Decreased incidence of acute graft-versus-host disease by continuous infusion of cyclosporine with a higher target blood level

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Cyclosporine A (CsA) is the mainstay of pharmacologic prevention of acute graft-versus-host disease (GVHD). We previously reported that continuous infusion of CsA with a target blood level between 250 and 400 ng/ml significantly increased the incidence of acute GVHD compared to twice-daily infusion with a target trough level between 150 and 300 ng/ml. Thus, we raised the target level of CsA continuous infusion to 450–550 ng/ml. We treated 33 patients with the higher target level (CsA500) and compared the efficacy and toxicity with those in the 33 historical control patients (CsA300 group). Other transplantation procedures were not changed. The patients' characteristics were equivalent. The average CsA concentration was adjusted around 500 ng/ml and the actual daily dose was maintained at the initial dose (CsA 3mg/kg/day). Toxicities were equivalently observed among the two groups. The incidence of grades II–IV acute GVHD was significantly lower in the CsA500 group (27 vs. 52%, $P = 0.033$). The target level of CsA was identified as an independent significant risk factor for grades II–IV acute GVHD ($P = 0.039$), adjusted for the presence of HLA mismatch. The incidence of chronic GVHD was also decreased in the CsA500 group (47 vs. 73%, $P = 0.016$). We conclude that the toxicity of the continuous CsA infusion with a target level of 450–550 ng/ml is acceptable and the efficacy to prevent acute GVHD is significant. A larger comparative study is warranted to confirm these findings. Am. J. Hematol. 83:226–232, 2008. © 2007 Wiley-Liss, Inc.

Introduction

Cyclosporine A (CsA) is one of the most commonly used immunosuppressive agents for the prevention of acute graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT). However, the dose, target blood level, and schedule of infusion vary among protocols and have not been optimized [1]. On the other hand, the importance of blood CsA concentration as well as administered dose has been shown in several reports [2–5]. We previously compared continuous infusion of CsA with a target blood level between 250 and 400 ng/ml and twice-daily infusion targeted to a trough level between 150 and 300 ng/ml in the early period after transplantation in a retrospective study [6]. The incidence of grades II–IV acute GVHD was significantly higher in patients who received the continuous CsA infusion, adjusted for the other significant factors. The actual daily dose of CsA in the continuous infusion group was decreased from the starting dose of 3–1.9 mg/kg/day on average at 4 weeks after transplantation, which might have adversely affected the incidence of acute GVHD. However, the incidences of renal dysfunction and relapse were significantly lower in these patients. The lower incidence of relapse in the continuous infusion group resulted in better disease-free survival in patients with high-risk diseases (43 vs. 16% at 2 years, $P = 0.039$), but not in standard-risk patients (72 vs. 80%, $P = 0.45$). We thus considered that the target CsA level of 250–400 ng/ml in the continuous infusion group was appropriate in high-risk patients, but too low in standard-risk patients. Therefore, we raised the target level of CsA to 450–550 ng/ml when we continuously infuse CsA in standard-risk patients [7]. In this report, we evaluated the safety and efficacy of the continuous infusion of CsA with this high target blood concentration at 500 ng/ml.

Results

Patient characteristics

We performed allogeneic HSCT for 33 standard-risk patients with the higher target CsA level at 450–550 ng/ml (CsA500 group). The historical control group treated with the original target CsA level at 250–400 ng/ml (CsA300 group) also included 33 patients [6]. The characteristics of the patients were equivalent between the two groups, except for the underlying disease (Table I). The number of patients with chronic myelogenous leukemia (CML) was only 2 in the CsA500 group, including one with chronic neutrophilic leukemia in uncontrollable leukocytosis, due to the introduction of imatinib in the treatment of such patients.

Blood concentration and actual daily dose of CsA

The dose of CsA was adjusted to maintain the blood CsA concentration between 450 and 550 ng/ml in the CsA500 group. All patients required repeated dose adjustments of CsA to maintain the targeted blood level. This adjustment was successful and the mean CsA concentration was 488 ±

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TABLE I. Characteristics of the Patients

	CsA500 group (n = 33)	CsA300 group (n = 33)	P-value
Sex			
Male	20	26	0.18
Female	13	7	
Age			
<40	16	17	>0.99
≥40	17	16	
Underlying disease			
AL	24	13	0.017
CML	2	12	
MDS	2	1	
NHL	3	6	
Others	2	1	
Donor			
Related	12	16	0.46
Unrelated	21	17	
HLA			
Match	28	25	0.54
Mismatch	5	8	
Stem cell source			
BM	25	26	>0.99
PB	8	7	
Regimen			
Non-TBI	4	9	0.21
TBI	29	24	
MTX dose			
<31mg/m^2	16	11	0.32
≥31mg/m^2	17	22	

BM, bone marrow; PB, peripheral blood; TBI, total body irradiation; AL, acute leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma.

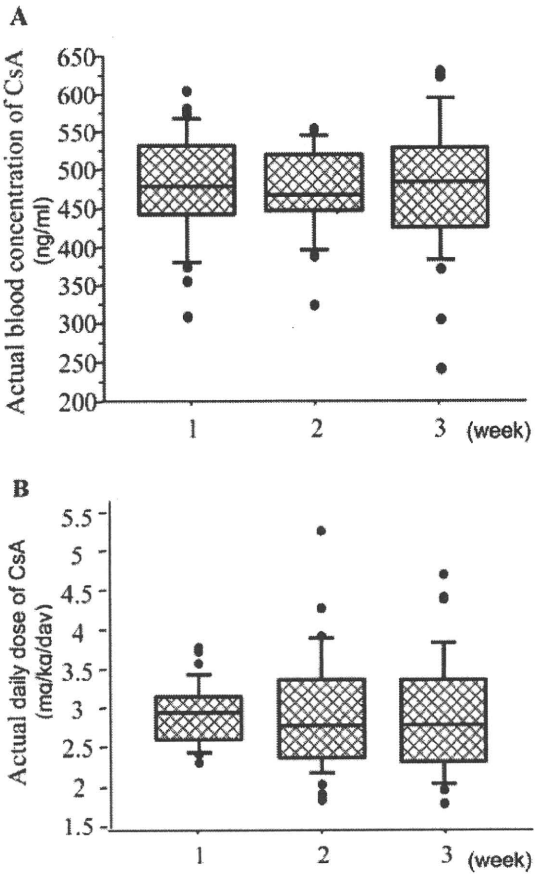

Figure 1. Actual blood concentration (A) and daily dose (B) of cyclosporine. The mean CsA concentration was 488 ± 89, 475 ± 41, and 482 ± 69 ng/ml and the actual dose was 2.9 ± 0.4, 2.8 ± 0.8, and 2.7 ± 0.7 mg/kg at the 1st, 2nd, and 3rd week after HSCT, respectively.

89, 475 ± 41, and 482 ± 69 ng/ml at the 1st, 2nd, and 3rd week after HSCT, respectively (Fig. 1A). The actual dose was 2.9 ± 0.4, 2.8 ± 0.8, and 2.7 ± 0.7 mg/kg at the 1st, 2nd, and 3rd week after HSCT, respectively (Fig. 1B). The median duration of intravenous cyclosporine was 41 days (range 16–74 days) after transplantation.

Toxicity

The incidence of renal dysfunction defined as elevation of the serum creatinine level above ×1.5 and ×2.0 the baseline value was equivalent between the CsA500 group and the CsA300 group (Table II, 24 vs. 24%, P = 0.96 and 15 vs. 13%, P = 0.71, respectively). Liver dysfunction defined as elevation of the total bilirubin level above 2 mg/dl was also similar (30 vs. 24%, P = 0.78). Thrombotic microangiopathy was not observed in any patients. No central nerves system toxicities were observed. In the CsA500 group, we decreased the target level of CsA to 300 ng/ml due to hyperbilirubinemia 9 days after HSCT in one patient and substituted prednisolone for CsA in another patient due to hyperbilirubinemia and renal dysfunction at day 21 after HSCT. The latter patient had already had liver cirrhosis classified to Child-Pugh A due to hepatitis C virus infection before HSCT.

TABLE II. Incidences of Adverse Events Due to Cyclosporine

	(−)	(+)	P-value
Incidence of serum creatinine > 1.5 × baseline value			
CsA500	25	8 (24%)	>0.99
CsA300	25	8 (24%)	
Incidence of serum creatinine > 2.0 × baseline value			
CsA500	28	5 (15%)	0.71
CsA300	30	3 (13%)	
Incidence of bilirubin > 2.0 mg/dl			
CsA500	23	10 (30%)	0.78
CsA300	25	8 (24%)	
Incidence of TMA			
CsA500	33	0 (0%)	>0.99
CsA300	33	0 (0%)	

TMA: thromobtic microangiopathy.

Incidences of acute and chronic GVHD

We performed a univariate analysis to evaluate the impact of potential confounding factors on the incidence of grades II–IV acute GVHD and identified two significant factors; the presence of HLA-mismatch including allele-mismatch and the target level of CsA (Table IIIA). As shown in Fig. 2A, the incidence of grades II–IV acute GVHD in the CsA300 group was significantly higher than that in the CsA500 group (52 vs. 27%, P = 0.033). Corticosteroids therapy for acute GVHD was more frequently required in the CsA300 group (39 vs. 15%, P = 0.051). The percentage of patients who received corticosteroids to treat GVHD was lower than the incidence of grades II–IV acute GVHD, because we did not use systemic corticosteroids for grades II acute GVHD with skin involvement only. The difference in the incidence of

TABLE III. Factors Associated the Incidences of Grades II–IV Acute GVHD and Nonrelapse Mortality

A. Univariate analyses

Factor	Acute GVHD	*P*-value	Nonrelapse mortality	*P*-value
Sex				
Male	20 (44%)	0.31	12 (30%)	0.020
Female	6 (30%)		0 (0%)	
Age				
<40 years	15 (46%)	0.30	4 (14%)	0.21
≥40 years	11 (33%)		8 (30%)	
Underlying disease				
CML	7 (50%)	0.25	2 (14%)	0.49
Non-CML	19 (37%)		10 (25%)	
Donor				
Related	11 (39%)	0.97	8 (36%)	0.052
Unrelated	15 (40%)		4 (13%)	
HLA				
Match	17 (32%)	0.0037	10 (23%)	0.78
Mismatch	9 (69%)		2 (18%)	
Stem cell source				
BM	19 (37%)	0.46	9 (21%)	0.68
PBSC	7 (47%)		3 (24%)	
Regimen				
Non-TBI	4 (31%)	0.56	5 (49%)	0.035
TBI	22 (42%)		7 (15%)	
MTX dose				
<31mg/m^2	12 (44%)	0.32	7 (19%)	0.87
≥31mg/m^2	14 (36%)		5 (24%)	
Target levels of CsA				
CsA500	9 (27%)	0.033	2 (8%)	0.051
CsA300	17 (52%)		10 (27%)	

B. Multivariate analyses

Factor	RR of acute GVHD	*P*-value	RR of nonrelapse mortality	*P*-value
Target levels of CsA				
CsA300	1.00	0.039	1.00	0.064
CsA500	0.43 (0.19–0.96)		0.24 (0.053–1.09)	
HLA				
Match	1.00	0.0062		
Mismatch	3.14 (1.39–7.14)			

grades II–IV acute GVHD between the two groups was more prominent in unrelated HSCT (Fig. 2B, 44 vs. 33% in related HSCT and 59 vs. 24% in unrelated HSCT).

Next, we performed a multivariate analysis to identify independent risk factors for the development of Grades II–IV acute GVHD. Two factors were independently significant with a relative risk (RR) of 3.14 (95% confidence interval [CI] 1.39–7.14, $P = 0.0062$) for the presence of HLA-mismatch and RR of 0.43 (95% CI 0.19–0.96, $P = 0.039$) for the CsA500 group, respectively (Table IIIB). The cumulative incidence of Grades III, IV acute GVHD was only 11%. The target level of cyclosporine (CsA500 vs. CsA300: 3 vs. 18%, $P = 0.045$) was identified as the only significant risk factor for the development of Grades III, IV acute GVHD.

The number of patients who developed limited and extensive chronic GVHD was 5 and 18, respectively, in the CsA300 group and 4 and 11, respectively, in the CsA500 group. The incidence of chronic GVHD was also significantly decreased in the CsA500 group (Table IV and Fig. 3, 47 vs. 73%, $P = 0.016$).

Transplantation outcome

The lower incidence of acute GVHD in the CsA500 group translated into the lower incidence nonrelapse mortality (Table III, 8 vs. 27%, $P = 0.051$). On the other hand, the incidence of relapse tended to be higher in the CsA500 group (Table V, 20 vs. 6%, $P = 0.065$), although this difference became smaller when we excluded patients with CML (19 vs. 10%, $P = 0.29$). Finally, there was no significant difference in disease-free survival between the CsA500 group and the CsA300 group (Fig. 4, 72 vs. 63%, $P = 0.68$).

Discussion

We successfully maintained the blood CsA concentration at around 500 ng/ml and the actual dose at around 3 mg/kg/day by twice a week monitoring for the first 3 weeks after transplantation. The preliminary data in these 33 patients suggested the feasibility and efficacy of the continuous infusion of CsA at this higher target level.

Several studies have reported the relationship between the blood concentration of CsA and the efficacy to prevent GVHD after allogeneic HSCT [2–5]. Especially, the area under the concentration–time curve (AUC) has been believed to be the most important pharmacokinetic parameter for the efficacy of calcineurin inhibitors [8,9]. The monitoring of AUC, however, requires frequent blood sampling and is not suitable for daily practice. Therefore, the trough concentration (C_{TL}) has been measured as a surrogate for AUC in twice-daily infusion of CsA, although recent reports

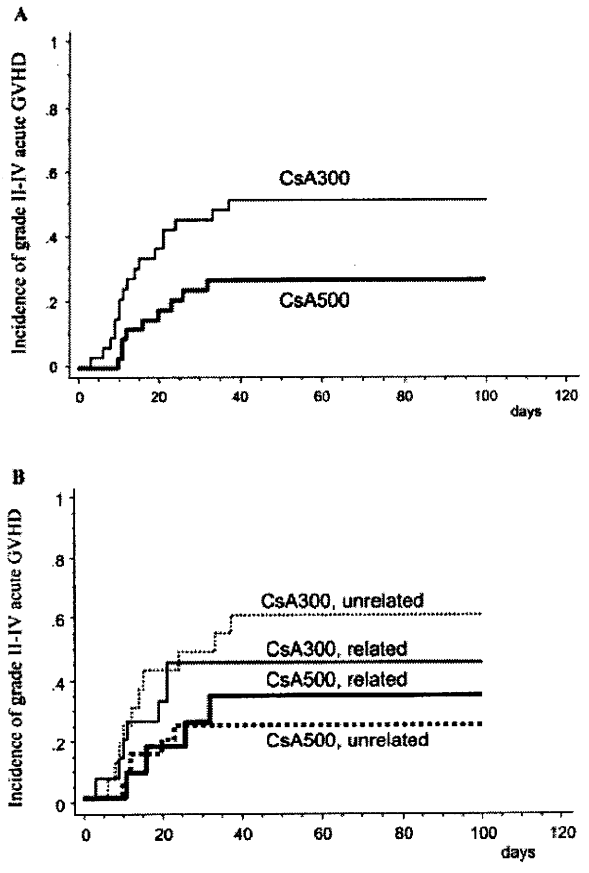

Figure 2. Incidence of Grades II–IV acute GVHD grouped according to the target level of cyclosporine. (A) all patients, (B) stratified by the donor type.

suggested that the measurement of blood concentration at 2–4 hr after infusion may be more appropriate [10]. In continuous infusion, the intradaily variation of the blood concentration of CsA should be minimal and we can evaluate the blood concentration regardless of the timing (steady-state concentration; C_{SS}). However, the relationship between Css in continuous infusion and C_{TL} in twice-daily infusion has not been clarified. Recently, Nakamura et al. reported that the target Css in the continuous infusion of CsA should be 2.55 times the C_{TL} to provide an equal AUC during the twice-daily infusion with a target C_{TL} [11]. Therefore, for example, the target C_{SS} in the continuous infusion of CsA should be 383–638 ng/ml to obtain a similar AUC during the twice-daily infusion with a target C_{TL} at 150–250 ng/ml, that is generally used in daily practice. However, the target blood concentration between 250 and 350 ng/ml is widely used in the continuous infusion of CsA [4]. The expected AUC will be far lower than that during the twice-daily infusion of CsA at the generally used target level. The target C_{SS} in this study at 500 ng/ml (450–550 ng/ml) would be appropriate according to the calculation model. In fact, the actual dose of CsA was maintained at 2.7 and 3.0 mg/kg on average. We had a concern that the incidence of renal dysfunction would be increased, since the relationship between the blood CsA level and drug-induced nephrotoxicity has been shown [12]. The incidence of renal dysfunction, however, was not increased by the dose adjustment and appropriate hydration when CsA levels above the target range were observed.

TABLE IV. Factors Associated the Incidence of Chronic GVHD

A. Univariate analyses

Factor	Chronic GVHD	*P*-value
Sex		
Male	24 (67%)	0.63
Female	10 (56%)	
Age		
<40 years	16 (60%)	0.31
≥40 years	18 (70%)	
Underlying disease		
CML	10 (77%)	0.12
Non-CML	24 (60%)	
Donor		
Related	14 (63%)	0.75
Unrelated	20 (66%)	
HLA		
Match	28 (64%)	0.74
Mismatch	6 (58%)	
Stem cell source		
BM	25 (60%)	0.20
PBSC	9 (81%)	
Regimen		
Non-TBI	27 (64%)	0.75
TBI	7 (65%)	
MTX dose		
<31mg/m^2	15 (64%)	0.81
≥31mg/m^2	19 (68%)	
Target levels of CsA		
CsA500	11 (47%)	0.016
CsA300	23 (73%)	

B. Multivariate analyses

Factor	RR	*P*-value
Target levels of CsA		
CsA300	1.00	0.014
CsA500	0.44 (0.23–0.85)	

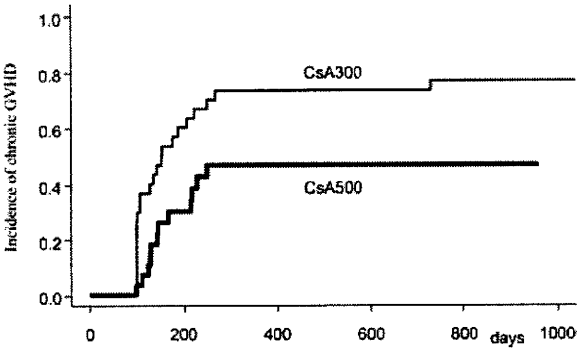

Figure 3. Incidence of chronic GVHD grouped according to the target level of cyclosporine.

Previous randomized control studies that compared continuous infusion of CsA and tacrolimus as GVHD prophylaxis had showed the superiority of tacrolimus to prevent acute GVHD [13–16]. However, these studies employed the lower target level of CsA between 150 and 400 ng/ml. Yanada et al. have also reported that tacrolimus-based regimen was better than cyclosporine-based regimen to prevent GVHD in unrelated bone marrow (BM) transplantation in Japan [17]. However, it was a retrospective analysis

TABLE V. Factors Associated the Incidence of Relapse and Disease-Free Survival

A. Univariate analyses

Factor	Relapse	*P*-value	Disease-free survival	*P*-value
Sex				
Male	5 (12%)	0.90	29 (58%)	0.054
Female	2 (15%)		18 (85%)	
Age				
<40 years	4 (16%)	0.72	25 (71%)	0.35
≥40 years	3 (10%)		22 (60%)	
Underlying disease				
CML	1 (7%)	0.51	11 (79%)	0.34
Non-CML	6 (15%)		36 (60%)	
Donor				
Related	1 (4%)	0.10	19 (60%)	0.53
Unrelated	6 (20%)		28 (67%)	
HLA				
Match	6 (13%)	0.74	37 (63%)	0.64
Mismatch	1 (10%)		10 (72%)	
Stem cell source				
BM	7 (16%)	0.15	35 (63%)	0.55
PBSC	0 (0%)		12 (76%)	
Regimen				
Non-TBI	0 (0%)	0.14	8 (51%)	0.41
TBI	7 (16%)		39 (69%)	
MTX dose				
<31 g/m^2	1 (4%)	0.071	21 (77%)	0.19
≥31 g/m^2	6 (21%)		26 (55%)	
Target levels of CsA				
CsA500	5 (20%)	0.069	26 (72%)	0.68
CsA300	2 (6%)		21 (63%)	

B. Multivariate analyses

Factor	RR of relapse	*P*-value	RR of disease-free survival	*P*-value
Target levels of CsA				
CsA300	1.00	0.065	1.00	0.68
CsA500	4.08 (0.92–18.1)		0.82 (0.32–2.12)	

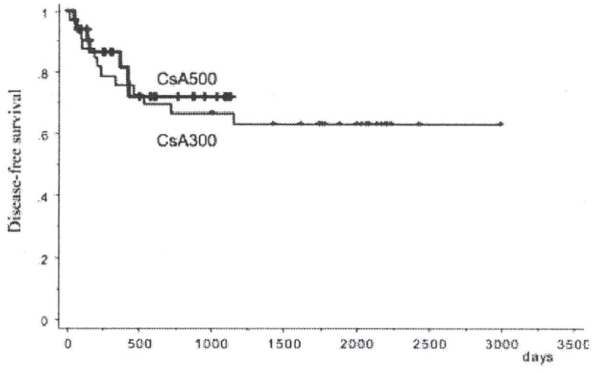

Figure 4. Disease-free survival grouped according to the target level of cyclosporine.

using the database of the Japan Society for Hematopoietic Cell Transplantation (JSHCT) and therefore the dose, target blood level, and infusion schedule of both cyclosporine and tacrolimus were various. Especially, the target level of CsA is generally low in the daily practice in Japan. Therefore, the results of these previous studies that compared CsA and tacrolimus as GVHD prophylaxis might have been affected by the target blood concentration [13–17].

The incidence of grades II–IV acute GVHD in the CsA500 group in this study was suppressed to 24% in unrelated HSCT including three HLA allele-mismatched transplants. This incidence was similar to that in the tacrolimus group of patients who underwent HSCT from an alternative donor (30 from an HLA-matched unrelated donor and 4 from the other alternative donor) in a Japanese randomized controlled trial (21%) [13]. Adverse drug reactions were more frequently observed in the tacrolimus group than in the CsA group in this Japanese randomized trial [13], whereas the toxicities in the CsA500 group were equivalent to those in the CsA300 group in the current study. Therefore, the continuous infusion of CsA with a target concentration at 500 ng/ml may provide similar efficacy of GVHD prophylaxis with less frequent toxicities compared to tacrolimus. Wingard et al. have reported that an important relationship between blood concentration of these agents and their efficacy and toxicity using data of a randomized controlled trial [16]. They showed that the efficacy of CsA to prevent GVHD could be improved by elevating the target blood concentration of CsA, whereas the toxicity of tacrolimus could be reduced by lowering the target blood concentration of tacrolimus. Therefore, a randomized controlled trial to compare CsA and tacrolimus with their appropriate target blood concentration is required to draw a definite conclusion.

Another concern about the elevation of the target concentration of CsA was the possible increase in the inci-

dence of relapse [18,19]. We previously showed that the incidence of relapse was significantly lower after the continuous infusion of CsA with the low target CsA concentration at 300 ng/ml compared to twice-daily infusions targeted to 150–300 ng/ml, because the actual dose of CsA was obviously decreased in the continuous infusion group [6]. In this study, the incidence of relapse tended to be higher in the CsA500 group (20 vs. 6%, $P = 0.065$), although there was no significant difference in disease-free survival. A possible explanation of the tendency toward higher relapse rate in the CsA500 group was the impaired graft-versus-leukemia effect due to the higher CsA concentration. Another explanation was the fact that the CsA300 group included significantly more patients with CML in the first chronic phase, the relapse rate of which is expected to be very low. Actually, the difference in the incidence of relapse became smaller when we excluded patients with CML. In addition, relapse in the CsA500 group mainly occurred in patients with relatively poor underlying diseases, including one with chronic neutrophilic leukemia in uncontrollable leukocytosis, one with acute myeloblastic leukemia with monosomy 7, and one with acute lymphoblastic leukemia with minimal residual disease detected by flow cytometry. Therefore, it might be important to make an appropriate definition of standard-risk disease. Currently, we are excluding acute leukemia in first remission with poor cytogenetic abnormalities, such as the presence of Philadelphia chromosome or monosomy 7, from standard-risk disease.

In conclusion, the continuous infusion of CsA with a target level of 450–550 ng/ml appeared to be safe and effective to prevent acute and chronic GVHD. A randomized controlled trial is being planned to confirm the appropriateness of this higher target level of CsA.

Patients and Methods

Patients

A continuous infusion of CsA with the target blood level between 450 and 550 ng/ml was started as GVHD prophylaxis for standard-risk patients at our institute in March 2003. We compared the safety and efficacy of this GVHD prophylaxis with those in the historical standard-risk patients in whom the blood CsA level was targeted to 250–350 ng/ml [6]. Standard-risk disease included acute leukemia in complete remission, CML in chronic phase, myelodysplastic syndrome without leukemic transformation, chemosensitive lymphoma, and nonmalignant disorders such as chronic active Epstein-Barr virus infection, while the others were considered high-risk diseases.

Transplantation procedure

Conditioning regimen 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). BM was exclusively used as stem cell source in unrelated HSCT, whereas peripheral blood (PB) or BM was chosen in HSCT from a relative. GVHD prophylaxis consisted of CsA and short term methotrexate (MTX). The dose of MTX was 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6 in HLA-matched related HSCT. MTX at 7 mg/m^2 was added on day 11 in HLA-mismatched related HSCT and HLA-matched unrelated HSCT. In HLA allele-mismatched unrelated HSCT, the doses of MTX were increased to 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11.

CsA was administered as a 24-hr continuous infusion. The concentration of CsA was measured twice a week by fluorescence polarization immunoassay with a specific monoclonal antibody, using whole blood samples [20]. The dose of CsA was adjusted based on the ratio of the measured blood concentration and the target blood concentration of cyclosporine at 500 ng/ml to maintain the blood CsA concentration between 450 and 550 ng/ml. For example, when the measured blood concentration was 400 ng/ml using a daily cyclosporine dose of 200 mg, we multiplied the dose of cyclosporine by the ratio and determined the next cyclosporine dose at 200 mg × 500/400 = 250 mg. The route of CsA administration was converted to oral at a ratio of 1:2 when patients were able to tolerate oral intake after engraftment. Acute GVHD was graded as previously described [21]. Prophylaxis against bacterial, fungal, and *Pneumocystis carinii* infection consisted of fluconazole, tosufloxacin, and sulfamethoxazole/trimethoprim or inhalation of pentamidine. As prophylaxis against herpes simplex virus infection, acyclovir was given from days 7–35. Pre-emptive therapy with ganciclovir for cytomegalovirus infection was performed by monitoring cytomegalovirus antigenemia. The initial dose of ganciclovir was 5 mg/kg once daily and the dose was elevated to 5 mg/kg twice daily, when an increasing antigenemia was observed [22]. Other supportive procedures were not changed.

Statistical considerations

Toxicities were evaluated until the route of CsA was changed to oral. Renal dysfunction was defined as elevation of serum creatinine level above ×1.5 or ×2.0 the baseline value. Liver dysfunction was defined as elevation of the total bilirubin level above 2 mg/dl. Dichotomous variables of the patients' characteristics in the two groups were compared using Fisher's exact test. Overall survival, disease-free survival, and the cumulative incidence of acute GVHD were calculated using the Kaplan–Meier method, whereas the cumulative incidences of relapse and nonrelapse mortality were calculated using Gray's method considering each other event as a competing risk [23]. Potential confounding factors for the analyses included age, sex, donor types (related or unrelated), stem cell sources (BM or PB), conditioning regimens (TBI or non-TBI), HLA-mismatch, total doses of MTX, and the target levels of CsA. To evaluate the influence of the confounding factors on these events, the log-rank test and proportional hazards modeling were used for univariate and multivariate analyses, respectively. Factors that showed at least borderline significance ($P < 0.10$) in univariate analyses were included in the multivariate analyses and stepwisely deleted from the model, although the target level of CsA was persistently stayed in the model. All P-values were two-sided and P-values of 0.05 or less were considered statistically significant.

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Case Report: Persistent Cytomegalovirus (CMV) Infection After Haploidentical Hematopoietic Stem Cell Transplantation Using In Vivo Alemtuzumab: Emergence of Resistant CMV Due to Mutations in the UL97 and UL54 Genes

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Addition of in vivo alemtuzumab to the conditioning regimen enabled 2- or 3-locus-mismatched hematopoietic stem cell transplantation with an acceptable incidence of graft-versus-host-disease. However, the procedure was associated with a high incidence of cytomegalovirus (CMV) reactivation. Although preemptive therapy with ganciclovir prevented successfully severe CMV diseases and CMV-related mortality, a patient developed persistent positive CMV antigenemia for more than 1 year after transplantation and CMV disease, despite the use of ganciclovir and foscarnet. The in vitro susceptibility assay showed that the clinical isolate was resistant to foscarnet, moderately resistant to ganciclovir, but sensitive to cidofovir. Therefore, cidofovir was administered. CMV antigenemia became negative within 2 weeks and never developed again. Nucleotide sequence of the UL54 and UL97 of the clinical isolate showed 4 amino acid substitutions (V11L, Q578H, S655L, and G874R) in UL54 and 2 mutations (A140V and A594V) in UL97 compared with the Towne and AD169 strains. Ganciclovir resistance was suspected to be caused by both A594V of UL97 and Q578H of UL54, whereas foscarnet resistance was due mainly to Q578H of UL54. In conclusion, the in vitro susceptibility assay as well as nucleotide sequence of clinical isolate is important to choose appropriate antiviral agents for patients who have persistent CMV reactivation after stem cell transplantation. ***J. Med. Virol. 80:1769–1775, 2008.***

KEY WORDS: CMV; resistance; mutation; hematopoietic stem cell transplantation; alemtuzumab

INTRODUCTION

Cytomegalovirus (CMV) disease is one of the major complications after allogeneic hematopoietic stem cell transplantation. Since the mortality rate is high, preemptive strategy by monitoring CMV reactivation with antigenemia assay or DNAemia assessed by the polymerase chain reaction have been developed for the management of CMV after hematopoietic stem cell transplantation [Boeckh et al., 1996; Kanda et al., 2002]. The risk factors for CMV disease after allogeneic hematopoietic stem cell transplantation include transplantation from an unrelated donor, the presence of HLA mismatch, the use of T-cell depletion, the development of acute graft-versus-host disease, the use of steroids, and so on [Nichols et al., 2001; Kanda et al., 2001a; Asano-Mori et al., 2005].

Alemtuzumab (Campath-1H) is a humanized monoclonal antibody directed against human CD52, that is

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expressed at a high density on B and T lymphocytes and dendritic cells, but not on hematopoietic stem cells [Gilleece and Dexter, 1993]. Therefore, the addition of alemtuzumab to the pretransplant conditioning regimen results in in vivo T-cell depletion and prevents acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation, even from HLA-mismatched donors [Kottaridis et al., 2000; Chakraverty et al., 2002; Kanda et al., 2005]. Although the use of alemtuzumab for in vivo T-cell depletion has been also reported to be a risk factor for CMV reactivation, preemptive therapy with ganciclovir or foscarnet can prevent an excessive incidence of CMV diseases and CMV-related mortality [Bainton et al., 2002; Nguyen et al., 2002; Laurenti et al., 2004; Kanda et al., 2005]. However, one patient developed persistent CMV antigenemia more than 1 year after haploidentical HLA-mismatched hematopoietic stem cell transplantation using in vivo alemtuzumab despite the use of ganciclovir and foscarnet. The clinical course of the patient and the results of the in vitro susceptibility assay and nucleotide sequence of the clinical isolate are described.

MATERIALS AND METHODS

Plaque Reduction Assay

The susceptibilities of the CMV isolate to ganciclovir, cidofovir, and foscarnet were examined by the plaque reduction assay [Cockley et al., 1988; Shiraki et al., 1990, 1991a,b; Yukawa et al., 1996]. Briefly, all assays were carried out in confluent HEL cell monolayers in 60 mm plastic dishes. The cells were infected with 100 plaque forming units (PFU)/0.2 ml of the CMV isolate and the Towne strain for 1 hr. Then, they were exposed to various concentrations of the drugs in 0.8% nutrient methylcellulose minimum essential medium supplemented with 2% fetal bovine serum, and incubated at 37°C as indicated below. The infected cells were fixed with neutral formalin and stained with methylene blue and then the number of plaques was counted. The inhibitory concentrations for 50% plaque reduction (IC_{50}) values were determined using the graph software MPM III-vs. 1.57 for Macintosh.

Sequence Determination of the UL57 and UL97 Genes

CMV DNA was prepared from the viral nucleocapsid as described previously [Shiraki et al., 1991; Ida et al., 1999; Yoshida et al., 2005]. The UL57 (DNA polymerase) and UL97 (phosphotransferase) genes were amplified by the primer sets of 5′-CCAACGAGCAGGCTTACC-3′ and 5′GTCGTCCTACGCGCGATACG-3′, and 5′-ACGCCTCTGTTCAGATTTTA-3′ and 5′-CCCACATGTAGATGGCGGCG-3′, respectively. The amplified fragments were sequenced by using ABI PRISM 3100 DNA sequencer according to the manufacture's procedures. The determined CMV UL54 and UL97 sequences were compared with the Towne (D14980 and U07355) and AD169 (X17403) strains and the nucleotide differences of the isolate common to the both were identified as the significant nucleotide changes.

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study have been deposited in the GenBank/DDBJ/EMBL database. The accession numbers of the CMV UL54 and UL97 genes from the patient were AB329634 and AB329635, respectively. The accession numbers of UL54 and UL97 used are D14980 and U07355 of the Towne strain and X17403 of the AD169 strain.

CASE REPORT

A 54-year-old man with myelodysplastic syndrome of refractory anemia with excess blasts (RAEB) with cytogenetic abnormalities including der(1;7)(q10;p10) participated in a clinical study of 2- or 3-locus- mismatched hematopoietic stem cell transplantation using in vivo alemtuzumab, because he did not have an available HLA-A/B/DR-matched related donor, 1-locus-mismatched related donor, or an HLA-matched unrelated donor. He had repeated episodes of infectious disease including pulmonary invasive aspergillosis due to sustained neutropenia. He was CMV-seropositive, but the donor was CMV-seronegative. The conditioning regimen consisted of alemtuzumab (0.2 mg/kg/day × 6 days, from day 8 to day 3), fludarabine (30 mg/m^2 × 6 days, from day 8 to day 3), busulfan (4 mg/kg/day × 2 days, from day 5 to day 4), and total body irradiation (2 Gy twice daily on day 1). Peripheral blood mononuclear cells were collected from his 3-locus-mismatched daughter, cryopreserved without ex vivo manipulation, and infused on day 0. The number of infused CD34 and CD3 positive cells was 5.24×10^6 cells/kg and 2.72×10^8 cells/kg of the recipient body weight, respectively. Post-transplantation prophylaxis against graft-versus-host disease was performed with continuous infusion of cyclosporine A (3 mg/kg) and short-term methotrexate (15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11). Neutrophil engraftment, defined as the first of 3 consecutive days with an absolute neutrophil count of at least 0.5×10^9/L, was documented on day 12. Though febrile neutropenia was observed for 9 days, no bacterial and fungal infection was documented during neutropenia. Acute graft-versus-host disease was not clinically observed.

CMV reactivation was first detected on day 19 after transplantation by the CMV antigenemia assay that was performed weekly after engraftment using C10/C11 antibody. Preemptive antiviral treatment with intravenous ganciclovir (5 mg/kg twice daily) was initiated [Kanda et al., 2001b]. The level of antigenemia was decreased gradually and the dose of ganciclovir was decreased to 5 mg/kg/day. However, antigenemia increased again, which was refractory to the re-increase in the dose of ganciclovir to 5 mg/kg twice daily. Antiviral agents were changed from ganciclovir to foscarnet at 50 mg/kg twice daily adjusted to his renal function at day 72 after transplantation, that was temporarily effective but antigenemia increased again

within 1 month. Although the combination therapy of ganciclovir and foscarnet was started on day 125, it was also ineffective.

He developed CMV retinitis in his right eye with unilateral blurring on day 204. An ocular injection of 5 mg ganciclovir once a week was started and conducted four times. Then, retinitis of the right eye was well controlled. Otherwise, he did not have any symptoms of CMV diseases. Also, he did not have the clinical manifestations of acute or chronic graft-versus-host disease.

The in vitro analyses of the clinical isolate were conducted using his urine and peripheral blood which were collected on day 224 after transplantation as described in Materials and Methods Section. Table I shows the susceptibilities of the Towne strain and the clinical isolate to ganciclovir, cidofovir, and foscarnet determined by the plaque reduction assay. Clinical isolate was resistant to foscarnet with IC_{50} greater than 100 μg/ml, but moderately resistant to ganciclovir with IC_{50} at 4.54 μg/ml. The isolate was sensitive to cidofovir with IC_{50} at only 0.20 μg/ml, while the Towne strain was sensitive to all three antiviral agents. Therefore, the dose of ganciclovir was increased to 20 mg/kg/day. CMV antigenemia decreased gradually without any additional toxicities, but did not completely disappear. Finally, cidofovir was administered at 5 mg/kg/day three times, which resulted in the clearance of CMV antigenemia without recurrence for more than a year.

Although the number of lymphocytes, especially CD4+ and CD8+ T-cells, was strongly suppressed within 100 days after transplantation, the numbers of T-cells gradually recovered after day 100. The serum IgG level was persistently higher than 1,200 mg/dl after transplantation. The quantitation of CMV-specific cytotoxic T-cells was performed with the tetramer assay. HLA-A*2402 restricted CMV-specific cytotoxic T-cells were detected at 0.12% of CD8+ T cells on day 90 after transplantation, but not detected thereafter (Fig. 1).

Nucleotide sequences of UL54 and UL97 of the clinical isolate were compared with those of the Towne and AD169 strains. The nucleotide difference of the clinical isolate was determined by comparing the sequence difference common to both the Towne and AD169 strains. There were nucleotide differences of the clinical isolate of 49 and 46 (95 in total among 7,458 bases, 1.27%) in the UL54 and 15 and 20 (35 in total among 4,248 bases, 0.82%) in UL97 to the Towne and AD169 strains, respectively (Table IIA). The nucleotides identical to neither the Towne nor AD169 strain resulted in four and two amino acid substitution in UL54 (V11L, Q578H, S655L, and G874R) and UL97 (A140V and A594V) of the clinical isolate as shown in Table IIB. Nucleotide variations of the isolate from the Towne and AD169 were 0.3% for UL54 and 0.26% for UL97 and 28 and 7 of 35 nucleotide variations were transition and transversion, respectively. The nucleotide variation from AT to GC and GC to AT were 24 and 10 in comparison to the isolates with the Towne and AD169 strains and thus favored the shift from AT to GC. One (Q578H) of the four amino acid substitutions in UL54 has been known to be responsible for the resistance to ganciclovir and foscarnet (Fig. 2B). One (A594V) of the two amino acid substitutions in UL97 has also been reported before as being responsible for ganciclovir resistance (Fig. 2A).

TABLE I. IC_{50} (μM) of Towne and CMV Isolate to Ganciclovir, Cidofovir, and Foscarnet

	Towne	Clinical isolate
Ganciclovir	3.92 + 0.20	17.8 + 4.0
Cidofovir	0.39 + 0.18	0.72 + 0.18
Foscarnet	110 + 25	>794 (100 μg/ml)

The IC_{50} values were expressed as the mean + SEM of four independent experiments.

DISCUSSION

Preemptive therapy with ganciclovir has dramatically reduced the incidence of CMV disease after allogeneic hematopoietic stem cell transplantation. However, approximately one-fourth of patients experience an increase in positive cells by the CMV antigenemia assay (rising antigenemia) despite the use of ganciclovir [Nichols et al., 2001; Asano-Mori et al., 2005]. The use of steroid has been identified as the strongest risk factor for the development of rising antigenemia, and the in vitro antiviral susceptibility assay showed that most of the isolates were sensitive to ganciclovir. Therefore, the delayed immune recovery, not the resistant virus, might be the major cause of rising antigenemia. Transplantation from a CMV-seronegative donor might also have contributed to the delayed CMV immunity in the current patient [Nichols et al., 2001]. Nevertheless, the emergence of resistant strains is not uncommon, especially in patients who require prolonged use of antiviral agents. In hematopoietic stem cell transplant recipients, children with immunodeficiency syndromes, the use of T-cell depleted grafts, and the development of graft-versus-host disease have been reported to be risk factors for the emergence of resistant CMV strains [Eckle et al., 2000, 2002; Wolf et al., 2003]. In addition, it was reported that ganciclovir resistance emerged in two adult haploidentical hematopoietic stem cell transplant recipients after prolonged preemptive therapy [Wolf et al., 2003]. Therefore, the current patient was at very high-risk for the development of the resistant CMV strain.

The in vitro susceptibility assay showed that the clinical isolate was resistant to foscarnet, moderately resistant to ganciclovir, and fully sensitive to cidofovir. The results were compatible with the clinical course that cidofovir was far more effective than ganciclovir or foscarnet to terminate CMV antigenemia. To clarify the mechanism of resistance to ganciclovir and foscarnet, the nucleotide sequence of the UL97 and UL54 genes of the clinical isolate were determined. UL97 encodes a phosphotransferase that is required to phosphorylate ganciclovir to its triphosphate form with antiviral

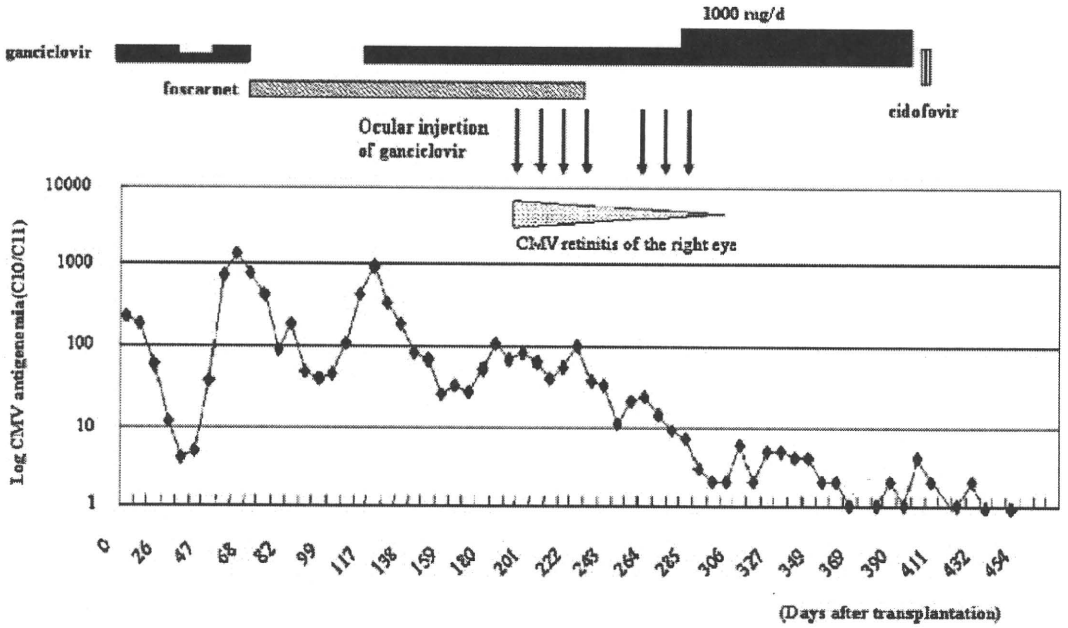

Fig. 1. A clinical course of the patient performed haploidentical transplantation using in vivo alemtuzumab. The CMV antigenemia was first detected early after transplantation and preemptive antiviral treatment with standard dose of intravenous ganciclovir was initiated. Because it was not effective, we changed antiviral agents from ganciclovir to foscarnet and then to the combination therapy of ganciclovir and foscarnet. After failing the increased dose of ganciclovir, we finally administered cidofovir, which resulted in the clearance of CMV reactivation.

activity [Chou, 1999]. Therefore, the mutations in UL97, especially at codons 460, 592, 594, and 595, are closely related to the resistance to ganciclovir [Chou et al., 1995; Chou, 1999]. On the other hand, UL54 encodes DNA polymerase, the main inhibitory target of antiviral agents including ganciclovir, foscarnet, and cidofovir. Therefore, the mutations in UL54 may be involved in resistance to all of these antiviral agents. However, ganciclovir resistance due solely to UL54 is rare [Smith et al., 1997], whereas foscarnet resistance is closely related to UL54 mutations. The appearance of UL54 mutations following UL97 mutations has been shown to be a higher level of resistance to ganciclovir [Erice et al., 1997; Smith et al., 1997; Wolf et al., 2003; Hantz et al., 2005]. The clinical isolate of the current patient showed four and two amino acid differences from the reference strains (Towne and AD169) in the UL54 and UL97 regions, respectively. The UL54 mutations included Q578H mutation, that is located in the δ-region C. The Q578H mutation has not been identified in a clinical isolate but has been reported to cause 10 folds resistance to foscarnet but only twice to ganciclovir in the isolate selected after in vitro passage under drug [Mousavi-Jazi et al., 2003]. However, the high-level foscarnet resistance suggested that the other three mutations (V11L, S655L, and G874R), which were not in the conserved

TABLE II. Difference Between Towne and AD169 Versus CMV Isolate

(A) Detected mutations between Towne and AD169 versus CMV isolate

Mutation	Number	%	Common mutations with the AD169 and Towne strains	%
T to C/A to G	56	43	19	54.3
C to T/G to A	56	43	9	25.7
C to A/G to T	11	7.70	1	2.9
G to C/C to G	4	3.10	1	2.9
T to G/A to C	3	2.30	5	14.3
T to A/A to T	0	0	0	0
Total	130	99	35	100
UL54	3,729 bases	95/7458 = 1.27%	24/7458 = 0.3%	
UL97	2,124 bases	35/4248 = 0.82%	11/4248 = 0.26%	

(B) Nucleotide and amino acid substitutions between Towne and AD169 versus CMV isolate

UL54	V11L, Q578H, S655L, G874R
UL97	A140V, A594V

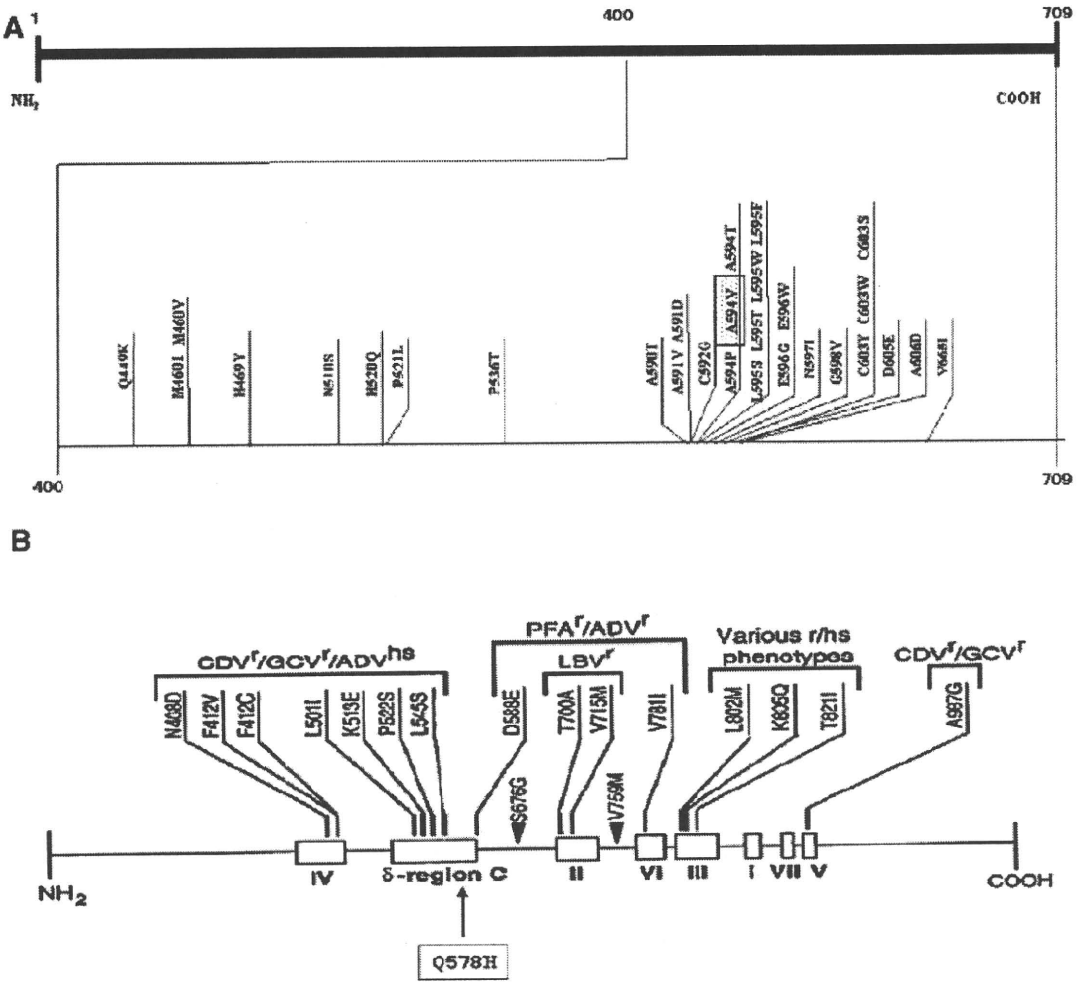

Fig. 2. Mutations responsible for drug resistance in the CMV gene. Many mutations responsible for drug resistance in the UL97 and UL54 genes were reported. **A**: Mutations responsible for resistance to GCV in the UL97 gene [Erice, 1999; Lurain et al., 2001; Eckle et al., 2004]. **B**: Mutations responsible for drug resistance in the UL54 gene. GCV, ganciclovir; ADV, adefovir; PFA, foscarnet; CDV, cidofovir; LBV, lobucavir.

regions of DNA polymerase among different herpesviruses, might also have affected the susceptibility to foscarnet. Mutations in the UL54 region may cause cross-resistance to all of these antiviral agents, but interestingly, the mutations in the UL54 region in this strain did not affect the susceptibility to cidofovir. Similar ganciclovir-resistant mutants with resistance to ganciclovir and foscarnet but sensitivity to cidofovir have been reported [Erice et al., 1997; Smith et al., 1997; Erice, 1999; Lurain et al., 2001; Ducancelle et al., 2004]. The UL97 mutations included A594V, that has been reported to be associated with ganciclovir resistance [Abraham et al., 1999; Erice, 1999; Gilbert et al., 2001; Lurain et al., 2001; Ducancelle et al., 2004; Eckle et al., 2004; Scott et al., 2004]. Therefore, ganciclovir resistance was mainly caused by A594V mutation in the UL97 region and probably enhanced by Q578H in the UL54 region. While the other four mutations in the UL 97 and UL54 regions might have been involved in the development of resistance, these mutations have not been reported before and further studies are required to clarify the impact of these substitutions.

In conclusion, the emergence of the resistant CMV strain was observed in a patient who had undergone haploidentical hematopoietic stem cell transplantation with in vivo T- and B-cell depletion. Profound immunosuppression as well as the prolonged use of antiviral agents might have affected the emergence of resistant strains. However, the CMV reactivation and CMV disease were successfully treated with cidofovir, selected according to the in vitro susceptibility assay. Therefore, as the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation recommended, resistance testing should be

performed for patients who failed first-line antiviral treatment allowing selection of the correct second-line antiviral therapy [Ljungman et al., 2004].

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Autologous Stem Cell Transplantation with PCR-Negative Graft Would Be Associated with a Favorable Outcome in Core-Binding Factor Acute Myeloid Leukemia

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Although core-binding factor acute myeloid leukemia (CBF-AML) is generally considered to be a low-risk form of AML, the survival rate is still 50% to 60%. To evaluate the effectiveness of autologous stem cell transplantation (ASCT) with a PCR-negative graft we analyzed a series of consecutive CBF-AML patients. Between 1997 and 2006, 18 patients aged <60 years were referred under a diagnosis of CBF-AML. Peripheral blood stem cells (PBSC) were collected after a second or further course of postremission therapy. When $>2.0 \times 10^6$/kg CD34-positive cells with minimal residual disease (MRD) undetectable by nested polymerase chain reaction (PCR) had been collected, ASCT was performed with busulfan, etoposide, and cytarabine combined with granulocyte colony-stimulating factor. Event-free survival (EFS) and complications of ASCT were then assessed. Fourteen of the 18 patients received ASCT. The median observation period was 4.4 years. The 5-year EFS was 93% for ASCT patients, despite the presence of adverse factors. In 8 of 10 patients who had detectable MRD in the bone marrow before ASCT, MRD became undetectable after ASCT. Neutrophils recovered promptly within 2 weeks, but platelets recovered relatively slowly. Half of the patients suffered from varicella zoster virus infection. Although 1 case of myelodysplastic syndrome occurred, there was no case of relapse. ASCT with a PCR-negative graft was associated with excellent EFS. For patients with CBF-AML, especially with adverse factors or remnant MRD in the bone marrow, this strategy is the treatment of choice.

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KEY WORDS: Core binding factor acute myeloid leukemia, Autologous stem cell transplantation, Minimal residual disease, Polymerase chain reaction

INTRODUCTION

Translocation (8;21) (q22;q22) or inversion (16) occurs in approximately 7% to 8% of patients with de novo acute myeloid leukemia (AML) [1]. These leukemia entities are associated with aberration of core-binding factors (CBF), which are heterodimeric transcriptional regulators containing a common β (CBFβ) and 1 of 3 α (CBFα) subunits. Translocation fuses the AML1 (CBFα2) gene located on chromosome 21 to the ETO (MTG8) gene located on chromosome 8. The CBFβ gene located at 16q22 fuses with the MYH11 gene located at 16p13. The AML1-ETO or CBFβ-MYH11 fusion protein represses and alters the function of CBF during normal differentiation [2].

Both cytogenetic groups (referred to as CBF-AML) have a relatively favorable prognosis compared with most other forms of adult AML [1,3-5]. In younger patients, repeated cycles of high-dose cytarabine (HDAC) therapy can prolong survival [6,7].

Prognostic factors of CBF-AML have been evaluated in several studies. In t(8;21) AML patients, inferior outcome has been associated with a high white blood cell (WBC) count [8], a low platelet count [8,9], a high WBC index [10], loss of sex chromosomes [8], expression of CD56 antigen [11], extramedullary disease [12], non-White race [9], and older age [9]. In inv(16) AML patients, a high WBC count [13,14],

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older age [9,15], a low platelet count [9,15], and absence of the additional aberration of trisomy 22 [8] have been considered to be adverse factors. In addition, c-*KIT* mutations have recently been identified as adverse factors for CBF-AML [16].

Although opinion about these adverse factors varies, disease-free survival (DFS) is estimated to be <50% [8-15] when patients with CBF-AML have at least 1 adverse factor. In addition, response rate and survival after first relapse are low and short in t(8;21) AML [8,9].

The European Group for Bone and Marrow Transplantation and the Japanese Society of Hematopoietic Stem Cell Transplantation have recommended autologous stem cell transplantation (ASCT) for selected AML patients achieving first complete remission (CR1) [17,18]. Based on these guidelines, ASCT has been performed at our center for younger AML patients with favorable risk other than acute promyelocytic leukemia or with intermediate risk without a human leukocyte antigen (HLA)-matched sibling. In addition, we have infused peripheral blood stem cells (PBSC) in which minimal residual disease (MRD) is undetectable using a nested polymerase chain reaction (PCR) (ASCT with a PCR-negative graft).

We have retrospectively analyzed a series of younger consecutive CBF-AML patients and evaluated ASCT using a PCR-negative graft.

MATERIALS AND METHODS

Patients

The analysis included all consecutive patients aged <60 years with CBF-AML diagnosed and treated at our institution between October 1997 and November 2006. In this survey, CBF-AML was defined by the presence of either t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22) chromosomal rearrangement, or by the presence of the AML1-ETO (MTG8) fusion gene or the presence of the CBFβ-MYH11 fusion gene confirmed by PCR. Although this study was not a formal clinical trial, all events and information were systematically recorded and available. This analysis was approved by our institutional review board.

Therapy

Induction therapy for younger patients aged <60 years was started with idarubicin (12 mg/m^2 days 1-3) and cytarabine (100 mg/m^2 days 1-7). After achievement of complete remission, the patients received postremission chemotherapy as follows: first postremission chemotherapy, idarubicin (12 mg/m^2, days 1-2) and cytarabine (2 g/m^2, every 12 hours, days 1-4); second therapy, enocitabine (200 mg/m^2, days 1-7), etoposide (100 mg/m^2, days 1-5), daunorubicin (50 mg/m^2, days 1-3) and mercaptopurine (70 mg/m^2, days 1-7); third therapy, mitoxantrone (10 mg/m^2, days 1-2), etoposide (100 mg/m^2, days 1-4), and cytarabine (1 g/m^2, every 12 hours, days 1-4); fourth to seventh therapy, cytarabine (3 g/m^2, every 12 hours, days 1,3,5).

If a patient had neither active infection nor sepsis, PBSC were mobilized by granulocyte colony-stimulating factor (G-CSF), and a collection of PBSC was attempted in the phase of recovery from myelosuppression after second or further postremission chemotherapy. One cycle of PBSC collection was defined as a sequential collection course after 1 chemotherapy course. PBSC collection was repeatedly attempted within a maximum of 3 cycles. When more than 2.0 × 10^6/kg CD34-positive cells in which MRD was undetectable by nested PCR had been collected, ASCT was attempted after a third or further session of postremission chemotherapy. Although the ideal doses were >2.0 × 10^6/kg CD34-positive cells, in fact, ASCT was performed when at least 1.5 × 10^6/kg CD34-positive cells were collected after 3 cycles of PBSC collections had been attempted.

The conditioning regimen for ASCT was G-CSF combined with BEA [19] as follows: busulfan (4 mg/kg/day for 4 days as 1 mg/kg four times a day for 16 doses on days −9∼−6), etoposide (20 mg/kg on days −5∼−4), cytarabine (100 mg/m^2 on days −10∼−4, 3 g/m^2 every 12 hours on days −3∼−2), and filgrastim 200 μg/m^2 on days −12∼−4). PBSC were administered on day 0. Filgrastim (300 μg) was started on day 1 until recovery of granulocytes. Prophylactic levofloxacin or tosufloxacin 300 mg/day, fluconazole 200 mg/day, and acyclovir 1000 mg/day were administered from day −7 until neutrophils had recovered to >0.5 × 10^9/L.

Minimal Residual Disease Monitoring

We evaluated the bone marrow (BM) and harvested PBSC for assessment of MRD. From 2001, we used quantitative real-time reverse transcriptase PCR (RQ-PCR) for detection of MRD in BM. Before 2000, MRD in BM was evaluated by fluorescence in situ hybridization or nested reverse transcriptase PCR (nested RT-PCR). MRD in PBSC were evaluated by nested RT-PCR.

For PCR, total RNA was extracted from mononuclear cells in BM and transcribed to cDNA in accordance with the manufacturer's instructions. RT-PCR assay [20-23] was performed by Taqman technology using the following primers: for AML1/MTG8 chimeric mRNA, forward 5′...GAG CCA TCA AAA TCA CAG TGG A ...3′, reverse 5′...ATG AAC TGG TTC TTG GAG CTC CTT ...3′, and probe 5′ FAM (6-carboxylfluorescein)...CAC CTG TGG

ATG TGA AGA CGC AAT CTA GGC TG...TAMRA (6-carboxy-tetramethyl-rhodamine) 3′; for CBFβ/MYH11 chimeric mRNA, forward 5′...CTC CAA AGA CTG GAT GGT ATG GGC ...3′, reverse 5′...CTT GGA CTT CTC CAG CTC ATG G ...3′, and probe 5′ FAM...TCT GGA GTT TGA TGA GGA GCG AGC CC...TAMRA 3′. Nested RT-PCR was performed in accordance with previous reports [21-23]. For RQ-PCR, the number of transcript copies was normalized relative to glyceraldehyde 3-phosphate dehydrogenase, and converted into molecules/μg RNA. The detection threshold of RQ-PCR was 50 copies/μg RNA and the sensitivity was 10^{-4}. The threshold of nested RT-PCR was 10^{-5}.

Mutational Analysis of c-*KIT*

Mutational analysis of the extracellular (EC) domain (exons 8 and 9), transmembrane (TM) domain (exon 10), juxtamembrane (JM) domain (exon 11), and the second intracellular kinase (TK) 2 domain (exons 17 and 18) of the *c-KIT* gene was performed with PCR followed by direct sequencing. The genomic DNA from Wright-Giemsa-stained or unstained blood smears was extracted with a Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany). TaKaRa LA Taq DNA polymerase (Takara, Shiga, Japan) was used to amplify the genes from genomic DNA. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced bidirectionally using the Big Dye Termination 3.1 kit and the ABI Prism 310 system (Perkin-Elmer Cetus, Norwalk, CT). Specific sequences of primers used for PCR and sequencing are available upon request. To validate the sequencing results, PCR products were inserted into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Recombinant plasmids isolated from 8 to 12 white colonies were sequenced.

Statistical Analysis

The major indicator of outcome was event-free survival (EFS), defined as the period from initial diagnosis to relapse (failure), secondary malignancy (failure), death because of any cause (failure), and alive at last follow-up (censored). Overall survival (OS) was also assessed, and was defined as the period from initial diagnosis to death because of any cause (failure), and alive at last follow-up (censored). Estimations of EFS and OS distributions were performed by the Kaplan-Meier method. Comparisons of patient characteristics were performed by χ^2 test for categorical variables and by the Mann-Whitney *U* test for continuous variables. A Cox hazard model was used for univariate analysis of prognostic factors. Estimates of hazard ratios (HR) and corresponding 95% confidence intervals (CIs) were obtained for each of the following variables: gender, age, WBC, hemoglobin, platelets, lactate dehydrogenase (LDH), percentage of blasts in peripheral blood or BM, WBC index, karyotype aberration, CD56 positivity, and total dose of cytarabine. WBC index was derived as the product of WBC and the ratio of marrow blasts at diagnosis [10]. To assess the impact on EFS of a cytarabine dosage exceeding 1 g/m^2, the actual cumulative dosage was calculated and entered as a continuous variable for univariate analysis. For all analyses, statistical significance was defined as a 2-sided value of $P < .05$. Statistical analyses were performed with StatView version 5.0.

RESULTS

Patient Characteristics

Between October 1997 and November 2006, 25 patients were diagnosed as having CBF-AML. Of these patients, 18 were <60 years old and eligible for ASCT. Sixteen had t(8;21) AML and 2 had inv(16) AML (Table 1). Fourteen patients actually received ASCT. The remaining 4 who did not received ASCT included 2 with relapse and secondary myelodysplasia during postremission chemotherapy, 1 with poor mobilization of PBSC, and 1 who withdrew consent to treatment. The relapsed and MDS patients received allogeneic transplantation at other hospitals and were lost to follow-up.

Between patients with and without ASCT, there were no significant differences in additional chromosome aberrations, CD56 positivity or WBC index. However, patients with ASCT had moderately lower platelet counts as well as more blasts in their peripheral blood and BM at diagnosis, and received a lower cumulative dose of cytarabine, although the differences were not statistically significant.

Survival Analysis

The median period of observation of survivors was 4.4 years. All of 18 patients achieved complete remission after induction therapy. The estimated 5-year EFS for these patients and the patients with ASCT was 83.0% ± 9.0% (±standard error) and 92.9% ± 6.9%, respectively (Figure 1). The estimated 5-year OS for the 18 patients was 100%, although follow-up details were lost for relapsed and MDS patients who later underwent allogeneic transplantation at other hospitals. Univariate analysis of prognostic factors for EFS showed that ASCT was the only significant factor (HR 12.9 [CIs: 1.05-157, $P = .045$]), and that age, WBC, cumulative dose of cytarabine, CD56 positivity, loss of sex chromosomes, and lower platelet count had no prognostic value for EFS. Analysis of OS was not done because none of the patients died.

Table 1. Patient Characteristics and Clinical Features

	Total	With ASCT	Without ASCT	P Value
Number of patients	18	14	4	
Gender				
male	15	11	4	*.80
female	3	3	0	
Age (years)				
median	44	44	51	†.2
range	20–59	20–59	43–53	
WBC ($\times 10^9$/L)				
median	5.5	5.5	6.8	†.91
range	1.7–82	1.7–82	2.5–37	
Hb (g/L)				
median	85.5	78	99	†.46
range	38–131	38–131	77–12.6	
Plt ($\times 10^9$/L)				
median	35	24	58	†.089
range	7.0–101	7.0–101	44–81	
LDH (IU/L)				
median	661	872	644	†.75
range	245–6090	245–6090	420–1865	
PB blast (%)				
median	47	49.5	32.8	†.22
range	13–90	24.5–90	18–82.5	
BM blast (%)				
median	67.3	71.2	61.25	†.24
range	26.4–84.4	26.4–84.4	50.4–64.3	
WBC index				
Low	5	5	2	*.80
Intermediate	6	6	1	
High	3	3	1	
Karyotype				
t(8;21)	16			*.50
only t(8;21)	7	5	2	
-X or -Y	8	7	1	
del(9q)	3	2	1	
inv(16)	2			
only inv(16)	2	2	0	
trisomy 22	0	0	0	
CD56‡				
+	8	5	3	*.67
–	4	3	1	
Extramedullary involvement	0	0	0	
Cumulative AC (g/m^2)	48 (12-114)	48 (12-78)	60 (30-114)	†.3

ASCT indicates autologous stem cell transplantation; WBC, white blood cell; Hb, hemoglobin; plt, platelet; LDH, lactate dehydrogenase; PB, peripheral blood; BM, bone marrow; cumulative AC, the actual cumulative dosage of cytarabine exceeding 1 g/m^2; WBC index, WBC x [% of marrow blast] low index <2.5, intermeditate index between 2.5 and 20; high index 20 or more.

P-value was calculated by x^2 exact test(*) or *U* exact test of Mann Whitney(†). P < .05 was considered as significant value. ‡ CD56 expression was investigated in 12 of total 18 patients.

ASCT and Clinical Features

Fourteen patients received ASCT using an identical conditioning regimen and PBSC with undetectable MRD. The median period from diagnosis to ASCT was about 9 months (Table 2). A median of 5 chemotherapy courses were given before ASCT. Neutrophil counts recovered to more than 0.5 × 10^9/L within a median period of 13 days (range: 11~36 days). Platelet counts recovered to >20 × 10^9/L without transfusion within a median period of 27.5 days (range: 12~217 days) and to >50 × 10^9/L within a median period of 4 months (range: 15-378 days). Infusion of more CD34 cells was associated with prompter platelet recovery (P = .027 for platelets >20 × 10^9/L and P = .038 for platelets >50 × 10^9/L, calculated by Pearson's correlation coefficient). As late adverse events, 5 patients suffered varicella zoster virus (VZV) reactivation, which was promptly resolved with acyclovir. These infections occurred at a median of 130 days after ASCT (range: 85-567 days). Although 1 case of secondary myelodysplasia was observed after ASCT, no relapse occurred.

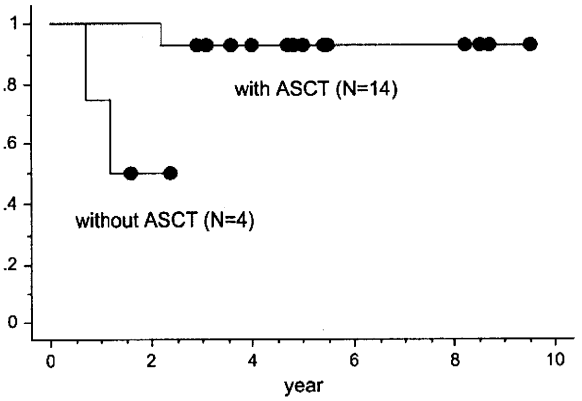

Figure 1. Kaplan-Meier curves of EFS of the subgroup of patients with (n = 14) and without (n = 4) autologous stem cell transplantation. ASCT: autologous stem cell transplantation.

Details of PBSC Collection Among ASCT Patients

In total, 23 cycles of PBSC collection were performed in 14 patients. A median of 1.85 × 10^6/kg CD34-positive cells were collected in each cycle. Eight cycles of PBSC collection were performed after the fourth session of postremission chemotherapy, 4 cycles after the fifth and sixth sessions, and 3 cycles after the second and third sessions. In 1 cycle, collections

Table 2. Clinical Features of Patients with ASCT

Total number	14 cases
Conditioning regimen Bu+VP16+AraC	14 cases
Median time to stem cell transplantation*	273 days (167–375)*
Median number of prior chemotherapy sessions*	5 times (3–8)*
Median number of cycle of PBSCH*	2 times (1–3)*
Median number of infused CD34 positive cells*	2.6×10^6/kg (1.5–4.4)*
Recovery of neutrophil count (>0.5 × 10^9/L)	13 days (11–36)*
Recovery of platelet count (>20 × 10^9/L)	27.5 days (12–217)*
Recovery of platelet count (>50 × 10^9/L)*	123 days (15–378)*
Median observation duration after transplantation*	4.3 years (1.3–8.9)*
Late adverse events	
MDS/sAML	1 case
VZV	5 cases
pneumonia	1 case
meningitis	1 case

PBSCH indicates harvest of peripheral blood stem cell; MDS/sAML, myelodysplastic syndrome/secondary acute myeloid leukemia.
*Ranges are shown in parentheses.

Table 3. Details of Timings and Doses of CD34 Positive Cells in Peripheral Blood Stem Cell Collections

	CD34 Doses/days of Collection in Each Cycle							
	Post-remission chemotherapy							
Pt	1st	2nd	3rd	4th	5th	6th	7th	Actually infused CD34 Cell Dose
1				1.0×10^6/kg/2	1.9×10^6/kg/1			2.9×10^6/kg
2					1.8×10^6/kg/2	0.28×10^6/kg/1		2.0×10^6/kg
3				0.68×10^6/kg/3	2.9×10^6/kg/2			3.4×10^6/kg
4				2.4×10^6/kg/1				2.4×10^6/kg
5				(×)1.2×10^6/kg/2	(×) 0.62×10^6/kg/3	1.5×10^6/kg/4		1.5×10^6/kg
6						(×) 4.6×10^6/kg/1	2.2×10^6/kg/2	2.2×10^6/kg
7						3.7×10^6/kg/4		3.7×10^6/kg
8		8.3×10^6/kg/3						2.7×10^6/kg
9			3.5×10^6/kg/2					3.5×10^6/kg
10				4.4×10^6/kg/2				4.4×10^6/kg
11				2.9×10^6/kg/2				2.9×10^6/kg
12		0.37×10^6/kg/3	0.88×10^6/kg/4	0.15×10^6/kg/1				1.6×10^6/kg
13			2.4×10^6/kg/1	1.3×10^6/kg/2				2.6×10^6/kg
14		2.0×10^5/kg/1						2.0×10^6/kg

Pt indicates patient number; (X) means that MRD was detected in collected peripheral blood stem cells.

were performed for median 2 days (range: 1~4 days) (Table 3).

The PBSC in 3 cycles (patients 5 and 6) were inappropriate as grafts because MRD was detected by nested RT-PCR, and the PBSC were therefore discarded. For collection of PBSC with undetectable MRD, 1 cycle of PBSC collection was sufficient for 7 patients, and 2 cycles were needed in 5 patients (Table 3). In the remaining 2 patients, 3 cycles were performed but no more than 2.0×10^6/kg CD34-positive cells could be collected.

MRD in BM was assessed by RT-PCR before chemotherapy in each of 20 cycles (Table 4). MRD remained in BM in 18 of the investigated 20 cycles. However, PBSC with undetectable MRD were collected in 15 of the 18 cycles. Finally, all 14 patients received PBSC with undetectable MRD.

MRD Monitoring of ASCT Patients

Table 4 shows the time courses of MRD and the timing of PBSC collections among ASCT patients. All of the patients sustained complete remission after ASCT, and MRD in BM remained at <100 copies/μgRNA in all 14 patients and became undetectable by RT-PCR in 12 patients (patients 1-3 and 6-14). MRD in BM became undetectable by RT-PCR after ASCT in 8 (patients 1-3, 6, 8, 11, 13, and 14) of 10 patients (patients 1-6, 8, 11, 13, and 14) whose MRD in BM before ASCT was detectable by RT-PCR.

Table 4. Time Course of Minimal Residual Disease (MRD) among Those with Autologous Stem Cell Transplantation (ASCT)

		Postremission Chemotherapy‡								After ASCT			
Pt	AML type	1st	2nd	3rd	4th	5th	6th	7th	ASCT‡	1 year	2 year	3 year	4 year
1	t(8:21)	•	⊗	⊗	⊗	⊗	—	—	⊗	○			
2	t(8:21)	NA	•	•	•	⊗	•	—	⊗	⊗	○		
3	t(8:21)	••	•	•	•	⊗	—	—	⊗	○	○		
4	t(8:21)	N.A	••	•	••	—	—	—	••	⊗	⊗	⊗	
5	t(8:21)	N.A	•	•	■	■	•	—	•	⊗	⊗	⊗	
6	t(8:21)	N.A	•	•	⊗	⊗	⊗	⊗	•	⊗	⊗	○	
7	t(8:21)	N.A	⊗†	⊗	○	○	○	—	○	○	○	○	○
8	t(8:21)	•	•	••	—	—	—	—	•	○	○		
9	t(8:21)	N.A*	•	N.A*	N.A*	—	—	—	N.A*	○	○	○	○
10	t(8:21)	N.A	N.A*	N.A*	⊗†	—	—	—	N.A*	○	○	○	○
11	t(8:21)	N.A*	N.A*	⊗†	⊗†	—	—	—	⊗†	○	○	○	○
12	t(8:21)	⊗†	⊗†	N.A	○	—	—	—	○	N.A	○	○	○
13	inv(16)	•	⊗	⊗	⊗	—	—	—	⊗	○	○	○	○
14	inv(16)	N.A	N.A	N.A	—	—	—	—	⊗†	○	○	○	○

Pt indicates patient number; AML, acute myeloid leukemia; ASCT, autologous stem cell transplantation; —, postremission therapy was not performed; ••, MRD was 10^3-10^4 copies by RQ-PCR; •, MRD was 10^2-10^3 copies by RQ-PCR; ⊗, MRD was less than 10^2 copies by RQ-PCR or detectable by nested PCR; †, detectable by nested PCR; ○, MRD was undetectable by nested PCR or RQ-PCR; N.A, PCR was not performed; *, negative for t(8:21) by FISH; ‡, PCR sample was obtained just before the start of each postremission chemotherapy and conditioning of ASCT; □, Harvest of peripheral blood stem cells with MRD undetectable by nested PCR; ■, Peripheral blood stem cells were not used because MRD was detectable by nested PCR.

Table 5. Analysis of c-kt Mutations of Leukemia Cells at Diagnosis

	Exon 8	Exon 10	Exon 11	Exon 12	Exon 13	Exon 17	Exon 18	Exon 19	Exon 20
Pt1	normal	normal	normal	normal	normal	normal	normal	normal	normal
Pt2	normal	normal	normal	normal	L813_A814 ins	normal	normal	normal	normal
Pt3	normal	normal	normal	normal	normal	normal	normal	normal	normal

Interestingly, in patients 2 and 6, molecular disappearance of AML1/ETO(MTG8) was confirmed by RT-PCR 2 and 3 years after each ASCT, respectively (Table 4). In the other 6 patients [patients 1, 3, 8, 11, 13, and 14) MRD was undetectable by RT-PCR 1 year after ASCT.

Analysis of c-*KIT* Mutations and MRD

c-*KIT* mutations were analyzed in 3 patients (patients 1-3). Pt.2 had c-*KIT* mutations on exon 17, and the others had no mutations (Table 5). All 3 patients remained in CR1. AML1/MTG8 chimera was undetectable by RQ-PCR in all 3 patients.

DISCUSSION

We analyzed the survival of 18 consecutive young patients with CBF-AML treated between 1997 and 2006 at our center, and revealed that EFS of ASCT with a PCR-negative graft was 93% with no incidence of relapse.

Neutrophils recovered promptly within 2 weeks, but platelets tended to recover more slowly, although severe hemorrhage was not a complication. After ASCT, half of the patients suffered late infections, especially VZV reactivation, at a median of 4 months after ASCT. Prolonged prophylaxis with acyclovir is reportedly effective for prevention of VZV reactivation [24]. The high rate of VZV reactivation in the present series may have been because of the short duration of prophylaxis.

Until more than 2.0×10^6/kg CD34-positive cells were collected, 1 cycle of PBSC collection was sufficient for half of the patients and 3 cycles were necessary for only 2. This number of collections seems average in comparison with other studies (median 2 times) [25,26]. Although MRD remained in the BM in most patients, a PCR-negative graft was obtainable, except in 3 cycles. This was consistent with other studies [27,28] in which MRD was observed less frequently in PBSC than in BM. Also in an animal model, leukemic contamination was reportedly not enhanced by G-CSF mobilization, and a different mechanism for mobilization of leukemic cells into peripheral blood was suggested [29].

Previous studies of ASCT in CBF-AML have indicated a survival of about 45% to 66% [8-15], which was not superior to that achieved with chemotherapy alone [8,9]. Our present result was excellent in comparison with previous studies. This may have been because of the characteristics of the patients; our series might include only patients without adverse factors [8-15] or c-*KIT* mutations [16]. In fact, however, those with ASCT had at least 1 adverse prognostic factor other than non-White race (Table 1), including a case showing c-*KIT* mutation of exon 17 (Table 5), which is associated with a high rate of relapse, although the number of cases analyzed was too small to allow any conclusion to be drawn. The main reason for the good outcome in our series was probably because our ASCT strategy was based on MRD in PBSC (ASCT with a PCR-negative graft). A gene-marking study has suggested that relapse after autologous bone marrow transplantation originates from the graft [30]. In addition, graft contamination of leukemic cells is associated with rapid relapse and poor prognosis [31]. We employed grafts in which absence of MRD was confirmed by nested RT-PCR, and this would have contributed to the good outcome.

As our analysis was retrospective and involved a very small population, it might have included variable bias. Although the present study included truly consecutive patients, there might have been an institutional bias because of the discrepancy in the number of patients between t(8;21)-AML and inv(16)-AML and because only 2 patients as yearly average were referred as having CBF-AML in our institution. In addition, in general, ASCT was used for patients with good performance status and good control of leukemia, which would have contributed to the good outcome. However, the actual OS and EFS for ASCT with a PCR-negative graft were surprisingly good in our series (100% and 93%, respectively), and EFS for patients overall exceeded 80%. Therefore, a prospective trial will be needed to investigate further confirmation of ASCT with a PCR-negative graft.

MRD in BM before ASCT was detectable in 10 of the 12 investigated patients. In 8 of these, MRD in BM became undetectable by RT-PCR after ASCT. Interestingly, in patients 2 and 6 (Table 4), the AML1/ETO(MTG8) fusion transcript disappeared 2 and 3 years after each ASCT, respectively, without further therapy. Although the reason for the late disappearance of MRD was unclear, 1 possibility was an enhanced and reconstructed immune response after ASCT. The myeloablative conditioning regimen would also have eradicated leukemic stem cells with