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ORIGINAL ARTICLE

Effectiveness and safety of rabbit anti-thymocyte globulin in Japanese patients with aplastic anemia

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Abstract Immunosuppressive therapy (IST) with antithymocyte globulin (ATG) and cyclosporine A is the standard treatment for aplastic anemia (AA). The ATG formulation in Japan was changed from horse ATG [Lymphoglobulin® (LG)] to rabbit ATG [Thymoglobulin® (TG)] in 2009. Since then, 12 patients with AA have been treated with TG. Here, we summarize the effectiveness and safety of TG in comparison with data from 14 AA patients treated with LG before April 2009. One subject treated with LG but none treated with TG terminated the treatment due to a grade III adverse effect. The overall 6-month response rate after IST was similar for LG and TG (67 and 75 %). Infection was noted in five (38 %) and four (33 %) subjects treated with LG and TG, respectively. The initial response rate was significantly higher in the early-treatment group treated within a year of diagnosis than in the late-treatment group, who were treated more than a year after diagnosis (85 vs. 29 %, respectively), as reported previously, without apparent differences between the LG and TG groups. We conclude that TG at a dose of 2.5 mg/kg/day for 5 days is effective and safe in Japanese patients with AA.

Keywords ATG · Aplastic anemia · Immunosuppressive therapy

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Introduction

Aplastic anemia (AA) is a rare disease pathologically characterized by a fatty bone marrow, in which hematopoietic cells are replaced by fat, resulting in pancytopenia [1]. Patients with severe AA occasionally develop life-threatening infections and experience decreased quality of life due to transfusion dependency and bleeding propensity. Hematopoietic stem cell transplantation is a treatment option. However, it is considered to be the first-line treatment only for young patients with human leukocyte antigen-identical sibling donors because of the high risk of adverse effects.

Immunosuppressive therapy (IST) using anti-thymocyte immunoglobulin (ATG) and cyclosporin A is a standard therapy for patients with moderate-to-severe AA [2–4]. Several ATG formulations that differ in the type of immunogens and immunized animals are available. ATG availability and preferences among practitioners also differ between countries. In Japan, an ATG formulation manufactured from horses [Lymphoglobulin® (LG)] was mainly used since 1995, but was withdrawn from the market in March 2009. Another formulation produced from rabbits [Thymoglobulin® (TG)] replaced LG; thereafter, hematologists have been increasingly incorporating this formulation in their practices without clear treatment guidelines.

Consequently, an optimal TG dose has not been established. A dose of 3.75 mg/kg/day for 5 days is routine in Europe [5], whereas a dose of 2.5 mg/kg/day or 3.75 mg/kg/day for 5 days is recommended by the Pharmaceutical and Medical Devices Agency of Japan.

We reviewed our experience with 12 patients with AA who were treated with TG at a dose of 2.5 mg/kg/day for 5 days at a single center along with our previous data of 14 patients with AA who were treated with LG in order to share our results with other specialists.

Patients and methods

We reviewed the medical records of 14 patients with AA who treated with LG from January 1997 to April 2009 and 12 patients with AA who were treated with TG from June 2009 to September 2011 at University of Tsukuba Hospital. LG and TG were administered at a dose of 15 mg/kg/day for 5 days and 2.5 mg/kg/day for 5 days, respectively. Cyclosporin A was initiated at a dose of 3 mg/kg every 12 h and continued for at least 6 months at a dose adjusted to maintain trough blood levels at 150–500 ng/ml.

The severity of AA was determined using criteria described by Guinan [6]. Red blood cells and neutrophils with decreased CD59 levels, known as paroxysmal nocturnal hemoglobinuria (PNH)-type blood cells, were detected using flow cytometry based on the method previously reported by Sugimori et al. [7].

Adverse events were evaluated according to the Common Terminology Criteria for Adverse Events version 4.0. If the criteria for febrile neutropenia and detection of causative microbes were fulfilled, the adverse event was classified as an infection.

Treatment responses were defined as follows [8]: complete response (CR) in any AA type with absolute neutrophil count $> 2.0 \times 10^9$/L, hemoglobin levels > 11 g/dL, and platelet count $> 100 \times 10^9$/L; partial response (PR) in severe AA with an improvement in blood counts no longer satisfying the criteria for severe AA but insufficient to meet the criteria for CR; and PR in nonsevere AA with transfusion independence (if previously transfusion-dependent), doubling or normalization of at least 1 cell line, or an increase above baseline in at least 1 cell line in hemoglobin by 3 g/dL (if initially < 6 g/dL), increase in neutrophil count by 500/μL (if initially < 500), or increase in platelet count by 20000/μL (if initially < 20000). Overall response included both CR and PR. Responses were determined by evaluating peripheral blood counts at 3, 6, and 12 months after initiating IST. Statistical analysis was conducted based on a group sequential trial design, using a 2-sided test at a 5 % significance level, 80 % power, and 1 interim analysis.

Results

There were no significant differences between the subjects in the LG- and TG-treated groups in terms of age, gender, disease severity, complications, time from diagnosis to the start of treatment, or the of positive PNH-type blood cells (Table 1). IST using LG had previously been employed in 1 of 14 subjects in the LH-treated group and 4 of 12 subjects in the TG-treated group. Treatments were administered within a year of the diagnosis in 8 of 14 subjects treated with LG and in 7 out of 12 subjects treated with TG. The median period of observation was 50 and 19 months in the LG- and TG-treated groups, respectively. The TG-treated group included 4 patients who received TG as a second course of ATG treatment because of AA relapse.

Of all treatment-induced adverse events (Table 2), the most common was fever. Abnormal liver function was also observed in 1 subject treated with LG, leading to termination of treatment. All the other subjects completed the ATG treatment. Infection was documented in 9 subjects; 4

Table 1 Patients' characteristics

Patient	LG	TG
Number	14	12
Median age (range)	50 (27–77)	54 (22–73)
Male:Female	7:7	7:5
Term	1997–2009	2009–2011
Disease status		
Severe	6	6
Non-severe	8	6
Time from diagnosis		
Median, day (range)	2453 (6–6746)	3025 (10–6455)
3 months	5	3
3–12 months	3	4
12 months	6	5
Times of ATG therapy		
First ATG	14	8
Second ATG	1	4
PNH clone		
Positive	6	6
Negative	1	6
Unexamined	7	0

LG horse ATG [Lymphoglobulin® (LG)], *TG* rabbit ATG [Thymoglobulin® (TG)]

Table 2 Adverse events

	LG ($n = 14$)	TG ($n = 12$)
Fever at administration		
Yes	7	4
No	7	8
Serum disease		
Yes	0	0
No	14	12
Liver injury		
Yes	1	0
No	13	12
Infection		
Yes	5	4
No	9	8

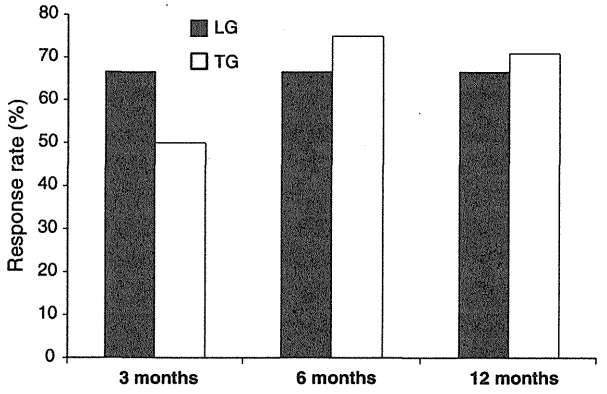

Fig. 1 Overall response to TG and LG at 3, 6, and 12 months

in the TG- and 5 in the LG-treated groups, respectively. Intravenous antibiotics were prescribed for all subjects. Cytomegalovirus infection was observed in 1 subject treated with TG. Although periodic inspection was not carried out in a complete manner, the EBV-related lymphoproliferative disorders were not detected in either group.

Because the relapsed patients were more likely to respond to the second course of ATG treatment, we analyzed these patients separately for overall response. The overall 6-month response rate after ATG administration was 67 % (8 of 12 subjects) with LG and 75 % (6 of 8 subjects) with TG (Fig. 1). Responses were observed after 3 months in 67 and 50 % subjects in the LG- and TG-treated groups, respectively. One subject treated with TG showed a response after 6 months, suggesting the possibility of a late response. A significant difference in the response rate was not detected between both groups.

In the similar evaluations only after the first ATG, the response rate at 6 months from ATG treatment in patients with severe AA (16 subjects) appeared to be higher than that in patients with non-severe AA [4 subjects (75 vs. 50 %)], although not statistically significant. The response rates tended to be higher in subjects treated with LG or TG earlier *after diagnosis than those in subjects treated later.* Treatment with LG and TG in 7 and 6 subjects, respectively, was initiated within a year of diagnosis (early treatment group), whereas treatment with LG and TG in the remaining 5 and 2 subjects, respectively, was initiated more than 1 year after diagnosis (late treatment group). Response rate after 6 months from ATG treatment was achieved more rapidly in the early treatment group than in the late treatment group (85 and 29 %, respectively; $p = 0.005$).

Again with the evaluations of the first ATG, the response rate at 6 months from ATG treatment tended to be higher in subjects with PNH-type blood cells (10 subjects) than in those without PNH-type blood cells (4 subjects), although the difference was not statistically significant

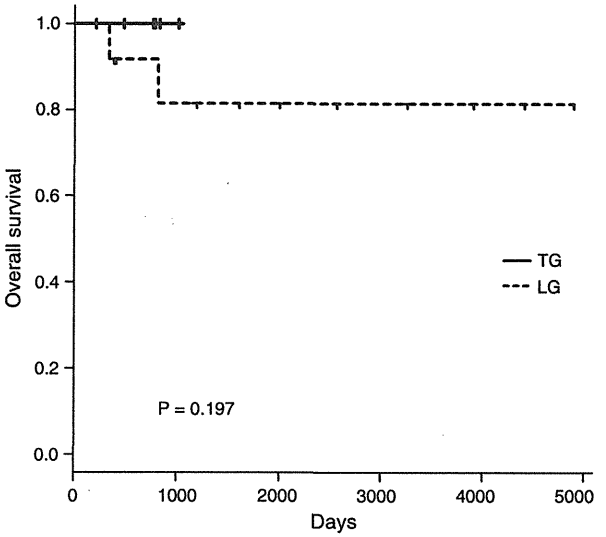

Fig. 2 Overall survival curves $p = 0.197$

(PNH-type cells positive and negative in 9 and 4 subjects, 70 and 50 %, respectively; $p = 0.63$).

With regard to the overall survival curves, no significant difference was detected between both groups, although the number of subjects is small (Fig. 2).

Discussion

We described the effectiveness and safety of TG in a small number of Japanese patients with AA. A recent multicenter prospective study comparing LG and TG in European countries demonstrated that LG was more effective than TG, with both formulations having a similar safety profile [9]. Another multicenter prospective randomized study in the US showed that horse ATG [ATGAM®] was more effective than TG [10].

Nevertheless, the availability of ATG formulations differs between countries [11]. The decision to change the ATG formulation in Japan was made by providers based on the similar efficacy of TG and LG in the second course of ATG treatment for patients who failed to respond to the first course with LG [12].

Given that ATG formulations differ between countries, it is necessary to perform a direct comparison between LG and TG in other ethnic groups in the Asia. A recent single-center retrospective study from Korea comparing LG and TG demonstrated that the overall survival rate and failure-free survival rate were not significantly different between the two groups, but failure-free survival rate tended to be higher in TG group [13, 14].

A prerequisite to that, however, is the dose adjustment of each formulation. For TG, the advantage of a dose of

3.75 mg/kg/day for 5 days over 2.5 mg/kg/day for 5 days was demonstrated in a European clinical study. However, a dose of 2.5 mg/kg/day or 3.75 mg/kg/day for 5 days is recommended in Japan because of insufficient evidence regarding an optimal dose [9, 15].

These circumstances embarrass hematologists who treat patients with AA in Japan, and thus, we need to share our experiences of treating this rare disease with TG. Given the lack of safety data, an initial dose of 2.5 mg/kg/day for 5 days was chosen to treat the 12 patients with AA. The response rate of 63 % was within our expectation or could exceed it, given the fact that the treatment with TG in the second course of IST in 4 of 12 subjects exceeded our expectation. The response rate was 56 % in 13 subjects treated with LG, suggesting that the effectiveness of TG at a dose of 2.5 mg/kg or LG at a dose of 15 mg/kg for 5 days is not substantially different. Adverse effects with TG formulations, such as infections, were also similar to those with LG formulations at these doses.

When the 26 subjects treated with LG or TG were analyzed together, we confirmed that the period from the diagnosis to the start of IST is a statistically significant predictor of the efficacy of IST, as reported previously [4, 16, 17]. We also observed a trend suggesting that the presence of PNH-type blood cells is an indicator of higher IST effectiveness in the same patient cohort, as reported previously [6, 18].

Based on these encouraging observations, we have increased the dose of TG from 2.5 to 3.75 mg/kg/day for 5 days in the current prescription for our patients with AA, expecting better responses without increasing the risk of adverse events. These small efforts may help us design future clinical trials of ATG-based IST for AA, not only in Japan but also throughout Asia.

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RESEARCH Open Access

Monitoring of minimal residual disease (MRD) is useful to predict prognosis of adult patients with Ph-negative ALL: results of a prospective study (ALL MRD2002 Study)

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Abstract

Background: Allogeneic hematopoietic stem cell transplantation (HSCT) for patients with Philadelphia chromosome (Ph)-negative acute lymphoblastic leukemia (ALL) in first complete remission (CR1) is much more intensive than multi-agent combined chemotherapy, although allogeneic HSCT is associated with increased morbidity and mortality when compared with such chemotherapy. Minimal residual disease (MRD) status has been proven to be a strong prognostic factor for adult patients with Ph-negative ALL.

Methods: We investigated whether MRD status in adult patients with ALL is useful to decide clinical indications for allogeneic HSCT. We prospectively monitored MRD after induction and consolidation therapy in adult patients with Ph-negative ALL.

Results: Of 110 adult ALL patients enrolled between July 2002 and August 2008, 101 were eligible, including 59 Ph-negative patients. MRD status was assessed in 43 patients by the detection of major rearrangements in *TCR* and *Ig* and the presence of chimeric mRNA. Thirty-nine patients achieved CR1, and their probabilities of 3-year overall survival and disease-free survival (DFS) were 74% and 56%, respectively. Patients who were MRD-negative after induction therapy (n = 26) had a significantly better 3-year DFS compared with those who were MRD-positive (n = 13; 69% vs. 31%, p = 0.004). All of 3 patients who were MRD-positive following consolidation chemotherapy and did not undergo allogeneic HSCT, relapsed and died within 3 years after CR.

Conclusions: These results indicate that MRD monitoring is useful for determining the clinical indications for allogeneic HSCT in the treatment of ALL in CR1.

Keywords: Acute lymphoblastic leukemia, Minimal residual disease, Hematopoietic stem cell transplantation, Adult

Background

Although more than 80% of adult patients with Philadelphia chromosome (Ph)-negative acute lymphoblastic leukemia (ALL) achieve complete remission (CR) with conventional induction therapy, their 5-year survival is only 30%–40%. Leukemia relapse is the most common cause of treatment failure in ALL [1-6]. Therefore, post-remission therapy is necessary and should be optimized in the treatment of adult ALL patients. If prognosis of patients with ALL in CR1 is estimated to be favorable, chemotherapy is usually continued to prevent leukemia relapse. However, patients with less favorable prognosis should be treated more aggressively [7]. Although allogeneic hematopoietic stem cell transplantation (HSCT) for patients with ALL in CR1 is much more intensive than multi-agent combined chemotherapy, it is associated with increased morbidity and mortality when compared with such chemotherapy.

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Minimal residual disease (MRD) status has been proven to be a strong prognostic factor for adult patients with Ph-negative ALL [8-14]. In this study, we prospectively monitored the MRD status after CR induction and consolidation chemotherapies in adult patients with Ph-negative ALL to determine the clinical indications for allogeneic HSCT.

Patients & methods

Patient eligibility criteria

A total of 110 adult ALL patients were enrolled in this study between July 2002 and August 2008 on the basis of the following eligibility criteria: non-L3 ALL, 16–65 years of age, an Eastern Cooperative Oncology Group performance status of 0–2, and adequate liver and kidney function (serum bilirubin, ≤2.0 mg/dl and serum creatinine, ≤2.0 mg/dl, respectively). Cytogenetic studies were performed on pretreated bone marrow or unstimulated blood samples using the standard banding technique. The treatment protocol was approved by the institutional review board of each participating hospital. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Of the 110 patients enrolled, 42 were excluded from the study because of Ph-positivity, 5 because of misdiagnosis, 2 because of infectious complications, and 1 each because of liver damage and protocol violation. The remaining 59 patients were Ph-negative.

Treatment

We used a modified CALGB 19802 [15,16] treatment protocol that comprised 6 courses of chemotherapy administered in the order of A-B-C-A-B-C regimens, followed by a maintenance phase. Induction chemotherapy (course A) was a 21-day course consisting of cyclophosphamide (CPM; 1200 mg/m^2 on day 1), daunorubicin (DNR; 60 mg/m^2 on days 1, 2, and 3), vincristine (VCR;1.3 mg/m^2 [maximum 2 mg] on days 1, 8, 15, and 22), L-asparaginase (3000 U/m^2 on days 9, 11, 13, 16, 18, and 20), and prednisolone (PSL; 60 mg/m^2 [max 100 mg]). Granulocyte-colony stimulating factor (nartograstim) was administered starting from day 4 and continued until neutrophil recovery. For patients aged 55 years or older, the doses of CPM and DNR were reduced to 500 mg/m^2 and 50 mg/m^2, respectively. Furthermore, PSL therapy was shortened to 7 days in these patients. The first consolidation therapy (course B) consisted of mitoxantrone (MIT; 10 mg/m^2 on days 2 and 3), cytarabine (AraC; 2000 mg/m^2/day on days 1, 2, 3, and 4) and intrathecal administration of methotrexate (MTX; 15 mg/body on day 1). For patients aged 55 years or older, the doses of MIT and AraC were reduced to 7 mg/m^2 and 1500 mg/m^2/day, respectively. The second consolidation therapy (course C) consisted of VCR (1.3 mg/m^2 [max 2 mg] on days 1, 8, and 15) and MTX (1500 mg/m^2 on days 1, 8, and 15) with leucovorin rescue and intrathecal MTX on days 1, 8, and 15. The patients received the following maintenance chemotherapy on a monthly basis: PSL, 60 mg/m^2 on days 1–5; VCR, 1.3 mg/m^2 (max 2 mg) on day 1; oral MTX, 20 mg/m^2 weekly; and oral 6-mercaptopurine, 60 mg/m^2 daily. MRD status was evaluated after the induction therapy (first course A) and after the second consolidation therapy (first course C). Patients with positive MRD following the second consolidation therapy were considered to be indicated for allogeneic HSCT as soon as possible. Eligible donors included HLA-identical related, HLA-identical unrelated donors from Japan Marrow Donation Program, and cord blood from Japan Cord Blood Bank Network. Conditioning before allogeneic HSCT and prophylaxis for graft-versus-host disease was performed according to each institutional standard.

MRD analysis

Real-time quantitative polymerase chain reaction (RQ-PCR) analysis of chimeric mRNA

mRNA from bone marrow cells were analyzed for the presence of major and minor *BCR/ABL, TEL/AML1, MLL/AF4, MLL/AF9, MLL/AF6, MLL/ENL, E2A/PBX1,* and *SIL/TAL1* chimeric genes. Samples were amplified by RQ-PCR and quantified by parallel amplification of serial dilutions of transcript-containing plasmids [17,18].

PCR analysis of TCR/Ig rearrangement

High-molecular weight DNA from marrow cells was initially screened for major rearrangement patterns of *TCRγ, TCRδ,* and *Igκ,* and secondarily screened for rearrangements in *Ig heavy chain* (*IgH*), using previously described primers [19-21]. Two-step (nested) PCR for MRD quantification was performed using allele-specific oligonucleotide (ASO)-primers based on the sequence of PCR screening products, which had clonal recombinations by heteroduplex analyses. Prior to PCR analysis, DNA samples from post-treatment bone marrow samples and DNA from the samples obtained at diagnosis were serially diluted (between 10^{-2} and 10^{-5}) with buffy coat DNA from eight healthy volunteers. Buffy coat DNA was also used as a control for nonspecific amplification of comparable *Ig/TCR* arrangements present in normal cells. All PCR reactions were performed simultaneously and analyzed using ethidium staining and agarose gel electrophoresis. MRD was quantified by comparing the intensities of band signals on an agarose gel stained with ethidium bromide without amplification of the background. MRD quantifications were performed using ASO-primers with a sensitivity of $\leq 1 \times 10^{-4}$, and MRD positivity was defined as a lower limit of detection of $\geq 1 \times 10^{-3}$.

Statistical analysis

Statistical analyses of the data accumulated throughout October 2011 were performed. Overall survival (OS) was

defined as the time between diagnosis and the end of the trial or death, and disease-free survival (DFS) was defined as the time from CR to relapse or death while still in CR. Survival curves were estimated using the Kaplan–Meier method, and the statistical significance of differences in survival was determined using the log-rank test.

The influence of prognostic factors including age, white blood cell (WBC) count, and MRD status on DFS was estimated with multivariate Cox regression analysis. The level of statistical significance was set at 0.05.

Results

Treatment outcome

The median follow-up time was 1134 days (range, 14–3248 days). A total of 59 patients were Ph-negative (29 males and 30 females), and their median age was 35 years ranging from 16 to 63. The median white blood cell count at presentation was 11.0 x 10^3/L (range 0.9–409). CR was achieved in 47 patients (80%). Six patients died during induction; their causes of death included sepsis (n = 3), pneumonia (n = 2), and other (n = 1). There were 29 survivors after the median follow-up period. The probability of 3-year OS and DFS in these patients with Ph-negative ALL was 59% and 52%, respectively (Table 1).

Relationship between MRD status and treatment outcomes

Among the 59 Ph-negative ALL patients, 43 patients (73%) could be monitored for MRD status, and the remaining 16 patients were not because 10 had no clonal *TCR/Ig* targets or chimeric mRNA and 6 did not provide sufficient DNA or RNA from their samples. The MRD status of 43 patients (21 males and 22 females; median age: 31 years, ranging from 17 to 63; median WBC count at presentation: 10.6 x 10^3/L ranging 1–409) was determined by PCR analysis of major gene rearrangements and/or chimeric mRNAs (15 were positive for *TCRγ*, 6 for *TCRδ*, 6 for *Igκ*, 11 for *IgH*, 1 for *TCRγ* and *TCRδ*, 1 for *TCRδ* and *IgH*, 1 for *E2A-PBX*, 1 for *MLL-AF4*, and 1 for *MLL-ENL*). CR was achieved in 39 of these 43 patients with known MRD status (91%). The median follow-up time was 1421 days (range, 162–3248 days). The probability of 3-year OS and DFS in the Ph-negative patients with known MRD status was 74% and 56%, respectively (Table 1). In terms of CR1 status, MRD-negative patients after induction chemotherapy A in the first course (n = 26) showed a better 3-year DFS (69%) compared with MRD-positive patients (n = 13; 31%), as shown in Figure 1. The difference was statistically significant (p = 0.004). MRD-negative patients also showed a significantly lower 3-year relapse rate compared with MRD-positive patients (28% vs. 58%, p = 0.031).

Table 1 Patient characteristics and clinical outcome

	Ph negative	Ph negative & known MRD status
Total No. patients	59	43
Sex, No. (%)		
Male	29 (49)	21 (49)
Female	30 (51)	22 (51)
Median Age, (range)	35 (16-63)	31 (17-63)
Median WBC count, x10^9/L, (range)	11.0 (0.9-409)	10.6 (1-409)
Immunophenotype, No. (%)		
B-lineage	45 (76)	36 (84)
T-lineage	14 (24)	7 (16)
CR rate, No. (%)	47 (80)	39 (91)
3-years OS (%)	59	74
3-years DFS (%)	52	56

MRD, minimal residual disease; *Ph*, Philadelphia chromosome; *CR*, complete remission; *OS*, overall survival; *DFS*, disease-free survival.

There was no patient who proceeded to allogeneic HSCT among 26 MRD-negative patients after induction therapy in CR. In contrast, patients who were MRD-positive after induction but became MRD-negative after consolidation chemotherapy C in the first course (n = 7) showed a significantly worse 3-year DFS compared with patients who were MRD-negative after induction chemotherapy A in the first course (29% vs. 69%, p = 0.004), as shown in Figure 2. Among 7 late-attained MRD-negative patients, three patients proceeded to allogeneic HSCT when MRD status became positive again under maintenance therapy. Six patients were MRD-positive after consolidation chemotherapy C in the first course, and 3 patients among them proceeded to allogeneic HSCT, while other 3 patients did not because of lack of a suitable donor (n = 1) and of patients' refusal to allogeneic HSCT (n = 2). All of 3 MRD-positive patients who did not undergo allogeneic HSCT, relapsed and died within 3 years after CR, whereas 2 of 3 patients those who received allogeneic HSCT gave DFS at 3 years. Table 2 shows the results of multivariate Cox regression analysis for DFS in 43 MRD-evaluable patients. The analysis indicates that age (≥35 years vs. <35 years: Hazard ratio (HR) 5.067, and p = 0.005) and MRD status after induction therapy (positive vs. negative: HR 8.769, and p < 0.001) were significant prognostic factors, whereas WBC count (≥30 x 10^9/L vs. <30 x 10^9/L: HR 1.496, and p = 0.505) or MRD status after consolidation therapy (positive vs. negative: HR 0.675, and p = 0.556) was not.

Discussion

Compared with treatments for childhood ALL, those for adult ALL are far less effective [22], and allogeneic HSCT is frequently recommended as the most potent post-remission therapy for ALL patients in CR1 [23].

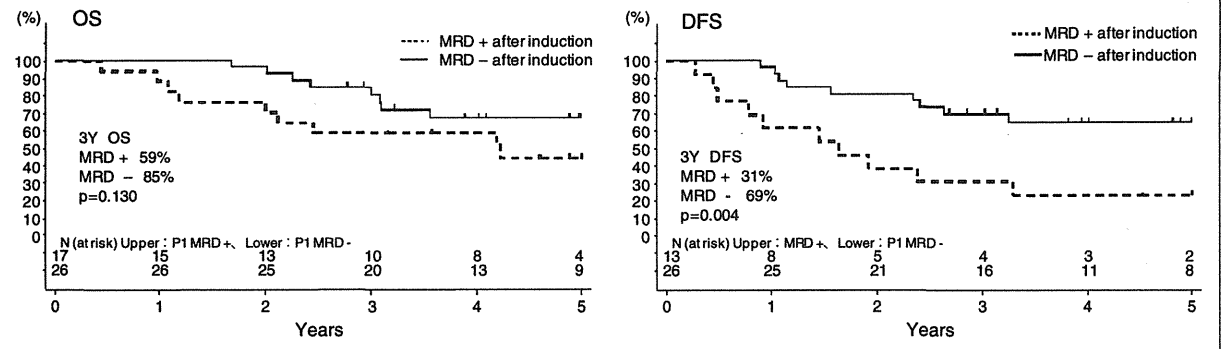

Figure 1 Impact of post-induction minimal residual disease (MRD) status on overall survival (OS) and disease-free survival (DFS). Patients who were MRD negative after induction therapy (first course A) (n = 26) had a significantly better 3-year DFS compared with those who were MRD positive (n = 13) (69% vs. 31%, p = 0.004).

Since relapse in ALL patients leads to very poor prognosis [24-26], the notion that allogeneic HSCT should be performed for all patients with ALL beyond CR1 is difficult to be realized in clinical situations [7].

The international ALL trial MRC UKALL XII/ECOG E2993 showed that allogeneic HSCT using matched related donors provided survival benefit for standard-risk adult patients with Ph-negative ALL in CR1 compared with chemotherapy, while there was no significant survival benefit for high-risk patients. Allogeneic HSCT is able to reduce relapse rates in both standard-risk and high-risk patients; however, there is a decrease in OS in the high-risk patients because of their higher rates of transplant-related mortality. The high-risk in this international study was defined as having as 1 of the following factors: age more than 35 years, a high WBC count at presentation (>30 × 10^9/L for B lineage and >100 × 10^9/L for T lineage) [23]. Age is a significant prognostic factor for ALL patients receiving allogeneic HSCT as well as chemotherapy [27]. Therefore, allogeneic HSCT may not be a recommended option for patients defined as high-risk because of their age being more than 35 years old [28,29].

Recent studies have shown that a pediatric-inspired ALL chemotherapy protocol significantly improves treatment outcome in relatively young adult ALL patients [30-34], and this patient population is at standard-risk in terms of age. Thus, the indication of allogeneic HSCT based on the risk stratification made by initial presentation needs to be tested, and more reliable indication for allogeneic HSCT in adult patients with Ph-negative ALL in CR1 is necessary.

MRD measurement in adult patients with Ph-negative ALL has been reported to be useful for identifying patients with a significantly high risk of relapse. The German Multicenter Study Group for adult ALL (GMALL) study used PCR analysis of antigen-receptor genes to assess MRD in standard-risk ALL patients. Low-risk patients

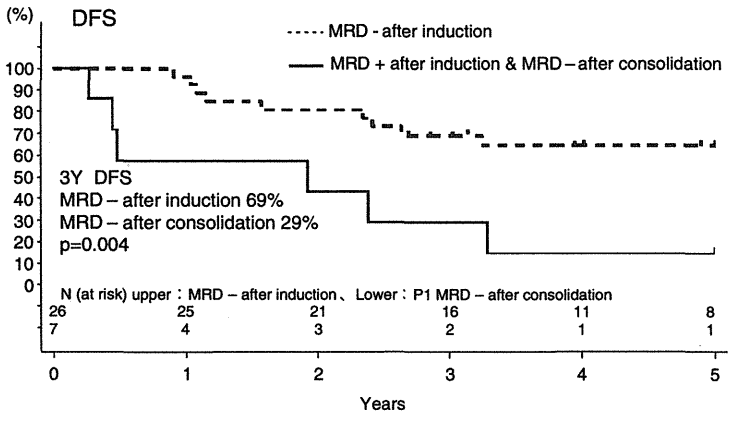

Figure 2 Effect of time taken to become minimal residual disease (MRD)-negative on disease-free survival (DFS). Patients who were MRD positive after induction and became MRD negative after consolidation chemotherapy C in the first course (n = 7) had a significantly worse DFS compared with patients who were MRD negative after induction chemotherapy A in the first course (n = 26) (69% vs. 29%, p = 0.004).

Table 2 Multivariate analysis for disease-free survival (Cox Regression Model)

	Hazard ratio	95% CI		P-value
Risk factors				
Age	5.067	1.616	15.885	0.005
WBC	1.496	0.457	4.897	0.505
MRD status after induction	8.769	2.465	31.196	<0.001
MRD status after consolidation	0.67	0.18	2.492	0.55

Age: ≥35 vs <35.
WBC: ≥30×10^9/L vs <30×10^9/L.
MRD after Induction: positive vs. negative.
MRD after consolidation: positive vs. negative

were those with MRD–negative on days 11 and 24 and had a 3-year relapse rate of 0%; high-risk patients were those with MRD-positive until week 16 and had a relapse rate of 94% [10]. The Northern Italy Leukemia Group-ALL 09/00 study found that MRD was the most significant predictor of relapse [13]. The estimated 5-year DFS was 72% in 58 MRD-negative patients at the end of consolidation and 14% in 54 MRD-positive patients.

Our results indicate that patients with MRD negativity after induction therapy provided excellent DFS without allogeneic HSCT, whereas patients with MRD positivity after several consolidation therapies showed very poor DFS if they did not undergo allogeneic HSCT. This observation is in line with above reports. Our analysis showed that late-attained MRD negativity could not lead to good prognosis, while other groups reported the MRD negativity at the end of consolidation to be associated with good prognosis. This controversy may reflect the sensitivity level of MRD measurement. We used semi-quantitative PCR analysis and defined MRD negativity as $<1 \times 10^{-3}$, whereas GMALL [10] and The Northern Italy Leukemia Group-ALL 09/00 study [13] analyzed MRD according to EuroMRD-ALL guidelines [35,36] and considered $<1 \times 10^{-4}$ as MRD negativity. In our study, MRD positivity after the second course of consolidation was seen in 6 of 37, while MRD positivity at the end of consolidation was observed in 54 of 112 in The Northern Italy Leukemia Group-ALL study 09/00 [13]. Thus, our MRD-negative patients had a possibility of MRD positivity if more sensitive MRD analysis was used. We suggest these late-attained MRD-negative patients were potential candidates for allogeneic HSCT.

In this study, the results of multivariate Cox regression analysis for DFS indicates that age (≥35 years vs. <35 years) and MRD status after induction therapy were significant prognostic factors, whereas WBC count ($\geq 30 \times 10^9$/L vs. $<30 \times 10^9$/L) or MRD status after consolidation therapy was not. Age is one of the most important prognostic factors in adult ALL patients, and age of our study population was median 31 years-old ranging 17 to 63 including adolescent and young adult patients. Thus, these relative young patients were supposed to have good prognosis with chemotherapy. Initial WBC count has been another important prognostic factor in adult ALL patients, but not in our study. Our chemotherapy regimen was modified CALGB 19802 with dose intensification of daunorubicin and cytarabine, and it might be possible that this intensive chemotherapy conquer negative impact of high initial WBC count. According to the recently reported result of CALGB 19802 [16], age (≥60 years vs. <60 years) was a significant prognostic factor for DFS, while initial WBC count ($\geq 30 \times 10^9$/L vs. $<30 \times 10^9$/L) was not. This report was in line with our observation. In our analysis MRD status after induction therapy (positive vs. negative: HR 8.769, and $p < 0.001$) was a very strong prognostic factor for DFS. Whether negative impact of MRD positivity could be overcome by allogeneic HSCT is the next consideration. There are two reports regarding the effect of prospective allocation for allogeneic HSCT based on MRD positivity in adult patients with Ph-negative ALL in CR1.

In the Northern Italy Leukemia Group-ALL study 09/00, for the MRD-positive patients at the end of consolidation, there was a significantly better 4-year DFS for 36 patients who had an allogeneic (n = 22) and autologous (n = 14) HSCT compared to 18 patients unable to undergo HSCT (33% vs. 0%, p = 0.0000) [13]. The GMALL reported that 5-year DFS for MRD-positive patients at week 16 with (n = 57) vs. without (n = 63) allogeneic HSCT were 44 ± 8% vs. 11 ± 4% respectively (p <0.0001) [37]. In our study, among MRD-positive patients following consolidation chemotherapy C in the first course, all of 3 patients without allogeneic HSCT relapsed while 1 of 3 patients with allogeneic HSCT did. The size of our study population was too small for statistical analysis. However, these three studies clearly indicate that MRD-positive patients at late phase of chemotherapy have little chance of DFS more than 10% without allogeneic HSCT [37]. These MRD-defined high-risk patients had much worse prognosis compared with conventional high-risk patients defined by initial presentation. Furthermore, allocation of allogeneic HSCT could improve the prognosis of MRD-defined high-risk patients.

The interpretation of our results may be affected by a limited number of adult ALL patients. A role of MRD measurement should be evaluated in relation with patients' geography, chemotherapy regimens used, and timing and sensitivity of MRD analysis. With our less sensitive MRD analysis compared to EuroMRD-ALL guidelines, we could identify patients with good early treatment response not indicated for allogeneic HSCT, while we could not identify patients with good late treatment response. In near future, the assessment of MRD status using standardized protocols and RQ-PCR [35,36] will be a valuable tool to stratify a risk of relapse in adult patients with Ph-negative ALL in CR1.

In conclusion, our data suggest that evaluation of MRD at least twice after induction and consolidation is very useful when considering clinical indication for allogeneic HSCT in adult patients with Ph-negative ALL in CR1.

Competing interests
The authors declare no competing financial interests.

Authors' contributions
All authors recruited and treated patients for this study. DNA-baed MRD analysis in this report were performed under supervision of SY. KN and MH were involved in the drafting of the manuscript. MH coordinated the study. All authors reviewed and approved the final draft of the manuscript.

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Double-Unit Cord Blood Transplantation after Myeloablative Conditioning for Patients with Hematologic Malignancies: A Multicenter Phase II Study in Japan

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A B S T R A C T

We analyzed the outcomes of 61 patients with hematologic malignancies who underwent double-unit cord blood transplantation (dCBT) after myeloablative conditioning performed as part of a prospective multicenter phase II study. The conditioning regimen for dCBT included total body irradiation, cyclophosphamide, and granulocyte colony-stimulating factor combined with cytosine arabinoside for myeloid malignancies and with total body irradiation and cyclophosphamide for lymphoid malignancies. The cumulative incidence of neutrophil engraftment after dCBT was 85% (95% confidence interval [CI], 73%-92%). All 51 of the patients who engrafted had complete chimerism derived from a single donor by day +60. Only the degree of HLA disparity in the host-versus-graft direction had an impact on unit dominance. The cumulative incidence of grade II-IV acute graft-versus-host disease was 25% (95% CI, 15%-37%), and that of chronic graft-versus-host disease was 32% (95% CI, 20%-44%). The 1-year cumulative incidence of relapse was 23% (95% CI, 13%-34%), and that of transplantation-related mortality was 28% (95% CI, 17%-39%). With a median follow-up of 41 months, event-free survival was 48% (90% CI, 37%-58%) at 1 year and 46% (90% CI, 35%-56%) at 3 years. Event-free survival at 3 years was 67% (95% CI, 46%-81%) for patients with standard risk and 29% (95% CI, 15%-45%) for those with advanced risk. This study suggests that dCBT after myeloablative conditioning is a promising alternative for adults and large children with hematologic malignancies who need stem cell transplantation but lack a suitable adult donor or an adequate single-unit cord blood graft.

INTRODUCTION

Cord blood (CB) is being increasingly used as an alternative source of hematopoietic stem cells for adults with hematologic malignancies requiring hematopoietic stem cell transplantation (HSCT) [1-5]. Although CB has advantages, including rapid availability [6] and low risk of severe acute graft-versus-host disease (GVHD) despite HLA mismatches, the low cell dose in a single CB unit contributes to high rates of graft failure and transplantation-related mortality (TRM), especially in adults and large children [7-9]. Double-unit CB transplantation (dCBT) was introduced to overcome these obstacles [10] and is becoming more widely applied [11-15]. We conducted a prospective multicenter Phase II study assessing the safety and efficacy of dCBT for patients with high-risk hematologic malignancies. We used relatively standard myeloablative conditioning regimens: total body irradiation (TBI) plus cyclophosphamide (CY) for lymphoid malignancies and TBI, CY, and granulocyte colony-stimulating factor (G-CSF) combined with cytosine arabinoside (ara-C) for myeloid malignancies. We used cyclosporine A (CyA) and short-term methotrexate (MTX) for GVHD prophylaxis.

PATIENTS AND METHODS

Thirty-nine centers participated in this study after approval by each pertinent Institutional Review Board (trial identifier: UMIN: C000000359, C-SHOT 0507). Written informed consent was obtained from all patients before transplantation.

Eligibility Criteria

Inclusion criteria were as follows: (1) age <55 years with a high-risk hematologic malignancy; (2) no HLA-matched or single antigen-mismatched related donor available; (3) no HLA-matched unrelated donor available, or requiring urgent transplantation even if an HLA-matched unrelated donor were available; (4) no 4-6/6 HLA-A, -B, or -DR serologically antigen-matched single CB unit containing a cell dose >2.5 × 10^7/kg; (5) no previous stem cell transplantation; (6) no active infection at the start of conditioning chemoradiotherapy; and (7) HIV-negative status. Patients with an Eastern Cooperative Oncology Group performance status ≥2, ejection fraction <50%, SaO_2 (arterial oxygen saturation) <93% in room air, serum creatinine of ≥1.3 mg/dL, total bilirubin ≥1.6 mg/dL, or glutamic-oxaloacetic transaminase ≥2 times the normal value were excluded. Patients with Down syndrome or Fanconi anemia were also excluded.

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CB Unit Selection Criteria

CB units were obtained from CB banks belonging to the Japan Cord Blood Bank Network. The criterion for CB unit selection was 4-6/6 HLA-A, -B, and -DR antigens matched to the recipient. One of the 2 units should contain a cell dose of at least 1.5×10^7/kg. The total cell dose of the 2 units had to be $>2.5 \times 10^7$/kg, and transplantation of 2 units each with a cell dose $>2 \times 10^7$/kg was not allowed.

Treatment

All patients received a myeloablative preparative conditioning regimen of 12 Gy TBI fractionated in 4 or 6 doses. Ara-C was given at a dose of 3 g/m^2 every 12 hours for 2 days (days -5 and -4). Recombinant human G-CSF was given by continuous infusion at a dose of 5 μg/kg/day; infusion was started at 12 hours before the first dose of ara-C and stopped at the completion of the last dose. CY was administered i.v. at 60 mg/kg/day for 2 days (days -3 and -2). A regimen of TBI, CY, and G-CSF combined with ara-C was used for patients with myeloid leukemias and myelodysplastic syndrome (MDS) [1]. TBI plus CY was used for those with lymphoid malignancies. At 2 days after completion of conditioning, CB units were thawed and then infused sequentially in an arbitrary order and nonmandatory time interval after premedication with hydrocortisone (100 mg) and hydroxyzine hydrochloride (25 mg).

GVHD prophylaxis was provided with CyA plus short-term MTX. CyA was given by continuous infusion at a dose of 3 mg/kg/day starting on day -1. MTX was given at 15 mg/m^2 i.v. on day +1 and at 10 mg/m^2 on days +3 and +6. Once oral intake could be tolerated, oral CyA was started at a dose ratio of 1:2.5 in 2 divided doses per day based on the last i.v. dose. In the absence of GVHD after day +60, CyA was tapered by 10% to 20% per week until it could be discontinued. The supportive care regimen, including prophylaxis for infection, was similar to that for single-unit CBT in each transplantation center. All patients received G-CSF starting on day 5 and continuing until the absolute neutrophil count (ANC) reached 5000/μL.

HLA Typing and Chimerism Analysis

HLA typing of the recipient and CB unit was determined by low-resolution (2 digits) and/or high-resolution (4 digits) DNA typing for HLA-A, -B, -C, and -DRB1. Donor chimerism was determined serially for bone marrow and/or blood at days +14, +30, +60, and +100 after dCBT, and at additional time points as needed. The analytic method used was based on the quantitative amplification of informative polymorphic short tandem repeat regions in the recipient and the donor.

Definitions

Patients who underwent dCBT in first or second remission of acute myelogenous leukemia (AML), in first remission of acute lymphoblastic leukemia (ALL) and malignant lymphoma (ML), or in the chronic phase of chronic myelogenous leukemia (CML) and refractory anemia of MDS were classified as standard risk. All others were classified as advanced risk.

Neutrophil recovery was defined as achievement of an absolute neutrophil count (ANC) of ≥500/μL for 3 consecutive days; platelet recovery was defined as a count of ≥50,000/μL without transfusion support. Primary engraftment failure was defined as the absence of donor-derived myeloid cells on the day of death or day +60 in patients surviving beyond day +2+8 after dCBT, or when a second stem cell transplantation was required for donor-derived myeloid recovery. Diagnosis and clinical grading of acute GVHD (aGVHD) were performed according to established criteria [16]. Relapse was defined as recurrence of the underlying hematologic malignancy. TRM was defined as death during a continuous remission. Disease-free survival (DFS) was defined as survival in a state of continuous remission. Event-free survival (EFS) was defined as survival in a state of remission without engraftment failure.

Statistical Analyses

The primary endpoint of this study was 1-year EFS; secondary endpoints were neutrophil and platelet engraftment, incidence of aGVHD and chronic GVHD (cGVHD), toxicity within 28 days, incidence of TRM and relapse, DFS, and overall survival (OS). The expected and threshold EFS at 1 year were estimated as 60% and 40%, respectively. With a statistical power of 90% and a 1-sided type I error of 5%, the number of eligible patients required for this study was calculated as 56 using a binomial analysis method. The projected sample size was 70 patients, assuming that 20% of patients would be ineligible. Primary endpoint analysis was performed using the Kaplan-Meier method to calculate the probability of EFS. Treatment was considered effective if the lower limit of the 90% confidence interval (CI) exceeded the threshold EFS (ie, 40%).

Cumulative incidence curves were used in a competing-risks setting to calculate the probabilities of neutrophil and platelet recovery, aGVHD, cGVHD, relapse, and TRM. For neutrophil and platelet recovery, death before recovery was the competing event; for GVHD, death without GVHD and relapse was the competing events; for relapse, death without evidence of relapse was the competing event; and for TRM, relapse was the competing event. OS, DFS, and EFS were calculated by the Kaplan-Meier method. The log-rank test was used for univariate comparisons. For multivariate analysis of prognostic variables affecting transplant outcomes, a Fine-Gray model was used to analyze transplantation outcomes with competing risks. A Cox proportional hazard regression model was used to analyze other outcomes. The following variables were considered: recipient cytomegalovirus (CMV) serology (positive versus negative), recipient age at enrollment (age ≥40 years versus <40 years; cutoff point was around the median), degree of ABO matching between recipient and engrafting unit (major mismatch versus matched or minor mismatch), sex matching between recipient and engrafting unit (mismatched versus matched), degree of HLA matching between donor and recipients (2 antigen- mismatched versus 0 or 1 antigen-mismatched, with HLA matching defined by the worst-matched of the 2 units), disease status at transplantation (advanced versus standard), cryopreserved TNC dose (median, $<3.52 \times 10^7$/kg versus $\geq 3.52 \times 10^7$/kg), CD34$^+$ cell dose (median, <1.04/kg $\times 10^5$/kg versus $\geq 1.04 \times 10^5$/kg), and cell dose difference [(TNC of large unit − TNC of smaller unit)/(TNC of large unit, ≥15% versus <15%)], and degree of HLA mismatch between the 2 units (≥3 antigen mismatches versus ≤2 antigen mismatches).

Variables found to affect outcome with a P value <.20 on univariate analyses were selected for the multivariate analyses. Variables were selected in a backward stepwise manner with a variable retention criterion of $P < .05$ for the final model. The Wilcoxon signed-rank test was used to evaluate the effect of cell dose and HLA compatibility on engraftment of a predominant single CB unit, and the McNemar test was used for evaluation of categorical factors. The median duration of follow-up of survivors was 41 months (range, 12 to 57.4 months). Results are reported as of March 2011. Calculations were performed using Stat View J version 5.0 and Stata version 11.1 (StataCorp, College Station, TX).

RESULTS

Patient and Graft Characteristics

A total of 70 patients were enrolled between April 2006 and January 2010. Nine patients did not undergo dCBT, 7 because of disease progression and 2 because they received a graft from another source. Patient and graft characteristics are summarized in Table 1. The 61 patients who underwent dCBT included 8 females and 53 males, with a median age of 37 years (range, 10 to 54 years) and a median body weight of 70.5 kg (range, 50.1 to 129.8 kg). Antibodies against CMV were detected in 75.4% of the patients; CMV antibody was not tested in 3 patients. The underlying malignancy was AML in 30 patients, ALL in 17 patients, CML in 6 patients, MDS in 5 patients, and ML in 3 patients. Disease status at dCBT was classified as standard risk in 27 patients and as advanced risk in 34 patients. The median TNC and CD34$^+$ cell doses (both units combined) at cryopreservation were 3.52×10^7/kg (range, 2.25 to 4.43×10^7/kg) and 1.04×10^5/kg (range, 0.39 to 2.67×10^5/kg), respectively. The median TNC doses of the larger and smaller units were 1.90×10^7/kg (range, 1.47 to 2.48×10^7/kg) and 1.60×10^7/kg (range, 0.74 to 1.97×10^7/kg), respectively. In 1 patient, the TNC dose of the larger unit was decreased from $>1.5 \times 10^7$/kg at registration to 1.47×10^7/kg at dCBT because of weight gain. Because 1.47 rounded off to 1 decimal place is 1.5, we decided to include this case in the analyses.

HLA matching for HLA-A, -B, and -DRB1 low- and high-resolution types between recipients and donors and between donors is described in Table 1. When the graft with fewest HLA mismatches was counted for each recipient, only 2 patients (3%) received a graft that contained at least 1 unit in which HLA-A, -B, and -DRB1 were matched at a low-resolution level to the recipient; 21 patients (34%) received a graft with at least 1 unit 5/6 HLA-matched to the recipient; and 38 patients (62%) received a graft with both units 4/6 HLA-matched to the recipient. Among the 58 patients with HLA-DRB1 typed by high-resolution DNA typing, 2 patients

Table 1
Patient and Graft Characteristics

Characteristic	Value
Number of patients	61
Sex, male/female, n	53/8
Age, years, median (range)	37 (10-54)
Body weight, kg, median (range)	70.5 (50.1-129.8)
Diagnosis and disease status at CBT, n	
ALL	17 (CR1, 8; CR2, 6; relapse, 3)
AML	30 (CR1, 6; CR2, 11; CR3, 1; relapse, 6; PIF, 4; no induction therapy, 2)
CML	6 (AP, 1; BC, 5)
MDS	5 (RA, 2; RAEB2, 3)
Non-Hodgkin lymphoma	3 (refractory, 3)
CMV antibody, positive/negative/unknown, n	46/12/3
Performance status, 0/1, n	48/13
Cell dose at cryopreservation, median (range)	
TNC, $\times 10^7$/kg	
Total	3.52 (2.25-4.43)
Large unit	1.90 (1.47-2.48)
Small unit	1.60 (0.74-1.97)
$CD34^+$ cells, $\times 10^5$/kg	
Total	1.04 (0.39-2.67)
Large unit	0.50 (0.12-2.41)
Small unit	0.46 (0.02-1.42)
GM-CFU, $\times 10^3$/kg	
Total	27.42 (0.42-100.9)
Large unit	11.94 (0.17-39.6)
Small unit	12.85 (0.25-88.98)
ABO compatibility, large unit/small unit, n	
Major mismatches	23/20
Minor mismatches	16/16
Matches	22/25
HLA compatibility, n	
-A, -B, and -DRB1 low resolution	
5/6 + 6/6	1
5/6 + 5/6	6
4/6 + 6/6	1
4/6 + 5/6	15
4/6 + 4/6	38
-A and -B low resolution, -DRB1 high resolution	
5/6 + 5/6	3
4/6 + 6/6	1
4/6 + 5/6	10
4/6 + 4/6	17
3/6 + 6/6	1
3/6 + 5/6	4
3/6 + 4/6	13
3/6 + 3/6	4
2/6 + 4/6	1
2/6 + 3/6	4
HLA compatibility to each unit, n	
-A, -B, and -DRB1 low resolution	
6/6	1
5/6	14
4/6	13
3/6	23
2/6	10
-A and -B low resolution, -DRB1 high resolution	
6/6	0
5/6	8
4/6	12
3/6	20
2/6	15
1/6	6

AP indicates accelerated phase; BC, blast crisis; CR, complete remission; PIF, primary induction failure; RA, refractory anemia; RAEB, refractory anemia with excess blasts.

(3%) received a graft that contained at least one 6/6 HLA-matched unit, 17 (29%) received at least one 5/6 HLA-matched unit, 31 (53%) received at least one 4/6 HLA-matched unit, 8 (14%) received at least one 3/6 HLA-matched unit. Three, 17, and 4 patients received a graft with both units 5/6, 4/6, and 3/6 HLA-matched to the recipient, respectively. The units were 6/6 HLA-A–, -B–, and DRB1–matched at low resolution to each another in 1 patient, 5/6 matched in 14 patients, 4/6 matched in 13 patients, 3/6 matched in 23 patients, and 2/6 matched in 10 patients. When HLA was typed by HLA-A and -B low-resolution and -DRB1 high-resolution typing, the units were 5/6 matched to each other in 8 patients, 4/6 matched in 12 patients, 3/6 matched in 20 patient, 2/6 matched in 15 patients, and 1/6 matched in 6 patients.

Survival

The median follow-up for survivors (n = 32) was 41 months. EFS at 1 year was 48% (90% CI, 37%-58%) (Figure 1A). One-year EFS at 1 year was 67% (95% CI, 46%-81%) in patients with standard risk and 32% (95% CI, 18%-48%) in patients with advanced risk at dCBT, and 3-year EFS was 67% (95% CI, 46%-81%) in patients with standard risk and 29% (95% CI, 15%-45%) in those with advanced risk ($P = .023$) (Figure 1B). One-year DFS was 49% (95% CI, 36%-61%), and 1-year OS was 57% (95% CI, 44%-69%). Three-year DFS was 47% (95% CI, 34%-59%), and 3-year OS was 54% (95% CI, 40%-65%). Disease status at transplantation was the sole prognostic factor affecting EFS (relative risk [RR], 2.71; $P = .011$). No other variable considered had a significant effect on EFS.

Toxicity Within 28 Days after dCBT

Toxicities occurring within 28 days after dCBT were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3. The most frequent grade 3-4 toxicity was infection, occurring in 43 of 61 patients (70.5%); other grade 3-4 toxicities included nausea/vomiting (17 patients; 27.9%), oral mucosa lesions (16; 26.2%), diarrhea (13; 21.3%), cardiac events (6; 9.8%), liver toxicity (6; 9.8%), bleeding (5; 8.2%), neurologic events (3; 4.9%), renal/urinary events (3; 4.9%), skin toxicity (3; 4.9%), lung toxicity (2; 3.3%), and thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (1; 1.6%). Grade 4 toxicities involving infections were seen in 5 patients (8.2%), and those involving the heart occurred in 2 patients (3.3%). Other grade 4 toxicities included bleeding and neurologic, lung, and liver toxicities, which were seen in 1 patient each.

Hematopoietic Recovery and Chimerism

The cumulative incidence of neutrophil recovery was 67% (95% CI, 53%-77%) at day +28 and 85% (95% CI, 73%-92%) at day +50 (Figure 2). The cumulative incidence of platelet recovery at day +180 was 77% (95% CI, 66%-89%). The median time to neutrophil recovery was 25 days (range, 17 to 49 days). A greater degree of HLA matching between the 2 units (≥3 antigen mismatches with increased risk of no neutrophil recovery compared with ≤2 antigen mismatches; RR, 0.53; $P = .023$) was the sole risk factor affecting neutrophil recovery.

Three patients (5%) died too early to allow evaluation of engraftment (2 patients on day +7 and 1 patient on day +12). Failure of primary engraftment occurred in 7 patients; 6 of these 7 patients underwent a second transplantation (single-unit CBT in 3, autologous peripheral blood stem cell transplantation in 2, haploidentical peripheral

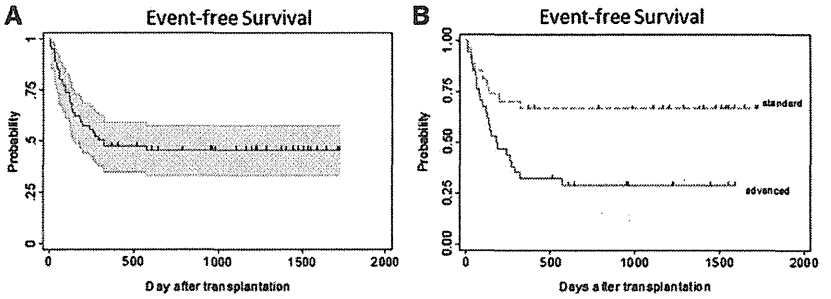

Figure 1. Kaplan-Meier analysis of EFS in patients with dCBT after myeloablative conditioning (A) and according to disease status (B). Patients with standard risk had significantly better posttransplantation survival than those with advanced risk ($P = .023$, log-rank test).

blood stem cell transplantation in 1) between day +27 and day +49. Only 1 of these patients survived beyond 1 year after dCBT.

All but 1 of the 51 patients with donor engraftment had complete chimerism derived from a single donor (median, 100%; range, 91.2% to 100%) by day 30 after dCBT. One patient demonstrated mixed chimerism from both donors (81.6% and 11.5%) at day +30, but this changed to complete chimerism of single-donor origin by day +60.

Predicting Factors Responsible for Unit Dominance

The degree of HLA disparity in the host-versus-graft (HVG) direction was associated with unit dominance (Table 2). Twenty of the 51 patients with donor engraftment received 2 units with varying degrees of HLA disparity (HLA-A, -B, and -DR antigen-level typing). Of these, the unit that was better HLA-matched to the recipient engrafted in 15 patients, whereas the more poorly matched unit predominated in 5 patients ($P = .0218$). Twenty-seven of 49 engrafted patients typed by HLA-A or -B antigen-level and -DRB1 high-resolution typing received 2 units with different degrees of HLA disparity; of these, the better-matched unit engrafted in 21 patients ($P = .0056$).

There was no correlation between unit dominance and cell dose (cryopreserved TNCs, $P = .4589$; cryopreserved $CD34^+$ cells, $P = .3823$; cryopreserved granulocyte macrophage colony-forming units (GM-CFU), $P = .6854$; infused TNCs, $P = .6114$; infused $CD34^+$ cells, $P = .3875$; infused GM-CFU, $P = .8405$). Other factors, including sex match ($P = .7003$), ABO match ($P = 1.0$), order of infusion ($P = .4838$), and graft viability ($P = .6152$), were not associated with unit dominance.

GVHD

aGVHD developed in 33 of the 61 patients (54%), classified as grade I in 18 patients, grade II in 11, grade III in 3, and grade IV in 1 (25% grade II-IV and 7% grade III-IV). cGVHD was observed in 18 of the 50 evaluable patients who survived for >100 days, and was extensive in 9 patients. The cumulative incidence of grade II-IV aGVHD was 25% (95% CI, 15%-37%), and that of cGVHD at 1 year was 32% (95% CI, 20%-44%) (Figure 3A and B). No risk factors for the development of grade II-IV aGVHD were identified in univariate and multivariate analyses including HLA disparities ($P = .327$).

Relapse

Relapse occurred in 15 patients, between 57 and 573 days (median, 135 days) after dCBT. The cumulative incidence of relapse at 1 year was 23% (95% CI, 13%-34%) (Figure 3C). Seven of 17 patients with ALL relapsed, compared with only 8 of 41 patients with myeloid malignancies (AML, 6 of 29; CML, 1 of 6; MDS, 1 of 6). In terms of disease status at transplantation, relapse occurred in 4 of 27 patients with standard risk and in 11 of 34 patients with advanced risk. No risk factors for relapse were identified by univariate and multivariate analyses, including disease status at CBT ($P = .291$) and HLA disparities ($P = .156$).

TRM and Cause of Death

The cumulative incidence of TRM was 15% (95% CI, 7%-25%) at day +100 and 28% (95% CI, 17%-39%) at 1 year (Figure 3D). No risk factors for TRM were identified on univariate and multivariate analyses. The causes of death are listed in Table 3. Disease progression was the leading cause of death. Of the 29 patients who died between 7 and 1368 days (median, 188 days) after dCBT, 15 died from causes other than relapse: graft failure in 5 (of whom 3 died from infection and 1 died from hepatic veno-occlusive disease after a second transplantation), infection without graft failure in 2, organ failure in 3, acute respiratory distress syndrome/interstitial pneumonia in 3, and cGVHD and bleeding in 1.

DISCUSSION

The present study is the first reported analysis of dCBT in Japan. In this multicenter Phase II study, greater HLA disparities between recipient and donor and between each of the 2 units were found compared with those reported in previous studies of dCBT, because we selected the 4-6/6 HLA-

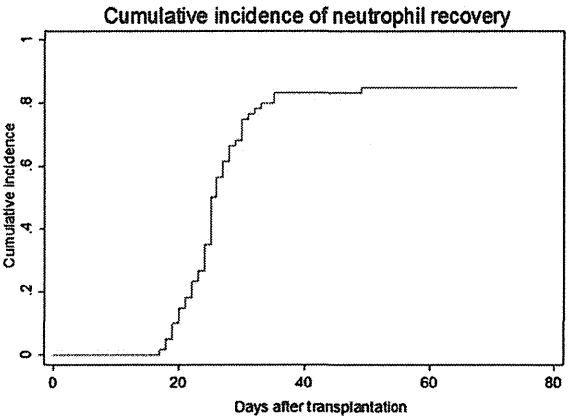

Figure 2. Cumulative incidence of neutrophil engraftment after myeloablative conditioning and subsequent dCBT.

Table 2
Degree of HLA Disparity in the HVG Direction and Unit Dominance

Number of HLA Mismatches in the HVG Direction for Winner/Loser	Difference in HLA Mismatches	Number of Patients with Sustained Engraftment	
		HLA-A, -B, and -DR at Low Resolution Level	HLA-A and -B at Low Resolution Level, HLA-DRB1 at High Resolution Level
1/1	0	11	3
2/2		20	15
3/3		0	4
0/1	−1	1	1
1/2		13	7
2/3		0	6
3/4		0	1
0/2	−2	1	1
1/3		0	4
2/4		0	1
1/0	1	1	0
2/1		4	2
3/2		0	2
4/3		0	1
3/0	3	0	1
		$P = .0218$	$P = .0056$

Analyses were performed with the Wilcoxon signed-rank test.

matched CB unit for the recipient by matching at the low-resolution DNA typing level of HLA-A, -B, and -DRB1, with no consideration of unit–unit match. The lower limit of the 90% CI did not exceed the threshold EFS by 3% in primary endpoint analyses. The threshold and expected EFS was estimated prior to study initiation according to survival results of single-unit CBT (EFS of 40% at 1 year; unpublished data, Japan Cord Blood Bank Network, 2005) and dCBT [17] (EFS of 64% at 1 year) for adults. In these studies, 21% and 36% of patients were received CBT in advanced-risk disease status, respectively, whereas 54% of patients in this study were in advanced-risk disease status at the time of dCBT. Our survival data are comparable to earlier reports of dCBT after myeloablative conditioning [10-15]; thus, we can confirm that dCBT after myeloablative conditioning is a promising alternative option for adults and large children with hematologic malignancies who need HSCT but do not have a suitable related/unrelated donor or an adequate single-unit CB graft. We have also shown that HLA disparity in the HVG direction helps determine which unit was engrafted. These data may provide clinically useful information to aid in the selection of CB units for dCBT.

Our cumulative incidence of neutrophil engraftment of 85% and median time to neutrophil recovery of 25 days are comparable to previously reported values for dCBT with myeloablative conditioning (ie, cumulative incidence of neutrophil engraftment, 80%-94%; median time to neutrophil recovery, 23-25 days) [10,12-15]. The degree of HLA disparity between the 2 units was the sole factor associated with neutrophil engraftment. On the other hand, unit–unit HLA match reportedly had no significant effect on sustained engraftment and speed of neutrophil recovery [18]. Further studies are needed to investigate the influence of cross-immunologic reactions between the 2 units on neutrophil engraftment.

Our results are in agreement with previously reported data, which indicated that 1 CB unit becomes predominant and supports sustained hematopoiesis in dCBT. The parameters that determine unit dominance have not yet been elucidated. In our analysis, only the degree of HLA disparity in the HVG direction was correlated with unit dominance. To our knowledge, this is the first report suggesting that host-versus-graft immune reactions play a role in determining the engrafting unit. There was no correlation between dominance and the doses of TNCs, $CD34^+$ cells, and GM-CFU or ABO, sex mismatch, cell viability, or order of infusion. Previous reports have implied that $CD3^+$, GM-CFU, and $CD34^+$ cell doses and the viability of $CD34^+$ cells were associated with the unit dominance [14,18-21], and that the presence of graft-versus-graft reactions mediated by $CD8^+$ T cells expanding from the dominant unit play a critical role

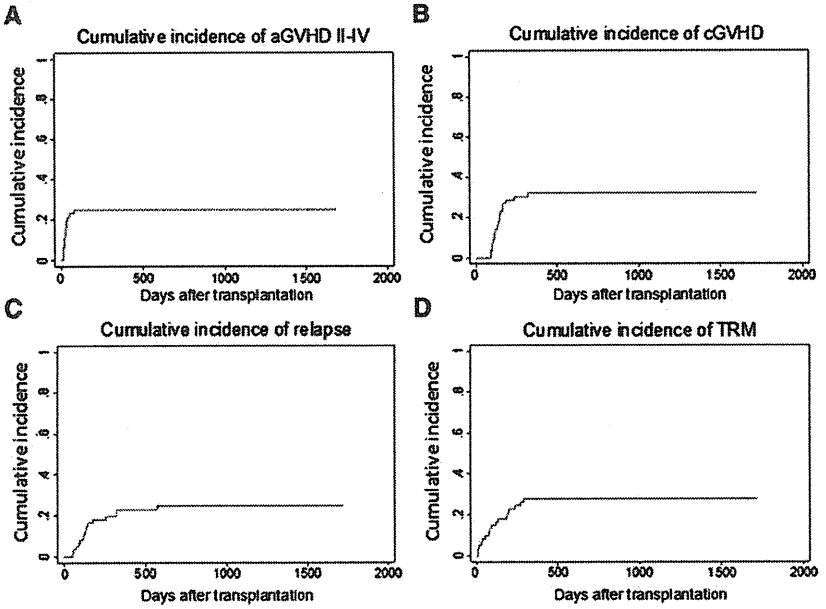

Figure 3. Cumulative incidence of grade II-IV aGVHD (A), cGVHD (B), relapse (C), and TRM (D).

Table 3
Primary Causes of Death after dCBT

Cause of Death	Number of Patients	Time of Death after dCBT (Day of Death)		
		<100 Days	100-365 Days	>365 Days
Relapse/disease progression	14	1 (day 12)	10 (days 134, 151, 197, 207*, 211, 225, 250†, 254, 266, and 296)	3 (days 419, 672, and 1368)
Graft failure	5	5 (days 28, 32‡, 53‡, 74§, and 90‡)		
Infection without graft failure	2		2 (days 132 and 183)	
Organ failure	3	1 (day 7)	2 (days 272 and 289)	
cGVHD	1		1 (day 239)	
Bleeding	1	1 (day 7)		
ARDS/IP	3	1 (day 82)	2 (days 118 and 188	

ARDS indicates acute respiratory distress syndrome; IP, interstitial pneumonia.
* Death by suicide after relapse on day +57.
† Death by relapse after second transplantation for graft failure.
‡ Death by infection after second transplantation.
§ Death by hepatic veno-occlusive disease after second transplantation.

in rejecting the other unit [22]. We could not verify the importance of $CD3^+$ cell count and $CD34^+$ cell viability because we did not assay these parameters in our study. Furthermore, there are reports concerning the effect of preformed donor-specific anti-HLA antibodies on graft failure or unit loss in dCBT [23]. Whether the unit dominance is influenced by immune reactions between the units or between the recipient and the units still remains to be determined. On the other hand, the use of 2 units might simply give the recipient a better chance of having a unit with a sufficiently high potential for engrafting. Further study is warranted.

In single-unit CBT, the doses of TNCs and $CD34^+$ cells at cryopreservation or at infusion have important effects on survival [7-9]. According to a recent report by Sanz et al. [24], in adults with AML, a low dose of TNCs ($<2 \times 10^7$/kg) at infusion was the only significant factor associated with lower leukemia-free survival (LFS at 4 years; $<2.0\times10^7$/kg versus $\geq2.0\times 10^7$/kg, 25% versus 75%) [24]. Based on the foregoing results, the suggested dose of TNC at cryopreservation should not be $<2.5 \times 10^7$/kg, given the anticipated cell loss of approximately 20% [25,26]. In this study, the TNC dose in the large unit was $<2.5\times10^7$/kg in all recipients and further that was $<2.0\times10^7$/kg in 38 patients (62%). Because dCBT was performed, these patients had a chance to undergo stem cell transplantation and achieved an EFS of 46% at 3 years, which is similar to or better than the survival seen with single-unit CBT [24,27]. The extent of the benefit has not been demonstrated by a matched-cohort analysis or prospective randomized trial, however.

The reported incidence of grade II-IV aGVHD is higher in patients undergoing dCBT compared with those undergoing single-unit CBT, although the frequency of severe-grade aGVHD is comparable in the 2 groups [28]. According to McMillan et al. [28], the increased risk for aGVHD after dCBT may be the result of a higher dose of T cells in the grafts when 2 units are used and/or a graft-versus-graft effect, although the precise mechanism is not clear. In our study, grade II-IV aGVHD developed in 25% of the patients and grade III-IV aGVHD occurred in 7%. The fact that the incidence of grade II-IV aGVHD seems to be lower than that reported by others (eg, 37%-65% incidence of II-IV aGVHD in dCBT with myeloablative conditioning), and the incidence of severe aGVHD was comparable [10-15], despite greater HLA disparities between recipients and donors and between the 2 units in our study. Our lower incidence of aGVHD is likely related to the different conditioning and GVHD prophylaxis regimens compared with previous studies [10-15,28]. The infused T cell doses in our study may have an influence on this finding, although the infused TNC dose was similar to that used in previous studies [10,12,13,28]. Ethnic differences also may contribute to these findings, as has been reported by Morishima et al. [29] and Oh et al. [30] Further studies with larger numbers of patients are needed to identify the effect of these factors, as well as of HLA matching, on GVHD.

Our relapse rate at 1 year of 23% is higher than reported in other studies (eg, relapse incidence of 15% at 5 years by Brunstein et al. [13], 19% at 5 years by Verneris et al. [12], 19% by Kang et al. [15], and 20% at 2 years by Kanda et al. [14] for dCBT after myeloablative conditioning). The relapse rate is reportedly lower in dCBT recipients compared with single-unit CBT recipients. Verneris et al. [12] analyzed the outcomes of CBT for acute leukemia and showed a significantly lower relapse rate among patients who received dCBT in remission compared with patients who underwent single-unit CBT (16% versus 31%). Rodrigues et al. [11] also reported a lower risk for relapse in patients with chronic lymphoid malignancies who underwent dCBT (13% versus 38% at 1 year). Although the precise mechanism is not known, these data suggest an enhanced graft-versus-leukemia effect in dCBT. The incidence of grade II-IV aGVHD in the present study was not higher than that reported in previous studies, and more patients with advanced risk at the time of dCBT were included compared with other reports [12-15], which might reflect the somewhat higher relapse rate.

The incidence of TRM (28%) in the present study was comparable with that reported in other studies of dCBT (29%-31%) [10-15]. Fifteen of the 29 patients who died did so from causes not related to relapse. Nine patients died within 100 days after dCBT, and all but 1 death were related to transplantation resulting mainly from graft failure. In addition, the major cause of death after day 100 was relapse. To improve survival, strategies to enhance engraftment and reduce relapse are required.

We demonstrated that survival outcomes and the incidence of engraftment, GVHD, relapse, and TRM seem to be comparable with that in other reports of dCBT and with our historical data from single-unit CBT. Interestingly, we also demonstrated that HLA disparity in the HVG direction has an impact on determining the grafting unit. Further studies with larger numbers of patients will be needed to clarify this and to develop guidelines for selection of units for dCBT.

In conclusion, we believe that myeloablative dCBT can be a feasible and effective alternative option for patients with

hematologic malignancies who need HSCT but have neither a suitable related/unrelated donor nor an adequate single-unit CB graft available. To validate the "double-cord" effect that is generally assumed to reduce the incidence of relapse, increase the incidence of aGVHD, and improve survival, a matched cohort study with a larger number of patients or a prospective randomized study (if possible), is needed.

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APPENDIX. TRANSPLANT CENTERS

The following transplant centers performed dCBT in this phase II study and produced follow-up reports: Department of Hematology, Hyogo College of Medicine; Department of Hematology, Toranomon Hospital; Department of Hematology and Oncology, Osaka Medical Center for Cancer and Cardiovascular Disease; Division of Internal Medicine, Sapporo Hokuyu Hospital; Division of Hematology and Oncology, Narita Red Cross Hospital; Department of Hematology, Osaka City University Graduate School of Medicine; Department of Hematology and Immunology, Tohoku University; Department of Hematology, Hokkaido University; Department of Hematology, Nippon Medical School Hospital; Department of Hematology, Tokai University School of Medicine; Division of Hematology, Osaka Red Cross Hospital; Division of Pediatrics, Red Cross Nagoya Daiichi Hospital; Division of Hematology, Kurashiki Central Hospital; Division of Transfusion Medicine, Nagaoka Red Cross Hospital; Division of Hematology, Kanagawa Cancer Center; Department of Pediatrics, Mie University; Department of Hematology and Oncology, Osaka Medical Center and Research Institute of Maternal and Child Health, Division of Hematology, Hyogo Cancer Center; Division of Internal Medicine, Hamanomachi Hospital; Department of Bone Marrow Transplantation, Niigata University Medical and Dental Hospital; Division of Pediatrics, Kyushu Cancer Center; Department of Hematology, Kanazawa University; Division of Hematology, Kumamoto Medical Center; Department of Hematology and Oncology, Okayama University; Department of Regenerative Medicine, Institute of Biomedical Research and Innovation Hospital; Division of Hematology, Red Cross Nagoya Daiichi Hospital.