transcriptional activity (19). Furthermore, functional modulation of AML1 such as ERK-dependent phosphorylation significantly alters the transforming activity of AML1 (34). To study a role of acetylation in the *in vivo* function of AML1, we compared transforming activities of the wild type and the lysine mutant of AML1. Replication-deficient retroviruses for AML1 and K24A/K43A were generated by hyperexpression of the corresponding plasmid in COS7 cells. NIH3T3 cells were infected with these retroviruses, and soft agar assays were performed on G418-resistant populations. As shown in Fig. 6, *C* and *D*, wild type AML1 rapidly produced a number of macroscopic colonies in soft agar. In contrast, replacement of Lys-24 and Lys-43 to alanines remarkably impaired the transforming activity of AML1, presumably because of the inability to bind to DNA efficiently. AML1 and K24A/K43A showed equivalent expression levels in NIH3T3 cells (data not shown). These results suggest that p300-mediated acetylation on Lys-24 and Lys-43 is also important for the biological activity of AML1 *in vivo*.

DISCUSSION

In this study, we showed that AML1 interacts with p300 and is acetylated on the two conserved lysines in the N terminus adjacent to the Runt domain. Acetylation increases sequence-specific DNA binding of AML1 and is needed for efficient transcriptional activation by AML1. Furthermore, acetylation plays a key role for the transforming activity of AML1 in fibroblasts.

Acetylation of proteins is shown to have both stimulatory and inhibitory effects on transcription (54, 55). As for histones, acetylation is reversible and affects the strength of protein-DNA or protein-protein interactions. In addition, acetylation of several transcription factors, such as p53 and MyoD, enhances transcription of their target genes (56, 57). In contrast, acetylation of *Drosophila* T-cell receptor, high mobility group protein I/Y, and activator of thyroid and retinoic acid receptor results in decreased transcription (58–60). Thus, acetylation plays bipartite roles in the regulation of gene expression. Recently, it was reported that acetylation of E2F enhances its function via multiple mechanisms including protein half-life other than the increased DNA binding activity and transcriptional activation. These findings suggest that acetylation may affect transcription factors at multiple steps (61). In the present case, one can envision several models for the regulatory mechanisms of AML1 by acetylation. First, p300-mediated acetylation may stabilize AML1 through a prolonged protein half-life. However, we could not observe that mutation of Lys-24 and Lys-43 causes a significant difference in protein stabilization when compared with wild type (Fig. 4). Secondly, acetylation may directly increase the affinity of AML1 for DNA. Significant in this regard is our demonstration that the residues of AML1 acetylated by p300 are located in the negative regulatory region for DNA binding N-terminal to the Runt domain (NRDBn) (25, 62). It might be expected that acetylation could induce a conformational change in NRDBn that unmasks the DNA-binding interface of the Runt domain, resulting in potentiation of sequence-specific DNA binding. Schematic model for this hypothesis is shown in Fig. 7. However, Gu *et al.* (63) reported the controversial results that sequences N-terminal to the Runt domain do not affect DNA binding, which does not support this hypothesis. Another possibility is that acetylation may cause an increase in heterodimerization of AML1 with PEBP2β (25, 62). However, substitution of Lys-24 and Lys-43 does not affect the affinity of AML1 to PEBP2β, indicating that acetylation of AML1 does not contribute to heterodimerization with PEBP2β (Fig. 6*A*). Therefore, the increase in DNA binding by p300-mediated acetylation would reflect the altered interaction of

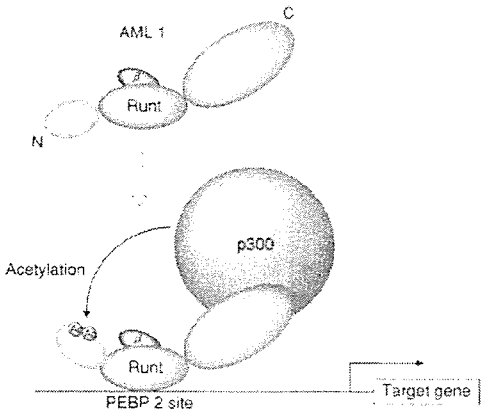

FIG. 7. **Schematic model for a regulatory mechanism of AML1 acetylation.** Physical interaction with p300 induces acetylation of AML1 at the two lysine residues in NRDBn, leading to the increase in DNA binding and transcriptional activation. NRDBn is a negative regulatory region for DNA binding N-terminal to the Runt domain.

AML1 itself with DNA rather than the lack of enhancing effect of the β protein. Finally, acetylation could affect protein-protein interactions, as described for binding of histone tails to the yeast transcriptional repressor Tup1 (64). Along these lines, further studies are in progress to elucidate the effect of AML1 acetylation on binding to other transcription factors such as Ets-1, CCAAT/enhancer binding protein-α, and PU.1.

In contrast to the remarkable effect of p300 acetylation on DNA binding of AML1, an impact on the transcriptional activation is relatively small. This discrepancy could be explained in several ways. First, AML1 manifests its transcriptional activation by participating in the assembly of a high order enhancer complex including other transcription factors as described above. These proteins in the complex may partially compensate for the decrease in DNA binding of AML1, which prevents a total loss of transcriptional activation. In this regard, it should be noticed that DNA binding of AML1 K24R/K43R can be detected to some extent in EMSA using nuclear extracts of COS7 cells, whereas it is much less detectable in EMSA using recombinant proteins (Fig. 5). Since many cooperating factors that associate with AML1 are supposed to exist in the nuclear extracts in contrast to highly purified recombinant proteins, it is reasonable to speculate that the formation of such a complex can partially compensate for decrease in DNA binding of AML1, which may blunt the effect of AML1 acetylation in transcriptional responses. Further investigation is needed to determine the existence of other possible intermolecular interactions. Another possibility is that other functional modifications of AML1, such as phosphorylation and methylation, may dampen the consequence of acetylation in transcriptional responses. In particular, phosphorylation is a critical modification that regulates the DNA binding activity, nuclear localization, protein interaction, and transactivation of various transcription factors. For example, p53 is phosphorylated in response to DNA damage, leading to stabilization and stimulated DNA binding *in vitro* (65, 66). Acetylation of C terminus of p53 is also observed in response to DNA damage. Furthermore, C-terminal acetylation of p53 has been shown to be regulated through its N-terminal phosphorylation induced by DNA damage, indicating an intimate cascade between phosphorylation and acetylation (56, 67, 68). Previously, we demonstrated that transcriptional activation of AML1 is regulated through phosphorylation by ERK at the specific serine residues (Ser-246 and Ser-266). Phosphorylation of AML1 is induced by cytokine stimulation in hematopoietic cells. Taken together with our present studies, it is now clear that AML1 undergoes

two types of posttranslational modifications (Fig. 7). A potential association between phosphorylation and acetylation of AML1 remains to be further investigated.

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Post-transplant complications

Predictors for severe cardiac complications after hematopoietic stem cell transplantation

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Summary:

The value of pre-transplant factors for predicting the development of cardiac complications after transplantation has been inconsistent among studies. We analyzed the impact of pre-transplant factors on the incidence of severe cardiac complications in 164 hematopoietic stem cell transplant recipients. We identified eight patients (4.8%) who experienced grade III or IV cardiac complications according to the Bearman criteria. Seven died of cardiac causes a median of 3 days after the onset of cardiac complications. On univariate analysis, both the cumulative dose of anthracyclines and the use of anthracyclines within 60 days before transplantation affected the incidence of severe cardiac complications ($P = 0.0091$ and 0.011). The dissociation of heart rate and body temperature, which reflects 'relative tachycardia', was also associated with a higher incidence of cardiac complications ($P = 0.024$). None of the variables obtained by electrocardiography or echocardiography were useful for predicting cardiac complications after transplantation, although the statistical power might not be sufficient to detect the usefulness of ejection fraction. On a multivariate analysis, the cumulative dose of anthracyclines was the only independent significant risk factor for severe cardiac complications. We conclude that the cumulative dose of anthracyclines is the most potent predictor of cardiac complications and the administration of anthracyclines should be avoided within two months before transplantation.

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Cardiac complications due to conditioning regimens are well recognized, and these include congestive heart failure, fatal arrhythmia, and cardiac tamponade. The incidence of such complications has varied among studies, from less than 1% to more than 26%.[1,9] The use of high-dose cyclophosphamide in the conditioning regimen has been considered to be the main cause of cardiac toxicity.[2,3,6,9] On the other hand, the usefulness of pre-transplant cardiologic evaluation for predicting cardiac complications is still controversial.[1,2,4,8,9] Braverman *et al*[3] and Fujimaki *et al*[5] showed that the incidence of severe cardiac complications was higher among patients with a low ejection fraction (EF), while Hertenstein *et al* found that there was no correlation between pre-transplant cardiac function and the development of life-threatening cardiac events.[8,9] Recently, Nakamae *et al*[8] and Akahori *et al*[1] reported that QTc dispersion and QTc interval, respectively, were good predictors for cardiac complications, which suggested that electrocardiography (ECG) before transplantation may be useful. In this study, we analyzed the impact of variables obtained by ECG and echocardiography (ultrasound cardiography; UCG) on the incidence of life-threatening cardiac complications after hematopoietic stem cell transplantation.

Patients and methods

Patients

Of the 207 adult patients who underwent hematopoietic stem cell transplantation for the first time between June 1995 and March 2003 at the University of Tokyo Hospital, Japan, we retrospectively reviewed the records of 164 patients for whom a standard 12-lead ECG and UCG within 3 months before transplantation was available. Patient characteristics are shown in Table 1. Acute leukemia in first or second remission, chronic myeloid leukemia in chronic phase, myelodysplastic syndrome with refractory anemia or refractory anemia with ringed sideroblasts, lymphoma or solid cancers in remission, and severe aplastic anemia were defined as standard-risk diseases, while others were considered high-risk diseases. In all, 132 patients underwent allogeneic transplantation, while 31 and one underwent autologous and syngeneic transplantation, respectively. Cyclophosphamide at more than 100 mg/kg was used in 129 patients (79.9%) and ifosfamide at 12 g/m^2 was used in one patient (0.6%). Total body irradiation was applied in 89 patients (54.2%).

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Table 1 Patient characteristics

	Severe cardiac complications		*P*-value
	Positive ($n=8$)	*Negative* ($n=156$)	
Sex (M/F)	3/5	102/54	0.14
Age > 40 years	3/8 (37.5%)	79/156 (50.6%)	0.72
Disease status (standard/high)	4/4	94/62	0.72
History of cardiac disease	0/8 (0%)	13/153 (8.4%)	>0.99
Ferritin level	738 (96.5–1379.9)	632.4 (204.9–1469.7)	0.40
Cumulative dose of anthracyclines			
Low (0–200 mg/m^2)	1	75	0.0031*
Intermediate (201–400 mg/m^2)	2	53	
High (>400 mg/m^2)	5	24	
Anthracycline within 60 days	5/8 (62.5%)	27/142 (19.0%)	0.011*
Radiation involving heart	1/8 (12.5%)	8/152 (4.6%)	0.39
ECG			
ECG abnormality	25.0%	14.7%	0.35
QT interval (ms)	370.3 (302.6–438.0)	379.1 (347.5–410.7)	0.47
QTc interval (ms)	416.4 (369.4–463.4)	424.4 (396.5–452.3)	0.45
QT dispersion (ms)	45.1 (28.2–62.0)	51.3 (30.4–72.2)	0.44
QTc dispersion (ms)	50.9 (33.3–68.5)	57.4 (34.0–80.8)	0.45
UCG			
EF (< 55%)	2/8 (25.0%)	16/142 (11.2%)	0.20
LAD (mm)	31.3 (23.5–39.1)	32.8 (26.9–38.7)	0.48
LVDd (mm)	48.0 (44.0–52.0)	48.0 (43.3–52.7)	0.99
LVDs (mm)	33.6 (28.7–38.5)	31.4 (26.9–35.9)	0.18
IVSth (mm)	8.5 (7.1–9.9)	8.8 (7.3–10.3)	0.55
PWth (mm)	8.4 (7.1–9.7)	8.6 (7.1–10.1)	0.64
E/A ratio	1.51 (0.71–2.31)	1.41 (0.83–1.99)	0.75
Vital			
Heart rate (beats/min)	80 (57–103)	76 (63–90)	0.50
Systolic blood pressure (mmHg)	108 (82–134)	112 (99–124)	0.46
HR–BT index (>25)	3/8 (37.5%)	8/138 (7.2%)	0.024*
Regimen			
Includes high-dose Cy or IFM	62.5%	80.8%	0.20
Includes TBI	4/8 (50.0%)	85/156 (54.5%)	>0.99
Stem cell			
Auto/allo	3/5	28/128	0.17
Bone marrow/peripheral blood	5/3	94/62	>0.99

*Statistically significant.

Evaluation of pre-transplant factors

QT intervals were measured manually from the beginning of the QRS complex to the end of the T wave. The average of two consecutive QT intervals was calculated as the QT interval for each lead and the QT interval for each patient was calculated as the mean QT interval of all available leads. QT dispersion was defined as the difference between the longest and shortest QT interval. Each value was corrected with Bazett's formula. QT dispersion could not be determined in three patients either because there were fewer than six readable leads ($n=2$) or due to frequent premature ventricular contractions ($n=1$).

Left ventricular EF and the E/A mitral Doppler ratio were evaluated by UCG as indices of systolic and diastolic functions, respectively. The cutoff of EF was determined as 55%, because the best *P*-value was obtained at this cutoff by univariate analyses. The following variables were also evaluated: left atrial dimension (LAD), left ventricular end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), end-diastolic intraventricular septal thickness (IVth), and left ventricular posterior wall thickness (PWth).

Blood pressure, heart rate (HR), and body temperature (BT) were calculated as the means of respective values measured on 2 consecutive days prior to the conditioning regimen. The dissociation of HR and BT, called the HR–BT index, was calculated to evaluate 'relative tachycardia' as follows, assuming that the normal HR was 80 beats/min at 37°C and increased by 20 beats/min with an increase in BT of 1°C:

$$\text{HR–BT (beats/min)} = \text{HR (beats/min)} - [80 + (\text{BT (°C)} - 37) \times 20]$$

The mean HR–BT calculated from values measured on 2 consecutive days was used for the analysis.

The cumulative dose of anthracyclines was calculated as the equivalent dose of native doxorubicin, assuming that the cardiac toxicity at an equal dose is 0.5, 0.8, 3.4, 0.6, 1.6, and 0.1 for daunorubicin, pirarubicin, mitoxantrone,

epirubicin, idarubicin, and aclarubicin, respectively.[10 13] The dose of anthracyclines was then categorized into low (0–200 mg/m^2), intermediate (201–400 mg/m^2), and high (>401 mg/m^2) groups. Other potential confounding factors considered in the analysis included the history of irradiation involving the heart, presence or absence of anthracycline administration within 60 days before transplantation, and pre-transplant serum ferritin levels.

Evaluation of regimen-related cardiac toxicity

Regimen-related cardiac toxicity was graded according to Bearman grade.[2] Only cardiac complications that developed within 28 days after transplantation were considered regimen-related cardiac toxicity. Grade III–IV cardiac complications were defined to be severe.

Statistical analysis

For univariate analyses, continuous variables in the two groups were compared using the unpaired *t*-test or the Mann–Whitney *U* test, whereas categorical variables were compared using the χ^2 test or Fisher's exact test. Factors associated with at least borderline significance ($P<0.10$) on univariate analysis were subjected to a multivariate analysis using backward stepwise logistic regression. *P* values of less than 0.05 were considered statistically significant.

Results

Severe cardiac complications after transplantation

Eight patients (4.9%) developed grade III–IV cardiac complications within 28 days after transplantation (Table 1). Characteristics of the eight patients are shown in Table 2. Manifestation of cardiac complications was mainly pulmonary congestion in five (patients 1, 2, 3, 7, and 8), while two had severe hypotension (patients 4 and 5). All had primary cardiac dysfunction, not secondary to other causes. Five developed cardiac toxicity during the preparative regimen and three of them died prior to hematopoietic stem cell transplantation. The remaining three patients developed cardiac toxicity 5, 6, and 11 days after transplantation, respectively. Seven died of cardiac causes a median of 3 days (range 0–45 days) after the onset of cardiac complications.

Risk factors for severe cardiac complications

The relationships between possible confounding factors and the development of severe cardiac complications are shown in Table 1. Patient age, sex, and disease status were not associated with cardiac complications. None of the patients who developed cardiac complications had a history of cardiac disease before transplantation, while 12 of 153 patients who did not develop such complications had a prior history of cardiac disease (0% *vs* 8.4%, $P>0.99$), including angina pectoris in two, arrhythmia in six, congestive heart failure in one, leukemic infiltration of the heart in two, and surgery for tetralogy of Fallot in one. Ten patients had diabetes mellitus, five had hypertension, and one had hyperlipidemia before transplantation, but none of them developed cardiac complications after transplantation. As for prior treatments, both the cumulative dose of anthracyclines and the use of anthracyclines within 60 days before transplantation affected the incidence of severe cardiac complications ($P=0.0091$ and 0.011, respectively; Figure 1).

There was no difference in the ECG findings, including QTc interval and QTc dispersion, between those who developed severe cardiac complications and those who did

Table 2 Cardiac complications during the first 30 days

	Age/sex	*Disease status*	*Anthracycline dose (mg/m^2)*	*EF (%)*	*Conditioning regimens*	*Onset*	*Outcome (Bearman grade)*
1	30/F	ALL CR2	1054	52	ETP 40 mg/kg CY 40 mg/kg fTBI 12 Gy/6	day 5	Severe CHF (IV), died on day 31
2	42/F	AML CR2	>800	45	BU 16 mg/kg FLU 120 mg/m^2	day −3	Severe CHF (IV), died on day 36
3	27/M	NHL NR	465	55	L-PAM 140 mg/m^2 BU 8 mg/kg fTBI 12 G/6 fr	day 6	Severe CHF (IV), died on day 51
4	24/M	GCT PR	0	79	IFM 12 g/m^2 CBDCA 1600 mg/m^2 ETP 1600 mg/m^2	day −4	Cardiogenic shock, (IV), died on day -2
5	31/F	NHL CR2	384	65	CBDCA 1600 mg/m^2 ETP 1600 mg/m^2 CY 100 mg/kg AraC 4 g/m^2	day −1	Cardiogenic shock, (IV), died on day-1
6	55/M	ALL CR1	307	59	CY 120 mg/kg fTBI 12 Gy/6 fr	day −1	Cardiac tamponade, (IV), died on day 2
7	46/F	NHL NR	520	69	CBDCA 1600 mg/m^2 ETP 1600 mg/m^2 CY 100 mg/kg AraC 4 g/m^2	day −2	Severe CHF, (IV), died on day −1
8	38/F	AML CR2	408	63	CY 120 mg/kg fTBI 12 Gy/6 fr	day 11	Severe CHF, (III), alive on day 686

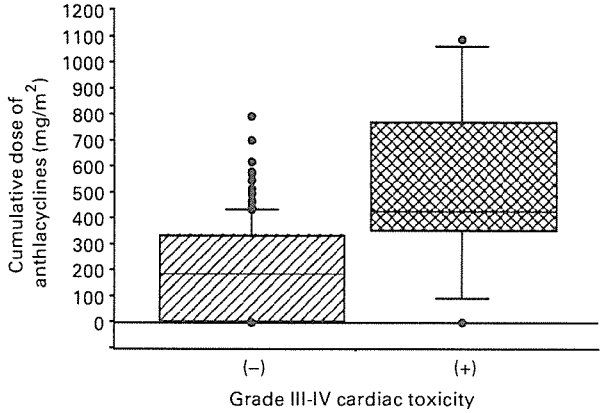

Figure 1 The cumulative dose of anthracyclines was compared in patients who developed grade III–IV cardiac complications and others. The box-and-whisker plot shows 10, 25, 50, 75, and 90 percentile values. Outliers are indicated by dots.

not. Furthermore, no difference was observed in the UCG findings, including EF and the E/A ratio, between the two groups. Impaired EF was more frequently observed in patients who developed severe cardiac complications, but the difference was not statistically significant (25% *vs* 11.2%, $P=0.25$). Heart rate and systolic blood pressure before transplantation were not correlated with the incidence of severe cardiac complications. However, a high HR–BT index, which reflected 'relative tachycardia', was associated with a higher incidence of cardiac complications (37.5% *vs* 7.2%, $P=0.024$).

The use of high-dose cyclophosphamide or ifosfamide was less frequent in patients who developed severe cardiac complications. This may have been because high-dose cyclophosphamide tended to be avoided in patients who were considered to be at higher risk for severe cardiac complications. The use of TBI did not affect the incidence of cardiac complications ($P>0.99$).

By multivariate analysis, the cumulative dose of anthracyclines was identified as the only independent significant risk factor for severe cardiac complications, with an odds ratio of 4.33 (95% CI 1.48–12.7, $P=0.0075$) for changes between categories.

Discussion

Cardiac toxicity due to the conditioning regimen is a well-recognized complication after hematopoietic stem cell transplantation.[2,3,9] The incidence of severe cardiac complications was 4.9% in this series, which is consistent with the values in previous reports (0.9%–26%).[1,9] We found that the cumulative dose of anthracyclines correlated independently with the development of grade III–IV cardiac complications. Five of the 29 patients (17.2%) who had received more than 400 mg/m^2 of anthracyclines developed severe cardiac complications. Furthermore, among this population, four of the 12 patients (33%) who had received anthracyclines within 60 days before transplantation developed severe cardiac complications, whereas these were seen in only one of 14 patients (7.1%) who had not received anthracyclines within 60 days, although this difference was not statistically significant ($P=0.15$).

The predictive value of pre-transplant cardiac evaluation has been inconsistent among studies. Braverman *et al* and Fujimaki *et al* showed that a reduced pre-transplant EF could be a predictive factor,[3,5] while Hertenstein *et al* showed that the incidence of life-threatening cardiac toxicity was not significantly increased in patients with reduced EF.[9] In this study, the incidence of severe cardiac toxicity was higher in the reduced EF (<55%) group, but this difference was not statistically significant (11.1% *vs* 4.5%, $P=0.25$). It is possible that the number of patients was too small to detect the difference; the statistical power of this study to detect the difference was only 15%. In addition, we tended to use less toxic regimens for patients with a reduced pre-transplant EF. This might also explain why a high-dose cyclophosphamide regimen was used less frequently in patients who developed severe cardiac complications.

In this study, we closely analyzed the correlation between severe cardiac complications and variables obtained by ECG or UCG, but none were useful for predicting cardiac complications after transplantation, although the usefulness of EF should not be excluded. On the other hand, relative tachycardia as shown by a high HR–BT index may reflect reduced cardiac reserve. In fact, a high HR–BT was associated with a higher incidence of severe cardiac complications on univariate analysis. Although this was not confirmed by multivariate analysis, it may be worthwhile to further evaluate the impact of this variable, since the HR–BT index can be determined easily without any cost. As another marker for cardiac reserve, Zangari *et al* showed that the increment of EF during excise was useful to predict overall peritransplant mortality, suggesting that pre-transplant cardiac reserve may be important in predicting transplant outcome.[14]

In conclusion, patients who had received a high cumulative dose of anthracyclines, particularly more than 400 mg/m^2, were at the highest risk for severe cardiac complications. Clinical interventions to prevent cardiac toxicity, such as the use of reduced-intensity conditioning or angiotensin-converting enzyme inhibitor as a cardioprotectant, should be evaluated in such patients.[15] Also, the administration of anthracyclines should be avoided within 2 months before transplantation.

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Graft-versus-host disease

Increased incidence of acute graft-versus-host disease with the continuous infusion of cyclosporine A compared to twice-daily infusion

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Summary:

We retrospectively compared the incidence of acute graft-versus-host disease (GVHD) before and after September 1999, when we changed the mode of cyclosporine A (CsA) administration from twice-daily infusions (TD) ($n = 58$) to continuous infusion (CIF) ($n = 71$). The incidence of grade II–IV acute GVHD in the CIF group (56%) was significantly higher than that in the TD group (27%, $P = 0.00022$). Multivariate analysis identified only two independent significant risk factors for the development of grade II–IV acute GVHD; CIF of CsA (relative risk 2.59, 95% CI 1.46–4.60, $P = 0.0011$) and the presence of HLA mismatch (2.01, 95% CI 1.15–3.53, $P = 0.014$). The incidence of relapse was significantly lower in the CIF group when adjusted for disease status before transplantation (0.41, 95% CI 0.18–0.95, $P = 0.038$), which resulted in better disease-free survival in high-risk patients (43 *vs* 16% at 2 years, $P = 0.039$), but not in standard-risk patients (72 *vs* 80%, $P = 0.45$). CIF of CsA with a target level of 250–400 ng/ml may not be appropriate for GVHD prophylaxis in standard-risk patients.

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Cyclosporine A (CsA) is a mainstay of treatment in the pharmacologic prevention of graft-versus-host disease (GVHD), and is usually combined with methotrexate (MTX). However, the dose, target blood level, and schedule of administration vary among protocols.[1] In particular, it has not been assessed whether CsA should be administered as a continuous infusion (CIF) or as twice-daily infusions (TD) in the early period after transplantation when patients cannot tolerate an oral intake. In September 1999, we changed the mode of CsA administration from TD to CIF, without major changes to other transplantation procedures. The aim of this study was to evaluate the impact of these two different modes of administration on the incidence of acute GVHD.

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Patients and methods

Patients

We retrospectively analyzed the records of adult patients who underwent allogeneic hematopoietic stem cell transplantation for the first time between June 1995 and May 2000 using a GVHD prophylaxis regimen consisting of CsA and MTX. During that time, this combination was the standard regimen for GVHD prophylaxis in our center, but CsA alone was used for patients who were at a very high risk for relapse, and a combination of tacrolimus and MTX was used for patients who had received a graft from an unrelated donor with at least one allele or antigen mismatch. Those who received a T-cell-depleted graft and those who received a reduced-intensity conditioning regimen or a conditioning regimen that included ATG or CAMPATH1-H were excluded. Otherwise, consecutive patients were included in the study. The data for 129 patients were analyzed. There were 95 males and 34 females with a median age of 38 years (range 18–60). Bone marrow (BM) was exclusively used in unrelated transplants, whereas 13 related donors chose a collection of G-CSF-mobilized peripheral blood stem cells (PBSC) rather than a BM harvest. BM was additionally harvested from poor mobilizers.

Transplantation procedure

Conditioning was mainly a combination of cyclophosphamide (60 mg/kg for 2 days) with either busulfan (4 mg/kg/day for 4 days) or total body irradiation (TBI; 2 Gy twice daily for 3 days). GVHD prophylaxis was with CsA and short-term MTX (10–15 mg/m^2 on day 1 and 7–10 mg/m^2 on days 3 and 6, and optionally on day 11), with a starting dose of CsA of 3 mg/kg/day. Before September 1999, CsA was administered as a 4 h infusion twice daily in equally divided doses. The dose of CsA was adjusted to maintain the trough blood CsA concentration between 150

and 300 ng/ml. After September 1999, CsA was administered as a CIF. The dose of CsA was adjusted to maintain the blood CsA concentration between 250 and 400 ng/ml. CsA concentration was measured at least once a week by fluorescence polarization immunoassay with a specific monoclonal antibody, using whole blood samples.[2] Metabolites of CsA were not measured by this method. The route of CsA administration was converted to oral at a ratio of 1:2 or 1:3 when patients were able to tolerate oral intake at least 3 weeks after transplantation. Acute GVHD was graded as previously described.[3] Prophylaxis against bacterial, fungal, and pneumocystis carinii infection consisted of fluconazole, tosufloxacin, and sulfamethoxazole/trimethoprim. As prophylaxis against herpes simplex virus infection, acyclovir was given from day −7 to 35. Pre-emptive therapy for cytomegalovirus infection was with ganciclovir, while monitoring cytomegalovirus antigenemia.[4]

Statistical considerations

Standard-risk disease was defined as acute leukemia in complete remission, chronic myelocytic leukemia in the first chronic phase, chemosensitive lymphoma, and myelodysplastic syndrome comprising refractory anemia or refractory anemia with ringed sideroblasts, while others were considered high-risk diseases. Renal dysfunction was defined as an elevation in serum creatinine level to above $\times 1.5$ or $\times 2.0$ the baseline value, except for that clearly caused by the administration of amphotericin B. Patients who received both BM and PBSC grafts were included in the PBSC group.

Probabilities and continuous variables in the two groups were compared using Fisher's exact test and the Mann–Whitney *U*-test, respectively. Cumulative incidences of acute GVHD and relapse were calculated using Gray's method, considering death without acute GVHD or relapse, as a competing risk.[5] Disease-free survival was estimated using the Kaplan–Meier method. Potential confounding factors considered in the analysis were age, sex, donor type (related or unrelated), stem cell source (BM or PBSC), disease risk, conditioning regimen, HLA mismatch, total dose of MTX, and mode of CsA administration.

Results

Patient characteristics

Of the 129 patients analyzed, 58 and 71 patients were in the TD and CIF groups, respectively. The CIF group included a significantly higher proportion of patients with high-risk disease ($P=0.021$), those transplanted from an unrelated donor ($P=0.004$), those who received an HLA-mismatched graft ($P=0.023$), and those who received a PBSC graft ($P=0.0061$) (Table 1). The total dose of MTX was significantly lower in the CIF group (median dose 35 mg/m^2 *vs* 33 mg/m^2, $P=0.0002$). Other characteristics were equivalent between the two groups.

Risk factors for grade II–IV acute GVHD

First, we performed a univariate analysis to evaluate the impact of potential confounding factors on the incidence of grade II–IV acute GVHD. As shown in Table 2, transplant from an unrelated donor, the presence of HLA mismatch, the use of a TBI-containing regimen, a lower total dose of MTX, and CIF of CsA were identified as significant risk factors for the development of grade II–IV acute GVHD. The incidence of grade II–IV acute GVHD in the CIF group (56%) was significantly higher than that in the TD group (27%, $P=0.00022$, Figure 1). Next, we performed a multivariate analysis using the backward stepwise selection method to identify independent risk factors for the development of grade II–IV acute GVHD. Only two factors, CIF of CsA (relative risk 2.59; 95% CI 1.46–4.60, $P=0.0011$) and the presence of HLA mismatch (2.01; 95% CI 1.15–3.53, $P=0.014$), were identified as independent significant risk factors (Table 3A). The impact of these two factors was significant even when adjusted for the total dose of MTX and donor type (Table 3B).

Table 1 Characteristics of the patients

	TD group (n = 58)	*CIF group (n = 71)*	*P-value*
Sex			
Male	42	53	0.84
Female	16	18	
Age			
<40 years	36	36	0.22
⩾40 years	22	35	
Risk			
Standard	39	33	0.021
High	19	38	
Donor			
Related	42	33	0.004
Unrelated	16	38	
HLA			
Match	50	9	0.023
Mismatch	8	22	
Stem-cell BM	57	59	0.0061
PBSC	1	12	
Regimen non-TBI	19	17	0.33
TBI	39	54	0.33

[a]BM = bone marrow, PBSC = peripheral blood stem cell, TBI = total body irradiation.

Renal toxicity

Renal dysfunction was significantly less frequent in the CIF group than the TD group: 27% *vs* 66% ($P<0.0001$) and 13% *vs* 41% ($P=0.0002$), when we defined renal dysfunction as an elevation of the serum creatinine level to above $\times 1.5$ and $\times 2.0$ the baseline value, respectively (Table 4).

Actual daily dose of CsA

We adjusted the dose of CsA to maintain the target blood level as described above. We compared the actual daily dose of CsA, excluding patients who were converted to oral administration. The actual daily dose of CsA in the CIF

Table 2 Impact of pretransplant factors on the incidence of acute GVHD by univariate analyses

	Incidence of acute GVHD	*P-value*
Sex		
Male	45%	0.55
Female	38%	
Age		
<40 years	44%	0.82
⩾40 years	42%	
Risk		
Standard	37%	0.15
High	51%	
Donor		
Related	36%	0.014
Unrelated	54%	
HLA		
Match	37%	0.0026
Mismatch	63%	
Stem cell		
BM	42%	0.33
PBSC	55%	
Regimen		
Non-TBI	30%	0.041
TBI	48%	
MTX		
$<35\,mg/m^2$	60%	0.0025
$\geqslant 35\,mg/m^2$	36%	
CsA		
TD	27%	0.00022
CIF	56%	

BM = bone marrow, PBSC = peripheral blood stem cell, TBI = total body irradiation, MTX = methotrexate, CsA = cyclosporine A, TD = twice-daily infusion, CIF = continuous infusion.

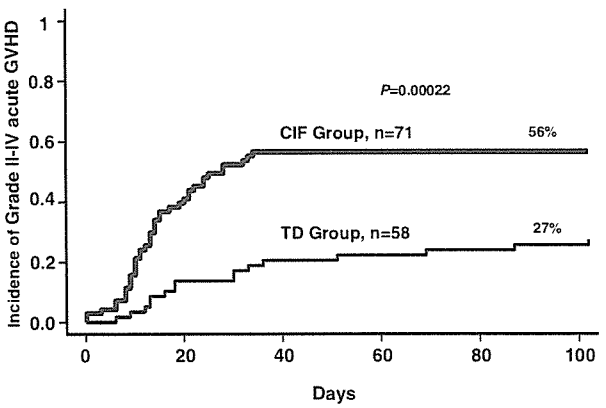

Figure 1 Cumulative incidence of grade II–IV acute GVHD grouped by the mode of CsA administration (TD = twice-daily infusion, CIF = contin-continuous infusion).

group was consistently significantly lower than that in the TD group during the first 4 weeks after transplantation (Figure 2).

Table 3 Impact of pretransplant factors on the incidence of acute GVHD by multivariate analysis: (a) independent significant risk factors identified by multivariate analysis using backward stepwise selection; (b) impact of HLA mismatch and the mode of cyclosporine A administration adjusted for the total methotrexate dose and the donor type (CsA = cyclosporine A, MTX = methotrexate, TD = twice-daily infusion, CIF = continuous infusion)

		Relative risk (95% CI)	*P-value*
	(A)		
HLA	Mismatch *vs* match	2.01 (1.15–3.53)	0.014
CsA	CIF *vs* TD	2.59 (1.46–4.60)	0.0011
	(B)		
HLA	Mismatch *vs* match	1.89 (1.04–3.45)	0.038
CsA	CIF *vs* TD	1.98 (0.98–4.00)	0.056
MTX	$\geqslant 35\,mg/m^2$ *vs* $<35\,mg/m^2$	1.51 (0.80–2.87)	0.20
Donor	Unrelated *vs* related	1.36 (0.78–2.38)	0.28

Table 4 Difference in the incidence of renal dysfunction by the mode of cyclosporine A administration (TD = twice-daily infusion, CIF = continuous infusion)

		(−)	*(+)*	*P-value*
Incidence of serum creatinine > 1.5 × baseline value				
CsA	TD	20	38 (66%)	<0.001
	CIF	52	19 (27%)	
Incidence of serum creatinine > 2.0 × baseline value				
CsA	TD	34	24 (40%)	0.0002
	CIF	62	9 (13%)	

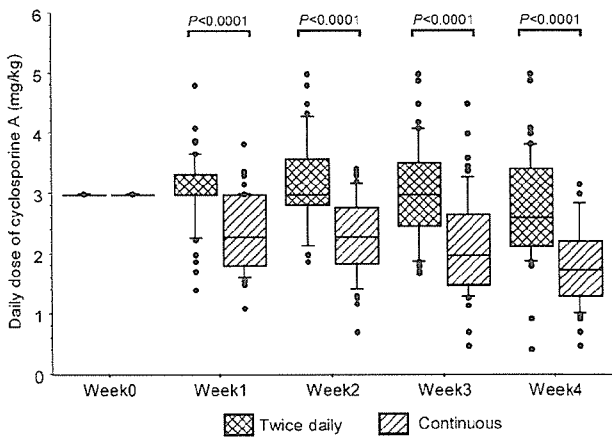

Figure 2 Actual daily dose of CsA grouped by the mode of administration. The box-and-whisker plot shows 10, 25, 50, 75, and 90 percentile values. Outliers are indicated by dots.

Transplant outcome

The CIF of CsA was shown to significantly decrease the incidence of relapse, after adjusting for disease status before transplantation (relative risk 0.41, 95% CI 0.18–0.95, $P = 0.038$). This resulted in significantly better disease-free

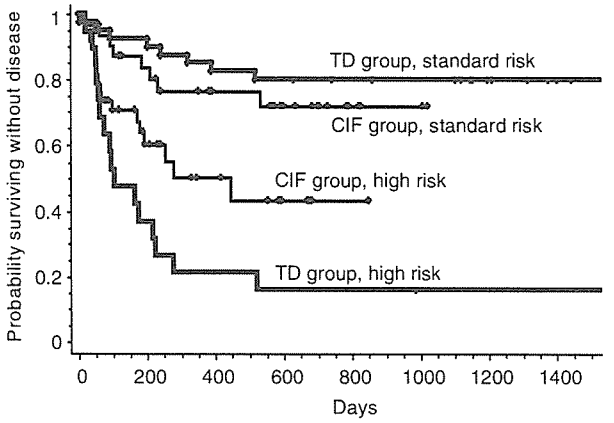

Figure 3 Disease-free survival grouped according to the mode of CsA administration, stratified by the disease status (TD = twice-daily infusion, CIF = continuous infusion).

survival in the CIF group than in the TD group among high-risk patients (43 *vs* 16% at 2 years, $P = 0.039$, Figure 3), whereas there was no significant difference in disease-free survival between the two groups among standard-risk patients (72 *vs* 80% at 2 years, $P = 0.45$).

Discussion

To summarize the findings of this study, the CIF of CsA with a target level of 250–400 ng/ml significantly increased the incidence of grade II–IV acute GVHD, but significantly decreased the incidences of renal dysfunction and relapse, which resulted in better disease-free survival in high-risk patients. However, disease-free survival was not improved in standard-risk patients, probably because the incidence of relapse was originally low in these patients. Therefore, this mode of CsA administration may not be appropriate for standard-risk patients.

There are at least two possible explanations for why the incidence of acute GVHD was higher in the CIF group. First, the total dose (or the area under the curve) of CsA may be important. Second, it may be important to achieve a peak CsA concentration. It was impossible to draw a definite conclusion from this study. However, considering that the actual daily dose of CsA was gradually decreased in the CIF group after transplantation, a target level of 250–400 ng/ml might be too low to prevent GVHD adequately, although this target level has been used in recent large randomized controlled trials.[6,7] Miller *et al*[8] adjusted the dose of CsA as a CIF to maintain the blood CsA level between 450 and 520 ng/ml. In this setting, the mean actual dose of CsA was maintained between 2.87 and 3.15 mg/kg during the first 4 weeks after transplantation. Therefore, this higher target level may be more appropriate when comparing the mode of CsA administration, although it needs to be confirmed by measuring the area under the curve.

The major shortcoming of this study was that this was not a randomized controlled trial and there were some uncontrolled variables that might have caused bias. However, the impact of the mode of CsA administration remained significant after adjusting for these uncontrolled variables, as shown in Table 3B. We are planning a randomized controlled trial to confirm these results.

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Stem cell leukemia protein directs hematopoietic stem cell fate

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Stem cell leukemia (SCL) protein has been shown to be an essential transcription factor during hematopoietic development in the embryo. In adult hematopoiesis, however, the role for SCL has remained largely unknown, whereas it is expressed in bone marrow hematopoietic stem cells (HSCs). In this study, we performed HSC transplantation and an in vitro HSC differentiation assay using retrovirally transduced HSCs with wild-type (WT) and dominant-negative (DN) SCL. The transplantation experiments showed that SCL does not affect the long-term repopulating capacity of HSCs but that WT SCL and DN SCL increase the short-term contribution of the transduced HSCs in myeloid and lymphoid lineages, respectively. An in vitro single-cell assay using a fetal thymus organ culture system further demonstrated that WT SCL facilitates HSCs to differentiate into the myeloid lineage but that DN SCL facilitates HSCs to differentiate into the lymphoid lineage. We conclude that the up-regulation or down-regulation of SCL directs HSCs toward myeloid or lymphoid lineage, respectively, although SCL does not affect their long-term repopulating capacity. (Blood. 2004;103: 3336-3341)

Introduction

Hematopoietic stem cells (HSCs) that can give rise to all lineages of blood cells are enriched in surface marker–defined populations, such as those that are lineage marker (Lin)–negative, c-Kit–positive, Sca-1–positive, and CD34–low/negative ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^{low-}$ [34^-KSL][1]) in adult mouse bone marrow. Recently, common myeloid progenitors (CMPs)[2] and common lymphoid progenitors (CLPs)[3] were identified by surface marker profiles as clonogenic cells that can generate all lineages of myeloid and lymphoid cells, respectively, without any potential to differentiate into one another. Therefore, the first step in blood production from an HSC must be initiated by commitment to a CMP[2] and a CLP.[3] This process, like other differentiation processes, is governed by an expression profile of transcription factors. Although a number of transcription factors are known to be involved in commitment and differentiation processes during hematopoiesis,[4] none have been identified that play roles in the progression from an HSC to a CLP and a CMP. It was reported that CLPs and CMPs differ in the expression pattern of transcription factors: stem cell leukemia (SCL), a basic helix-loop-helix (bHLH) motif–containing transcription factor,[5] and GATA2, a zinc finger motif–containing transcription factor, are expressed in HSCs and CMPs but not in CLPs.[2]

SCL appears to play roles in hematopoietic cell differentiation into myeloid, erythroid, megakaryocyte, and mast cell lineages because it is preferentially expressed during early cell stages in these lineages.[6-12] Studies in knockout mice have revealed that SCL is indispensable in the early stages of primitive embryonic hematopoiesis, when it functions in the specification of ventral mesoderm to blood cells and in the formation or maintenance of immature blood progenitors.[13,14] Studies using embryonic stem cells in vitro have demonstrated that SCL is necessary for the development of bipotent progenitors of blood and endothelial cells in vertebrates.[15,16] Furthermore, ectopic expression of SCL promotes the proliferation and inhibits the apoptosis of 32D, a late myeloid progenitor cell line; these effects are dependent on the bHLH domain.[17] Essential roles for SCL in megakaryopoiesis and erythropoiesis in the adult mouse bone marrow were recently demonstrated by conditional targeting of the *SCL* gene.[18,19]

To clarify the roles of SCL in the commitment process of an HSC to a CLP and a CMP, we performed HSC transplantation and an in vitro HSC differentiation assay using retrovirally transduced HSCs with wild-type (WT) and bHLH domain–deleted—that is, dominant-negative (DN)—SCL.[9,20] Our data show that the overexpression of WT SCL in HSCs strikingly promotes myelopoiesis. In sharp contrast, the overexpression of DN SCL (down-regulation of SCL) in HSCs results in a marked bias toward lymphopoiesis.

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†Hisamaru Hirai died suddenly on August 23, 2003. His students, fellows, and colleagues will greatly miss his energetic and nurturing leadership in the field of hematology. We dedicate this paper in his memory.

An Inside *Blood* analysis of this article appears in the front of this issue.

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Materials and methods

Mice

C57BL/6 (B6-Ly5.2) mice were purchased from SLC (Tokyo, Japan). Mice congenic for Ly5 locus (B6-Ly5.1) were bred and maintained at the University of Tsukuba Animal Research Center (Tsukuba, Japan).

Purification of HSCs and flow cytometric analysis

We purified 34$^-$KSL cells as HSCs (Figure 1A) from murine bone marrow cells and analyzed the surface phenotypes of cells from peripheral blood, bone marrow, spleen, and thymus as described.[21] All antibodies were purchased from PharMingen (San Diego, CA), as follows: biotinylated and unmodified sets of rat immunoglobulin G2b (IgG2b) antilineage markers Gr-1, B220, CD4, CD8, Mac1, and Ter119; fluorescence-labeled antibodies phycoerythrin (PE)–Gr-1, PE-Mac1, PE-B220, allophycocyanin (APC)–B220, APC-Thy1.2, PE-Ly5.1, PE-Sca-1, PE-CD71, APC-c-Kit, and fluorescein isothiocyanate (FITC)–antimurine CD34; biotinylated antimurine α subunit of the receptor for interleukin-7 (IL-7Rα); APC-streptavidin and peridin chlorophyll protein (PerCP)–Cy5.5-streptavidin. All cytokines except for recombinant mouse IL-7 were from Kirin Brewery Research Laboratory (Takasaki, Japan). IL-7 was purchased from PEPROTECH EC (London, United Kingdom).

Plasmid construction

The cDNA fragment for *SCL* was obtained from a library made from an embryonic day-14 (E14) mouse embryo (a gift from Kirin Brewery Research Laboratory) by using a polymerase chain reaction (PCR) method based on the published sequence. *DN SCL* devoid of the DNA-binding domain (amino acid residues 180-233) was constructed with 2 PCR fragments. One of the PCR fragments was generated with primers 1 and 2 (ACAGAATTCTCTAAATATGCCCCAGGATGACGGAG and CACTACTTTGGTGTGAGGACCATCAG); the other fragment was generated with primers 3 and 4 (AAGTACATCAATTTCCTGGCCAAGTTACT and AAACTCGAGTCACCGGGGGCCAGCCCCATCA). A Flag sequence 5′-GACTACAAAGACGATGACGATAAATGA-3′) was fused in-frame to the 3′ end of the cDNAs. The obtained *SCL* and *DN SCL* fragments were cloned into pMY IRES-GFP,[22] which allows the expression of green fluorescence protein (GFP) from an internal ribosomal entry site (IRES) (Figure 1B).

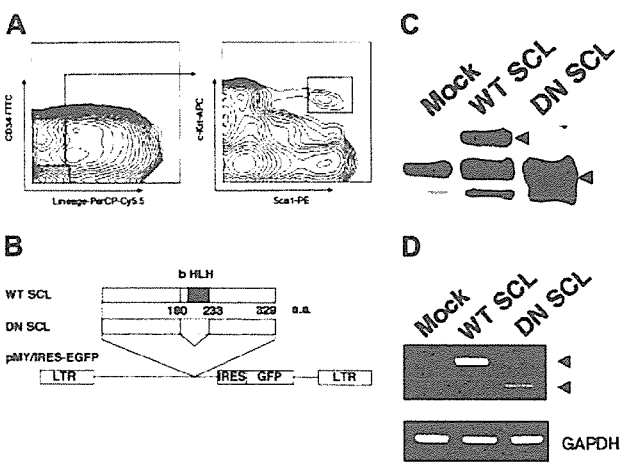

Figure 1. Construction of retroviral vector and target HSC for gene transduction. (A) Flow cytometric analysis of murine hematopoietic stem cells in adult bone marrow. Staining profile of lineage marker versus CD34 for lineage-depleted cells (left) and Sca-1 versus c-Kit in lineage$^-$/CD34$^{-/low}$–gated populations (right) in bone marrow is shown. (B) Schematic representation of the retroviral vector, pMY/WT SCL and pMY/DN SCL, encoding WT SCL and DN SCL linked by IRES to a cDNA encoding enhanced GFP (EGFP). (C) Expression of WT SCL and DN SCL proteins in retrovirally transduced 32D cells. Arrowheads indicate approximately 37 kDa (lane WT SCL) and 29 kDa (lane DN SCL). The other bands commonly seen in all the lanes, including the Mock control lane, represent nonspecific reaction of the antibody. (D) Expression of SCL mRNA in retrovirally transduced HSCs. Arrowheads indicate 308–base pair (bp) (lane WT SCL) and 170-bp (lane DN SCL) transcripts, the latter of which resulted from the deletion of a 138-bp sequence corresponding to the bHLH domain.

Retroviral gene transfer

We produced WT and DN SCL retroviruses and the Mock GFP retrovirus from a packaging cell line, ψMP34. The 34$^-$KSL cells[1] were infected with these retroviruses under stimulation with mouse stem cell factor (SCF) and thrombopoietin (TPO), as previously described.[21]

Western blot analyses and reverse transcription–polymerase chain reaction

32D cells, which were infected with retrovirus and were selected by the expression of GFP, were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) with 10 ng/mL mouse interleukin-e (IL-3). They were harvested in TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA [ethylenediaminetetraacetic acid], 1% NP-40) with protease inhibitor cocktail (Sigma, St Louis, MO) and were sonicated by Handy Sonic HR-20P (Tomy Seiko, Tokyo, Japan). Cell lysates were immunoprecipitated with anti-FLAG M2-Agarose Affinity Gel (Sigma), and Flag fusion proteins were eluted by competition with Flag peptide (Sigma) according to the manufacturer's instructions. The proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using anti-Flag M2 monoclonal antibody (Sigma) and were detected by antimouse IgG antibody conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark).

Reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed as described previously.[21] Primer pairs were: GAPDH, 5′-GCATTGTGGAAGGGCTCATG-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG-3′; SCL, 5′-CTAGGCAGTGGGTTCTTTGG-3′ and 5′-TCCTCCTCCTGGTCATTGAG-3′.

Colony, noncompetitive and competitive reconstitution, and MLP assays

Colony and transplantation assays were performed as previously described.[21] To perform a competitive long-term reconstitution (CLTR) assay and to evaluate whether SCL affects the long-term repopulating capacity of 34$^-$KSL cells, competitor cells were prepared by culturing 34$^-$KSL cells without retroviral transduction in the same conditions used for transducing 34$^-$KSL cells. Competitor cells and GFP-sorted transduced cells (test cells) (1000 each) were then transplanted into irradiated recipient mice and were analyzed for the contribution of test cells against competitor cells (ie, ratio of GFP$^+$ cells among donor [Ly5.1] cells) in the recipients' blood at various times. For noncompetitive assays, we transplanted 2000 transduced cells and 10 000 lineage-depleted fresh bone marrow cells into lethally irradiated recipient mice. A multilineage progenitor (MLP) assay was performed according to the original method previously described.[23] In brief, to prepare hematopoietic cell–depleted fetus thymic lobes, thymi obtained from B6 fetuses 15 days after coitus were cultured on polycarbonate filters (pore size, 8 μm) (Nuclepore, Pleasanton, CA) floating on culture medium containing dGuo (1.35 mM) for 6 days. A single HSC placed in each well of a 96-well V-bottom plate was cultured together with a washed deoxyguanosine (dGuo)–treated lobe in the medium supplemented with recombinant murine IL-7 (rmIL-7) (100 U/mL). Plates were placed into a plastic bag (Ohmi Oder Air Service, Hikone, Japan) in which the air had been replaced by a gas mixture (70% O_2, 25% N_2, 5% CO_2) and were incubated for 12 days.

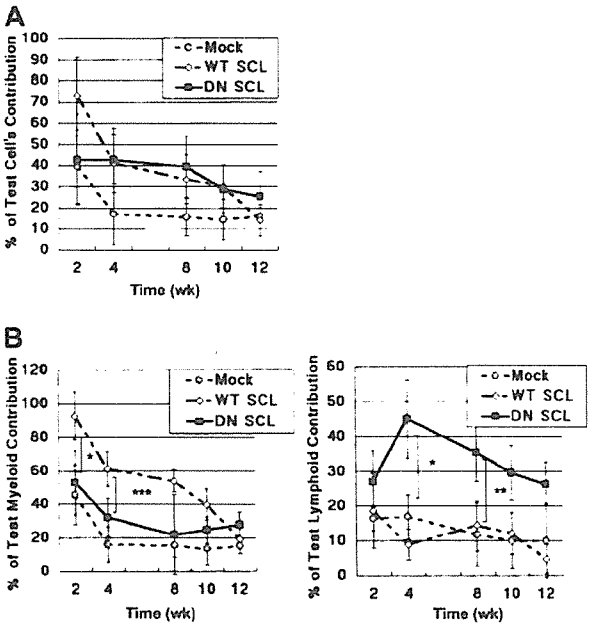

Figure 2. Effects of retrovirally mediated WT SCL and DN SCL on stem cell activities. (A) Analysis of total peripheral blood after competitive reconstitution assay. (B) Lineage-specific contribution in peripheral blood from recipient mice. (left) Percentage of GFP$^+$ cells in the donor-derived Mac1$^+$/Gr1$^+$ cells. (right) Percentage of GFP$^+$ cells in the donor-derived Thy1.2$^+$/B220$^+$ cells (percentage of GFP$^+$/CD45.1$^+$/Mac1$^+$/Gr1$^-$ or CD45.1$^+$/Thy1.2$^+$/B220$^+$ cells of recipient mice). Numbers of recipient mice in each group were between 6 and 9 in panels A and B. Plots are shown as the mean ± SD. *$P < .1$; **$P < .01$; and ***$P < .0001$.

Results

Effects of retrovirally transduced WT SCL and DN SCL on the properties of HSCs

First, we confirmed the expression of Flag-tagged WT SCL and DN SCL proteins in retrovirally transduced 32D cells (Figure 1C). The predicted molecular weight from an amino acid sequence of WT SCL is 34276.6 Da, and from an amino acid sequence of DN SCL it is 28764.46 Da. We next examined the expression of mRNA in retrovirally transduced HSCs (Figure 1D). In WT SCL–transduced HSCs, the level of SCL mRNA expression was higher than that of Mock GFP–transduced HSCs. mRNA for DN SCL was detected only in DN SCL-transduced HSCs.

In a CLTR assay, the contribution of WT SCL– and DN SCL–transduced HSCs was slightly greater than that of Mock GFP–transduced HSCs when the chimerism was accessed shortly after transplantation (Figure 2A). However, the ratios of GFP$^+$ cells gradually decreased, and, at 12 weeks after transplantation, they were as low as approximately 20%, irrespective of introduced genes (Figure 2A). We previously compared in detail the number of the GFP$^+$ cells and congenic marker (Ly5.1$^+$) cells at different time points after transplantation. Silencing of GFP expression did occur, but its ratio was less than 20% by 3 months after transplantation (data not shown). Thus, the decrease in GFP$^+$ cells in the current study is attributed mainly to the decrease in donor-derived cells rather than to the silencing of GFP expression, indicating that the long-term repopulating capacity of retrovirally transduced (ie, GFP$^+$) HSCs was less than that of nontransduced HSCs, regardless of whether WT SCL, DN SCL, or Mock virus was introduced.

We found, however, that the contribution of WT SCL–transduced cells was greater than that of the DN SCL– or the Mock GFP–transduced cells in the myeloid lineage (Figure 2B, left) and, conversely, that the contribution of DN SCL–transduced cells was greater than that of the WT SCL– or the Mock GFP–transduced cells in the lymphoid lineage (Figure 2B, right) until 12 weeks after transplantation if the chimerism was separately characterized in myeloid (Mac1$^+$ or Gr1$^+$) and lymphoid (Thy1.2$^+$ or B220$^+$) lineages.

In a methylcellulose colony-forming assay using the retrovirally transduced GFP$^+$ cells, we found that neither WT SCL nor DN SCL affected the number of immature progenitor-derived colonies compared with the Mock GFP vector (Figure 3A). WT SCL–transduced cells, however, gave rise to progenitors that formed 4 times as many mature myeloid and erythroid colonies than those of Mock GFP–transduced cells (Figure 3B-C).

Up-regulation or down-regulation of SCL in HSCs influences the distribution of progeny in recipient mice

CLTR assay results suggested that SCL had no effect on the maintenance of multipotent HSCs but that it affected the extent of contribution to mature cells from myeloid or lymphoid lineage progenitors. To focus on the lineage commitment of HSCs, we next performed a noncompetitive reconstitution assay. In the blood from recipients of transplanted Mock GFP-transduced HSCs, the ratio of Mac1$^+$ or Gr1$^+$ (hereafter Mac1$^+$/Gr1$^+$) cells in the GFP$^+$ cells was essentially the same as that in total blood cells 6 weeks after transplantation (Figure 4A, upper left panel). In GFP$^+$ cells from recipients of WT SCL–transduced HSCs, however, the Mac1$^+$/Gr1$^+$ cell ratio was markedly higher than that in the total blood cells (Figure 4A, upper middle panel). This corresponded to the decreased ratio of the sum of B220$^+$ cells and Thy1.2$^+$ cells (Figure 4A, lower middle panel). In contrast, the ratio of Mac1$^+$/Gr1$^+$ cells in the GFP$^+$ cells was markedly lower than that in the total blood cells from the recipients of transplanted DN SCL–transduced HSCs (Figure 4A, upper right panel), which corresponded to the increase in the sum of B220$^+$ cells and Thy1.2$^+$ cells (Figure 4A, lower right panel). These results were consistent in all recipients (Figure 4B).

Next, we examined the myeloid and lymphoid cell populations in each hematopoietic organ. In the GFP$^+$ bone marrow cells 6 weeks after transplantation, more than 75% Mac1$^+$/Gr1$^+$

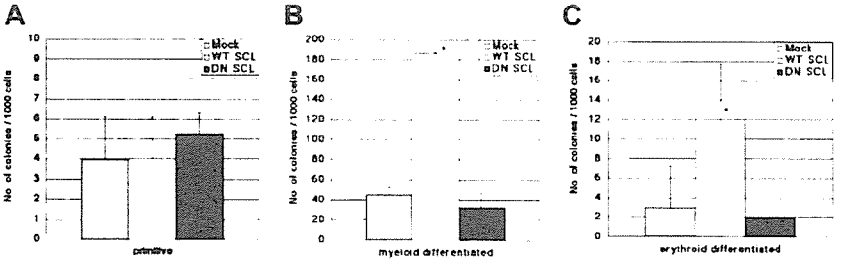

Figure 3. In vitro colony-forming potential of retrovirally gene-transduced cells. Numbers of mixed (A), myeloid (B), and erythroid (C) colonies are shown. Data show the mean ± SD of triplicates. Similar data were obtained in 2 independent experiments.* $P < .1$.

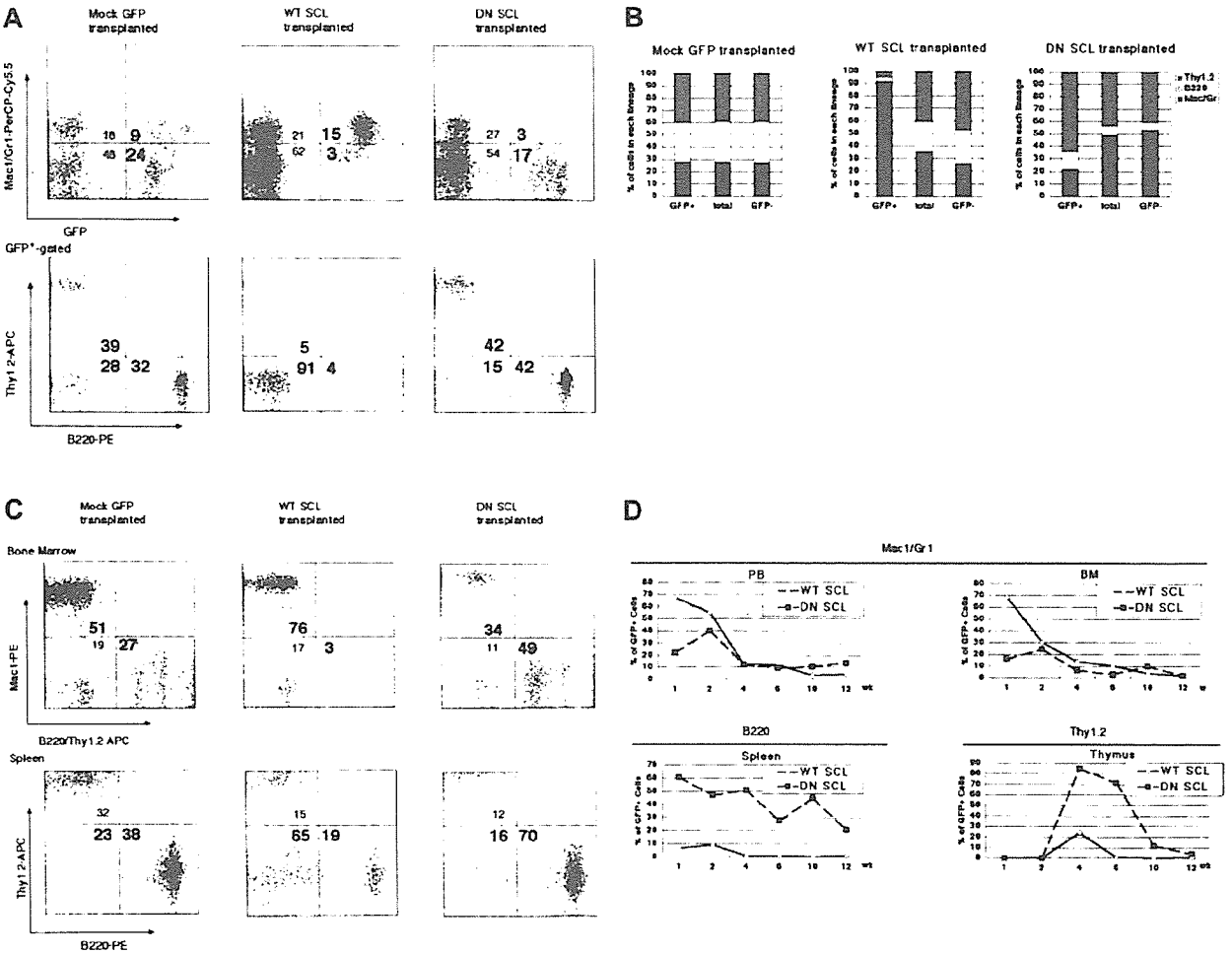

Figure 4. Contribution of WT SCL– and DN SCL–transduced donor-derived cells in myeloid and lymphoid lineages 6 weeks after transplantation. (A) Results of representative FACS analysis of peripheral blood. Profiles for GFP and Mac1 or Gr1 in peripheral blood cells (upper panels) and B220 and Thy1.2 in GFP$^+$-gated peripheral blood subpopulations (lower panels) are shown. (B) Mean ratios of T, B, and myeloid lineage cells in GFP$^+$-gated, nongated, or GFP$^-$-gated peripheral blood subpopulations in recipient mice receiving Mock GFP–transduced cells (SD = 0.02-0.04), WT SCL–transduced cells (SD = 0.19-0.60), and DN SCL–transduced-cells (SD = 0.21-0.22). (C) Results of representative FACS analysis of bone marrow and spleen. (D) Chimerism of GFP$^+$ cells in myeloid cells from peripheral blood and bone marrow (upper panels), in B220$^+$ cells from spleen (lower left), and in Thy1.2$^+$ cells from thymus (lower right). (A-D) Three, 7, and 5 recipient mice were analyzed during Mock GFP–transduced, WT SCL–transduced, and DN SCL–transduced cell transplantation, respectively. Numbers in each quadrant (A,C) represent the percentage of cells in the respective quadrants.

cells—less than 3% B220$^+$ or Thy1.2$^+$ (B220$^+$/Thy1.2$^+$) cells and less than 35% Mac1$^+$/Gr1$^+$ cells—more than 45% B220$^+$/Thy1.2$^+$ cells were observed in the recipients of transplanted WT SCL– and DN SCL–transduced HSCs, respectively, whereas 51% Mac1$^+$/Gr1$^+$ and 27% B220$^+$/Thy1.2$^+$ cells were in the GFP$^+$ cells from Mock GFP–transduced HSCs (Figure 4C, upper panel). In most of the recipients of DN SCL–transduced HSCs, the ratio of B220$^+$ cells was increased in the GFP$^+$ population compared with that in the total population of spleen cells (Figure 4C, lower panel). Changes in the ratios of Mac1$^+$/Gr1$^+$ cells in the blood and bone marrow, B220$^+$ cells in the spleen, and Thy1.2$^+$ cells in the thymus, collectively, showed that WT SCL and DN SCL facilitated the contributions from donor cells to myeloid and lymphoid lineages, respectively (Figure 4D). It was suggested that persistent up-regulation or down-regulation of SCL in HSCs influences their commitment to myeloid or lymphoid progenitors. Furthermore, we found marked decreases and increases in the number of cells expressing IL-7Rα in the GFP$^+$ bone marrow cells from recipients receiving WT SCL– and DN SCL–transduced HSCs (Figure 5A-B). This observation supports the possibility that SCL influences the cell fate of an HSC at the bifurcation of myeloid and lymphoid lineages because IL-7Rα is expressed in immature progenitor cells and because IL-7Rα is one of the key markers discriminating CLPs from CMPs.

In vitro single-cell progenitor assay demonstrates that WT SCL and DN SCL direct HSCs to opposite lineages

To obtain further evidence that SCL affects the commitment process of HSCs rather than the expansion of myeloid progenitors, we examined the developmental capacity of individual hematopoietic progenitors to generate T, B, and myeloid (M) cells using an MLP assay.[23] Cell growth sufficient to perform fluorescence-activated cell sorter (FACS) analysis was achieved in approximately 50% of the wells (data not shown), in each of which a single sorted cell was cultured together with a dGuo-treated fetal thymic lobe. A single cell was designated as p-M, p-T, p-B, p-MT, p-MB, p-TB, or p-MTB if the cell population in the well expressed only Mac1/Gr1, Thy1.2, B220, Mac1/Gr1 and Thy1.2, Mac1/Gr1 and

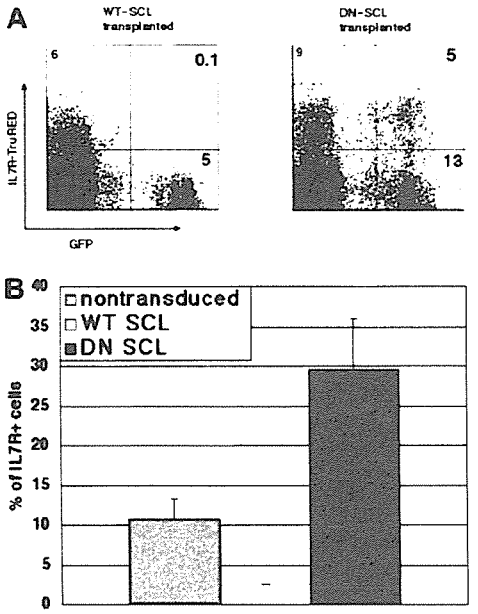

Figure 5. IL-7Rα–expressing cells in the donor-derived cells from bone marrow of recipients of transplanted WT SCL– and DN SCL–transduced HSCs. (A) Results of representative FACS analysis 10 weeks after transplantation. Numbers in quadrants represent the ratio of cells in the respective quadrants. (B) Mean of IL-7α–expressing cells in GFP$^+$-gated or nongated subpopulations in lineage-depleted bone marrow cells from recipients. Data show the mean ± SD of 2 to 4 mice from 2 independent experiments.

B220, Thy1.2 and B220, or Mac1/Gr1, Thy1.2, and B220, respectively. WT SCL–transduced HSCs generated a higher number of p-M and a lower number of p-T than Mock GFP–transduced HSCs (Figure 6A-B). Indeed, p-T was not detected in the cells derived from WT SCL–transduced HSCs in repeated experiments. Conversely, DN SCL–transduced HSCs generated a higher number of p-T and a lower number of p-M than Mock GFP–transduced HSCs (Figure 6A-B). Generation of p-MT and p-MB was not affected by the introduction of WT SCL or DN SCL into HSCs. If only the expansion of myeloid or lymphoid progenitors takes place and the myeloid or lymphoid commitment is not affected by the up-regulation or down-regulation of SCL, the ratio of the number of p-M–containing wells to that of p-T–containing wells would not be altered, and only the cell number of each well would be affected. Results of the MLP assay combined with those of the reconstitution assay described here strongly suggest that the up-regulation of SCL actively promotes an HSC to the myeloid lineage and that the down-regulation of SCL results in the facilitated lymphoid commitment of HSCs.

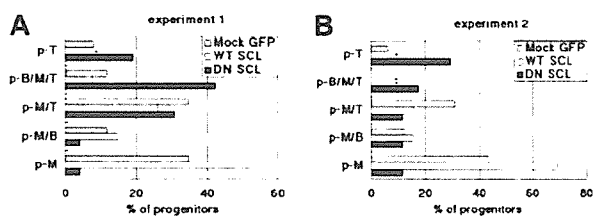

Figure 6. Frequency of different types of progenitors in WT SCL– and DN SCL–transduced HSCs. Progenitor types were determined by analyzing the cells generated from a single cell. Results of 2 independent experiments (A-B) are shown. Asterisks indicate the wells in which the indicated type of progenitor was undetectable. At least 3000 hematopoietic cells per well were analyzed using FACS.

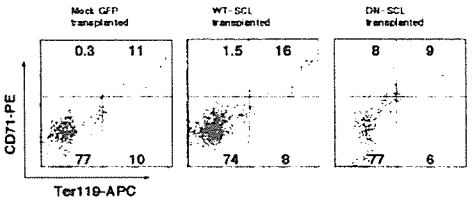

Figure 7. Perturbed erythropoiesis in bone marrow of recipients of transplanted DN SCL–transduced HSCs. FACS analysis of single-cell suspensions of GFP$^+$-gated bone marrow cells immunostained with Ter119 and CD71 antibodies is shown. Numbers in quadrants represent the ratio of cells in the respective quadrants.

Erythroblast maturation of transduced HSCs in recipient bone marrow

SCL expression is closely associated with erythroid maturation. We analyzed bone marrow cells from mice that underwent transplantation for erythroid differentiation markers. We found an increase in the frequency of Ter119^{low} CD71$^+$ cells representing an immature erythroid population[24] in GFP$^+$ bone marrow cells from recipients receiving DN SCL–transduced HSCs (Figure 7). In GFP$^+$ cells derived from WT SCL–transduced HSCs, however, the frequency of the Ter119$^+$CD71$^+$ cells, more mature erythroid cells, was slightly increased (Figure 7), suggesting that SCL also affects the maturation of erythroid cells, as expected from the previous report.[18,19]

The hypothesis of lineage commitment of an HSC through SCL signaling is consistent with the expression pattern of SCL, which is positive in HSCs, CMPs, and their progeny but is negative in CLPs.[2] The results demonstrated in this report suggest that SCL is the most obvious candidate molecule determining the cell fate of HSCs toward CMPs or CLPs.

Discussion

Mice without the *SCL* gene in adult HSCs were recently established by a conditional gene-targeting method.[18,19] In these studies, *SCL* was shown to be dispensable for HSC properties, such as long-term repopulating activity and multipotency, but not for proper erythroid and megakaryocyte generation. In the current study, we observed similar abnormalities induced by DN SCL during erythroid differentiation (Figure 7), but megakaryocytic differentiation was not changed by the transduction of WT SCL or DN SCL, as judged by the expression of CD61 (data not shown). Our major observation that the down-regulation of SCL results in predominant lymphoid commitment is also supported by results of a conditional knockout (cKO) mouse study.[19] Cre-mediated excision of *SCL* resulted in a significant increase in the number of T cells in the peripheral blood of HSC recipients. The number of B cells did not appear to be influenced in the cKO mice, and this result could represent a difference from our result. However, the number of mice analyzed in the cKO study[18,19] does not appear to be sufficiently large. Therefore, we speculate that the decrease in the number of B cells in the cKO mice might be observed if the number of mice examined were large enough, given that we observed large mouse-to-mouse variation in T- and B-cell ratios in the GFP$^+$ population. If so, data described in the cKO studies are very similar to ours using DN SCL–transduced HSCs, including the findings that continued expression of *SCL* is not essential for the maintenance of HSCs.

Our study, however, is the first to shed light on the fact that SCL influences the polarity of an HSC toward myeloid or lymphoid

commitment because WT SCL and DN SCL guide HSCs to opposing commitment pathways. Previously, the exogenous expression of SCL was reported to support proliferation and to inhibit apoptosis of 32D myeloid cells[17] and $CD34^+$ bone marrow cells[11,12] or to stimulate erythromegakaryocytic lineage progenitor cells and to inhibit monomyelocytic lineage cell differentiation. These data and ours apparently show a discrepancy. We used the most highly purified HSCs from bone marrow, which might be a plausible cause of the discrepancies, while others used less purified mouse cells or human cells. It is likely that we observed early differentiation events from HSCs rather than mature lineage differentiation events from progenitors.

Thus, we propose that SCL controls the generation of CMPs and CLPs from HSCs without affecting the self-renewal activity of HSCs. It is of interest whether other transcription factors such as GATA-2, NF-E2, and C/EBPα, which are expressed in CMP but not in CLP, and Aiolos and GATA-3, which are expressed in CLP but not in CMP, play a similar role.

The authors of the reports describing *SCL* cKO mouse characterization did not conclude that lymphoid commitment was facilitated at the expense of myeloid commitment.[18,19] This could suggest that DN SCL might be stronger than *SCL* cKO in influencing HSCs. If so, it is possible that DN SCL interferes with bHLH motif–containing transcription factors other than SCL and that such transcription factors substitute for the function of SCL in *SCL* cKO mice. Direct comparison of mice receiving DN SCL–transduced HSCs with *SCL* cKO mouse–derived HSCs could help to address this issue.

Although CMPs and CLPs as the direct progeny of HSCs has been described, no molecular backgrounds controlling the transition from HSC to progenitor has been characterized. Changes in the expression profiles of transcription factors, including bHLH motif–containing ones, must represent the most important determinant. Our data indicate that a change in SCL activity is the first candidate of such a determinant.

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Incidence of Invasive Aspergillosis after Allogeneic Hematopoietic Stem Cell Transplantation with a Reduced-Intensity Regimen Compared with Transplantation with a Conventional Regimen

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ABSTRACT

To evaluate the clinical characteristics of invasive aspergillosis (IA) after reduced-intensity stem cell transplantation (RIST) compared with those after conventional stem cell transplantation (CST), we examined the medical records of 486 CST and 178 RIST recipients. The overall incidence of IA after allogeneic transplantation was 35 (5.3%) of 664, which gave a 3-year cumulative incidence of 5.6%. The estimated 3-year incidence of IA in CST and RIST was 4.5% and 8.2% ($P = .045$), respectively, but the mortality rates were similar (76% and 86%). The median onset of IA after RIST (day 127) occurred significantly later than that after CST (day 97). A multivariate analysis revealed that IA was associated with age older than 50 years (relative risk, 2.12; 95% confidence interval, 1.08-4.17; $P = .03$) and the presence of acute and/or chronic GVHD (relative risk, 6.2; 95% confidence interval, 2.4-16.4; $P = .0002$). IA remains an important complication after allogeneic transplantation, regardless of the type of conditioning regimen.

KEY WORDS

Invasive fungal infection • Invasive aspergillosis • Reduced-intensity stem cell transplantation • Graft-versus-host disease • Allogeneic stem cell transplantation

INTRODUCTION

Over the past few decades, many changes in transplantation strategies and supportive measures have been developed, and these may influence the epidemiology of fungal infection. Since prophylactic use of fluconazole for the prevention of candida infection has become a common practice in allogeneic hematopoietic stem cell transplantation (HSCT), candida and molds that are resistant to azoles have become the chief pathogens in HSCT [1]. Invasive fungal infection (IFI) has become a frequent complication of HSCT, and *Aspergillus* species are the most common causative organisms [2]. The reported incidence of invasive aspergillosis (IA) after HSCT has been 6.3% to 15.1% [3,4]. Reported risk factors for IA include neutropenia, transplantation without laminar air flow equipment, transplantation from a matched unrelated donor, presence of graft-versus-host disease (GVHD), use of corticosteroids, older age, and underlying diseases [3,4]. Routine screening with high-resolution chest computed tomography scanning [5] and the development of non–culture-based diagnostic methods [6,7] have improved our ability to diagnose IA. Furthermore, new investigational agents with impressive activity against *Aspergillus* species have also been

†*Hisamaru Hirai died in August 2003.*

shown to be useful in early clinical trials [8,9]. Despite these advances in diagnostic and treatment modalities for IA [5-7], its prognosis still remains poor, with high mortality rates ranging from 50% to >90% [3,4,10].

Reduced-intensity HSCT (RIST) is a new transplantation strategy that was developed to decrease regimen-related toxicity while preserving an adequate antitumor effect [11]. Different pioneering conditioning regimens for RIST have been investigated, including a combination of purine analogs [12] and total body irradiation with potent immunosuppressants [13]. It is assumed that elderly patients, who are not eligible for conventional stem cell transplantation (CST) with a myeloablative regimen, may be able to benefit from RIST. Favorable results have been reported in patients with various hematologic malignancies, including chronic myelocytic leukemia [14] and malignant lymphoma [15]. Among solid tumors, metastatic renal cell carcinoma has been shown to respond well to RIST [16], and on the basis of these early clinical results, studies are currently under way to evaluate the efficacy of RIST in a wide variety of hematologic and nonhematologic malignancies.

However, it remains unknown whether RIST carries the same risk of IA, because it is associated with a shorter neutropenic period and less regimen-related toxicity. Several studies have suggested that IA still occurs frequently after RIST [17,18], whereas others have reported no increase in IFIs despite an increase in the rate of cytomegalovirus (CMV) infection [19]. All of these studies on IA after RIST were small, with 12 to 163 patients, and there is still only limited information on this complication. In this study, we investigated the clinical characteristics of IA after RIST, with regard to its incidence, characteristics, and risk factors, in comparison to those after CST.

PATIENTS AND METHODS

Patients

The medical records of all of the patients who underwent allogeneic HSCT at the National Cancer Center Hospital, Toranomon Hospital, Tokyo Metropolitan Komagome Hospital, and the University of Tokyo Hospital between January 1999 and November 2002 were reviewed. Among the 664 patients, 486 underwent CST and 178 underwent RIST (Table 1). The patients in the RIST group were significantly older, and their malignancies were in more advanced stages than those in the CST group ($P < .01$ and $P < .01$, respectively). The 3-year non-IA mortality rates after CST and RIST were 41.2% and 50.2%, respectively ($P = .24$).

The factors that we considered to be associated with low risk for transplantation were as follows: acute myeloblastic leukemia or acute lymphoblastic leukemia in first or second complete remission, chronic myelogenous leukemia in chronic phase, and myelodysplastic syndrome refractory anemia. All other patients were defined as having high-risk diseases. All of the patients with solid tumors were incurable with conventional treatments.

Stem Cell Sources

In the CST group, 423 and 63 patients received transplants from an HLA-identical and 1 antigen–mismatched donor, respectively (Table 1). In the CST group, 96 patients received granulocyte colony-stimulating factor–mobilized peripheral blood stem cells, whereas 390 received bone marrow. In the RIST group, 153 patients had an HLA-identical donor and 25 had a mismatched donor, and the stem cell source was peripheral blood (n = 155) or bone marrow (n = 23).

Preparative Regimens

Conventional preparative regimens consisted of cyclophosphamide (CY) and total body irradiation (n = 295) or of busulfan and CY (n = 191), as shown in Table 1. Reduced-intensity preparative regimens included fludarabine/CY-based (n = 21), fludarabine/busulfan-based (n = 119), fludarabine/melphalan-based (n = 13), and cladribine/busulfan-based regimens (n = 25). Antithymocyte globulin was added to the preparative regimen in 7 CST and 74 RIST patients. Total body irradiation (4 to 8 Gy) was further added in 14 RIST patients.

Prophylaxis and Treatment of GVHD

Cyclosporine (CSP) and short-term methotrexate were primarily used for GVHD prophylaxis in the CST group (Table 1), whereas most of the RIST patients received CSP 3 mg/kg alone. The diagnosis of GVHD was clinically made in concert with biopsy of the skin or gastrointestinal tract. Acute and chronic GVHD were graded according to the consensus criteria [20,21]. Grade II to IV acute GVHD was treated with methylprednisolone 2 mg/kg/d in addition to CSP.

Management of Infections

Most of the patients were cared for in reverse isolation in a laminar airflow-equipped room, and they received prophylaxis with trimethoprim/sulfamethoxazole or pentamidine inhaler and ciprofloxacin against *Pneumocystis carinii* and bacterial infection, respectively. Herpesvirus prophylaxis with acyclovir was also given as previously described [22]. CMV pp65 antigenemia was routinely monitored once a week. When antigenemia was detected, preemptive therapy with ganciclovir was initiated as previously reported [22].

Absorbable antifungal agents, usually fluconazole,

Table 1. *Characteristics of Patients*

Variable	CST (n = 486)	RIST (n = 178)	*P* Value
Age, y, median (range)	36 (1-58)	53 (4-70)	<.01
Sex (male/female)	309/177	110/68	.67
Underlying diseases			
Acute myeloid leukemia	154	44	<.01
Acute lymphoblastic leukemia	111	5	
Chronic myelogenous leukemia	129	12	
Myelodysplastic syndrome	43	27	
Malignant lymphoma	31	36	
Solid tumors*	0	34	
Others	18	20	
Risk for transplantation (low/high)†	290/196	55/123	<.01
Stem cells (peripheral blood/bone marrow)	96/390‡	155/23	<.01
HLA (matched/mismatched)	423/63	153/25	.72
Donor (related/unrelated)	224/262	154/24	<.01
Conditioning regimen			
TBI/CY-based	295		
BU/CY-based	191		
2-CdA/BU-based		25	
Flu/BU-based		119	
Flu/CY		21	
Flu/Mel		13	
GVHD prophylaxis			
CSP	9	118	
CSP MTX	426	57	
FK506	3	0	
FK506 sMTX	46	0	
Others	2	3	

CST indicates conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; TBI, total body irradiation; CY, cyclophosphamide; BU, busulfan; 2-CdA, cladribine; Flu, fludarabine; Mel, melpharan; CSP, cyclosporine; sMTX, short-term methotrexate.

*All the patients with solid tumors had documented progressive lesions despite prior therapy.

†We divided the risk of transplantation into 2 groups. The low-risk group was as follows: acute myeloid or lymphoid leukemia in first and second remission, chronic myelogenous leukemia in chronic phase, and myelodysplastic syndrome refractory anemia. The other patients were defined as having high-risk diseases.

‡Four patients were infused with both peripheral and bone marrow. These patients were assigned to the bone marrow group.

were used for antifungal prophylaxis at a dose ranging from 200 to 400 mg/d. Neutropenic fever was treated according to the recommendation of Pizzo [23]. Briefly, we empirically started both a β-lactam antibiotic and an aminoglycoside at the time of the first febrile episode. Intravenous administration of amphotericin B at 0.5 mg/kg/d was added when the fever persisted for more than 5 to 7 days. If the diagnosis of aspergillus infection was confirmed, the dosage of amphotericin B was increased to 1.0 mg/kg/d. We used blood tests, enzyme-linked immunosorbent assay for galactomannan antigen, (1-3)-beta-D-glucan assay, and chest computed tomography for the early diagnosis of IA, as previously reported [5].

Diagnostic Criteria for IA

IA was categorized as proven, probable, or possible IA, according to the established European Organization for Research and Treatment of Cancer (EORTC)/National Institutes of Health (NIH)/Mycology Study Group (MSG) criteria [24]. Briefly, we diagnosed patients as having proven IA when there was histologic evidence of tissue invasion by small uniform, dichotomously branching hyphae, septate at regular intervals, and/or culture findings positive for *Aspergillus* species from autopsy specimens, mostly the lung, and a compatible clinical presentation. Probable IA was defined as radiographic findings compatible with IA on computed tomography with clinical symptoms and positive findings in enzyme-linked immunosorbent assay for galactomannan antigen. Aspergillus antigen positivity was defined as an optical density index of galactomannan >1.5 in 2 consecutive blood samples by an enzyme-linked immunosorbent assay (Platelia *Aspergillus* EIA; Bio-Rad Laboratories, Hercules, CA). Possible IA was not included in this study.

End Points and Statistical Analysis

The primary end point of this study was the incidence of IA after allogeneic HSCT, and the probability of IA after RIST was compared with that after CST. Another objective was to investigate the char-