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CELL THERAPY AND ISLET TRANSPLANTATION

Hyperglycemia During the Neutropenic Period Is Associated With a Poor Outcome in Patients Undergoing Myeloablative Allogeneic Hematopoietic Stem Cell Transplantation

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Background. Recipients of allogeneic hematopoietic stem cell transplantation (HSCT) frequently require support with parenteral nutrition and immunosuppressive drugs, which introduce the risk of hyperglycemia. Van den Berghe et al. showed that the strict glucose control improved the outcome of patients treated in the intensive care unit, and this point was evaluated in this study in a HSCT setting.

Methods. A cohort of 112 consecutive adult patients treated by myeloablative allogeneic HSCT between January 2002 and June 2006 was reviewed retrospectively. Twenty-one patients were excluded due to graft failure, preexisting infectious diseases, preexisting neutropenia or previous allogeneic HSCT. The remaining 91 patients were categorized according to mean fasting blood glucose (BG) level in the neutropenic period after conditioning: normoglycemia (BG <110 mg/dL, n=28), mild hyperglycemia (110 to 150 mg/dL, n=49), and moderate/severe (>150 mg/dL, n=14). The primary endpoint was the occurrence of febrile neutropenia (FN) and documented infection during neutropenia, and the secondary endpoints included organ dysfunction according to the definition used by van den Berghe, acute graft-versus-host disease (GVHD), overall survival, and nonrelapse mortality (NRM).

Results. Although the incidence of FN or documented infections was similar between the three groups, hyperglycemia was significantly associated with an increased risk of organ dysfunction, grade II–IV acute GVHD, and NRM.

Conclusions. While the results suggested an association between the degree of hyperglycemia during neutropenia and an increased risk of posttransplant complications and NRM, the possibility that intensive glucose control improves the outcome after HSCT can only be confirmed in a prospective randomized trial.

Keywords: Allogeneic transplantation, Hyperglycemia, Nonrelapse mortality, Acute graft-versus-host disease.

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Van den Berghe et al. showed with patients nursed in the intensive care unit (ICU) that the rigid control of hyperglycemia with intensive insulin therapy to keep the blood glucose level at 80–110 mg/dL reduced morbidity, including infections, and mortality compared to patients who received standard care maneuvers that maintained the level at <200 mg/dL (*1–3*). Although these results have been confirmed in several subsequent studies (*4–7*), the precise mechanism that underlies this association is unclear. In animal models, it has been shown that insulin itself has a direct inhibitory effect on the inflammation process (*8, 9*). However in human studies, it has been suggested that these benefits could be directly attributed to intense glucose control rather than to any pharmacological activity of administered insulin per se (*3, 4*).

Recipients of allogeneic hematopoietic stem cell transplantation (HSCT) suffer from serious complications including infection, graft-versus-host disease (GVHD) and organ dysfunction. They are also at higher risk of hyperglycemia due to the use of steroids for the treatment of graft-versus-host disease (GVHD), prolonged total parenteral nutrition (TPN), immunosuppressive drugs, and infectious complications (*10, 11*). This makes them susceptible to numerous serious complications, including multiple organ failure (*12–14*). In this study, we evaluated whether hyperglycemia during the cytopenic pe-

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riod after conditioning for HSCT could be a significant risk factor for the subsequent clinical course.

PATIENTS AND METHODS

Patient Characteristics

A cohort of 112 consecutive adult patients who received myeloablative allogeneic HSCT between January 2002 and June 2006 at the National Cancer Center Hospital (Tokyo, Japan) was reviewed retrospectively. Twenty-one patients were excluded due to graft failure, pre-existing infectious diseases or neutropenia before HSCT, and previous allogeneic HSCT. The remaining 91 patients were subjected to further analysis, and their characteristics are listed in Table 1. Their median age was 36 years (range, 18–57 years), and their diagnosis included acute myeloid leukemia (AML, n=41), acute lymphoblastic leukemia (ALL, n=21), non-Hodgkin lymphoma (NHL, n=13), myelodysplastic syndrome (MDS, n=10), and chronic myelogenous leukemia (n=6). Standard-risk patients included those with acute leukemia in first complete remission, chronic leukemia in first chronic phase, MDS in refractory anemia, and NHL in complete remission, and the remaining patients were categorized as high-risk. Forty-six and 45 patients received a graft from a related donor and an unrelated donor, respectively. Stem cell sources included bone marrow (n=46), peripheral blood (n=41), and cord blood cells (n=4). In this study, only two patients were diagnosed as type 2 diabetes mellitus before HSCT, which reflects the low prevalence of this condition in Japan, especially in younger patients who can be the target of allogeneic HSCT with a myeloablative conditioning regimen. These two diabetic patients were included in the moderate and severe hyperglycemia group. None of the patients, including these two patients, had major organ dysfunction or diabetic complications before HSCT. For the transplantation procedure, signed informed consent was obtained according to the Declaration of Helsinki.

Transplantation Procedures

All patients received a myeloablative conditioning regimen that included oral busulfan (BU) plus cyclophosphamide (CY, n=45), CY plus 12 Gy total body irradiation (TBI, n=43) or cytarabine (CA) plus CY plus TBI (n=3: Table 1). GVHD prophylaxis included cyclosporine- (n=62) and tacrolimus-based regimens (n=29), with an additional short course of methotrexate (MTX) in 89 patients. Granulocyte

TABLE 1. Patient characteristics

Variable	Normoglycemia (<110 mg/dl)	Mild hyperglycemia (110−150 mg/dl)	Moderate and severe hyperglycemia (>150 mg/dl)
N	28	49	14
Blood glucose, median mg/dl (range)	104 (81–109)	120 (110–150)	168 (150–211)
Age, median years (range)	31 (21–52)	36 (18–57)	45 (30–57)
<40	20 (71)	32 (65)	4 (29)
≥40	8 (29)	17 (35)	10 (71)
Sex			
Male	9 (32)	34 (69)	8 (57)
Female	19 (68)	15 (31)	6 (43)
Disease risk			
Standard	16 (57)	18 (37)	6 (43)
High	12 (43)	31 (63)	8 (57)
Conditioning			
TBI-containing	11 (39)	26 (53)	9 (64)
Non-TBI-containing	17 (61)	23 (47)	5 (36)
GVHD prophylaxis			
Cyclosporine-based	24 (86)	33 (67)	5 (36)
Tacrolimus-based	4 (14)	16 (33)	9 (74)
Relation to donor			
Related	19 (68)	24 (49)	3 (21)
Unrelated	9 (32)	25 (51)	11 (79)
Stem cell source			
Bone marrow	11 (39)	24 (49)	11 (79)
PBSC	16 (57)	22 (45)	3 (21)
Cord blood	1 (4)	3 (6)	0 (0)
HLA match			
Match	25 (89)	34 (69)	10 (71)
Mismatch	3 (11)	15 (31)	4 (29)

Data are n (%) unless noted.

TBI, total body irradiation; GVHD, graft-versus-host disease; PBSC, peripheral blood stem cells; HLA, human leukocyte antigen.

colony-stimulating factor (G-CSF) was administered in all patients from day +6 after transplantation until engraftment. Most patients received ciprofloxacin (200 mg orally three times daily) for bacterial prophylaxis until neutrophil engraftment. Fluconazole (100 mg once daily) was administered for fungal prophylaxis. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and varicella zoster virus until the cessation of immunosuppressive agents. Prophylaxis against *Pneumocystis jiroveci* infection consisted of trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day −3 of transplantation, and from day +28 until day +180 or the cessation of immunosuppressive agents. Patients who developed fever during the neutropenic period were treated with cefepime, and additional agents including vancomycin, aminoglycosides and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the absolute neutrophil count exceeded 0.5×10^9/L.

Grouping of Patients

Patients were categorized according to the mean blood glucose (BG) level in the preengraftment neutropenic period: normoglycemia BG maintained at <110 mg/dL (group 1, n=28), mild hyperglycemia at 110–150 mg/dL (group 2, n=49), and moderate/severe hyperglycemia at >150 mg/dL (group 3, n=14). Blood glucose level was routinely tested in the morning at least three times a week. Daily caloric intake was calculated by dietitian following the chart record.

Outcome Measures

The primary outcome measure was the occurrence of febrile neutropenia (FN) and documented infection including bacteremia, pneumonia and central venous catheter infection in the neutropenic period. Secondary outcome measurements were organ dysfunction in the neutropenic period, acute GVHD, overall survival (OS) and nonrelapse mortality (NRM). Organ dysfunction was defined with reference to van den Berghe (*5–7*) as follows: 1) hypercreatininemia: serum creatinine level ≥2.0 mg/dL or more than twice the baseline; 2) hyperbilirubinemia: serum total bilirubin level ≥2.0 mg/dL; and 3) increased inflammatory markers: serum C-reactive protein (CRP) level ≥15 mg/dL. Acute GVHD was graded by the Consensus Criteria (*15*).

Statistical Analyses

Standard descriptive statistics were used. The Student's *t*-test, chi-square, and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. Multiple logistic regression analysis was conducted to ascertain odds ratios (ORs) and 95% confidence intervals (CIs). OS was estimated using Kaplan-Meier curves. The cumulative incidences of NRM were estimated based on a Cox regression model for the cause-specific hazards by treating progressive disease or relapse as a competing event. Cox proportional hazard models were used for multivariate analysis of variables on NRM and OS after HCT. Clinical factors that were assessed for their association with NRM and OS included patient age, sex, conditioning regimen (TBI-based vs. non-TBI-based), donor [human leukocyte antigen (HLA)-matched vs. HLA-mismatched, related vs. unrelated], GVHD prophylaxis (cyclosporine-based vs. tacrolimus-based) and disease risk (standard vs. high). Factors with $P<0.10$ in the univariate analyses were subjected to a multivariate analysis. A level of $P<0.05$ was defined as statistically significant. All *P* values are two-sided. All analyses were performed using SPSS 10.0 statistical software (Chicago, IL).

RESULTS

Patients and Transplantation Characteristics

The median ages of the patients in the normoglycemia, mild hyperglycemia, and moderate/severe hyperglycemia groups were, respectively, 31, 36, and 45 years. The percentages of patients who received graft from an unrelated donor were 32%, 51%, and 79%, and the percentages of patients who received GVHD prophylaxis with tacrolimus were 14%, 33%, and 74%. To clarify the risk factor to be included in moderate and severe hyperglycemia group, logistic analysis was performed, which showed older age and GVHD prophylaxis with tacrolimus were associated with moderate and severe hyperglycemia [$P=0.04$, OR 3.9 (1.1–14.0), and $P=0.01$, OR 5.5 (1.5–20.3), respectively], and there was a trend that patients who received stem cell from unrelated donor were associated with moderate and severe hyperglycemia [$P=0.07$, OR 3.6 (0.9–14.2)]. Multiple logistic analysis showed age more than 40 years old and GVHD prophylaxis with tacrolimus were associated with moderate and severe hyperglycemia [$P=0.042$, OR 4.1 (1.1–15.7), and $P=0.01$, OR 5.8 (1.5–22.1), respectively].

Although in practice we generally keep the parenteral glucose dose relatively low to avoid severe metabolic complications including hyperglycemia and hyperlipidemia during the acute phase of allogeneic HSCT, the possibility that the dose of parenteral nutrition affects the blood glucose level should be explored. We calculated the total caloric intake by combining both oral and parenteral nutrition. Although the mild hyperglycemia group received significantly more parenteral nutrition than the normoglycemia group (group 1 694+322 kcal/day vs. group 2 969+383 kcal/day), overall there was no essential difference in caloric intake between the three groups (1070+303 kcal/day, 1190+393 kcal/day, 1045+530 kcal/day, respectively). The median duration of the follow-up time in surviving patients was 809 days (range, 132-1530 days) in group 1, 369 days (105–1550 days) in group 2, and 587 days (170–774 days) in group 3. Described as hydrocortisone-equivalent dose, the median dose of corticosteroid used during neutropenia was 0 mg (0–1610 mg) in group 1, 100 mg (0–9700 mg) in group 2, and 375 mg (0–2468 mg) in group 3. Statistically more dose of corticosteroid was used in group 2 and group 3, compared with group 1.

Primary Endpoints

The incidence of FN and documented infections is summarized in Table 2. The incidences of FN and documented infections including bacteremia, pneumonia, and central venous catheter infection in groups 1, 2 and 3 were, respectively, 89% and 32% (25%, 4% and 11%), 88% and 20% (16%, 6% and 6%), and 98% and 43% (36%, 14% and 14%). Overall, no statistically significant difference was observed between the three groups in the incidence of infectious episodes, including FN and documented infections.

TABLE 2. Endpoints

Variable	Normoglycemia (<110 mg/dl)	Mild hyperglycemia (110−150 mg/dl)	Moderate and severe hyperglycemia (>150 mg/dl)
N	28	49	14
Febrile neutropenia	23 (89)	43 (88)	13 (98)
Documented infection	9 (32)	10 (20)	6 (43)
Bacteremia	7 (25)	8 (16)	5 (36)
Pneumonia	1 (4)	3 (6)	2 (14)
Central-venous catheter infection	3 (11)	3 (6)	2 (14)
Organ dysfunction			
Hypercreatininemia	1 (4)	4 (8)	4 (29)
Hyperbilirubinemia	3 (11)	11 (22)	6 (43)
Increased inflammatory markers	4 (14)	15 (31)	9 (64)

Data are n (%).

Hypercreatininemia, serum creatinine level ≥2.0 mg/dl or more than twice of baseline; hyperbilirubinemia, serum bilirubin level ≥2.0 mg/dl; increased inflammatory markers, serum C-reactive protein level ≥15 mg/dl.

Secondary Endpoints

The incidence of hypercreatininemia was 4% in group 1, 8% in group 2 and 29% in group 3, as summarized in Table 2, and that in group 3 was significantly higher than those in group 1 (OR 10.8, 95% CI 1.1–108.6; $P=0.018$) and group 2 (OR 4.5, 95% CI 1.0–21.1; $P=0.043$). The incidence of hyperbilirubinemia was, respectively, 11%, 22% and 43%, in the three groups, and that in group 3 was significantly higher than that in group 1 (OR 6.3, 95% CI 1.3–30.9; $P=0.017$). The incidence of increased inflammatory markers was, respectively, 14%, 31% and 64%, and that in group 3 was significantly higher than those in group 1 (OR 10.8, 95% CI 2.4–49.5; $P<0.001$) and group 2 (OR 4.1, 95% CI 1.2–14.3; $P=0.022$). Multiple logistic regression analysis showed that the degree of hyperglycemia was associated with hypercreatininemia, hyperbilirubinemia, and increased inflammatory markers (Table 3).

TABLE 3. Multiple logistic regression analysis for organ dysfunction and multiple variate analysis for acute GVHD, nonrelapse mortality, and overall survival

Outcomes and variables	Odds/hazard ratio	95% CI	P value
Multiple logistic regression analysis			
Hypercreatininemia			
Hyperglycemia	5.2	1.1–24.6	0.039
Hyperbilirubinemia			
Hyperglycemia	4.9	1.6–14.9	0.005
Increased inflammatory markers			
Hyperglycemia	6.7	2.2–20.3	0.001
Tacrolimus-based	6.9	1.6–30.5	0.011
Multivariate analysis (Cox-proportional hazard model)			
Acute GVHD			
Hyperglycemia	2.3	1.2–4.3	0.013
Disease risk (high)	2.3	1.0–5.1	0.047
HLA mismatch	2.8	1.3–5.9	0.009
Nonrelapse mortality			
Hyperglycemia	2.9	1.2–6.6	0.013
Disease risk (high)	2.7	0.9–8.7	0.091
Overall survival			
Hyperglycemia	2.0	1.1–3.6	0.019
TBI-containing	2.3	1.1–5.0	0.035
Disease risk (high)	1.9	0.9–4.1	0.10

Odds ratios are presented for multiple logistic regression analysis; hazard ratios are presented for multivariate analysis.

GVHD, graft versus host disease; TBI, total body irradiation.

The cumulative incidence of grade II–IV acute GVHD is shown in Figure 1. The degree of hyperglycemia was associated with a higher incidence of grade II–IV acute GVHD

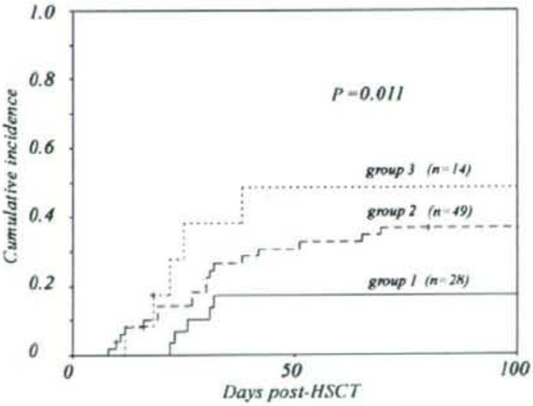

FIGURE 1. Cumulative incidence of acute GVHD grade II–IV stratified according to the mean glucose level during neutropenia. Group 1 included patients with normoglycemia, group 2 included patients with mild hyperglycemia, and group 3 included patients with moderate and severe hyperglycemia.

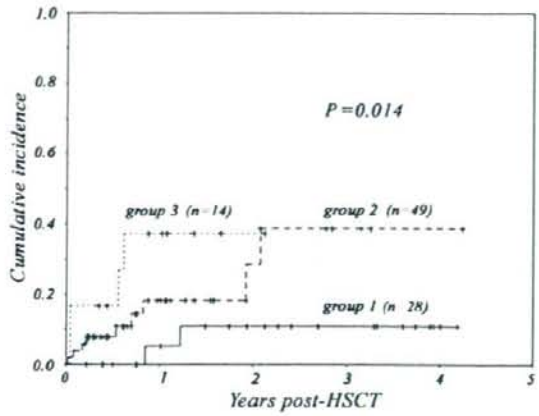

FIGURE 2. Cumulative incidence of treatment-related mortality stratified according to the mean glucose level during neutropenia.

($P=0.002$). A Cox proportional hazard model showed that hyperglycemia, high-risk underlying disease, and HLA mismatch were risk factors for grade II-IV acute GVHD (Table 3).

The cumulative incidence of NRM was, respectively, 5%, 17%, and 35% at 1 year, and was significantly related to the degree of hyperglycemia ($P=0.014$; Fig. 2). The probability of OS was, respectively, 88%, 70%, and 56%, and was significantly associated with hyperglycemia ($P=0.008$; Fig. 3). A Cox proportional hazard model showed that the degree of hyperglycemia was associated with NRM and OS (Table 3).

DISCUSSION

In this study, we evaluated whether hyperglycemia during the cytopenic period after conditioning for HSCT could be a significant risk factor for the subsequent clinical course. Infectious diseases remain a major cause of morbidity and mortality in patients who receive HSCT, and we speculated that this might be exaggerated in the presence of hyperglycemia. Alternatively, hyperglycemia can be caused by infectious diseases and also aggravates infectious diseases to lead to a vicious cycle, with resultant morbidities that include organ dysfunction and mortality. Theoretically, strict glucose control should prevent this vicious cycle and help to reduce morbidity and mortality in patients after HSCT, as shown previously in ICU settings (*1, 2*). However, in this study the incidences of FN and documented infections were not different among the three groups. On the other hand, we found that hyperglycemia was associated with organ dysfunction and increased inflammatory markers, which was consistent with previous reports that demonstrated the impact of hyperglycemia on clinical outcomes of patients suffering from nonhematological diseases (*1–3, 12–14*). Additionally, a multivariate analysis showed that hyperglycemia was a risk factor for acute GVHD.

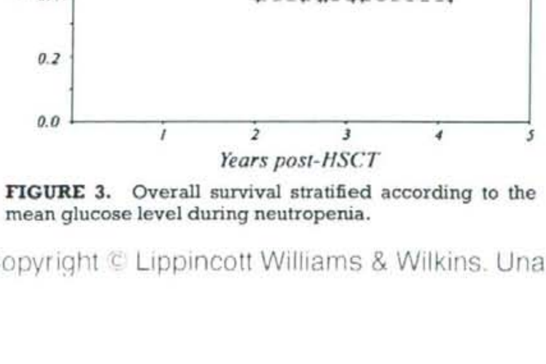

FIGURE 3. Overall survival stratified according to the mean glucose level during neutropenia.

The reason for the association between early hyperglycemia and late complications needs to be clarified. The increase in the levels of circulating cytokines due to hyperglycemia may further aggravate hyperglycemia itself (*16–21*). Therefore, this condition which occurs during the critical period of neutropenia before engraftment may influence the afferent phase of acute GVHD, as suggested by Ferrara et al. Elevated cytokine levels during the afferent phase then lead to subsequent acute GVHD in the effector phase (*22, 23*). Teshima et al. reported that the effector phase of acute GVHD is not antigen-specific and inflammatory cytokines mediate target destruction (*24*), and other reports have shown that inflammatory cytokines were required in acute GVHD and these molecules can cause tissue damage (*25–27*). With these reports in mind, it is reasonable to speculate that the aggravated production of inflammatory cytokines by hyperglycemia may be a risk factor in the pathogenesis of acute GVHD and organ dysfunction.

This study has several limitations, including heterogeneous patient populations and a retrospective nature. First, hyperglycemia can be caused by infection itself and it has been previously shown that the level of hyperglycemia was correlated with the severity of illness (*4*). In this retrospective study, we could not confirm whether hyperglycemia directly influenced organ dysfunction or increased inflammatory markers. Furthermore, statistically more corticosteroid was used in the group of moderate and severe hyperglycemia, and statistically more parenteral nutrition was used in the group of mild hyperglycemia. However, the observation that hyperglycemia and the severity of illness were independently associated with a worse prognosis has been well confirmed in the ICU setting (*4*), and several prospective studies have shown that intensive glucose control reduced both morbidity and mortality (*1, 2*). Considering these findings, we suggest that our data still support the possibility that the degree of hyperglycemia was associated with morbidity and mortality in the allogeneic HSCT setting. Second, we must consider that the patients who developed moderate and severe hyperglycemia included older patients, those who received more unrelated grafts, and those who received tacrolimus compared to other groups. In terms of immunosuppressive drugs, tacrolimus has recently become a preferred immunosuppressive drug for GVHD prophylaxis in unrelated or HLA-mismatched HSCT, based on the results of two Japanese studies, which showed that, compared to cyclosporine, tacrolimus was associated with a lower incidence of acute GVHD and better overall survival, which were similar to those in related HSCT, even after HSCT with alternative donors, including unrelated donors (*28, 29*). Therefore, the effect of unrelated graft and tacrolimus on the incidence of acute GVHD and NRM might not be significant in this study.

The effects of tacrolimus on hyperglycemia, hyperbilirubinemia, and hypercreatininemia need to be clarified. It is well known that hyperglycemia occurs more often in patients receiving tacrolimus than in those receiving cyclosporine (*30–32*). In the present study, patients receiving tacrolimus were more likely to have moderate to severe hyperglycemia. However, the association of hyperbilirubinemia with tacrolimus has not been previously reported and two other studies (*33, 34*) showed that cyclosporine was more likely to cause hyperbilirubinemia than tacrolimus after allogeneic HSCT or kidney transplantation. Although the relative nephrotoxicity attributed to tacrolimus compared to cyclosporine has been controversial (*30, 33, 35*), studies that have reported such nephrotoxicity used a higher target tacrolimus level (>20 ng/ml) (*30, 35*). On the other hand, it has been reported that the use of lower levels of tacrolimus (10–15 ng/ml in our hospital) was associated with reduced complications in allogeneic HSCT (*36, 37*), with no difference in the incidence of hypercreatininemia compared to cyclosporine (*33*). Based on a consideration of all of these results, we think that tacrolimus might not be the direct cause of hypercreatininemia in this study. Finally, due to the nature of this retrospective study, during the period evaluated we did not apply any consistent protocol for glucose control and nutritional support, although we tried to avoid severe hyperglycemia (BG ≥ 200 mg/dl), which certainly biases the interpretation of the data, although it has been reported that the overall glucose level, rather than the dose of insulin administered, directly influenced the outcome of patients (*3*).

Even with these limitations, we believe that our observation is still of value in considering the clinical impact of the strict control of hyperglycemia during the early phase of HSCT. To confirm our preliminary observation, a prospective pilot study is underway to assess the effect of intensive glucose control after HSCT. If this pilot study shows a beneficial effect of intensive glucose control, a prospective randomized trial would be warranted to confirm the possibility that intensive glucose control improves the outcome after HSCT. Additionally, in this ongoing pilot study, we evaluate the diurnal blood glucose and insulin levels, including postprandial levels, to detect hyperglycemia more precisely before transplantation since the level of HgA1c is affected by both the blood glucose level and the turnover rate of red blood cells, and would not precisely correlate with the true mean blood glucose level in patients who received courses of blood transfusion for anemia.

In conclusion, the association of the degree of hyperglycemia during neutropenia and an increased risk of posttransplant complications and NRM was suggested, but the possibility that intensive glucose control improves the outcome after HSCT would only be confirmed in a prospective randomized trial.

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WILEY InterScience
DISCOVER SOMETHING GREAT

Prospective phase II trial to evaluate the complications and kinetics of chimerism induction following allogeneic hematopoietic stem cell transplantation with fludarabine and busulfan

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This prospective trial assessed the safety and efficacy of allogeneic hematopoietic stem cell transplantation from a HLA-matched donor with a reduced-intensity regimen (RIST) consisting of iv fludarabine 30 mg/m^2 for 6 days and oral busulfan 4 mg/kg/day for 2 days in patients older than 50 years with hematological malignancies. Cyclosporine alone or cyclosporine with short-term methotrexate was randomized for graft-versus-host disease prophylaxis. After 30 patients had been enrolled, an interim analysis was performed, and this report focuses on a precise evaluation of the toxicity profile and chimerism kinetics. Sustained engraftment in all patients, no severe regimen-related toxicity (RRT) within 20 days, and no transplant-related mortality through Day 100 were observed. T-cell (CD3+) full-donor (over 90%) chimerism was observed in 22 of the 30 patients, while the remaining eight had mixed-donor chimerism over 77% on Day 90. Thereafter, five subsequently converted to full-donor chimerism without donor lymphocyte infusion by day 120 ($n = 4$) or Day 180 ($n = 1$). Two showed persistent mixed chimerism without relapse through Day 180. Grade III–IV acute graft-versus-host disease and extensive chronic graft-versus-host disease occurred in 10% and 73%, respectively. With a median follow-up of 1.5 years, overall survival and disease-free survival at 1 year was 83% and 62%, respectively. Seven patients hematologically relapsed overall, and five of them had myelodysplastic syndrome with poor prognostic factors. In older patients, RIST with fludarabine and busulfan was associated with acceptable toxicities and a satisfactory antileukemia effect, regardless of the early chimerism status. Am. J. Hematol. 00:000–000, 2007.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment of choice for hematological malignancies. However, many centers limit HSCT to younger patients because of the threat of a higher risk of treatment-related toxicities including graft-versus-host disease (GvHD), nonrelapse mortality, and lower disease-free survival (DFS) in the older population, although the median age of onset of chronic myeloid leukemia (CML) is in the sixth decade of life, and the peak incidence of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) is in the seventh decade. To overcome this obstacle, allogeneic HSCT with a reduced-intensity (RIST) or nonmyeloablative conditioning regimen has recently been explored for patients who are ineligible to receive conventional myeloablative HSCT (CIST) due to age limits or comorbidities. Many studies suggested that RIST is a reasonable option for older patients or patients with comobidities with acceptable treatment-related complications or morbidity, while preserving adequate antitumor effects [1–12]. However, these studies mostly pursued different variables including disease types, stages [1,4–6, 8,12], donor type [1,2,5,10], graft source [1,2], conditioning regimens [4,5,7,9], and/or GvHD prophylaxis [1,4,9]. This

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TABLE I. Patient and Donor Characteristics

UPN	Patient age/sex	Donor age/sex	Stage and diagnosis	IPSS/cytogenetic risk in MDS patients	CD34+ cells (10^6)	Blood type patient/donor
1	60/M	46/F	MDS (RA)	Intermediate-2/Poor	3.92	A/A
2	61/M	54/F	MDS (RAEB)	Intermediate-2/Poor	5.84	O/O
3	67/M	60/F	AML (M4) in 2CR		2.74	B/O
4	60/M	55/F	AML (M2) in 2CR		4.58	B/B
5	63/M	60/M	CML in 2CP		12.59	O/O
6	54/M	59/F	MDS (RAEB)	High/Intermediate	5.4	O/A
7	52/M	55/F	AML (M2) in 1CR		6.77	A/A
8	61/M	54/M	AML (M1) in 1CR		3.29	B/A
9	58/F	64/F	CML in 1CP		2.9	A/AB
10	64/F	59/F	ALL (L2) in 1CR		5.54	A/A
11	55/M	44/M	AML (M1) in 1CR		3.13	A/A
12	55/F	51/F	CML in 1CP		4.94	A/O
13	52/F	42/M	AML (M4) in 1CR		3.59	A/A
14	59/M	64/M	MDS (RAEB)	Intermediate-2/intermediate	3.58	A/AB
15	59/M	56/M	MDS (RA)	Intermediate-1/Good	3.58	AB/A
16	53/F	55/F	MDS (RA)	Intermediate-2/Poor	2.2	O/O
17	55/F	68/M	AML (M3) in 2CR		2.63	A/A
18	54/M	50/M	MDS (RA)	Intermediate-1/Poor	3.74	O/B
19	51/M	44/F	AML (M1) in 1CR		4.86	AB/A
20	64/F	66/M	CML in 2CP		3.59	O/A
21	68/F	64/M	MDS (RAEB)	Intermediate-1/Good	3.56	B/B
22	53/M	44/M	MDS (RAEB)	High/Intermediate	7.2	B/B
23	60/F	53/M	AML (M2) in 1CR		2.83	A/B
24	59/M	62/M	AML (M4) in 2CR		5.47	A/O
25	51/F	47/F	MDS (RAEB)	Intermediate-2/Poor	5.93	A/A
26	59/M	62/F	MDS (RA)	Intermediate-2/Poor	4.02	B/O
27	59/M	48/M	AML (M2) in 2CR		4.94	B/A
28	56/M	62/F	MDS (RAEB-t)	High/Good	4.38	AB/A
29	53/F	62/F	AML (M2) in 1CR		3.06	O/O
30	54/F	63/M	AML (M2) in 1CR		6.47	A/O

M, male; F, female; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEB-t, refractory anemia with excess blasts in transformation; CR, complete remission; CP, chronic phase. All donors were HLA-matched siblings.

makes overall interpretation of studies difficult. Additionally, there has been no study to prospectively assess whether RIST consisting of 180 mg fludarabine plus 8 mg/kg busulfan without antithymocyte globulin actually produces less significant organ toxicities and treatment-related toxicities in an older patient population. Information regarding the impact of the speed and degree of lineage-specific donor chimerism on clinical outcomes after RIST in older patients has been limited [3,8,13–17]. Moreover, even studies evaluated with more homogeneous patient population, type of GvHD prophylaxis and/or tempo of withdrawal of immunosuppressive agents varied depending on transplant centers and a feasible prophylaxis regimen for acute GvHD has not been well evaluated in RIST, which is considered to require a sophisticated balance between GvHD and a graft-versus-leukemia (GvL) effect.

To address these points, we conducted a prospective randomized clinical trial to evaluate the safety and efficacy of RIST with fludarabine and oral busulfan in patients aged over 50 years and with appropriate GvHD prophylaxis. In this report, the results of an interim analysis, including clinical outcomes, complications, and chimerism kinetics, were compared with those previously published in the literature.

Patients and Methods

Patient eligibility and accrual

Eligible patients ranged in age from 50 to 69 years (median 58.5, range 51–68 years) and had a hematological malignancy, including AML or acute lymphoblastic leukemia (ALL) in 1st or 2nd complete remission (CR), CML in 1st or 2nd chronic phase (CP), and MDS. They were required to have an HLA-identical related donor. The study protocol was reviewed and approved by the institutional review boards of the participating transplantation centers (Appendix). Eligible patients and their donors gave written informed consent before enrollment. The enrollment criteria included a performance status (PS) of the Eastern Cooperative Oncology Group (ECOG) of less than two, a serum creatinine concentration of less than 2.0 mg/dl, a cardiac ejection fraction of more than 50%, arterial oxygen saturation without supplemental oxygen of more than 93%, liver function tests less than fourfold the upper limit of normal, total bilirubin less than 2.0 mg/dl, no active infection, and no previous allergy for drugs used for conditioning or GvHD prophylaxis. Donors were required to have a normal physical examination, and normal values in the serum chemistry and blood counts, and negative results of serologic testing for human immunodeficiency virus and hepatitis B. The patient and donor characteristics are shown in Table I. Those with AML/ALL in 1st CR, CML in 1st CP, or MDS in refractory anemia were defined as low risk, and the others were defined as high risk. All 12 patients with MDS except one (UPN 22) were transfusion dependent, and all those were grouped according to the International Prognostic Scoring System (IPSS) into intermediate or high risk at the time of transplantation: Intermediate-1, $n = 3$; intermediate-2, $n = 6$; high risk, $n = 3$. By IPSS criteria, 3 patients had good-risk, 3 had intermediate-risk, and 6 had poor-risk cytogenetics.

Donor selection and blood stem cell harvest

Related donors were selected based on compatibility of HLA-A, B and DRB1 by intermediate- or high-resolution DNA typing. After G-CSF treatment, apheresis procedures were performed daily until at least 2.0 × 10^6 CD34+ cells per kilogram of the recipient's body weight, up to three times, and all of the collected cells were cryopreserved until stem cell infusion.

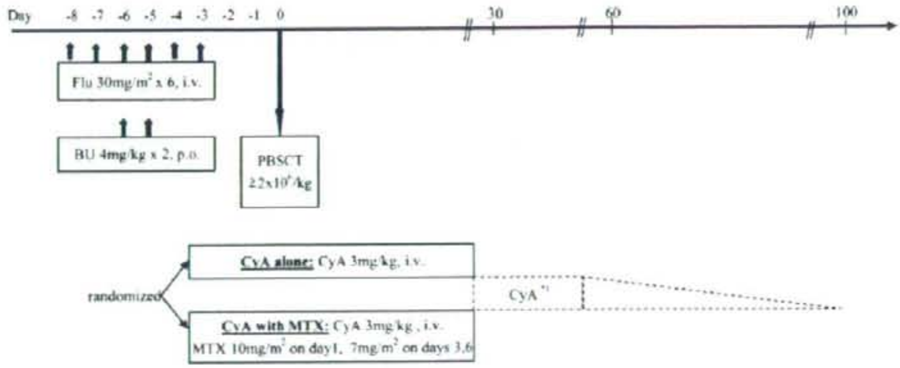

Figure 1. Treatment schedule. CyA; cyclosporine, MTX; methotrexate. *1: When acute GvHD was not observed, CyA was tapered by 10% a week starting at Day 28, and was eliminated by Day 100. When mixed chimerism was seen without active acute GvHD over Day 60, CyA was tapered and discontinued within 2 weeks. Patients who did not convert to complete chimerism after CyA withdrawal received donor lymphocyte infusion.

Treatment schedule

The treatment schedule is shown in Fig. 1. The conditioning regimen consisted of fludarabine (30 mg/m²/day) infused over 30 min once a day on Days 8, 7, 6, 5, 4, and 3, and oral busulfan (4 mg/kg/day) on Days 6 and 5. To prevent seizures, the patients received oral valproate sodium, at a dose of 600 mg divided into 3 doses 2 days before busulfan administration, and this was continued until 24 hr after the last dose of busulfan.

Patients were randomized to receive either cyclosporine (CyA) alone or CyA plus short-term methotrexate (MTX) for GvHD prophylaxis. Randomization was performed by stratifying according to disease (AML, ALL, CML or MDS), transplant center, age (less than 60 years or more than or equal to 60 years), and sex (male or female). All patients received 3 mg/kg/day CyA by continuous iv infusion daily from Day 1 to maintain a therapeutic trough level of 250–400 ng/ml, and thereafter orally in an attempt to maintain a therapeutic trough level of 150–250 ng/ml. The patients who were assigned to CyA plus short-term MTX received a dose of 10 mg/m² iv MTX on Day +1, and 7 mg/m² on Days +3 and +6 after stem cell infusion. CyA was tapered starting at Day 28 in the absence of acute GvHD and was discontinued by Day 100 after transplantation. When a patient did not achieve complete donor chimerism by Day 60, CyA was tapered rapidly and discontinued within 2 weeks if clinically feasible, since anti-leukemic effect was presumed to occur after development of complete donor chimerism [14]. Cases of Grade II–IV acute GvHD were treated with 2 mg/kg/day of methylprednisolone in addition to CyA.

Supportive care

The following infection prophylaxis was recommended: prophylactic antibiotics (fluoroquinolones) were given during cytopenia, fluconazole (200 mg/day) was given at the start of conditioning and continued until the discontinuation of immunosuppressant, and oral acyclovir (1,000 mg/day) or iv acyclovir (750 mg/day) was given for prophylaxis of herpes simplex virus (HSV) and varicella zoster virus (VZV) from Day −7 to Day 35. Prophylaxis against *Pneumocystis carinii* was consisted of trimethoprim-sulfamethoxazole after neutrophil engraftment ($\geq 0.5 \times 10^9$ L^{-1}) and was continued until the discontinuation of immunosuppressant. During the first 100 days after transplantation, cytomegalovirus antigenemia assay with HRP-C7 or C10/C11 monoclonal antibody was performed weekly after neutrophil engraftment until Day 100 after transplantation. Pre-emptive therapy with ganciclovir was recommended upon the detection of positive antigenemia and was continued until it became negative. Patients were treated with G-CSF from Day +6 to neutrophil engraftment.

Chimerism analysis

Hematopoietic chimerism was evaluated with regard to peripheral T cell (CD3+) fraction by an analysis of DNA microsatellite polymorphisms by polymerase chain reaction (PCR) with D18S51, D20S471, and D22S684 fluorescence-labeled primers, which identified differences between patient and donor (on the basis of polymorphisms found in pretransplant patient/donor samples) using an BECKMAN COULTER CEQ8000 GENETIC ANALYSIS SYSTEM. T cell (CD3+) chimerism studies post HSCT were performed on Days 30, 60, 90, 120, and thereafter every other month through 1 year.

Assessment of response

Day 0 was defined as the day of stem cell infusion day. The day of neutrophil engraftment was defined as the first of two consecutive days on which the patient's absolute neutrophil count was above 0.5×10^9 L^{-1}. The day of platelet engraftment was defined as the first of seven consecutive days on which the platelet count was above 20×10^9 L^{-1} without platelet transfusion.

Regimen-related toxicity (RRT) was graded using the Seattle criteria [18] on the day before the initiation of conditioning regimens and at least 3 days a week until Day 20 after transplantation. All other observed adverse events were graded according to the National Cancer Institute Common Toxicity Criteria version 2.0 (NCI-CTC ver. 2.0) until Day 100 after transplantation. Infectious diseases were diagnosed based on any positive blood culture or histologic evidence of tissue invasion.

To evaluate the general condition of patients associated with the toxicity profile, PS, and dietary oral intake were also reported at least three times a week during the initial hospitalization and once a week afterwards up to Day 100 post-transplant.

The diagnosis and grading of acute and chronic GvHD was made based on the date of onset (within or beyond 100 days) and clinical findings in conjunction with biopsy of the skin and digestive tract using the published criteria [19,20]. Patients who survived 100 days or longer were evaluable for the assessment of chronic GvHD.

Pharmacokinetic studies of fludarabine phosphate and busulfan

Blood sampling for pharmacokinetic studies was done on Day −5 to investigate the effect of concomitant busulfan administration on the pharmacokinetics of 2-fluoro-ara A (2F-ara-A), which is the major metabolite of fludarabine phosphate. Blood samples for determining the 2F-ara-A plasma level were collected at 0, 0.5, 1, 2, 5, and 23.5 hr after the 4th infusion of fludarabine. We also obtained blood samples for determining the busulfan plasma level at 0, 0.5, 1, 1.5, 2, 3, and 6 hr after the sixth administration of busulfan (1 mg/kg/dose for 8 times). Blood samples were taken in tubes containing heparin and erythro-9-(2-hydroxy-3-nonyl)adenine. Plasma was obtained by centrifugation, and then transported to the laboratory and were stored at −20°C until analysis. Plasma levels of 2F-ara-A and busulfan were determined using high-performance liquid chromatography with fluorescence and UV detection, respectively. The accuracy and precision of the assays for 2F-ara-A and busulfan were confirmed by measuring QC samples of both before this study. The maximum concentration of drug in plasma after drug administration (C_{max}, C_{peak}) and the time to reach the maximum concentration following drug administration (T_{max}) were observed. The area under the plasma concentration-versus-time curve (AUC) for 2F-ara-A or busulfan was calculated by dividing the administered dose by the final plasma clearance estimate, whereas the plasma clearance was determined by modeling all plasma concentration versus time data. Terminal half-lives ($T_{1/2}$) were calculated from the primary parameters.

Statistical analysis

The primary endpoint of this study was to determine the percentage of patients who were alive at 100 days after transplantation with complete donor chimerism (over 90%) achieved by Day 90. Secondary endpoints included the time to engraftment of neutrophils and platelets, the incidence and severity of RRT, the incidence and severity of acute and chronic GvHD, the anti-leukemia effect, DFS, and overall survival (OS). A descriptive statistical analysis was performed to assess patient baseline characteristics and disease. Time to engraftment, complete chimerism, acute or chronic GvHD, OS, and DFS were calculated using the Kaplan–Meier method. OS was defined as the time between stem cell infusion to death from any cause. DFS was defined as the time between stem cell infusion to relapse and death from any cause, whichever occurred first. After 30 patients had been enrolled in the study, a data and safety monitoring committee undertook an interim analysis. This analysis, completed in October 2004, included data for the primary endpoint, i.e. survival at Day 100 and chimerism status at Day 90, and data on acute and chronic GvHD, survival, chimerism status, and anti-tumor effect through Day 180. Neither of the predefined criteria for stopping the study was met; however, a review of available safety data including incidence and severity of RRT and Day 100 mortality indicated that this conditioning regimen was adequately safe for older patients. According to the recommendation of the committee, we decided to continue the study and published an interim report when 30 patients were enrolled and evaluated without comparing the two different GvHD prophylaxis procedures. This report includes data on these 30 patients with all available follow-up data through December 2005, and does not include the results of a comparison of the two different GvHD prophylaxis procedures.

Results

Engraftment and chimerism analysis

The results are summarized in Table II. One and four patients were not evaluated for neutrophil and platelet engraftment, respectively, because they did not show a nadir. The remaining patients achieved sustained engraftment and none experienced graft failure. The median number of days to achieve a neutrophil count $\geq 0.5 \times 10^9$ L^{-1} was 13 (range, 10–25 days), and this was 18 (range, 11–24 days) for a platelet count $\geq 20 \times 10^9$ L^{-1} without transfusion. Full-donor (over 90%) T-cell (CD3+) chimerism was observed in 2 and 9 of the 30 patients on Day 30 and Day 60, respectively (median [range], Day 30:71 [40 to $\geq$90] %, day 60:81 [41 to $\geq$90] %). Twenty-two patients achieved full-donor chimerism, while the remaining eight patients had mixed chimerism ranging from 78% to 88% on Day 90. Among those with mixed chimerism on Day 90, five subsequently converted to full-donor chimerism without early CyA withdrawal because of the severe acute GvHD ($n = 2$: UPN 1 and 15) and/or donor lymphocyte infusion (DLI) by day 120 ($n = 4$) or day 180 ($n = 1$). One achieved full-donor chimerism on Day 120 after DLI since the patient did not respond to the discontinuation of immunosuppressive drugs, and two had persistent mixed chimerism without relapse through 180 days after transplantation (71% and 75% donor-type chimerism on Day 180). The diagnoses of two patients with persistent mixed chimerism through Day 180 were CML and MDS, and they had not received proceeding cytotoxic chemotherapy; the patient with CML (UPN 12) received immunomodulators, imatinib mesylate and hydroxyurea, and the patient with MDS (UPN 21) received low-dose cytarabine and aclarubicin in combination with granulocyte colony stimulating factor before RIST.

Regimen-related toxicities, complications, and general condition

The frequencies of Grade I–IV organ toxicities within 20 days after transplantation are listed in Table III. Although non-fatal toxicities including Grade I/II were seen in all 30 patients, all of the observed episodes were reversible and in no case required suspension of fludarabine. Stomatitis was the most frequently observed organ toxicity (57%, 17/30), with 47% of them (8/17) had Grade II events. None of the patients experienced veno-occlusive disease of the liver (VOD). Twenty patients had at least one episode of infectious complications within the first 100 days, with a total of 44 documented episodes (median, 2; range, 1–7 episodes) within the first 100 days after transplantation. These included proven bacterial infection (1 episode), suspected bacterial infection (1), suspected fungal infection (2), cytomegalovirus antigenemia (6), HSV infection (1), suspected viral infection (1), and uncertain causes (33). All infectious complications were recovered with or without appropriate antibiotic therapy.

The median PS for the first 28 days was 0 (range, 0–3). The worst PS of 2 ($n = 5$) or 3 ($n = 2$) within the first 28 days was experienced temporarily due to infection ($n = 2$), Grade III GvHD ($n = 1$), and nausea/vomiting ($n = 4$). Those ($n = 6$) observed from Day 29 to Day 100 were all caused by Grade II or III acute GvHD. A one-thirds reduction in dietary oral intake was temporarily seen in 20 and 11 patients within the first 28 days and from 29 days to 100 days post HSCT, respectively, which resulted from nausea/vomiting ($n = 18$) and treatment-related mucositis ($n = 2$) within Day 28, and Grade II–III acute GvHD ($n = 9$), prolonged infection with Grade II acute GvHD ($n = 1$) and gastroesophageal reflux disease ($n = 1$) between Day 29 and Day 100.

GvHD

Grade I–IV acute GvHD at 100 days was documented, respectively, in 5 (17%), 15 (50%), 3 (10%), and 0 (0%) patients. The median time to the occurrence of Grade II–IV acute GvHD was 74 days (range, 18–100 days). All 30 patients survived beyond Day 100 and were evaluated for chronic GvHD. Twenty-six of the 30 patients (87%) developed chronic GvHD (limited type in four cases and extensive type in 22 cases) with the onset at a median of 123 days after transplantation (range, 116–217 days).

Disease response, survival, and cause of death

No patient died within the first 100 days, and the median follow-up period was 555 days (149–1114 days) after transplantation. Twenty-nine of the 30 patients achieved CR within 100 days after transplantation, but two of them with MDS, who had poor-risk cytogenetics and were classified into intermediate-2, subsequently relapsed on Day 141 (UPN 26) and Day 156 (UPN 25). One was treated with DLI (UPN 25) and showed a temporary response, but died because of the disease progression on Day 401. The other patient (UPN 26) did not respond to DLI and died of progressive disease on Day 412. One patient (UPN 22) with MDS with high risk IPSS achieved full-donor chimerism on Day 90, but could not achieve CR on Day 98 and died with progressive disease on Day 306. This patient showed full-donor chimerism through Day 180. Five other patients died between 100 days and 1 year after transplantation (149, 151, 169, 187, and 354 days). In six patients who died within the first year, two patients were over 60 years and four patients were classified into high risk disease group. Causes of death included progressive disease of MDS with poor IPSS in 1, GvHD and/or its complications in 4, and recurrence of interstitial pneumonia in 1. In four patients, who died of GvHD and/or its complications, all had experienced

TABLE II. Summary of Clinical Outcomes

UPN	Chimerism analysis			Post transplant DLI (reason)	GvHD		Infection until day 100 (etiological agent)	Relapse	Outcome (Cause of death)	Follow up
	Day 90(%)	Day 120(%)	Day 180(%)		Acute	Chronic				
1	88.40	≥90	≥90		Gr II (S, G)	Extensive	–	–	Alive	1,114
2	85	≥90	≥90	Yes (d662, relapse)	Gr II (S)	Extensive	Yes (unknown)	Yes (d402)	Dead (recurrent disease and its complication)	652
3	≥90	≥90	≥90		–	Extensive	Yes (S. maltophilia, unknown)	–	Alive	735
4	≥90	≥90	D		Gr II (S)	–	–	–	Dead (IP)	169
5	≥90	≥90	≥90		–	Extensive	–	–	Alive	731
6	≥90	≥90	≥90		Gr II (L)	Extensive	–	–	Alive	716
7	≥90	≥90	≥90		Gr II (S, G)	Extensive	Yes (CMV antigenemia)	–	Dead (GvHD)	354
8	≥90	≥90	≥90		Gr III (S, G, L)	Extensive	Yes (bacteremia susp., fungal susp., CMV antigenemia, unknown)	–	Alive	431
9	≥90	≥90	≥90		–	Extensive	Yes (unknown)	–	Alive	592
10	≥90	≥90	≥90		Gr II (S, G)	Extensive	Yes (unknown)	–	Dead (GvHD)	757
11	≥90	≥90	≥90		Gr II (S)	Extensive	–	–	Alive	360
12	88	79	71		Gr III (S, L)	Extensive	Yes (unknown)	–	Alive	720
13	≥90	≥90	≥90		Gr I (S)	Extensive	Yes (HSV, unknown)	–	Dead (GvHD)	517
14	≥90	≥90	≥90		Gr II (S, G, L)	Limited	Yes (fungal susp., unknown)	–	Dead (GvHD and its complication)	187
15	85	88	≥90		Gr III (S, G)	–	–	–	Alive	702
16	84	88	≥90		–	Limited [a]	Yes (CMV antigenemia)	–	Alive	642
17	80	≥90	D	Yes (d98, mixed chimerism)	Gr II (S, G)[b]	–	–	–	Dead (GvHD and its complication)	149
18	≥90	88	≥90		Gr II (S)	Extensive	Yes (CMV antigenemia)	–	Alive	729
19	≥90	≥90	≥90		Gr I (S)	Limited	–	–	Alive	737
20	≥90	≥90	≥90		Gr II (G)	–	Yes (CMV antigenemia)	Yes (d147)[c]	Alive	688
21	78	77	75		Gr II (S, L)	Extensive	–	Yes (d364)	Dead (BOOP)	593
22	≥90	≥90	≥90		Gr II (S, G)	Extensive	Yes (unknown)	Yes (d98)	Dead (progressive disease)	306
23	≥90	≥90	D		–	Extensive	Yes (unknown)	–	Dead (GvHD and its complication)	151
24	≥90	≥90	≥90		Gr II (G)	Extensive	–	Yes (>d365)[d]	Dead (recurrent disease)	825
25	≥90	87	≥90	Yes (d186, d238, relapse)	Gr II (S)	Extensive	Yes (CMV antigenemia)	Yes (d156)	Dead (recurrent disease)	401
26	≥90	≥90	≥90	Yes (d204, relapse)	Gr I (S)	Extensive	Yes (unknown)	Yes (d141)	Dead (recurrent disease and its complication)	412
27	84	≥90	≥90		–	Extensive	Yes (unknown)		Alive	371
28	≥90	≥90	≥90		Gr II (S)	Extensive	Yes (viral susp., unknown)	–	Alive	365
29	≥90	≥90	≥90		Gr I (S)	Extensive	Yes (unknown)	–	Alive	366
30	≥90	≥90	≥90		Gr I (S)	Limited	Yes (unknown)	Yes (d370)	Alive	370

ND, not done; D, dead; DLI, donor lymphocyte infusion; Gr, grade; GvHD, graft-versus-host disease; GvHD site codes, S-skin, G-gut, L-liver; CMV, cytomegalovirus; susp., suspected; unknown, no microbiological evidence despite symptoms; IP, interstitial pneumonia.

[a] This patient developed a GvHD starting on day 112 after receiving DLI for mixed chimerism.

[b] This patient developed gut GvHD starting on day 92.

[c] CNS relapse without hematological relapse.

[d] This patient relapsed after day 365, but the exact date of relapse is unknown.

TABLE III. Regimen-Related Toxicities Within 20 Days After HSCT According to the Seattle Criteria in 30 Patients

	Grade			
Toxicity	1	2	3	4
Heart	1	0	0	0
Bladder	0	1	0	0
Kidney	5	1	0	0
Lung	2	0	0	0
Liver	8	0	0	0
CNS	1	0	0	0
Stomatitis	9	8	0	0
GI toxicity	4	1	0	0

HSCT, hematopoietic stem cell transplantation; CNS, central nervous system; GI, gastro-intestinal.

gut GvHD, three of those developed extensive chronic GvHD and all were treated with corticosteroid.

The Kaplan-Meier estimated probability of OS and DFS at 1 year was, respectively, 83% and 62% (Fig. 2). Both patients age ($\leq$55 years versus >55 years) and CD34+ cell dose ($>5.0 \times 10^6\ \mathrm{kg}^{-1}$ versus $\leq 5.0 \times 10^6\ \mathrm{kg}^{-1}$) were not associated with better outcomes by a stratified analysis (data not shown).

Pharmacokinetic results for fludarabine and busulfan

2F-ara-A and busulfan PK parameters were calculated from data obtained from blood samples from six consenting patients (UPN 1, 3–7). After the start of the 4th infusion of fludarabine phosphate (30 mg/m^2/dose), the maximum plasma level of 2F-ara-A was 3.12 ± 1.08 nmol/ml, with a subsequent decline to $T_{1/2}$ of 8.59 ± 1.57 h. The AUC (0–24 hr) and CL were 17.7 ± 2.82 nmol hr/ml and 78.9 ± 13.1 ml/min/m^2, respectively. After the 6th administration of busulfan (1 mg/kg/dose for eight times), the maximum plasma level of busulfan was 1.37 ± 0.34 nmol/ml, with a subsequent decline to a $T_{1/2}$ of 2.88 ± 0.65 hr. The AUC (0–6 hr) and CL were 4.85 ± 1.07 nmol hr/ml and 3.60 ± 0.88 ml/min/m^2, respectively. Since these parameters are similar to those in a previous study with the repeated administration of fludarabine phosphate alone at 15, 20, and 25 mg/m^2/dose (data not shown), combination with busulfan seemed to have no effect on the pharmacokinetics of 2F-ara-A. The steady-state plasma level of busulfan (808 ± 178 ng/ml) was observed to remain within a therapeutic level (600–900 ng/ml) in adults [21].

Discussion

In this prospective study, we showed that a combination of fludarabine (180 mg/m^2) and oral busulfan (8 mg/kg), despite the omission of antithymocyte globulin from the original regimen by Slavin et al. [6], can be successfully used to help prepare patients older than 50 years with hematological malignancies for HSCT from an HLA-matched related donor: All patients achieved sustained engraftment without graft failure, only an insignificant occurrence of RRT and treatment-related complications were seen, and PS and dietary intake were well maintained, which agrees with published observational studies on RIST with fludarabine and busulfan [16,22,23].

The rapid induction of complete donor-type chimerism was considered as an essential part of the RIST procedure. Although all of our patients rapidly developed conventional neutrophil and platelet engraftment, two of the 30 patients without preceding cytotoxic chemotherapy remained in mixed T-cell chimerism during the first 6 months after transplantation. A more rapid induction of T-cell chimerism has

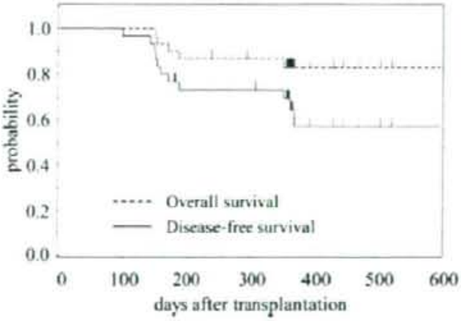

Figure 2. Kaplan–Meier product estimates of overall survival and disease-free survival.

been observed in other studies of RIST in patients who had been previously treated with chemotherapy for diseases other than CML or MDS [24]. Although a close association between the occurrence of acute GvHD and the induction of higher levels of donor T-cell chimerism has been reported [14], in our experience over 50% of patients did not achieve complete chimerism at the onset of acute GvHD, demonstrating that mixed chimerism status did not provide absolute protection from GvHD, which is in agreement with data published by Baron et al. [15]. We speculate that differences in the conditioning regimen and GvHD prophylaxis may result in different observations.

While our less intensive regimen was associated with less toxicity, this strategy will only work if modifications to the conditioning regimen intensity that allow early clinical benefits do not also lead to reduced induction of GvL effect or other complications that increase relapse rate or result in worse survival in later time period [25]. A recent observational study from European Group of Blood and Marrow Transplantation Registry compared treatment-related mortality (TRM) and other outcomes between 315 RIST recipients and 407 CIST recipients, who were over 50 years and transplanted from a HLA matched sibling donor [26], and suggested that lower TRM but higher relapse rate were seen in RIST recipients. Given the fact that all three patients, who relapsed within 6 months after transplantation, were MDS with poor prognostic factors, the incidence of relapse in our study seems to be no higher than that in published data for CIST [27–30]. Taussig et al. evaluated the feasibility and safety of the fludarabine based RIST regimen in 16 patients with standard risk diseases [31]. In this study, TRM rate within 100 days was 0%, however, OS and DFS at 1 year read from Fig. 2 were 69% and 56%, respectively, where most of the patients included in this study had early stage diseases and over 30% of patients were aged less than 50 years. Despite the older patient population, our data showing no treatment-related mortality (TRM) within the first 100 days after transplantation and OS and DFS at 1 year of 83% and 62%, respectively, was encouraging.

In a previous report, we suggested that the development of GvHD is not essential for the control of low-risk myeloid malignancies, and that GvHD and infection, rather than relapse, are more important problems to be addressed in these patients [25]. Although our data showed favorable outcomes, six patients with four low risk disease and three patients aged less than 55 years died of GvHD or its complication within the first year should be interpreted with care. The incidence of Grade II–IV acute GvHD in this study was somewhat higher than that in published literature and our own observational data with elder patients and high risk diseases [25]. However, Grade III–IV acute GvHD was infrequent and none died from acute GvHD. The incidence of chronic GvHD was higher than that in our previous experience (56%) [32] or in other reports [31,33] even after considering inevitable differences in the ethnicity, GvHD prophylaxis and matching practice of HLA, or disease risk. G-CSF mobilized peripheral blood stem cells may have been associated with an increased incidence of GvHD, particularly in its chronic form [34,35]. Conditioning regimen excluded antithymocyte globulin was also a possible explanation of this finding [23]. Most importantly, patients undergoing RIST are usually older than those undergoing CIST, which leads to a higher risk for GvHD [36,37]. Early CyA withdrawal regulation to get speedy achievement of complete donor chimerism after RIST in our protocol might have influenced the increased incidence of Grade II–IV acute GvHD, which might have affected the rate of chronic GvHD [33,35,38,39]. Although severe GvHD will be unavoidable for some patients including MDS with poor prognostic factors [40,41], the balance between GvHD and GvL is a significant concern in RIST and we should seriously evaluate the type and tapering speed of immunosuppressive agents after RIST. Current findings suggested GvHD control might be improved simply by extending the duration of CyA administration. Additionally, we noticed that the clinical features of GvHD are different in RIST than in CIST, i.e. a syndrome compatible with acute GvHD occurs well after Day 100. Hence, the current grading system for GvHD, which was developed on the basis of experience in ablative settings, may not be an optimal tool for assessing GvHD after RIST. We observed a late onset of acute GvHD and an early onset of chronic GvHD, and therefore believe that a significant number of late-onset acute GvHD may have been judged as chronic GvHD in this study simply because the onset of GvHD was over 100 days after transplantation. Our results support the current proposition by Mielcarek and Storb concerning the abandonment of the traditional Day 100 cutoff for separating acute from chronic GvHD [35].

In this prospective study, we confirmed the short-term safety and efficacy of our RIST procedure for hematological malignancies in the elderly. Long-term follow-up of patients to evaluate disease control and the consequence of therapy is mandatory, and the development of optimal GvHD prophylaxis, with the use of novel assessment criteria, will be of primary importance for the wider application of the RIST procedure. RIST may also be beneficial in young patients, since organ damage, including infertility, might be milder and less frequent in RIST than in CIST, which should be confirmed by further prospective clinical trials. Although the number of patients studied was limited, the analysis of fludarabine pharmacokinetics has for the first time provided reliable information on the interaction of key drugs, and we found no evidence to suggest that synergic or specific toxicities were associated with increased exposure to the concomitant use of busulfan, or vice versa. This information should be useful in future studies in which different drugs are combined with fludarabine.

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Appendix

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Case Report

T-Cell Large Granular Lymphocyte Leukemia of Donor Origin After Cord Blood Transplantation

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Abstract

We report the first case of T-cell large granular lymphocyte leukemia of donor origin after a second cord blood transplantation for acute myeloid leukemia, and review the literature regarding rare cases of T-cell–origin posttransplantation lymphoproliferative disorders.

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Key words: Bone marrow, Epstein-Barr virus, Polymerase chain reaction, Posttransplantation lymphoproliferative disorders, T-cell receptor

Introduction

T-cell large granular lymphocyte leukemia (LGL; LGLL) is characterized by the monoclonal proliferation of CD3+, and CD8+ LGLs, with abundant cytoplasm and fine or coarse azurophilic granules.[1,2] Reactive expansion of LGL in the peripheral blood has been occasionally reported during viral infection and in recovery phase of allogeneic hematopoietic stem cell transplantation (HSCT).[3,4]

Posttransplantation lymphoproliferative disorder (PTLD) is a characteristic lymphoid proliferation or the development of lymphoma in a setting of decreased T-cell immune surveillance, typically in recipients of solid organ transplantation or allogeneic HSCT. Most reported cases of PTLD are of B-cell origin, in association with Epstein-Barr virus (EBV) infection, which leads to monoclonal or, less frequently, polyclonal proliferation of B cells. Most of the rare cases of T-cell PTLD were reported after solid organ transplantation, with very rare cases after allogeneic HSCT.

In this report, we describe the unique clinical and laboratory findings of a patient with γδ T-cell LGLL of cord donor origin after a second cord blood transplantation for acute myeloid leukemia.

Case Report

A 58-year-old Japanese man with acute myeloid leukemia (French-American-British classification; M2) in second complete remission received allogeneic HSCT from an unrelated female cord blood donor. The conditioning regimen consisted of total body irradiation of 12 Gy in 6 fractions from day −6 to −4, and cyclophosphamide 60 mg/kg once daily intravenously on days −3 to −2 (total dose, 120 mg/kg). He received human leukocyte antigen–loci mismatched (2 by serology and 2 by DNA typing) unrelated cord blood, which contained 3.03×10^7 nucleated cells/kg in January 2003. Cyclosporine and short-term methotrexate were used as graft-versus-host disease prophylaxis. However, hematologic recovery was not observed up to day 40, and we concluded that this was a case of primary graft failure without leukemia relapse because the results of interphase fluorescence in situ hybridization analysis on days 23, 30, and 37 on bone marrow (BM) samples were negative. Because his condition remained good, we planned a second cord blood transplantation with a reduced-intensity regimen, which consisted of fludarabine 30 mg/kg once daily intravenously from days −8 to −3 (total dose 180 mg/kg), busulfan 4 mg/kg orally on days −6 and −5 (total dose 8 mg/kg), and total body irradiation of 4 Gy in 1 fraction on day −1. Cyclosporine and mycophenolate mofetil 15 mg/kg twice daily were administered. On day 51 of the initial transplantation in March 2003, human leukocyte antigen–loci mismatched (2 by serology and 3 by DNA typing) male cord blood, containing 2.6×10^7/kg nucleated cells, was infused. Neutrophil engraftment was observed by day 33 after second transplantation. Acute and chronic graft-versus-host disease did not develop, and cyclosporine was tapered off in November 2003.

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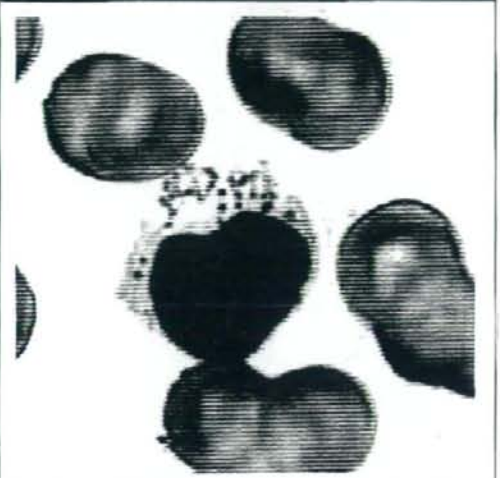
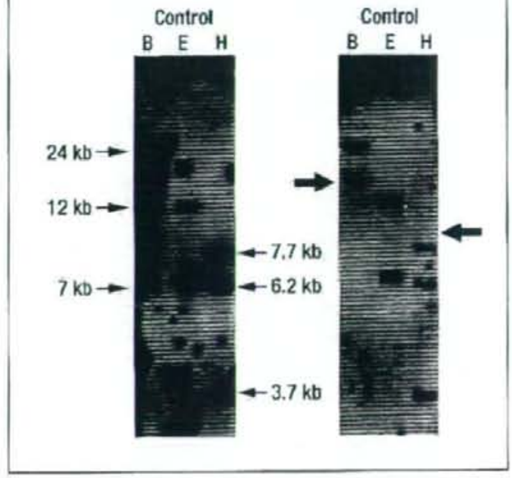
Figure 1 T-Cell Large Granular Lymphocyte Leukemia Stained with May-Giemsa on the Peripheral Blood Smear



The predominant cells were typical of LGLs with abundant cytoplasm and fine or coarse azurophilic granules.
Hematoxylin and eosin stain; original magnification ×1000.

Figure 2 Southern Blots of T-Cell Receptor β-Chain Gene Rearrangements



DNA from BM of this patient was hybridized with a TCR Cβ1 probe.
Arrows indicate rearranged bands.
Abbreviations: B = Bam H1; E = Eco R; H = Hind III

In February 2004, 10 months after the second cord blood transplantation, he developed anorexia, abdominal distention with fluid accumulation, and edema in the lower extremities. A computed tomography scan showed gross ascites and mild pleural effusion but no sign of enlarged lymph nodes or hepatosplenomegaly. The peripheral white blood cell count was 10,300/μL (10.3 × 10^9/L), and 30% of the cells had a morphology of medium to large lymphocytes with abundant azurophilic granules in the cytoplasm, as shown in Figure 1. The hemoglobin level was 8.8 g/dL (88 g/L), and the platelet count was 192 × 10^3/μL (1.92 × 10^9/L).

A retrospective review of the peripheral blood smears disclosed that the appearance of LGL coincided with the tapering off of immunosuppression 3 months before the admission.

Flow cytometry examination of the peripheral blood mononuclear cells showed a homogeneous population of T-cell LGLs positive for CD2, CD3, CD8, CD56, and T-cell receptor (TCR)–γδ, but negative for CD4 and TCR-αβ. The BM biopsy specimen histologically showed 10% of hypocellular gelatinous marrow with diffuse infiltration of medium to large lymphoid cells. Immunoperoxidase studies on sections of BM showed strong expression of T-cell–restricted intracellular antigen-1, partially positive staining of CD8 and granzyme B, but no expression of CD3 or CD20. Southern blot analysis of the BM cells revealed a clonal rearrangement of the TCR-β chain, as shown in Figure 2 and TCR-δ chain (data not shown).

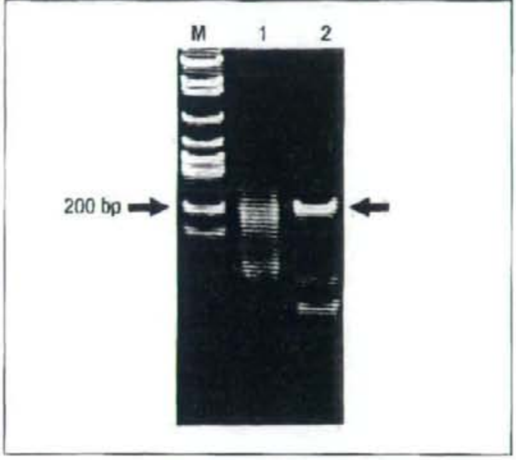
Abdominal paracentesis was performed with milky chylous fluid, and a flow cytometry examination showed results similar to those in the peripheral blood. Multiprimer-based polymerase chain reaction (PCR) analysis of ascitic cells also showed clonal rearrangement of the TCR-δ chain, as shown in Figure 3. The primer sets were used in the following locations: Vδ1, 5'-AAA GTG GTC GCT ATT CTG TC-3'; Vδ2A, 5'-GCA CCA TCA GAG AGA GAT GA-3'; Jδ, 5'- TGG TTC CAC AGT CAC ACG GG-3'; Dδ3B, 5'-TTG TAC CAC CGT GCG TAT CC-3'. The amplified 200 base-pair PCR products of the TCR-δ chain were then cloned into the pCR-TOPO vector. The DNA sequences of 3 clones amplified by vectors were identical and had high homology to TCR-δ chain including a 197 base-pair sequence (data not shown). This sequence also involved the forward and reverse primers Vδ1 and Jδ, respectively, described previously.

The results of all of the previously mentioned studies indicated the clonal expansion of T cells compatible with a diagnosis of T-cell LGLL with γδ T-cell phenotype involving peripheral blood, BM, and ascites.

Donor-recipient DNA chimerism was analyzed by comparing the short tandem repeat findings for the donor blood sample and pretransplantation recipient samples. Eleven short tandem repeat loci were analyzed by PCR using an AmpFISTR SGM Plus® kit. The peripheral blood sample (containing 30% T-LGL) and the second cord blood sample showed the same peaks at the locus (D16S539), as shown in Figure 4. These results further confirmed that the expanded γδ T-LGL cells were exclusively of second cord blood transplantation donor origin.

Serologic examination showed no evidence of viral infection. Real-time PCR analysis revealed a high load of EBV (7.9 × 10^3 copies/10^6 cells). However, in situ hybridization studies of BM cells did not reveal EBV-encoded small RNA, and Southern blot analysis of BM cells also showed no band for

Figure 3 Polymerase Chain Reaction for T-Cell Receptor δ Gene Rearrangement



(1) Negative control, and (2) patient's sample of frozen neoplastic lymphoid cells in ascites. A clonal band was identified at approximately 200 base pairs.
Abbreviations: bp = base pairs; M = molecular weight marker

clonal EBV genomes. Chromosomic analysis demonstrated a normal 46, XY karyotype in all 20 cells examined.

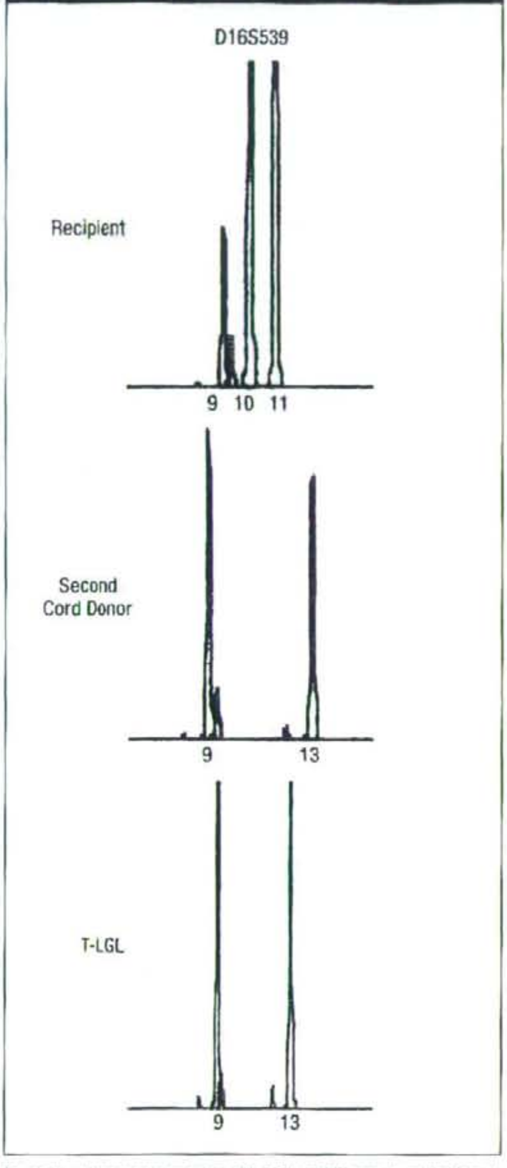
After admission, his abdominal distention and dyspnea with hypoxemia progressed rapidly with spiking fever. A computed tomography scan demonstrated acute respiratory distress syndrome. Because we found no evidence of bacterial or fungal infection or drug-induced pneumonia, cyclosporine and methylprednisolone were started immediately but with no effect, and he died of acute respiratory failure 1 week later. A postmortem lung biopsy showed extensive diffuse alveolar damage without the T-LGL cell's involvement; on the other hand, the leukemic cell involvement in Glisson's sheath was shown by a liver biopsy.

Discussion

In this case, the increase in LGLs developed 7 months after the second cord blood transplantation, and the kinetics of LGLs correlated with the tapering off of immunosuppression, which suggested the possibility that lymphocytosis might have been associated with reactive expansion because of viral infection or an alloimmune reaction. However, our case showed *TCR-β* and *TCR-δ* gene rearrangement by Southern blot analysis and *TCR-δ* gene rearrangement by PCR and cytotoxic T-cell immunophenotype, which were compatible with T-cell LGLL.

Most cases of PTLD, usually of B-cell origin, are associated with EBV infection and represent the EBV-induced monoclonal expansion of B cells in conditions with decreased T-cell immune surveillance.[5,6] Although there have been some reports of EBV-associated PTLD after cord blood transplantation,[7-10] the incidence of PTLD of T-cell origin has been reported to be only 4%-14% with a less frequent association with EBV.[6,11]

Figure 4 Donor-Recipient DNA Chimerism Analysis by Comparing the Short Tandem Repeat



The peripheral blood sample (containing 30% T-LGL) and the second cord blood sample showed the same peaks at the locus (D16S539).

In our case, because a high viral load of EBV was detected by real-time PCR analysis, we initially speculated that γδ T-LGLL was EBV-associated PTLD, but this was later denied based on the results of EBV-encoded small RNA in situ

Table 1A Literature Review of T-Cell Posttransplantation Lymphoproliferative Disorder After Hematopoietic Stem Cell Transplantation[7,13-16]

Study	Case Number	Age/Sex	Donor	Diagnosis	Origin	Involved Organ
Zutter et al[13]	1	14/Male	Sibling*	Lymphoblastic lymphoma	Recipient	Lymph node, BM
Zutter et al[13]	2	9/Male	Sibling*	Lymphoblastic lymphoma	ND	Pericardium, pleura
Zutter et al[13]	3	2/Female	Father	NHL (polymorphic)	Donor	Lung, liver, spleen
Wang et al[14]	4	13/Male	Sibling*	NHL (diffuse large)	Recipient	Lymph node
Sirvent et al[7]	5	ND/ND	ND	LGL ($\alpha\beta$)	ND	PB, BM
Collins et al[15]	6	11/Male	ND	NHL (polymorphic)	ND	Lymph node, brain
Au et al[16]	7	39/Male	Unrelated	LGL	Donor	PB, BM
Our Case	8	58/Male	UCB	LGL ($\gamma\delta$)	Donor	PB, BM, ascites, liver

*Human leukocyte antigen–matched sibling.
Abbreviations: ND = not determined; NHL = non-Hodgkin lymphoma; PB = peripheral blood; UCB = unrelated cord blood

Table 1B Literature Review of T-Cell Posttransplantation Lymphoproliferative Disorder After Hematopoietic Stem Cell Transplantation[7,13-16]

Study	Case Number	Time to PTLD* (Days)	EBER-ISH	Rearrangement	Survival† (Days)
Zutter et al[13]	1	1290	Not determined	TCR-γ (SB)	851
Zutter et al[13]	2	630	Not determined	Not determined	180
Zutter et al[13]	3	39	Not determined	Polyclonal	11
Wang et al[14]	4	601	Negative	TCR-γ (PCR)	> 1170
Sirvent et al[7]	5	300	Negative	TCR-β (SB)	> 690
Collins et al[15]	6	90	Negative	Not determined	29
Au et al[16]	7	180	Negative	TCR-γ (PCR)	134
Our Case	8	330	Negative	TCR-β (SB), TCR-δ (SB, PCR)	30

*Time from transplantation to PTLD.
†Survival time from diagnosis of PTLD.
Abbreviations: EBER-ISH = EBV-encoded small RNA in situ hybridization; SB = Southern blotting

hybridization stains and Southern blot EBV terminal repeat analysis. Therefore, the clinical significance of EBV infection in this case remains undetermined.

Most previously reported cases of T-cell PTLD developed after solid organ transplantation,[12] and there have been only 7 previously documented cases of T-cell PTLD after allogeneic HSCT, as summarized in Table 1.[7,13-16] Posttransplantation lymphoproliferative disorder was of donor origin in 3 of 8 total cases, including our case, of recipient origin in 2, and of undetermined origin in the remaining 3. No correlation has been demonstrated between EBV and T-cell PTLD after HSCT.

Generally, most cases of B-cell posttransplantation lymphoproliferative disorder after HSCT develop within the first 5 months, because the balance between proliferating EBV-infected B cells and cytotoxic T cells cannot be controlled with the unrecovered lymphocyte components.[17] In solid organ transplantation, EBV-positive cases tend to occur earlier than EBV-negative cases, ie, a median interval of 6-10 months compared with 4-5 years.[6,7] Some cases of T-cell PTLD have a longer interval between the day of transplantation and the occurrence of PTLD than in B-cell PTLD. The donor source of transplantation included sibling (3 cases), father (1 case), unrelated (1 case), cord (our case), and not described (2 cases). Therefore, whereas there has been very little experience with cases after cord blood transplantation, all 8 cases of PTLD in the literature are of B-cell origin.[8-11] Our case is the first report of PTLD of T-cell origin after cord blood transplantation and might reflect very intense immunosuppression passing through consecutive cord blood transplantation.

It has been reported that T-cell PTLD has a worse prognosis than B-cell PTLD in a solid organ transplantation setting. In 1 series of 6 cases presenting with T-cell non-Hodgkin lymphoma as PTLD, pulmonary involvement was reported in 5 cases and marrow infiltration in 4 cases. All patients showed aggressive courses.[18] Of importance is that of 8 patients with T-cell PTLD after HSCT: 3 patients who died within 30 days had extranodal involvement in the lung, liver, spleen, brain, and/or ascites.