unrelated donors. The available evidence suggests that all of these factors would result in greater numbers of patients with cGVHD. Thus, management of cGVHD is one of the greatest challenges to physicians practicing HSCT.

In the present study, we evaluated infectious complications associated with cGVHD in patients who received an RIC or a CIC regimen before undergoing PBSC transplantation (PBSCT) from a human leukocyte antigen (HLA)-matched relative (related PBSCT) or bone marrow transplantation (BMT) from an HLA-matched unrelated volunteer (unrelated BMT).

Patients and methods

Patient characteristics

We retrospectively analyzed data from 145 consecutive adult patients with hematologic malignancies who had received allogeneic HSCT with an RIC ($n = 91$) or CIC ($n = 54$) regimen between January 2000 and December 2004 at our institution. All of these 145 patients had sustained engraftment, had survived for >100 days following transplantation, and had developed cGVHD. The following types of patients were excluded: patients who suffered from disease progression before the development of cGVHD and received donor lymphocyte infusion, and patients with a history of previous allogeneic HSCT. Significant differences were observed between the RIC and CIC groups in terms of the age of the patients and donors, the gender of the patients, diagnosis, disease risk (8), time from diagnosis to transplantation, donor type and source of stem cells, and GVHD prophylaxis. The patient characteristics are summarized in Table 1. Typing for HLA-A, -B, and -DR antigens of the donor and recipient was performed using low-resolution DNA typing. The frequency with which allogeneic PBSCT is performed in Japan has been increasing since it became eligible for reimbursement from health insurance organizations in the year 2000, and our banking system only approves donation of bone marrow. The clinical characteristics of cGVHD, including use of immunosuppressive drugs at diagnosis and initial treatment, are summarized in Table 2. The present study was approved by the Ethics Committee of our institution, and all 145 subjects provided informed consent.

Conditioning regimen and supportive care

The CIC regimen consisted of cyclophosphamide (CY, 120 mg/kg), in combination with either 12 Gy total-body irradiation (TBI, $n = 25$) or busulfan (BU, 16 mg/kg; $n = 29$). The RIC regimen consisted of BU (8 mg/kg) in combination with either fludarabine (Flu, 180 mg/m^2; $n = 70$) or 2-chlorodeoxyadenosine (2-CdA, 0.66 mg/kg; $n = 21$); 14 patients received either anti-thymocyte globulin (ATG, 5–10 mg/kg; $n = 6$) or 4 Gy TBI ($n = 8$). All patients received cyclosporine (CSP, 3 mg/kg/day; $n = 137$) or tacrolimus (TAC, 0.03 mg/kg/day; $n = 8$), with ($n = 78$) or without ($n = 67$) short courses of methotrexate (MTX; related PBSCT, 10 mg/m^2 on day 1, and 7 mg/m^2 on days 3 and 6; unrelated BMT, 10 mg/m^2 on days 3, 6, and 11) as GVHD prophylaxis. All patients received prophylactic ciprofloxacin (200 mg orally 3 times daily) for prevention of infections until neutrophil recovery. Trimethoprim-sulfamethoxazole (80 mg of trimethoprim once daily) was administered for the prevention of *Pneumocystis* pneumonia and encapsulated bacterial infection, from the first day of the conditioning regimen until day 3, and from day +30 until 6 months after transplantation, or for prolonged periods in patients with cGVHD. Patients also received oral or intravenous fluconazole (100 mg once daily) for prevention of infection by *Candida* species, and low-dose acyclovir (600 mg until engraftment, and then 100 mg/day orally), starting at the same time as the conditioning regimens and continuing until cessation of administration of immunosuppressive drugs (9). Cytomegalovirus (CMV) antigenemia was monitored weekly until cessation of the administration of immunosuppressive drugs. Testing for CMV antigenemia consisted of direct immunoperoxidase staining of leukocytes with a peroxidase-labeled monoclonal antibody. Quantitative real-time polymerase chain reaction was not performed.

Definition of outcome

Patients with grades II–IV acute GVHD were treated with prednisolone (PSL) according to a standard regimen (10). Chronic GVHD was assessed and graded according to the standard criteria (11). The diagnosis and staging of cGVHD were also assessed according to the working report published by the National Institutes of Health Consensus Development Project (12). Relapse was defined either by morphologic evidence of the disease in the peripheral blood, marrow, or extramedullary sites, or by recurrence and persistence of pre-transplant chromosomal abnormalities in cytogenetic analysis of the marrow cells.

Infectious complications

A documented infection was defined as signs and symptoms associated with microbiological documentation of a pathogen from the site of infection. Culture-documented bacteremia, fungemia, or viremia was considered to be a definite infection, regardless of symptoms. On the other hand, clinical infection was defined as signs or symptoms consistent with an infection, but without microbiological confirmation. Central venous catheter (CVC)-related

Patient characteristics and transplant outcomes

	RIC ($n = 91$)	CIC ($n = 54$)	P
Median age of patients (range)	55 (26–68)	37 (18–53)	< 0.0001
Median age of donors (range)	50 (17–69)	34 (19–54)	< 0.0001
Male/female patient	57[1]/34	22/32	0.015
Female donor for male patient	19	10	0.83
Diagnosis AML (+ MDS)	27 (9)	17 (4)	0.0029
MDS	17	4	
CML	7	12	
ALL	1	8	
ML	36	13	
Others[2]	3	0	
Disease risk group (standard/advanced)[3]	14/77	21/33	0.0023
Median time interval[4] (range), (months)	19 (2–178)	10 (1–100)	0.014
KPS[5] $\leq$ 80%	10	5	0.41
HCT-SCI[6] $\geq$ 2	13	7	0.99
Prior infectious complications	6	3	0.99
Prior autologous transplantation	5	2	0.99
Donor type and source of stem cells			
Related PBSC/Unrelated BM	82/9	34/20	0.0002
GVHD prophylaxis			
CSP or TAC alone/MTX with CSP or TAC	66/25	1/53	< 0.0001
Acute GVHD grade II/III/IV	24/23/3	18/8/2	0.072
Median onset day (range) of grades II–IV acute GVHD[7]	39 (12–97)	32 (14–91)	0.48
Prior use of PSL for acute GVHD			
0.5– < 1.0/1.0– < 2.0/ $\geq$ 2.0 mg of PSL/kg	5/34/18	4/13/9	0.27
Relapse/progressive disease following cGVHD	16	10	0.99
Cause of death	30	20	0.27
Infection	15[8]	7[8]	
Chronic GVHD	9[8]	8[8]	
Lungs/gastrointestinal tract/MOF/Others[9]	3/1/3/2	3/3/2/0	
Others[10]	3	6	
Progression	8	2	
Median follow-up (range), (months)	39 (5–73)	45 (15–79)	0.20

[1]Number of patients, unless indicated otherwise.
[2]Others = myelofibrosis ($n = 1$), chronic lymphocytic leukemia ($n = 1$), and multiple myeloma ($n = 1$).
[3]Patients who were considered standard risk with a diagnosis of AML + MDS or AML, or ALL in first complete remission, CML in first chronic phase, or untreated refractory anemia in MDS. All other conditions were considered to indicate advanced risk.
[4]Time from diagnosis to transplantation.
[5]KPS was evaluated before the start of the conditioning regimen, and was graded according to Karnofsky performance status score.
[6]HCT-SCI was evaluated before the start of the conditioning regimen, and was graded according to hematopoietic cell transplantation-specific comorbidity index (ref. 8).
[7]Time from occurrence of grades II–IV acute GVHD to transplantation.
[8]Total number of patients differs because 8 patients (RIC, 5; CIC, 3) died of both infection and chronic GVHD.
[9]Others = renal ($n = 1$) and liver ($n = 1$).
[10]Others = RIC: cerebral infarction ($n = 1$), secondary hepatocellular carcinoma ($n = 1$), infection following secondary allogeneic cord blood stem cell transplantation; CIC: acute myocardial infarction ($n = 1$), cerebral infarction ($n = 1$), drug-induced interstitial pneumonia ($n = 1$), infection following chemotherapy ($n = 1$), and suicide ($n = 2$).
RIC, reduced-intensity regimen; CIC, conventional-intensity regimen; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma; PBSC, peripheral blood stem cell; BM, bone marrow; CSP, cyclosporine; TAC, tacrolimus; MTX, methotrexate; GVHD, graft-versus-host disease; PSL, prednisolone; cGVHD, chronic graft-versus-host disease; MOF, multiple organ failure.

Table 1

Clinical characteristics of cGVHD

	RIC	CIC	*P*
Median onset day (range)[1]	100 (79–479)	109 (93–348)	0.51
Limited/extensive	5/86	1/53	0.41
De novo/quiescent/progressive	27/38/26	24/9/21	0.16
KPS score 1/2/3	61/16/8	46/2/2	0.045
Skin score 1/2/3	27/33/8	17/12/3	0.15
Mouth score 1/2/3	40/29/3	23/9/1	0.87
Eyes score 1/2/3	30/14/7	15/9/1	0.38
Gastrointestinal tract score 1/2/3	28/4/12	19/1/7	0.84
Liver score 1/2/3	7/23/44	6/13/24	0.89
Lungs score 1/2/3	6/8/4	8/7/1	0.26
Joints and fascia score 1/2/3	13/2/0	8/5/1	0.13
Genital tract score 1/2/3	1/0/0	0/0/0	0.99
Eosinophilia $>0.5 \times 10^9$/L	30	22	0.37
Platelets $<100 \times 10^9$/L	26	20	0.36
Others[2]	5	2	0.39
Immunosuppressive drugs at diagnosis of cGVHD			
CSA/TAC	66/3	37/3	0.76
<0.5/0.5–<1.0/1.0–<2.0/ $\geq$ 2.0 mg of PSL/kg	17/9/11/2	9/2/3/1	0.57
Initial treatment for cGVHD			
Addition or increased dose of CSA/TAC	69/7	41/4	0.99
<0.5/0.5–<1.0/1.0–<2.0/ $\geq$ 2.0 mg of PSL/kg	18/14/15/4	10/7/6/2	0.74
Median follow-up from diagnosis of cGVHD (range) (months)	39 (5–73)	45 (15–79)	0.26

[1]Time from occurrence of cGVHD to transplantation.
[2]Others = pleural effusion ($n = 4$), pericardial effusion ($n = 3$), ascites ($n = 3$), and polymyositis ($n = 1$).
RIC, reduced-intensity regimen; CIC, conventional-intensity regimen; cGVHD, chronic graft-versus-host disease; KPS, Karnofsky performance status; CSP, cyclosporine; TAC, tacrolimus; PSL, prednisolone.

Table 2

infections consisted of exit site infections without bacteremia. Bacterial pneumonia was included in the category of definite infections, and was diagnosed by chest x-ray examination or computed tomography (CT) and identification of a bacterial pathogen on culture of sputum, bronchoalveolar lavage fluid, pleural fluid, or blood specimen. Fungal infections, including proven or probable invasive fungal infections, were diagnosed by identification of a fungal pathogen on culture or *Aspergillus* antigen and CT examination according to consensus criteria (13). Pneumonia of unknown origin was included in the category of undefined pneumoniae, which were diagnosed by chest x-ray and/or CT. There was no significant difference in CMV serostatus between the RIC and CIC groups (data not shown). A polymicrobial infection of 1 organ or several adjacent organs was considered to be a single infection. Death associated with a documented infection was defined as the death of a patient with findings consistent with an infection, or as detection of the pathogen in an autopsy specimen.

Statistical analysis

Comparisons of variables were performed using the 2-tailed Fisher exact test or the χ^2 test. Continuous variables were compared by the Mann–Whitney *U*-test. All *P*-values were 2-sided, and the type I error rate was fixed at $P < 0.05$.

Results

Transplant outcomes

The transplant outcomes are summarized in Table 1. Twenty-two patients (RIC, $n = 15$; CIC, $n = 7$) died of infections, of whom 8 patients (RIC, $n = 5$; CIC, $n = 3$) died of

both infections and chronic GVHD, with cGVHD at a median follow up of 40 months from transplantation (RIC, 39 vs. CIC, 45 months). The median onset of cGVHD was 112 days (RIC, 100 vs. CIC, 109 days), and 47 patients (RIC, $n = 26$; CIC, $n = 21$) developed progressive-type cGVHD at a median follow up of 32 months from diagnosis of cGVHD (RIC, 39 vs. CIC, 45 months). The severity of the Karnofsky performance status (KPS) score was significantly greater in the RIC group ($P = 0.045$).

Infectious complications

A total of 134 infectious episodes occurred in 83 patients (RIC, 51 vs. CIC, 32; $P = 0.73$), as shown in Table 3. Of these, 28 patients (RIC, 18 vs. CIC 10; $P = 0.83$) developed bacteremia, the causative organisms (43 positive cultures) of which are summarized in Table 4. Gram-positive bacteremia (27 positive cultures) was more common than gram-negative bacteremia (16 positive cultures). The bacteremia was caused by 2, 3, and 4 types of organisms in 4, 4, and 1 patient, respectively. The incidence of bacteremia was significantly higher in patients with the following factors: cGVHD including progressive types ($n = 15$, $P = 0.0027$), a KPS score ≥ 2 ($n = 11$, $P = 0.0062$) and a gastrointestinal (GI) score ≥ 2 ($n = 13$, $P < 0.0001$); PSL dose ≥ 1 mg/kg at the time of diagnosis ($n = 9$, $P = 0.00090$) and for the initial treatment of cGVHD ($n = 11$, $P = 0.0050$). CVC-related infections ($n = 11$) were caused by *Staphylococcus epidermidis* ($n = 4$), *Staphylococcus* species ($n = 2$), *Stenotrophomonas maltophilia* ($n = 2$), *Acinetobacter iwoffii* ($n = 1$), *Corynebacterium* species ($n = 1$), or methicillin-resistant *Staphylococcus aureus* (MRSA, $n = 1$). The incidence of CVC-related infections was significantly higher in patients with PSL dose ≥ 1 mg/kg at the time of diagnosis of cGVHD ($n = 4$, $P = 0.026$). Bacterial pneumonia was observed in 4 patients, and the isolated organisms were as follows: *Pseudomonas aeruginosa* ($n = 1$), *Hemophilus influenzae* ($n = 1$), *S. epidermidis* ($n = 1$), and *Staphylococcus* species ($n = 1$). The incidence of bacterial pneumonia ($n = 4$) was significantly higher in patients with PSL dose ≥ 1 mg/kg at the time of diagnosis ($n = 3$, $P = 0.0051$) and for the initial treatment of cGVHD ($n = 3$, $P = 0.021$). Invasive aspergillosis (IA) and *Candida* infections developed in 7 and 3 patients, respectively. All patients with IA had been given ≥ 0.5 mg of PSL/kg at the time of diagnosis of cGVHD. The incidence

Infectious complications associated with cGVHD

	Total (median onset, range, days)	RIC	CIC	*P*
Bacterial infections				
Bacteremia	28 (175, 104–1629)	18 (5)[1]	10 (2)	0.83
CVC-related	11 (123, 101–1774)	5 (0)	6 (0)	0.33
Pneumonia	4 (311, 101–1045)	3 (2)	1 (1)	0.99
Others[2]	16 (302, 102–1065)	7 (4)	9 (2)	0.11
Fungal infections				
Candida infection	3 (128, 101–358)	1 (0)	2 (0)	0.56
Invasive aspergillosis	7 (181, 112–1232)	6 (0)	1 (0)	0.26
Viral infections				
Adenoviral hemorrhagic cystitis	8 (192, 111–538)	5 (0)	3 (0)	0.99
CMV colitis	1 (343)	0 (0)	1 (0)	0.37
Cutaneous VZV	18 (502, 106–1684)	12 (0)	6 (0)	0.80
Influenza	4 (483, 355–898)	1 (0)	3 (0)	0.15
Others[3]	2 (133, 103–164)	1 (0)	1 (0)	0.99
CMV antigenemia	15 (140, 104–448)	11 (0)	4 (0)	0.42
Pneumoniae of unknown origin	32 (283, 101–1735)	18 (4)	14 (4)	0.41

[1]Number of infectious episodes (number of deaths) is shown.
[2]Others = sepsis of unknown origin (4 episodes), dermatitis (3), hemorrhagic cystitis (2), otitis media (2), meningitis (2), cholecystitis (1), pseudomembranous enterocolitis (1), and urinary tract infection (1).
[3]Others = herpes simplex viral esophagitis (1 episode) and meningitis (1).
cGVHD, chronic graft-versus-host disease; RIC, reduced-intensity regimen; CIC, conventional-intensity regimen; CVC, central venous catheter; CMV, cytomegalovirus; VZV, varicella zoster virus.

Table 3

Bacteremia associated with cGVHD

	RIC ($n = 18$)	CIC ($n = 10$)
Gram-positive organisms	16[1]	11
Staphylococcus epidermidis	7	2
Streptococcus species	2	3
Enterococcus species	3	0
Staphylococcus species	0	3
Bacillus species	0	1
Corynebacterium species	1	0
MRSA	0	1
Gram-positive cocci	3	1
Gram-negative organisms	10	6
Bacteroides species	3	2
Pseudomonas aeruginosa	2	2
Klebsiella species	2	0
Enterobacter species	0	1
Escherichia coli	0	1
Gram-negative rods	3	0

[1]Number of positive cultures.
cGVHD, chronic graft-versus-host disease; RIC, reduced-intensity regimen; CIC, conventional-intensity regimen; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 4

of IA was significantly higher in patients with cGVHD including a GI score ≥ 2 ($n = 4$, $P = 0.015$), PSL dose ≥ 1 mg/kg at the time of diagnosis ($n = 4$, $P = 0.0037$), and for the initial treatment of cGVHD ($n = 7$, $P < 0.0001$). Eighteen patients developed cutaneous varicella zoster virus (VZV); all responded promptly to acyclovir. Eight patients developed adenoviral hemorrhagic cystitis (HC); 2 of these 8 patients developed continuously complicated lethal bacteremia. The incidence of adenoviral HC was significantly higher in patients with cGVHD including a KPS score ≥ 2 ($n = 5$, $P = 0.0071$) and a GI score ≥ 2 ($n = 4$, $P = 0.026$); PSL dose ≥ 1 mg/kg at the time of diagnosis ($n = 4$, $P = 0.0069$); and for the initial treatment of cGVHD ($n = 5$, $P = 0.0060$). *De novo* CMV antigenemia before or after development of cGVHD was observed in 62 and 15 patients, respectively. Sixteen and 8 patients, respectively, died of bacterial infections and pneumoniae of unknown origin.

Discussion

In the present retrospective analysis, 57% (83/145) of patients with cGVHD developed infections, with a mortality rate of 27% (22/83). Although the limitations of this study were the retrospective study design and the differences in baseline characteristics in both the RIC and CIC groups, these results illustrate the importance of establishing more effective management of infectious complications associated with cGVHD, which are predictive of poor outcome for both RIC and CIC regimens.

In patients with cGVHD, the major source of bacteremia was heterogeneous, gram-positive organisms such as *S. epidermidis* and *Streptococcus* species, which were more common than gram-negative organisms, and bacteremia caused by *Pseudomonas aeruginosa*, including multidrug-resistant *P. aeruginosa*, occurred only in patients with cGVHD involving a GI tract score ≥ 2. Additionally, *Streptococcus pneumonia* sepsis was a risk factor for non-relapse mortality, as reported previously (4), and pneumococcal vaccination of transplant recipients was found to be relatively ineffective in the presence of cGVHD. In other studies with RIC regimens, the incidence of bacteremia appeared to be significantly lower than in the present study, but this may a result of the shorter follow-up periods in those studies (14, 15). Moreover, 29% (7/24) of the present patients with cGVHD involving a GI tract score ≥ 2 had received ≥ 2 mg of PSL/kg before developing cGVHD, and all 7 of these patients developed bacteremia. Although 50% (14/28) of patients with bacteremia received antibiotic

drugs and all 14 of these patients received intravenous immunoglobulin to maintain IgG levels at >400 mg/dL for prophylaxis of encapsulated bacteria and *Pneumocystis*, these results suggest that patients with cGVHD having a GI tract score ≥ 2, especially after high-dose PSL, are more likely to develop bacteremia than patients with cGVHD not having a GI tract score ≥ 2. This was probably due to colonization of the GI tract resulting from translocation into the bloodstream or disruption of the ecologic GI equilibrium involving GI bacterial overgrowth (e.g., use of antibiotic decontamination), increased permeability of the GI mucosal barrier (e.g., GVHD-induced mucosal damage), or deficiencies in the host immune defenses (e.g., use of immunosuppressive drugs). Thus, a review of strategies for prevention of bacteremia may lead to improvement of patient outcomes after allogeneic HSCT. That is, in patients with cGVHD having a GI tract score ≥ 2, restrictions on the use of broad-spectrum antibiotics may help reduce GI bacterial overgrowth, including overgrowth by antibiotic-resistant organisms, resulting from failure of the GI barrier. In contrast, we recognize the difficulty in identifying bacteremia using culturing blood. Our patients were immunocompromised hosts who presented with undifferentiated fever; therefore, blood culture results were often delayed well into the course of empirical therapy. There is a need to develop suitable strategies for screening of bacteremia associated with cGVHD in patients who receive allogeneic HSCT with either RIC or CIC regimens.

Most of the present patients with cGVHD who developed *Candida* infection or IA received ≥ 0.5 mg of PSL/kg before developing cGVHD and the incidence of IA was significantly higher in patients with cGVHD having a GI score ≥ 2, especially after high-dose PSL. The number of patients with fungal infections was small, but high-dose PSL may be effective for improving the prophylaxis for such infections. Furthermore, the duration of prophylaxis still remains unclear as randomized clinical trials have yet to be conducted.

All the present patients with adenoviral HC developed grades II–IV acute GVHD and received PSL for GVHD therapy, which differs considerably from what has been reported previously(16). The incidence of adenoviral HC was significantly higher in patients with cGVHD having a KPS score ≥ 2, a GI score ≥ 2, and high-dose PSL at the time of diagnosis and for the initial treatment of cGVHD. Although the present study was limited in its ability to detect risk factors for adenoviral HC, because of low patient numbers and lack of prospective investigation of viral infection, the present results suggest that patients who receive high-dose PSL before and after developing cGVHD should be frequently checked for abdominal and urinary symptoms, and that urinary tests should be regularly performed during ongoing use of immunosuppressive drugs. In addition, we identified only 1 patient with cGVHD who suffered from CMV colitis, indicating that it is useful to monitor and treat CMV antigenemia intensively in patients receiving immunosuppressive drugs, especially before development of cGVHD. In contrast, 12% of the present patients with cGVHD developed cutaneous VZV with a median onset of 502 days (range, 106–1684), despite low-dose acyclovir prophylaxis during at least the first year after allogeneic HSCT. Nonetheless, there were no cases of breakthrough VZV infection. This suggests that low-dose acyclovir prophylaxis effectively prevented breakthrough VZV infection, but that reestablishment of antiviral therapy was needed to protect against cutaneous VZV in patients with cGVHD.

In summary, the present data indicate that infections associated with cGVHD, especially after high-dose PSL, are predictive of poor outcome, whether RIC or CIC is used. Accordingly, there is a need for clinical trials to develop new strategies for screening and prevention of infections associated with cGVHD in patients who receive allogeneic HSCT with either RIC or CIC regimens.

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Impact of T Cell Chimerism on Clinical Outcome in 117 Patients Who Underwent Allogeneic Stem Cell Transplantation with a Busulfan-Containing Reduced-Intensity Conditioning Regimen

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Within the concept of reduced-intensity stem cell transplantation (RIST) there is a wide range of different regimens used, and little information is available on the clinical impact of chimerism status in patients conditioned with a busulfan-containing regimen. Therefore, we retrospectively reviewed lineage-specific chimerism and the subsequent clinical outcome in 117 patients (median age, 55 years; range: 29-68) who underwent busulfan-containing RIST. The conditioning regimen consisted of busulfan (oral 8 mg/kg or i.v. 6.4 mg/kg) and fludarabine (180 mg/m^2, n = 64) or cladribine (0.66 mg/kg, n = 53), with or without 2-4 Gy total-body irridiation (TBI) (n = 26) or antihuman T-lymphocyte immunoglobulin (ATG; 5-10 mg/kg; n = 31). Chimerism was evaluated with peripheral blood samples taken on days 30, 60, and 90 after transplantation by polymerase chain reaction (PCR)-based amplification of polymorphic short tandem repeat regions. The median follow-up of surviving patients was 1039 days (153-2535). The percent donor-chimerism was significantly higher in granulocyte than T cell fraction throughout the entire course, and the median (mean) values were, respectively, 100% (96%) versus 95% (83%), 100% (98%) versus 100% (89%), and 100% (98%) versus 100% (91%) at days 30, 60, and 90 after RIST. In a multivariate analysis, having received <2 types of chemotherapy regimens before RIST was the only factor that was significantly associated with low donor T cell chimerism (<60%) at day 30 (hazard ratio [HR]: 6.1; 95% confidence interval [CI], 2.1-18.4; $P < .01$). The median percentage of donor T cell chimerism at day 30 was 9% (0%-63%) in 5 patients who experienced graft failure, which was significantly lower than that (97%; 15%-100%) in the rest of the patients ($P < .01$). No correlation was found between the kinetics of T cell chimerism and the occurrence of acute or chronic GVHD (aGVHD, cGVHD). The stem cell source and the addition of TBI or ATG were not associated with the degree of T cell chimerism, overall survival (OS) or event-free survival (EFS). In a Cox proportional hazard model, low donor T cell chimerism of <60% at day 30 was associated with both poor OS (HR: 2.2; 95% CI, 1.1-4.5; $P = .02$) and EFS (HR: 2.0; 95% CI, 1.1-3.8; $P = .02$). In conclusion, we found that 43% of the patients retained mixed donor T cell chimerism (<90% donor) at day 30, whereas 92% achieved complete chimerism in granulocyte fraction. Low donor T cell chimerism of <60% at day 30 may predict a poor outcome, and a prospective study to examine the value of early intervention based on chimerism data is warranted.

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KEY WORDS: Reduced-intensity stem cell transplantation, Chimerism, Busulfan

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) with a reduced-intensity conditioning (RIC) regimen has been increasingly used in patients with hematologic diseases who cannot be candidates for conventional HSCT because of age, medical comorbidities, or prior failed myeloablative SCT. Many different RIC regimens are currently in use, but most of them

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incorporate fludarabine (Flu) as a background agent in combination with other drugs including cyclophosphamide (Cy) [1], melphalan (Mel) [2], busulfan [2,3], low-dose total body irradiation (TBI) [4], antithymocyte globulin (ATG) [3], and alemtuzumab [5].

RIC regimens have been investigated in the hope of reducing toxicity, whereas their engraftment potential and antileukemia effect rely mainly on the expansion of donor-derived cells and subsequent immune-mediated graft-versus-leukemia (GVL) effects [6,7]. In this setting, lineage-specific chimerism analysis to assess the origin of lymphohematopoietic cells becomes particularly important for identifying patients at risk for graft failure/rejection, graft-versus-host disease (GVHD), and relapse or progressive disease (PD) [4,8,9]. Because the posttransplantation chimerism status is based on a fine balance between the cytotoxicity or immunosuppressive potential of the regimen used and the recipient's reserve immunocompetence, each RIC regimen should be evaluated individually for chimerism kinetics [1,4,10-13].

Compared with a regimen that includes Flu and Me, it has been reported that the combination of Flu and i.v. Bu was associated with improved survival in patients transplanted in remission, which was more frequently associated with mixed chimerism [2]. However, very little information is currently available on the clinical impact of lineage-specific chimerism status in patients who are conditioned with a Bu-containing RIC regimen. Therefore, we examined the correlation between specific patterns of lineage-specific chimerism and subsequent clinical outcomes.

PATIENTS AND METHODS

Patients and Transplantation Procedures

We retrospectively reviewed the medical records of 117 patients who had various hematologic malignancies and underwent allogenic HSCT with Bu-containing RIC at our hospital from January 2000 to December 2006. The reasons for selecting RIC regimens included older patient age, medical comorbidities, and prior failed myeloablative SCT. The patients' characteristics are summarized in Table 1. The median age of the patients was 52 years (range: 29-68 years), and the hematologic malignancy included acute myelogenous leukemia (AML) (n = 23), AML evolving from a myelodysplastic syndrome (MDS) (n = 16), acute lymphoblastic leukemia (ALL) (n = 5), malignant lymphoma (n = 44), MDS (n = 16), chronic myelogenous leukemia (CML) (n = 9), chronic lymphocytic leukemia (CLL) (n = 1), multiple myeloma (MM) (n = 1), and atypical CML (n = 2).

The conditioning regimen consisted of Bu (oral 8 mg/kg or i.v. 6.4 mg/kg) and Flu (180 mg/m^2, n = 64) or cladribine (0.66 mg/kg, n = 53), with or without 2-4 Gy TBI (n = 26) or antihuman T-lymphocyte immunoglobulin (Fresenius Biotech GmbH, Germany) (ATG; 5-10 mg/kg, n = 31).

In Japan, only bone marrow is permitted as a stem cell source in transplantation from an unrelated healthy volunteer donor. In the setting of nonmyeloablative SCT from an unrelated donor, the sustained engraftment rate has been reported to be lower for recipients of bone marrow than for those given granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells (G-PBMC) [14]. Therefore, low-dose TBI was also added to the conditioning regimen in 25 of the 32 patients who underwent reduced intensity stem cell transplantation (RIST) from an unrelated bone marrow donor to facilitate engraftment. Recipients of HLA-mismatched grafts tended to receive ATG-containing conditioning regimens (20 of the 27 recipients of HLA-mismatched grafts [74%] versus 11 of the 90 recipients of HLA-matched grafts

Table 1. Association between patients characteristics and donor T-cell chimerism at day 30

Characteristics	Total (n=117)	T cell chimerism at day 30 <60% (n=18)	T cell chimerism at day 30 ≥60% (n=99)
Patient age, years			
Median (range)	55 (29-68)	57 (35-66)	54 (29-68)
<55	56 (48%)	6 (33%)	50 (51%)
≥55	61 (52%)	12 (67%)	49 (49%)
Diseases type			
Acute leukemia	44 (38%)	5 (28%)	39 (39%)
Lymphoma	46 (39%)	6 (33%)	40 (40%)
MDS/MPD	27 (23%)	7 (39%)	20 (20%)
Disease risk			
High	91 (78%)	15 (83%)	76 (77%)
Low	26 (22%)	3 (17%)	23 (23%)
No. of prior chemotherapy regimens			
≥2	77 (66%)	6 (33%)	71 (72%)
<2	40 (34%)	12 (67%)	28 (28%)
Donor			
Unrelated	32 (27%)	2 (11%)	30 (30%)
Related	85 (73%)	16 (89%)	69 (70%)
HLA			
Match	90 (77%)	15 (83%)	75 (76%)
Mismatch	27 (23%)	3 (17%)	24 (24%)
Stem cell source			
G-PBMC	81 (69%)	13 (72%)	68 (69%)
Bone marrow	36 (31%)	5 (28%)	31 (31%)
Conditioning regimen			
2CdA/Bu	24 (21%)	4 (22%)	20 (20%)
2CdA/Bu/ATG	18 (15%)	4 (22%)	14 (14%)
2CdA/Bu/TBI	11 (9%)	1 (6%)	10 (10%)
Flu/Bu	38 (32%)	8 (44%)	30 (30%)
Flu/Bu/ATG	11 (9%)	1 (6%)	10 (10%)
Flu/Bu/ATG/TBI	2 (2%)	0 (0%)	2 (2%)
Flu/Bu/TBI	13 (11%)	0 (0%)	13 (13%)

Acute leukemia (n=44): acute myelogenous leukemia (AML; n=23), AML evolving from a myelodysplastic syndrome (n=16), and acute lymphoblastic leukemia (ALL; n=5); Lymphoma (n=46): malignant lymphoma (44), chronic lymphocytic leukemia (CLL; n=1) and multiple myeloma (MM; n=1); MDS/MPD (n=27): MDS n=16 and MPD including chronic myelogenous leukemia (n=9) and atypical CML (n=2); G-PBMC indicates granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells; 2CdA, cladribine; Bu, busulfan; Flu, fludarabine; ATG, antihuman T-lymphocyte immunoglobulin; TBI, total-body irradiation.

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

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

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

Informed consent was obtained according to the Declaration of Helsinki.

Definitions

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

Chimerism Analysis

We assessed donor-recipient chimerism by the polymerase chain reaction (PCR)-based amplification of a polymorphic short tandem repeat region. Chimerism was evaluated using peripheral blood samples on days 30, 60, and 90 after transplantation. Samples were separated using Ficoll-hypaque into mononuclear cells and a precipitate that included red blood cells and granulocytes. Mononuclear cells were further separated into CD3-positive and -negative fractions with immunomagnetic beads (CD3 Magnetic Particles-DM, BD Pharmingen, San Diego, CA). Granulocytes were collected by lysing red blood cells in the precipitate. Briefly, DNA was extracted from selected cells using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Multiplex PCR was performed using primer sets (AmpFISTR Identifiler Kit, Applied Biosystems, Foster City, CA). Five-color fluorescence detection was performed on an ABI 3100-Avant Genetic Analyzer (Applied Biosystems). For each STR allele, the area under the curve for the corresponding signal was automatically processed using GeneScan 3.7 software (Applied Biosystems). The percentage of donor cells was calculated as (area signal donor)/(area signal donor + area signal recipient). The range of the error of chimerism was regarded as 5% at our laboratory (Heike et al., unpublished data).

Statistical Analysis

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

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

RESULTS

Kinetics of Chimerism

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

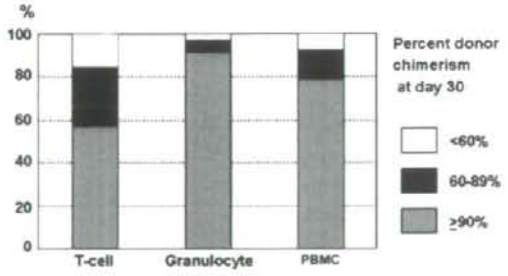

Figure 1. Distribution of chimerism status at day 30 after RIST.

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

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

Graft Composition and Donor Chimerism

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

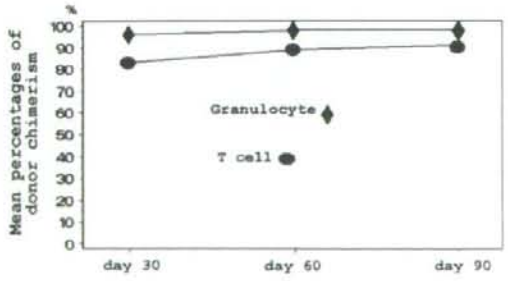

Figure 2. Kinetics of chimerism status after RIST (mean percentages of donor chimerism levels). Percent donor cell chimerism was significantly higher in granulocyte than T cell fraction throughout the entire course, and the mean values were, respectively, 96% versus 83%, 98% versus 89 %, and 98% versus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

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

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

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

Graft failure

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

Whereas 4 of the 5 patients (80%) who experienced graft failure received HLA-mismatched grafts, 23 of the 112 patients (21%) who did not experience graft failure received HLA-mismatched grafts ($P = .01$). In a multivariate analysis, however, neither day 30 T cell chimerism below 60% nor HLA mismatch was associated with an increased risk of graft failure. Among 18 patients with <60% donor T cell chimerism at day 30, HLA mismatch was significantly associated with an increased risk of grafts failure (3 of 3 who received HLA-mismatched graft versus 1 of 15 who received HLA-matched grafts, $P = .005$). In contrast, HLA mismatch was not associated with an increased risk of graft failure in 99 patients with 60% or more donor T cell chimerism at day 30 (1 of 24

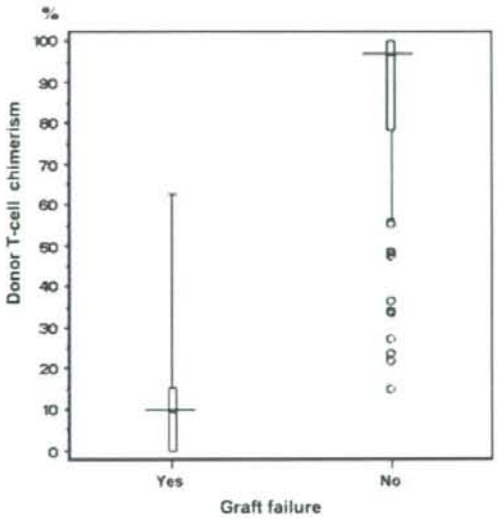

Figure 3. Donor T cell chimerism levels at day 30 in patients with or without subsequent graft failure. Five of the 117 patients (4%) who experienced graft failure had a significantly lower donor T cell chimerism level than the other engrafted patients (n = 112) (donor T cell chimerism, median 9% [range: 0%-63%] versus 97% [range: 15%-100%], respectively) ($p < .01$). Horizontal lines, median; boxes, 25-75 percentile; vertical lines, 10-90 percentile; circles, individual data outside the 10-90 percentile.

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

GVHD

Grade II-IV aGVHD occurred in 54 patients (46%), and cGVHD occurred in 63 patients (64%). No correlation was found between the kinetics of T cell chimerism and the occurrence of aGVHD or cGVHD, as shown in Table 3.

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

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

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

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

NRM and PD

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

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

Cause of death

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

OS and EFS

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

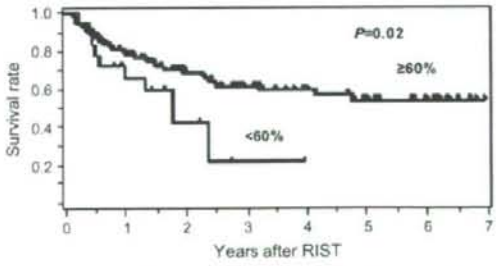

Figure 4. OS stratified according to donor T cell chimerism at day 30. OS was significantly better in patients who achieved 60% or more donor T cell chimerism at day 30 than in those who did not ($P = .02$).

Table 4. Multivariate analysis: factors associated with clinical outcome

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

Introduction

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

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

Results

Engraftment

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

Clinical features of acute GVHD

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

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

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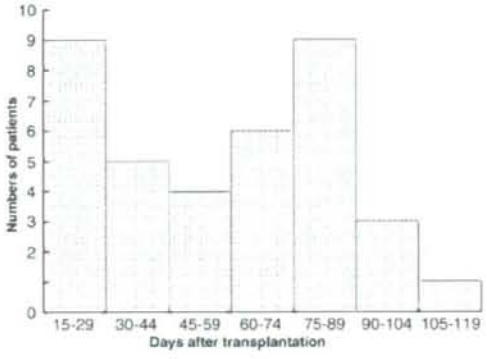

Figure 1. Onset of acute GVHD following RIST. Thirty-six patients developed Grade II–IV acute GVHD. Its median onset was day 58 (range, 17–109), while it was bimodal, peaking days 15–29 and days 75–89 after transplantation.

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

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

Chronic GVHD

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

PFS and its prognostic factors

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

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

Discussion

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

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

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

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

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

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

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

[a] Oxygen saturation <92%.

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

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

[d] These included secondary cancer and interstitial pneumonitis.

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

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

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

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

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

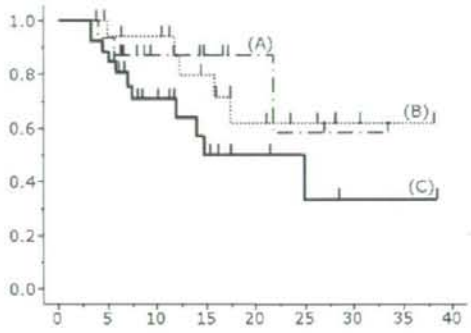

Figure 2. Progression-free survival according to the types of acute GVHD. (A) Patients with early-onset GVHD (n = 17). (B) Patients with late-onset GVHD (n = 18). (C) Patients without acute GVHD (n = 24). Patients who were surviving without relapse at day 100 after RIST were included in the analysis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

Patients and Methods

Data collection and transplantation procedures

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

Transplantation procedures

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

Definition

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

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

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

GVHD, graft-versus-host disease.

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

[b] Statistically significant.

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

Objectives and statistical analysis

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

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

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