (CI). For the assumption of proportional hazards over time, acute GVHD (aGVHD) and cGVHD were treated as time-dependent covariates (13). For multivariate analyses, we decided to include covariates with a *P*-value of <0.1 in univariate analyses. In addition, we included conditioning regimens and GVHD in these models to evaluate their effects on the outcome. The statistical analysis was performed with R-Project (version 2.2.1; http://www.r-project.org/).

Results

Patient characteristics

The characteristics of a total of 115 patients are summarized in Table 1. The median age was 55 yrs (range: 19–68) and the median follow-up of surviving patients was 40 months (range: 4–130). Eighty one patients (70%) received RIC regimens, whereas 34 (30%) received MAC regimens. According to the FAB stage at peak, the proportions of patients with refractory anemia (RA)/refractory anemia with ringed sideroblasts (RARS), refractory anemia

Table 1 Patient characteristics

No. of patients	All $N = 115$	MAC $N = 34$	RIC $N = 81$
Period of HCT (%)			
2000–2004	71 (62)	18 (53)	53 (65)
2005–2009	44 (38)	16 (47)	28 (35)
Age at HCT, median (range)	55 (19–68)	46 (23–57)	57 (19–68)
Age at HCT, yrs			
≧50 yrs (%)	84 (73)	10 (29)	74 (91)
Patient sex, male (%)	82 (71)	24 (71)	58 (72)
FAB stage at diagnosis (%)			
RA/RARS	45 (39)	13 (38)	32 (40)
RAEB/CMMoL	44 (38)	12 (36)	32 (40)
RAEB-T/AML-MLD	26 (23)	9 (26)	17 (20)
IPSS at diagnosis (%)			
Low/Intermediate-1	37 (32)	13 (38)	24 (30)
Intermediate-2/High	64 (56)	16 (47)	48 (59)
Unknown	14 (12)	5 (15)	9 (11)
FAB stage at peak (%)			
RA/RARS	22 (19)	6 (18)	16 (20)
RAEB/CMMoL	38 (33)	10 (29)	28 (34)
RAEB-T/AML-MLD	55 (48)	18 (53)	37 (46)
IPSS at peak (%)			
Low/Intermediate-1	24 (21)	6 (18)	18 (22)
Intermediate-2/High	77 (67)	23 (68)	54 (67)
Unknown	14 (12)	5 (14)	9 (11)
Cytogenetic risk group (%)			
Good/Intermediate	75 (65)	27 (79)	48 (59)
Poor	40 (35)	7 (21)	33 (41)
BM blasts at HCT, median (range)	5 (0–78)	3 (0–46)	4 (0–78)
≦4%	60 (52)	18 (53)	42 (52)
5–19%	38 (33)	10 (29)	28 (35)
≧20%	10 (9)	3 (9)	7 (8)
Unknown	7 (6)	3 (9)	4 (5)
Disease duration, months, median (range)	9 (1–200)	8 (2–200)	10 (1–172)
Karnofsky score at HCT (%)			
90–100	96 (83)	29 (85)	67 (83)
Transfusion dependence (%)	89 (77)	27 (79)	62 (77)
Prior chemotherapy (%)	68 (59)	22 (65)	46 (57)
Donor (%)			
Related	55 (48)	12 (35)	43 (53)
Unrelated	60 (52)	22 (65)	38 (47)
HLA matching (%)			
HLA match (6/6)	101 (88)	31 (91)	70 (86)
HLA mismatch (5/6)	14 (12)	3 (9)	11 (14)
Source of stem cells (%)			
Peripheral blood	52 (45)	11 (32)	41 (51)
BM	63 (55)	23 (68)	40 (49)
Sex mismatch (%)			
Female donor/Male recipient	36 (31)	13 (38)	23 (28)
Other combination	79 (69)	21 (62)	58 (72)
Follow-up duration for survivors, months, median (range)	40 (4–130)	40 (4–130)	47 (4–125)
Conditioning regimen			
MAC (%)			
CY/TBI		15 (44)	
Bu/CY		19 (56)	
Reduced intensity conditioning			
Flu/Bu-based			65 (80)
2-CdA/Bu-based			16 (20)
TBI-containing			23 (28)
ATG-containing			26 (32)
GVHD prophylaxis (%)			
CSP			26 (32)
CSP+MTX		24 (71)	37 (46)
TAC			2 (2)
TAC+MTX		10 (29)	16 (20)

(continued)

Table 1. (contiuned)

MAC, myeloablative conditioning; RIC, reduced intensity conditioning; HCT, allogeneic hematopoietic cell transplantation; 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; mons, months; CY, cyclophosphamide; TBI, total body irradiation; Bu, busulfan; ATG, antithymocyte globulin; Flu, fludarabine; 2-CdA, cladribine; CSP, cyclosporine; MTX, methotrexate; TAC, tacrolimus; GVHD, graft-versus-host disease; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome.

with excess blasts (RAEB)/chronic myelomonocytic leukemia (CMMoL), and refractory anemia excess blasts in transformation (RAEB-T)/AML-MLD were 19%, 33%, and 48%, respectively. According to the cytogenetic risk at diagnosis, the proportions of patients with good/intermediate and poor risk were 65% and 35%, respectively. According to the IPSS risk at peak, the proportions of patients with Low/Intermediate-1 and Intermediate-2/High were 21% and 67%, respectively, and 12% of the patients did not have evaluable data. BM blast counts at allo-HCT were 4% or less in 52%, 5–19% in 33%, 20% or higher in 9%, and not evaluable in 6%. The RIC group was significantly older than the MAC group (median, 57 vs. 46 yrs, $P < 0.001$) and included more patients with poor cytogenetic risk (41% vs. 21%, $P = 0.03$).

Conditioning regimen and GVHD prophylaxis

The conditioning regimen and GVHD prophylaxis are shown in Table 1. The MAC group included either Bu/CY or TBI/CY, followed by a combination of methotrexate and tacrolimus or cyclosporine. The RIC group included Flu/Bu or 2-CdA/Bu, followed by either cyclosporine or tacrolimus alone or a combination of either of the calcineurin inhibitors and methotrexate.

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,

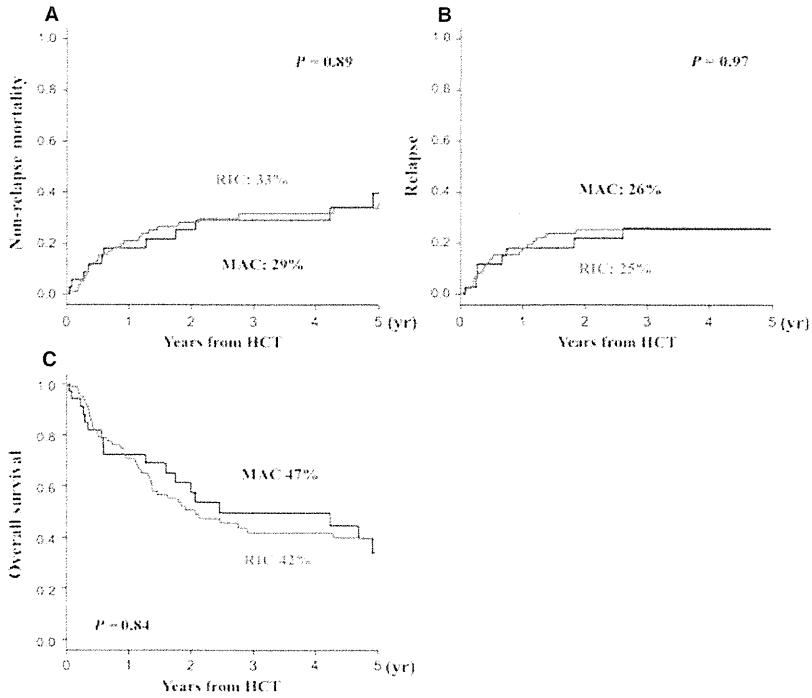

Figure 1 Outcomes stratified according to the intensity of the conditioning regimens. non-relapse mortality (A), Relapse (B) and overall survival (C) of patients with myelodysplastic syndrome receiving allo-hematopoietic cell transplantation after myeloablative conditioning or reduced-intensity conditioning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

	NRM		Relapse		OS	
Variable	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

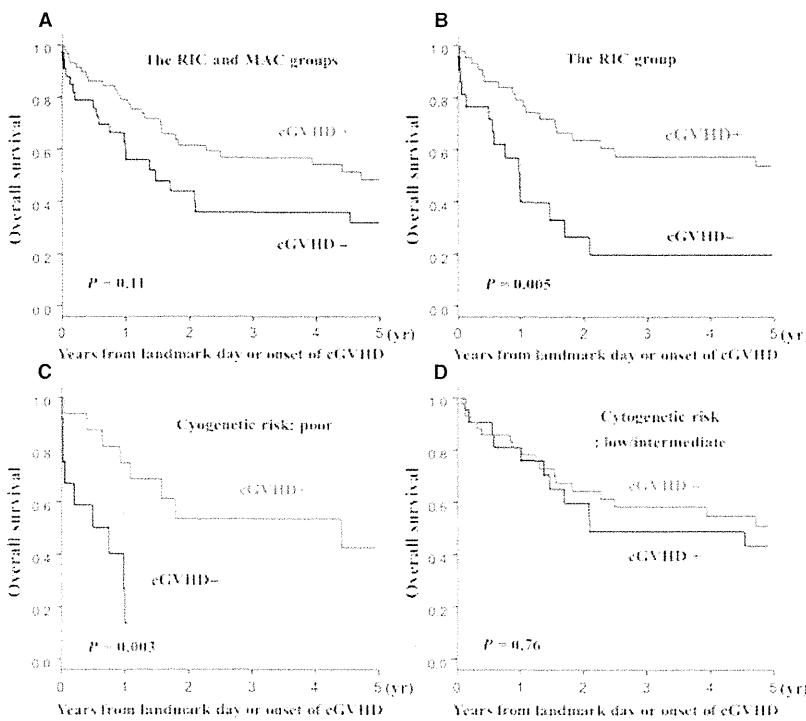

Figure 2 Semilandmark plots illustrating the impact of chronic graft-versus-host disease (GVHD) on overall survival (OS) of patients with myelodysplastic syndrome receiving allo-hematopoietic cell transplantation. OS curves of patients with or without chronic GVHD are shown for the entire population (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

Diagnosis and evaluation of intestinal graft-versus-host disease after allogeneic hematopoietic stem cell transplantation following reduced-intensity and myeloablative conditioning regimens

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Abstract Colonoscopic evaluation of mucosal tissues after allogeneic hematopoietic stem cell transplantation (HSCT) is very useful in evaluating pathogenesis and diagnosis of intestinal graft-versus-host disease (GVHD). However, information on the timing and sites of biopsies and the immunohistological evaluation of mucosal tissues for diagnosing intestinal GVHD, especially following reduced-intensity (RIC) regimens, remains very limited. A total of 33 patients with histologically proven GVHD after allogeneic HSCT with RIC ($n = 23$) and myeloablative conditioning (MAC, $n = 10$) regimens were enrolled in the present study. Colonoscopy was performed due to gastrointestinal symptoms, especially diarrhea and anorexia. Sites of biopsies with the worst histopathological grading were the terminal ileum in 67 % of patients. In the RIC group, the onset of diarrhea prior to colonoscopy examination was later (median: RIC, 57 vs. MAC, 27 days) and the number of patients who developed abdominal pain tended to be higher (RIC, 70 % vs. MAC, 30 %). A lower number of CD4+ cells and a higher ratio of Foxp3+ cells to CD4+ cells were detected in the involved lesions of intestinal GVHD following RIC. These differences in the RIC and MAC groups suggest that regimen-specific therapeutic strategies are required for diagnosing intestinal GVHD.

Keywords Intestinal graft-versus-host disease · Allogeneic hematopoietic stem cell transplantation · Reduced-intensity regimen · HLA-matched donor · Colonoscopy

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Introduction

Acute graft-versus-host disease (GVHD) develops when donor alloreactive T cells recognized alloantigens expressed on host antigen-presenting cells and attack host target epithelium [1]. Many other immune cells, such as Foxp3+ regulatory T (Treg) cells, NKT cells and NK cells, are involved in the pathogenesis of GVHD [2–6]. In addition, damage to host tissue, particularly the gastrointestinal (GI) tract, mainly caused by the conditioning regimen, results in the translocation of endotoxin across damaged mucosal barriers, which augments the cytokine cascade [7, 8]. According to this notion, the use of reduced-intensity conditioning (RIC) regimens could have a favorable impact on the incidence and characteristics of GVHD. In fact, it has been reported that the incidence of GVHD was lower in patients after RIC than myeloablative conditioning (MAC, [9]).

Precise pathological evaluation of the GI tract may provide valuable information about the occurrence of GVHD. Hence, in the present study, we examined biopsy samples obtained by colonoscopy from patients, who suffered from intestinal GVHD after allogeneic hematopoietic stem cell transplantation (HSCT) with RIC or MAC regimens, to evaluate the characteristics of cells infiltrating into the intestinal mucosa.

Patients and methods

Patient characteristics

This study included 258 adult Japanese patients with hematologic malignancies who had undergone their first allogeneic HSCT from a human leukocyte antigen (HLA) matched related ($n = 168$) or unrelated donor ($n = 90$) between January 2002 and May 2006 at the National Cancer Center Hospital in Japan. Typing for HLA-A, -B, and -DR antigens of the donor and recipient was performed by low-resolution DNA typing. RIC regimens consisted of the combination of busulfan (BU, 8 mg/kg) and fludarabine (Flu, 180 mg/m^2; $n = 95$) or 2-chlorodeoxyadenosine (2-CdA, 0.66 mg/kg; $n = 41$) with antithymocyte globulin (ATG, 5–10 mg/kg; $n = 5$) or 2–4 Gy total body irradiation (TBI, $n = 21$), whereas MAC regimens consisted of cyclophosphamide (CY, 120 mg/kg) in combination with either 12 Gy TBI ($n = 38$) or BU (16 mg/kg, $n = 51$) with cytarabine ($n = 3$). GVHD prophylaxis included cyclosporine (CSP) or tacrolimus (TAC) from day −1, with 159 patients receiving short courses of methotrexate (MTX).

Definition of transplant outcomes

Neutrophil engraftment was defined as an absolute neutrophil count (ANC) exceeding 0.5×10^9/L for 3 consecutive days after transplantation. The day of neutrophil engraftment was determined to be the first of these 3 consecutive days. Regimen-related toxicity (RRT) of organ systems which developed within 100 days was graded according to the criteria proposed by the National Cancer Institute-Common Terminology Criteria for Adverse Events v4.0 (CTCAE v4.0). GI symptoms were graded similarly. The onset of acute GVHD and its severity was graded by consensus criteria [10]. Classic or late-onset acute GVHD was distinguished by the time of onset, i.e., whether it developed within or later than 100 days of transplantation, respectively [11]. Patients with Grades II–IV acute GVHD were treated with prednisolone (PSL) according to a standard regimen [12].

Colonoscopic evaluation

All colonoscopies were performed using an Olympus colonoscope (PCF-Q240ZI; Olympus Optical Co., Ltd, Tokyo, Japan). Biopsies of 65 allografted patients were taken at a first colonoscopy examination after transplantation when neutrophil recovery occurred and GVHD was suspected due to GI symptoms including nausea, vomiting, abdominal pain, anorexia and diarrhea. In all cases, microbiologic standard evaluation of stool including screening for *Clostridium difficile* toxin and surveillance with cytomegalovirus (CMV)-antigenemia tested by direct immunoperoxidase staining of leukocytes with peroxidase-labeled monoclonal antibody was performed. Patients receiving preemptive antiviral drugs for CMV-antigenemia or suffering from CMV-colitis were defined as clinical CMV infection. As controls, we evaluated 36 samples from 18 patients who had colorectal cancer and underwent colorectostomy after endoscopic mucosal resection. We confirmed that all these latter samples were negative for cancer cells.

Histological evaluation and immunohistochemistry

Biopsy samples were stained with hematoxylin and eosin, and graded microscopically based on a scale adapted from histological criteria [13] as follows: Grade I, single cell necrosis and apoptosis noted on medium power; Grade II, evidence of epithelial damage by crypt/glandular abscess, epithelial flattening or crypt/glandular dilation; Grade III, dropout of one or more crypts/glands; and Grade IV, total epithelial denudation.

Immunostaining was performed based on the dextran polymer method by use of the Envision + kit (DakoCytomation). Briefly, serial 4 μm sections were cut, deparaffinized and subjected to a heat-induced epitope retrieval step using the autoclave technique before incubation with antibodies. The sections were rinsed in cool running water, washed in phosphate-buffered saline, and incubated with antibodies. These were mouse monoclonals against CD3 (clone PS1, 1:50 dilution, Novocastra, UK), CD4 (clone 1F6, 1:50, Novocastra), CD8 (clone 4B11, 1:50, Novocastra), CD25 (clone 4C9, 1:1000, Novocastra), CD56 (clone Lu-243, 1:200, Novocastra), TIA-1 (clone 2G9A10F5, 1:1000, Beckman Coulter, USA), Granzyme B (clone GrB-7, 1:200, DakoCytomation), Foxp3 (clone ab22510, 1:50, Abcam, UK), and anti-CMV antigens (clones CCH2 + DDG9, 1:50 dilution, DakoCytomation). Bound antibodies were visualized by the 3,3′-diaminobenzidine tetrahydrochloride reaction. All slides were counterstained lightly with hematoxylin and mounted for microscopic examination, and then coded and read in a blinded fashion. Each observer estimated the number of

positive cells per 10 high-power fields (×400 microscopic fields, 10 HPF), using an Olympus BX40 microscope (Olympus, Tokyo, Japan). Samples of reactive lymphoid hyperplasia in the tonsil served as a positive control. Mouse N-universal negative control (cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM; DakoCytomation) was run concurrently. In all cases, the clinical records and the results of routine histology performed at diagnosis were reviewed.

A retrospective analysis was performed on paraffin wax-embedded tissue samples from 65 patients, obtained from multiple sites where there appeared to be mucosal abnormalities. This study was approved by the Ethics Committee, and all patients provided informed consent.

Statistical analysis

Comparisons of variables were performed using the two-tailed Fisher exact test or the Chi-square test. Continuous variables were compared by the Mann–Whitney *U* test. Results of cell enumerations were expressed as means ± SEM. All *P* values were two-sided with type I error rate fixed at 0.05.

Results

Clinical features

Thirty-three patients (51 %, 33/65) were diagnosed as histologically proven GVHD by biopsy samples (RIC, 17 %; 23/136 vs. MAC, 11 %; 10/89; $P = 0.081$). Fourteen patients (22 %, 14/65) as histologically proven GVHD with clinical CMV infection, 12 (18 %, 12/65) as clinical CMV infection without GVHD, 5 (8 %, 5/65) as histologically proven GVHD with both clinical CMV and *Clostridium difficile* infections, or 1 (1 %, 1/65) as histologically proven GVHD with *Clostridium difficile* infection were excluded. Patient characteristics and transplant outcomes in 33 patients with histological proven GVHD are summarized in Table 1. There were significant differences between the RIC and MAC groups in the number of related donors (RIC, 83 % vs. MAC, 40 %; $P = 0.035$), the number of patients who were given MTX for GVHD prophylaxis (RIC, 35 % vs. MAC, 100 %; $P = 0.00050$), the time to neutrophil engraftment (median: RIC 12 vs. MAC 15 days) and the incidence of RRT Grade 3 (RIC, 9 % vs. MAC, 50 %). Prednisolone (PSL) for the treatment of acute GVHD was administered after colonoscopy with

Table 1 Patient characteristics and transplant outcomes

	RIC ($n = 23$)	MAC ($n = 10$)	*P*
Median age of patients (range)	53 (27–63)	48 (25–57)	0.12
Median age of donors (range)	48 (22–65)	37 (29–54)	0.072
Male/female patient	19/4	7/3	0.65
Female donor for male patient	6	2	0.99
Diagnosis			
Acute myeloid leukemia	3	4	0.28
Acute lymphoblastic leukemia	1	2	
Chronic myeloid leukemia	3	0	
Malignant lymphoma	11	2	
Myelodysplastic syndrome	4	2	
Myelofibrosis	1	0	
Donor type (related/unrelated)	19/4	4/6	0.035
Stem cell source (BM/PBSC)	7/16	6/4	0.14
GVHD prophylaxis (CSP or TAC alone/MTX with CSP or TAC)	15/8	0/10	0.00050
Median day to neutrophil engraftment (range)	12 (9–20)	15 (10–31)	0.036
RRT (Grade 3/4)	2/0	5/0	0.016
Acute GVHD (Grade II/III/IV)	4/13/1	6/4/0	0.12
Median onset day (range) of Grade II–IV acute GVHD	28 (14–99)	22 (11–66)	0.10
Use of PSL for GVHD therapy			
0.5 to <1.0/1.0 to <2.0/≥2.0 mg of PSL/kg	2/10/6	3/1/3	0.20
Stage of GI tract 1/2/3/4	3/4/9/1	4/2/2/0	0.45

RIC reduced-intensity regimen, *MAC* myeloablative regimen, *BM* bone marrow, *PBSC* peripheral blood stem cell, *CSP* cyclosporine, *TAC* tacrolimus, *MTX* methotrexate, *RRT* regimen-related toxicity, *GVHD* graft-versus-host disease, *PSL* prednisolone, *GI* gastrointestinal

Table 2 Presenting gastrointestinal symptoms, laboratory data, and clinical and histological diagnosis at the time of biopsy

	RIC	MAC	*P*
Median day of biopsy (range)	63 (14–158)	32 (11–120)	0.11
Gastrointestinal symptoms			
Diarrhea (Grade 1/2/3)	2/4/17	2/3/5	0.40
Median (range) onset day	57 (10–145)	27 (9–103)	0.050
Median (range) duration day[a]	5 (1–16)	5 (2–23)	0.40
Stools/day, median (range)	7 (2–16)	6 (1–14)	0.15
Nausea (Grade 1/2/3)	2/2/7	2/4/2	0.090
Vomiting (Grade 1/2/3)	5/3/0	5/2/0	0.16
Abdominal pain (Grade 1/2/3)	2/6/8	2/1/0	0.054
Anorexia (Grade 1/2/3)	1/0/22	0/2/8	0.073
Laboratory data, median (range)			
WBC ($\times 10^9$/L)	5.2 (1.0–24.2)	4.1 (2.5–23.8)	0.36
Hemoglobin (g/dL)	10.3 (5.6–14.2)	8.2 (7.1–13.0)	0.14
Albumin (g/dL)	3.0 (1.8–4.2)	3.1 (1.7–3.8)	0.98
Total serum bilirubin (mg/dL)	0.7 (0.3–11.8)	0.8 (0.3–3.1)	0.98
Acute GVHD at the biopsy (Grade II/III/IV)	5/15/1	4/4/0	0.42
Classic/late-onset	16/7	8/2	0.69
Stage of GI tract 1/2/3/4	5/6/8/1	4/2/2/0	0.71
Median onset day (range) of acute GVHD with the GI tract	27 (10–99)	22 (11–66)	0.31
Worst histological grading (Grade I/II/III/IV)	9/0/4/10	3/0/5/2	0.14
Sites of biopsies (Terminal ileum/Colon/Rectum)	15/6/2	7/3/0	0.63

[a] Time from onset of diarrhea to colonoscopy examination

biopsies. In patients with histologically proven GVHD, presenting GI symptoms, laboratory data at the time of biopsy, and clinical and histological GVHD diagnosis are summarized in Table 2. In these patients, colonoscopy was performed for GI symptoms including diarrhea ($n = 33$), nausea ($n = 19$), vomiting ($n = 15$), abdominal pain ($n = 19$) and anorexia ($n = 33$). The onset of diarrhea was significantly later in the RIC than the MAC group (median: RIC, 57 vs. MAC, 27 days; range, 10–145 vs. 9–103, respectively; $P = 0.050$). The number of patients who developed abdominal pain tended to be higher in the RIC than the MAC group (RIC, 70 % vs. MAC, 30 %; $P = 0.054$).

Infiltrating cell type in the intestinal mucosa

All 167 samples were classified as histologically proven GVHD and each of the biopsy samples diagnosed as having the worst histopathological grading [13] was selected for the evaluation of infiltrating cells. In 67 % of patients, the sites of biopsies with the worst histopathological grading were the terminal ileum.

Using standard immunohistological techniques, the number and phenotype of mucosa-infiltrating cells was determined in intestinal biopsy samples from the 33 allografted patients with histologically proven GVHD. The number of CD4+ cells was significantly lower in the RIC than the MAC group (RIC, 333 ± 122 vs. MAC, 522 ± 142 per 10 HPF, respectively; $P = 0.042$: Table 3 and Fig. 1a). The ratio of Foxp3+ cells to CD4+ cells was higher in the RIC than the MAC group (RIC, 61 ± 16 vs. 7 ± 2 per 10 HPS, respectively; $P = 0.034$: Fig. 1b).

Table 3 Phenotype of infiltrating cells in intestinal biopsy samples measured by immunoenzymatic labeling

	RIC	MAC	*P*	Control
Cell type				
CD3+	922 ± 158[a]	1126 ± 230	0.35	661 ± 53
CD4+	333 ± 122	522 ± 142	0.042	334 ± 44
CD8+	583 ± 65	609 ± 139	0.84	293 ± 33
CD25+	77 ± 21	76 ± 22	0.83	51 ± 11
CD56+	29 ± 7	23 ± 8	0.60	32 ± 4
TIA-1+	443 ± 68	323 ± 71	0.33	181 ± 38
Granzyme B+	10 ± 2	5 ± 2	0.38	4 ± 1
Foxp3+	79 ± 22	63 ± 26	0.54	49 ± 11
Foxp3+/CD4+ ($\times 10^{-2}$)	61 ± 16	7 ± 2	0.034	7 ± 1
Foxp3+/CD8+ ($\times 10^{-2}$)	14 ± 3	19 ± 9	0.99	18 ± 4

[a] Mean ± SEM

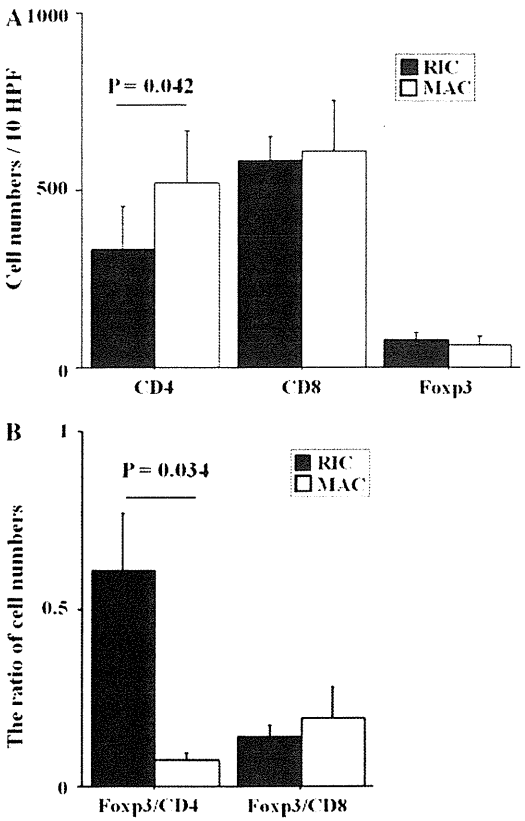

Fig. 1 Comparison of the cell numbers per 10 HPF in intestinal control and biopsy samples. Paraffin-embedded intestinal biopsy samples with signs of GVHD following RIC ($n = 23$) and MAC ($n = 10$) regimens were stained for CD4, CD8, and Foxp3 by immunohistochemistry. The numbers of CD4+, CD8+, and Foxp3 cells (**a**) or the ratios of Foxp3+ cells to CD4+ and CD8+ cells (**b**) were counted per 10 high-power fields (×400, 10 HPF). The results show the mean ± SEM

Discussion

Precise information on the timing and sites of biopsies and the immunohistological evaluation of mucosal tissues after intestinal GVHD, especially in the RIC group, which is very useful in evaluating pathogenesis and diagnosis of GVHD, has been very limited. The results of the present study provide some evidence supporting the notion that there are some differences in intestinal GVHD between the RIC and MAC groups, such as the onset of diarrhea prior to colonoscopy examination was significantly later in the RIC group and the number of patients who developed abdominal pain tended to be higher in the RIC group. The number of CD4+ cells was decreased and the ratio of Foxp3+ cell to CD4+ cells was increased in the RIC group.

In the patients who received colonoscopy, all these patients took an examination when neutrophil recovery occurred and GVHD was suspected due to GI symptoms, especially diarrhea and anorexia. Further studies are needed to detect the marker including the suitable timing of biopsies, i.e., GI symptoms, such as the onset of diarrhea and abdominal pain, for diagnosing an early stage of intestinal GVHD following RIC regimens in all patients who received allogeneic HSCT with and without intestinal GVHD, but prolonged or intensified periods for GVHD prophylaxis may be important to prevent the later onset of intestinal GVHD in the RIC group.

In this study, the sites of biopsies selected for the evaluation of infiltrating cells were those with the worst histopathological grading. This was the terminal ileum in 67 % of patients regardless of RIC and MAC groups. In addition, all samples were classified as histologically proven GVHD. These results suggest that GVHD following both RIC and MAC regimens is possible to be diagnosed at any sites of colon, while samples obtained from the terminal ileum by colonoscopy are more useful for finding severe GVHD lesions than those obtained from other sites, in agreement with previous reports [14].

There were significant differences in the characteristics of infiltrating CD4+ cells between the RIC and MAC groups probably due to the use of adenine nucleoside analog, i.e., fludarabine or chlorodeoxyadenosine, which has a suppressive effect on T cells, predominantly CD4+ cells [15]. Our data could not show the data that agree with murine studies that RIC regimens are associated with less tissue damage and cytokine secretion [16]. However, we should recognize the possibility of overestimating the incidences of intestinal GVHD in the RIC group because there was no difference in the incidence of intestinal GVHD between the RIC and MAC groups probably due to the differences in the patient characteristics, such as the number of related donors and the number of patients given MTX for GVHD prophylaxis, which may influence the repairing process in the intestinal mucosa. The Foxp3+/CD4+ ratio in infiltrating lymphocytes was higher in the RIC than MAC group, indicating that we should not reduce the intensity of GVHD prophylaxis and omit MTX for GVHD in the RIC groups. Because intestinal CD4+ cells and Foxp3+ Tregs play an important role in immunological homeostasis preventing uncontrolled inflammation against intestinal bacteria, whereas the effector phase with damage in the RIC regimen may be different from that in the MAC regimen. Although Foxp3+ Tregs are important for preventing intestinal GVHD [6, 17], the number of infiltrating Foxp3+ Tregs may not be enough to prevent intestinal GVHD, but the balance of infiltrating CD4+ cells and Foxp3+ Tregs might be important. To compare the characteristics of cells infiltrating into the intestinal mucosa, studying the pathophysiological feature of infiltrating lymphocytes may be a useful, but not appear to support these previous data, because of the limitation of colonoscopy examination, such as timing and sites of biopsies.

In summury, our results suggest that GI symptoms, not only diarrhea but abdominal pain, are possible to have been useful for diagnosing intestinal GVHD using colonoscopy, especially in the RIC group. Compared with the MAC group, later onset, lower CD4+ cells, and higher Foxp+/CD4+ ratio in the RIC group indicate that we need to adjust the therapeutic procedures of intestinal GVHD after RIC regimen, including prolonged or intensified periods for GVHD prophylaxis and treatment, using conventional MTX and PSL as well as additional secondary therapies at the beginning, i.e., MMF or ATG.

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Short Article

4. 免疫療法の臨床試験を行う際に考えること

平家勇司

免疫療法の開発においては，治療薬剤の非臨床での有効性・安全性の評価方法，品質管理法が従来の抗腫瘍薬剤と大きく異なっており，また臨床での治療効果の表れ方，安全性のプロファイルも大きく異なっている．immune checkpoint antibodiesの早期臨床試験の結果は目を見張るものがあり，多くの腫瘍内科医の注目を集めることとなったが，免疫療法に適した臨床開発手法は，試行錯誤の状態である．今後，腫瘍内科医と免疫研究者が協力し，新たな開発手法を確立していくことが必要である．

はじめに

長い期間にわたり，腫瘍内科医はがん免疫療法に対して懐疑的な見方をしてきた．しかし，この数年でがん免疫療法は，腫瘍内科医が注目を集める治療法となった．その理由は，抗CTLA-4抗体や抗PD-1抗体，抗PD-L1抗体に代表される，immune checkpoint antibodies※1の目覚ましい治療効果によっている（その詳細については，**玉田の稿**を参照されたい）．特に，2012年のASCOでの発表と同時にNEJM誌に掲載された抗PD-1抗体，抗PD-L1抗体の早期臨床試験の結果は，多くの腫瘍内科医の注目を集めた[1)2)]．その結果を受けて現在行われている第Ⅲ相試験は，今，腫瘍内科医が最も注目している臨床試験の1つである．

前記の試験が注目される理由は，従来の抗がん剤治療と同等あるいはそれ以上の有効性が得られる可能性が強く示唆されたことに加え，腫瘍におけるPD-L1の発現状況と治療効果が相関し，proof of conceptが確立されるとともに，治療効果の予測マーカーが見つかったことに由来する．これらの結果は，免疫治療に興味がある研究者のみならず，化学療法一辺倒であった腫瘍内科医にとっても衝撃的であった．今後，多くの免疫に関連した新規抗腫瘍薬剤が開発されてくると思われる．

それらの背景を踏まえ，本稿では，免疫療法の臨床開発に関し総論的に解説した後，immune checkpoint antibodies，ペプチドワクチン，細胞免疫療法それぞ

[キーワード＆略語]
immune checkpoint antibodies，RECIST，irRC，先進医療B

RECIST：Response Evaluation Criteria in Solid Tumors
irRC：immune-related response criteria
CIC：Cancer Immunotherapy Consortium
CIMT：Association for Cancer Immunotherapy

※1 immune checkpoint antibodies
過剰な免疫反応を制御する分子（immune checkpoint分子：CTLA-4やPD-1など）に対する抗体．immune checkpoint分子は，がんが免疫から逃れる機序の1つであり，抗体を用いてこの分子の働きを阻害することで，がんに対する免疫反応が誘導・増強される．

Basic knowledge for immunotherapy clinical trials
Yuji Heike：Hemotopoietic Stem Cell Transplantation Service, National Cancer Center Hospital（国立がん研究センター中央病院造血幹細胞移植科）

れに関し，特に製剤の品質保証の点からコメントを加える．

なお，従来から存在する腫瘍表面上に発現する抗原（Her2/neu，EGFRなど）に対する抗体療法は，NK細胞などを介した免疫療法ではあるものの，その開発手法はすでに確立されたものとして本稿では割愛する．

1 免疫療法の非臨床試験

免疫治療は，患者の免疫機能を賦活化させることで抗腫瘍効果を誘導することから，腫瘍や腫瘍血管に直接作用する抗腫瘍薬剤と，非臨床における評価方法が大きく異なる．*in vitro*や動物を用いた*in vivo*非臨床安全性試験・有効性試験は，ヒトでのon targetの有効性や安全性の確認には必ずしもつながらないことが指摘されている．免疫治療薬剤の非臨床試験においては，臨床試験に登録される患者の安全性を最大限に確保することを前提にしたうえで，何を実施し，何を実施しないかを，科学的根拠を示しながら判断していくことが必要である．

2 臨床試験の進め方

標準的な臨床試験の進め方は，第Ⅰ相・第Ⅱ相・第Ⅲ相試験という段階を踏んだ開発である．第Ⅰ相試験では用量毒性を指標に，第Ⅱ相以降で使用する薬剤の至適用量を決定する．薬物動態解析も併せて実施する．第Ⅱ相試験は比較的少数の患者を対象に，安全性，有効性の検証を行う試験である．第Ⅲ相試験は，上市後に対象となる患者を対象にした大規模確認試験である．

免疫治療の多くは，その毒性が用量依存性ではないため，毒性を指標とした至適用量の決定ができないうえ，有効性の指標となるバイオマーカーも開発されていない．したがって，臨床第Ⅱ相試験以降の至適投与量は，動物実験から類推される有効量や，物理的な限界量，時に経済的な限界量などで暫定的に決められることも多い．

さらに，近年の傾向として，「第Ⅰ相試験＋拡大第Ⅰ相試験と「第Ⅲ相試験」の二本立ての開発も行われている．このような開発では，第Ⅰ相試験部分で安全性の確認と用量設定を行ったうえで，予想がつかない有害事象や反応性の洗い出しを行うために，拡大第Ⅰ相試験として追加症例を入れるのが原則である．その際，拡大第Ⅰ相で登録する患者を，次相以降の対象症例に絞って登録することで，安全性に加え有効性の感触をつかみ，第Ⅲ相にいきなり進む例がみられている．抗PD-1抗体や抗PD-L1抗体の開発においても，第Ⅰ相試験＋拡大第Ⅰ相試験としてそれぞれ296例，207名の患者が登録され，その後悪性黒色腫，非小細胞肺がん，腎細胞がんを対象とした第Ⅲ相試験が行われている．

3 抗腫瘍効果の評価方法（患者有用性のsurrogate marker）

抗腫瘍薬剤の早期臨床開発における有用性評価方法のゴールデン・スタンダードはRECISTである．RECISTは，「抗がん剤が直接腫瘍を縮小させ，その腫瘍縮小が生存延長につながる」ことを前提としている．しかし，免疫治療では，治療効果が表れるまでに新たな病巣が現れたり，さらに治療効果が表れる時点で炎症が誘導され，所属リンパ節や腫瘍病巣が画像上一時的に増悪しているようにみられる例が散見される．

それらの免疫治療の特徴を踏まえた抗腫瘍効果の評価法として，irRC（immune-related response criteria）※2が提唱された[3]．RECISTとの大きな違いは，新たな測定不能病変の出現はPDとしないこと，さらに標的病変の増大によるPD判定を「25％以上の増大が4週間隔以上をあけて連続して起こること」とした点である．現時点では，腫瘍内科医のすべてがirRC受け入れるには至っていないことから，臨床開発においては，RECISTとirRCでの評価を並行して行い，どちらがより有用であるかを臨床試験のなかで検証していくことが必要と考える．

4 免疫療法は安全か

長い間，免疫治療は安全な治療法といわれてきた．しかし，2006年に起きた，TGN1412（抗CD28スーパーアゴニスト抗体）に関する事例から，免疫治療が

※2 irRC (immune-related response criteria)

RECISTやWHOの評価基準をもとに，免疫治療の特徴を取り入れて提唱された，臨床評価基準．治療効果が得られるまでに時間を要することを考慮し，一定の条件のもとに腫瘍の一時的増悪を許容しているのが特徴である．現時点では，腫瘍内科医のコンセンサスが完全に得られているわけではない．

安全であるとの考えはなくなった[4]．その試験のなかでは，TGN1412が投与された健常ボランティア6名全員が，多臓器不全のためICUに入り人工呼吸器管理となった．その原因は，TGN1412によるヘルパーT細胞の強力な賦活化による，サイトカインストームと結論付けられた．

現在注目を集めている，抗CTLA-4抗体や，抗PD-1抗体，抗PD-L1抗体でも，強い自己免疫反応の誘導がみられている．さらに，最近注目を集めているCD19CAR遺伝子導入T細胞治療においても，サイトカイン放出症候群，B細胞形成不全，マクロファージ活性化症候群など，通常の抗がん剤治療ではみられず，かつ致死的な有害事象が多数みられている[5]．これらのことは，免疫療法は必ずしも安全なものではないということを示している．したがって，免疫療法の臨床開発における安全性確保のためには，従来の抗がん剤治療における副作用対策とは異なった観点での対策が必要となる．また，免疫モニタリングを行うことで，副作用出現を予測しうるバイオマーカーを探索する試みも必要と考えられる．

5 治療薬剤の品質保証

新規治療の臨床開発を行ううえで，使用薬剤の品質保証がなされていることは前提条件である．抗体や遺伝子組換えタンパク質は生物製剤として品質保証が可能であり，化学合成でつくられるペプチドは化学合成品としての品質保証が可能である．

一方，免疫細胞療法で用いられる細胞製剤の品質保証法はまだ確立されていない．原材料となる細胞や，最終製品の滅菌ができないため，ウイルス感染の危険性が完全には否定できない．さらに，動物実験を含めたon targetの有効性・安全性試験が困難であることなど，多くの制約がある．また，原材料のばらつきによる，最終製品の有効性，薬効性のばらつきの問題も存在する．これらの問題は規制当局も十分認識しており，それらに対する基本的考えは，薬食発第0208003号「ヒト（自己）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」，ならびに薬食発第0912006号「ヒト（同種）由来細胞や組織を加工した医薬品又は医療機器の品質及び安全性の確保について」に記載されている．重要なことは，開発品目を最も理解している開発者自身が，安全性を可能な限り確保する原則を堅持したうえで，実施可能なこと・不可能なことを明確にして開発を進めて行くことである．

6 臨床開発の制度（先進医療Bと治験）

わが国での未承認薬の臨床開発の道筋は，2つのフレームに分けて考えることができる．1つは，「先進医療B」であり，その先には「治験」が控えている．先進医療Bは，保険との併用を認めるための制度であるが，同時に，先進医療技術審議会にて技術的妥当性ならびにその試験実施計画などを審査し，わが国の研究の質を向上させ，承認へ向けた治験にスムーズに移行させることも目標としている．その際には，実施組織が，「臨床研究に関する倫理指針」を順守して行うことが求められており，ハードルは決して低くはない．

最近，再生医療推進法が成立し，細胞製剤を用いた臨床開発が加速化されることになった．細胞免疫療法もその流れを受け，今後，積極的な開発が行われることが期待される．一部の研究者からは安全性が確認できた段階で早期承認を行い，その後有効性を確認すればよいとの声も聞こえてくる．しかし，治療法の開発がグローバルとなる現在，わが国だけで承認される治療方法を開発することは意味がない．長い目でみて，開発初期の段階から，国際的に評価される治療法を開発して行くことが重要と感じている．そのために，開発者が海外の規制にも気を配るとともに，わが国の考えが国際的な基準に反映されるように，規制当局の国際化への頑張りにも期待したいところである．

7 免疫モニタリングの重要性

免疫治療は，ここ数年で抗腫瘍薬開発の主流に躍り出たものの，その臨床開発手法はいまだ模索状態である．それに加えて，proof of concept確立のために重要な免疫モニタリング法も標準化されておらず，研究者ごとに解析法，評価方法ともまちまちなのが現状である．科学的，客観的な開発を進めるうえで，これは重大な問題であり，現在CIC（Cancer Immunotherapy Consortium：米国）やCIMT（Association for Cancer Immunotherapy：欧州）が中心となって，免疫モニタリングの標準化が精力的に行われている．今後，わが国においても，個々の研究者の努力ととも