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Successful Engraftment with Fludarabine, Cyclophosphamide, and Thymoglobulin Conditioning Regimen in Unrelated Transplantation for Severe Aplastic Anemia: A Phase II Prospective Multicenter Study

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Antithymocyte globulin (ATG) has been used in severe aplastic anemia (SAA) as part of the conditioning regimen. Among the many kinds of ATG preparations, thymoglobulin had been found to be more effective for preventing graft-versus-host disease (GVHD) and the rejection of organ transplants. After the promising results of our preliminary study, we conducted a phase II prospective multicenter clinical trial using a fludarabine (Flu), cyclophosphamide (Cy), and thymoglobulin conditioning regimen to allow good engraftment in patients who underwent unrelated transplantation for SAA. Twenty-eight patients underwent bone marrow (N = 15) or mobilized peripheral blood (N = 13) transplantation from HLA-matched unrelated donors with Cy (50 mg/kg once daily intravenously (i.v.) on days −9, −8, −7, and −6), Flu (30 mg/m^2 once daily i.v. on days −5, −4, −3, and −2), and thymoglobulin (2.5 mg/kg once daily i.v. on days −3, −2, and −1). Donor-type hematologic recovery was achieved in all patients. The estimated survival rate (SR) was 67.9%, and all the events were treatment-related mortality (TRM), which included thrombotic microangiopathy (N = 2), pneumonia (N = 1), myocardiac infarction (N = 1), posttransplantation lymphoprolifarative disease (N = 3), and chronic GVHD-associated complications (N = 2). The SR of patients who received bone marrow (60.0%) was not different from that of patients who received mobilized peripheral blood (76.9%) ($P = .351$), but the SR of patients who received more than 15 units of red blood cells before transplantation (45.5%) was significantly lower than that of the other patients (82.4%) ($P = .048$). The Flu, Cy, and thymoglobulin conditioning regimen achieved promising results for successful engraftment, but the TRM was high. This study was registered at www.clinicaltrials.gov (NCT00737685), and now we are performing a new multicenter study (NCT00882323) to decrease the TRM by reducing the dose of Cy.

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INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) with a matched related donor is a curative therapy for severe aplastic anemia (SAA), and cyclophosphamide (Cy)-based conditioning with or without antithymocyte globulin (ATG) is known to be optimal for HSCT with a matched related donor [1,2]. However, many patients have no appropriate related donor, and they need another treatment such as immunosuppressive therapy (IST) and/or stem cell transplantation (SCT) with an alternative donor. Transplantation with a matched unrelated donor (MUD) is associated with a high incidence of rejection, and the conditioning regimen for related donor transplantation is insufficient for HSCT with an MUD [3]. Two kinds of strategies have been developed to improve the engraftment by increasing the immunosuppressive activity of the conditioning regimen, but only 2 multicenter prospective studies have been conducted on this. One multicenter study in United State was conducted with adding total body irradiation (TBI) to Cy and ATG [4,5], and another was done by the European Group for Blood and Marrow Transplantation Severe Aplastic Anemia Working Party (EBMT-SAAWP) using the combination of fludarabine (Flu), Cy, and ATG [6].

These 2 studies included ATG, which has been commonly used for SAA as a part of the conditioning regimens. Among the many kinds of ATG, thymoglobulin (a rabbit-derived antithymocyte polyclonal antibody) is known to be more potent than the other available preparations, and it has been found to be more effective for preventing GVHD and the rejection of organ transplants [7-9]. Previously, we have reported the promising preliminary results of a Flu, Cy, and thymoglobulin conditioning regimen in matched unrelated transplantation for SAA [10]. After the promising results of our pilot study, the phase II prospective multicenter clinical trial was first conducted in Asia with a Flu, Cy, and thymoglobulin conditioning regimen to achieve good engraftment in unrelated transplantation for SAA.

PATIENTS AND METHODS

Patient and Donor Selection

From February 2006 to May 2008, 28 patients with SAA received HSCT from HLA matched unrelated donors at multicenter in Korea. Those patients, with the diagnosis of SAA, patients without prior HSCT, patients who had ECOG 0-2 performance status, patients who were free of significant functional deficits in major organs, and patients without any active viral infections or active fungal infection, were included in this study. Pregnant or nursing woman, patients with a malignant or nonmalignant illness that was uncontrolled or whose control had been jeopardized by complications of study therapy, patients with a psychiatric disorder that would preclude compliance, and patients with congenital AA including Fanconi anemia, were excluded. Transplantation performed at median 13 (3-210) months after the diagnosis of SAA. The clinical characteristics of patients are summarized in Table 1. The selection of donors was based on HLA serologic typing performed for class I antigens and HLA molecular typing for the DRB1 loci. HLA-A, -B, -C, and -DRB1 were confirmed by a high-resolution molecular method for all patients and donors. All patients received the designed conditioning regimen after obtaining informed consents from them or their guardians. This study was approved by the institutional review board of each center and registered at www.clinicaltrials.gov (NCT00737685).

Conditioning Regimen

The conditioning regimen was the same as that of the previous pilot study; the regimen was composed of Cy (50 mg/kg once daily intravenously [*i.v.*] on days −9, −8, −7, and −6), Flu (30 mg/m^2 once daily *i.v.* on days −5, −4, −3, and −2), and thymoglobulin (SangStat, Lyon, France and Genzyme, Cambridge, MA) (2.5 mg/kg once daily *i.v.* on days −3, −2, and −1) [10]. Patients received adequate hydration during the conditioning chemotherapy, and they also received mesna to prevent hemorrhagic cystitis. Unmanipulated bone marrow (BM) or mobilized peripheral blood (PB) harvest was infused on day 0 of the conditioning regimen.

Graft-versus-Host Disease (GVHD) Prophylaxis and Supportive Care

We allowed the use of each institution's protocol for GVHD prophylaxis. Patients received combination of cyclosporine (CsA) or tacrolimus (FK), and methotrexate (MTX), with or without posttransplant low-dose thymoglobulin (pATG: 1.25 mg/kg once daily *i.v.* on days 7, 9, and 11) (Table 2) [11,12]. Supportive care was done according to the guidelines for each institution.

Assess Engraftment and Toxicities

Myelogenous engraftment was defined as the first of 3 consecutive days with an absolute netrophil count (ANC) of 0.5 $\times$ 10^9/L, and platelet recovery was defined as the day the platelet count was 20 $\times$ 10^9/L without platelet transfusions. The BM was examined for morphology and cellularity at 1, 3, and 6 months, and 1 year after transplantation. Hematopoietic chimerism was evaluated by molecular analysis. Secondary graft failure was defined as engraftment followed by severe neutropenia (ANC $<$ 0.5 $\times$ 10^9/L) or the absence of donor cells in the BM or blood as demonstrated by

Table 1. Clinical Characteristics of Enrolled Patients

Median age, Years (range)	13.5 (1-30)
Sex, No. (%)	
Male	17 (60.7)
Female	11 (39.3)
Interval from diagnosis to transplantation, No. (%)	
<1 year	13 (46.4)
≥1 year	15 (53.7)
History of pervious IST, No. (%)	
Yes	14 (50.0)
No	14 (50.0)
History of RBC transfusion, No. (%)	
<15 units	17 (60.7)
≥15 units	11 (39.3)

IST indicates immune suppressive therapy (with regimens including antithymocyte globulin); RBC, red blood cells.

a chimerism assay without subsequent improvement occurring either spontaneously or after treatment with growth factor. BM or PB stem cell (PBSC) reinfusion, which was carried out at any time after day 0 because of inadequate hematopoietic function, was taken as a definitive indication of graft failure regardless of ANC values and BM cellularity. The regimen-related toxicity until 42 days after transplantation was graded according to the NCI Common Toxicity Criteria for BM transplant (BMT) recipients (CTC 2.0).

Statistics

This trial was designed to detect improvement of the engraftment rate and a 2-stage optimal design proposed by Simon was used for this trial ($P = .05$, power 80%). The engraftment rate in treatment groups was estimated by the proportion of the responders and the target engraftment rate was 95% (a 15% increases over the historic rate of 80%) [13]. If 7 or fewer engraftments were observed among 9 patients in first stage, accrual would stop with the conclusion that the regimen did not hold promise for further study. Otherwise, accrual continued to a total of more than 29 patients. At the end of second stage if the engraftment rate is ≤26/29, the regimen was considered to have no benefit of engraftment potential. Differences between categoric variables were analyzed by chi-square test, Fisher's exact test, or linear by linear association test. Differences between means of continuous variables were calculated with Student's *t*-test. Cumulative incidences (CI) were estimated for acute GVHD (aGVHD), chronic GVHD (cGVHD), cytomegalovirus (CMV) infection, Epstein-Barr virus (EBV) infection, and treatment-related mortality (TRM) to take competing risks into account. Kaplan-Meier method and log-rank univariate comparisons were used to estimate the probability of survival. Cox propotional hazard regression model was used for the multivariate analysis of prognostic factors affecting survival. SPSS version 17.0 was used for all statistical analyses and statistical significance was accepted for $P < .05$.

RESULTS

Engraftment Data

The median infused cell dose of nucleated cells and CD34-positive cells were 6.8×10^8/kg (1.3-39.9 $\times$ 10^8/kg) and 5.2×10^6/kg (1.2-27.0 $\times$ 10^6/kg), respectively. The median number of days required for ANC

Table 2. Transplantation Data of Patients Received Bone Marrow (BM) and Mobilized Peripheral Blood (PB)

Stem cell sources	BM (N = 15)	PB (N = 13)	*P*-Value
Infused cells dose, (range)			.000
TNC, $\times 10^7$/kg	3.1 (1.3-9.6)	17.0 (6.8-26.5)	
CD34, $\times 10^5$/kg	2.5 (1.2-6.3)	8.6 (4.7-23.8)	
HLA disparity, 8/8 (HLA-A, B, C and DR by high-resolution molecular method)			.244
0/8	9	10	
1/8	5	3	
3/8	1		
GVHD prophylaxis			.014
CsA + MTX + pATG*	9	2	
CsA + MTX	4	5	
FK + MTX	2	6	
Engraftment, median days (range)			.000
ANC of more than 0.5 $\times$ 10^9/L	16 (14-32)	12 (10-15)	
Regimen related toxicities†			.433
Grade III-IV	11	7	
Acute GVHD			.699‡
Grade II	6	4	
Grade III	1	1	
Grade IV	1		
Chronic GVHD/patients survived 100 days			.065‡
Limited	3/12	1/13	
Extensive	3/12	1/13	

CsA indicates cyclosporine; CMV, cytomegalovirus; FK, FK506/tacrolimus; GVHD, graft-versus-host disease; MTX, methotrexate; PTLD, posttransplantation lymphoproliferative disease; TNC, total nucleated cell.

*pATG: posttransplantation low-dose thymoglobulin (1.25 mg/kg once daily i.v. on days 7, 9, and 11).

†Toxicities assessed 42 days after transplantation.

‡Estimated by cumulative incidence.

of more than 0.5×10^9/L and 1.0×10^9/L were 15 days (10-35 days) and 16 days (11-40 days), respectively. Spontaneous platelet recovery to more than 20×10^9/L required a median of 22 days (22-182 days) except for 1 patient who died before recovery. Myelogenous engraftment in the mobilized PBSC transplantation (PBSCT) was significantly faster than that in the BMT (P <.001) (Table 2). All patients become transfusion independent, and they achieved donor-type hematologic recovery (donor-type chimerism of >90%). Until the day of analysis (November 2009), there was no report of secondary graft failure.

GVHD and Prophylaxis

Among the 3 kinds of GVHD prophylaxis, FK was administered more frequently to the PBSCT than the BMT (P = .014) (Table 2). Grade II–IV aGVHD occurred in 13 patients and the CI was 46.4%, with the majority being grade II. Grade III-IV aGVHD occurred in only 3 patients and the CI was 10.9%. The CI of grade II-IV aGVHD in patients receiving BM (53.3%) was not significantly different from that of patients who received PB (38.5%) (P = .699).

cGVHD occurred in 8 patients and the CI was 34.8%. Extensive cGVHD occurred in 4 patients and CI was 17.4%. The CI of cGVHD in patients received BM (54.6%) was not significantly different from that of patients who received PB (16.7%) (P = .065).

Toxicity

No patients experienced veno-occlusive disease, but grade III-IV regimen-related toxicity until 42 days after transplantation occurred in 18 (64%) patients. Grade IV pulmonary toxicity with pneumonitis/pulmonary infiltrates and dyspnea occurred in 2 patients, including 1 with proven respiratory syncytial virus infection. Grade IV SGOT/SGPT elevation occurred in 4 patients but was not associated with aGVHD. Grade IV diarrhea, vomiting, bilirubin, and skin rash occurred in a patient with grade IV aGVHD confirmed by biopsy. Toxicities are presented in Table 3.

Infection and TRM

CMV infection developed in 19 patients with 69.1% of the CI, and CMV disease occurred in 2 patients (pneumonia and enteritis) with 7.3% of the CI. Five patients developed posttransplantation lymphoproliferative disorder (PTLD) with 22.5% of the CI and 2 of them developed after the treatment of GVHD. The CI of PTLD in patients with previous history of IST before SCT (16.4%) was not different from that of others (29.1%) (P = .696). Patients received treatment including rituximab for PTLD.

Nine patients died of TRM and the CI was 32.1%. The causes of TRM were thrombotic microangiopathy (N = 2), pneumonia (N = 1), myocardiac infarction (N = 1), PTLD (N = 3), and cGVHD-associated complications (N = 2). Myocardiac infraction occurred in a 14-year-old female at 75 days after transplantation diagnosed by the echocardiogram and cardiac enzymes, but the cause was not proven. The cause of death associated with PTLD was progressive disease in 2 patients and sepsis after chemotherapy for lymphoma in 1 patient. One patient who received 8 doses of rituximab for steroid refractory cGVHD (NCT00472225) died of fungal sepsis and another died of pulmonary complication of cGVHD.

Table 3. Regimen-Related Toxicities

Body System	Grade I	Grade II	Grade III	Grade IV
Bladder				
Hematuria	3	3	6	1
Cardiac				
Pericardiac effusion	1			1
CNS				
Depressed level of consciousness	1	1		
Seizure				3
Coagulation				
Thrombotic microangiopathy				2
Constitutional				
Weight gain			1	
GI				
Diarrhea	8	3		1
Vomiting	6	5	6	1
Stomatitis	4	5	3	
Hepatic				
Bilirubin	11	2		1
SGOT/SGPT	10	1		4
Infection				
Febrile neutropenia			7	2
Infection (documented microbiologically) with neutropenia			3*	1†
Catheter-related infection	1	1		
Pulmonary				
Dyspnea		1		2
Pneumonitis/pulmonary infiltrates	2	1		2
Renal				
Proteinuria	2	2		
Creatinine	2	3	2	1
Skin				
Rash/desquamation	4	3		1

CNS indicates central nervous system; GI, gastrointestinal.
Data assessed 42 days after transplantation according to the NCI Common Toxicity Criteria (CTC 2.0).
*Respiratory syncytial virus, *Pseudomonas aeruginosa, Stenotrophomonas maltophilia.*
†*Streptococcus pneumoniae.*

Survival Data

The estimated survival rate (SR) of 28 patients was 67.9% (Figure 1). The SR of 13 patients who received PB (76.9%) was not different from that of the others who received BM (60.0%) (P = .351), and the SRs according to each GVHD prophylaxis regimen (CsA + MTX + rATG: 72.7%, CsA + MTX: 66.7%, FK + MTX: 62.5%) were not significantly different (P = .946). The SR of 19 patients with 8/8 HLA

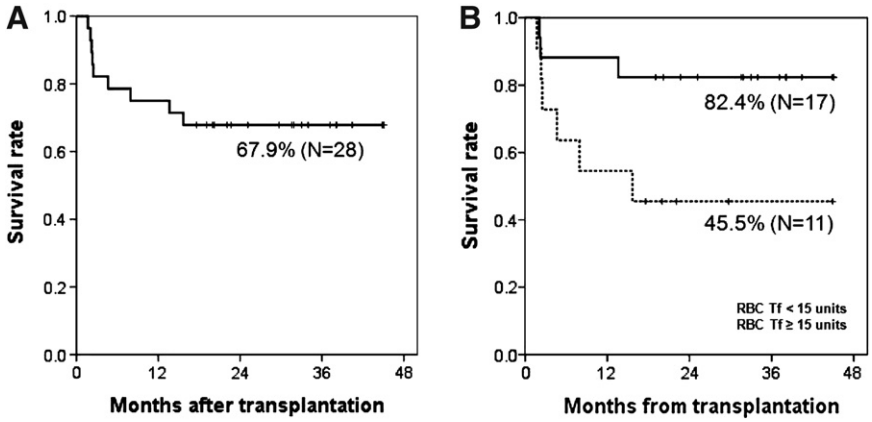

Figure 1. The survival data. (A) The estimated survival rate (SR) of enrolled 28 patients was 67.9%. (B) The SR of patients who received more than 15 units of RBC before transplantation (45.5%) was lower than that of the other patients (82.4%), significantly ($P = .048$).

matched by a high-resolution molecular typing (68.4%) was not different from that of mismatched patients (66.7%) ($P = .731$). The SR of 14 patients with the history of IST (57.1%) was not significantly different from that of patients who received first-line unrelated SCT (78.6%) ($P = .162$). The interval from diagnosis (<1 year: 76.9%, ≥1 year: 60.0%) did not affect survival ($P = .366$). Yet, the SR of 11 patients who received more than 15 units of RBC before transplantation (45.5%) was lower than that of the other patients (82.4%), significantly ($P = .048$) (Figure 1). Among the factors affecting the outcomes, including the stem cell source, history of previous IST, and the interval from diagnosis to SCT, only the amount of transfusion before transplantation (≥15 units) was a significant prognostic factor for survival on multivariate analysis (Table 4). The amount of transfusion before transplantation (≥15 units) was not associated with the interval from diagnosis to SCT ($P = .934$)

DISCUSSION

The data on matched unrelated transplant in SAA is still insufficient with the previous studies showing a 28% to 94% of overall survival (OS), and the optimal conditioning regimen has not yet been established [13,14]. Intensification of the conditioning regimens by the addition of irradiation has improved the outcome with enhanced engraftment rate [4,5,15-17], and a recent systemic review presented the use of radiation in the majority of patients [18]. Yet radiation has a deteriorative effect on growth, development, endocrine function, fertility, and so on [19,20], and the incidence of secondary malignancy is higher for the irradiation-containing conditioning regimen [21,22]. To avoid these problems, the nonradiation fludarabine-based conditioning regimen has been studied with promising results [6,10,23-25]. Among the studies that have used Flu, Cy, and ATG for unrelated stem cell transplantation (SCT) in SAA, our previous preliminary report and an EBMT-SAAWP study used the same kind of ATG preperation, namely, thymoglobulin [6,10].

Table 4. Multivariate Analysis of Factors Affecting Survival

Outcome measure (number of patients)	Relative Risk (95% CI)	*P*-Value
RBC transfusion ≥15 units	5.767 (1.212-27.434)	.028
History of IST	3.167 (0.702-14.289)	.134
HLA mismatch ≥1/8	2.060 (0.472-8.933)	.337
Diagnosis to transplant ≥1 year	0.706 (0.123-4.129)	.706
Stem cell source: BM versus PB	0.355 (0.059-2.134)	.258

BM indicates bone marrow; IST, immune suppressive therapy; PB, mobilized peripheral blood; CI, confidence interval; RBC, red blood cell.

The rationale for the selection of thymoglobulin was presented in our previous report [10]. Briefly, thymoglobulin had been found to be more effective and potent than the other anti-T cell antibody preparations for preventing GVHD and graft failure, and it was used as part of a conditioning regimen for aplastic anemia with promising results [6,7,15,26,27].

In our previous preliminary study and our present phase II multicenter study, a total of 33 patients achieved donor type engraftment, which implies that engraftment can be successfully achieved without radiation [10]. The study of EBMT-SAAWP reported 18% of graft failure. Although these studies used the same combination of Flu, Cy, and thymoglobulin, we started our study before the report of the EBMT-SAAWP using a higher dose of Cy (200 mg/kg versus 1,200 mg/m^2) and a lower dose of thymoglobulin (7.5 mg/kg versus 15 mg/kg) than that of the EBMT-SAA study, although the dose of Flu was same (120 mg/m^2).

The characteristics of the present study were the relatively younger age of the enrolled patients, a high proportion (46%) of PBSCT, we allowed each institution's GVHD prophylaxis regimen, we allowed first-line HSCT, we included only matched (6/6 in serologic typing for HLA A/B and low-resolution

molecular typing of HLA DR) unrelated transplant, and we excluded mismatched related transplantation.

The recipient age has been proven to be a potential prognostic factor in many studies, and the younger patients showed better engraftment and outcomes [18]. Although the majority of patients in our study were young as this was a multicenter study of the Korean Society of Pediatric Hematology-Oncology, 4 patients were over the age of 18 years and 3 of them alive now including a 30 years old female. This result implied that similar conditioning regimen could be applicable to adults.

Worse outcomes and more cGVHD were reported when performing transplantation with PB than BM in HLA-matched sibling donor transplants for young patients with SAA [28], and the use of BM as a stem cell source rather than PB has been strongly recommended for SAA as the GVL effect is not necessary [14]. In our study, the results of transplant with PB and BM were not different and FK was administered more frequently to the PBSCT patients than to the BMT patients. FK has been known to be more potent than CsA in preventing GVHD [29], and it has been shown superior survival in the setting of unrelated BMT [30]. Recently, many donors have preferred PB rather than BM, owing to the convenience after the official allowance of unrelated PBSCT by the government in Korea. Although BM is strongly recommended for transplantation to treat SAA, if only PB donor is available, it should not be avoided, and the use of intense GVHD prophylaxis agents such as FK might improve the outcome of PBSCT for SAA, and this should be clarified in future.

First-line unrelated transplants were performed without previous IST in half of our enrolled patients. Although IST has remained first-line therapy for patients without a matched related donor, IST has achieved limited improvement in survival and with the development of transplantation technique, promising results of unrelated transplantation (first-line or salvage after failure of IST) that were comparable to those of transplantation from a matched related donor were reported in SAA [31,32]. Early intervention was recommended for better outcomes, whatever the first-line therapy, owing to the improvement of both first-line-related and unrelated transplantation [33]. We decided not to exclude first-line HSCT for patients with appropriate unrelated donors as IST could increase the number of the required transfusions, which might induce allo-immunization and effects that are deteriorative to the success of unrelated HSCT. As HSCT with a matched related donor has become a standard therapy for SAA after the development of an excellent regimen, matched unrelated HSCT could become one of standard options for those patients without a matched related donor if optimal conditioning regimens and GVHD prophylaxis are developed.

Among the factors affecting the outcomes, the amount of RBC transfusion was significant in this study, but not the interval from diagnosis to transplantation. Multitransfused AA patients are known to have increased rates of graft rejection after HSCT because of alloimmunization, which adversely impacts survival rates [34,35]. But the amount of transfusion did not affect engraftment in our study. Iron overload by transfusion is known to increase complications, infections, and TRM after HSCT [36,37]. Although we did not assess the exact amount of iron overload before transplantation, it might have affected the outcome of our study. As reported previously, introduction of intensive iron chelating therapy before SCT could improve the outcome [37].

The engraftment was successful in our study and veno-occlusive disease did not occur. But high-grade regimen-related toxicities were observed in more than half of patients and incidence of the CMV infection and PTLD were also high, which implies that our conditioning regimen might have been somewhat too intensive. After we achieved statistically significant engraftment potential of our conditioning regimen, we closed this study before the planned enrollment of 29 patients.

Now we are performing a newly designed multicenter study (NCT00882323) to decrease the TRM by reducing the dose of Cy (120 mg/kg) with the hope of achieving better outcomes of unrelated transplantation for SAA.

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