Early hematopoietic recovery after single unit unrelated cord blood transplantation in adults supported by co-infusion of mobilized stem cells from a third party donor

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Background and Objectives. Our objective was to improve the outcome of cord blood (CB) transplantation in adults, by overcoming the limitations imposed by the low number of stem cells present in CB units.

Design and Methods. We combined single CB units and co-infusion of third party donor (TPD)-derived peripheral blood mobilized hematopoietic stem cells (MHSC) following myeloablative conditioning with reduced extra-hematologic toxicity.

Results. Twenty-seven eligible patients with high-risk hematologic malignancies (age 16-60 years, median 29, weight 43-78 kg, median 67) received CB units (median nucleated cell count 2.37×10^7/kg, median $CD34^+$ cells 0.11×10^6/kg) co-infused with TPD-derived MHSC (2.30×10^6/kg $CD34^+$ cells; $<1\times10^4$/kg $CD3^+$ cells). Neutrophil engraftment ($>0.5\times10^9$/L) occurred 10 days (9-36) post-transplant and was initially of TPD-origin in all patients except for four who received maternal MHSC, and then became of stable CB-origin. Median times to CB-derived neutrophil count $>0.5\times10^9$/L and full CB-chimerism were 22 and 55 days, respectively. The maximum cumulative incidence for engraftment, CB-engraftment and full CB-chimerism was 0.93 (95%CI: 0.83-1.00). The median time to reach unsupported platelet counts $>20\times10^9$/L was 33 days, with a maximum cumulative incidence of 0.74 (95%CI: 0.59-0.93). Transplant-related morbidity was associated primarily with non-neutropenic phase infections. Co-infusion of TPD-cells was well tolerated, with only 14.8% of recipients developing acute graft-versus-host disease (grade III-IV) and 20% developing a chronic (limited) form. The predicted 4-year overall survival was 69% for the whole group and 77% for the 23 patients receiving non-maternal TPD.

Interpretation and Conclusions. Our strategy offers prompt engraftment with a low rate of complications in a feasible alternative protocol that overcomes the current limitations of a single CB-transplant in adults.

Key words: cord blood, transplantation.

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Experience accumulated since 1988 has shown that cord blood (CB) transplants may effectively restore hematopoiesis in both children and adults, with a relatively low risk of graft-versus-host disease (GVHD).[1-6] For adults transplanted because of leukemia, retrospective observational studies based on registry data have shown higher rates of early transplant-related mortality (TRM) among CB recipients than among patients undergoing bone marrow transplantation.[7-9] Although better results have recently been reported in several single center studies, low rates of engraftment, delayed hematopoietic reconstitution and infections during post-transplant neutropenia are recognized as factors contributing to the early TRM of CB transplants.[10-13] Different strategies are being investigated to overcome slow hematopoietic recovery, which is the major deterrent to the use of CB transplants in adults.[14-17] We have developed a strategy consisting of a single unit of CB following myeloablative conditioning, supported by the co-infusion of a relatively low number of T-cell-depleted, mobilized hematopoietic stem cells (MHSC) from a third party donor (TPD).[15] We report the results of 27 patients consecutively transplanted in our center using this strategy, with a median follow up of 10 months (range 1-75) and 17 months for the 19 patients alive when the study was closed.

Design and Methods

Design

This was a phase II exploratory, open label, clinical study. The clinical protocol was approved by the Institutional Research and Ethics Committee. Written informed consent, in accordance with the Declaration of Helsinki, was obtained from all patients or their legal guardians. Patients were eligible for enrollment from the age of 15 years if they had a hematologic neoplasm at high risk of relapse, including chronic myelogenous leukemia beyond initial chronic phase; acute leukemia beyond first remission with high-risk cytogenetics or poor response to induction chemotherapy; severe myelodysplasia and high-grade lymphomas with poor response to chemotherapy.

HLA typing and donor selection

Patients and potential donors were HLA-typed in the Department of Immunology at our Center HLA-A and HLA-B antigens were determined serologically and HLA-DRB1 alleles were identified using high resolution molecular typing. CB units were selected on the basis of HLA A-B-DRB1 typing provided by several CB banks, the number of total nucleated cells and, when available, $CD34^+$ cell count at the time of storage. No preference was given to transplantation from an HLA-compatible unrelated donor if a CB unit was readily available with: (i) no more than 2/6 A-B-DRB1 HLA mismatches and (ii) a minimum of 1.5×10^7 total nucleated cells and 0.1×10^6 $CD34^+$ cells/kg recipient body weight before freezing.

Third party donors were selected based on their suitability to donate and to undertake an MHSC collection procedure with granulocyte colony-stimulating factor (G-CSF) mobilization, negative serological cross-match with the patient, cytomegalovirus (CMV) serology, age and gender. Young male donors were preferred and negative CMV serology was required for CMV-negative patients. HLA-haploidentical relatives were also preferred, as were candidates with ABO compatibility or minor incompatibility.

Collection of TPD cells

The donors' hematopoietic stem cells were mobilized with G-CSF.[15] The MHSC given to two patients were, in part, selected as $CD34^+$ and, in part (1/2 and 2/3), as $CD133^+$ using the CliniMACS immuno-magnetic method for both selections (Miltenyi Biotech, Bergisch, Gladbach, Germany). The selected T-cell-depleted MHSC were frozen in the usual manner until the day of the CB transplant.

Preparative regimen, GVHD prophylaxis and supportive care

For most patients, the preparative regimen consisted of fractionated total body irradiation to a total dose of 10 Gy in five doses in three days (-8 to -6) with lungs shielded at 8 Gy, fludarabine i.v., total dose of 120 mg/m^2 (30 mg/m^2/day, days -5 to -2), cyclophosphamide i.v., 120 mg/kg total dose (60 mg/kg/day over 1 hr, days -3 and -2) and equine antithymocyte globulin (Linfoglobulina®, Imtix, Sangstat) 30 mg/kg on day -1. Busulfan at a total dose of 8 mg/kg substituted total body irradiation when this latter was contraindicated. In these cases, busulfan was given orally 4 mg/kg/day on days -5 and -4, fludarabine on days -7 to -4, and cyclophosphamide and antithymocyte globulin as in the total body irradiation regimen. Cyclosporine was begun on day -5 at an initial i.v. dose of 3 mg/kg daily and adjusted to a serum level of 180-250 ng/mL. The drug was then given orally as soon as oral tolerance allowed and continued until full CB engraftment was achieved, at which point tapering was immediately initiated. If there were no signs of GVHD, methylprednisolone was given i.v., at a dose of 1 mg/kg from days -1 to days +10 to +14, when rapid tapering was initiated, unless there was a clinical indication to the contrary. G-CSF was initiated on day +1 at a dose of 30 MU daily and continued until a sustained absolute neutrophil count (ANC) higher than 1×10^9/L was reached. The dose was then adjusted as required to maintain the ANC above this level. Corticosteroid-based treatment (methylprednisolone 1-2 mg/kg/day) was given when grade II or higher acute GVHD developed.

Patients were nursed in positive pressure air-filtered rooms. Gut decontamination using ciprofloxacin was initiated on day -8 and continued until the ANC dropped below 0.5×10^9/L , when patients were switched to i.v. meropenem. Patients also received daily i.v. trimethoprim-sulfamethoxazole (from day -8 to day -2), i.v. fluconazole (from day -8 until ANC recovery), i.v. immunoglobulin (400 mg/kg weekly from day -3 to +60), and i.v. acyclovir (from day -8 to day +35, when it was switched to the oral route). Chemoprophylaxis against toxoplasmosis consisted of oral azithromycin 1 g twice a week until CB engraftment and then pirimetamine-sulfadioxine (Fansidar®) and folic acid, continued until day +180. This chemoprophylaxis was introduced in the last year of the study for seropositive patients and for those seronegative patients receiving MHSC from a seropositive TPD.[18] Post-transplant prophylaxis for *Pneumocystis carinii* infection with trimethoprim-sulfamethoxazole was used in all patients except those receiving prophylaxis for toxoplasmosis. Policies for transfusion support and treatment of infections were as previously described.[15]

Definitions and assessments of hematopoietic recovery, chimerism and transplant-related events

Hematologic recovery was assessed on the basis of peripheral blood ANC, platelet count and transfusion requirements. The time to granulocyte recovery was defined as the interval to the first of a minimum of three consecutive days with an ANC>0.5×10^9/L and the time to platelet recovery as the interval to the first day of platelet counts >20×10^9/L or >50×10^9/L without transfusion support for a minimum of one week. Engraftment failure was defined as the absence of durable ANC recovery at day +30 in surviving patients.

Chimerism was evaluated in bone marrow and/or peripheral blood cells (granulocytes and mononuclear cells) at least once a week from day +7, until day +28, and thereafter once or twice a month. This evaluation was carried out by HLA allele analysis using the RSCA method and/or by quantitative molecular genotype analysis using 15 markers of DNA short tandem repeats tagged with four fluorochromes and analyzed by an ABI PRISM 3100 sequencer and Gene Mapper 3.0 software.[19-20] This method was also used to analyze DNA from biopsy material taken from GVHD skin lesions to evaluate the presence of donor cells.

The time to CB engraftment was defined as the time to a sustained ANC of CB origin (CB-ANC)>0.5×10^9/L. This was determined as a function of the total ANC and the percentage of CB DNA, evaluated by the above methods in the DNA obtained from granulocytes after the transplant. Acute GVHD was graded 0 to IV and chronic GVHD as limited or extensive according to established criteria. TRM was defined as death from any cause except relapse or unrelated factors. Relapse was defined as evidence of disease in peripheral blood, marrow or extramedullary sites by morphological or, when applicable, cytogenetic or molecular criteria.

Statistical analysis

Cumulative incidences of hematopoietic recovery and relapse were estimated by competing risk analysis.[21] Death and relapse without recovery were regarded as competing events. No hematopoietic recovery was considered as a censored observation. The Mann-Whitney non-parametric U test was used to compare times to engraftment. Overall survival and disease-free survival were estimated by the Kaplan-Meier method.

Results

Characteristics of the patients

The study includes data from 27 consecutive patients (19 males and 8 females) transplanted in our Center between March 1999 and April 2005 (Table 1). The median age and weight of these patients were 29 years and 67 kg, respectively. Fourteen patients had acute lymphoblastic leukemia; of these seven were Philadelphia-chromosome-positive (all in first complete remission), two had myeloid markers, two were in partial remission and four were in second or higher complete remission. Six had acute myeloid leukemia; two were in partial remission and four in complete remission (one after presentation with extramedullary granulocytic sarcoma and one after relapse following auto-transplantation). One had acute leukemia of dendritic cells. Four had chonic myeloid leukemia, one in second chronic phase and two in accelerated phase (one of these following auto-transplantation). Two patients had high-grade non-Hodgkin's lymphoma, one was in unstable remission. Altogether, seven patients were not in stable complete remission. Pre-transplant CMV serology was positive in 24 patients (89.9%) and serology to toxoplasma was known to be negative in 11. Four patients known to be seronegative for toxoplasma received MHSC cells from a known seropositive donor. Seven patients were positive for HBsAb prior to the transplant without any other positive markers for hepatitis B virus.

Transplanted cord blood units

The units were provided by CB Banks within the international network. Cellularity and compatibility data of the transplanted units are shown in Table 2. The number of total nucleated cells of the transplanted units after thawing ranged from 1.31 to 3.70×10^7/kg of patient body weight (median 2.37). Pre-freezing $CD34^+$ cell count was available for 24 units and ranged from 0.035 to 0.37×10^6/kg (median 0.11). Patient vs CB mismatches in the six HLA A-B-DRB1 antigens were 0-2 in the graft-versus-host direction and 0-3 rejection-wise.

Third party donor cells

The third party donor (TPD) was the mother in four cases, other haploidentical relatives in 19 and a donor of no shared haplotype in the other four. The median purity of the TPD-selected MHSC was 98% The median number of infused MHSC (selected as $CD34^+$, or as $CD34^+$ plus $CD133^+$) was 2.3×10^6/kg and the median number of infused $CD3^+$ cells was 2.3×10^5/kg. Patient vs TPD mismatches in the six HLA A-B-DRB1 antigens were 2-5 both

Table 1. Characteristics of the patients.

Total Number (N)	*27*
Gender distribution	
Male	19
Female	8
Age (years)	
Median	29
Range	16.0-60.0
Weight (kg)	
Median	67.0
Range	43.0-87.0
CMV serological status	
Negative	3
Positive	24
Toxoplasma serological status	
Negative	11
Positive	8
Unknown	8
HBVsAb status	
Positive	7
Negative	20
Diagnosis	
Acute myeloid leukemia	6
Acute lymphoblastic leukemia	14
Acute leukemia (dendritic)	1
Chronic myeloid leukemia	4
Non-Hodgkin's lymphoma (high grade)	2
Added poor risk factors	
Acute myeloid leukemia patients	
Partial remission	2/6
Chloromas	1/6
Auto-transplantation	1/6
Acute lymphoblastic leukemia patients	
Philadelphia chromosome-positive	7/14
Myeloid markers	2/14
Partial remission	2/14
CR-2 or higher	2/14
Chronic myeloid leukemia patients	
2nd chronic phase	1/4
Accelerated phase	2/4
Prior autotransplant	1/4
Non-Hodgkin's lymphoma/Unstable remission	1/2

Table 2. Data of the transplanted CB units.*

Total number of units (N)	*27*	
TNC: median (range)×10^7/kg	2.37 (1.31-3.7)	
CD34+cells: Median (range)×10^6/kg	0.11 (0.035-0.370)	
CB vs recipient ABO match		
Matched	9	
Minor mismatch	7	
Major mismatch	9	
Major & minor mismatch	2	
CB vs recipient HLA mismatch	*GVHD*	*Rejection*
0/6	6	4
1/6	10	12
2/6	11	10
3/6	0	1

**For the patient obtaining engraftment with the unit transplanted 34 days after the initial transplant, which did not have viable progenitors, the entered data are those of the second transplanted unit. TNC: total nucleated cells; CB: cord blood.*

Table 3. Data on the TPD transplant products.*

Total Number (N)	*27*	
TPD		
Mother	4	
Other haploidentical relative	19	
No shared haplotype	4	
TPD infused cells: median (range)		
MHSC(*)×10^6/kg	2.3 (1.05-2.58)	
MHSC puritiy (%)	98 (91-99.1)	
$CD3^+$×10^3/kg	2.3 (0.5-9.8)	
ABO matches		
Recipient vs TPD ABO match		
Matched	19	
Minor mismatch	6	
Major mismatch	2	
CB vs TPD ABO match		
Matched	11	
Minor mismatch	12	
Major mismatch	3	
Major & minor mismatch	1	
HLA mismatches		
Recipient vs TPD	*GVHD*	*Rejection*
2/6	8	10
3/6	15	13
>3/6	4	4
CB vs TPD	*TPD rejection*	*CB rejection*
1/6	1	1
2/6	6	8
3/6	10	11
>3/6	10	7

**Positively selected as $CD34^+$ cells (n=25) or as $CD34^+$ plus $CD133^+$ cells (n=2)*
CB: cord blood; TPD: third party donor; MHSC: mobilized hematopoietic stem cells.

graft-versus-host and rejection-wise. CB vs TPD mismatches were 1-6 in both directions (Table 3).

Preparative regimen

Twenty-two patients received a preparative regimen containing total body irradiation: the first three patients of the series received a 12 Gy dose (lungs shielded at 8 Gy) and did not receive fludarabine. In order to reach the objective of reducing extra-hematologic toxicity for the other 19 patients, the dose of irradiation was reduced to 10 Gy (lungs also shielded at 8 Gy) and fludarabine was introduced. With the aim of reducing post-transplant immune deficiency, antithymocyte globulin was omitted in the first two. As severe GVHD occurred in one, antithymocyte globulin was maintained in all the following patients at a single dose of 30 mg/kg on day -1. Four patients received the busulfan-containing regimen because of myeloproliferative disease or previous irradiation. One patient, who was in an unstable remission of a high-grade previously irradiated non-Hodgkin's lymphoma, was conditioned with an individualized regimen consisting of cytosine-arabinoside 2000 mg/m^2/d for 5 days (-8 to -4), fludarabine 30 mg /m^2/d for 5 days (-8 to -4), cyclophosphamide 60 mg/m^2/d for 2 days (-3 and -2), and antithymocyte globulin 30 mg/kg and total body irradiation 200 cGy, both on day -1. All patients received cyclosporine A (Table 4).

Table 4. Transplantation data.

Total Number (N)	*27*
Conditioning	
TBI (12Gy) + CTX+ ATG	3
TBI (10Gy) + CTX + FDB	2
TBI (10Gy) + CTX + FDB + ATG	17
Busulfan + CTX + FDB + ATG	4
AraC + CTX + FDB + ATG	1
GVHD Prophylaxis: CsA + Pred	27
G-CSF administration	
From day +1	26
From day +5	1
Follow-up time (months)	
All patients (N=27), median (range)	10 (1-75)
Survivors (N=19), median(range)	17 (3-75)
ANC recovery: median (range)	
Time to ANC recovery (days):	10 (9-36)
MCI of ANC recovery	0.93 (95%CI: 0.83-1.00)
CB-ANC recovery: median (range)	
Time to CB-ANC recovery (days)	21 (13-53)
MCI of CB-ANC recovery	0.93 (95%CI: 0.83-1.00)
Final full CB chimerism median (range)	
Time to full CB chimerism (days)	55 (11-96)
MCI full CB chimerism	0.93 (95%CI: 0.83-1.00)
Platelet recovery: median (range)	
Time to >20×10^9/L recovery (days)	33 (13-96)
MCI of platelet >20×10^9/L recovery 0.74	95%CI: 0.59-0.93)
Time to >50×10^9/L recovery (days)	57 (14-240)
MCI of platelet >50×10^9/L recovery	0.69 (95%CI: 0.54-0.89)

TBI: total body irradiation; BUS: busulfan; CTX: cyclophosphamide; FDB: fludarabine; ATG: antithymocyte globulin; AraC: cytosine arabinoside; CsA: cyclosporine A; Pred: Methylprednisolone; ANC: absolute neutrophil count; MCI: maximal cumulative incidence; CI: confidence interval; CB: cord blood.

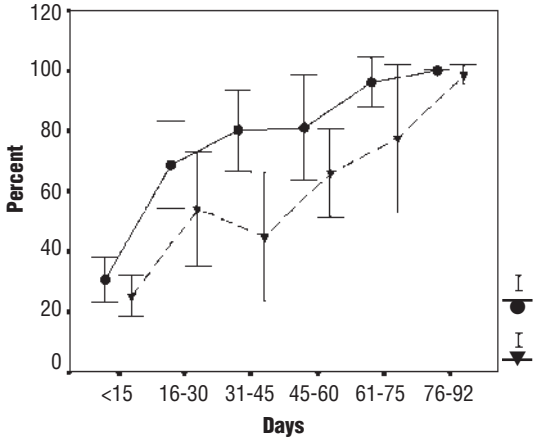

Figure 1. Progression of cord blood (CB) engraftment. ●. Proportion of CB DNA in mononuclear cells. ▼. Proportion of CB DNA in granulocytes.

Hematopoietic recovery and engraftment

G-CSF was initiated at the standard dose of 30 MU on day +1 after the transplant in all cases, except in one when it was initiated on day +5. Sequential evaluation of chimerism showed initial predominance of TPD DNA, both in granulocytes and mononuclear circulating and marrow cells, followed by progressive replacement by cells from the CB transplant. Figure 1 shows the progression of the proportion of CB DNA in the DNA obtained

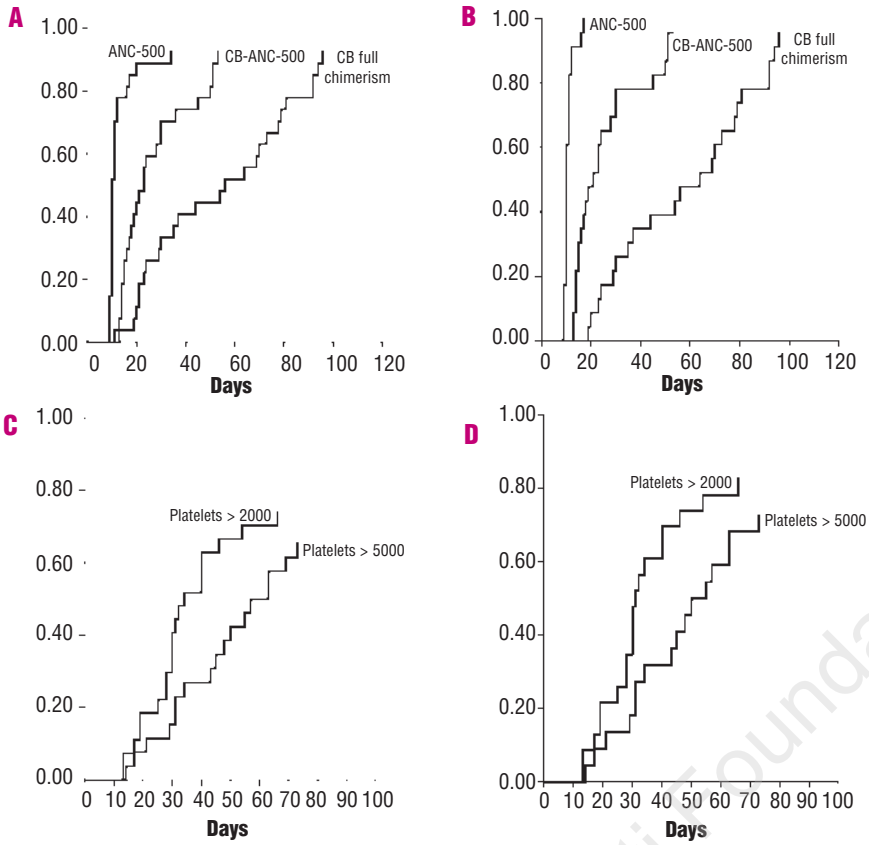

Figure 2. Cumulative incidence of engraftment: A and B show neutrophil recovery in all patients (A) and in those receiving non-maternal third party donor cells (B). Corresponding platelet recoveries are shown in panels C and D.

from granulocytes and mononuclear cells after the transplant and Figure 2 shows cumulative engraftments. The time to an ANC>0.5×10⁹/L ranged from 9 to 36 days, with a median of 10 and a maximum cumulative incidence (MCI) of 0.93 (95% CI, 0.83-1.00). MHSC of maternal origin did not lead to early engraftment in any of the four cases in which they were used. For the 23 patients who received non-maternal MHSC, the median time to ANC>0.5×10⁹/L was 9.5 days (range 9 to 17) and the MCI 1.00 (95% CI, 1.00-1.00). The patient whose ANC recovered on day +17 was the one in whom the start of G-CSF was delayed until day +5. The estimated time to CB-ANC>0.5×10⁹/L ranged from 13 to 55 days, with a median of 22 and a MCI of 0.93 (95% CI 0.83-1.00). For patients who received non-maternal MHSC, the corresponding data ranged from 13 to 53 days (median 22) and MCI 1.00 (95% CI 1.00-1). Final full CB chimerism was achieved in 25 patients for a MCI of 0.93; 95% CI 0.83-1.00 for the whole group and of 0.96 (95% CI 0.88-1.00) for the patients who received non-maternal MHSC. The time to full CB chimerism ranged from 11 to 96 days with the median being 55.

The homogeneity of intervals to granulocyte recovery (ANC>0.5×10⁹/L) in patients who received non-maternal MHSC implies independence of HLA and ABO mismatches between recipient and TPD. Regarding CB engraftment, the only significant correlation observed was between the number of CB-CD34⁺ cells/kg of patient body weight and time to CB-ANC>0.5×10⁹/L.The median interval to CB-ANC>0.5×10⁹/L was significantly shorter for patients who received CB-CD34⁺ cells above the median of 0.11×10⁶/kg.

Table 5. Post-transplant complications and survival data.

GVHD	*N*	*(%)*
Acute GVHD		
Evaluable patients	27	
Grade		
0	9	(33.0)
I	11	(40.0)
II	5	(18.5)
III	3	(11.1)
IV	1	(3.7)
Time to acute GVHD (days)		
Median	24	
Range	11-55	
Chronic GVHD		
Evaluable patients	20	
Limited	4	(20)
Extensive	0	(0)

Probability of survival (Kaplan-Meier)	*All patients (N=27)*	*Non-maternal donor (N=23)*
Overall survival	0.69	0.77
Disease-free survival	0.56	0.61
Primary causes of death		
Graft failure	1	
Infection	3 (2 CMV, 1 Toxoplasmosis)	
Acute GVHD	2	
Toxicity	2 (1 multiorgan failure, 1 veno-occlusive disease)	

GVHD: graft-vs-host disease.

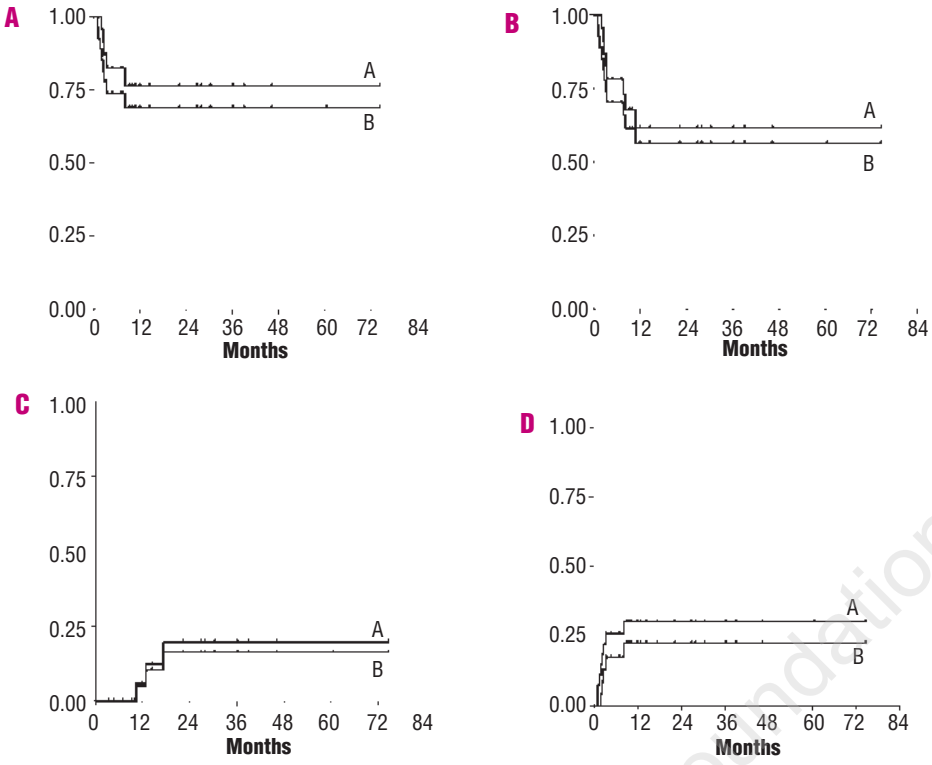

Figure 3. Survival and mortality curves for all patients and non-maternal TPD cases. Panel **A**: overall survival (Kaplan-Meier). Panel **B**: disease free survival (KM). Panel **C**: cumulative incidence of relapses. Panel **D**: cumulative incidence of non-relapse mortality.

FGC, 6/03

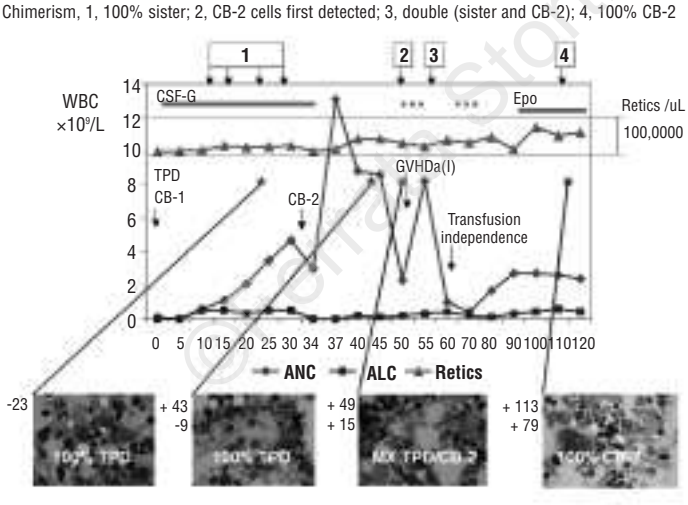

Figure 4. Graphic representation of the post-transplant course of patient FGC, a 28-year old male, who first received TPD MHSC from a sister, with whom he did not share any haplotype, and a CB unit that proved to lack viable progenitor cells as ascertained by post-thaw CD34⁺ counting and CFU control cultures. ANC recovery and full TPD chimerism were evidenced from day +10. On day +23 the bone marrow was morphologically normal, although there was no evidence of CB engraftment. By then he also reached independence of transfusion support, with lymphocyte counts remaining low (<500/μL). He had no manifestations of GVHD and no evidence of CB engraftment had been detected by day +30. He then received fludarabine (FDB) (30 mg/kg×3 days) and ATG (30 mg/kg×3 days) followed by a second CB unit on day +34. Following this he continually maintained normal blood counts and bone marrow cellularity. Progressive replacement of the TPD cells by cells from the second CB unit was documented from day +15 after the second CBT. Time to CB-ANC >500/μL was 49 days, and time to full CB chimerism 70 days from the second CBT. The patient was alive and well more than three years after the second transplant. (*for abbreviations see text*).

Median time to sustained platelets >20×10⁹/L and 50×10⁹/L, were 33 and 57 days, with MCI of 0.74 (95% CI 0.59-0.93) and 0.69 (95% CI 0.54-0.89%) for the whole group. For patients who received non-maternal MHSC, medians were 30 and 50 days with MCI 0.83 (95% CI 0.69-0.99) and 0.77 (95% CI 0.62-0.97) as shown in Figure 3. The initial unit transplanted in one patient showed a lack of viable progenitor cells, as ascertained by post-thaw counts and CFU control cultures and did not engraft. The patient achieved early recovery of the ANC and morphologically normal bone marrow exclusively derived from his TPD, who did not share a common haplotype. He also reached independence of transfusion support but lymphocyte count remained low (<0.5×10⁹/L). He received a second CB unit on day +34. This was followed by continually sustained blood counts and bone marrow cellularity with progressive replacement of the TPD cells by cells from the second CB unit. Time to CB-ANC>0.5×10⁹/L was 49 days and time to full CB chimerism was 70 days after the second CBT (Figure 4).

Morbidity, mortality and survival

The follow-up time ranged from 1 to 75 months (median 10), with 19 patients alive at the close of the study on

May 15, 2005. The median follow-up time for the 19 patients alive was 17 months with eight (40%) surviving more than 24 months (Table 5). Patients transplanted after the intensity of the conditioning regimen was reduced did not develop severe mucositis and requirements for parenteral alimentation were very limited. No major neutropenia-related infections were observed during the period until ANC recovery, and most patients had temperatures below 38°C. The main infections occurred after ANC recovery. The most frequent were CMV reactivations and hemorrhagic cystitis related to polyoma-virus. Conversion to HBVsAg positivity occurred in three out of eight patients who had previously been HBVsAb-positive, HbVsAg-negative. The development of toxoplasma infection in three patients prompted us to introduce chemoprophylaxis for this agent and no new cases of toxoplasma infection were observed thereafter. Twenty patients (74%) had manifestations of acute GVHD of any grade which began from 11 to 55 days after the transplant and in four cases (14.8%) reached grade III-IV. DNA analysis of skin biopsy material of grade >I acute GVHD lesions in five patients detected CB DNA in two. TPD DNA was not detected in any of the cases. Most of the patients with acute GVHD responded to corticosteroid therapy. There were two deaths primarily related to acute GVHD. One of these occurred in one of the two patients who did not receive antithymocyte globulin as part of the preparative regimen. Only four out of 20 patients developed manifestations of chronic GVHD, which was limited in all. These results compare favorably to those of other hematopoietic transplant procedures. Altogether, there were eight non-relapse deaths. Two were due to toxicity and occurred in heavily pre-treated patients who received preparative treatment with 1.2 Gy total body irradiation without fludarabine: one developed multiorgan failure and the other veno-occlusive disease. One was primarily due to graft failure in another previously heavily pre-treated patient with chronic myeloid leukemia whose treatment had included an auto-transplant. Three other deaths were due to opportunistic infections (one toxoplasmosis, two CMV) and another two were primarily due to acute GVHD>II. In the whole group of patients, the probability of overall survival at 4 years was 0.69. It was 0.77 after excluding the four patients who received maternal TPD cells. Relapses occurred in three patients, resulting in a 4-year cumulative incidence of 0.2 (95% CI, 0.07-0.54), The 4-year probability of disease-free survival was 0.56 for the whole group and 0.61 for the 23 patients who received non-maternal TPD cells (Figure 3).

Discussion

The slow hematopoietic recovery, low rate of engraftment, infections during the long-lasting post-transplant neutropenia and toxicity of preparative regimens are the main recognized factors for the early TRM of CB transplantation. Strategies that are being investigated to enhance engraftment include two-unit transplants, sub-myeloablative conditioning and *ex vivo* expansion of CB progenitor cells.[14-17]

We theorized that co-infusion of CB cells and selected MHSC (in a number low enough to carry a total number of T-cells below the threshold for GVHD), would be safe in terms of the risks of GVHD and rejection of the CB transplant, and might have the capacity for early engraftment in an intensively immuno-suppressed recipient. To comply with this, we chose the conservative threshold of 10^4/kg $CD3^+$ cells. This MHSC dose we use is much lower than the megadose used in transplants from haploidentical donors.[22] The fate of the eventual engraftment of these TPD cells would be either to persist or to fade out. On the other hand, we presumed that CB cells would have a competitive advantage for final chimerism over the TPD MHSC.[23] The expected main role of the TPD MHSC would be to provide early neutrophil engraftment during the window period prior to engraftment of the primitive hematopoietic stem cells of the CB transplant.

The conditioning regimen was designed to provide an intense anti-tumor effect, not to rely only on the graft-versus-leukemia effect of CB transplantation and to induce strong immuno-suppression in the recipient to minimize the risk of rejection, given the low number of progenitor cells in the CB unit, without producing risky extra-hematologic toxicity. For this purpose, modifications were introduced stepwise, maintaining the regimen myeloblative. The pursued objectives appear to have been reasonably well-achieved. In fact, excluding the first three patients who received a higher dose of irradiation without fludarabine, we did not observe significant manifestations of mucositis, lung toxicity, veno-occlusive disease or multiorgan failure and most patients went through the procedure without, or with only minimal need for, parenteral alimentation. On the other hand, engraftment rates were high and the relapse rate low. Antithymocyte globulin was maintained at a reduced dose after attempting its total suppression with a discouraging result (one death due to severe acute GVHD).

Our results support the hypothesis of a short-term engraftment advantage for the TPD MHSC resulting in prompt post-transplant recovery of the ANC (median 10 days) and a final advantage for the CB graft to competitively achieve sustained engraftment and full CB chimerism. It is notable that the 21-day median time to CB-ANC $>0.5\times10^9$/L observed in our patients is similar to the 20-25 day median time to ANC$>0.5\times10^9$/L observed in different series of patients receiving a single unit CB transplant after myeloablative conditioning.

The low incidence of post-transplant neutropenia-related infections and the high rate of survival beyond the first six months (0.68-0.87) are clearly benefits that may be the result of the combined effects of the supporting action of early granulocyte recovery and the low extra-hematologic toxicity of the conditioning regimens used. This low incidence of neutropenia-related infections may be a contributing factor to the relatively low incidence and severity of GVHD. In addition, the early recovery of the ANC allows the patients to withstand treatment with gancyclovir and other drugs with myelosuppressive effects in case of need.

The beneficial supportive effect of the co-infused TPD MHSC was clearly observed in the patient whose initially-

transplanted CB unit lacked viable progenitor cells. The early and persistent engraftment of the TPD cells allowed the patient to survive in good condition until the engraftment of the second CB unit, which was transplanted 34 days later (Figure 4). The progressive replacement of the TPD graft by the second CB transplant is highly suggestive of a mechanism of immune rejection of the TPD cells by the CB graft. Also significant was the serendipitous observation in this same patient of the capacity for engraftment of T-cell-depleted MHSC from a TPD of no shared haplotype with the recipient, which was later ascertained in three other cases. These observations widen the possible availability of a suitable TPD and may also allow searching for donors who may be particularly advantageous. For example, for a patient with AML, a donor KIR-compatible with the CB graft and KIR-incompatible with the recipient, might provide immunotherapeutic effects through the addition of TPD NK cells.

Immune rejection of the TPD cells by the CB engraftment seems the likely mechanism of effacement of the TPD cells. Neverthelss, evaluation by *in vitro* testing is difficult because of the slow recovery of circulating T cells after the transplant. The lack of engraftment of the MHSC cells from the maternal TPD cannot be explained on the basis of rejection by the recipient due to prenatal immunization against non-inherited maternal HLA antigens (NIMA). A possibility is immunization to minor histocompatibility antigens.[24] Obviously, the possibility of a casual coincidence cannot be ruled out.

Data from our patients are indicative of TPD graft contribution to platelet and red cell production, but not to the point of resulting in early transfusion independence. (In order to explore the possibility of improving results in this regard, we have started using TPD MHSC partially or totally selected as $CD133^+$ cells).[25]

The low number of relapses, despite the high-risk features of the transplanted patients, suggests a significant graft-versus-leukemia effect. Nevertheless, poor recovery of protective immunity is denoted by the frequent opportunistic infections occurring after engraftment. Additional improvement of the overall outcome of CB transplantation may result from strategies directed to further facilitate engraftment, reduce toxicity of the preparative regimens, enhance antitumor effects and recover protective immunity, as well as from the development of more efficient methods for treating specific infections. It is conceivable that benefits of this kind may be possible, on the basis of CB transplantation supported by co-infusion of MHSC from aTPD donor, supplemented with other selected/expanded subpopulations of cells that may act on the hematopoietic microenvironment or as immuno-modulators. Candidates are mesenchymal stem cells, regulatory cells and subpopulations of lymphoid effector cells.

In conclusion, by improving the engraftment rate, the strategy of co-infusion of MHSC from a TPD can make CB transplantation feasible for a large number of patients and merits further investigation.

EM: managed the database, did the statistical analysis and wrote the manuscript; CR: processed the transplant products and performed cell culture studies; IS: obtained third party donor cells and supervised patients transfusion support; RC: directed patients' clinical management; ER: clinical management of patients and clinical data collection; GB: clinical management of patients and clinical data collection; SG: performed cytology diagnostic studies; RF: performed fluorocytometric analyses; IM: directed statistical analyses; JAG-M: performed cytogenetic and molecular genetic studies to evaluate residual disease and chimerism; AM: performed HLA molecular genetic analises for evaluation of chimerism; MNF: designed the project, directed research, analyzed data and directed the manuscript writing.

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